LABORATORY OPTIMIZATION OF A PROTEASE EXTRACTION AND PURIFICATION PROCESS FROM BOVINE PANCREAS IN PREPARATION FOR INDUSTRIAL SCALE UP

By

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Thesis presented in partial fulfilment of the requirements for the degree Master of Science in Biochemistry at the University of Stellenbosch

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December 2012

DECLARATION

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BRIEF SUMMARY

This study describes:

a) Characterization of traditional methodologies and testing methods used to purify and quantify trypsin and α-chymotrypsin

b) Re-engineering / development of a new method for purifying trypsin and α-chymotrypsin that delivered higher product yields and improved control exercised over the process by investigating:

   i. Extraction methods
   ii. Centrifugation
   iii. Ultrafiltration
   iv. Chymotrypsinogen and trypsin crystallization
   v. Column chromatography
   vi. Investigation into different raw material sources for pancreatic enzyme production

c) Development of kinetic and ELISA testing methodologies for in-process QC analysis.

OPSOMMING

Hierdie Studie beskryf:

a) Karakterisering van die ou prosessering metodes en toets metodes wat gebruik word om Tripsien en Alpha-chimotripsien te suiwer en te kwantifiseer.

b) Herontwerp / ontwikkeling van 'n nuwe metode vir die suiwering Tripsien en Chimotripsien wat ‘n hoër opbrengs lewer en meer kontrole oor die proses uit oefen deur ondersoek in te stel na:

   i. Ekstraksie- metodes
   ii. Sentrifugering
   iii. Ultrafiltrasie
   iv. Chymotripsienogeen - en tripsien kristallisasie
   v. Kolom chromatografie
   vi. Ondersoek na verskillende rou materiaal bronne vir die produksie van pankreas ensieme.

c) Die ontwikkeling van kinetiese- en ELISA toets metodes vir die in-proses kwaliteitkontrole.

ACKNOWLEDGEMENTS

I hereby wish to express my sincere gratitude to the following persons and institutions:

My Heavenly Father to whom belongs all the glory. (1 Corinthians 10:31)

Sunandi de Wet my wife for all the moral support, patience, coffee and food she prepared whilst writing this thesis.

My parents for all the motivation and support in the world.

Prof. P. Swart for his expert advice and guidance.

Dr J. Carney for his assistance and expert advice.

Dr M Graz for always questioning everything I did.

Dr S Clark for support throughout the writing of this thesis.

Gabriel Mashabela for his generous support in the laboratory.

Almero Barnard for entertaining and inspiring.

BBI Enzymes for financial support.

Desmond February for always being willing to give a helping hand throughout this project.

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ABBREVIATIONS

BBI	-	British Biocell International
PDF	-	Pancreas Derived Factor (s)
QC	-	Quality Control
kDa	-	Kilo Dalton
CTG	-	Chymotrypsinogen
DNase	-	Deoxyribonuclease
RNase	-	Ribonuclease
DNA	-	Deoxyribonucleic acid
RNA	-	Ribonucleic acid
A/S	-	Ammonium Sulphate
MBR	-	Master Batch Record
A_{280}	-	Absorbance at 280 nanometres
TRIS	-	Tris(hydroxymethyl)aminomethane
RML	-	Recovery Mother Liquor
BAEE	-	N-Benzoyl-L-Arginine Ethyl Ester
ATEE	-	N-Acetyl-L-Tyrosine Ethyl Ester
SDS PAGE	-	Sodium Dodecyl Sulphate Poly Acrylamide Electrophoresis
UF	-	Ultrafiltration
FTU	-	Formazin Turbidity unit
TFF	-	Tangential Flow Filtration
MWCO	-	Molecular Weight Cut-Off
TMP	-	Trans Membrane Pressure
NWP	-	Normalised Water Permeability
PES	-	Poly Ether Sulfone
HIC	-	Hydrophobic interaction Chromatography
CM	-	Carboxy Methyl
pABA	-	p-Amino Benzamidine
RO	-	Reverse Osmosis
pI	-	Isoelectric Point
IPC	-	In Process Control
ELISA	-	Enzyme-linked immunosorbent assay
L-BAPNA	-	Na-Benzoyl-L-Arginine 4-nitroanilide Hydrochloride
DMF	-	Dimethyl Formamide
PBS	-	Phosphate Buffered Saline
BSA	-	Bovine Serum Albumin
HRP	-	Horseradish Peroxidase
TMB	-	Tetramethylbenzidine
BU	-	Billion Units

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CHAPTER 1

1. INTRODUCTION

This project was part of an investigation at BBI Enzymes South Africa to optimize the output of a protein purification process from acid treated bovine pancreas. Although there are a number of different enzymes that were purified from bovine pancreas such as deoxyribonuclease, ribonuclease, trypsin, chymotrypsin and chymotrypsinogen, the focus of this thesis was to describe the process improvements of only the trypsin, chymotrypsin and chymotrypsinogen (CTG) processes due to their annual monetary value to the company. The aim of this project was to develop a high yielding controlled protein purification process for trypsin and alpha chymotrypsin (ogen) by making use of adapted, optimized laboratory techniques. These methods needed to be simple, cost effective and should consistently be able to produce high quality enzymes. The laboratory based research had to yield a process that could be scaled up to an industrial production process. The outcome of this study will have major financial benefits for the company, and was considered an extremely high priority within BBI Enzymes.

BBI Enzymes is one of the largest natural enzyme producing companies in the world. The traditional methods for enzyme purification used at the facility in Cape Town were time consuming, out-dated, inconsistent and inefficient. The time required to produce the enzymes was not financially viable, and the company was thus in need of new methods to rapidly and consistently purify high quality enzymes. The traditional extraction and purification process of pancreatic proteases was extremely long due to a series of crystallizations at various stages during the process, and low yields were achieved at final product, due to inefficient extraction and poor control over the process. Because of the complexity of the manufacturing process, and the out-dated in-process testing methodologies, BBI was only able to predict the yield of the process during the latter stages of the process.

In this study, the three focus areas were:

1. Characterization of the traditional manufacturing process, and the enzymes

2. Re-Engineering/development of a new method for purification of the pancreatic proteases.

3. Development of new testing methodologies for in-process Quality Control (QC) analysis

1. Characterization of the existing manufacturing process

By making use of the developed assays for trypsin and chymotrypsin allowed us to gain a better understanding of the limitations of the current manufacturing methodologies. Analysis of the final products revealed additional information on efficiency of current methods to purify specific enzymes. A determination of the maximal amount of enzyme that could be extracted per mass of raw material input would determine if the outcome of the study was achievable, in terms of product yield.

2. Re-engineering/development of a new method for purification of pancreatic protease

After a detailed study of each of the processing steps, and after the main process inefficiencies had been identified, a new process was designed. Laboratory scale experiments would determine the optimal conditions for plant scale production, all laboratory based studies needed to be reproducible on plant scale.

Laboratory scale trials included the following:

2.1. Improved extraction of pancreatic proteases by better maceration of frozen pancreas and more vigorous agitation of extraction medium.

2.2 Optimized clarification techniques using high-speed centrifuges and diatomaceous earth filters.

2.3 Ultrafiltration technology as a means of protein purification and volume reduction. Investigating different types of membranes optimal performance.

2.4 Investigating different conditions to establish the optimal conditions for crystallization of chymotrypsinogen and trypsin.

2.5 Chromatography development to separate trypsin from chymotrypsin.

2.5.1 Affinity chromatography of trypsin.

2.5.2 Hydrophobic Interaction chromatography

2.5.3 Ion exchange chromatography

2.6 Usage of untreated bovine pancreas as an alternative source of enzyme production.

3. Development of testing methodologies for in-process QC analysis

To allow BBI Enzymes to perform proper protein purification, well established, robust testing methodologies were required to quantify the amount of enzyme (and precursor) present at every stage in the process, and to track the effectiveness of the methods and processes. These assays needed to be rapid, reliable, sensitive, specific and repeatable in order to feedback real time information to the process operators. Enzyme specific assays (microtitre assays) would be used to test the activity of the active protease during the latter stages of the process. These assays would include a set of standards and a control against which the in-process sample could be tested.

Because the precursor enzymes (zymogens) did not have activity, these testing methods could not be considered to test for the presence of the zymogens. In addition to the use of commercial reagents, immunoassays would be developed to quantify the zymogens during the initial stages of the process.

These testing methodologies would give a better indication of the amount of enzyme present in the initial stages of the process. Quantitative analysis would allow for characterization of the material at all stages during the process, and would facilitate tracking the outcome of every individual stage. The new testing methods would primarily be used to track the development of the new process, and would eventually allow us to compare the new method against the one previously used.

CHAPTER 2

2. THE PANCREAS AS A COMPLEX SOURCE OF ENZYMES

The pancreas is a complex organ and a rich source of different digestive enzymes. These enzymes include trypsin, chymotrypsin, ribonuclease, deoxyribonuclease, elastase, amylase to name only a few. Some of these enzymes have been identified to have industrial value, especially in the pharmaceutical industry. The study of pancreatic enzymes dates back to late 1876 when Kuhne *et al.* started to investigate the proteolytic properties of pancreatic juice. The work of Kunitz *et al.* revolutionized the way in which pancreatic enzymes were studied when they started crystallizing bovine trypsin and chymotrypsin (Northrop, 1948).

The predominant two enzymes in the bovine pancreas are trypsin and chymotrypsin. These are the two best defined and characterised enzymes and are of great commercial significance. This study focussed primeraly on the purification of trypsin and chymotrypsin(ogen).

2.1 ENZYMES OF THE BOVINE PANCREAS

The process described in this study did not allow the purification of trypsin and chymotrypsin only, but also of two other pancreatic enzymes, deoxyribonuclease and ribonuclease. The pancreas is a rich source of digestive enzymes that are all secreted into the duodenum to facilitate hydrolysis of proteins, nucleic acids, carbohydrates and fats. These enzymes include:

2.1.1. DEOXYRIBONUCLEASE (EC # 3.1.21.1)

Deoxyribonuclease (DNase) is a 31.3 kilo Dalton (kDa) endonuclease enzyme consisting of 282 amino acids that hydrolyses phosphodiester bonds adjacent to pyrimidine nucleotides of deoxyribonucleic acid (DNA) yielding 5'-phosphate terminated polynucleotides with a free hydroxyl group at the 3' position (Chen, 2006). DNase plays an important role in apoptosis and in the regulation of actin polymerization in cells. DNase I has been used as a treatment for cystic fibrosis and systemic lupus erythematous. DNase greatly decreases the viscosity of cystic fibrosis sputum, transforming it from a gel into a liquid after incubation, and this viscosity reduction is accompanied by a reduction in sputum DNA strand size (Thomson, 1995, Chen, 2006).

2.1.2. RIBONUCLEASE (EC # 3.1.27.5)

Ribonuclease (RNase) consists of 124 amino acid residues with a molecular mass of 16 kDa and includes four disulphide bonds. RNase is classified as an endonuclease, which specifically cleaves phosphodiester bonds at the 3'-end of pyrimidine nucleosides and at the 5'-ribose of a nucleotide, ribonucleic acid (RNA) (Smyth, 1963). RNase operates in an optimum pH range of 7.0 - 7.5. A major application for RNase is the removal of RNA from preparations of plasmid DNA. RNase demonstrates a series of important biological functions, such as killing tumour cells and inhibiting viruses by degradation of RNA. Several Ribonucleases are known to be toxic to tumour cells (Kim, 2009).

2.1.3. AMYLASE (EC # 3.2.1.1)

Amylase is a 60 kDa enzyme with the primary function of hydrolysis of dietary starch into di- or tri–saccharides, which are subsequently further hydrolysed into primary sugars. Amylase is not produced in the pancreas only, as there are other sources of amylase, such as saliva and the liver. Plant, bacterial and fungal amylase have been identified. Compared to the proteases, the amylase concentration in the pancreas is very low, and constitutes less than 2% of the total protein of the pancreas (Keller, 1958). Amylases are widely used in the industry to convert starch into sugars and syrups. These hydrolytes are often used as carbon sources during fermentation processes (Aiyer, 2005).

2.1.4. CARBOXYPEPTIDASE (EC # 3.4.17.1)

Carboxypeptidase is a 47 kDa protease that is secreted into the small intestine, and serves as the activator of trypsin by cleaving off the activation peptide of trypsinogen, causing a conformational change that leads to the activation of trypsin. Carboxypeptidase performs its function by hydrolysing the first peptide or amide bond at the carboxyl or C-terminal end of proteins or peptides (Cox, 1962).

2.1.5. ELASTASE (EC # 3.4.21.71)

Elastase is a 25 kDa serine protease that exerts its function by hydrolysing amides and esters in elastin (mainly), but also in other proteins (Schotton 1973). It has the ability to release soluble peptides from insoluble elastin fibers. Elastase is also produced in the pancreas as an inactive enzyme (like trypsinogen and CTG), and is called proelastase. Proelastase is activated by trypsin when it reaches the duodenum. Elastase is also classified as a serine protease.

2.1.6. LIPASE I (EC # 3.1.1.3)

Triglycerides cannot be absorbed into the blood stream from the small intestine if they are not hydrolysed. Lipases hydrolyse triglycerides (lipids) into fatty acids and glycerol for uptake from the gut. Unlike the zymogens, trypsinogen and chymotrypsinogen, lipase is produced as an active enzyme and secreted into the pancreatic juice. Pancreatic lipases have a molecular mass of approx. 45 – 50 kDa (Verger, 1969).

2.1.7. CHOLESTEROL ESTERASE (EC # 3.1.1.13)

Cholesterol esterase catalyses the hydrolysis of sterol esters into sterols and fatty acids. The enzyme is primarily found in the pancreas, but has been detected in other tissues as well. Bile salts, such as chelate and its conjugates, are required to stabilize the enzyme in its native polymeric form and to protect it from proteolytic hydrolysis in the intestine. Cholesterol esterase finds clinical applications in the determination of serum cholesterol (Allain, 1974).

THE PROTEASES OF INTEREST: TRYPSIN AND CHYMOTRYPSIN

The focus of this study, however, was on the purification of trypsin, chymotrypsin and chymotrypsinogen because of the commercial interest and the monetary value of these enzymes for BBI Enzymes. These enzymes contribute up to 50% of the revenue of BBI Enzymes, and were considered a high priority.

Trypsin and chymotrypsin are classed as serine proteases, and are synthesized and excreted by the acinar cells of the exocrine pancreas as inactive pro-enzymes (trypsinogen and chymotrypsinogen-A). The zymogens are stored in zymogen granules acting as intracellular storage sites (Greene, 1963).

Having inactive precursors in storage is a way for the cells to safely express and process these enzymes. Trypsin inhibitor is also found within the secretory vesicles and serves as an additional safeguard should some of the trypsinogen be activated to trypsin. Being encapsulated in a vesicle, the local concentration of trypsin inhibitor is relatively high. When the proteolytic enzymes are secreted and released into the lumen of the small intestine, trypsin inhibitor is diluted out and becomes ineffective.

Once secreted into the lumen of the duodenum, trypsin and chymotrypsin digests proteins into peptides of various sizes. These enzymes are, however, incapable of digesting proteins and

peptides to single amino acids. The hydrolysis of peptides into individual amino acids is executed through carboxypeptidase and mainly through peptidases on the surfaces of small intestinal epithelial cells from where the amino acids are absorbed into the blood stream (Roxas, 2008).

2.1.8. TRYPSIN (EC# 3.4.21.4)

Trypsinogen, secreted into the duodenum as a 26.3 kDa pro-enzyme, is activated when Enterokinase (Kunitz, 1939), secreted by the duodenal mucosa, cleaves the peptide bond between Lysine 15 and Isoleucine 16 resulting in active trypsin with a molecular mass of 25.8 kDa (Bringer, 1986). The activation of trypsin by enterokinase disrupts the hydrogen bond between His-40 and Asp 194. This causes a conformational change within the enzyme, and allows Asp 194 to associate with the N-terminus of Isoleucine (Ile)-16. This conformation change allows the creation of the active pocket of trypsin, and aligns the catalytic triad (His-57-Asp-102-Ser-195) (Hedstrom, 1996).

Trypsin has a high degree of substrate specificity as it catalyses the hydrolysis of peptide bonds on the carboxyl terminus of positively charged amino acids such as Lysine (Lys) and Arginine (Arg). The optimum pH of trypsin is pH 8. The presence of (minimum 20 mM) $CaCl_2$ is required for optimal enzyme activity and stability. Trypsin has a high affinity calcium binding site that is essential for the enzyme's stability. Auto-degradation rapidly occurs once this calcium binding site is mutated (Higaki, 1985). The catalytic efficiency (k_{cat}/K_m) of trypsin for substrates with Lys and Arg is 10^5 times higher than that for any other amino acids (Craik, 1985). Binding of substrate to the active site of trypsin influences both the K_{cat} and the K_m. The binding of the substrate to the active site is the rate limiting step in the hydrolysis reaction (Corey, 1992). Once trypsin is activated, it can act on trypsinogen by cleaving the peptide bond at Lysine 15, thus resulting in autocatalysis and accelerated activation within the duodenum, thereby facilitating protein digestion and ultimately enhancing protein absorption. Trypsin is also responsible for the activation of chymotrypsinogen and pro-elastase in the duodenum. Trypsin is very stable at pH 3.0 or as a lyophilized powder (Corey, 1992).

Trypsin possesses anti-inflammatory as well as potent proteolytic properties that can be used in molecular research and as an active pharmaceutical ingredient in various anti-inflammatory medicines (Swamy, 2008). Trypsin may be useful in removing dead tissue, and might alter the fibrous structure of blood clots. Localization of tissue damage, a cardinal aspect of the inflammatory process, is in part due to fibrin deposition, with the consequent formation of a

mechanical barrier (connective tissue) in the tissue spaces. Trypsin facilitates breakage of these blockages, and allows passing of blood and nutrients to inflamed areas (Martin, 1957). See Appendix1 for the complete amino acid sequence of bovine trypsin. Trypsin is reversibly inhibited by protein inhibitors such as ecotin, soybean trypsin inhibitor, α1-proteinase inhibitor, benzamidine (pABA) and the natural pancreatic trypsin inhibitor (Bringer, 1986).

2.1.9. CHYMOTRYPSIN (EC# 3.4.21.1)

Chymotrypsinogen (CTG) is activated when trypsin cleaves the peptide bond between Arg-15 and Ile-16, and will undergo structural modification to form/expose the substrate binding site. Chymotrypsin (25 kDa) specifically catalyses the hydrolysis of peptide bonds formed by hydrophobic amino acid residues such as Tyrosine, Phenylalanine and Tryptophan (Boeris, 2009). Activation of three different isozymes of chymotrypsin have been described (alpha, beta and gamma); however, this study will merely focus on the extraction and purification of α-chymotrypsin (Hudaaky, 1999, Folk, 1965). Depending on the mechanism of CTG activation, this would dictate which isoform of chymotrypsin is formed. Two primary modes of activation have been described for CTG; slow activation by trypsin only would yield the γ and α-isoforms, and fast activation by both trypsin and chymotrypsin would yield the β isoform (Desnuelle, 1960, Neurath, 1949). α-Chymotrypsin is a serine protease of the peptidase S1 family consisting of 241 amino acid residues. The molecule has three peptide chains: an A chain consisting of 13 residues, a B chain consisting of 131 residues, and a C chain consisting of 97 residues. α-chymotrypsin is the predominant form of active enzyme produced from its zymogen, chymotrypsinogen A. There is a striking similarity between trypsin and chymotrypsin with regards to synthesis, structure, activation, function, molecular mass and isoelectric points (Walsh, 1964) .

Chymotrypsin possesses anti-inflammatory properties that enable it to hasten the resorption of inflammatory oedema, as well as post-operative and post-traumatic haematoma. Chymotrypsin also possesses proteolytic properties that enable to *in situ* destroy the fibrinous formations resulting from sub-acute or chronic inflammatory processes in-situ. See Appendix1 for the complete amino acid sequence of bovine chymotrypsin.

Trypsin and chymotrypsin are both classed as serine proteases. The tertiary structure of these two enzymes are nearly identical, however there is a 50% difference in the primary structure. Even though there are such similarities in the tertiary structure of these two enzymes, they

have different substrate specificities (Higaki, 1985). The position of the catalytic triad (His-Asp-Ser) of both trypsin and chymotrypsin is located at the same position. The fact that these enzymes are secreted as inactive zymogens is an important characteristic trait that is used during the purification process. It is essential to maintain these enzymes in the zymogen state during the primary processing, as activation into the active forms of the enzymes will lead to auto activation and eventually to product loss. Activation as well as the catalytic activity of the enzymes is pH dependant. To understand the rational for some of the processing steps described in Chapter 3, an understanding of the mode of action of the Serine proteases is required. The pH of the solution in which the enzyme is solubilised in will have an effect on the catalytic activity of the enzymes. Trypsin activates trypsinogen and chymotrypsinogen into the respective active forms of the enzyme. Trypsin does not distinguish between itself, CTG or any other protein or peptide.

2.2 MODE OF ACTION OF THE SERINE PROTEASES

Both trypsin and chymotrypsin belong to the greater enzyme family called the serine proteases. The name is derived from a serine residue located within the active site of the enzyme that facilitates the catalytic mechanism of serine proteases (Outzen, 1996). Serine proteases can hydrolyse either ester or peptide bonds (Northrop, 1948).

Serine proteases all share three amino acids within the active site, which function together to hydrolyse a specific bond. These amino acids are Serine-195 (Ser-195), Histidine-57 (His-57) and Aspartate-102 (Asp-102) (Keller, 1958). Peptide bond hydrolysis occurs in four steps (see figure 1). For the reaction to occur, Ser-195 is deprotonated by His-57 converting it into a strong nucleophile (Northrop, 1948). To prevent the His-57 from being deprotonated immediately, the Asp-102 residue is positioned to stabilize the deprotonated His-57 (Keller, 1958).

In the first reaction, the nucleophilic oxygen in the side chain of Ser-195 attacks the electrophilic centre (carbonyl carbon) of the substrate scissile bond, and forms a tetrahedral intermediate. During the second reaction, the tetrahedral intermediate decomposes to form an acyl-enzyme intermediate with the assistance of His (proton transfer to the new amino terminus). The third reaction sees the nucleophilic attack of water (reaction occurs in an aqueous medium) on the acyl-enzyme intermediate with assistance of His-57 and the

formation of another tetrahedral intermediate. The final step is a reversal of the first step, and yields a carboxyl product and an active enzyme (Outzen, 1996).

At a relatively low pH (< 3.00), Ser-195 remains protonated and His-57 cannot abstract a proton from Ser-195. This will effectively prevent the enzyme from being able to hydrolyse any peptide bonds (the enzyme remains inactive), which is the main reason why the pH is maintained between 2.0–2.2 during isolation. At higher pH values, Ser-195 will be deprotonated by His-57, and both trypsin and chymotrypsin will be catalytically active (Outzen, 1996). This was the reason why the activation of both these enzymes was carried out at higher pH values (Chymotrypsin activation at pH 7.6, and trypsin activation at pH 8.0), to facilitate the catalytic mechanism of trypsin to activate trypsin and chymotrypsin.

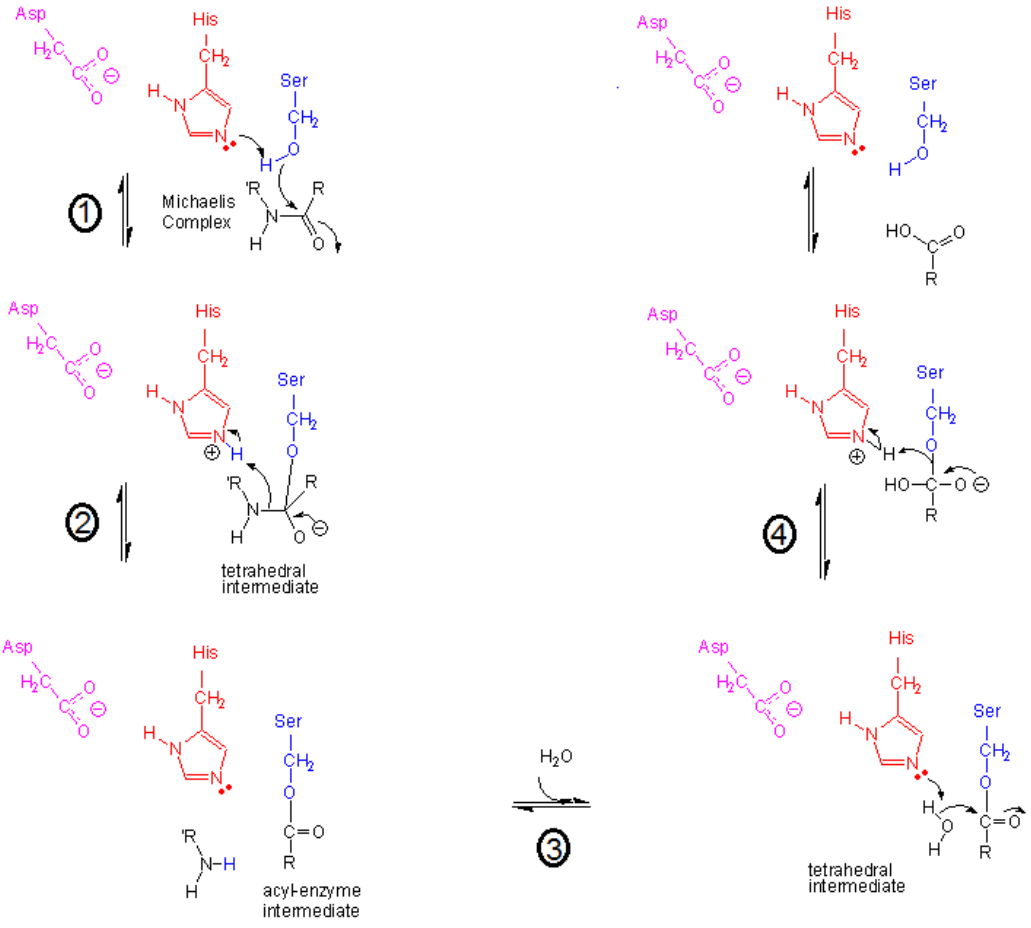

Figure 1. Catalytic mechanism of the serine proteases as it occurs in four steps (Graf, 2003, Keller, 1958).

With an overview of the complexity of the pancreas, and the main enzymes produced and secreted by this complex organ, it is clear that trypsin and chymotrypsin are considered valuable enzymes in the pharmaceutical industry, as well as having great financial importance for BBI Enzymes in Cape Town.

Apart from the enzymes listed above, numerous other nonspecific proteins are released when a pancreas is finely minced and extracted in a defined extraction medium. The aim of the purification process is to eliminate as many of these nonspecific proteins and to purify trypsin and chymotrypsin as final products. During the primary stages of the purification process, it is essential to prevent the activation of trypsinogen, as this will lead to further activation of both trypsin and chymotrypsin, and eventually cause autolysis and product loss.

Throughout the description of the purification processes used, the pH of the product is emphasised. Having gained an understanding of the mode of action of the serine proteases, the pH of the environment the enzymes are processed in would be a major contributing factor to the success of the outcome of the process. It is clear that an acidic environment (pH<3) will prevent any proteolytic activity which can lead to auto activation of trypsin and eventually to product loss. Chapter 3 provides an overview of the processing steps used to eliminate the nonspecific proteins that are present in the pancreas, and the specific techniques used to purify chymotrypsin and trypsin as final lyophilized products.

The traditional processing methodologies used to purify trypsin and chymotrypsin was considered time consuming and low final lyophilized product yield was achieved. To gain a better understanding of the process used to purify trypsin and chymotrypsin, chapter 3 will give an overview of the methods used and will elaborate on the inefficiencies observed in the process.

CHAPTER 3

3. OVERVIEW OF THE TRADITIONAL TRYPSIN AND CHYMOTRYPSIN PROCESSING METHODOLOGIES

The pancreas is a complex organ, and a rich source of enzymes. When the pancreas is homogenized and extracted, multiple enzymes are released. As described in Chapter 2, the two enzymes with the highest commercial value are trypsin and chymotrypsin. Traditional processing methodologies used to purify these two enzymes have been used for more than 50 years at BBI Enzymes. This section will describe these processing methodologies used to purify pancreatic proteases on an industrial scale at BBI Enzymes. Although there have been numerous process changes from the original methodologies applied when the process was started, the process described here was the one followed at the time of this study. This section will only describe the production of chymotrypsinogen, chymotrypsin and trypsin. Although deoxyribonuclease and ribonuclease are also co-purified with this method, they do not form part of this process description.

The method described below cannot deliver the enzymes at sufficient yields for the company to be sustainable, and was in need of review, this method differs significantly from the original methodologies applied and which were derived from the work of Kunitz *et al.* (1936). The trypsin and chymotrypsin processes are divided into primary and secondary purification processes. The primary processing of both enzymes is identical, and consists of a sulphuric acid extraction followed by a series of ammonium sulphate (A/S) precipitation steps. The purpose of the A/S precipitations is to 1) selectively remove nonspecific proteins and 2) to selectively precipitate the two zymogens. The zymogen precipitate generated during the primary processing is transferred to the secondary processing. Two different processes are described for the secondary processing of trypsin and chymotrypsin (see figure 2). The onset of the secondary processing is marked by the separation of the two zymogens during a crystallization of chymotrypsinogen (referred to as the zymogen separation).

Chymotrypsin and trypsin are purified separately. During the CTG purification, the CTG crystals obtained during the zymogen separation are washed, dissolved and prepared for lyophilisation. The Chymotrypsin purification process is similar to the CTG purification process, but includes a chymotrypsin activation stage prior to the product being lyophilised.

Trypsin purification starts with the supernatant of the zymogen separation. The trypsin is activated and subsequently crystallized to obtain pure trypsin crystals. The trypsin crystals are dissolved and prepared for lyophilisation. Figure 2 is a flowchart that illustrates the processing of the pancreatic enzymes. There is no difference in the primary processing of trypsin and chymotrypsin, but different purification methods for these two enzymes are described.

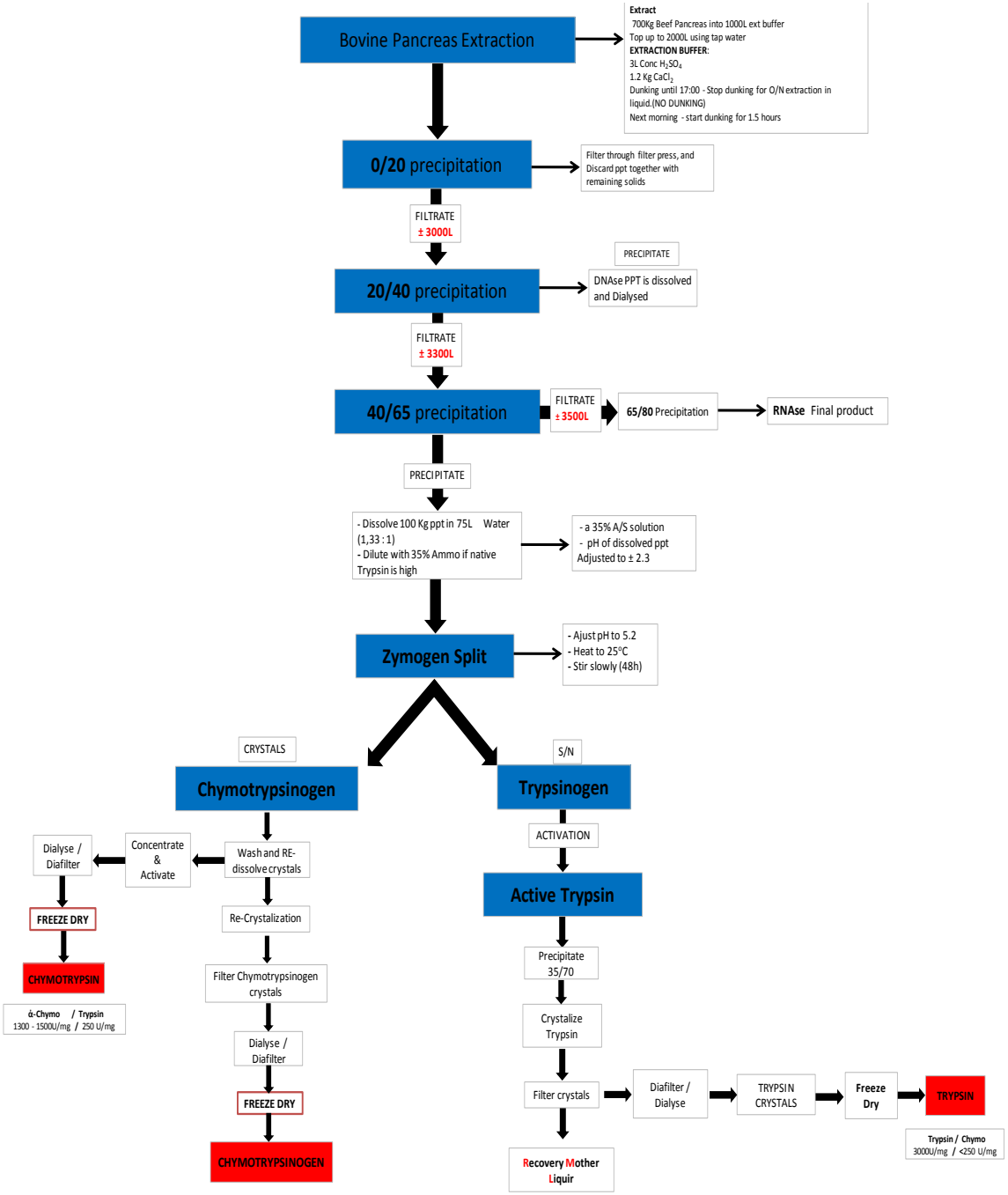

Figure 2. Flowchart of the entire traditional extraction and purification process for chymotrypsin, CTG and trypsin. The primary processing of both enzymes is similar. The two different processes for the purification of trypsin and chymotrypsin(ogen) are indicated.

3.1 PRIMARY PROCESSING

Deep frozen acid dipped bovine pancreas were semi thawed overnight. Semi-thawed blocks (20 kg) were flaked into baskets which were submerged into an acidic (acidified with H_2SO_4) extraction medium containing 1000 L water (37^oC) and $CaCl_2$ (1.2 g/l). The flaker is a machine that cuts the frozen pancreas into flakes 2-20 mm thick (see figure 3). The flaker used a hydraulic arm that forced a block of frozen pancreas into a rotating blade that shaved off flakes of pancreas that fell into big perforated baskets submerged into the extraction medium. The pH of the extraction medium was adjusted to between 1.8 and 2.2 using concentrated H_2SO_4.

Figure 3: Frozen blocks of beef pancreas lined up to be flaked into the perforated basket submerged in extraction medium. The frozen block is forced into a rotating blade that slices the pancreas into flakes.

The baskets were repeatedly submerged into the extraction medium for 3 hours with a crane mounted on a platform adjacent to the tanks, and then completely submerged in the extraction medium for a 16-hour static extraction. At the end of the extraction period (16 hours), the baskets were removed from the extraction medium (see figure 4) and the extraction medium was pumped to a holding tank, where the tissue was re-extracted in fresh extraction buffer by continuously submerging the basket containing the tissue into the fresh extraction buffer for a further 30 – 60 minutes.

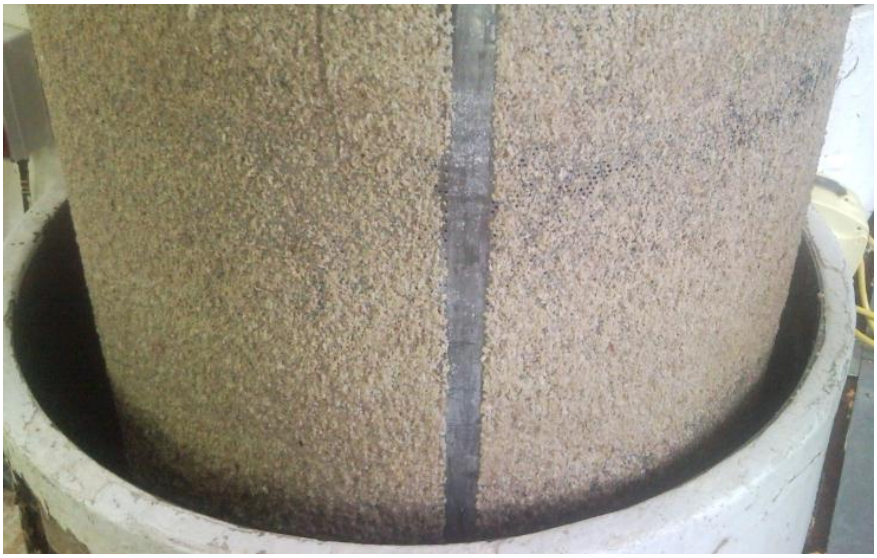

Figure 4. Perforated basket filled with tissue debris after submersion in extraction medium for 16 hours. This basket is submerged into the extraction medium with a hydraulic arm.

At BBI Enzymes, the A/S concentration of a solution is expressed as percentage saturation. Percentage saturation is calculated as described by Dawson *et al* (1989). A/S precipitation tables were used to determine the amount of solid A/S to add to a defined volume of liquid to obtain the desired final % A/S saturation. From the A/S precipitation table, the desired % A/S saturation corresponds to a certain amount of solid A/S to be added to the liquid. When a % A/S is quoted, it is referring to the % saturation of that product. To achieve a 20% saturated A/S solution, solid A/S (114 g/l) was added to the extract, and stirred until all the A/S had dissolved.

There is specific terminology used at BBI Enzymes to describe the different stages of A/S precipitation. The precipitate formed when the % A/S saturation of an extract (containing no A/S) is raised to 20% is referred to as a 0/20 precipitate. This implies that the % A/S saturation was increased from 0% to 20%. Typically, the precipitate formed during that specific precipitation stage would be removed, and the clear supernatant (containing 20% A/S) will be further processed.

After re-extraction, the tissue debris was discarded as solid waste, and the liquid phase of the re-extract was used as the extraction medium for the next batch.

The liquid from the extracts was then transferred to a holding tank where it would be precipitated with 20% ammonium sulphate. When all the salt was dissolved and the 20% saturation point had been achieved, the pH of the solution was adjusted back to 1.8 – 2.2

using 2.5M H_2SO_4, as there was an increase in pH because of the A/S addition. A/S dissociates in water to form ammonium and sulphates. The ammonium is primarily responsible for the increase in pH observed. This increase in pH after A/S addition was observed with every precipitation step during the primary processing, and the pH of the extract was subsequently adjusted to 1.8 – 2.2. As described in section 2.2, this was performed to prevent the auto activation of trypsin during the primary purification stages.

The proteins that precipitated at 20% A/S were removed by depth filtration using diatomaceous earth as a filtration medium in a filter press. The clear supernatant was transferred to a holding tank where it was further precipitated with A/S. The solid waste and 20% protein precipitate, removed by the filter press, was discarded as solid waste.

The A/S concentration of the liquid was further raised to 40% saturation by the addition of solid A/S (123 g/L) to the clear supernatant. During this step, deoxyribonuclease is precipitated (referred to as the 20/40 precipitate). This precipitate was removed by draining the tanks onto large filters colloquially called “coffin filters” where the precipitate was retained on a filter bed under vacuum. Coffin filters are used very successfully to separate precipitate from the supernatant (see figure 5). A thin filter bed of diatomaceous earth was prepared on filter paper lining the bottom of the filter. A vacuum was applied to the sump of the filter, drawing the liquid through the filter bed, and retaining the precipitate on the filter bed.

Figure 5. Coffin filter filled with precipitate being dried under a vacuum. On the right, the perforated bottom of the coffin filter is visible.

The clear supernatant collected from the coffin filters (at 40% A/S saturation) were transferred to another holding tank for further A/S precipitation. Solid A/S (168 g/l) was added to the 40% supernatant to increase the % A/S saturation to 65%. The precipitate formed

with this precipitation step (referred to as the 40/65 precipitate) contains the proteases, trypsinogen and CTG. The precipitate was removed by draining the tanks onto coffin filters where the precipitate was filtered and dried under vacuum as described above for further processing to purify trypsinogen and CTG. The 40/65 precipitate was collected and stored in a freezer (at -20^{o}C) until further processing was required. This step marked the end of the primary processing stages. The supernatant was collected and further processed to purify ribonuclease.

3.2 SECONDARY PROCESSING

At the start of the secondary processing, the enzymes (trypsinogen and CTG) are both still present as zymogens as the conditions of the primary processing were designed to prevent the activation of trypsin that would lead to the rapid activation of both trypsinogen and CTG. If the pH and temperature during the primary processing was not properly controlled, and allowed to exceed the limits specified in the Master Batch Records (MBR's), conditions would be favourable for trypsinogen activation, and the native trypsin activity in the final 40/65 precipitate would be very high, which would affect the secondary processing. If the native trypsin activity of the dissolved 40/65 precipitate was >300 U/ml, CTG would not crystallize, as the majority of the CTG would be converted to chymotrypsin, and could not be separated from trypsin(ogen) under the specified conditions. The traditional practice applied to reduce the trypsin specific activity was to dilute the trypsin with a 35% saturated A/S solution to reduce the trypsin activity to < 300 U/ml.

To separate the zymogens from each other, CTG was selectively crystallized out of solution during a process referred to at BBI Enzymes as the "Zymogen separation". The 40/65 precipitate was dissolved in 0.75 times (m/v) potable water (1 Kg precipitate – 750 ml water) to achieve a final % A/S saturation of 35%. The dissolved precipitate was transferred into a temperature-controlled vessel where CTG would undergo crystallization (see figure 6). The pH of the suspension was raised to 5.2 using NaOH, and the temperature adjusted to 25^{o}C by an element that was fixed to the bottom of the incubation vessel.

Figure 6. Zymogen separation incubation vessel in which the CTG was crystallized for 48 hours.

When the optimum conditions were achieved (25^{o}C and pH 5.2), CTG crystals (referred to as "seed" crystals) were added to the suspension whilst it was gently stirred to initialize the crystallization process. The CTG was given 48 hours to crystallize with gentle stirring. During the 48-hour period, the majority of the alpha-chymotrypsinogen would crystallize. After 48 hours, the crystals were removed by centrifugation. Using a vertical axis centrifuge the solid CTG crystals were separated from the supernatant. The supernatant contained mainly trypsinogen and some CTG that did not crystallize.

Based on the customer demand for chymotrypsin or CTG, the CTG crystals could either be processed to produce CTG final product, or it would be activated and purified as active chymotrypsin as described in section 3.2.1 and 3.2.2.

3.2.1. PURIFICATION OF CHYMOTRYPSINOGEN

The harvested CTG crystals were washed thoroughly with an A/S solution (35% saturation or concentration) to remove all entrained supernatant containing trypsinogen and other non-specific proteins. The crystals were dissolved in 3x water (m/v) at pH 3 and clarified using diatomaceous earth.

The clear liquid containing CTG was re-crystallized by increasing the pH of the solution to 5.2. A saturated A/S solution at 40°C was added to the suspension whilst stirring until protein flocculation and a white haze was observed. With constant stirring at a pH of 5.2 and at ambient temperature, the suspension was allowed to re-crystallize for 16 hours. After 16

hours, the crystals were harvested by filtration. The harvested crystals were washed with a 35% saturated A/S solution to remove any non-specific proteins entrained between the crystals.

CTG crystals were dissolved in water and dialysed until salt free in MC 110 dialysis tubing against acidified tap water (pH 2.0, acidified with 1M H_2SO_4) in a cold room (2 – 8 ^oC). The salt free solution was clarified and prepared for lyophilisation.

3.2.2. PURIFICATION OF CHYMOTRYPSIN

The harvested CTG crystals were washed thoroughly with 35% A/S to remove all entrained supernatant containing trypsinogen and other non-specific proteins. The crystals were dissolved in water and clarified using diatomaceous earth.

CTG was activated by adding 26.1 g/L of K_2HPO_4 to the clarified liquid, and raising the pH to 7.6. The activation of CTG was reliant on native trypsin present in the mixture. If the native trypsin activity in the solution was < 300 U/ml, lyophilized trypsin was added to the solution to initialize the chymotrypsin activation process. The chymotrypsin activity was monitored over a 4 – 6 hour period, and was assayed every hour. The completion of the chymotrypsin activation was marked by a plateau or decline in the specific activity of chymotrypsin (>750 U/A_{280}). The pH of the solution was subsequently lowered to 3.0 by the addition of 0.5M H_2SO_4 to terminate the activation.

The solution was immediately precipitated with 70% A/S by the addition of 472 g/L solid A/S. All chymotrypsin was precipitated at 70% Ammonium Sulphate. The precipitate was collected on a coffin filter under a vacuum, dissolved in water and dialysed against acidified tap water (pH 2.0, acidified with 1 M H_2SO_4) for 3 days until salt free. The salt free solution was clarified using diatomaceous earth and prepared for lyophilisation.

It is important to note that at BBI Enzymes, the protein content of a solution was determined as the absorbance of the solution at 280 nm using a spectrophotometer (referred to as an A_{280}). Although this was not an absolute protein concentration determination, it was used as a quick convenient reference (Smith, 1985) are too time consuming, and could not be used as an in-process measurement of total protein. Unless otherwise specified, protein concentration was always expressed as $A_{280.}$

Protein concentration is expressed as **A_{280}.** To determine the protein concentration of a solution, the sample is appropriately diluted to obtain an absorbance reading at 280 nm of 0.8 – 1.2 over a 1cm light path. This absorbance reading is multiplied by the dilution factor to obtain the A_{280} units for the solution. To determine the total amount of protein or total A_{280} of the solution, the A_{280} value is multiplied by the total volume of the solution. The term **U/A_{280}** describes the specific activity of an enzyme preparation. This is calculated as the U/ml of the solution divided by the A_{280} of the solution.

3.2.3. PURIFICATION OF TRYPSIN

Two grades of trypsin can be purified during the secondary purification stages, but only one of the two can be prepared from any single batch processed, both grades could not be prepared simultaneously. The first was a product that contained both trypsin and chymotrypsin in a ratio of trypsin: chymotrypsin of 6:1. A purer grade of trypsin could also be prepared which involved further processing that contains less than 1% chymotrypsin and a trypsin specific activity of >3000 U/mg. This study will only focus on the preparation of purer grade of trypsin with a specific activity of >3000 U/mg.

After the CTG crystals were harvested and washed with a 35% saturated A/S solution, the supernatant of the zymogen separation and the washings of the crystals were combined and further processed to purify trypsin. 2.42 g/L tris(hydroxymethyl)aminomethane (TRIS) (0.02 M) and 2.94 g/L $CaCl_2$ (0.02M) was added to the trypsinogen containing solution in preparation for trypsin activation.

While stirring, the pH of the trypsinogen liquid was slowly raised to 8.0 with 1M NaOH to initialize the trypsin activation at 5^{o}C. The liquid was continually assayed for trypsin activity to monitor the activation sequence. If the starting trypsin activity was <100 U/ml, 100 g of lyophilized trypsin (3000U/mg) was added to the stirring liquid. The activation was monitored hourly (assayed for trypsin activity (U/ml) and A_{280}), to monitor the increase in the trypsin specific activity. The activation was completed when the trypsin activity (U/ml) reached a plateau and the specific activity (U/A_{280}) of trypsin was between 900 to 1000.

The activation was terminated by lowering the pH of the activation mixture to 3.0 with 2.5M H_2SO_4 followed by immediate A/S precipitation by increasing the A/S saturation to 75% by adding solid A/S (278 g/L) to the liquid. The precipitate was removed by filtration using a coffin filter. This precipitate is referred to as the 35/75 precipitate, indicating this is the

precipitate that formed when the % A/S saturation of the material after the activation was increased to 75%.

In preparation for trypsin crystallization, the 35/75 precipitate was dissolved in 0.4 M borate buffer at a pH of 9.0 in a cold room (8^{o}C). The trypsin was left to crystallize at 2-8^{o}C for 7 days whilst gently stirring the liquid. After the 7th day, the trypsin crystals were harvested by filtration on a coffin filter. The crystals were washed with 0.4 M borate buffer, 35% A/S, pH 9 to remove any entrained proteins. After the crystals were thoroughly washed, they were dissolved in reverse osmosis water (RO water) at pH 3 (acidified with H_2SO_4). The liquid was either diafiltered or dialyzed until salt free and prepared for lyophilisation.

The supernatant of the trypsin crystallization and the washings of the crystals were combined and referred to as Recovery Mother Liquor (RML). This liquid contained any un-crystallized Trypsin and any chymotrypsin that carried through from the CTG crystallization. The RML was precipitated with 70% A/S and stored in the freezer at -20^{o}C.

The processes described in section 3.2.1 – 3.2.3 was capable of producing high quality final products, however, the yields (expressed as kilograms of final product produced per ton of raw material input) achieved were extremely low. This was as a result of some inefficient proceeding steps, and methodologies that were applied. These shortcomings of the traditional processing methods are described in section 3.3.

3.3 SHORTCOMINGS OF THE TRADITIONAL PROCESSING METHODOLOGIES

Although the traditional processing methodologies were based on the original work of Kunitz *et al*. (1936) there have been many changes to the original process, leading to a process that was not properly controlled and could not consistently deliver high quality product at high yields. Following an process overview, and investigating every step of the process, it was possible to specify which processing steps could be improved.

3.3.1. EXTRACTION

The first major processing inefficiency observed was the extraction process of the bovine pancreas. The mechanism of tissue maceration was executed by means of forcing flozen blocks of tissue through a rotating cutter that shaves off flakes of tissue which fell directly into the extraction medium. Observing the efficiency of the maceration of the Flaker, it was clear that there were still large pieces of intact pancreas present at the end of the extraction process. This indicated that the efficiency of maceration of the pancreas was not optimal, and the flaker could not deliver a consistent output (particle size of the macerated tissue). The second observation was that the flaking process was extremely time consuming. To flake 1.4 tonnes of pancreas took approximately 1 – 2 hours. This allowed additional time for pancreas to thaw, and pancreatic juice containing valuable enzymes was lost.

The 16 hour static extraction also did not allow for the proper extraction of all the enzymes. This was revealed by A_{280} spectophotometric measurements[1] when the protein concentration of an extract sample was compared to the protein concentration of different extraction methods, see section 4.1.

There was inconsistent exposure of the tissue to the extraction medium throughout the basket and as a result the enzymes could not be extracted efficiently. This problem was identified by inspecting the appearance of the tissue at the centre of the basket post extraction. Pancreas that had been in contact with the acidified extraction medium had a pale light brown colour, whilst pancreas that have not been exposed to acid had a light pink colour (see figure 7). The majority of the tissue found at the centre of the basket was light pink in colour. The macerated pancreas, after being exposed to acidic medium, formed strings of tissue and fat. This stringy tissue caused the perforations in the baskets to block and did not allow for proper flow of extraction medium into and out of the basket and through the flaked pancreas.

[1] See note on page 19 for the calculation of the total protein content of a solution my means of measuring absorbance at 280 nm.

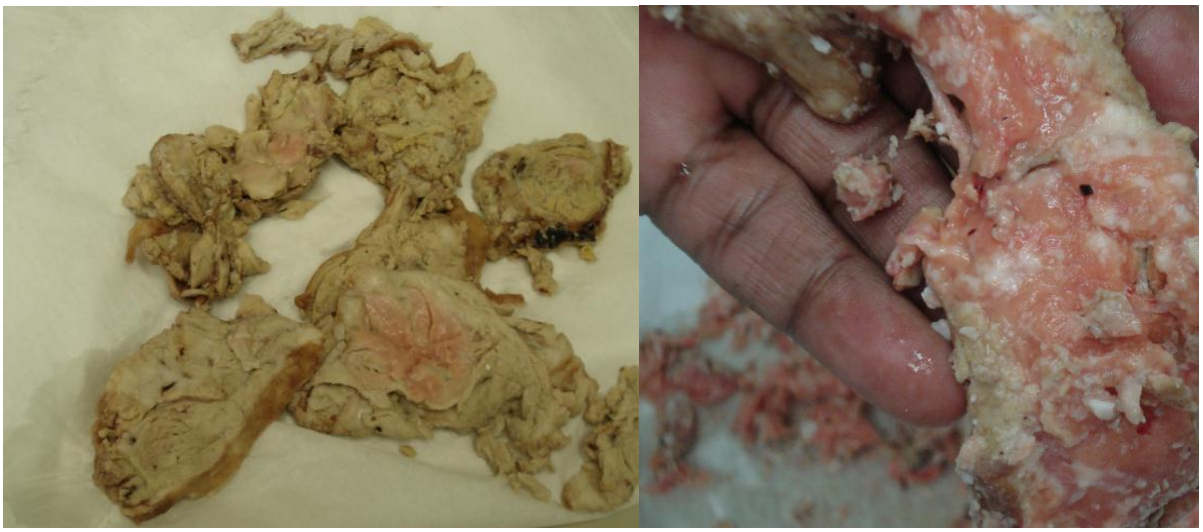

Figure 7. Large pieces of pancreas collected after extraction. The pink colour of the pancreas indicates that the tissue was not fully exposed to the extraction medium.

3.3.2. AMMONIUM SULPHATE PRECIPITATION

The supernatant of the extraction was precipitaed with 20% A/S to remove non-specific proteins. The method used to add solid A/S into the tanks was completely unregulated. The stainless steel tanks have a capacity of >15000 L of which only the first 3000 L was utilised to precipitate the extract. The volume measurement was inaccurate which lead to major miscalculations of the total amount of solid A/S to be added to the liquid. Volume was determined by lowering a measuring tape into the tank until it reached the surface of the liquid which often had a layer of foam which made the volume measurement extremely inacurate.

Based on the dimensions of the tank, the volume of liquid was calculated from the measurement taken. The point from which the tank measurement was taken was too high, and was not an acurate determination of the volume of the tank (see figure 8 for an illustration of the dimensions of the tank and how the volume was measured). The point from which the volume was measured was 41 cm higher than that of the actual tank height. This measurement was used to calculate the volume of the tanks. The implication was that, during all the A/S precipitation stages, the incorrect amount of A/S was added to the liquid.

Figure 8. Closed tops of the precipitation tanks complicated the volume determinations, and lead to major volume miscalculations because of faulty measurements.

The additional volume that was mistakenly added whist calculating the amount of solid A/S to be added into the tank could be quantified based on the dimensions of the tank. A total of 1288 L of liquid was added to the calculation as a result of the incorrect volume determination methodology used. For a 0/20% A/S precipitation (113 g/L) this would imply adding 145 kg more solid A/S to the batch than what was required, and for a normal production batch (4000 L) this would in reality be a 0/26% precipitation. When this batch was precipitated 20/40 (121 g/L) and the same error was calculated a total of 155 kg of additional A/S was added to the batch and the % A/S saturation would be 47%, and would precipitate some of the proteases which would be discarded with the 20/40 precipitate. The cumulative effect of these miscalculations was not only detrimental because protease enzymes were discarded as solid waste, but that the total A/S consumption per batch increased with 646 kg. See table 1 for a summary of the errors introduced as a result of faulty volume determinations.

Table 1. Additional A/S added per processing step as a result of miscalculations of the volume of the tanks

Processing Step	**Additional A/S (kg) added at each stage of the process**
0/20	154
20/40	155
40/65	206
65/80	131
TOTAL	**646**

CTG precipitate at 40% A/S saturation. This is evident from figure 9 lane 7 when a DNase sample was loaded. The protein band visible at 25 kDa corresponds to the molecular weight of the proteases. This would imply that more proteases would precipitate when the % A/S saturation was raised higher than 40% as a result of volume miscalculations. All subsequent precipitation stages were not accurately executed, as a result of the miscalculation of the volume of the tanks.

The rate at which solid A/S was added to the product also lead to process instability. When adding A/S in large quantities into a stirring tank with liquid product, it was importaint not to add the solid A/S too rapidly, as this caused an accumulation of solid A/S at the bottom of the tank, and lead to local supersaturation at the bottom of the tank which in turn leads to an accumulation of non-specific precipitation of proteins at the bottom of the tank.

After A/S precipitation, when the liquid was transferred onto the coffin filters to remove the precipitate, solid A/S crystals were often found that had not dissolved. This implied that A/S precipitation was not accurately carried out, and the % A/S saturation was not homogenous throughout the tank. This lead to incomplete precipitation of certain proteins and precipitation of unwanted proteins resulting in major batch to batch process variation.

3.3.3. pH MEASUREMENT

The pH of the extract during the entire primary processing stage was maintained between 1.9 – 2.2. This was to prevent trypsin activation (see section 2.2). This could have had a direct impact on the yield of any production batch because of the proteolytic action of trypsin. As trypsin and chymotrypsin are both unstable at a pH < 1.6, this would imply that the lower pH limit of the process was too close to the pH at which the enzymes could be destabilized (Outzen, 1996).

Operators would sometimes overcompensate while adjusting the pH of a solution, or pH meter calibrations were not carried out properly, which often lead to observed pH values well below 1.6, severely compromising the stability of the proteins.

The liquid product, as it was processed during the primary processing, had a high fat content, and often contained small tissue debris particles. The pH probes used on the factory floor often got blocked or coated in a layer of fat. The pH probes were never properly cleaned with

a pepsin solution to remove any proteins that coated the probe. This lead to major variation in the pH measured throughout the process.

3.3.4. CHYMOTRYPSINOGEN CRYSTALLIZATION (ZYMOGEN SEPARATION)

Inefficiencies observed at this stage were related to the way in which heat was applied to the tanks to regulate the temperature. An element, linked to a temperature control unit, was mounted in the bottom of the tank. The control unit could switch the element on when the core temperature of the liquid was below 25^{o}C, and off when the core temperature of the liquid was above 25^{o}C. Being an element, this strip would get extremely hot (>70^{o}C), and burned the product that got into direct contact with it. This was identified by a visible strip of protein / crystals that got burnt to the walls of the tank where the element was in contact with the tank (see figure 6).

During the CTG crystallization process, the protein content of the supernatant was continously reduced as a result of CTG crystal formation. This had a major effect on the efficiency of the zymogen separation, as the crystallization process was dependent on protein concentraion (Garcia-Ruez, 2003). The two day crystallization procedure did not compensate for the major decline in the protein content of the supernatant and as a result CTG crystallization efficiency was significantly reduced.

The second CTG crystallization step had no benefit for the production of CTG (with regards to yield specifically). The purpose of the step was to further purify the CTG and to remove any contaminating trypsin and non-specific proteins found in the supernatant of the first zymogen separation. The conditions for this crystallization were, however, also not optimal for CTG crystallization and an overnight crystallization was insufficient to crystallize all the CTG present. Major losses were observed during this processing step with no real gain in enzyme purity.

3.3.5. TRYPSIN CRYSTALLIZATION

Trypsin was allowed to crystallize for 7 days. Before the onset of the crystallization the total amont of trypsin was quantified by testing the trypsin activity and expressed as millions of units (MU's) of trypsin. The total trypsin at the onset of crystallization could be used to determine the efficiency of the crystallization process. This was calculated by quantifying the

total activity recovered when the trypsin crystals were resuspended divided by the total trypsin content at the onset of the crystallization. Alternatively, the total trypsin content of the supernatant was determined which gave an indication of the total amount of trypsin that did not crystallize during the 7 day crystallization step. The inefficiency of the seven day crystallization process was clearly illustrated when the total trypsin content of the supernatant post trypsin crystallization (referred to as the recovery mother liqor or RML) was taken into consideration. Up to 50% of the total trypsin was found present in the RML as a result of inefficient crystallization conditions. The conditions did not allow for proper crystallization of trypsin, and the long time period allowed for crystallization also allowed for autolysis of trypsin (indicated by the loss of total trypsin content before and after this crystallization step).

Protein crystallization is dependant on the protein concentration of the material (Chayen, 2004). During the crystallization process, the protein content of the supernatant continously decreased as solubilised proteins were converted to protein crystals (Garcia-Ruez, 2003). This implied that the efficiency of the crystallization process decreased as the process continued. The decreasing protein concentration was not being accounted for, and was considered an area for improvement.

Although the products that were produced when the traditional processing methods were executed conformed to the final product specifications, these methods did not consistently deliver product at a yield that was profitable. The products produced by the traditional methods were analysed and characterized to set a benchmark for the quality of the products. The products produced by the improved process needed to be equal to or of a higher quality than those being produced by the traditional methods.

3.4 CHARACTERIZATION OF PRODUCTS PRODUCED BY TRADITIONAL PROCESSING METHODOLOGIES

The products produced during the traditional processing methods were characterized by Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS PAGE) analysis and a variety of assay methods used in BBI Enzymes Cape Town to assess compliance to the specifications (see tables 2 and 3). The SDS PAGE gave an indication of the purity of the products, and was used to confirm that inefficiency of the A/S precipitation described in section 3.3.2. (This is visible when inspecting the final deoxyribonuclease sample analyses in lane 7 of figure 9). A clear protease band (corresponding to the molecular weight of chymotrypsin) is visible at approximately 25 kDa. Deoxyribonuclease is purified from the 20/40 precipitate, which contained a large amount of protease enzymes. Final lyophilized products were used for this analysis. The trypsin and chymotrypsin samples were compared to that of an industrial standard, Sigma (Sigma life sciences, St. Louis, USA).

For all SDS PAGE analysis during this study, the following materials and methods were used.

The samples were prepared (10 mg/ml) in deionised water and diluted 1:1 with sample buffer (Bio-Rad, Hercules, USA, product # 161-0737) containing β mercapthoethanol. Samples were further denatured by heating for 5 minutes at 100^{o}C prior to loading onto the gel. 10 well 4 – 20% precast gels (Thermo scientific, Rockford, USA, product # 25204) were used for SDS PAGE analysis. Tris-HEPES-SDS Running buffer (Thermo scientific, Rockford, USA, prod # 28398) was used as the electrophoresis buffer. A broad protein molecular weight marker (Takara, 3-4-1, Otsu, Shiga 520-2193, Japan, product # 3452) was used for the analysis. Samples were loaded at 5 µg/ml, and the gel was run for 3 hours at 21 mV. The gel was stained using Gelcode blue safe protein stain (Thermo scientific, Rockford, USA, product # 1860957) for 1 hour, and de-stained with deionised water for 16 hours.

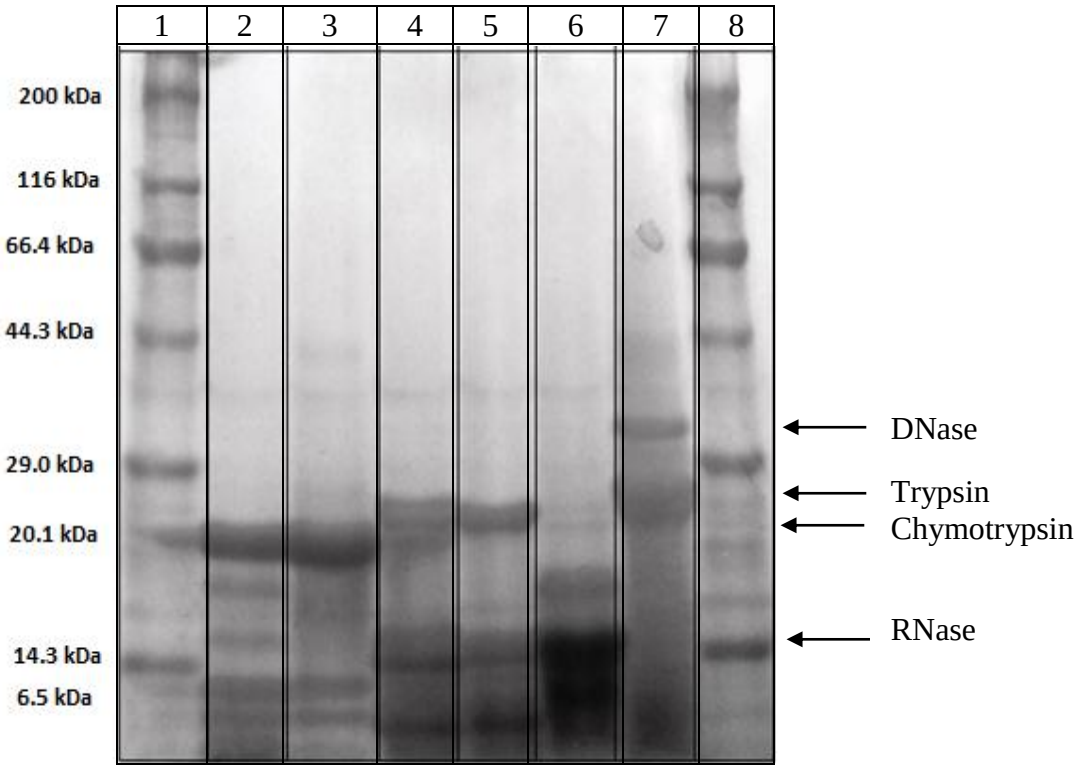

Figure 9: SDS PAGE analysis of all the products produced by the traditional processing method, trypsin and chymotrypsin as compared to an industrial standard (Sigma). All samples were prepared as 10 mg/ml protein, and 8 µl of sample was loaded per well. *Lane 1:* Molecular weight marker, *Lane 2:* BBI trypsin, *Lane 3*: Sigma trypsin, *Lane 4*: BBI chymotrypsin, *Lane 5*: Sigma chymotrypsin, *Lane 6:* BBI ribonuclease, *Lane 7:* BBI deoxyribonuclease, *Lane 8:* Molecular weight marker.

The final product is released after testing according to a customer specification. The tests used to qualify the products are listed as appendices at the end of the document. The trypsin and chymotrypsin, prepared by the traditional methodologies, complied with the specifications. These were again used to set a benchmark for the products to be produced by the newly developed method.

Table 2. Lyophilized chymotrypsin Quality Control analysis of product produced by traditional processing methodologies according to product specification.

Aspect	Description	Units	Limits	L12260AC	Method*
Description	Physical form as per specification	N/A	**As per specification**	White buff powder	Visual
Activity	Chymotrypsin	µkatal/mg	**>5.4**	6.2502	APPENDIX 2
	Chymotrypsin	NFU/mg	**≤1307**	1691.5	APPENDIX 3
	Trypsin BP	%	**<1**	< 1	APPENDIX 4
Additional Data	Moisture (4hours @ 60 °C)	%	**≤5**	0.3	APPENDIX 5
	Sulphated Ash	%	**≤2.5**	0.4	APPENDIX 6
	pH in distilled H_2O (10 mg/ml)	N/A	**3.0-5.0**	3.1	APPENDIX 7
	Absorbance	281 nm	**18.5-22.5**	18.791	APPENDIX 8
	Absorbance	250 nm	**<8**	6.83	APPENDIX 8
	Opalescence	N/A	**≤Soln 11**	between I & II	APPENDIX 9
	Enzymatic Activity A	%	**Reddish**	red colour	APPENDIX 10
	Enzymatic Activity B	%	**No colour**	no red colour	APPENDIX 10
	Trypsin Identification	N/A	**No colour**	no colour	APPENDIX 11
	Trypsin	NFU/mg	**Record**	22.6	APPENDIX 12
Solubility	Distilled H_2O	10 mg/ml	**Soluble**	Soluble	APPENDIX 13
Microbiological Data	Total Aerobic Microbial Count	cfu/g	**≤1000**	85	APPENDIX 14
	Total Combined Yeast & Mould	cfu/g	**<100**	<10	APPENDIX 15
	Salmonella	cfu/10g	**0**	0	APPENDIX 16
	Pseudomonas aeruginosa	cfu/g	**0**	0	APPENDIX 17
	Staphylococcus aureus	cfu/g	**0**	0	APPENDIX 18

Table 3. Lyophilized Trypsin Quality Control analysis of product produced from by traditional processing methodologies according to product specification.

Aspect	Description	Units	Limits	1031	Methods*
Description	Physical form as per specification	N/A	Buff coloured powder	Buff coloured powder	Visual
Activity	Trypsin	NFU/mg	>3000	3946	APPENDIX 12
	Trypsin	µKatal/mg	≥0.831	1.03	APPENDIX 19
Additional Data	Trypsin Identification A	N/A	Purple	Purple	APPENDIX 20
	Trypsin Identification B	N/A	No colour	No Colour	APPENDIX 20
	Chymotrypsin	pH	>Reference	Complies	APPENDIX 21
	Loss on drying (0.5 g for 2 hrs @60°C)	%m/m	≤5.0	1.00	APPENDIX 23
	pH (10 mg/ml)	N/A	3.0 – 6.0	3.3	APPENDIX 7
	Absorption 280 nm	N/A	13.5-16.5	15.2	APPENDIX 22
	Absorption 250 nm	N/A	≤7.0	5.2	APPENDIX 22
	Opalescence (0.10 g in 10 ml water)	N/A	≥ Ref Sol II	Similar to Ref I	APPENDIX 9
Microbiological Data	*Salmonella*	Count/10g	0	0	APPENDIX 16
	E. coli	Count/g	0	0	APPENDIX 24
	Total Aerobic Microbial Count	cfu/g	≤1000	5	APPENDIX 14
Solubility	Distilled water	10 mg/ml	Sparingly soluble	Soluble	APPENDIX 13

*All assay methods are given in Appendices.

This chapter described the traditional processing methodologies used to purify trypsin and chymotrypsin. The methods used were considered out-dated and were unable to consistently deliver high yielding products. This was because of the inefficiencies observed during the manufacturing processes. These inefficiencies include the inefficient extraction where all the enzymes were not extracted, the inaccurate A/S precipitation and pH measurements and inefficient crystallization of both CTG and trypsin. These inefficiencies were investigated, and improved. New technologies were also considered to reduce the overall production time. Chapter 4 describes the work done to improve each one of the inefficiencies identified, and subsequently, in Chapter 5, new techniques and methods considered to purify trypsin and chymotrypsin are discussed.

CHAPTER 4

4. RE-ENGINEERING A NEW PROCESS FOR PURIFICATION OF BOVINE TRYPSIN AND CHYMOTRYPSIN

From the discussion in Chapter 3 it is evident that the traditional methods followed at BBI enzymes for the production of trypsin and chymotrypsin were inefficient and not economically sustainable. The discussions in this section will therefore focus on the development of more efficient and effective isolation and purification techniques which could be up scaled and implemented in a large scale production facility. The aim of all of these method improvements was to increase the process yields obtained at lyophilized stage, and to reduce production costs by reducing raw material input costs and the total production time. Therefore each experiment executed and described in this chapter was focussed on either yield improvement or cost reduction.

4.1 IMPROVED EXTRACTION OF PANCREATIC PROTEASES

In section 3.3.1, the inefficiencies in the extraction of trypsin and chymotrypsin from the pancreas were highlighted. In summary, the methodology used was time consuming, and did not allow for optimal protein extraction (determined by A_{280} measurements). In this section the methodologies applied to investigate the inefficient process, and the work done to improve the extraction methodology to increase the extraction efficiency and allow for maximal liberation of enzymes at the onset of the process are explained. The aim of these trials were to increase overall process efficiency and process yields.

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4.1.1. MATERIALS AND METHODS

To substantiate the claims that not all the enzymes were extracted during the flaking / dunking process, a simple comparison study was carried out where an extraction sample from the factory floor was compared with a sample generated in the laboratory. The laboratory sample was prepared by collecting flaked pancreas from the factory flaker and was further macerated by blending it in a Hamilton Beach commercial 1L blender. Acidified potable water (pH 2.0,

acidified with 2.5 M H_2SO_4) was used to facilitate the blending process. The solid: liquid ratio was kept consistent with that of the factory process as described in the master batch record (MBR) as 1:2 (solid: liquid). 1 kg of flaked pancreas was blended with a desktop blender in 2 L of acidified water for 3 minutes. After the pancreas was blended, the pulp was extracted by continuous stirring using a top entry mixer for 12 hours. The A_{280} content (as described on page 19) was determined for both samples at the end of the extraction process.

Following this small scale comparison, a simple comparative study was carried out in the factory. Twenty batches were extracted using two different extraction methods. Ten batches were extracted according to the traditional processing method (flaking, as described in section 3.1), and ten batches were minced through a frozen tissue mincer housing a 10 mm and a 8 mm hole plate and extracted by continuous stirring of the extraction medium (see figure 10 for the difference between pancreas that has been macerated by a mincer and those that were macerated using a flaker).

A **B**

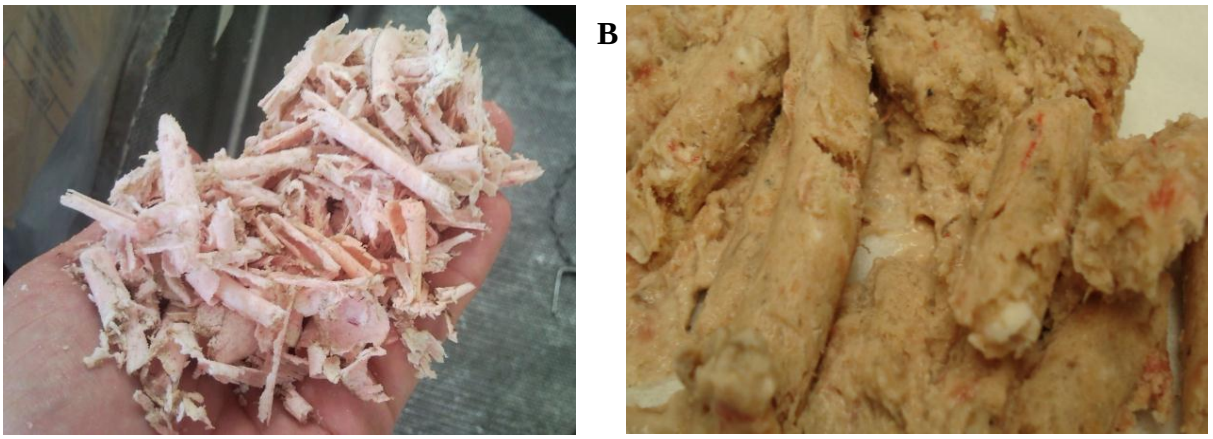


Figure 10. Difference between the two maceration methods. The pancreas flakes (*A)* were very thin, and were not uniform as large lumps of tissue were still present after the mincing. Flaked pancreas were collected and minced through a 10 mm and an 8 mm hole-plate. The texture of the mince obtained *(B)* was very fine and consistent compared to the flaked tissue *(A)*, and the particle size was uniform. No lumps of tissue were found after mincing.

The total A_{280} and total amount of trypsin (total trypsin content was determined by activity measurements as described in chapter 6.) were quantified following an overnight (16 hours)

extraction. The two extraction methods were executed in two different factories with two different extraction tank configurations (see figure 11 for the difference between the two extraction methods and the different tanks used during this investigation).

The two extraction methods differed in that the flaked tissue was flaked into a basket that was dunked into the extraction medium, while the minced tissue was added to an extraction medium, and stirred continuously. Both samples were acidified using 2.5 M H_2SO_4 to adjust the pH of the solution to 1.8 – 2.2. pH measurements were conducted using portable Crison pH 25 pH meters.

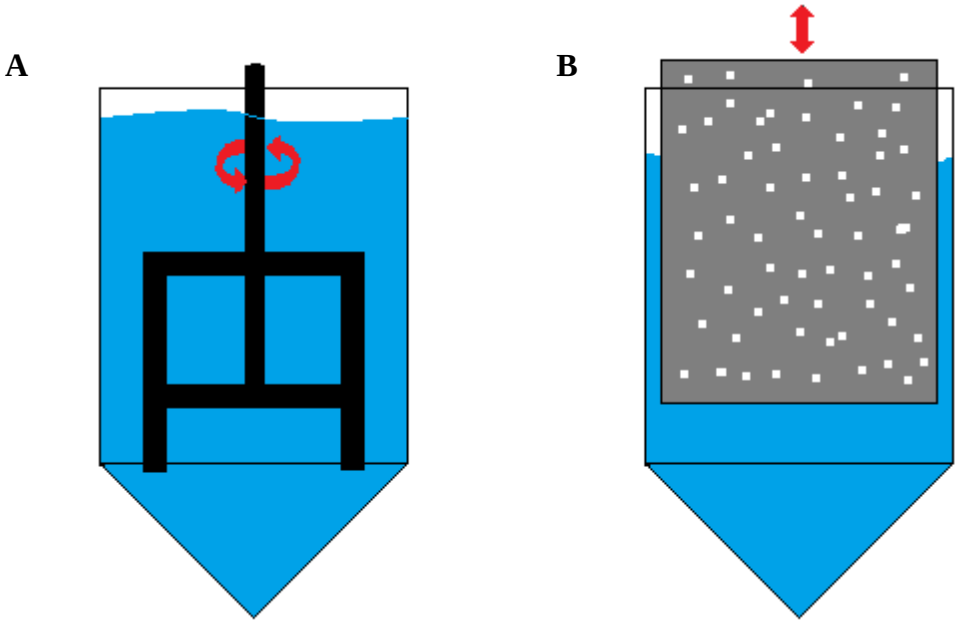


Figure 11. Schematic presentation of the two different extraction methodologies compared. (A) Represents the method whereby minced tissue was stirred vigorously and (B) represents the method whereby flaked tissue was dunked into a basket which was submerged into the extraction medium.

The use of $CaCl_2$ was also questioned in the extraction medium. The aim of the calcium was to stabilize trypsin, and to facilitate the prevention of the activation thereof. The problem this posed was the formation of insoluble $CaSO_4$ in the presence of A/S as shown in the following reaction:

$$CaCl_2 + (NH_4)_2SO_4 \rightarrow (NH_4)_2Cl_2 + CaSO_4\downarrow$$

4.1.2. RESULTS

4.1.2.1. THE USE OF $CaCl_2$ IN THE EXTRACTION MEDIUM

The addition of $CaCl_2$ to the extraction medium did not have any additional advantages compared to a batch that did not contain $CaCl_2$ at the extraction stage. This claim was supported by the fact that there was no difference between the qualities of the final lyophilized material delivered by the two extraction methods (based on final lyophilized product QC analysis carried out by the QC department). The quantity of the material delivered by the mincing method (without $CaCl_2$) was more than that delivered by the flaking method (with $CaCl_2$) i.e. the 40/65 intermediate precipitate weight was more, and the final lyophilized weight of both trypsin and chymotrypsin was higher (see table 4 and 5).

This study supported the claim made in section 3.4.1 that the dunking method supplemented with $CaCl_2$ was the inferior method for the extraction of the proteases.

4.1.2.2. LABORATORY SCALE INVESTIGATION

To investigate the efficiency of the dunking extraction method compared to a method where the pancreas were further (and better) macerated, the two samples were processed separately and the protein content measured at the end of the 16 hour extraction. See Table 4 below for the results.

Table 4. Comparison of the total protein content of the two different extraction methods for the same batch. Flaking referrers to the production scale batch and mincing referrers to the laboratory scale extraction using the same raw material as the flaking method (with additional maceration by means of blending in a desktop blender). (n=3)[2]

Method	Average total A_{280} at the end of extraction
Flaking method including $CaCl_2$	29.60 (±6.7)
Mincing method excluding $CaCl_2$	45.31(±5.5)

The additional maceration of the pancreas clearly had an effect on the extraction efficiency, as more protein was extracted at the end of the 16 hour extraction when compared with the production scale batch.

[2] "n" refers to the total amount of repeats of each experiment.

4.1.2.3. PLANT SCALE INVESTIGATION

Following these laboratory trials, further production trials were conducted to investigate these findings on a larger scale. The A_{280} of twenty in-process batches was compared at the extraction stage, ten of which were extracted using the flaker method and ten of which were extracted using a mincer fitted with a 10 mm and an 8 mm hole-plate, and continuously stirring the extraction mixture. The 40/65 intermediate precipitate weight was also used as an indication of the recovery of proteases at the end of the primary purification stage. The final lyophilised products were used to compare the two extraction methods. The results of this experiment is summarised in table 5 below. The flaking method included $CaCl_2$ in the extraction, and the mincing method excluded $CaCl_2$ from the extraction process.

Table 5. Summary of the results where two different extraction methods were compared. The traditional flaking method compared with a mincing method where minced tissue was extracted by stirring the quoted values is averages of ten batches of each extraction method. (n=10)[3]

Method	A_{280} at extraction	40/64 precipitate weight (Kg)	Final trypsin weight (Kg/t)	Final chymotrypsin weight (Kg/t)
Flaking	30.66 (±6.8)	35.5 (±5.6)	0.7 (±1.1)	1.40 (±1.0)
Mincing	40.51(±5.5)	41.3 (±6.1)	0.82 (±0.9)	1.92 (±0.8)

4.1.3. CONCLUSION

Major differences were observed between the two extraction methods. The protein content of the minced pancreas at the end of the extraction was higher than what was observed for the flaking method, indicating increased protein liberation as a result of improved cell maceration. This was supported by other findings in the process. The protease precipitate (40/65% precipitate) increased in weight from 35.5 –41.3 kg precipitate per 1.4 ton batch, which indicated that at this stage of the process, more proteases or protein were present.

Other areas where increases were observed (indicating improved liberation of enzymes) when the improved mincing method was implemented:

- CTG crystal weight (after Zymogen separation) increased from 30 to 40 kg per batch.
- Total amount of chymotrypsin after the activation stage increased from 12 to 14 BU.

[3] "n" refers to the total amount of individual batches monitored for each extraction method.

- Total amount of trypsin after the trypsin activation increased from 29 to 36 BU.
- Lyophilized weights of both trypsin and chymotrypsin increased with approximately 20%.
    - Trypsin (increased from 0.7 kg/t to 0.82 kg/t)
    - Chymotrypsin (increased from 1.4 kg/t to 1.92 kg/t)

After these trials were completed, it was recommended that the use of the flaker and baskets be terminated and that extraction of pancreas was to be carried out using a mincer and a stirring extraction mixture (as described in this section above).

4.2 OPTIMIZED CLARIFICATION TECHNIQUES

The aim of these investigations was to improve processing efficiency. The sole purpose of this processing step was to ensure that the clarity of the liquid extract prior to ultrafiltration was high, which was fundamental for optimal performance of the ultrafiltration system and to increase the lifespan of the membranes installed on the unit.

The clarification process started as early as the decanting step in the process. The removal of the bulk of the tissue debris, fat and the insoluble particles was achieved by a single machine.

During the traditional processing method, this process step was achieved by removal of the perforated baskets from the extraction medium, resulting in large pieces of tissue passing through to the rest of the process, and removal of fat content from the extraction liquid.

Centrifugal separation of solids and insoluble material was considered as clarification mechanisms in the process. The advantage of centrifugal separation was that there are no additional pre-treatment of the product required, compared to a series of filtration steps where the liquid needs to be prepared by adding diatomaceous earth to the liquid to assist the filtration process. See figure 12 for an illustration of the inside of a clarifying centrifuge.

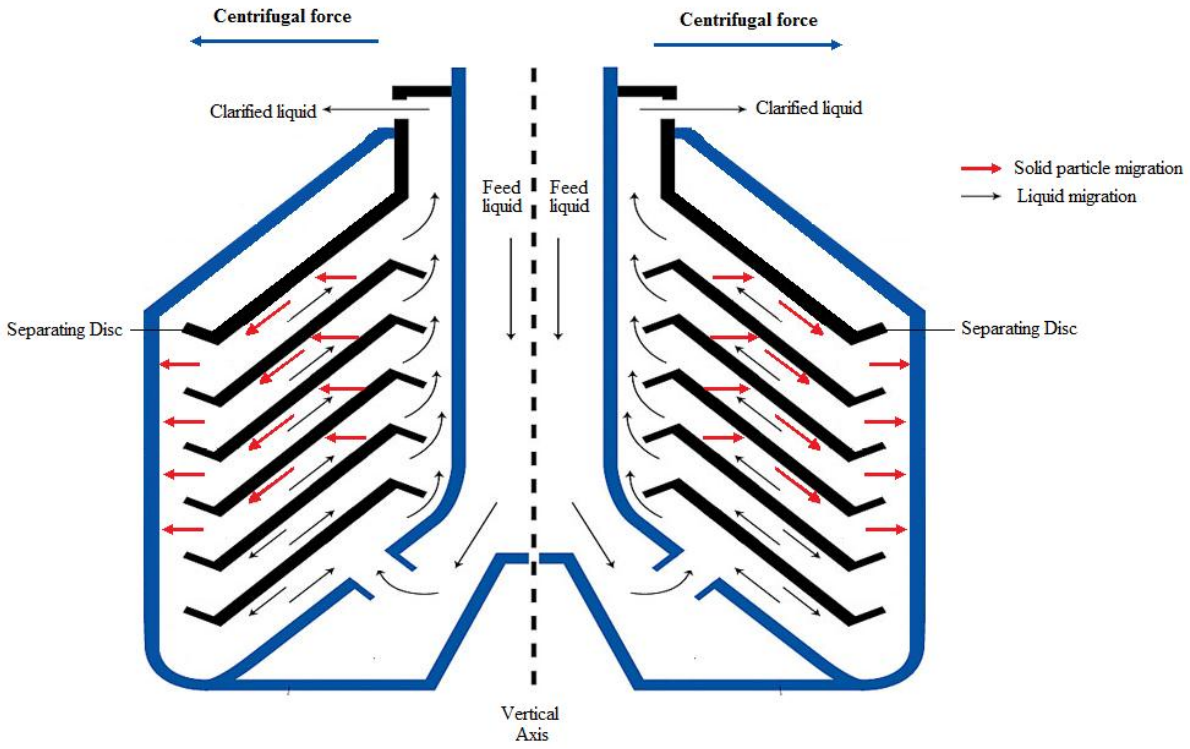


Figure 12. Principle of solid removal by centrifugal force using a stacked disc centrifuge. Stacked discs increased the settling area for the solids, and enhanced the solid separation.

Continuous flow centrifuges, capable of removing large quantities of solids from most liquid sources, have wide spread applications ranging from the wine industry to sewerage treatment. During continuous flow centrifugation the feed material is fed into the separator at a certain rate. Because the rotation speed of the bowl remains constant, it is important that the feeding rate of the liquid into the machine is controlled. The time that the liquid spends inside the bowl of the centrifuge, before it leaves the outlet ports, is termed the "dwell time". The longer the dwell time of any given particle inside the centrifuge, the higher the probability that the solid will be removed by centrifugal force. A two stage centrifugal separation was envisaged where stage one would be removal of the bulk of the tissue extract, using an industrial decanter (horizontal axis centrifuge), and stage two would entail the removal of fine insoluble particles and fats using a stacked disc centrifuge.

The solid content specification (v/v) of the input material into the second centrifuge was 1.5% (v/v) solids. This solid content of the liquid was determined by "spin tests" using calibrated centrifuge tubes and a desktop centrifuge. A 10 ml sample was spun in a calibrated centrifuge tube at 13000 RPM for 5 minutes to separate solids from the liquid phase, and to determine the solid content (%) of the liquid material. This implied that the solid content specification of

the output material from the decanter needed to be ≤1.5% to justify the installation of a high speed disc centrifuge in series with the decanter for optimal solids removal.

A decanter is a continuous flow horizontal axis centrifuge used to remove the bulk of the solids from a liquid suspension (can process liquid with a solid content of up to 70% solids). A stacked disc centrifuge is a continuous flow centrifuge used to remove insoluble particles from a liquid suspension. This is a vertical axis centrifuge that uses a slightly different application of centrifugal force to separate solids from liquid phase. A disc stack centrifuge separates solids from liquids in a single continuous process, using extremely high centrifugal forces. As the liquid is fed into the disc centrifuge, dense solids are subjected to the extremely high centrifugal force generated by the rotating bowl, and are forced outward against the rotating bowl of the centrifuge whilst the less dense liquid phase form concentric inner layers around the vertical axis. By inserting special plates (the "disc stack") this provides additional surface settling area for the solids, which contributes to speeding up the separation process dramatically.

The use of the high speed disc centrifuge was initially considered to completely remove the diatomaceous earth filtration step. This was, however, not possible, as the fatty nature of the material prevented the disc centrifuge to completely remove all the solids to a clarity specification of <5 Formazin Turbidity Units (FTU's). The installation of a disc centrifuge was then considered to reduce the workload placed on the downstream clarification equipment.

After considering two of the world's leading manufacturers of centrifuges, Alfa Laval and Westfalia, both companies made their test units available to test the performance of the machines on bovine pancreas. An Alfa Laval NX 418 decanter and a fully automated Westfalia XSC 15-06-177 disc centrifuge were purchased as a secondary centrifuge installed in series with the Alfa Laval decanter disc centrifuge. The reason for the purchase of these units from two different suppliers, rather than from a single supplier, was the cost benefits and the value for money each of the different machines offered. The budget for this project also did not allow the purchase of both units from the same supplier as the decanter from Westfalia and the disc centrifuge from Alva Laval were very expensive units.

4.2.1 ALFA LAVAL DECANTER CENTRIFUGE OPTIMIZATION

The Alfa Laval decanter was a semi-automated unit. Once the machine was started, the bowl would spin at a constant speed (3250 rpm) for the duration of the production run. There were two variables that could be changed to optimize the performance of the unit. The optimal performance was achieved when the solids discharged from the machine had a very low liquid content, and when the solid content of the output material was ≤ 1.5%.

The first parameter that could be changed was the rate at which product was fed into the machine. The second parameter was the "pond depth" which is the level of liquid retained within the bowl of the centrifuge whilst in rotation. The pond depth was controlled by discs placed over the outlet ports of the decanter. The deeper the pond depth, the longer the dwell time of the liquid inside the machine, which resulted in a cleaner liquid output.

The machine was capable of delivering a maximum water flux of 10 000 L/h when operated at optimal conditions. A cautious approach was taken when assessing the performance of this unit on a pancreas extract. To test the performance of this unit when pancreas extract was used, the feed pump was set to deliver 5000 L/h as a starting point to the optimization, and the solid content of the output material was measured. The machine was capable of delivering 10 000 L/h with pure water, the product optimization trials were started at 50% of the maximum). Throughput was another important consideration that needed to be considered when these trials were executed. For this machine to be a suitable replacement for the traditional methodologies, a minimum feeding rate of 1000 L/h was set.

Following a series of trials on process equipment, it was concluded that a feeding rate of 1200 L/h yielded optimal performance, which resulted in a dry solids discharge and a supernatant with a solid content (v/v) of 1.3 %.

4.2.2 DISC CENTRIFUGE OPTIMIZATION

Following the optimization of the decanter, it was clear that a solid content of <1.5% was achievable with the Alfa Laval decanter.

The aim of the disc centrifuge optimization was to reduce the solids and fat content to as low as possible and to yield a liquid product that was clear and contained no fats. The bowl of the disc centrifuge rotated around a vertical axis at a constant speed of (12000 RPM / 24000 g). The same principle that applied to the decanter centrifuge regarding the dwell time of the

product inside the bowl of the centrifuge, applied to the disc centrifuge where a longer dwell time of the particles in the bowl resulted in a higher probability of removal by centrifugal force. The disc centrifuge had a maximum water capacity of approximately 10 000 L/h.

A second variable with this machine was the time interval between solid discharges. A discharge is when the bowl periphery was cleared form the build-up of solids by a jet of high pressure water. The more frequent the discharges, the lower the risk of these accumulated particles to be re-introduced into the liquid.

The optimization process was started using a feeding rate (50% of maximum capacity) of 5000 L/h and a discharge interval of 10 minutes. The clarity of the product was not sufficient (did not comply with the clarity specification of < 5FTU's), and caused the filter press to block up immediately. This meant that not sufficient fines/fats were removed from the liquid. The discharge time was investigated, and it was found that a more frequent discharge time improved the clarity of the liquid after centrifugation. Three different discharge times were investigated (3, 5 and 10 minute intervals).

It was found that a three minute discharge time resulted in the clearest liquid. The feeding rate was also investigated. The slower the feeding rate, the longer the retention time inside the bowl of the centrifuge, which in turn would mean a higher probability that the solid particles would be removed by centrifugal force. The feeding rate was reduced to 1200 L/h. The discharge interval was further reduced to every 1 minute. This was all controlled automatically from a central control panel. The best performance was achieved when the solid content was reduced from 1.5% to 0.5%.

It was concluded that the stacked disc centrifuge would not be able to yield product that would comply with the final clarity specification of <5 FTU's, but would reduce the burden placed on the downstream clarification equipment but would increase the production time. Following the two stage centrifugal separation and a single pass through a filtration system utilizing diatomaceous earth as a filter aid, a very clear supernatant was consistently achieved that met the specification.

4.2.3 CONCLUSION

Proper clarification techniques were identified to improve the process efficiency and to improve the quality of the product being applied to the ultrafilter. Two different centrifuges were installed in series, an Alpha Laval NX 418 Decanter to remove extracted tissue debris, and a Westfalia high speed disc centrifuge to remove small particles and fats.

The installation of these two centrifuges ensured a consistent product to be supplied to the clarification equipment (cloth filter press) and reduced the load placed on these machines. The processing time was increased with 2 hours per batch as a result of the installation of these machines.

4.3 INVESTIGATING DIFFERENT CRYSTALLIZATION CONDITIONS FOR CHYMOTRYPSINOGEN AND TRYPSIN

4.3.1. INTRODUCTION

The aim of this investigation was to improve the protein crystallization conditions, and ultimately increase the overall process yields of trypsin, chymotrypsin and CTG.

Protein crystallization, still one of the best methods of protein purification (Chayen, 2004), is a process whereby solid protein crystals are formed from a homogeneous protein solution. Protein crystal formation is initiated by the process called nucleation, which is the formation of clusters of molecules that display a high degree of structural order. Nucleation is an important key to the initiation of crystallization, and controls the structure of the crystallizing phase and the number of particles appearing in a crystallization system (Garcia-Ruez, 2003).

Supersaturation, a state in which a solution contains more protein molecules than would normally dissolve, should be achieved for the initialization of crystallization. Increasing concentration of a precipitant increases the saturation state of a solution until it reaches a supersaturation point. Most commonly used precipitants are salts, organic polymers and alcohols. During this study A/S was used to bring the solution to a state of supersaturation (Chayen, 2004). The thermodynamics of a supersaturated solution will dictate that the solution must return to equilibrium by segregating a solid phase until equilibrium is achieved.

Once the supersaturation point is exceeded, spontaneous precipitation of proteins will occur. (Garcia-Ruez, 2003)

As explained by Garcia-Ruez *et al* (2003), proteins (being monomers or oligomers) freely moving in a solution can be described as individual growth units. These growth units will form the constituents of a growing crystal by aggregating in an ordered cluster (see figure 13). The probability of these clusters being dissolved is governed by the external forces that influence it. These parameters include the degree of supersaturation, the temperature, pH and concentration of precipitant in the solution. These clusters (also named the nucleus of critical size) have an equal probability of being dissolved as of growth. An energetic barrier (ΔG*) must be exceeded for stable nuclei to form.

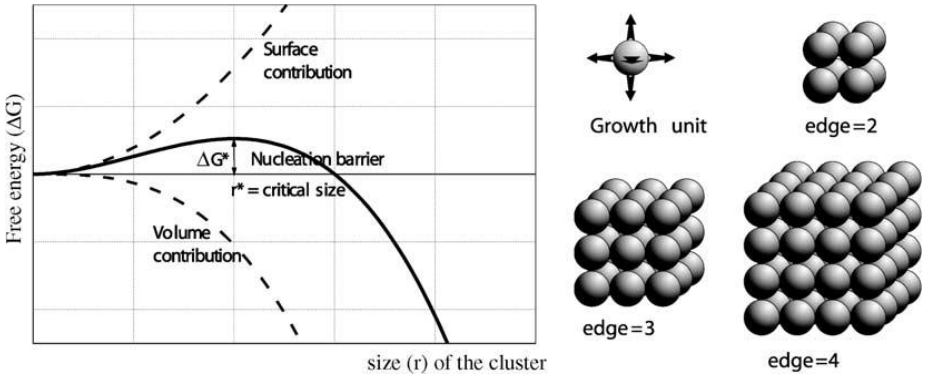


Figure 13. The principle of protein crystal formation. The onset of protein crystallization is the formation of nuclei. For stable nuclei to form, a threshold energy barrier needs to be exceeded (Garcia-Ruez, 2003).

Protein crystallization is a process whereby proteins undergo phase transition(s). This can be illustrated by a phase diagram (see figure 14) which indicates the different states or phases a protein would be stable in under a variety of parameters. These states include the liquid, crystalline or amorphous solid (precipitate) states. A crystallization phase diagram is categorized into four zones, each representing a different degree of supersaturation. These zones are (1) *High supersaturation* in which proteins will precipitate, (2) *moderate supersaturation*, in which spontaneous nucleation will occur, (3) *the Metastable zone*, which is of lower supersaturation where crystals are stable and they can grow (the conditions in this zone ate optimal for crystal growth, not for nucleation). The last (4^{th}) zone is the zone of *under saturation* in which a protein is fully dissolved, and will never crystallize.

The degree of supersaturation can be varied by controlling the concentration of protein and precipitant (salt), the pH and temperature. During a crystallization experiment, the conditions should always promote nucleation first, followed by a condition that would promote protein growth for the duration of the crystallization, or until the maximal amount of crystals have been achieved. To achieve this, the protein solution is undersaturated with precipitant in the beginning of the experiment, and is gradually increased until it reaches a state of supersaturation by the addition of crystallizing agents throughout the duration of the experiment. Seeding is a process whereby nucleation of the specific protein is facilitated by the addition of crystals of the specific protein of interest into the solution under metastable conditions.

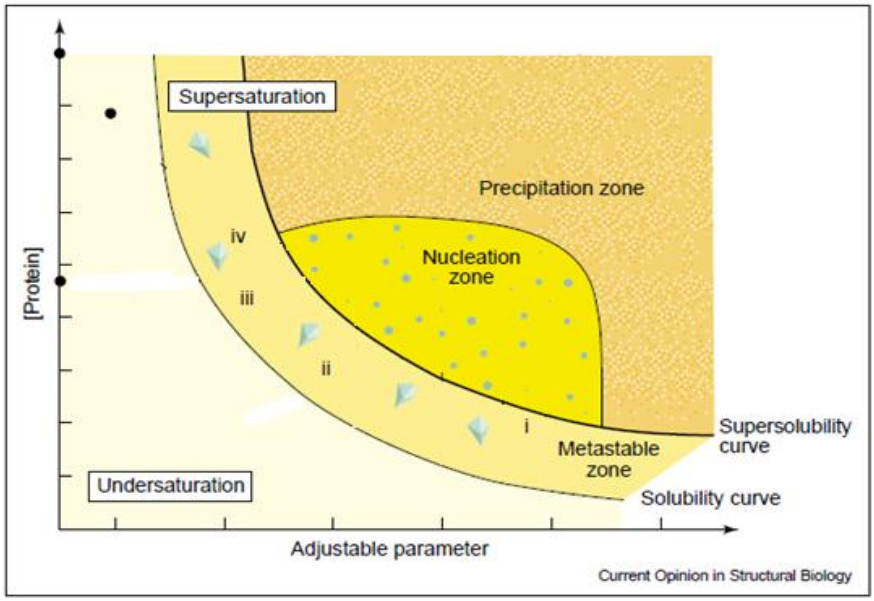


Figure 14. Protein phase diagram indicating the four different phases, (1) high supersaturation, (2) moderate supersaturation, (3) metastable zone and (4) under-saturation. The adjustable parameters can be concentration of precipitant, pH or temperature (Chayen, 2004).

4.3.2. CRYSTALLIZATION AT BBI ENZYMES

The crystallization techniques used at BBI Enzymes were based on the original work of Kunitz *et al*. (1934), but the methods used at BBI Enzymes have changed over the years and resulted in poor product recovery. The crystallization conditions and the ability to maintain a metastable phase were questioned and a new way to facilitate crystallization was described.

For both CTG and trypsin, the addition of saturated A/S during the duration of the crystallization process was investigated to facilitate crystal growth and to maintain a stable metastable protein growth phase for the duration of the crystallization. As highlighted in section 3.3.4 and 3.3.5, the conditions for protein crystallization were adequate to induce crystallization. They were, however, not sustainable for the duration of the crystallization process as a decreased crystallization efficiency during the latter phases of crystallization was observed. Crystal mass was measured throughout the crystallization process.

4.3.3. CHYMOTRYPSINOGEN CRYSTALLIZATION

The Zymogen separation

Trypsinogen and CTG are the two zymogens extracted from acid treated pancreas and from which the active forms trypsin and chymotrypsin respectively are produced. The two zymogens cannot be separated by simple A/S precipitation, a technique used to separate the zymogens from DNase and RNase. Instead, zymogens are separated from each other by a crystallization process.

As mentioned earlier, protein crystallization is an extremely delicate process and success depends on optimization of a number of factors. These factors include optimal temperature, pH, concentration of contaminants, high protein concentration, and optimal precipitant concentration. The zymogen separation was performed at the optimal temperature range of 22-25^{o}C, pH 4.9-5.2 in a highly concentrated protein solution with an A_{280} of more than 240 (see note on page 21 for the determination of the A_{280} value). There were insignificant amounts of contaminants as the two zymogens were the predominant proteins in solution at this stage.

For the zymogen separation, all the conditions mentioned above had been optimized except for the concentration of the precipitant. The precipitant of choice for the zymogen separation was A/S and, for the traditional process, it was estimated to be at 33-35% saturation. This saturation point did not appear to be optimal for maximum CTG crystallization because high amounts of chymotrypsin were still observed in trypsin samples. As a result, this study was conducted to determine the optimum A/S saturation during the zymogen separation in order to obtain optimal CTG crystallization (see section 4.3.3), a process referred to as A/S saturation.

The success criterion of this study was to increase the yields (final lyophilized weight) of trypsin, chymotrypsin and CTG (see table 6) without decreasing their specific activities as set out in the product specification. An efficient zymogen separation means less chymotrypsin contamination in trypsin samples, therefore an additional objective was to achieve at least a 6:1 trypsin to chymotrypsin ratio at the end of trypsin activation stage.

Table 6: Yields of pancreatic derived factors (enzymes) affected by zymogen separation.

Product	**Yield (March 2010) (kg /ton*)**	**Target Yields (kg/ton)**	**Specific Activity (U/mg) (USP specification.)**
Trypsin	0.7	**1.4**	2500 (≤50 Chymotrypsin)
Chymotrypsin	1.4	**2.8**	1250 (≤1% Trypsin)
Chymotrypsinogen	1.4	**2.8**	1200 (Potential Chymotrypsin activity)

*Raw Material Input

The yields of enzymes before A/S saturation of the zymogen separation was introduced are reflected in table 6 above. The A/S saturation exercise was aimed at producing an efficient zymogen separation, which would directly increase the yields of chymotrypsin and CTG and indirectly increase the yield of trypsin, by reducing chymotrypsin content in the trypsin crystallization mixture, as chymotrypsin is considered a contaminant during the trypsin crystallization stage.

The principle of A/S saturation during crystallization step was proven successfully during the experiments conducted with the trypsin crystallization (see section 4.3.4). The work carried out on the CTG A/S saturation was conducted on in-process material in accordance with the company's change control procedure. Three independent production batches were used in a trial to evaluate the effect of A/S saturation during the zymogen separation. Any single batch can only be processed to either CTG or chymotrypsin. CTG and chymotrypsin can never be produced from the same batch. This is because CTG is activated to chymotrypsin, and the material is always fully activated. The first batch was processed to CTG, and the last two batches were both processed to chymotrypsin. The trypsin portion of each batch was processed to trypsin. Table 7 gives a summary of the final products produced from each production batch. The company sales forecast determined whether the CTG was purified further to chymotrypsin or purified as CTG.

Table 7: Batches affected by A/S saturation and products produced during the study

Batch	Final Product
Batch 1	Chymotrypsinogen /Trypsin
Batch 2	Chymotrypsin / Trypsin
Batch 3	Chymotrypsin / Trypsin

4.3.3.1 MATERIALS AND METHODS

Saturated A/S solution (759 g/L at 22^{o}C) was slowly added to the product with the use of a Seko PR-4 peristaltic pump feeding from a reservoir (feeding rate of 1 L/hr.). Because there was no rapid assay available at the time to determine the A/S concentration of a batch, a set of standards were prepared with different A/S concentrations with corresponding conductivity measurements. These standards were prepared by adding increasing amounts of solid A/S to a clarified 0/20 sample that was concentrated 10 fold to obtain a final $A2_{280}$ of 240. The samples were stirred continuously for 1 hour to allow all A/S to dissolve. After 1 hour, the samples were filtered using 0.45 µm syringe filters to remove any insoluble particles and the conductivity of the clear liquid was measured using a Eutech PC650 multimeter. See table 8 for a summary of the A/S concentrations with corresponding conductivity measurements. The conductivity of a solution is affected by the protein concentration. An increasing protein concentration will reduce the conductivity measurement. The standards were all prepared using product with a high protein concentration (A_{280} of 240 – 280, see footnote page 20), and a pH of 5. This is the protein concentration at the onset of the zymogen separation.

The concentration of A/S was monitored by conductivity measurements, using a Eutech PC650 multimeter, to ensure that the parameter being changed could be accurately monitored.

Table 8. Set of A/S standards and their corresponding a conductivity measurements.

% A/S saturation	Corresponding conductivity (milliSiemens)
30	80 – 90
35	100 – 110
40	120 – 125

At the end of the CTG crystallization step, the suspension was diluted with 40% (244 g/L at 22^{o}C) saturated A/S 1:1 (v/v), to facilitate better centrifugation, and centrifuged on a vertical axes centrifuge to harvest the crystals. The harvested crystals were washed with a 40% A/S solution and recovered by filtration through Viking filter paper on a coffin filter.

The CTG crystals were processed either to CTG or chymotrypsin (see table 7) while the combined supernatant was further processed to trypsin. The processing of CTG was performed as per the master batch record that describes CTG production (CTGP1, revision 11). Further trypsin processing was performed following normal trypsin protocol as described in the master batch record for trypsin production (TP 1, Revision 12).

Three different final conductivity measurements were investigated to see at which % A/S saturation the crystallization process was most effective. Table 9 gives a summary of the initial and final conductivity measurements for each one of the batches.

Table 9: Conductivities and volumes of saturated ammonium sulphate added

Batch	Starting Volume (L)	Volume (L) Sat. A/S added	Initial Conductivity (mS/cm)	Final Conductivity (mS/cm)
Batch 1	170	8.5	80.1	100
Batch 2	170	8.7	86	108
Batch 3	240	22.25	84	125

4.3.3.2 RESULTS

The outcome of these trials was determined by the lyophilized weights achieved for each of the batches. From the results presented in table 10, it was clear that the yield of the chymotrypsin(ogen) increased with increasing A/S saturation. The highest yield was achieved at a conductivity of 125 mS/cm for both chymotrypsin and trypsin. This indicated that when the decrease in protein concentration of the supernatant (as a result of protein crystallization) was compensated for by systematically increasing the A/S concentration of the supernatant, the crystallization efficiency was improved. The specific activity of all three trypsin batches was above specification. This implied that the implementation of A/S saturation did not affect the quality of the trypsin produced. The specific activity of the chymotrypsin produced (batch 2 and 3) also complied with the specification, indicating that the implementation of A/S saturation did not affect the quality of the final chymotrypsin product either.

Table 10. Yields and specific activities of CTG, trypsin and chymotrypsin as a result of varying final % A/S saturations.

Batch #	Product	Final Conductivity	Yield (kg/ton)	Yield Targets (kg/ton)	Specific Activity achieved (U/mg)	Target Specific Activity (U/mg)
Batch 1	Chymotrypsinogen	100 mS/cm	1.53	**2.8**	1268.7	**1250**
	Trypsin		1.00	**1.4**	4600.4	**2500**
Batch 2	Chymotrypsin	108 mS/cm	1.79	**2.8**	1650.4	**1250**
	Trypsin		1.1	**1.4**	4388.6	**2500**
Batch 3	Chymotrypsin	125 mS/cm	2.38	**2.8**	1751.4	**1250**
	Trypsin		1.13	**1.4**	4080.5	**2500**

Other than the yield of CTG/chymotrypsin, the efficiency of the zymogen separation was also judged by the amount of chymotrypsin found in trypsin at the end of the trypsin activation. The objective was to achieve a 6:1 ratio of trypsin to chymotrypsin after activation of trypsin. During the trypsin crystallization process, chymotrypsin is considered the biggest contaminant. The crystallization efficiency of trypsin would increase as the concentration of contaminating proteins (chymotrypsin) decreased. Improved CTG crystallization during the zymogen resulted in a higher trypsin: chymotrypsin ratio after completion of the trypsin activation. The target ratio was almost achieved in batch 2 and exceeded in batch 3, where the conductivity was at 125 mS/cm (see table 11).

Table 11: Total trypsin and chymotrypsin activities after completion of trypsin activation and the trypsin: chymotrypsin ratio achieved as an indication of the CTG crystallization efficiency.

Batch	Trypsin total activity ($x10^9$U)	Chymotrypsin total activity ($x10^9$U)	Trypsin/Chymotrypsin ratio
Batch 1	36.17	6.74	5.37
Batch 2	37.78	6.34	5.96
Batch 3	47.06	5.71	8.24

4.3.3.3 CONCLUSION

A/S saturation of the zymogen separation by the addition of saturated A/S increased the efficiency of CTG crystallization compared to the traditional crystallization method. The best results were obtained at the final conductivity of 125 mS/cm, where an 8:1 trypsin: chymotrypsin ratio was achieved at the completion of the trypsin activation stage. High yields of both trypsin and chymotrypsin were also achieved at a conductivity of 125 mS/cm, both achieving specific activities well above the BBI enzymes product specifications. This also confirmed the theory that a decreasing supernatant protein concentration, as a result of protein crystallization, can be compensated for by the addition of a precipitant (ammonium sulphate) to stimulate crystal growth. No further repetitions of this experiment were conducted, as the data was sufficient to prove that the A/S saturation was effective to increase the crystallization efficiency. Time constraints for this project also did not allow further research to be conducted on CTG crystallization. A/S saturation was implemented immediately with great success.

The recommendation following this study was:

1. That the zymogen separation should be conducted using a saturated A/S solution to a final conductivity of 125 mS/cm, and
2. That no second CTG crystallization step was to be performed as specified in the traditional processing method.

4.3.4. TRYPSIN CRYSTALLIZATION

Trypsin purification at BBI was executed following the master batch record TP1 (revision 11). Trypsin was crystallized at approximately 35% A/S saturation in the presence of magnesium sulphate. By using this procedure, approximately 0.7 kg/ton of lyophilized trypsin, with average specific activity of 3600 U/mg was produced. In order to meet the higher trypsin demands, it was important to optimize critical stages in this process. A critical concern with the traditional trypsin process was the inefficiency of trypsin crystallization. It was confirmed that approximately 60% of the total trypsin present at the onset of crystallization did not crystallize under the conditions described in TP (Rev 11). This was determined by quantifying the total trypsin before the onset of the crystallization, and comparing that to the total amount of trypsin recovered at the end of the trypsin crystallization. The mass balance of the trypsin that did not crystallize was found in the

recovery mother liquor (the supernatant of the trypsin crystallization). A different method for trypsin crystallization was therefore required.

According to TP1, (revision 11), ammonium sulphate salt was the precipitant of choice for trypsin crystallization at BBI at 35% saturation. It was however believed that at 35% saturation, the trypsin solution was only in the early metastable phase that only allows slow crystal growth. In an effort to increase the efficiency of the crystallization, a trial was conducted in which trypsin crystallization was performed by increasing the saturation of A/S until a labile supersaturation phase was achieved. At this phase rapid crystal initiation and crystal growth occurred, leading to efficient crystallization.

4.3.4.1 MATERIALS AND METHODS

The 75% trypsin precipitates (obtained after trypsin activation) from selected batches were dissolved in 0.4 M borate buffer, pH 9.0 in a 2: 1 (w/v) ratio (2 kg ppt in 1L buffer). The A/S concentrations of the solutions were reduced to 35% by diluting with 0.4 M borate buffer followed by addition of 1 M calcium chloride solution (20 ml/L), and the pH was adjusted to 7.0 using 1 M NaOH. The crystallization was initiated by seeding the product (90 g/L) with trypsin crystals from previous bathes.

After overnight incubation, when crystallization was observed, a saturated A/S solution (767 g/L) was added to the stirring solution slowly (0.5 L/h) using a Seko PR-4 peristaltic pump to increase the A/S saturation in the solutions from 35% to 40% or 45% and the crystallization was allowed to continue for 7 days. The crystals were recovered by filtration in a coffin filter and washed with a 40% saturated A/S solution to remove any entrained proteins. The washed crystals were re-dissolved in RO water and diafiltered until salt free in preparation for freeze drying using a 10 kDa PALL diafiltration system.

4.3.4.2 RESULTS

The 7 batches used for these trials were all crystallized as described in section 4.3.4.1 above, and the results of these trials are summarised in tables 12 – 14. Batch 1 and 2 both had a final % A/S saturation of 40%, whilst batches 3 – 7 had a final % A/S saturation of 45%.

Crystallization efficiency was calculated as the difference between the total trypsin before and after the crystallization remaining in the supernatant, expressed as a percentage. An increase in crystallization efficiency was observed at a higher final A/S saturation (45%). The highest total trypsin present at the onset of the trypsin crystallization had the best recovery of trypsin. This indicated that trypsin crystallization efficiency was also dependant on the total trypsin content relative to non-specific proteins. The higher the non-specific protein concentration, the lower the crystallization efficiency (see table 12).

Table 12: Trypsin crystallization efficiency of 7 consecutive batches was calculated to investigate the effect A/S saturation on trypsin crystallization.

Batch	Raw material input (Ton)	Final A/S saturation	Pre Crystal (Total units)	Post Crystal (Total units)	Crystallization Efficiency
Batch 1	1.33	40%	$16.4x10^9$	$6.4x10^9$	59.5%
Batch 2	2.8	40%	$20x10^9$	$8.34x10^9$	58.3%
Batch 3	4.2	45%	$32.29x10^9$	$6.898x10^9$	79%
Batch 4	4.2	45%	$37.57x10^9$	$12.68x10^9$	66%
Batch 5	4.2	45%	$36.43x10^9$	$13.75x10^9$	62%
Batch 6	4.2	45%	$49.59x10^9$	$6.68x10^9$	86.52
Batch 7	4.2	45%	$68.48x10^9$	$6.88x10^9$	90.2

*Post crystal activities refer to the activity of trypsin in the supernatant.

Crystallization efficiency was described as the total trypsin content of the supernatant (S/N) divided by the pre crystallization activity. The trypsin crystallization efficiency increased by up to 70% with increasing A/S saturation, which implied that more trypsin was crystallized.

The outcome of the trial was determined by the weight of the lyophilized product, and the specific activity (U/mg material) of the trypsin. Table 13 gives an overview of the final yield (kg of product/ton of raw material input) achieved for the trials.

Table 13: Trypsin Yields achieved for batches that underwent A/S saturation during the trypsin crystallization process.

Batch	Raw Material input (ton)	Final A/S sat.	Lyophilized weight (Kg)	Yield achieved (Kg/ton)	Target yield (Kg /ton)	Deficit
Batch 1	1.33	40%	0.94	0.71	**1.05**	49%
Batch 2	2.8	40%	2.28	0.81	**1.05**	34%
Batch 3	4.2	45%	3.63	0.86	**1.05**	27%
Batch 4	4.2	45%	4.05	0.96	**1.05**	9%
Batch 5	4.2	45%	3.93	0.94	**1.05**	10%
Batch 6	4.2	45%	4.58	1.09	**1.05**	-
Batch 7	4.2	45%	5.2	1.24	**1.05**	-

The higher crystallization efficiency translated in to higher trypsin yields achieved per ton of raw material (pancreas) input. Not only were the yields achieved higher than before the A/S saturation was introduced, the specific activity (see table 14) of the trypsin also increased dramatically as the efficiency of crystallization increased.

Table 14: Trypsin quality of the final lyophilized products. According to the specification, the trypsin specific activity should be > 2500 U/mg, and the chymotrypsin should be less than 50.

Batch	Trypsin Specific Activity (U/mg)	Chymotrypsin Specific Activity (U/mg)
Batch 1	4008	55.5
Batch 2	4163	10.8
Batch 3	3886	105.0
Batch 4	4202	24.0
Batch 5	4167	61.4
Batch 6	4112.6	8.78
Batch 7	4560.4	16.05

To prove that the trypsin quality was not compromised by increased A/S saturation during crystallization, a denaturing SDS PAGE was performed (see figure 15) to investigate if any additional proteins were co-purified as a result of the % A/S saturation.

The quality of the trypsin produced by the improved crystallization method was higher than the set trypsin specification of 3000 U/mg. There was no marked difference between the samples that underwent A/S saturation and the control. No additional protein bands were observed in these samples, indicating that the A/S saturation did not cause additional non-specific proteins to precipitate (see figure 15).

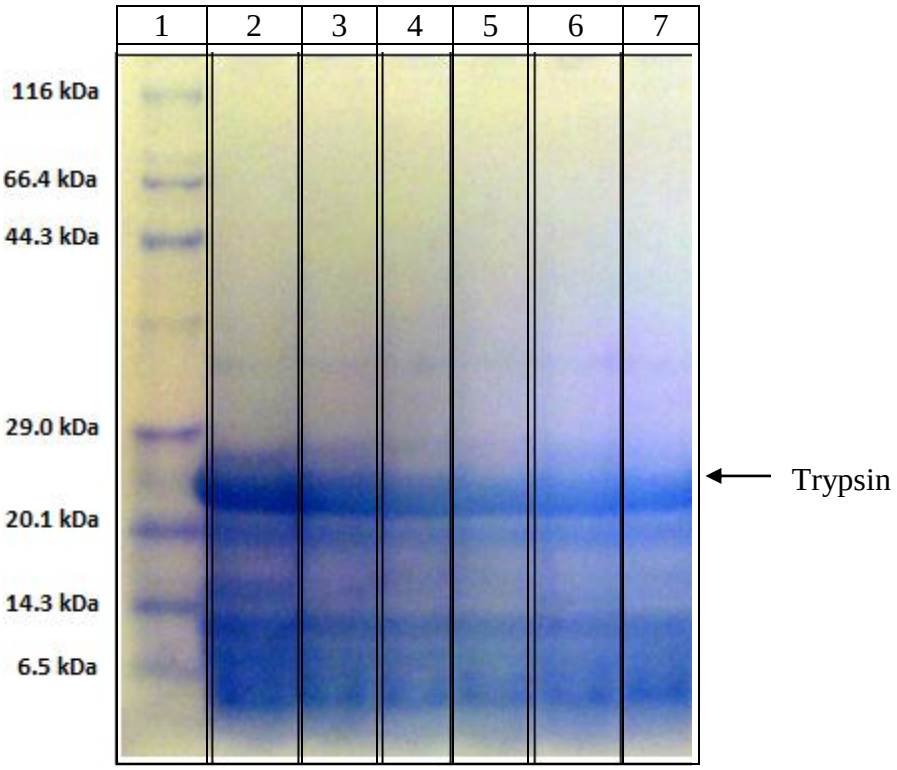


Figure 15: SDS PAGE of trypsin produced through A/S saturation. *Lane 1* Takara Molecular weight marker. *Lane 2* (batch 1), *Lane 3* (batch 2), *Lane 4* (batch 3), *lane 5* (batch4), *Lane 6* (batch5) and *Lane 7* (Batch 01810, control sample).

The SDS PAGE confirmed the presence of trypsin with a molecular weight of about 25 kDa. There was no apparent difference on SDS page profiles between trypsin produced by TP1 method and the ones produced in this study. This indicated that no extra undesirable impurities precipitated at 45% A/S saturation; Figure 16 is an indication of trypsin crystal growth after 7 days of crystallization.

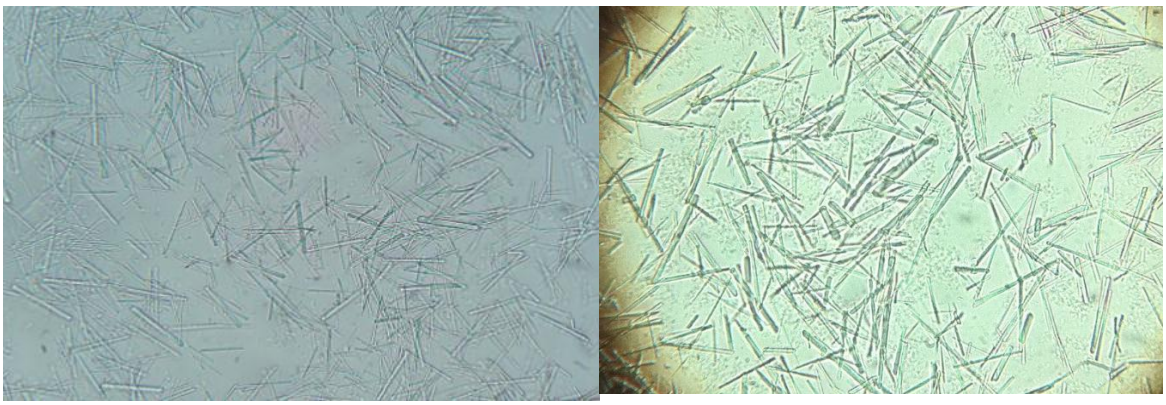


Figure 16 Trypsin crystals (400x magnification) after 7 days of crystallization under the newly defined conditions.

4.3.4.3 CONCLUSION

The results of this work proved that gradually increasing the % A/S saturation from 35% to 45% increased efficiency of trypsin crystallization and did not compromise the trypsin quality. The quality of the trypsin improved as a result of improved crystallization conditions. Specific activity of the final lyophilized product increased to > 4100 U/mg. The amount of trypsin remaining in the RML also decreased as a result of improved trypsin crystallization. The trypsin in the RML was not considered a value-adding product, as it could not be sold to many customers. By slowly increasing the precipitant (A/S), the solution was maintained within the metastable zone which promotes the formation of crystals. Based on the results of this study, it was recommended that trypsin crystallization be performed at 45% A/S saturation in order to increase trypsin yields.

The implementation of % A/S saturation proved to be successful to increase the crystallization efficiency of both trypsin and CTG. During the CTG crystallization, the A/S concentration is expressed as a conductivity reading that corresponds to A/S saturation. The best CTG crystallization was achieved at a final conductivity of 125 mS/cm. The best trypsin crystallization was achieved at a 45% final A/S solution. Future work to further optimize trypsin crystallization can be to investigate the crystallization efficiency at an oil – water interface (Chayen, 2004).

Chapter 4 described the improvements made to the existing processes used, and how the shortcomings identified in section 3.3 were addressed to improve the efficiency of each of the process steps identified. Improved extraction of the pancreas resulted in immediate increases

in protein liberated. These improvements resulted in an increased final yield of the products. The advances made in the improved clarification techniques by the implementation of new centrifuges increased the efficiency of the clarification stages, and resulted in a reduction in the total processing time, and as a result of new equipment installed, the mechanical strain placed on the diatomaceous earth filtration equipment was reduced. Improved crystallization techniques allowed for better crystallization of the specific enzymes, and resulted in an increase in final product yield for both trypsin and chymotrypsin. In addition to the improvements made to the existing processing methodologies, new innovative techniques were evaluated (as described in chapter 5) to optimize the trypsin and chymotrypsin purification processes. These improvements were investigated in an attempt to reduce overall process time and cost and will be described in Chapter 5.

CHAPTER 5

5. NEW TECHNIQUES AND METHODS CONSIDERED FOR TRYPSIN AND CHYMOTRYPSIN PURIFICATION

Further to the process improvements described in Chapter 4, this chapter describes the new techniques considered to further improve the extraction and purification process of trypsin and chymotrypsin. The main aim of the process re-engineering strategy was to reduce the overall production cost to produce trypsin and chymotrypsin, and to reduce the time in which these enzymes were manufactured. Ultrafiltration technology was investigated as a means to reduce the volumes of product handled during the primary process. Ultrafiltration would also reduce the time of the primary processing, and the total amount of A/S used during the primary processing. The benefits of introducing ultrafiltration technology were thus two-fold; a reduction in the overall process time and a cost saving in ammonium sulphate. Chromatography development was considered as a protein purification technique to reduce the overall processing time of the secondary processing. The time-consuming crystallization stages during the secondary purification of both trypsin and chymotrypsin could be shortened by implementation of column chromatography. The advantage of implementing column chromatography as a protein purification technique was a reduction in the overall secondary processing of both trypsin and chymotrypsin. This was also a more elegant approach to purify the enzymes as supposed to the methods described in the traditional methods.

The use of non-acid treated pancreas as a raw-material source was investigated to reduce the raw material costs of the process. The acid treated pancreas constituted the biggest portion of the process raw material costs, whereas non-acid treated pancreas could be processed at a lower cost.

5.1 ULTRAFILTRATION TECHNOLOGY AS A MEANS OF PROTEIN PURIFICATION AND VOLUME REDUCTION

5.1.1. INTRODUCTION

One of the major challenges faced by BBI Enzymes was that the price of the raw materials used in the processes had increased over time, and the processing methods used did not adapt or change to accommodate for this factor. Because of the nature of the traditional processing method, large quantities of A/S were used to perform precipitation steps. The price of A/S increased by 297% over the last 13 years, and has become one of the biggest expenses of the process. To eliminate the usage of large quantities of A/S, a concentration step, in which the volume of the extract was reduced, was investigated. The aim of the ultrafiltration step was to reduce the working volumes to better controllable volumes, to reduce the raw material input cost (A/S), to reduce the overall process time and to minimise the chances of product loss due to inaccurate A/S precipitation (as described in section 3.3.2 above).

Tangential flow filtration (TFF) is the pressure driven process whereby liquid is circulated through a series of membranes that only allows the passage of water and molecules of a certain size. Depending on membrane porosity, the applied process can be classified as a microfiltration or ultrafiltration process. Microfiltration membranes, are generally used for clarification, sterilization, and removal of micro particles (10 μm > pore sizes > 0.1 μm). Ultrafiltration membranes have much smaller pore sizes between 0.001 and 0.1 μm and are used for concentrating and desalting dissolved molecules such as proteins, peptides and nucleic acids. Ultrafiltration membranes are classified by molecular weight cut-off (MWCO) rather than pore size. The process described in this study only focuses on the usage of ultrafiltration as a means to reduce the working volumes.

Ultrafiltration is the pressure driven process whereby liquid is circulated through a series of semi permeable ultrafiltration membranes where liquid and small e.g. proteins, peptides and salts permeate through the membranes as a result of different hydrostatic forces. The main driving force behind the passage of liquid and proteins and or peptides across a membrane is the trans membrane pressure (TMP). The resulting pressure difference across the membranes serves as the driving force for liquid and proteins to permeate though a membrane. When a certain volume of liquid is circulated through an ultrafiltration system, the volume thereof

would be reduced over a time period. This is the process of concentration. See figure 17 for an illustration of a typical ultrafiltration system.

TMP is calculated as the sum of the inlet and outlet pressures, divided by 2. The inlet pressure is measured before the inlet to the membrane, and the outlet pressure is measured directly after the membrane (see figure 17). The TMP can be regulated by adjusting the feeding rate into the system or by adjusting the backpressure generated by the system.

Liquid passing through the membranes is called the filtrate, and the liquid that remains in circulation is called the retentate. The retentate is re-circulated through the membrane system at a rate called the crossflow rate and is qualified as litres of filtrate produced per square meter of membrane surface area per hour ($L/m^2/hr$). In the process of re-circulation, the total volume of the feed material is reduced over time (see figure 18 A).

Depending on the membrane pore size, contaminants and proteins larger than the membrane pore size are being rejected by the membrane and retained in the retentate. A build-up of retained proteins on the inside of the membrane would lead to membrane fouling, and as a result less effective filtration. Consequently, the rate of filtrate production would decrease (See figure 18 B).

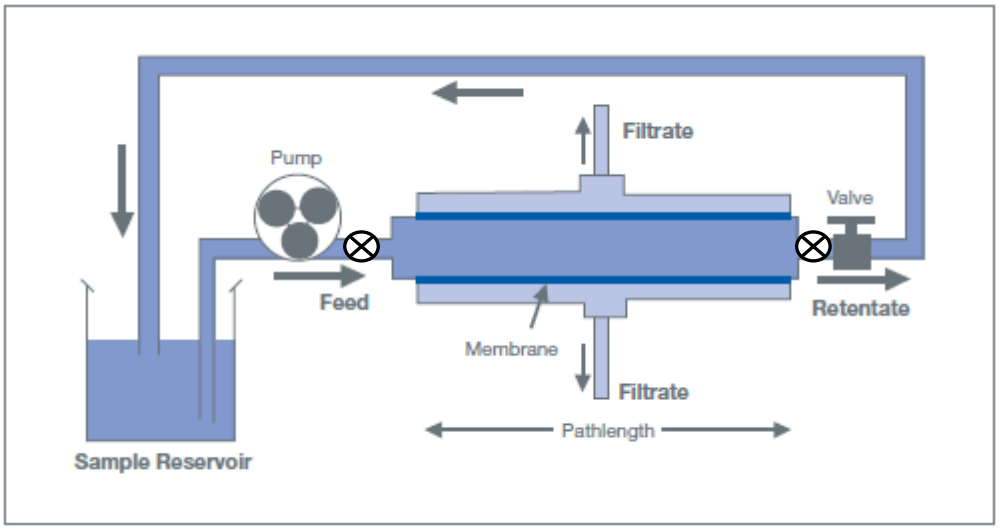


Figure 17. A simple flow path diagram of a typical TFF system. The flow path is indicated by the arrows. The pressure gauges before and after the membrane are indicated by a ⊗ sign (Schwartz, 1999).

This technology can be used successfully in a protein purification processes to reduce the total volume of a protein mixture, and retain the protein of interest, in the process removing proteins and molecules smaller than MWCO of the membrane. It is important that the correct MWCO of the membrane is selected for the process so that the protein of interest is not lost into the filtrate.

It is recommended to use a membrane with MWCO of 4 times less than the molecular weight of the protein of interest to avoid passage of the protein of interest into the filtrate (Schwartz, 1999). High concentrations of contaminants (proteins and organic foulants) in a membrane system can lead to fouling of the membranes which will compromise the performance of the membrane, i.e. reduce the rate at which filtrate is generated (referred to as the filtrate flux).

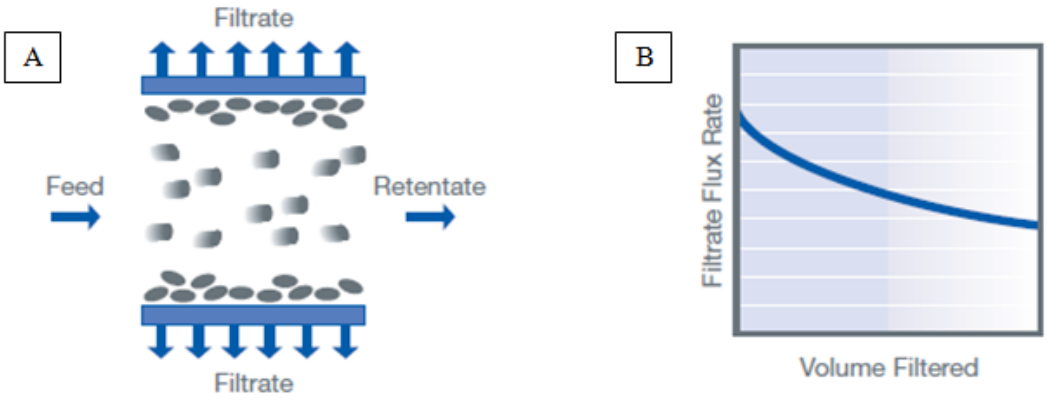


Figure 18. (A) Liquid passes through the feed channel and along (tangent to) the surface of the membrane as well as through the membrane. The crossflow prevents build-up of molecules at the surface that can cause fouling. (B) The TFF process prevents the rapid decline in flux rate seen in direct flow filtration allowing a greater volume to be processed per unit area of membrane surface (Schwartz, 1999).

The temperature of the circulating liquid could alter the performance of the membrane. Elevated temperatures (35 – 40^{o}C) allow the Poly Ether Sulfone (PES) membrane to expand, and subsequently increased pore size of the membrane, resulting in enzyme leaching into the filtrate. It was thus important to maintain the operating temperature of the liquid to < 21^{o}C to avoid any potential enzyme loss into the filtrate. It was also observed that elevated temperatures during concentration of the zymogens (>25^{o}C) spontaneously denatured the zymogens, and forced them out of solution.

In an industrial protein purification process, when ultrafiltration technology is used to reduce the volume of a batch, it is important that this process should be completed in the shortest possible time. The end of an ultrafiltration cycle is marked by the achievement of a certain concentration factor, or when a defined protein concentration of the retentate is achieved. In this study, protein concentration was determined by measuring the A_{280} of the concentrated solution, see note on page 21.

To achieve the optimal concentration factor, the operating conditions for the system were to be determined beforehand. Important considerations when determining the process parameters includes the MWCO of the membranes, the amount of membrane modules needed (total surface area of membranes) to achieve the desired concentration within a specified time frame, the cross flow and backpressure required to achieve a certain TMP, the crossflow flux rate (crossflow rate per unit area of membrane; $L/m^2/hr.$) and the TMP to achieve minimal membrane fouling. The design of a TFF system is easily scalable, and can be trailed on pilot scale and scaled up directly for use in an industrial process.

Concentration polarization is the accumulation of the retained molecules (gel layer) on the inner surface of the membrane caused by a combination of the following factors: trans-membrane pressure, crossflow rate, sample viscosity and solute concentration (Bauser, 1986). This is why it is important to always maintain a high crossflow and TMP during a concentration cycle. Such organic foulants can be removed by the circulation of 0.4 M NaOH solution at 40^oC at high crossflow through the system with a maximum contact time of 50 minutes (as recommended by the supplier).

Inorganic fouling of the membranes by e.g. silica is extremely hard to remove. Prior to any ultrafiltration process would be a clarification process to ensure the liquid clarity is suitable for concentration. In the industry, diatomaceous earth is commonly used as a filtration medium. When membranes are fouled with filter aid (diatomaceous earth), it is impossible to remove all the silica residues from the membrane. It is thus always recommended to have a proper in-line pre-filtration system (5 μm pore size pre-filter) installed upstream of the membrane to avoid any particulate materials passing into the membrane and causing irreversible membrane fouling.

After a cleaning cycle was performed, the normalised water permeability (NWP) of the membrane was determined to indicate the effectiveness of the cleaning cycle that was

executed. The NWP is also referred to as the clean water flux, where clean water is passed through the system (after the system was cleaned) at a constant TMP and crossflow. As the membrane ages, and irreversible fouling starts to block the membrane, the NWP will decrease over time.

5.1.2. MATERIALS AND METHODS

PALL life sciences supplied two models of cassettes in the TFF range of membranes, a T-series and an F-series. Both these cassettes were made out of PES. PES membranes are hydrophilic, are highly resistant to chemicals such as NaOH and are not prone to protein binding. The difference between the PALL T-series and F-series cassettes was that the T-series was designed to create additional turbulence as the liquid passed through the membrane channel, and thereby reduce the possibility of membrane fouling. This was achieved by an irregular membrane surface (bumps) that generated turbulence as the liquid passed through the channel. Because the ultrafiltration process was considered to be used early in the primary purification process, the risk of concentration polarization was high as the protein concentration was high (the only proteins that were removed at this stage were the proteins precipitated with 20% A/S).

The use of 10 kDa PALL[4] T-series Centramate® cassettes were considered for the trials carried out in this study. The centramate cassettes are lab scale equivalents of the large-scale membranes, and were designed for laboratory trials in preparation for industrial scale up. It was recommended that a MWCO of four times less than the size of the protein of interest should normally be used indicating that a 5 kDa membrane was required for these trials. Based on experience with industrial scale 5 kDa membranes, the use of such low MWCO membranes was discouraged, as the filtrate flux obtained by these membranes was too low. As a result, the 10 kDa membrane was considered. Two different kinds of PALL 10 kDa membranes were considered, PES hollow fibre and PES cassette membranes. The usage of PES hollow fibre membranes was discouraged following a trial with a small-scale 10 kDa PALL hollow fibre membrane concentrating a solution of ribonuclease. The process at BBI

[4] PALL is a leading manufacturer of tangential flow membranes. The Centramate cassette is a laboratory replica of the T-series cassettes that is used on industrial scale.

Enzymes required RNase to be purified as a further product, and 90% of the RNase that was used for this trial passed through the hollow fibre membrane, but did not pass through a 10 kDa cassette. The nominal cut-off on cassette membranes is closer to the quoted MWCO than hollow fibre membranes. This statement was supported by the supplier (PALL).

The Pall T-series Centramate® membrane could withstand a maximum pressure of 6 Bar, and a maximum TMP of 4 Bar. The pH range of this membrane was 2 – 14 which allowed the liquid extract from the process with a pH of 2 – 2.2 to be concentrated. This membrane had a 0.1 m^2 surface area, and was fitted to a PALL cassette housing. A peristaltic pump fitted with a WEG frequency drive was used to control the circulation speed of the liquid through the membrane. The liquid product used for these trials (clarified 0/20 material) was collected from the production plant, and when necessary, it was re-clarified to avoid excessive membrane fouling. To control the temperature of the extract the sample reservoir, containing the extract, was cooled with ice and water.

The aim of these trials was to define the operating conditions for the large-scale process, and to determine the amount of cassettes required to achieve the concentration factor required in a set time period. In the production scale process, the average volume at clarified 0/20 stage was approx. 4000 L which needed to be concentrated down to a approx. 200 L (20x concentration factor) to achieve a final A_{280} of 240 – 260. This A_{280} was equivalent to the protein concentration of the dissolved 40/65 precipitate (pre zymogen separation) from the traditional process. The aim was to concentrate the clarified 0/20 material to the defined A_{280} value and immediately perform the zymogen separation directly thereafter. The maximum process time allowed for this step was 8 hours, which meant that an average filtrate flux of 500 L/h was required to concentrate the 4000 L down to approx. 200 L.

There were two reasons for implementing the ultrafiltration step directly after the clarification of the 20% A/S precipitation. Firstly because this was the first stage in the process where the clarity of the product was sufficient (< 15 FTU's), and secondly because this was the point in the process with the lowest A/S concentration. The viscosity of the liquid would increase as the % A/S saturation increased which would have a negative effect on the flow dynamics of the TFF system (Viscosity is one of the factors that could accelerate membrane fouling). A/S is very corrosive, and could potentially cause damage to the machinery used during the ultrafiltration process. Any steel structures on the system were at risk to start rusting which would be detrimental to the process.

In all of the experiments conducted, when the performance of a membrane was assessed, the samples were properly clarified to avoid false negative results. The clarified product was re-circulated through the UF system using a peristaltic pump. The pressures (inlet and outlet) were controlled by valves installed in the system (as described in figure17). Pressure readings, filtrate fluxes and crossflows were measured over the concentration period until a certain concentration factor was achieved. The temperature of the product was read using a digital thermometer, and adjusted by the addition of ice around the vessel containing the recirculating liquid.

The filtrate of the experiment was all collected and assayed for enzyme activity at the end of the experiment to assess if there was any leakage of the enzymes through the membrane. SDS PAGE analysis was also conducted to verify the passage of any of the proteins of interest.

5.1.3. RESULTS

The first experiment was carried out to investigate the optimal TMP and crossflow required to give the best and most stable filtrate flux. Three different crossflows and TMPs were considered. Table 15 gives a summary of the parameters that were monitored during the duration of the concentration run, and figure 19 is the graphical translation of table 15 where the filtrate flux and TMP was measured over time. This experiment to investigate the optimal TMP was only conducted once due to time constraints for UF membrane optimization.
During the lab scale investigations, it was easier to express the filtrate flux as $L/m^2/min$ as the fluxes were considerably lower than those achieved on a large-scale membrane (where filtrate flux is measured as $L/m^2/min$).

Table 15. Summary of the parameters controlled during the first optimization trials. The crossflow and the TMP were varied to investigate the optimal performance.

Time (min)	P_{in} (Bar)	P_{out} (Bar)	Feeding rate (Hz)	Crossflow (L/min/m^2)	Filtrate flux (L/min/m^2)	Temp (^{o}C)	TMP
0.00	1.2	0.8	7.57	4.00	0.355	19.7	1
15.00	1.2	0.8	7.57	4.00	0.32	21	1
25.00	1.2	0.8	7.57	4.00	0.305	22.3	1
35.00	1.2	0.8	7.57	4.00	0.28	23.6	1
40.00	2	2	7.57	4.00	0.375	23.6	2
50.00	2	2	13.4	8.33	0.35	25.2	2
55.00	2	1	13.4	7.67	0.32	25.2	1.5
65.00	1.5	1.5	13.4	8.67	0.31	26	1.5
150.00	2	1	13.4	8.67	0.265	26.5	1.5
210.00	2	1	13.4	8.67	0.26	27.1	1.5

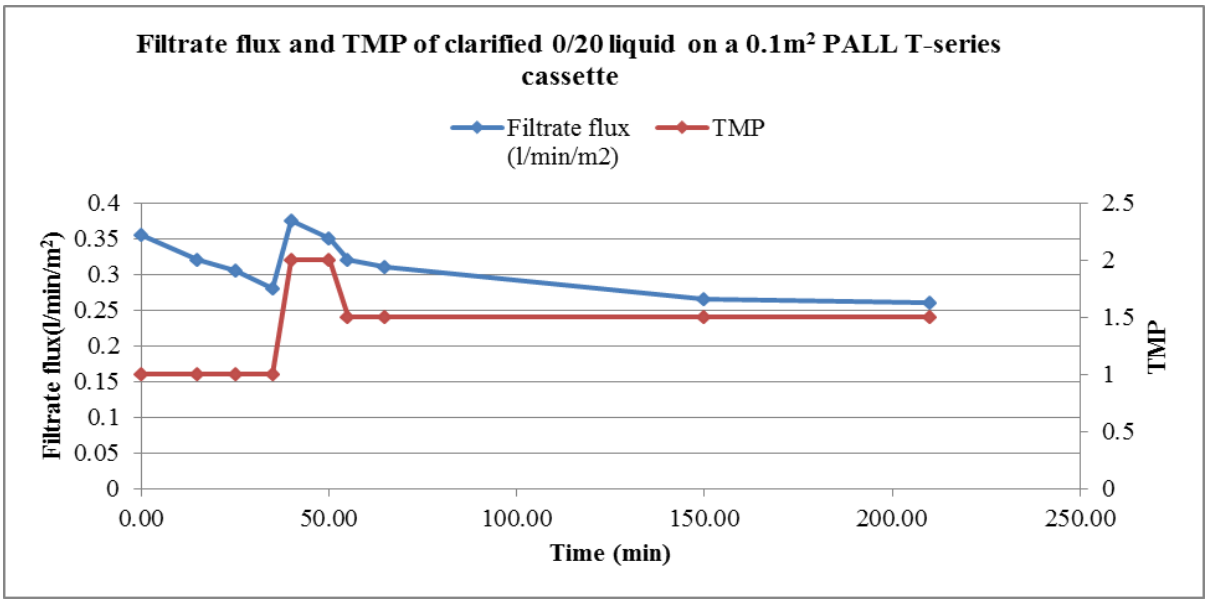


Figure 19. Three different TMP's and crossflows were investigated to determine the optimal conditions for the best filtrate flux. Filtrate flux (L/min/m2) and TMP (Bar) was plotted against time.

At a TMP of 1, the filtrate flux decreased rapidly. This indicated that this TMP was too low to sustain a proper production run, and on a production scale process, the membranes would require cleaning during the concentration cycle.

The low crossflow allowed fouling of the membrane to occur. When the TMP was increased to 2, a similar finding was observed where there was a sharp decline in the filtrate flux. When the TMP was adjusted to 1.5 bar, the filtrate flux stabilised, and the decline was not as rapid. This could have been as a result of the initial fouling.

Following the first trial, the membrane was cleaned with 0.4 M NaOH at 40^{o}C for 45 minutes, and flushed with water. The NWP was 3.4 L/min/m^2. The second trial investigated the effect of a constant TMP of 1.5 on the filtrate flux. The findings of the second trial are summarised in table 16 and figure 20.

Table 16. Summary of the second trial where clarified 0/20 material was concentrated at a constant TMP of 1.5

Time (min)	**P_{in} (Bar)**	**P_{out} (Bar)**	**Feeding rate (Hz)**	**Crossflow (L/min/m^2)**	**Filtrate flux (L/min/m^2)**	**Temp (^{o}C)**	**TMP**
0.00	2	1	13.37	8.50	0.53	19.5	1.5
5.00	2	1	13.37	8.50	0.48	19.5	1.5
10.00	2	1	13.37	8.43	0.39	22	1.5
20.00	2	1	13.37	8.33	0.34	23	1.5
30.00	2	1	13.37	8.27	0.32	25	1.5
60.00	2	1	13.37	8.20	0.25	24.2	1.5
120.00	2	1	13.37	8.17	0.19	22.3	1.5
180.00	2	1	13.37	8.10	0.16	23	1.5

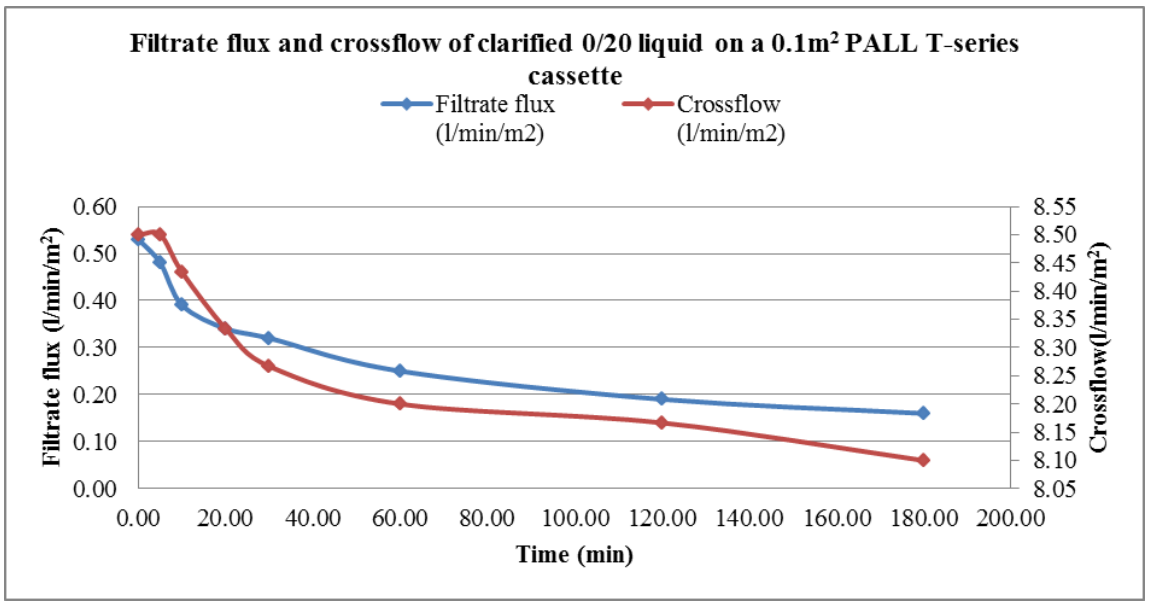


Figure 20. The second trial carried out on clarified 0/20 material at a constant TMP of 1.5. A sharp initial decrease in both filtrate flux and crossflow indicated that the membrane was slightly fouled until a certain point was reached after an hour where both fluxes stabilised, and the decrease in flux was gradual (as expected).

The average filtrate flux for this run was 0.33 L/min /m^2. This could be directly scaled to an industrial scale process. The large T-series cassettes had a total surface area of 2.5 m^2 per cassette. Extrapolating the laboratory scale data, a total of 10 cassettes were required to concentrate the total volume of 4000 L down to approx. 200 L in 8 hours.

The NWP of the membrane after cleaning was 3.3 ℓ/min/m^2, which was similar to the NWP obtained after the first trial, indicating that the cleaning regime used was successful to remove any organic foulants.

The third trial served as verification for the second trial where a constant TMP of 1.5 was maintained throughout the production run. For this trial, a higher crossflow was investigated to see if this would improve the performance of the membrane. The results of the third trial are summarised in table 17 and figure 21.

Table 17. Summary of the third trial where clarified 0/20 material was concentrated at a constant TMP of 1.5, but at higher crossflow.

Time	P_{in}	P_{out}	Feeding rate (Hz)	Crossflow (L/min/m^2)	Filtrate flux (L/min/m^2)	Temp (^{o}C)	TMP
0.00	2	1	8	9.17	0.58	21.00	1.5
10.00	2	1	8	9.17	0.54	22.30	1.5
20.00	2	1	8	9.08	0.50	24.70	1.5
30.00	2	1	8	8.97	0.48	25.70	1.5
40.00	2	1	8	8.83	0.45	24.00	1.5
50.00	2	1	8	8.53	0.38	21.70	1.5
60.00	2	1	8	8.20	0.35	20.60	1.5
90.00	2	1	8	8.03	0.34	25.70	1.5
120.00	2	1	8	7.97	0.33	25.80	1.5
150.00	2	1	8	7.91	0.27	24.93	1.5
180.00	2	1	8	7.68	0.24	25.22	1.5

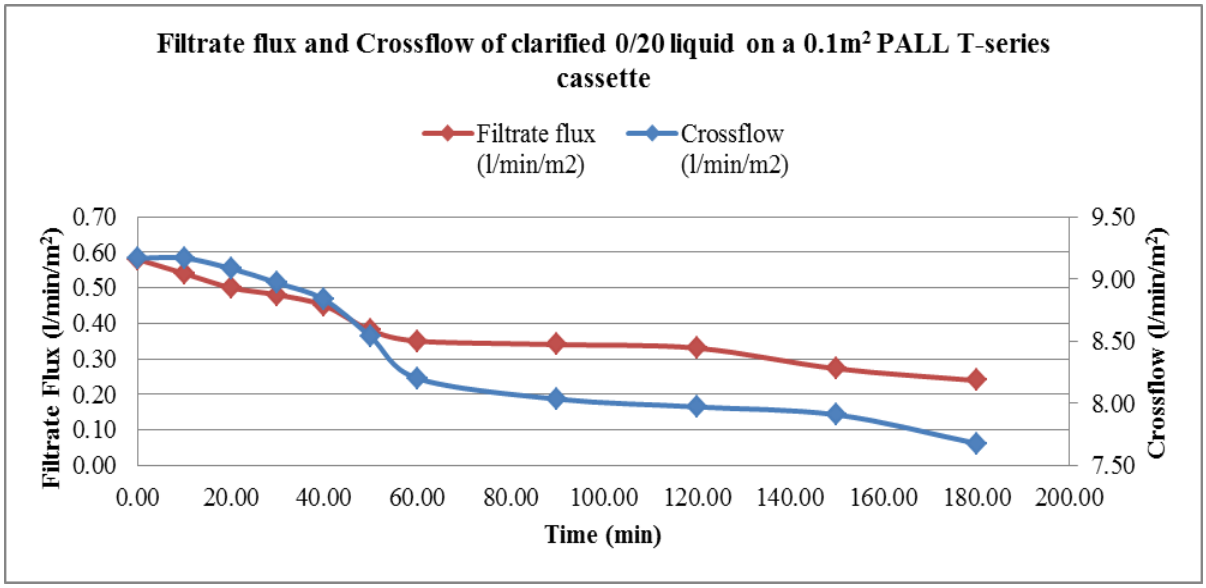


Figure 21. Third trial carried out on clarified 0/20 material at a constant TMP of 1.5, but at higher crossflow. The flux decrease was not as rapid as observed during the second trial, and a higher average filtrate flux was observed.

The average filtrate flux achieved for this run was 0.37 L/min/m^2. Extrapolating the laboratory scale data, a total of 9 cassettes were required to concentrate the total volume of

4000 L down to approximately 200 L in 8 hours. This compared well with the results obtained in the second trial (see figure 20). The NWP of the membrane after cleaning was again 3.3 L/min/m^2 which was again similar to the NWP obtained the first trial, and exactly the same as that measured for the second trial, confirming that the cleaning regime used was successful.

The filtrate was tested for trypsin, deoxyribonuclease and ribonuclease activity. No activity could be indicated for any of these enzymes showing that there was no passing of these enzymes through the membrane, proving that the membrane successfully retained all the desired enzymes.

5.1.4. CONCLUSION

The 10 kDa PALL T-series cassette was capable of fully retaining the zymogens under the defined conditions, even when the clarified 0/20 material was concentrated up to 20 fold. The flow characteristics of the 0.1 m^2 test membrane indicated that it was capable of achieving the desired volume reduction within a 16-hour period by using 15 membranes. The running conditions were defined at a constant TMP of 1.5 with an inlet pressure of 2 Bar and an outlet pressure of 1 Bar. After multiple concentration cycles, the NWP remained constant, indicating that no irreversible membrane fouling was observed as a result of the nature of the liquid product.

It was recommended that appropriate pre-filtration system was to be installed prior to the cassette inlets. The risk of damage to the membranes was high as there was upstream clarification using diatomaceous earth as a filtration medium, which could cause irreversible membrane fouling. The installation of an ultrafiltration unit had major financial benefits as the overall raw material inputs (A/S) were reduced significantly, and the processing time was reduced with 5 days.

5.2 CHROMATOGRAPHY DEVELOPMENT TO SEPARATE TRYPSIN(OGEN) FROM CHYMOTRYPSIN(OGEN)

5.2.1. INTRODUCTION

The traditional purification methodologies used to purify both trypsin and chymotrypsin were extremely time-consuming due to a series of crystallization and precipitation stages, in the case of trypsin being up to 7 days, and in the case of chymotrypsinogen a total of three days of crystallization. With the increased demand for these products, it was essential to find new ways to increase the throughput during the secondary purification stages. Liquid chromatography was a technique already used elsewhere within the organization with great success, and was considered for the purification of both trypsin and chymotrypsin.

Figure 22 illustrates the three different kinds of liquid chromatography techniques were considered for purification of the enzymes: Affinity chromatography using P-amino-Benzamidine resin to purify trypsin specifically, Hydrophobic Interaction chromatography (Phenyl Sepharose) and Ion Exchange chromatography (weak cation exchange resin, Carboxy Methyl (CM) sepharose).

Affinity Chromatography focussed on the separation of activated trypsin from chymotrypsin as a means to purify trypsin, Hydrophobic Interaction chromatography (HIC) focussed on the separation of the zymogens as a potential replacement of the zymogen separation and Ion Exchange chromatography focussed on the separation of both the active enzymes and the zymogens from each other (the zymogens were separately investigated from the active enzymes).

The investigation of the implementation of column chromatography during the secondary purification process was performed to devise methods to increase the overall processing time (to replace the crystallization stages) and to recover more enzymes during the secondary purification that would ultimately contribute to higher yields being achieved. The aim was to develop a purification strategy that would be able to produce products that are in compliance with the product specifications as set out in tables 2 and 3.

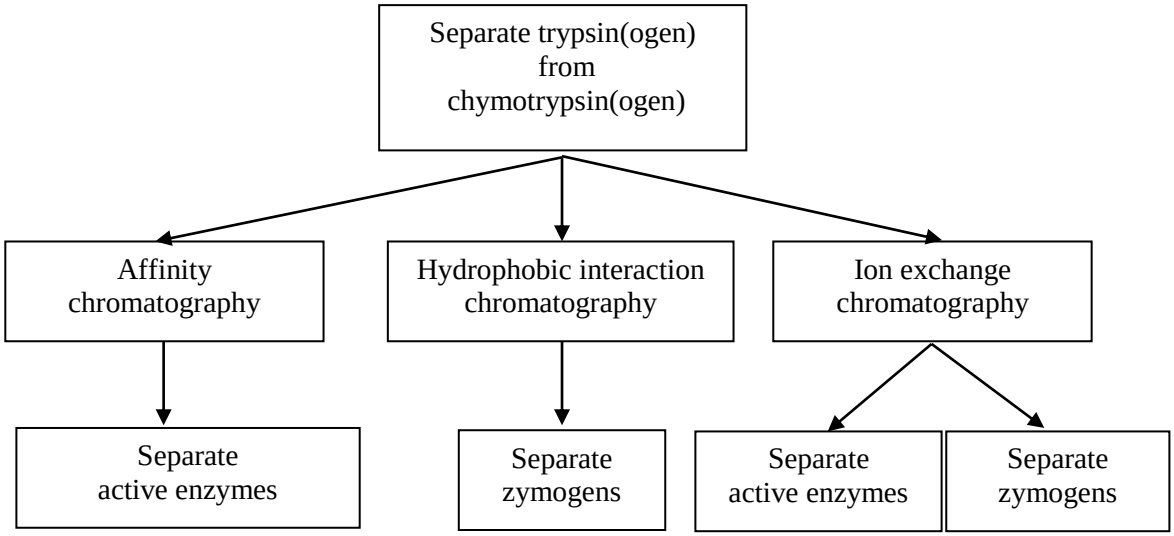


Figure 22. Experimental design of the chromatography development aiming to separate trypsin(ogen) from chymotrypsin(ogen) using Affinity chromatography (Benzamidine), Hydrophobic Interaction chromatography (Phenyl Sepharose) and Ion Exchange chromatography (CM sepharose).

5.2.1.1 AFFINITY CHROMATOGRAPHY

Affinity chromatography is the separation of a specific enzyme (protein) from a mixture of different proteins by the reversible binding of the enzyme to a ligand, substrate or co-factor that is coupled to a stationary phase. Separation of the enzyme from a mixture of proteins is achieved when a (crude) sample is applied to a column (where the specific substrate is covalently linked to the resin) under conditions that favour the (reversible) binding of the substrate to enzyme, and other nonspecific proteins are eluted from the column.

Elution of the target protein is achieved by applying an elution buffer that would change the conditions (pH, ionic strength or polarity) in such a way that it would not favour enzyme – substrate binding. The target protein is typically eluted in a sharp peak, which is a highly concentrated form of the target protein (GE Healthcare, 2007). See Figure 23 for an illustration of a typical affinity chromatography elution profile.

This technique has been applied to purify trypsin (Jameson, 1973) with great success, and was considered as a mechanism to purify trypsin during the secondary purification stages and as a replacement for the 7-day trypsin crystallization.

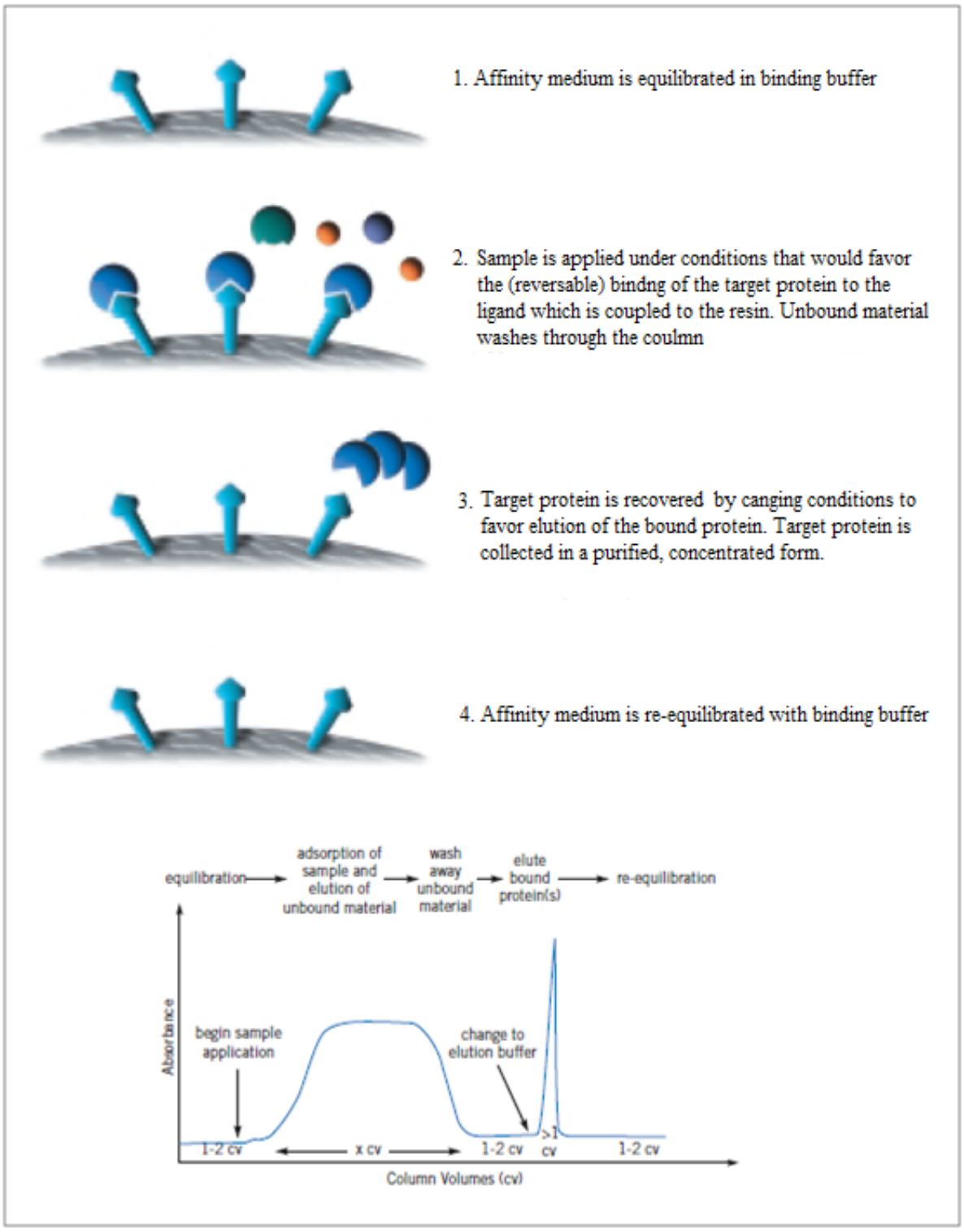


Figure 23. Basic principles of affinity chromatography where the enzyme substrate / ligand is immobilized onto a resin. When crude mixture is applied to the resin, only the specific protein will bind to the ligand / substrate, and the nonspecific proteins will elute. Elution of the specific target protein as achieved by changing the buffer and the conditions that would not favour the binding of the protein to its ligand/substrate (Amersham Pharmacia Biotech, 2001).

The aim was to introduce the affinity column immediately after the product was activated (see figure 2). The advantage of the affinity resin was that the product could be loaded whilst still

containing ammonium sulphate, which implied that the product could be loaded onto the column directly after the trypsin activation step.

Benzamidine (see figure 24) is a synthetic trypsin ligand and serves as a model system for the basic amino acids lysine and arginine, which are both affiliated with trypsin specific activity. At physiological pH (7.3 to 7.4), benzamidine, arginine and lysine are fully protonated (Talhout, 2001). Benzamidine binds in the close vicinity of the trypsin catalytic triad (His-57, Asp-102, Ser-195) in the active site (stabilized by hydrogen bonds), and in this way it serves as an competitive inhibitor and prevents the binding of the substrate to the active site (Figure 25) , and also shields the active site from water molecules (Essex, 1997). See section 2.2 for the mode of action of the serine proteases.

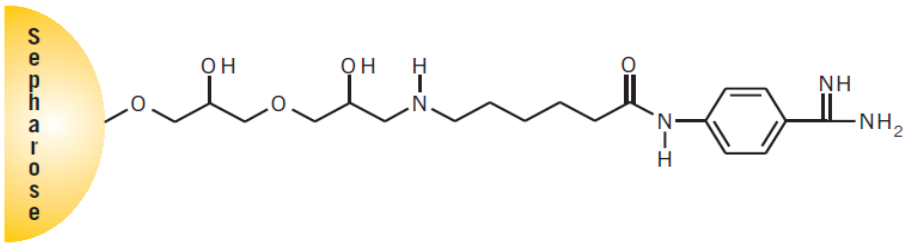


Figure 24. Benzamidine linked to sepharose fast flow (High sub) resin via stable ether linkages. (Amersham Pharmacia Biotech, 2001)

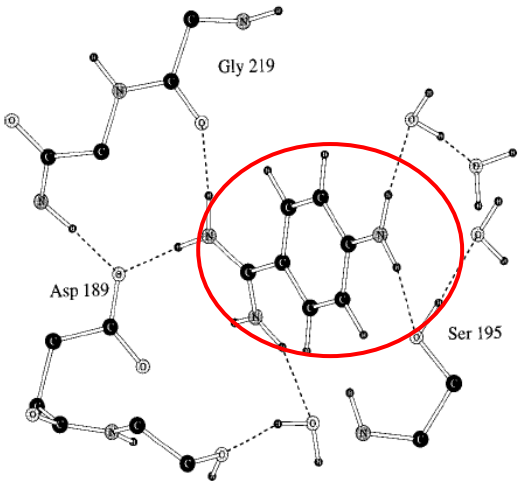


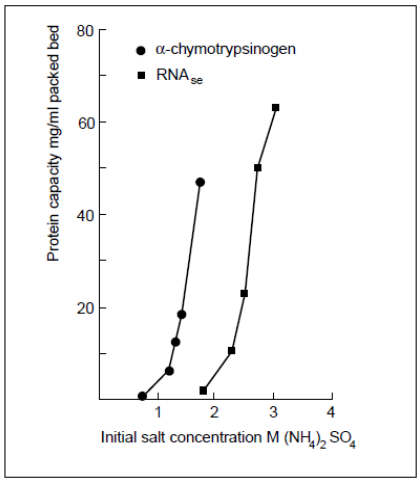
Figure 25. The structure of the trypsin – pABA complex formed when trypsin binds to the immobilized Benzamidine on the Sepharose resin. The benzamidine is stabilized by hydrogen bonds (Essex, 1997).

5.2.1.2 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

The first consideration for HIC was to separate the two enzymes as early as the zymogen separation stage. The intent was to replace the 48-hour zymogen separation step with a single chromatography step that would separate the zymogens within a few hours. Phenyl Sepharose resin was considered for this step as this was a resin that was used elsewhere within the organization with great success, and the BBI staff was familiar with the resin and its properties. The advantage of using HIC at this early stage in the process was that the liquid could be directly loaded onto the phenyl column following the 35% A/S precipitation without having to prepare a salt free product (by dialysis or diafiltration) before loading it onto the resin. The % A/S saturation could be as high as 80%, and proteins would still bind to phenyl resin. This made HIC an attractive option to use in combination with protein precipitation using A/S. The aim was to implement this HIC step directly after the ultrafiltration stage where the product is concentrated until a A_{280} of 240 – 260 was achieved.

HIC is the separation of proteins when the hydrophobic regions on the protein interact differentially with hydrophobic molecules that are immobilized on the surface of the hydrophilic stationary phase such as agarose or sepharose beads (Jennissen, 2002). The presence of salts plays an important role in the binding of hydrophobic regions on the proteins to the resin (Tiselius, 1948) and to stabilize the protein structures (Amersham Pharmacia Biotech, 2000). It is generally considered that the higher the salt concentration, the better the binding of the protein to the resin. See figure 26 where binding capability of CTG and ribonuclease are plotted against the A/S concentration. For this reason, a protein that is soluble at high % A/S saturations would bind strongly to phenyl resin. The driving force behind hydrophobic interactions is the extrusion of an orderly fashioned monomolecular layer of water molecules that covers the surface of two neighbouring hydrophobic surfaces into less ordered bulk water with an increase in entropy. It is thus an entropy driven attraction between non-polar groups in an aqueous medium (Jennissen, 2002).

The Sepharose resin used in HIC is usually a very hydrophilic resin that would prevent any interaction of the proteins with the resin itself. The binding of proteins occur between the hydrophobic surface of the protein and the conjugated group (in this case a phenyl group) which is linked onto the resin (Jennissen, 2002).



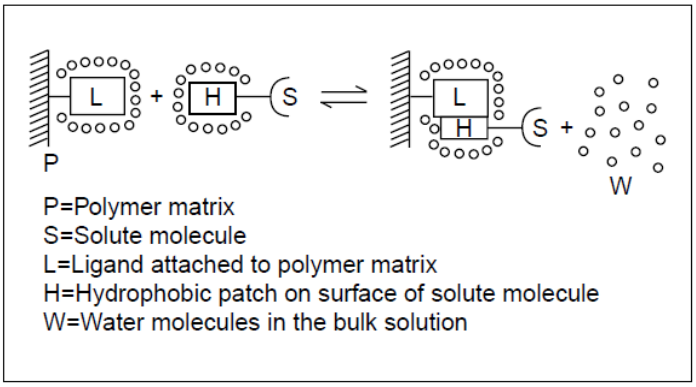


Figure 26. Binding capacity of CTG and ribonuclease as a function of salt concentration. A proposed mechanism for the hydrophobic interaction between protein and the resin (Hjerten, 1977) where water molecules are highly ordered on the surface of the protein and the hydrophobic ligand and appears to shield off the hydrophobic ligand and protein. Higher salt concentration would interact with the water and reduces the "shielding" effect of the water leaving the hydrophobic regions exposed to the hydrophobic ligands on the resin, and facilitates binding (Amersham Pharmacia Biotech, 2000).

5.2.1.3 ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography involves the reversible binding of a charged protein to an oppositely charged resin. The pH and the ionic strength of the buffers play a vital role in the binding of a particular protein to the charged resin. Negatively charged Carboxymethyl (CM) Sepharose resin (a weak cation exchange resin) was considered for this application due to the ease of use in a production environment, and the ease of scalability from laboratory scale to plant scale. In addition, CM Sepharose can give high resolution with a very high capacity. A major feature of ion exchange is its power to concentrate a bound component and it is often used to capture and concentrate very dilute protein samples.

Separation of different proteins is obtained as a result of the different degrees of interaction different proteins have with the ion exchange resin because of differences in charges at specific pH's, and distribution of charge on their surfaces. The two main contributing factors that dictate binding to the resin are the pH and the ionic strength of the buffers applied during the chromatographic run. The difference in the charges of proteins at the conditions at which the column is run is the main reason for separation of the different proteins.

Figure 27 illustrates how the net charge of a protein can be changed to either positive or negative depending on the pH of the buffer the protein is dissolved in.

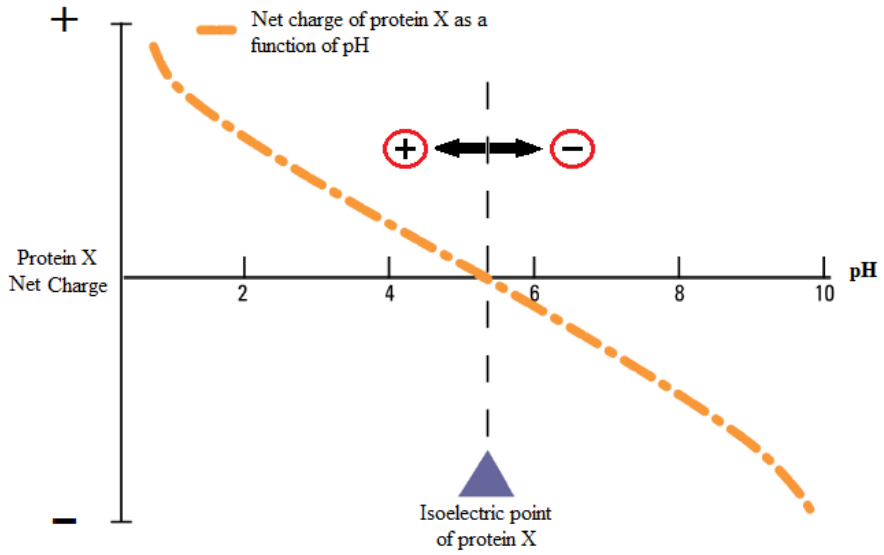


Figure 27. Net charge of the protein of interest is dependent on the pH of the buffer it is solubilised in relative to its pI. When the pH of the buffer is higher than the pI of the protein, the protein will have a net Negative charge. When the pH of the buffer is lower than the pI of the protein, the protein will have a net Positive charge. The greater the difference between the pI and the pH, the greater the charge of the protein.

The greater the net difference between the pI of the protein and the pH of the buffer, the greater the charge of the protein would be. The greater the charge of the protein, the stronger the protein would bind to the resin. The resin used for purification of the enzymes was CM sepharose resin that has a net negative charge, and would thus bind positively charged proteins. CM Sepharose resin is an agarose bead with a carboxymethyl group conjugated to its surface. The use of Ion Exchange chromatography was considered for both the active and the inactive (zymogen) form of the enzymes. The intent in both scenarios was to reduce the total production lead-time by the implementation of a single or a two-stage chromatographic separation during the secondary processing.

The differences between the pI values of the various forms of trypsin(ogen) and chymotrypsin(ogen), allowed the potential separation of these enzymes using ion exchange chromatography. Table 18 gives an overview of the pI values of the proteases.

Table 18. Summary of the iso-electric points of the different enzymes that formed part of this investigation (Uniprot, 2002, Hirs, 1953).

Enzyme	Iso-electric point (pI)
Trypsin	9.3
Trypsinogen	10.1 – 10.5
α-Chymotrypsin	8.75
Chymotrypsinogen - A	9.1
Chymotrypsinogen - B	5.2

The investigation was divided into two stages of development namely separation of the active enzymes trypsin and chymotrypsin, and secondly the separation of the inactive enzymes trypsinogen and CTG. Different strategies were used in both cases to separate these proteins. The differences in iso-electric points of the enzymes (table 18) were big, implying it might be difficult to separate the enzymes from each other using ion exchange chromatography.

A summary of the strategy applied to investigate the use of ion exchange chromatography is illustrated in figure 28. Different binding and elution strategies were considered.

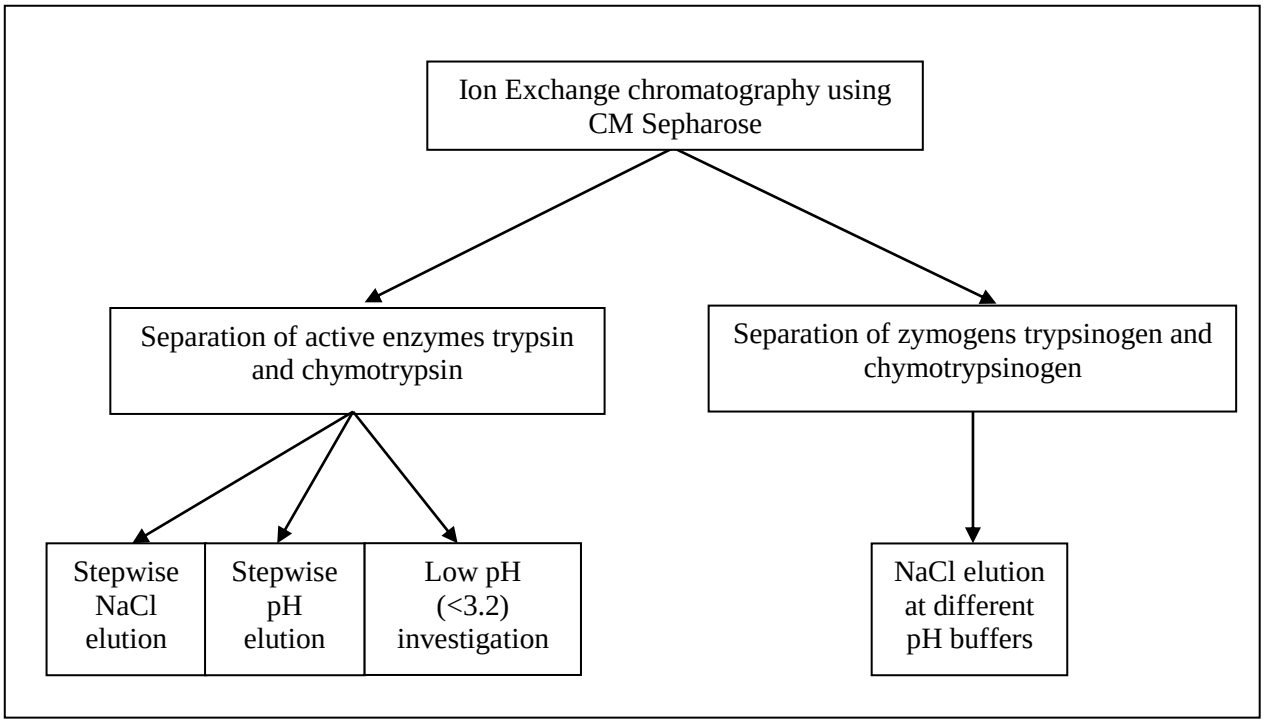


Figure 28 Flow diagram of the experimental design of the Ion exchange chromatography for the separation of trypsin(ogen) from chymotrypsin(ogen).

5.2.2. MATERIALS AND METHODS

5.2.2.1 AFFINITY CHROMATOGRAPHY

Benzamidine Sepharose 4 fast flow (high sub) resin was used for the affinity chromatography development of trypsin. Resin samples were obtained from GE Healthcare (GE healthcare, Uppsala, Sweden, product # 17-5123-10). The binding capacity of the affinity resin was ≥35 mg trypsin/ml resin as specified by GE Healthcare. The implication thereof was that for production scale processing, approximately 56 L of resin was required for a 1.4 t extraction batch at a yield of 1.4 kg/t.

A 20 ml benzamidine column was packed in a clear 1.5 X 20 cm Perspex column under gravity. The benzamidine resin was equilibrated with 10 column volumes of equilibration buffer consisting of 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4. All chemicals used during this investigation were obtained from Sigma (Sigma Life sciences, St. Louis, USA). The column was fed using a Gilson MINIPLUS 3 peristaltic pump at a constant feeding rate of 1.5 ml/min. Fractions (6.5 ml) were collected using a Pharmacia Biotech FRAC-100 fraction collector and assayed for protein content (A_{280}) with a Schimadzu UV-1601 spectrophotometer. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. This instrument was used for indication purposes only, to visualise the elution profile in real-time. The A_{280} values obtained of each fraction were used to plot elution profiles of the enzymes. Trypsin activity assays were performed using the trypsin microtitre assay that was developed for this project (described in section 6.4).

Two different loading and elution conditions were investigated for the purification of trypsin. Both methods were derived from the instruction manual booklet that came as an appendix with the resin.

Experiment 1

The first experiment was conducted exactly according to GE Healthcare prescribed method (GE Healthcare, 2007) to mimic the elution of trypsin as described by the supplier.

Commercial preparations of lyophilised trypsin and chymotrypsin were obtained from Sigma (Sigma life sciences, St. Louis, USA, trypsin product# T8003-1G, chymotrypsin product#

C4129). The samples were prepared (10 mg/ml) in equilibration buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4), and 10 ml was loaded onto the column at a constant feeding rate of 1.5ml/min using a Gilson MINIPLUS 3 peristaltic pump. After the sample was loaded onto the column, the column was washed with washing buffer (0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4) at 1.5 ml/min until the A_{280} of the eluate had reached a stable baseline (as visualised by the in-line UV detector). Once a baseline was reached, the column was eluted at a flow rate of 1.5 ml/min with elution buffer (0.05 M Glycine buffer, pH 3.00) until the A_{280} of the eluate had reached a stable baseline.

Fractions (6.5 ml) were collected for A_{280} determinations with a Pharmacia Biotech FRAC-100 fraction collector, and the A_{280} of the fractions was determined using a Schimadzu UV-1601 spectrophotometer. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. The A_{280} values of the collected fractions were plotted to establish an elution profile for both enzymes.

Experiment 2

After an elution profile was obtained using the prescribed method as specified by GE Healthcare, the elution profile of trypsin in the presence of 35% A/S was investigated. The aim of this investigation was to determine if there was any difference between the two elution profiles with and without A/S present. This experiment was conducted to investigate the implementation of a benzamidine column step directly after trypsin was activated in the process (see figure 2). Activated trypsin material collected from the factory floor (containing 0.02 M Tris-HCl, 0.02 M $CaCl_2$, 35% A/.S, pH 8.0) was used for this trial.

The column was equilibrated with equilibration buffer (0.05 M Tris-HCl, 0.5 M NaCl, 35% A/S, pH 7.4). The trypsin sample obtained from the factory floor was loaded onto column using a Gilson MINIPLUS 3 peristaltic pump at a constant feeding rate of 1.5 ml/min. The column was washed with washing buffer (0.05 M Tris-HCl, 0.5 M NaCl, 35% A/S, pH 7.4) until the A_{280} of the eluate had reached a stable baseline (as visualised by the in-line UV detector). Once a baseline was reached, the column was eluted at a flow rate of 1.5 ml/min with Elution buffer: 0.05 M Glycine buffer, pH 3.00 (Sigma Aldrich, St Louis, USA product # 410225) until the A_{280} of the eluate had reached a stable baseline.

The fractions collected during experiment 1 and 2 were assayed for protein content and prepared for lyophilisation. The samples were dialyzed in RO water until salt free prior to

lyophilisation. The lyophilised samples were assayed according to the QC procedures for trypsin and chymotrypsin content. (Trypsin: appendix 12, Chymotrypsin: appendix 3). After a chromatographic run, the column was regenerated by washing the column with 2 column volumes 1 M NaCl, and washed with water until no NaCl was present in the flow through. Thereafter the column was equilibrated with the loading buffer.

Due to the time constraints placed on the development of this process, each experiment was only conducted once. If there was a significant finding, the experiment was repeated.

5.2.2.2 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Phenyl Sepharose, fast flow (High sub) resin obtained from GE Healthcare (GE Healthcare, Uppsala, Sweden, Product # 17-0973-10) was used for the trials conducted during the trials. A 10 ml (1,2 x 10 cm) column was packed with 7.5 ml resin under gravity and fed with a Gilson MINIPLUS 3 peristaltic pump at 1.5 ml/min. For all the experiments, 6.5 ml fractions were collected using a Pharmacia Biotech FRAC-100 fraction collector, and assayed for protein content (A_{280}) with a Schimadzu UV-1601 spectrophotometer. These values were used to plot elution profiles of the enzymes. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. This instrument was used for indication purposes only, to visualise the elution profile in real-time.

Purified preparations of trypsinogen and CTG samples were purchased as lyophilized powders from Sigma (Sigma life sciences, St. Louis, USA. Trypsinogen product # T1143-1G, CTG product # C4879-1G). Trypsinogen and CTG samples were prepared (2.5 mg/ml) in equilibration buffer (0.05 M Tris-HCl buffer, 35% A/S, pH 8.00.) and were loaded separately onto two separate columns to determine the individual elution profiles of the pure enzymes. The two elution profiles of the pure enzymes were superimposed to determine what level of separation was achieved.

The elution buffer for the two pH studies differed. To investigate HIC at higher pH values, a Tris-HCl buffer was prepared at pH 8.00, and for the lower pH trials, a sodium Acetate buffer at pH 3.00 was prepared. Both buffers contained 35% A/S to mimic the reaction conditions on the factory floor. All experiments were conducted at 23^{o}C in a temperature-controlled laboratory. All buffers were prepared in advance and stored at 2 – 8 ^{o}C, but were allowed to acclimatise before application to the columns.

Experiment 1

The starting point for the investigation was to investigate the elution profiles of trypsinogen and CTG at a high pH value (pH 8.00). The column was equilibrated with Tris-HCl buffer, 35% A/S, pH 8.00. The samples were individually prepared (2.5 mg/ml) in 10 ml of a 0.05 M Tris-HCl buffer, pH 8.00 in 35% A/S. The samples were individually loaded onto two separate columns with a Gilson MINIPLUS 3 peristaltic pump at a consistent feeding rate of 1.5 ml/min and the column washed with 0.05 M Tris-HCl, 35% A/S, pH 8.00 until a baseline was achieved (as visualised by the in-line UV detector). Thereafter the samples were eluted with RO water at 1.5 ml/min until a baseline was achieved. Fractions (6.5 ml) were collected with a Pharmacia Biotech FRAC-100 fraction collector and the A_{280} values were determined for each individual fraction using a Schimadzu UV-1601 spectrophotometer.

The A_{280} values of each of the fractions were plotted on a graph to establish an elution profile for each of the enzymes. The elution profiles of trypsinogen and CTG were superimposed to establish if the two enzymes could be separated under the conditions.

Experiment 2

The second part of the investigation was to investigate the elution profile of trypsinogen and CTG at a low pH buffer, pH 3.00. The column was equilibrated with 35% A/S, pH 3.00. The samples were individually prepared (2.5 mg/ml) in 10 ml of a 0.05 M sodium acetate buffer, pH 3.00 in 35% A/S. The samples were individually loaded onto two separate columns with a Gilson MINIPLUS 3 peristaltic pump at a consistent feeding rate of 1.5 ml/min. The column was washed with 0.05 M sodium acetate buffer until a baseline was achieved (as visualised by the in-line UV detector), followed by elution with RO water at 1.5 ml/min. Fractions (6.5ml) were collected with a Pharmacia Biotech FRAC-100 fraction collector and tested for protein content using a Schimadzu UV-1601 spectrophotometer.

The A_{280} values of each of the fractions were plotted on a graph to establish an elution profile for each of the enzymes. The elution profiles of trypsinogen and CTG were superimposed to establish if the two enzymes could be separated under the conditions.

5.2.2.3 ION EXCHANGE CHROMATOGRAPHY OF TRYPSIN AND CHYMOTRYPSIN USING CM SEPHAROSE RESIN

To determine the elution profile of the active enzymes, purified preparations of trypsin and chymotrypsin were purchased from Sigma (Sigma life sciences, St. Louis, USA) as lyophilized powders.

A 10 ml CM sepharose column was packed under gravity in a 1.2 x 10 cm clear Perspex column housing with 7.5 ml of resin. CM Sepharose (fast flow) resin was obtained from GE healthcare (GE Healthcare, Uppsala, Sweden, Product # 17-0719-10) as a free sample. The column was fed with a Gilson MINIPLUS 3 peristaltic pump at a consistent feeding rate of 1.5 ml/min. 6.5 ml fractions were collected with a Pharmacia Biotech FRAC-100 fraction collector, and the A_{280} of the fractions was determined using a Schimadzu UV-1601 spectrophotometer. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. This instrument was used for indication purposes only, to visualise the elution profile in real-time.

All samples were prepared from a fresh weighing of lyophilized powder obtained from Sigma (Sigma life sciences, St. Louis, USA. Trypsin product # T8003-1G, chymotrypsin product # C4129-1G). All experiments were conducted at 23^{o}C in a temperature-controlled laboratory. All buffers were prepared in advance and stored at 2 – 8 ^{o}C, but were allowed to acclimatise before application to the columns. After every chromatographic run, the column was regenerated using 1 M NaCl and 1 M NaOH to remove any bound proteins, and subsequently equilibrated with the loading buffer of the next experiment.

The different techniques used to load and elute the enzymes included; 1) a stepwise NaCl elution where the ionic strength of the buffer was changed and the pH of the buffer kept constant. Increasing concentrations of NaCl in the elution buffer was considered in an attempt to separate trypsin and chymotrypsin. 2) A stepwise pH elution where the pH of the buffer was changed and the ionic strength was kept constant and 3) using $CaCl_2$ as an elution buffer.

Experiment 1

The starting point for the investigation was to investigate the elution profile of trypsin and chymotrypsin with a stepwise NaCl elution at low pH (pH 3.2). Trypsin and chymotrypsin samples were prepared (2.5 mg/ml) in 10 ml of 0.05 M sodium acetate buffer, pH 3.2 and were loaded separately onto two separate columns at a flow rate of 1.5 ml/min using a Gilson MINIPLUS 3 peristaltic pump. Three different eluting buffers were prepared containing increasing concentrations of NaCl (100 mM, 125 mM and 1 M NaCl in a 0.05 M Sodium acetate buffer, pH 3.2). The column was eluted with the eluting buffers. After each elution, the A_{280} was allowed to stabilise before applying the next elution buffer. Fractions (6.5ml) were collected and the A280 of each of the fractions was determined and plotted on a graph to establish an elution profile for each of the enzymes. The elution profiles were superimposed to establish if the two enzymes could be separated under these conditions.

Experiment 2

The effect of a stepwise (increasing) pH elution was investigated. A chymotrypsin sample was prepared (2.5 mg/ml) in 10 ml of 0.05 M sodium acetate, 0.05 M NaCl, pH 3.2 buffer. The column was equilibrated with 0.05 M sodium acetate, 0.05 M NaCl, pH 3.2 buffer. Prepared chymotrypsin sample was loaded onto the column at a flow rate of 1.5 ml/min using a Gilson MINIPLUS 3 peristaltic pump. Four different eluting buffers were prepared with varying pH values ranging from 4.0 – 5.5. A: 50 mM Na/Ac, 50 mM NaCl, pH 4.0 buffer, B: 50 mM Na/Ac, 50 mM NaCl, pH 4.5 buffer, C: 50 mM Na/Ac, 50 mM NaCl, pH 5.0 buffer, D: 50 mM Na/Ac, 50 mM NaCl, pH 5.5.

The column was eluted with the eluting buffers. After each elution, the A_{280} was allowed to stabilise before applying the next elution buffer. Fractions (6.5ml) were collected and the A_{280} of each of the fractions was determined and plotted on a graph to establish an elution profile for chymotrypsin. The protein content of each of the fractions was determined and plotted on a graph to establish an elution profile for each of the enzymes. The elution profile of chymotrypsin was assessed to see if separation from trypsin was possible.

Experiment 3

Following the stepwise pH elution trials, this experiment was conducted to investigate the elution profile of trypsin and chymotrypsin with a stepwise NaCl elution at pH 5.5 with increasing concentrations of NaCl ranging from 75 mM to 1 M NaCl. Trypsin and chymotrypsin samples were prepared (2.5 mg/ml) in 10 ml of 0.05 M sodium acetate, 0.05 M NaCl buffer, pH 5.5. Trypsin and chymotrypsin samples were loaded separately onto two separate columns at a flow rate of 1.5 ml/min using a Gilson MINIPLUS 3 peristaltic pump.

The column was equilibrated with 0.05 M sodium acetate, pH 5.5 buffer. Four different eluting buffers were prepared with increasing NaCl concentrations ranging from 75 mM to 150 mM. Elution buffer A: 0.05 M sodium acetate, 0.075 M NaCl, pH 5.5, elution buffer B: 0.05 M sodium acetate, 0.1 M NaCl, pH 5.5, elution buffer C: 0.05 M sodium acetate, 0.125 M NaCl, pH 5.5 and elution buffer D: 0.05 M sodium acetate, 0.15M NaCl, pH 5.5.

The column was eluted with the eluting buffers as specified. After each elution, the A_{280} was allowed to stabilise before applying the next elution buffer. Fractions (6.5 ml) were collected and the A_{280} of each of the fractions was determined and plotted on a graph to establish an elution profile for each of the enzymes. The protein content of each of the fractions were determined and plotted on a graph to establish an elution profile for each of the enzymes. The elution profiles were superimposed to establish if the two enzymes could be separated under the chromatographic conditions.

Experiment 4

Trypsin is at risk of auto activation at higher pH values. The elution profile of trypsin and chymotrypsin at low pH (pH <3.00) prevented the activation of trypsin. This experiment investigated the ability of CM resin at a very low pH to separate trypsin and chymotrypsin.

The column was equilibrated with 0.05 M Glycine-HCl buffer, pH 3.2. Three different eluting buffers were prepared with decreasing pH values ranging from 3.2 – 2.6. Buffer A: 50 mM Glycine-HCl buffer, pH 3.2, buffer B: 50 mM Glycine-HCl buffer, pH 2.8 and buffer C: 50 mM Glycine-HCl buffer, pH 2.6.

Trypsin and chymotrypsin samples were prepared at 2.5 mg/ml in 10 ml of 50 mM Glycine-HCl buffer, pH 3.2, and were loaded separately onto two separate columns at a flow rate of

1.5 ml/min using a Gilson MINIPLUS 3 peristaltic. The enzymes were eluted in a stepwise manner with 50 mM Glycine-HCl buffer at decreasing pH's.

The column was eluted with the eluting buffers as specified. After each elution, the A_{280} was allowed to stabilise before applying the next elution buffer. Fractions (6.5 ml) were collected and the A_{280} of each of the fractions was determined and plotted on a graph to establish an elution profile for each of the enzymes. The elution profiles were superimposed to establish if the two enzymes could be separated under the chromatographic conditions.

5.2.2.4 ION EXCHANGE CHROMATOGRAPHY OF TRYPSINOGEN AND CHYMOTRYPSINOGEN USING CM SEPHAROSE RESIN

The only technique used during the investigation of separation of the zymogens using Ion Exchange chromatography was considering different elution conditions of increasing concentrations of NaCl at three different pH values. The intent of this step was to replace the zymogen separation to allow rapid and efficient separation of the zymogens using ion exchange chromatography. A new 7.5 ml CM Sepharose column was packed under gravity in a
1.5 x 15 cm clear Perspex column with fresh resin for these experiments. CM Sepharose (fast flow) resin was obtained from GE healthcare (GE Healthcare, Uppsala, Sweden, Product # 17-0719-10) as a free sample.

Sigma lyophilized material (Sigma life sciences, St. Louis, USA. Trypsinogen product # T1143-1G, CTG product # C4879-1G) was used for the preparation of all the samples in order to establish elution profiles for the pure enzymes before sample material from the factory was used.

Two pH buffers were considered for the separation of trypsinogen and CTG using CM sepharose. Sodium phosphate buffers were used for the preparation of the samples because of the buffering capacity. The buffering capacity of Sodium phosphate buffer was higher than that of Sodium acetate buffer, as shown below.

- Buffering range of sodium acetate: pH 3.7 - 5.6. (Sigma Chemicals, 2003)
- Buffering range of sodium phosphate: pH 5.8 – 8.5 (Sigma Chemicals, 2003)

Experiment 1

The first experiment was the investigation of the elution profile of trypsinogen and CTG when loaded onto a CM Sepharose column at high pH values (pH 8.45) and eluted with increasing concentrations of NaCl.

Trypsinogen and CTG were individually prepared at 13.3mg/ml in 15 ml of a 0.05 M sodium phosphate buffer, pH 8.45 and were loaded separately onto two separate columns at a flow rate of 1.5 ml/min using a Gilson MINIPLUS 3 peristaltic pump.

The column was equilibrated with 0.05 M sodium phosphate buffer, pH 8.45 and after the samples were loaded, the column was washed at a flow rate of 1.5 ml/min with 0.05 M sodium phosphate buffer, 0.05 M NaCl, pH 8.45 and eluted at a flow rate of 1.5 ml/min with increasing NaCl concentration in the elution buffer.

The NaCl concentration range of the elution buffers investigated was 50 mM – 1 M (elution buffer. A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 8.45, buffer B: 0.05 M sodium phosphate, 0.2 M NaCl, pH 8.45 and buffer C: 1 M NaCl solution). After application of each eluting buffer, the A_{280} was allowed to stabilise before applying the next elution buffer. Fractions (7.5 ml) were collected for A_{280} determinations with a Pharmacia Biotech FRAC-100 fraction collector, and the A_{280} of the fractions was determined using a Schimadzu UV-1601 spectrophotometer. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. This instrument was used for indication purposes only, to visualise the elution profile in real-time.

The A_{280} values of the collected fractions were plotted to establish an elution profile for both enzymes. The elution profiles were superimposed to establish if the two enzymes could be separated under the chromatographic conditions.

Experiment 2

Following the investigation of zymogen elution at a high pH (pH 8.45), the elution profiles of the zymogens was investigated at a low pH (pH 6.1). The same strategy as experiment 1 was applied where the column was eluted in a stepwise fashion with increasing NaCl concentrations of the elution buffers at a set pH value.

The column equilibrated with 0.05 M sodium phosphate buffer, pH 6.1. The zymogens were individually prepared at 13.3 mg/ml in 15 ml of a 0.05 M sodium phosphate buffer, pH 6.1 and were loaded separately onto two separate columns at a flow rate of 1.5 ml/min using a Gilson MINIPLUS 3 peristaltic pump.

The column was equilibrated and washed at a flow rate of 1.5 ml/min with 50 mM podium phosphate buffer, pH 6.1 and eluted at a flow rate of 1.5 ml/min with increasing NaCl concentration in the elution buffer. The NaCl concentration range of the elution buffer was 50 mM – 1 M (elution buffer. A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 6.1, buffer B: 0.05 M sodium phosphate, 0.2 M NaCl, pH 6.1 and buffer C: 1 M NaCl solution).

Fractions (7.5 ml) were collected for A_{280} determinations with a Pharmacia Biotech FRAC-100 fraction collector, and the A_{280} of the fractions was determined using a Schimadzu UV-1601 spectrophotometer. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. This instrument was used for indication purposes only, to visualise the elution profile in real-time. The A_{280} values of the collected fractions were plotted to establish an elution profile for both enzymes.

Experiment 3

The elution profiles of trypsinogen and CTG were investigated at a pH value between that of experiment 1 and 2. Separation of the zymogens was investigated at pH 7.2 using a 0.05 M sodium phosphate buffer with increasing concentrations of NaCl in the elution buffers.

Trypsinogen and CTG were individually prepared at 13.3 mg/ml in 15 ml of a 0.05 M sodium phosphate buffer, pH 7.2 and were loaded separately onto two separate columns at a flow rate of 1.5 ml/min using a Gilson MINIPLUS 3 peristaltic pump.

The column was equilibrated with 0.05 M sodium phosphate buffer, pH 7.2 and after the samples were loaded, the column was washed at a flow rate of 1.5 ml/min with 0.05 M Sodium Phosphate buffer, 0.05 M NaCl, pH 7.2 and eluted at a flow rate of 1.5 ml/min with increasing NaCl concentration in the elution buffer.

The NaCl concentration range of the elution buffers investigated was 50 mM – 1 M (elution buffer. A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 7.2, buffer B: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2 and buffer C: 1 M NaCl solution). After application of each eluting buffer, the A_{280} was allowed to stabilise before applying the next elution buffer.

Fractions (7.5 ml) were collected for A_{280} determinations with a Pharmacia Biotech FRAC-100 fraction collector, and the A_{280} of the fractions was determined using a Schimadzu UV-1601 spectrophotometer. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. This instrument was used for indication purposes only, to visualise the elution profile in real-time

The A_{280} values of the collected fractions were plotted to establish an elution profile for both enzymes. The elution profiles were superimposed to establish if the two enzymes could be separated under the chromatographic conditions.

Experiment 4

This experiment was a repeat of experiment 3 where the zymogens were separated at pH 7.2 with increasing concentrations of NaCl in the elution buffers. The difference between experiment 3 and 4 was that trypsinogen and CTG were prepared together in a single preparation and loaded onto a single column.

Trypsinogen and CTG were prepared at 13.3 mg/ml in 15 ml of a 0.05 M sodium phosphate buffer, pH 7.2 and loaded onto the CM column at a flow rate of 1.5 ml/min using a Gilson MINIPLUS 3 peristaltic pump.

The column was equilibrated with 0.05 M sodium phosphate buffer, pH 7.2 and after the samples were loaded, the column was washed at a flow rate of 1.5 ml/min with 0.05 M sodium phosphate buffer, 0.05 M NaCl, pH 7.2 and eluted at a flow rate of 1.5 ml/min with increasing NaCl concentration in the elution buffer.

The NaCl concentration range of the elution buffers investigated was 50 mM – 1 M (elution buffer. A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 7.2, buffer B: 0.05 M Sodium phosphate, 0.15 M NaCl, pH 7.2 and buffer C: 1 M NaCl solution). After application of each eluting buffer, the A_{280} was allowed to stabilise before applying the next elution buffer. Fractions (7.5ml) were collected for A_{280} determinations with a Pharmacia Biotech FRAC-100 fraction collector, and the A_{280} of the fractions was determined using a Schimadzu UV-1601 spectrophotometer. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. This instrument was used for indication purposes only, to visualise the elution profile in real-time.

The A_{280} values of the collected fractions were plotted to establish an elution profile for both enzymes from the same column.

Experiment 5

A clarified 0/20 sample from the production line was loaded onto the CM column to investigate the ability of the CM column to separate the zymogens at pH 7.2 using representative material.

The sample was dialysed against 0.05 M sodium phosphate buffer, pH 7.2 in preparation for column chromatography and subsequently concentrated using a 10kDa ultrafiltration system until the product had a A_{280} of 245.

The column was equilibrated with 0.05 M sodium phosphate buffer, pH 7.2 and after the samples were loaded, the column was washed at a flow rate of 1.5 ml/min with 0.05 M sodium phosphate buffer, 0.05 M NaCl, pH 7.2 and eluted at a flow rate of 1.5 ml/min with increasing NaCl concentration in the elution buffer. The NaCl concentration range of the elution buffers investigated was 50 mM – 1 M (elution buffer. A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 7.2, buffer B: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2 and buffer C: 1 M NaCl solution). After application of each eluting buffer, the A_{280} was allowed to stabilise before applying the next elution buffer. Fractions (7.5ml) were collected for A_{280} determinations with a Pharmacia Biotech FRAC-100 fraction collector, and the A_{280} of the fractions was determined using a Schimadzu UV-1601 spectrophotometer. An in-line UV detector (Gilson 112 UV/VIS) was installed to monitor the elution profile. This instrument was used for indication purposes only, to visualise the elution profile in real-time

The A_{280} values of the collected fractions were plotted to establish an elution profile for both enzymes from the same column and the ability of the column to separate the zymogens by using representative product from the factory floor.

5.2.3. RESULTS

5.2.3.1 AFFINITY CHROMATOGRAPHY

Experiment 1

For experiment 1 the elution profile of Sigma trypsin was obtained by plotting the A_{280} values obtained for each fraction collected (see figure 29). Elution of trypsin was achieved as described in the protocol recommended by GE Healthcare (GE Healthcare, 2005).

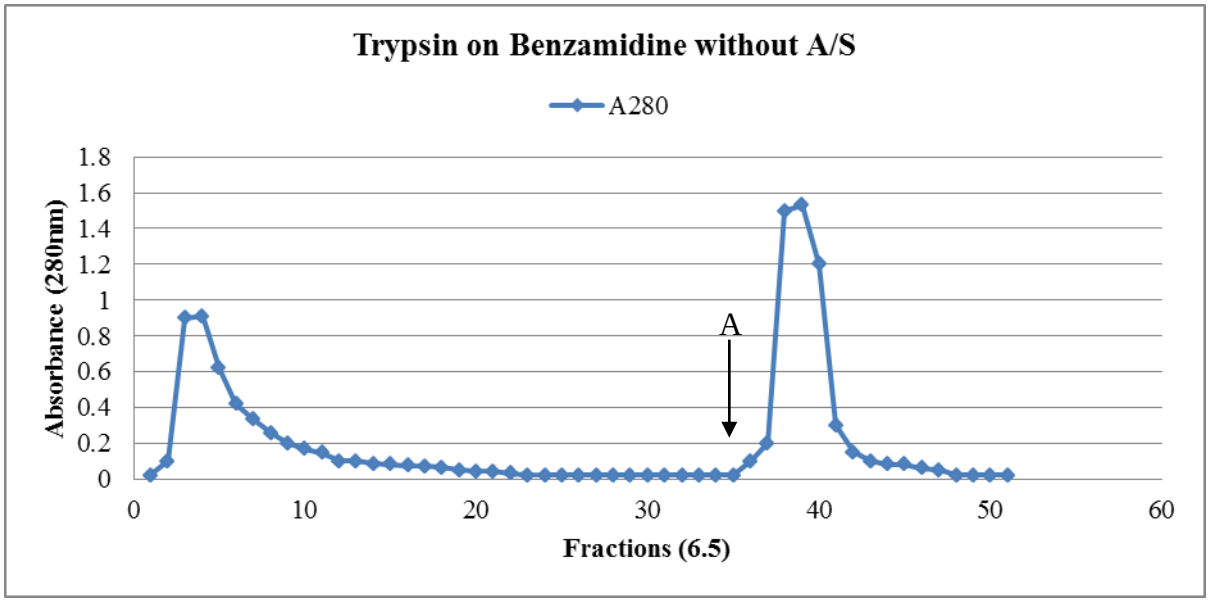


Figure 29. Elution profile of Sigma trypsin using benzamidine sepharose as an affinity resin. The sample was prepared and loaded onto the column in 0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4, and eluted with elution buffer A: 0.05 M Glycine buffer, pH 3.00.

Experiment 2

The elution of trypsin from a benzamidine column was investigated in the presence of 35% A/S to mimic the material on the factory floor. Activated trypsin from the production line was collected and loaded onto the column. Figure 30 shows the elution profile of the trypsin obtained from the production line. Elution of trypsin was achieved as described in the protocol recommended by GE Healthcare (GE Healthcare, 2005).

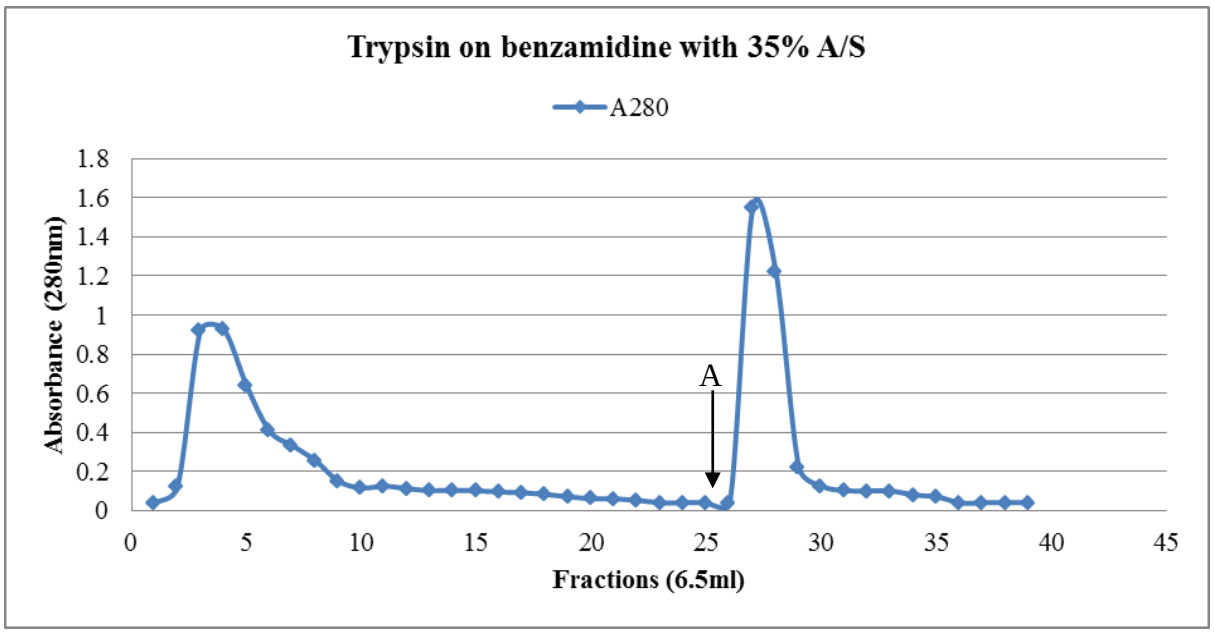


Figure 30. Elution profile of trypsin when loaded onto Benzamidine affinity column in the presence of 35% A/S. The column was loaded and washed with 0.05 M Tris-HCl, 0.5 M NaCl, 35% A/S, pH 7.4, and eluted with elution buffer A: 0.05 M Glycine buffer, 35% A/S, pH 3.00.

In both these experiments, separation of trypsin from chymotrypsin was achieved. There was no marked difference in the elution profiles between the pure preparations of trypsin and chymotrypsin compared to that of the material obtained from the production line. The specific activity of the material that was eluted from the columns is summarized in table 19 below. Chymotrypsin did not bund to the column , and was collected in the flow through, whereas trypsin eluted after application of buffer A.

Table 19. Summary of the activity assay results of the fractions collected from the Benzamidine column.

Experiment	Trypsin Specific activity (U/mg) (Fraction A)	Chymotrypsin specific activity(U/mg) (Flow through)
Experiment 1	2304.8	661.5
Experiment 2	3252.9	448.6

5.2.3.2 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Experiment 1

The starting point for the investigation was to investigate the elution profiles of trypsinogen and CTG at a high pH value (pH 8.00). Trypsin and CTG samples were individually prepared in 0.05 M sodium acetate buffer, pH 8.00 in 35% A/S. The zymogens were loaded onto two separate columns and were eluted with RO water. The elution profiles of trypsinogen and CTG were obtained by plotting the A_{280} values of each fraction, and the two elution profiles were superimposed to assess the degree of separation that was possible (see figure 31).

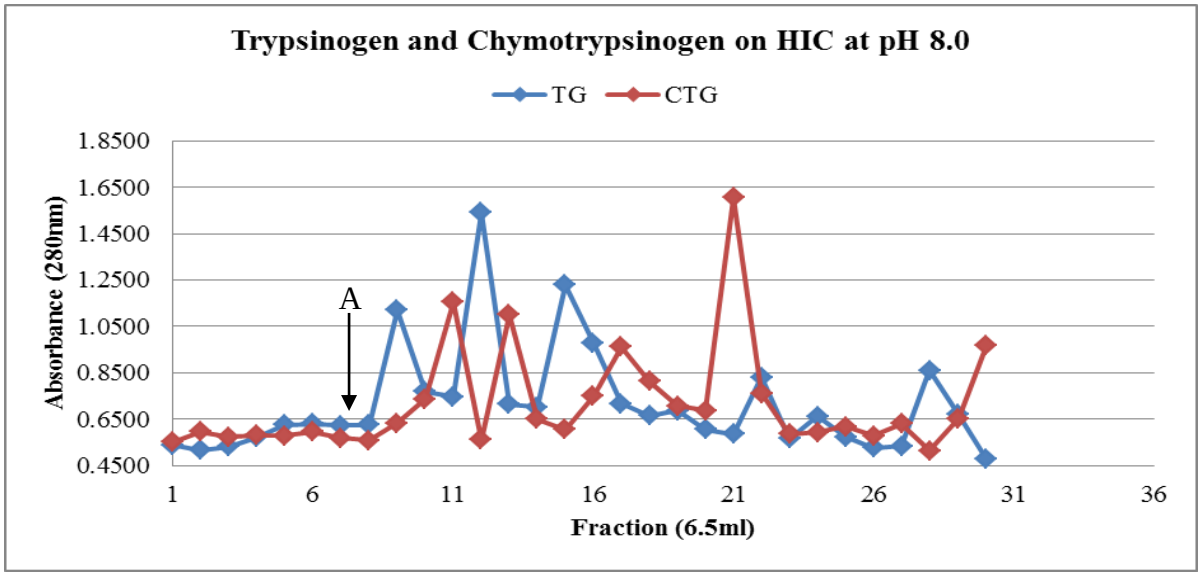


Figure 31. Superimposed elution profiles (A_{280}) of the two individual experiments when the zymogens were loaded onto phenyl sepharose resin at high pH (pH 8.00). The column was eluted with RO water (A) at a constant rate of 1.5ml/min.

Experiment 2

The second part of the investigation was to investigate the elution profile of trypsinogen and CTG at a low pH buffer, pH 3.00. Figure 32 shows the superimposed elution profiles of zymogens using HIC chromatography where samples were individually loaded onto two columns. Both enzymes were prepared and loaded in 0.05 M sodium acetate buffer, pH 3.00 in 35% A/S, and was eluted with RO water. The elution profiles of trypsinogen and CTG were obtained by plotting the A_{280} values of each fraction, and the two elution profiles were superimposed to assess the degree of separation that was possible (see figure 32).

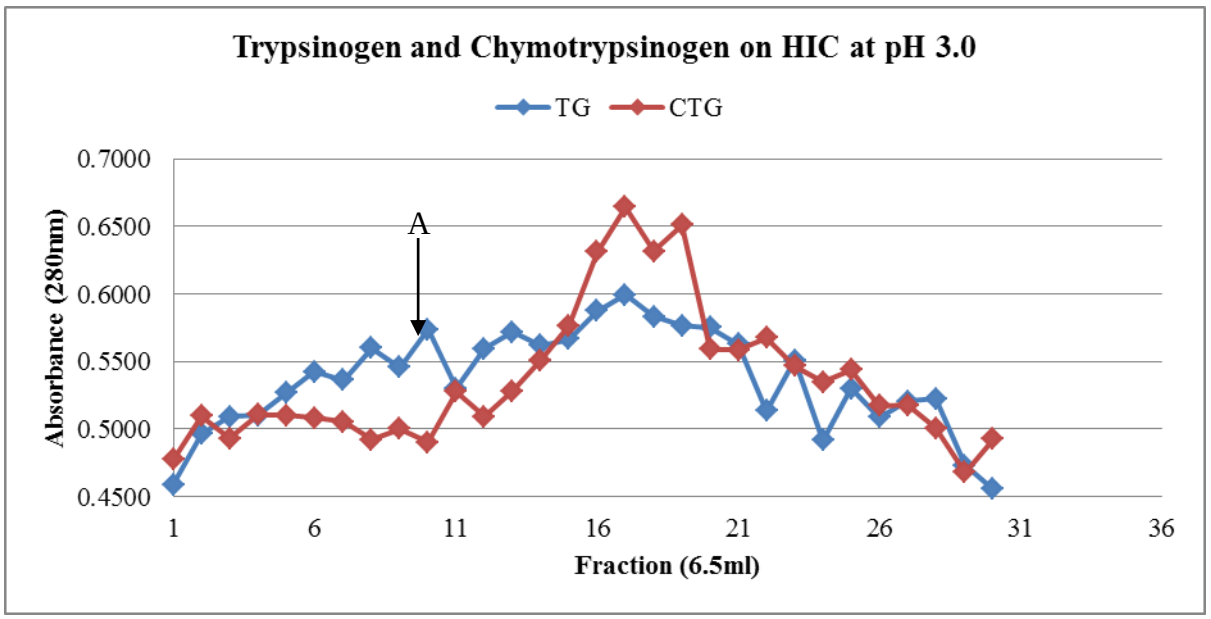


Figure 32. Superimposed elution profiles (A_{280}) of the two individual experiments when the zymogens were loaded onto Phenyl Sepharose resin at low pH (pH 3.00). Column was eluted with RO water (A) at a constant rate of 1.5 ml/min.

Both elution profiles obtained in experiment 1 and 2 indicate that the zymogens cannot be separated using HIC at pH 8.00 and 3.00 when loaded with a 0.05 M sodium acetate buffer in 35% A/S. The elution profiles of trypsinogen and CTG overlapped, and no clear separate peaks were observed.

5.2.3.3 ION EXCHANGE CHROMATOGRAPHY OF TRYPSIN AND CHYMOTRYPSIN

Experiment 1

The starting point for the investigation was to investigate the elution profile of trypsin and chymotrypsin with a stepwise NaCl elution at low pH (pH 3.2). Trypsin and chymotrypsin samples were individually prepared and loaded onto two separate columns. Three different eluting buffers were prepared containing increasing concentrations of NaCl (100 mM, 125 mM and 1M NaCl in a 0.05 M sodium acetate buffer, pH 3.2). The elution profiles of trypsin and chymotrypsin were obtained by plotting the A_{280} values of each fraction, and the two elution profiles were superimposed to assess the degree of separation that was possible (see figure 33).

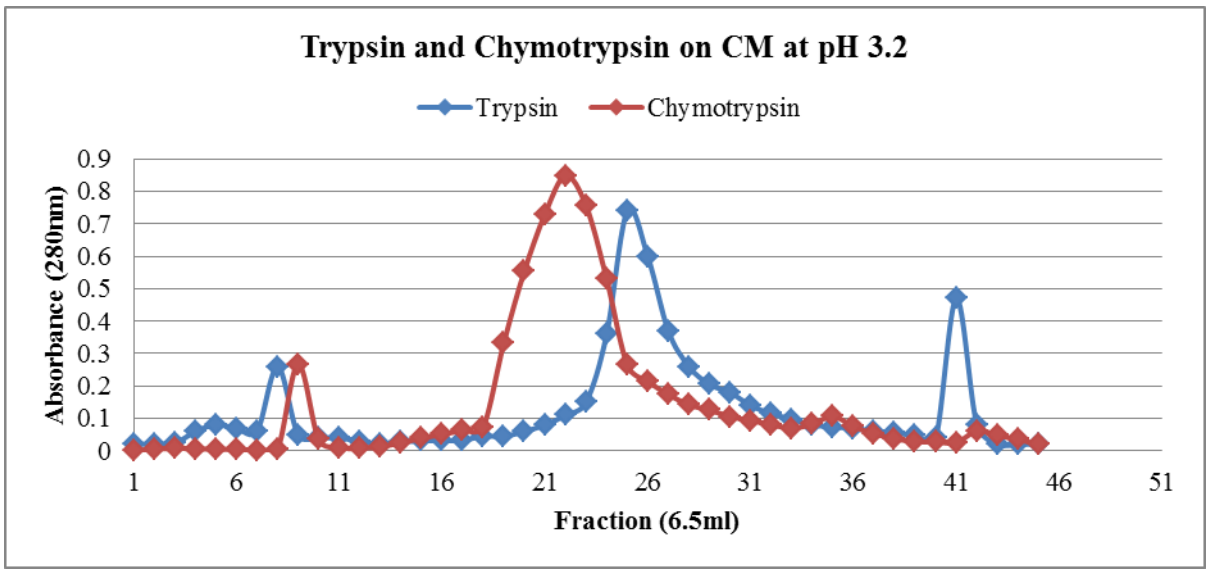


Figure 33. Superimposed elution profile of Sigma trypsin and chymotrypsin on CM-Sepharose loaded with 50 mM sodium acetate buffer, pH 3.2 and eluted with the same buffer containing increasing concentrations of sodium chloride.

No separation of trypsin and chymotrypsin was achieved when the column was run under these conditions. The two enzymes had near identical elution profile as shown in figure 34.

Experiment 2

The effect of a stepwise (increasing) pH elution was investigated to separate trypsin and chymotrypsin. Four different eluting buffers were prepared with varying pH values. Elution buffer A: 50 mM Na/Ac, 50 mM NaCl, pH 4.0 buffer, B: 50 mM Na/Ac, 50 mM NaCl, pH 4.5 buffer, C: 50 mM Na/Ac, 50 mM NaCl, pH 5.0 buffer, D: 50 mM Na/Ac, 50 mM NaCl, pH 5.5. Figure 34 shows the protein elution profile (A_{280}) of the pH stepwise elution of chymotrypsin.

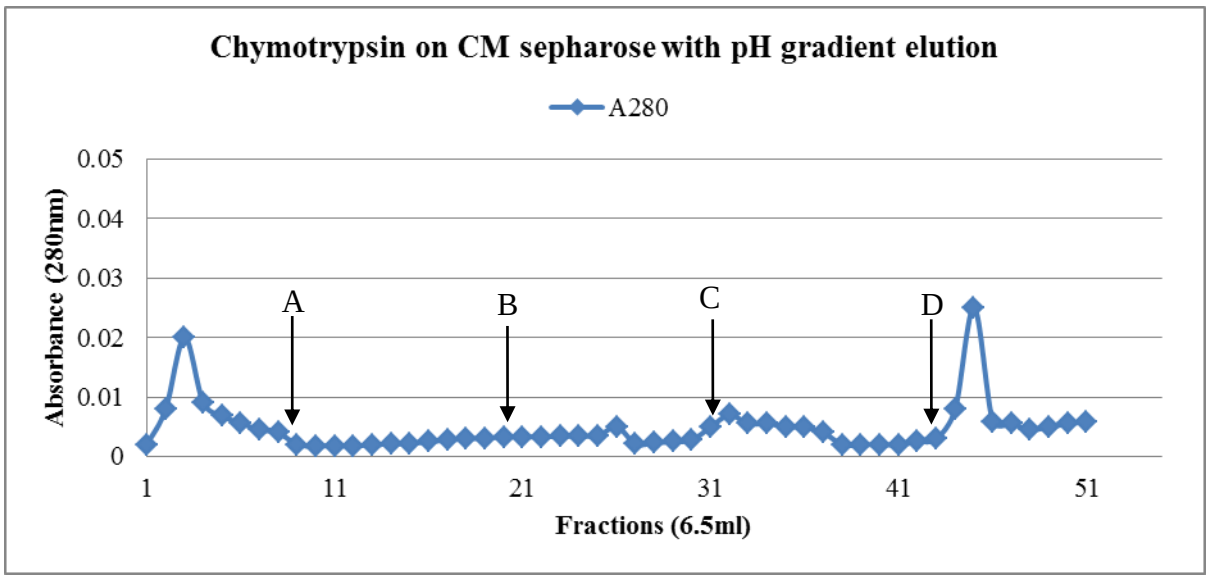


Figure 34. Elution profile of chymotrypsin when loaded onto a CM Sepharose column in 0.05 M sodium acetate buffer, 0.05 M NaCl, pH 3.5, and elution with increasing pH of the eluting buffer. Elution buffer A: 50 mM sodium acetate, 50 mM NaCl, pH 4.0, Elution buffer B: 50 mM sodium acetate, 50 mM NaCl, pH 4.5, Elution buffer C: 50 mM sodium acetate, 50 mM NaCl, pH 5.0, Elution buffer D: 50 mM sodium acetate, 50 mM NaCl, pH 5.5.

No clear chymotrypsin peak was observed as a result of one of the elution buffers applied. Therefore, the conditions used as described above were not suitable for separation of trypsin from chymotrypsin.

Experiment 3

Following the stepwise pH elution trials, this experiment was conducted to investigate the elution profile of trypsin and chymotrypsin with a stepwise NaCl elution at fixed pH of 5.5 with increasing concentrations of NaCl in the elution buffers ranging from 75 mM to 1 M NaCl. The elution profiles of trypsin and chymotrypsin were obtained by plotting the A_{280} values of each fraction, and the two elution profiles were superimposed to assess the degree of separation that was possible. Figure 35 shows the superimposed A_{280} elution profile of trypsin and chymotrypsin.

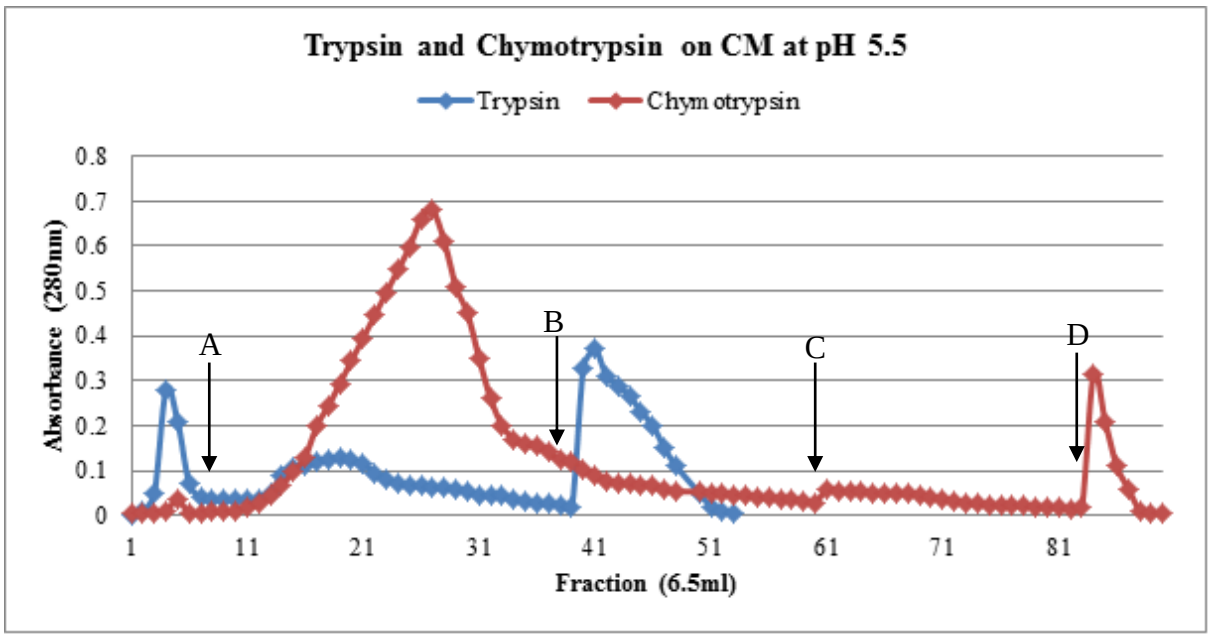


Figure 35: Superimposed elution profile of trypsin and chymotrypsin on CM-Sepharose loaded in 0.05 M sodium acetate, 0.05 M NaCl buffer, pH 5.5 and eluted with increasing concentration of NaCl in the elution buffers. Four different eluting buffers were prepared with increasing NaCl concentrations. Elution buffer A: 0.05 M sodium acetate, 0.075 M NaCl, pH 5.5, elution buffer B: 0.05 M sodium acetate, 0.1 M NaCl, pH 5.5, elution buffer C: 0.05 M sodium acetate, 0.125 M NaCl, pH 5.5 and elution buffer D: 0.05 M sodium acetate, 0.15M NaCl, pH 5.5.

Fractions eluted with 0.075 M NaCl (buffer A) had little protein content as detected by A_{280} but had significant amount of trypsin activity in the same area where chymotrypsin eluted. The high trypsin activity in this area was concerning as the activity of trypsin will cause autolysis of chymotrypsin. These conditions were therefore not suitable for the separation of trypsin from chymotrypsin.

Experiment 4

Trypsin is at risk of auto activation at higher pH values. The elution profile of trypsin and chymotrypsin at low pH (pH <3.00) prevented the activation of trypsin. This experiment investigated the ability of CM resin at a very low pH to separate trypsin and chymotrypsin. Figure 36 shows the protein elution profile (A_{280}) of the investigation of CM chromatography at a low pH range using glycine buffer. Samples were prepared in 0.05 M Glycine-HCl buffer, pH 3.2, and were loaded onto the column that had been equilibrated with the same buffer. The enzymes were eluted in a stepwise manner with 50 mM Glycine-HCl buffer at decreasing pH's. Three different eluting buffers were prepared with decreasing pH values ranging from

3.2 – 2.6. Buffer A: 50 mM Glycine-HCl buffer, pH 3.2, buffer B: 50 mM Glycine-HCl buffer, pH 2.8 and buffer C: 50 mM Glycine-HCl buffer, pH 2.6.

The elution profiles of trypsin and chymotrypsin were obtained by plotting the A_{280} values of each fraction, and the two elution profiles were superimposed to assess the degree of separation that was possible (see figure 36).

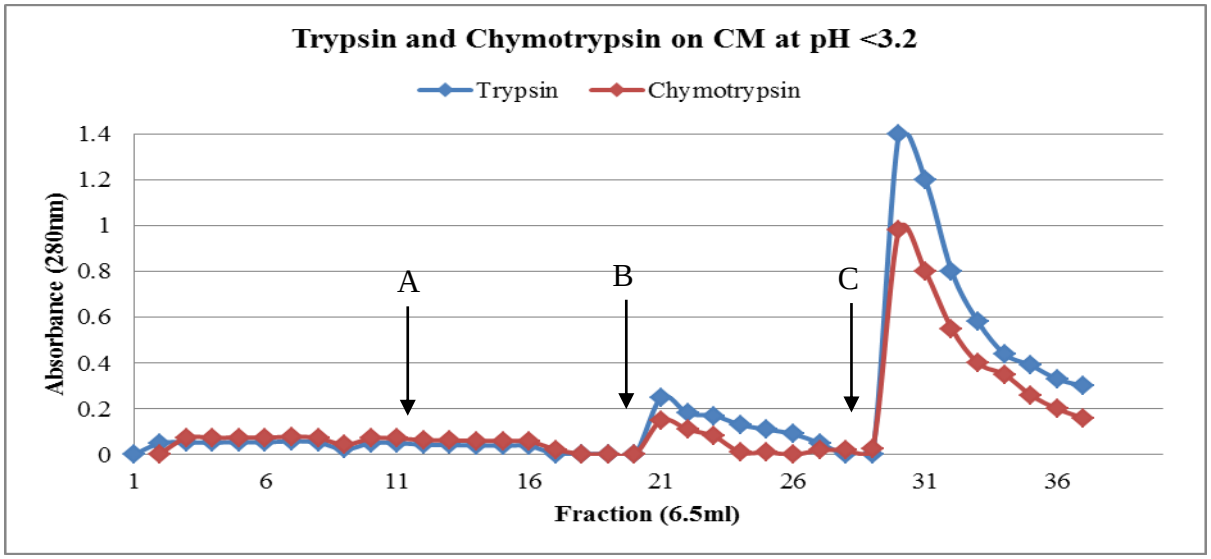


Figure 36. Elution profile of trypsin and chymotrypsin on CM-Sepharose at low pH (<3.00). The enzyme dissolved in 50 mM Glycine- HCl buffer, pH 3.2 was charged on the column that had been equilibrated with the same buffer. The enzyme was eluted in stepwise manner with 50 mM Glycine-HCl buffer at decreasing pH: A: pH 3.00, B: pH 2.8, C: pH 2.6.

The elution profiles of the two enzymes were very similar and no separation of the two enzymes was achieved under the conditions described. If the two peaks observed could be further resolved, these eluting conditions had potential to further separate the two enzymes.

None of the above mentioned experiments was able to successfully separate trypsin from chymotrypsin. This does not imply that it is not possible to separate the two enzymes using Ion Exchange chromatography. Future investigations of chromatographic separation of trypsin from chymotrypsin using Ion Exchange chromatography might include the use of the weak anion exchange DEAE resin.

5.2.3.4 BINDING STUDIES OF TRYPSINOGEN AND CHYMOTRYPSINOGEN TO CM SEPHAROSE RESIN

The only technique used during the investigation of separation of the zymogens using ion exchange chromatography was considering different elution conditions of increasing concentrations of NaCl at three different pH values.

Experiment 1

The first experiment was the investigation of the elution profile of trypsinogen and CTG when loaded onto a CM column at high pH values (pH 8.45) and eluted with increasing concentrations of NaCl in the elution buffers. Figure 37 shows the superimposed A_{280}elution profile of the zymogens at pH 8.45 using a 50 mM sodium phosphate buffer with increasing concentrations of NaCl.

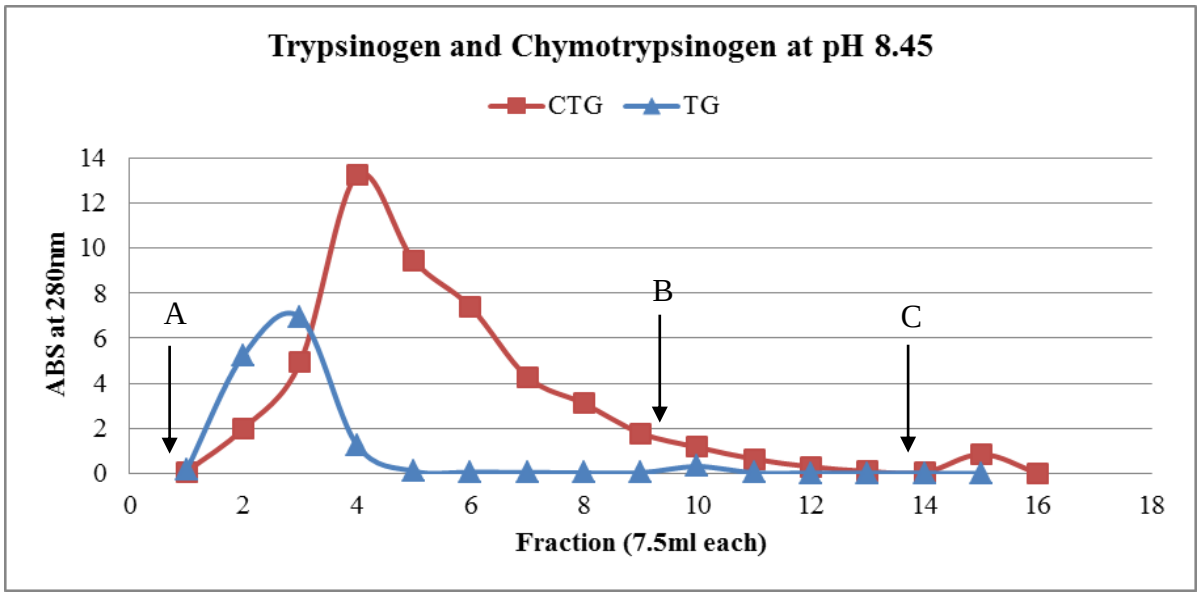


Figure 37. Elution profile of trypsinogen and CTG at pH 8.45 with increasing concentrations of NaCl in the elution buffer. Elution buffer A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 8.45, elution buffer B: 0.05 M sodium phosphate, 0.2 M NaCl, pH 8.45 and buffer C: 1 M NaCl solution.

In both cases, trypsinogen and CTG eluted with the initial elution buffer at 0.05 M NaCl. An increase in buffer molarity up to 1 M NaCl did not lead to more protein eluting from the column. This indicated that trypsinogen and CTG could not be separated when loaded in 50 mM sodium phosphate, pH 8.45.

Experiment 2

Following the investigation of zymogen elution at a high pH (pH 8.45), the elution profiles of the zymogens was investigated at a low pH (pH 6.1). The same strategy as experiment 1 was applied where the column was eluted in a stepwise fashion with increasing NaCl concentrations of the elution buffers at a set pH value. Figure 38 and 39 shows the protein A_{280} elution profile of the zymogens at pH 6.1 using a 50 mM sodium phosphate buffer with increasing concentrations of NaCl.

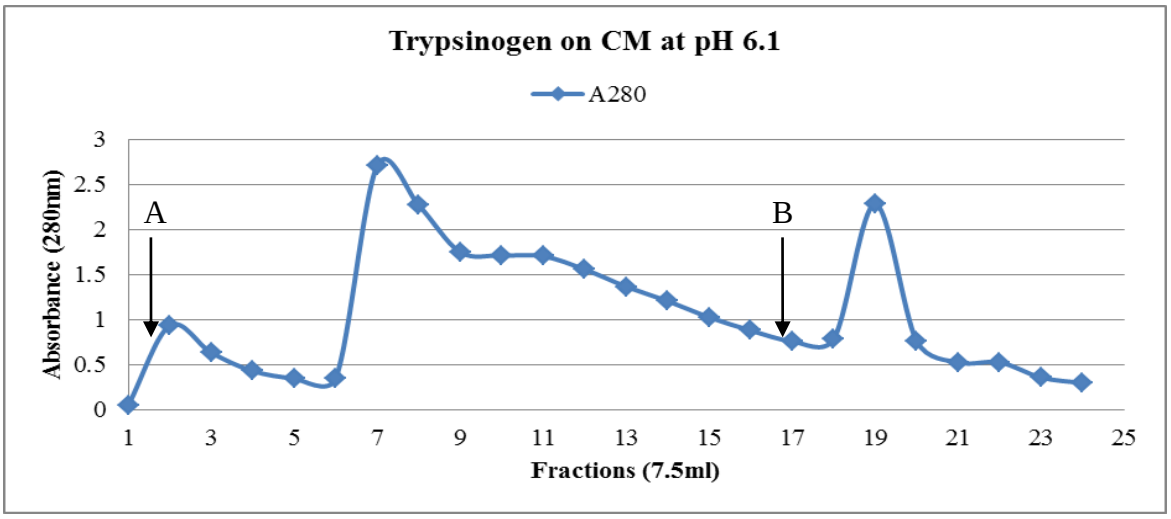


Figure 38. Elution profile of trypsinogen using a sodium phosphate buffer, pH 6.1 with increasing concentrations of NaCl in the elution buffer. Elution buffer A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 6.1, elution buffer B: 0.05 M sodium phosphate, 0.2 M NaCl, pH 6.1.

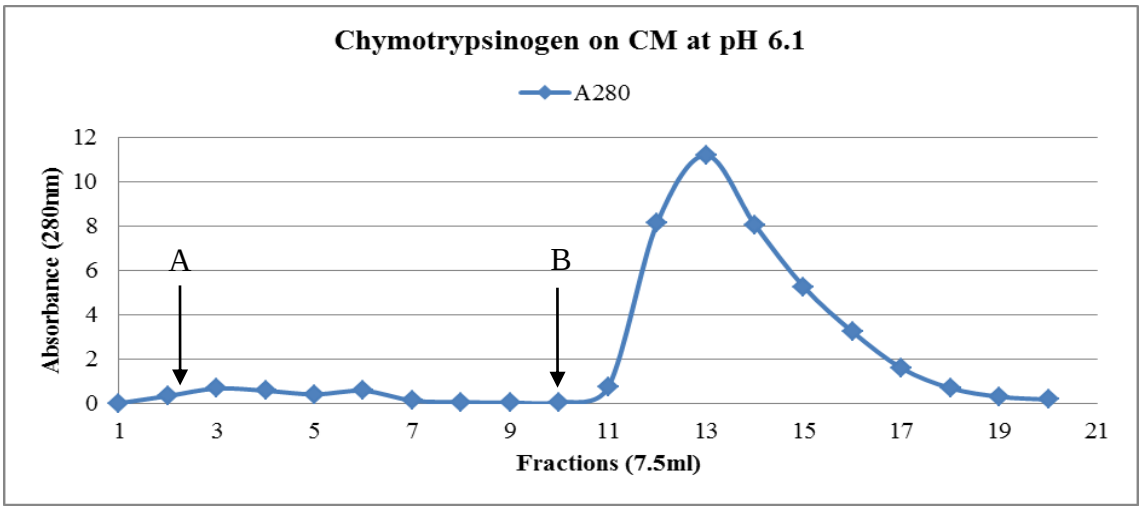


Figure 39. Elution profile of chymotrypsinogen using a Sodium Phosphate buffer, pH 6.1 with increasing concentrations of NaCl in the elution buffer. Elution buffer A: 0.05M sodium phosphate, 0.05 M NaCl, pH 6.1, elution buffer B: 0.05 M sodium phosphate, 0.2 M NaCl, pH 6.1.

It was not possible to completely separate trypsinogen from CTG under these conditions as the trypsinogen peak slightly overlapped with the CTG peak. The CTG elution profile looked promising, as there was clearly no elution of CTG at NaCl concentration < 150 mM. There was however a difference between the elution profiles compared to that of experiment 1 that was conducted at pH 8.45.

Experiment 3

The elution profiles of trypsinogen and CTG were investigated at a pH value between that of experiment 1 and 2. Separation of the zymogens was investigated at pH 7.2 using a 0.05 M sodium phosphate buffer with increasing concentrations of NaCl in the elution buffers.

The trypsinogen elution profile of experiment 1 (figure 37) and the CTG elution profile of experiment 2 (figure 39) indicated that it was possible to separate these two enzymes.

Figure 40 shows the A_{280} elution profile of the investigation of the separation of the zymogens at pH 7.2 using a 50 mM Sodium phosphate buffer with increasing concentrations of NaCl.

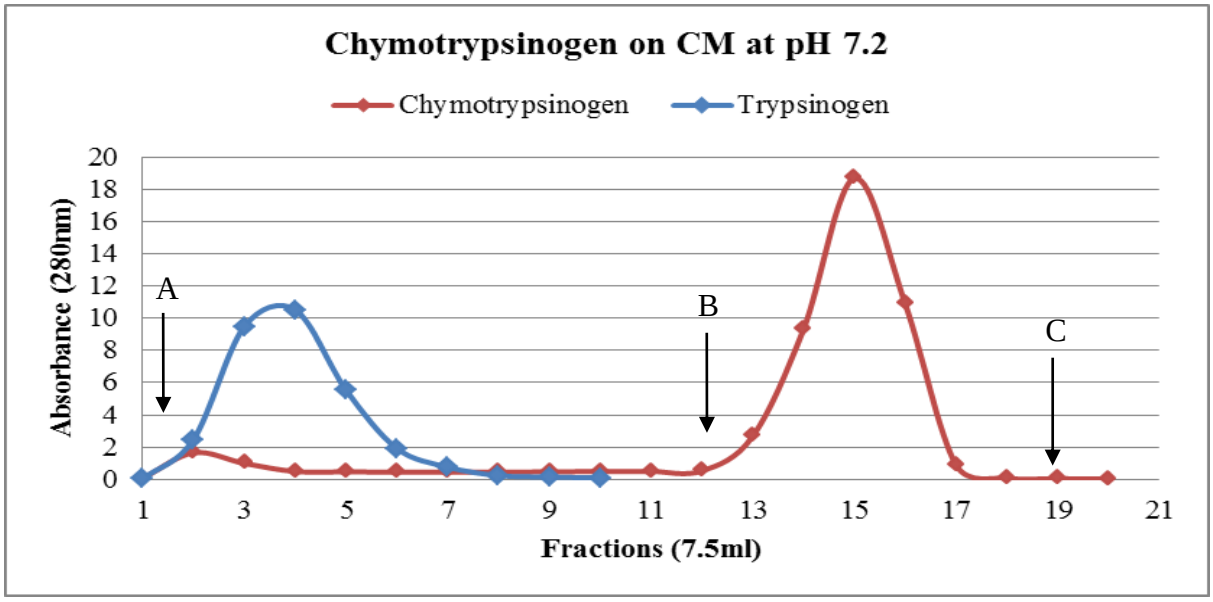


Figure 40. Elution profile of trypsinogen and CTG on a CM sepharose column at pH 7.2 with increasing NaCl concentrations. Elution buffer. A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 7.2, buffer B: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2 and buffer C: 1 M NaCl solution.

The elution profiles in figure 40 indicated that when the column was loaded with 50 mM Sodium Phosphate buffer, pH 7.2 and eluted in two simple steps with 0.05 M and 0.15 M NaCl in the loading buffer, the two zymogens could be separated. The results were obtained

using pure Sigma material. Two further trials using these conditions were conducted to evaluate the finding of experiment 3.

Experiment 4

This experiment was a repeat of experiment 3 where the zymogens were separated at pH 7.2 with increasing concentrations of NaCl in the elution buffers. The difference between experiment 3 and 4 was that trypsinogen and CTG were prepared together in a single preparation and loaded onto a single column. Figure 41 shows the A_{280} elution profile of the combined zymogens at pH 7.2 using a 0.05 M sodium phosphate buffer with two concentrations of NaCl, first with 0.05 M followed by an elution with 0.15 M NaCl.

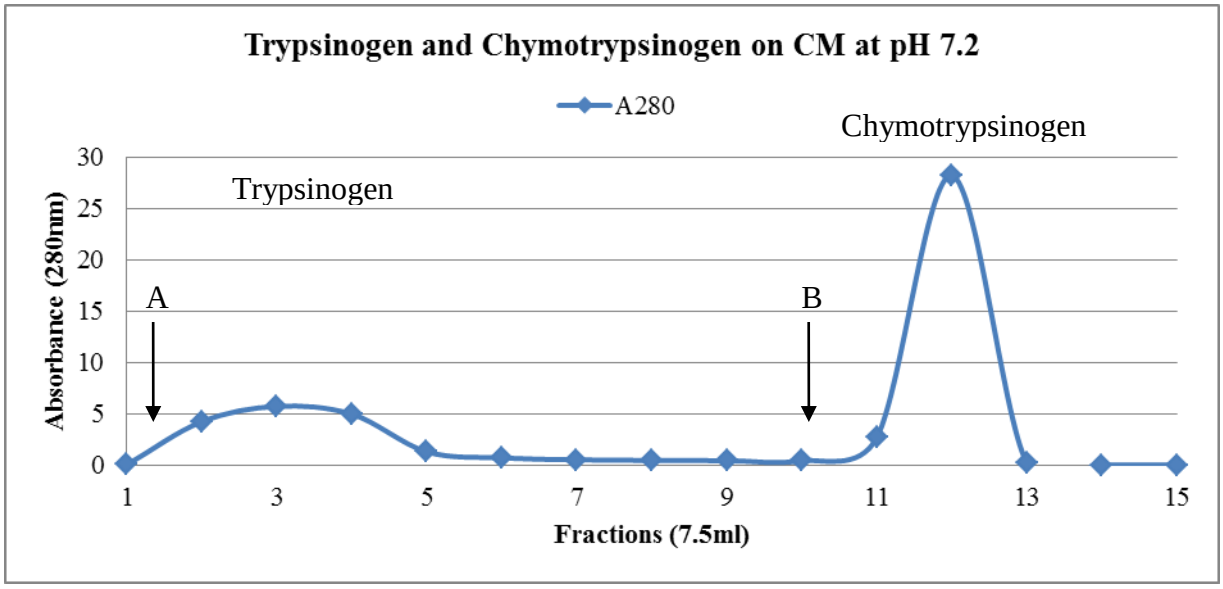


Figure 41. Elution profile of a mixture of trypsinogen and CTG using a sodium phosphate buffer, pH 7.2 and eluting only with 0.05 M NaCl and 0.15 M NaCl in the elution buffer. Fractions 1 – 4 was trypsinogen and fractions 11 – 13 was CTG. Elution buffer A: 0.05 M sodium phosphate, 0.05 M NaCl, pH 7.2, elution buffer B: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2.

From the elution profile in figure 41 it was evident that it was possible to separate the two zymogens from each other using the conditions as described. Two separate peaks were observed and there was no overlap between the two.

Experiment 5

This experiment was carried out using material obtained from the factory floor. 0/20 clarified material was collected, and was concentrated until the material reached an A_{280} of 245. The material was loaded onto CM resin according to the method described. The two fractions were collected and diafiltered until salt-free in preparation for lyophilisation.

The column was eluted with 0.05 M sodium phosphate, 0.05 M NaCl, pH 7.2 and 0.05 M sodium phosphate 0.15 M NaCl, pH 7.2. Figure 42 shows the A_{280} elution profile of the investigation of the separation of the clarified 0/20 product at pH 7.2. The lyophilized product was dissolved and activated as described in section 3.2.3, and lyophilised. The lyophilized product was assayed as per the assay methods for both trypsin and chymotrypsin.

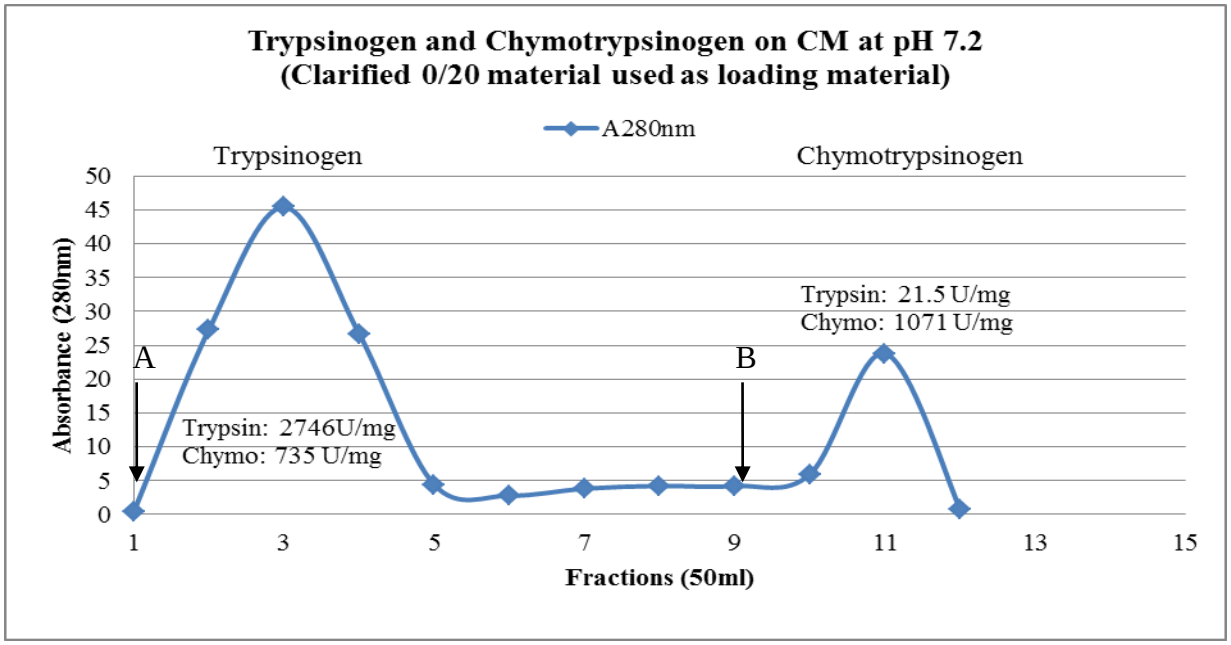


Figure 42. Elution profile of a clarified 0/20material from the factory floor using a Sodium Phosphate buffer, pH 7.2 and eluting only with 50 mM NaCl and 150 mM NaCl in the elution buffer. Fractions 1 – 3 was trypsin and fractions 11 – 13 was CTG. Elution buffer A: 0.05M NaCl and buffer B: 0.15 M NaCl.

As seen in experiment 4, two separate peaks observed when this production sample was separated on CM resin under the described conditions, indicating successful separation of two enzymes. It was possible to separate trypsinogen from CTG under the conditions described. The chymotrypsin activity observed in the trypsin fraction could be β-chymotrypsinogen (Delaage, 1968) which did not elute with the rest of the α-chymotrypsinogen; however the presence of β-chymotrypsinogen in the supernatant still needed to be confirmed by iso-electric focussing.

5.2.4. CONCLUSION

The aim of the investigation was to develop chromatographic methods to purify trypsin(ogen) from chymotrypsin(ogen). Three different chromatographic strategies were investigated in an attempt to separate the enzymes, Affinity, hydrophobic interaction and ion exchange chromatography.

5.2.4.1 AFFINITY CHROMATOGRAPHY

The material obtained from affinity chromatography was not pure enough (see table 19) to satisfy the requirement according to the product specification for pure trypsin, the chymotrypsin content in the trypsin samples was too high (specification: <50 U/mg). It was, however a satisfactory purification result, as trypsin could be purified in a single chromatographic step. The material would have been sufficiently pure to market as a lower grade of trypsin, but not for the pure grade trypsin (>2500 U/mg trypsin and <50 U/mg chymotrypsin). These results implied that the benzamidine ligand was not sufficiently specific for trypsin and also bound chymotrypsin, which eluted with the trypsin. This was not an uncommon occurrence, as it was also experienced by Evans *et al*. (1982) .

GE Healthcare indicated that the lifespan of benzamidine resin for industrial application was approximately 300 purification cycles. This implied that the resin needed to be replaced after every 300 purification cycles. Affinity resins are extremely expensive, and this was thus not a financially viable option as the resin (56 L) needed to be replaced annually at a cost of R160,000/L, resulting in a total cost of replacement of R9 million. Even though proper separation and a semi-pure trypsin product was obtained using benzamidine affinity chromatography, this was not a viable production scale purification methodology.

The use of p-Aminobenzamidine was investigated as a purification technique for trypsin specifically. This step was envisaged to be implemented after trypsin activation as a potential replacement for the 7-day trypsin crystallization. Although we were able to purify trypsin using benzamidine resin, the resultant product did not comply with the specific activity criteria (specifically the chymotrypsin content). The chymotrypsin content (U/mg material) was too high as a result of some competitive binding to the resin. The product that was eluted from this column did comply with the specifications of a lower grade of trypsin (Trypsin: Chymotrypsin 6:1).

The capital investment required to purchase approx. 56 L of benzamidine resin was outside the budget of the entire project that made this a less favourable option. According to the

manufacturer, the lifespan of the resin would be approx. 2 years of continual usage, which was another financial constraint, as the cost to replace the resin would be approximately R9 million.

Benzamidine affinity resin was thus not a viable option to purify trypsin from chymotrypsin for our industrial application.

5.2.4.2 HYDROPHOBIC INTERACTION CHROMATOGRAPHY

According to the results of both these experiments, it was concluded that it was not possible to separate the zymogens using HIC with the conditions described as there were overlapping elution profiles (of trypsinogen and CTG) and no separate enzyme peaks observed. This does not imply that it is impossible to separate the two zymogens using HIC, as there are still more loading and elution conditions that were not investigated.

Hydrophobic interaction chromatography was investigated specifically as a potential replacement of the 48-hour CTG crystallization step. The intent was only to separate the zymogens. None of the strategies applied to investigate HIC was successful in separating the zymogens as a replacement for the Zymogen separation step. This did not imply that it was impossible to separate the zymogens using HIC. The timeframe for the investigation did not allow the investigation of further HIC development.

HIC (using the defined conditions) was thus also not a viable option to separate the zymogens as a replacement for the zymogen separation step.

5.2.4.3 ION EXCHANGE CHROMATOGRAPHY

Ion exchange chromatography was the last method investigated to separate both the active and the inactive enzymes. None of the conditions investigated to separate the active enzymes was successful in separating the active enzymes. This did not imply that it was impossible to separate the two active enzymes.

The intent to separate the inactive enzymes was to replace the 48-hour Zymogen separation. A sodium Phosphate buffer, pH 7.2 and eluting buffers 50 mM NaCl and 150 mM NaCl were used to separate the zymogens using a weak cation exchange resin.

The specific activities of the material produced using this method did not reach the required levels of trypsin and chymotrypsin. Further processing was required to produce a product that complied with the required specification. It was, however, a successful separation of the two zymogens, and the methodology could be applied in the production environment. The risk associated with working at the relative high pH (pH 7.2) was that the trypsin could potentially activate over a period of time, which could lead to reduced recoveries over the step. This was verified when the column was operated in a cold room for extended periods and the recovery over the step was approximately 50%. This could be overcome if the CM column is operated at $<3^{o}C$ to prevent the catalytic effect of trypsin (Outzen, 1996).

5.3 INVESTIGATING THE FEASIBILITY OF USING NON ACID DIPPED PANCREAS AS A RAW MATERIAL SOURCE FOR PROTEASE ENZYME PRODUCTION

5.3.1 INTRODUCTION

The aim of this investigation was to determine the feasibility of using quick frozen non acid dipped bovine pancreas for the production of pancreas derived factors (PDF) at BBI Enzymes' Cape Town production facility. The investigation was driven by the need to reduce the cost of the raw material input to the PDF process. Treated bovine pancreas was available at $2.14 per kg whereas untreated pancreas could be purchased at $ 1.70 per kg, which made untreated pancreas an attractive option as a raw material.

Simple laboratory techniques, such as protein determination (A_{280}), activity assays and SDS PAGE analysis were used to characterize the product as it was processed. The aim of the laboratory investigation was to establish if there were significant differences in the processing and the amount of trypsin present from both these sources, and to qualify untreated bovine pancreas as a raw material source for the production of trypsin and chymotrypsin.

5.3.2. MATERIALS AND METHODS

To investigate the feasibility of processing quick frozen non-acid dipped bovine pancreas, a small-scale laboratory investigation was carried out. Acid dipped and quick frozen non-acid dipped pancreases were purchased from a local supplier (Jack Grey tissue supplies) from the

Free State in South Africa. The untreated pancreases were frozen immediately after removal from the animals to eliminate the possibility of trypsin activation, which could lead to activation of CTG and potentially caused autolysis during processing. The acid treated pancreas were dipped in 0.25 M H_2SO_4 for 30 min before they were frozen.

A) Extraction

Frozen pancreas (acid dipped and non-acid dipped) was cut into thin slices by first flaking large frozen blocks into smaller flakes using an industrial scale flaker, and afterwards blending the flaked pancreas pieces with a desktop blender (using water as a liquid phase) into a pulp to liberate maximal amounts of protein. The blended pancreas was extracted overnight (16 h) in acidic water containing H_2SO_4 (pH 1.9 – 2.2) with continuous stirring. The pH of both extraction media was adjusted to 2.01 for the extraction process. An extraction ratio (m/v) of 1:2 (pancreas: extraction liquid) was used to extract the proteins to mirror the conditions of the production scale process. One kg of macerated pancreas was extracted in 2 L of acidified water. At the end of the extraction process, the material was left to settle for 3 hours and analysed for any noticeable differences in texture, pH and fat content. The untreated pancreas was expected to have a higher pH at the end of the overnight extraction period because of the alkaline nature of the pancreas (Patel, 1995).

B) Clarification and 20% A/S fractionation

The first step of the clarification process was to remove the solid mince debris. The minced bovine pancreases were removed from the extraction liquid using different sized sieves to retain all the tissue particles. This was done to mimic the decanting process used in the production facility.

A diatomaceous earth filter aid suspension was used to prepare a thin filter cake on a 30 cm Buchner filter. A coarse filter aid (Celite 545) was used to remove insoluble particles (10 – 50 μm), and fats, to mimic the BRPX centrifugation step. The protein content (A_{280}) of the supernatant liquid was determined with a Schimadzu UV-1601 spectrophotometer to see if there was a difference between the total protein content of the two sources. The supernatant of the initial clarification step was fractionated with 20% A/S (114 g/l solid A/S). At 20% A/S saturation, non-specific proteins were precipitated and subsequently enhanced the final clarification step. The 0/20% suspension was filtered through a thin Celite Hyflo filter bed on a 30 cm Buchner filer to remove any insoluble particles and protein precipitate (1 – 5 μm) to

mimic the filter press clarification step. The protein content (A_{280}) of the clear supernatant was measured to establish the non-specific protein loss in both samples.

C) 20/80% Ammonium Sulphate fractionation

To allow for further quantitative and qualitatively analysis, the samples were further concentrated. Both samples were fractionated with 80% A/S by the addition of 424 g/l solid A/S to precipitate all trypsinogen and CTG. The resulting 20/80 precipitates were collected using a Whatman filter paper in a 30 cm Buchner filter. The precipitates were dried under vacuum and removed from the filter paper. The precipitate was re-dissolved in a 0.75 (m/v) ratio in acidified water (pH 2.00, acidified with 1 M H_2SO_4) to obtain a highly concentrated protein solution. This solution contained deoxyribonuclease, ribonuclease, trypsinogen and CTG.

D) Activation of the 20/80 precipitate to quantify and investigate trypsin content of the samples

The activation experiment was used to determine the amount of total trypsin present in the two samples. The native trypsin content was an important factor at this stage of the process, as this determined the success of the zymogen separation step (refer to section 4.3.3). Both samples were activated in accordance with the activation procedure described section 3.2.2 – 3.2.3. A 10g aliquot of 20/80 trypsinogen precipitate of each source was dissolved for trypsin activation in 20 mM Tris buffer pH 8, 20 mM $CaCl_2$. 20 mg of pure lyophilized trypsin was added to both samples to initiate the activation. The trypsin activity was assayed every hour using trypsin activity assays (Schwert, 1955) and plotted against time to create an activation plot. At the end of the activation, when the trypsin content had reached a plateau, the activation was terminated by lowering the pH to 3 using 2.5 M H_2SO_4.

5.3.3. RESULTS

A) Raw materials and extraction

There was a distinct difference between the raw materials used. These differences are summarized in Table 20 below.

The acid treatment thus had a significant effect on the pancreatic fat. It facilitated fat aggregation and separation from the extraction liquid. Considering the role of bile acids in the

small intestine in their facilitation of fat absorption (Miettinen, 1972), fats were better emulsified in the acid treated sample which simplified downstream processing (Figure 43).

Table 20. Comparison between the two pancreas sources before and after the extraction, comparing physical appearance, pH before and after extraction and fat content.

Acid dipped pancreas	**Non acid dipped pancreas**
Physical appearance of pancreas and initial pH of extraction	
Light brown colour (an indication that the outer surface of the pancreas had been in contact with H_2SO_4 which denatured the tissue)	Dark pink colour
Initial pH before pH adjustment was 3.5	Initial pH before the pH adjustment pH 6.5
After 16 hour extraction	
Supernatant was clear and had a dark brown appearance with a thick layer of fat floating at the surface.	Supernatant had a distinct milky appearance and did not have any fat floating at the surface.
The fat completely separated from the extraction medium and aggregated at the surface of the beaker.	The fats were still entrained within the extraction medium / macerated pancreas.
Aggregated fats at the surface had a thick consistency and were easily removed	It was not possible to remove the fat entrained within extraction medium.

Figure 43. A Comparison of the two extraction media after the completion of the extraction process. The supernatant of the untreated pancreas (left) had a distinctive milky appearance (because of fats entrained and not disrupted). The supernatant of the treated pancreas (right) was much clearer and all the fats aggregated at the surface of the extraction medium. No fats were visible within the extraction medium of the treated pancreas. The aggregated fats at the surface had a thick consistency and were easily removed.

B) Clarification

Initial clarification using a coarse (10 – 50 µm) filter aid (Elite 545) indicated that the untreated pancreas was more difficult to process, as the entrained fats rapidly blocked the filter immediately and multiple filter beds were prepared to complete the clarification process. The treated pancreas provided no resistance on initial clarification, with only a single filter bed required to clarify the entire sample.

C) Protein content

20 ml Aliquots were removed to determine the protein content (A_{280}) of the extract. All samples were spun at 14000 xg in a microfuge for 5 minutes and micro filtered through a 0.45 µm pore size filter to obtain a clear liquid for protein analysis (see table 21).

The A_{280} of the clear supernatant was measured using a Schimadzu Schimadzu UV-1601 spectrophotometer.

Table 21. Summary of the total protein content and precipitate weight of the two samples as they were processed.

Processing stage	**Treated pancreas**	**Untreated pancreas**
	Protein content (A_{280})	
Extraction	75.9	38.6
0/20 Supernatant	67.4	30.24
20/80 Supernatant	43.56	15.18
	Precipitate weight (g)	
20/80 precipitate	28.5	36.25

The protein content of the treated pancreas was almost double that of the untreated sample at the extraction and clarified 0/20 stages. More small protein fragments, as observed using SDS PAGE, were observed in the treated pancreas sample, potentially as a result of the acid treatment. 57% of the total protein observed in the treated sample was observed in the 20/80 supernatant, indicating these were not intact proteins or this could be trypsin inhibitors. These fragments did not precipitate at 80% A/S saturation.

The low pH value of the extraction buffer could lead to the denaturation of many non-specific proteins. This could serve as a purification step in the process to eliminate non-specific proteins that cannot stand these harsh conditions. SDS PAGE analysis was conducted to compare the different processing stages of the two raw materials (see figure 44). The treated samples indicated pseudo high protein content, as the high protein content could be ascribed to small protein fragments. These samples were loaded onto the gel at the same protein concentration (5 µg/µl, 35 µg protein / well).

Even though the samples were loaded at the same concentration (5 µg/µl), the treated samples did not develop a defined protein band of the same intensity as that of the untreated samples, indicating that there were several protein fragments present that increased the total protein content, but which were all non-specific, or could be trypsin inhibitors.

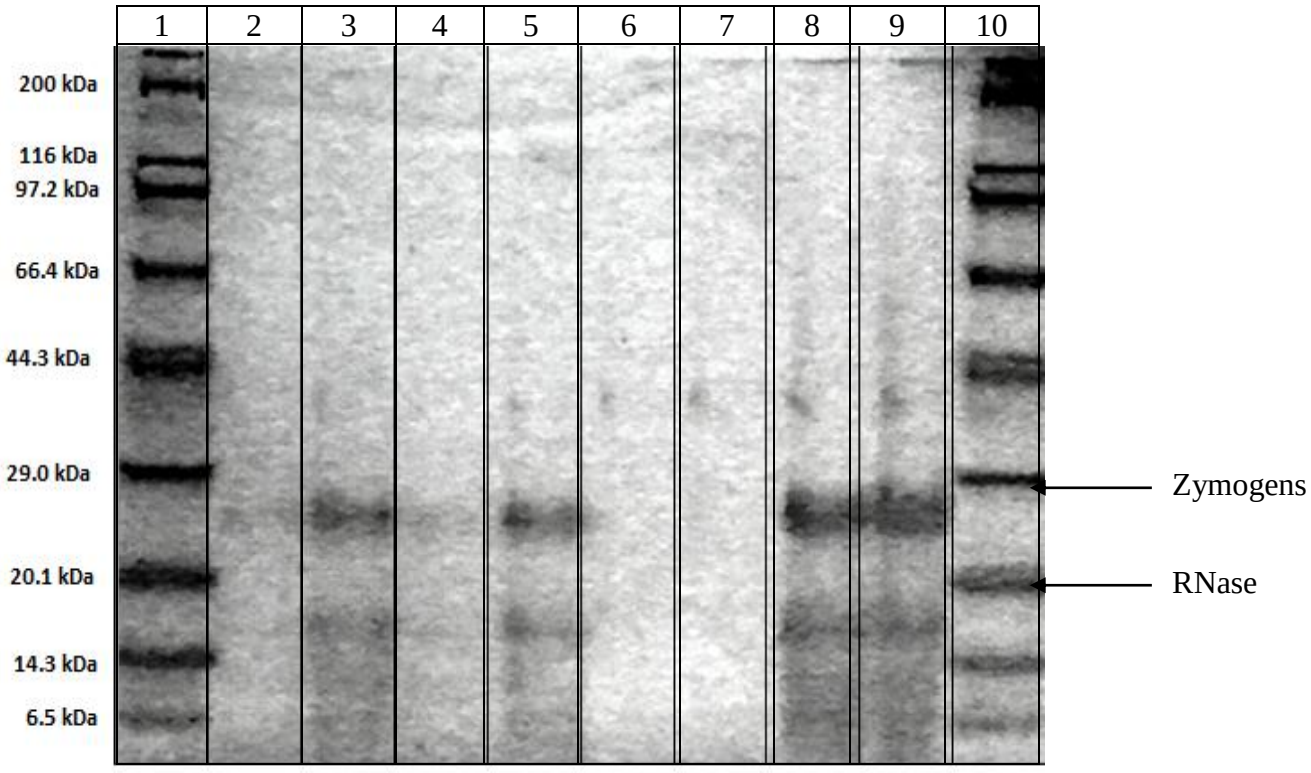


Figure 44. SDS PAGE Analysis comparing three different process stages of the small scale purification. *Lane 1*; Molecular weight marker (6.5 – 200 kDa). *Lane 2*; Extraction sample (Treated). *Lane 3*; Extraction sample (Untreated). *Lane 4*; Clarified 0/20 S/N (Treated). *Lane 5*; Clarified 0/20 S/N (Untreated). *Lane 6*; 20/80 supernatant (Treated). *Lane 7*; 20/80 S/N (Untreated). *Lane 8*; Dissolved 20/80 precipitate (Treated). *Lane 9*; Dissolved 20/80 precipitate (Untreated). *Lane 10*; Molecular weight marker (6.5 – 200 kDa).

D) Activation of the 20/80 precipitate to quantify the trypsin content of the samples

Both samples were activated as described in section 3.2.2 – 3.2.3. The native trypsin activity in the untreated sample was extremely high (842 U/ml) compared with the 150 U/ml of the treated sample, indicating that the acid treatment had a significant effect in conserving the zymogen state of trypsin after harvesting of the pancreas. The pH values of both these trials were kept within operating limits (1.9 – 2.2) for the duration of the processing which could not have promoted trypsinogen activation (see figure 45).

The total amount of trypsin extrapolated from these activation experiments indicated that there was no significant difference between the total trypsin content of treated and untreated

pancreas. There was however a difference in the rate of activation between the two samples with the activation rate of the non-acid dipped pancreas being higher than that of the acid treated pancreas. The high native trypsin present in the untreated pancreas may have led to problems with trypsin stability throughout the process and could have affected the zymogen separation negatively. Effective zymogen separation does not occur if the native trypsin in the solution was high. If the native trypsin content is >350 U/mg, trypsin would activate CTG to chymotrypsin and less chymotrypsin would be able to crystallize as the majority of the CTG would be converted to chymotrypsin.

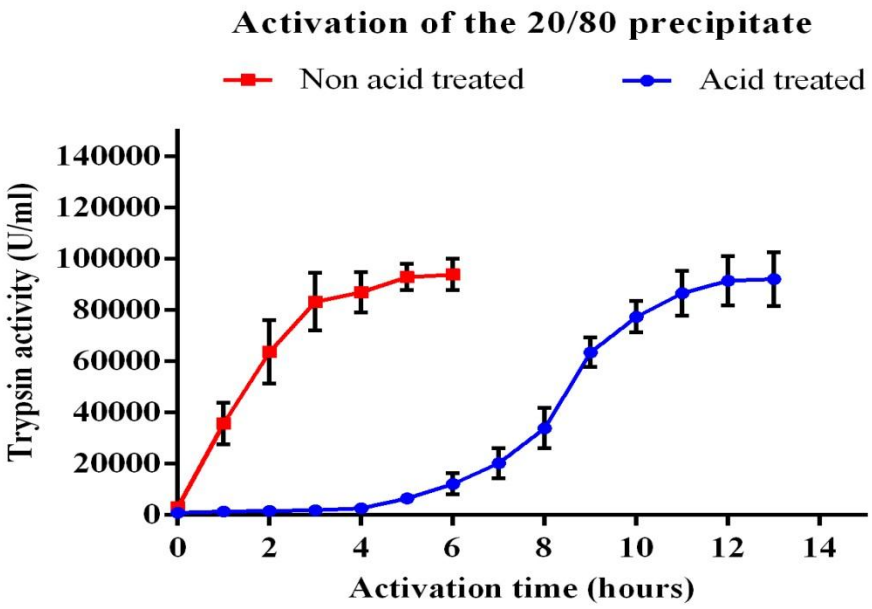


Figure 45. Activation plots for both trials. The untreated pancreas had very high native trypsin (842 U/ml), which accelerated trypsin activation. The treated pancreas with lower native trypsin content was slow to activate, but both samples activated to the same amount of total trypsin (approx 78 BU).

The high activity of active native trypsin present in the untreated pancreas explained the rapid activation and supported the hypothesis that the additional non-specific proteins present in the treated pancreas could act as trypsin inhibitors. This study indicated that there was no major difference between the total amount of trypsin present in treated and untreated pancreas. The high native trypsin content of the untreated pancreas suggested that this material could not be used to purify trypsin and chymotrypsin, using the existing methodologies where CTG was crystallized to separate the zymogens.

5.3.4. PROCESSING A LARGE SCALE BATCH TO INVESTIGATE THE EFFECT OF USING NON ACID DIPPED PANCREAS ON THE ZYMOGEN SEPARATION

A plant scale trial was initiated to further investigate the effect of the high native trypsin activity in observed the untreated pancreas on the zymogen separation. This was also conducted to confirm the findings of the laboratory trials (difficulty in clarification as a result of non-emulsified fat and high native trypsin observed at the end of primary processing). Seven hundred kg of untreated pancreas were processed to investigate the possibility of the usage of untreated pancreas for the production of PDF's. The 700 kg of non acid dipped pancreas was purchased and processed according to the prescribed processing records.

5.3.4.1 RESULTS

At the extraction stage, the non acid treated pancreas extract had a milky appearance with an initial pH of 6.28. The extract also had a light pink colour, similar to what was found in pancreas used for routine production that had high starting pH values (see figure 46).

The batch had an extremely high fat content (visibly) after being processed through a BRPX centrifuge and the high fat content severely complicated clarification of the batch on the filter press. Two separate filter cakes were prepared to process this specific batch. In contrast, for a normal 1.4 t production scale batch, the operators prepared 2, or possibly 3, filter cakes on the filter press to clarify the batch.

No abnormalities were observed during the 0/20 % precipitation, and the concentration with a 10 kDa MWCO ultrafiltration system did not appear to be any more time consuming when compared with a routine production batch. It was possible to concentrate the full volume (1700 L) down to 60 L without any difficulty.

The aim of this trial was to determine the feasibility of crystallizing CTG using material from untreated pancreas.

Figure 46. Light pink colour observed during the initial stages of the extraction of the untreated bovine pancreas. This colour was only observed when pancreas was extracted with very high pH values above 6. Routinely the extract has a light brown colour as a result of the low pH of the extract.

The aim of this trial was to determine the feasibility of crystallizing CTG with material from untreated pancreas. Normally, if the native trypsin activity of a batch was >350 U/ml, CTG crystallization does not occur.

As expected the native trypsin activity exceeded 500 U/ml at the zymogen separation, and the material could not be processed to trypsin or chymotrypsin and needed to be activated and processed to a very low grade of trypsin where the trypsin: chymotrypsin ratio was approx. 1:1. The final Lyophilized yield was only 480 grams. The assay results for the lyophilized material were 552.0 U/mg trypsin and 1810.0 U/mg chymotrypsin.

These results confirmed the laboratory scale findings on, and proved that untreated bovine pancreas was not a suitable source for producing PDFs.

5.3.5. CONCLUSION

Untreated pancreas, for the production of PDFs, was shown not to be a viable raw material source whilst using the current technology. The high pH of the material during the early stages of processing allowed for trypsin activation that led to protein degradation. The high fat content of the material complicated the processing of the material. High native trypsin activity at the zymogen separation stage did not allow for CTG crystallization. It was thus concluded that it was not possible to purify CTG/chymotrypsin or pure trypsin from untreated pancreas. Further exploration is necessary to investigate possibilities to prevent the activation of trypsin during the harvesting and freezing of the pancreas. The consideration of the usage of non-acid treated pancreas was an innovative recommendation to reduce the cost of the raw material, which was the biggest contributor to the production cost.

Chapter 5 described innovative methodologies considered to improve the production throughput and reduce the overall production time and cost. The implementation of ultrafiltration technology reduced the overall production time, and reduced the amount of A/S used per batch. Column chromatography presented a new and innovative platform for zymogen separation and had the potential to decrease the production of trypsin and chymotrypsin.

The final aspect of the protein purification process at BBI that was investigated was the testing methodologies used to quantify the amount of enzyme at various stages during the production of trypsin and chymotrypsin. Both these enzymes had catalytic activities that could be quantified by spectrophotometric assays. Chapter 6 will give an overview of the testing methodologies used and present new methodologies that were considered to quantify the active and inactive enzymes.

CHAPTER 6

6. DEVELOPMENT OF IN-PROCESS QC ANALYSIS TO QUANTIFY THE TOTAL AMOUNT OF ENZYME AT DIFFERENT STAGES OF THE PROCESS

6.1 OVERVIEW OF THE TESTING METHODOLOGIES USED

The ability to successfully purify an enzyme relies on the ability to test for the presence of the enzyme throughout the process. It was essential that real-time information was fed back to the operators working with the product, as failure to communicate these results in real time could have lead to major product losses. The only two assays used during the production of trypsin and chymotrypsin were two kinetic assays for both enzymes. In both these 5 minute kinetic assays the substrate was digested by the proteases and the change in absorbance of the reaction mixture was measured at a certain wavelength and plotted against time. Although these two enzymes indicate a high level of structural similarities (Walsh, 1964) , they do have completely different substrate affinities (Neurath, 1949). N-benzoyl-L-arginine ethyl ester (BAEE) was used as a substrate for trypsin activity measurement, and N-acetyl-L-tyrosine ethyl ester (ATEE) was used as substrate for chymotrypsin activity measurement (Schwert, 1955).

6.1.1. DETERMINATION OF TRYPSIN ACTIVITY

The trypsin assay method was one suggested by Schwert and Takenaka (Schwert, 1955) in which BAEE is hydrolysed at the ester linkage causing an increase of absorbance measured at 253 nm and 25°C (see Figure 47).

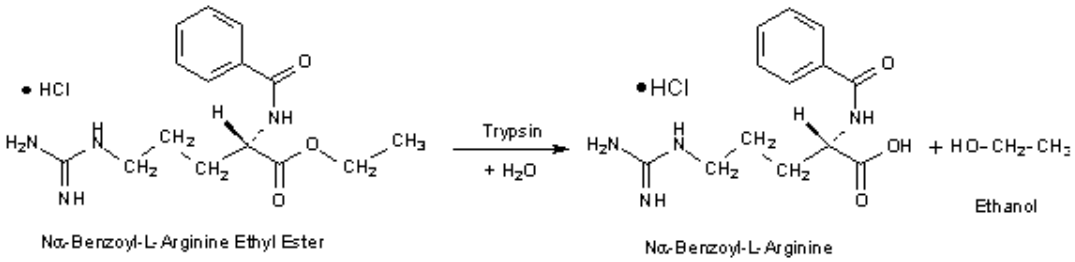


Figure 47. Schematic presentation of the cleavage of BAEE by trypsin, resulting in Nα-Benzoyl-L-Arganine and ethanol. The reaction volume was 3.2 ml, the light path was 1cm, and the approximate reaction time was 5 minutes. The reaction is carried out at 25°C where one trypsin unit will produce a change in absorbance of 0.001 per min at 253 nm (ΔA_{253}) (pH 7.6 and 25 °C) (Schwert, 1955).

6.1.2. DETERMINATION OF CHYMOTRYPSIN ACTIVITY

A method for the activity measurement was published and used with great success until today (Schwert, 1955). N-Acetyl-L-Tyrosine Ethyl Ester [ATEE] is hydrolysed at the ester linkage causing a decrease of absorbance measured at 237 nm and 25°C (see figure 48).

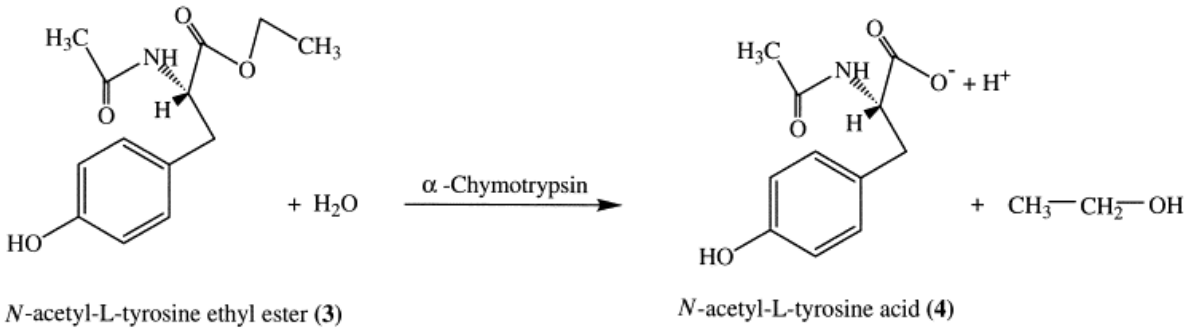


Figure 48. Schematic presentation of the cleavage of ATEE by chymotrypsin, resulting in -acetyl-L-tyrosine acid and ethanol. The reaction is carried out at 25°C where one chymotrypsin unit will produce a change in absorbance of 0.0075 per min at 237 nm (ΔA_{237}) (pH 7.6 and 25 °C). The reaction volume was 3.2 ml, the light path was 1 cm, and the approximate reaction time was 5 minutes.

6.2 SHORTCOMINGS OF THE TRADITIONAL TESTING METHODOLOGIES

The testing methodologies used to determine the activity of the enzymes during isolation and purification were not inadequate, as they were reliable and robust activity assasys. The one major limitation of the traditional testing methodologies was the inability to monitor primary process efficiency. Because both the enzymes are expressed as zymogens and remained in their zymogen state during the primary processing, there was no assay to monitor primary process efficiency, and no way in which to quantify the total amount of enzyme present. For both trypsin and chymotrypsin, the first opportunity to quantify the total amount of enzyme (units)[5] for a single production batch was immediately after the activation stages during the latter stages of the secondary purification. If a particular batch showed low trypsin or chymotrypsin activity, there was no way to determine if this was as a result of direct process loss or if the raw material used had a low protease content to start off with. It became apparent

[5] **Unit Definition**: That amount of enzyme causing a decrease in absorbance at 237 nm of 0.0075 per minute at 25°C under the specified conditions.

that there was a need to monitor the primary processing stages before enzyme activation took place. The number of sampling points across the purification process needed to increase to gain better insight into the efficiency of every production step. A rapid method was also required that allows for real-time results to be fed back to the oparatos on the production floor.

6.3 DEVELOPMENT OF NEW TESTING METHODOLOGIES

The ability to purify an enzyme properly from a complex mixture relies on the ability to monitor the performance of all the purification stages in the process and to quantify the protein of interest throughout the purification process. Kinetic assays were used successfully to quantify the presence of an active enzyme such as trypsin or chymotrypsin in a complex mixture of proteins.

In-process controls (IPC) are checks that are routinely performed on the product before the manufacturing process is completed (see figure 49). The functions of IPC's are to monitor the performance, and when necessary, adapt the process to produce a product that fully complies with the final product specification (see Table 2 and 3).

Proper in-process QC testing provides a means to ensure that a stable process is maintained. A stable process is a controlled process that can consistently deliver the same end product (that conforms to the specification) with little or no step-to-step variability.

This implies that for equal amounts of raw material processed, a similar final yield is consistently to be achieved and that losses observed across a certain process step never exceed the accepted tolerances. The process parameters should be tightly controlled to minimize specific protein losses.

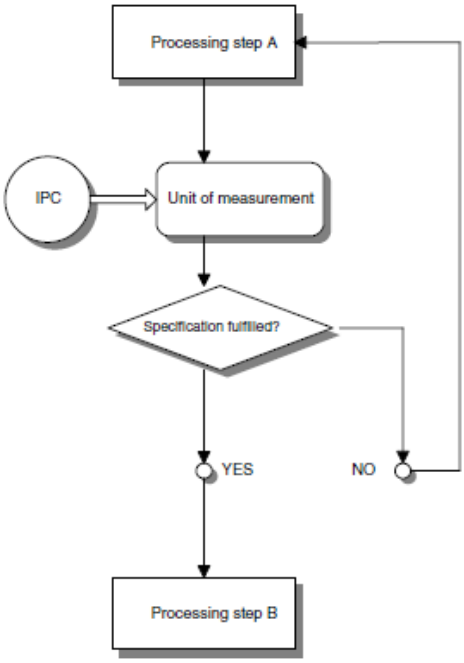


Figure 49. Process control by means of in process controls as described by the GMP manual (Gausepohl, C., Mukherji, P. © Maas & Peither AG, 2007).

One of the difficulties in monitoring the efficiency of the primary processing was to quantify the presence of the zymogens as they progressed through the process. The zymogens are inactive forms of the enzymes and activation assays are not always suitable for in-process QC analysis, as they may be lengthy (up to 6 hours per assay). One of the other enzymes in the extraction mixture, ribonuclease, does possess kinetic activity and was used as an indicator of step-to-step variability. A rapid 3-minute activity assay for ribonuclease was available which made this a suitable assay to use to track the performance of the major processing steps during primary processing.

The reasoning behind the use of ribonuclease as an in-process measurement of process stability, was that there should be a proportional equivalent protein loss of ribonuclease across a particular step as there would be of trypsinogen or CTG. These processing steps included: Decanting (first centrifugation step to remove the bulk of the extracted pancreas), BRPX centrifugation (second high-speed disc centrifugation to remove small insoluble particles and fats), 0/20% A/S precipitation and filter press clarification. None of these were steps that specifically removed any of the proteases or ribonuclease. This assay gave an indication of the reaction conditions during the process and indicated total protein loss across the processing steps, but did not specifically quantify or indicate a loss of trypsinogen. Ribonuclease was also a much hardier enzyme and thus may not necessarily have reflected where other enzymes

(trypsinogen) may have lost activity. This assay could not indicate if the reaction conditions during the process were in any way unfavourable to the zymogens.

Immunosorbent Assays

There was thus a need for an assay that could quantify the zymogens during the primary purification stages of the process to indicate process efficiency. Immunochemical techniques may readily detect small quantities of specific proteins in a complex mixture of proteins and provide a means by which it may be possible to quantify the zymogens and provide better insight into the efficiency of the primary processing stages.

Figure 50 is an illustration of an Enzyme-Linked Immunosorbent Assays (ELISA's) which have been used with great success to quantify the amount of specific protein present in a mixture. This study only focussed on the development of an ELISA for trypsinogen. If trypsinogen was damaged or lost during a certain processing step, it was anticipated that CTG would also be affected in the same way.

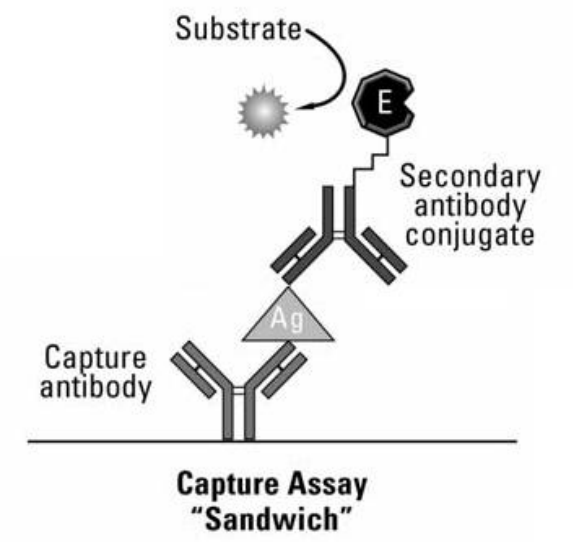


Figure 50. Diagram indicating the principle of a sandwich ELISA where the primary antibody is coated onto a 96 well micro plate (Thermo Scientific Tech Tip #65 ELISA Technical guide and protocol).

An ELISA must be simple, repeatable and robust and must be able to detect the presence of the zymogen at very low concentrations.

Kinetic Assays

Kinetic assays have been used extensively over the years at BBI enzymes to quantify the amount of active enzyme during the secondary processing stages. The shortcoming of these kinds of assays was that they only considered a single dilution per sample, and there were no control samples included when assaying any sample. These assays could not be used to quantify the zymogens during the primary processing. The setup of these assays was extremely time consuming, and required a great deal of preparation. Although the assays used during the secondary processing were extremely accurate, the lack of a control sample and the ability to perform the assays in duplicate raised concerns regarding assayists to assayists variance.

The use of kinetic microtitre assays for monitoring in process control was considered, and a kinetic assay for trypsin was developed. This assay included a standard curve and required each sample to be assayed in duplicate. Each sample was assayed at three dilutions, and multiple samples could be assayed with a single assay. In the same way as the ELISA, this assay needed to be simple, reproducible and accurate.

Defining a standard curve

A standard curve is a quantitative research tool that may be used to determine the concentration of a specific protein. A dilution series of defined protein concentration is prepared by diluting a standard and measuring the absorbance thereof at a certain wavelength. A standard curve is obtained when the absorbance at a certain wavelength is plotted against a defined protein concentration or activity. When an unknown sample is analysed under the same conditions as the standards, and an absorbance value is obtained which falls within the absorbance range of the standard curve, it can directly be correlated to a corresponding protein concentration or enzyme activity. If the absorbance value obtained for the unknown sample falls outside of the specified absorbance range, the sample can be diluted accordingly so that the absorbance of the diluted sample falls within the defined absorbance range. The corresponding protein concentration is multiplied by the dilution factor to obtain an accurate protein concentration or enzyme activity of the unknown protein sample.

Another important consideration when defining a standard curve is the curve fit or R^2 value. This gives an indication of the linearity of the data points when looking at distribution of data points. An R^2 value of 1.00 indicates a perfect curve fit, being linear or quadratic polynomial.

6.4 DEVELOPMENT OF A MICROTITRE KINETIC ASSAY FOR TRYPSIN

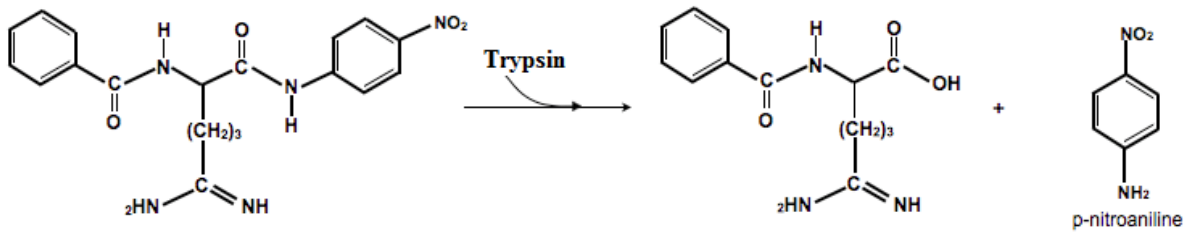
6.4.1 INTRODUCTION

The major advantage of microtitre-based assays over conventional spectrophotometer based kinetic assays was the number of samples that could be tested simultaneously. Another advantage of microtitre assays was the consistency and accuracy of the assay, with a control sample included with every assay, and the fact that every sample was assayed in duplicate at three different dilutions. These advantages therefore indicated that microtitre based assays were an attractive assay method for the in-process testing of trypsin. There was no commercial test kit available that would suit the needs of the process at BBI Enzymes, which necessitated the development of an in-house microtitre based assay for trypsin.

Selecting a substrate

The main requirements when selecting a substrate was that the substrate had to be trypsin specific, and because this was a colorimetric assay, the product formed needed to be visible within the visible light spectrum to allow quantification using a standard filter set on a microtitre plate reader.

Na-Benzoyl-L-arginine 4-nitroanilide hydrochloride (L-BAPNA) was one of the few substrates found that satisfied these criteria. L-BAPNA was a colourless, chromogenic substrate for proteolytic enzymes. Hydrolysis of the L-BAPNA at the bond between the arginine and the *p*-nitroaniline moieties released the chromophore *p*-nitroaniline (see figure 51), which could be detected by colorimetric analysis at 405 nm (Somorin, 1978).



N-Benzoyl –L Arginine 4-Nitroalanine Hydrochloride
(L-BAPNA)

Figure 51. Reaction mechanism of trypsin hydrolysis of L-BAPNA, yielding the substrate *p*-nitroalanine that could be quantified at 405 nm.

6.4.2 MATERIALS AND METHODS

For the development of the microtitre assay, pure trypsin was purchased from Sigma (Sigma life sciences, St. Louis, USA, product # T8003), (specific activity 13400 U/mg BAEE) and used to prepare the dilution series and standards. The substrate (L-BAPNA) was also purchased for Sigma (Sigma Aldrich, St. Louis, USA, product # B3279).

A Thermo Fisher Multiskan FC series plate reader was used for the incubation and reading of the plates. Skanit software (version 2.5.1) was used to operate the plate reader and to analyse the results.

Buffer and reagents

A 50 mM Tris-HCl, pH 8.4 buffer was prepared as assay buffer and was used as dilution buffer for the substrate. A 5 mM Glycine-HCl buffer, pH 3.0 containing 30% glycerol was prepared to prepare and store trypsin samples for assay.

Trypsin stock solutions were prepared (2 mg/ml) in 5 mM Glycine-HCl buffer, pH 3.0 containing 30% glycerol.

Trypsin substrate stock (5 mg/ml L-BAPNA) was prepared in dimethyl formamide (DMF) and stored at -20^{o}C. For a single assay, the substrate stock solution was diluted 1:18 with assay buffer (50 mM Tris-HCl, pH 8.4) and mixed well before use. The substrate was light sensitive, and needed to be prepared in amber glass bottles and stored in the dark when not used.

Preparation of a standard curve

The following dilutions were made from trypsin stock solution (2 mg/ml) using a 5 mM Glycine-HCl buffer, pH 3.0 containing 30% glycerol: 1/6, 1/12, 1/24, 1/48 and 1/96.

The diluted enzyme solutions were transferred into duplicate microtitre wells (20µl/well). The substrate solution (200 µl/well) was added into each well to obtain a final volume of 220 µl. The reaction mixture was shaken (medium shaking) for 10 minutes on a microtitre instrument before absorbance was read at 405 nm.

6.4.3 RESULTS

Standard curve

Standard curves were plotted of the Absorbance at 405 nm against the corresponding enzyme concentrations. Total dilutions were calculated as follows: *Dilutions made under enzyme dilutions (see general method)* x *Dilution in the reaction mixture (1/20).*The results of these standard curves are summarized in table 22 and figure 52 below.

Table 22. Absorbance at 450 nm of 4-nitroaniline produced by trypsin enzymatic cleavage of L-BAPNA substrate after a 10 minute incubation. (n=3)

Total dilutions	Absorbance 405 nm	Trypsin Activity (U/ml)
1/120	1.552 (±0.051)	223.8
1/240	0.884 (±0.040)	111.9
1/480	0.483(±0.05)	55.6
1/960	0.270 (±0.081)	28.1
1/1920	0.155(±0.064)	14.1

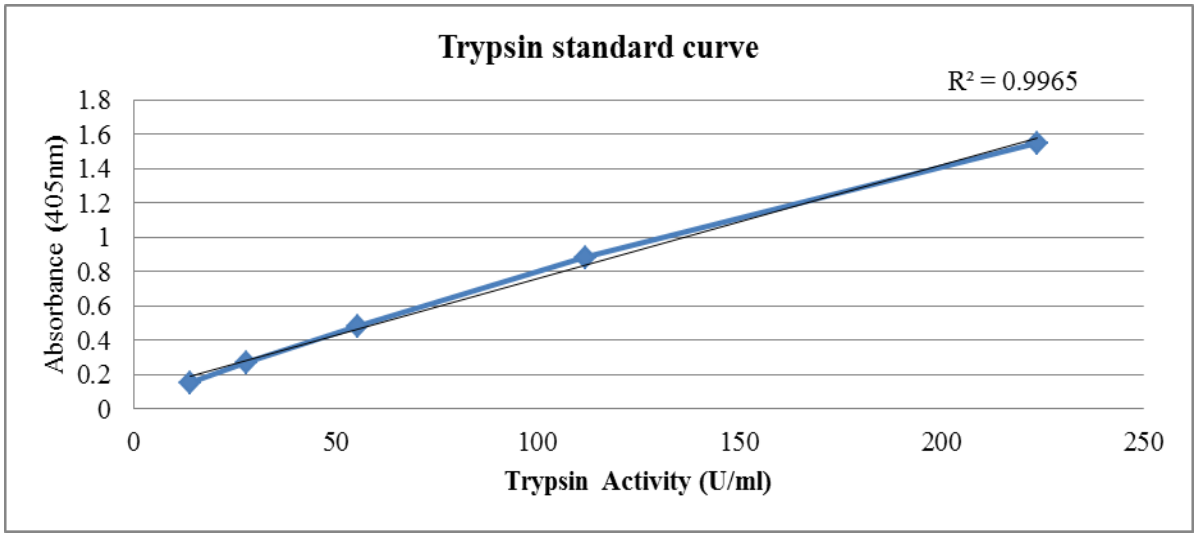


Figure 52: Standard curve obtained for the trypsin activity assay. Sigma trypsin was used for the assay.

6.4.4 INVESTIGATION OF CROSS REACTIVITY OF CHYMOTRYPSIN IN THE TRYPSIN ASSAY

Since trypsin and chymotrypsin were both isolated from the same source it was apparent that an in-process sample of trypsin would contain chymotrypsin as a contaminating enzyme and vice versa. It was therefore imperative to investigate if chymotrypsin hydrolyzed trypsin specific substrate. The assay method used was similar to one the used in construction of standard curve (see above).

6.4.4.1 RESULTS

When chymotrypsin samples were prepared (200 ng/ml) and was used to breakdown trypsin specific substrate L-BAPNA, it showed no activity towards the substrate. The same negative response was observed even when chymotrypsin concentration was increased 4 fold to 800 ng/ml. It was thus concluded that chymotrypsin did not cross react with L-BAPNA in the trypsin assay, and did not put the assay at risk of a false positive result (see figure 53).

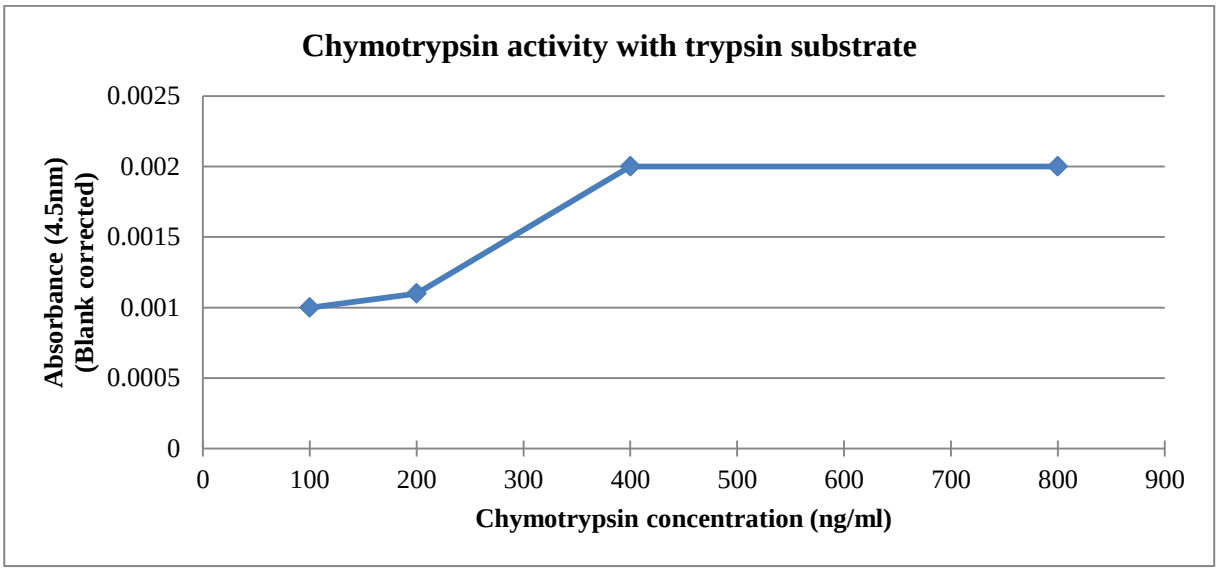


Figure 53: Chymotrypsin assayed using trypsin specific substrate. The Ab405 were corrected by subtracting a blank Ab405 reading from the sample Ab405 reading. The graph showed no cross reactivity of chymotrypsin on trypsin substrate.

6.4.5 CONCLUSION

The trypsin standard curve had an R^2 value close to 1, suggesting that this assay could be used to determine the unknown activities of trypsin in in-process samples. The stability of the standards would have to be established before these assays are used for in-process samples. The trypsin assay was found not to be sufficiently sensitive at low concentrations. As a result, trypsin assays could not be used to determine activity of in-process pancreatic extracts prior to activation because the activity of native trypsin in the extracts was always less than 100 U/ml. An efficient microtitre assay for analysing the activity of trypsin was developed. With suitable standards the assay could be used for analysis of in-process samples for trypsin at the secondary stage of purification, after the activation stage

6.5 ELISA DEVELOPMENT FOR TESTING TRYPSINOGEN CONTENT

6.5.1 MATERIALS AND METHODS

The following materials have been purchased from KALON Biological (Kalon, Guildford, UK):

Assay buffer (PBS pH 7 containing 1% BSA and 0.05% Tween 20), used as a diluent for the trypsinogen standards and the antibody conjugates. Wash buffer concentrate (dilute 40x with dH_2O before use), used for intermittent plate wash steps between additions of samples and conjugates. Anti-bovine trypsinogen coated 96-well microtitre plates (10 µg/ml, 100 µl per well), used as a platform for the ELISA. 1000 ng/ml trypsinogen standard, horseradish peroxidase (HRP)-conjugated anti-Bovine trypsinogen solution (Dilute 20x with assay buffer before use) used to selectively bind bovine trypsinogen. Tetramethylbenzidine (TMB) Chromagen (Dilute 40x with substrate buffer before use) and TMB Chromagen substrate buffer used to quantify the HRP activity.

A Thermo Fisher Multiskan FC series plate reader and a Thermo Fisher plate washer were used for the incubation and washing of the plates. Skanit software (version 2.5.1) was used to operate the plate reader and to analyse the results.

The starting point of the development of the ELISA was to setup a standard curve against which the protein concentration of the zymogen could be measured. In order to establish the concentration range for the standard curve (to define the linear range of the curve), a dilution series of trypsinogen (1000 ng/ml) was prepared and assessed for linearity. For each of the

concentrations of the dilution series, a corresponding absorbance value was obtained which was used to plot on a standard curve.

The most linear and reproducible part of the curve obtained was further investigated and the protocol adjusted to obtain a standard curve that was reproducible, had an R^2 value of >0.99 with an absorbance range between 3.5 – 0.1 absorbance units at 450 nm. .

Given the target yield for trypsin was 1.4 kg lyophilized product/tonne of pancreas processed and the average volume after extraction was approximately 3000 L for a standard 1.4 tonne batch, it implied that, after extraction, the trypsinogen concentration was approximately **653 µg/ml**. This was the lowest concentration of trypsinogen that the assay needed to be able to detect, as the specific protein concentration increased as it progressed through the process due to concentration steps. This was easily achieved since the trypsinogen standard supplied was 1µg/ml. The implication thereof was that the starting dilutions for the assay were approximately **1:10000** to obtain absorbance values that fell within the limits of the standard curve.

Following the development of the ELISA assay, the method needed to be validated according to the requirements as prescribed by the QA department of BBI Enzymes before this assay could be used within the production.

Assay protocol used during method development

On a separate (non coated) 96-well plate, a standard curve dilution series was prepared by double diluting a 125 ng/ml trypsinogen sample 8 times with assay buffer. (Concentration range: 125 – 0.98 ng/ml.) All sample dilutions were prepared on the same plate. The starting dilution for the samples was always 1:10000. All sample dilutions were prepared in duplicate. 100 µl of both the standard curve and the sample were transferred onto an anti-trypsinogen pre-coated 96-well plate. The samples and standards were incubated for 12 minutes at room temperature with brief (30 seconds) initial shaking. During this interval, a 15x dilution of the secondary antibody-HRP conjugate was prepared in assay buffer. After the 12 min incubation, the plate was washed 4 times with washing buffer, and the plate tapped dry to remove any excess buffer from the wells after washing. A 100 µl of suitably diluted (15x) conjugate was transferred into each well. The HRP conjugate was incubated for 12 minutes at room temperature with brief (30 seconds) initial shaking. During this interval, the enzyme substrate solution was prepared by preparing a 40x dilution of the TMB Chromagen in substrate buffer. After the 12 min incubation, the plate was washed 4 times with washing buffer, and the plate

tapped dry to remove any excess buffer from the wells after washing. A 100 µl of suitably diluted (40x) HRP Chromagen was transferred into each well. The HRP Chromagen was incubated for 12 minutes at room temperature with brief (30 seconds) initial shaking. 100 µl of stop solution (0.25 M H_2SO_4) was transferred into each well to terminate the reaction. The plate was read at 450 nm using a microtitre plate reader.

6.5.2 RESULTS

Preparation of a standard curve

After assessing the protein standards provided (1000 ng/ml stock solution) for linearity, it was found that the best working range for the standard curve lay between 125 – 0.98 ng/ml. Concentrations greater than 125 ng/ml did not have any higher absorbance values, indicating that a level of saturation was achieved. The highest standard for this assay thus needed to be ≤125 ng/ml.

A second dilution series of 125 – 0.98 ng/ml was prepared to assess linearity (see figure 54). The standard curve had a sigmoidal shape, but did not plateau as the higher protein concentrations (>125 ng/ml) did, indicating that a level of saturation was not achieved. Absorbance values for this concentration range were evenly distributed with the absorbance of the upper standard at 3.7 and the lowest absorbance value at 0.26.

Figure 54 below shows the standard curve obtained between 125 – 0.98 ng/ml. This dataset was obtained by multiple (8 times) repetitions of the standard curve at the specified conditions. Error bars indicate little / no variance, indicating that the standard curve was extremely reproducible, the average standard deviation was 0.02.

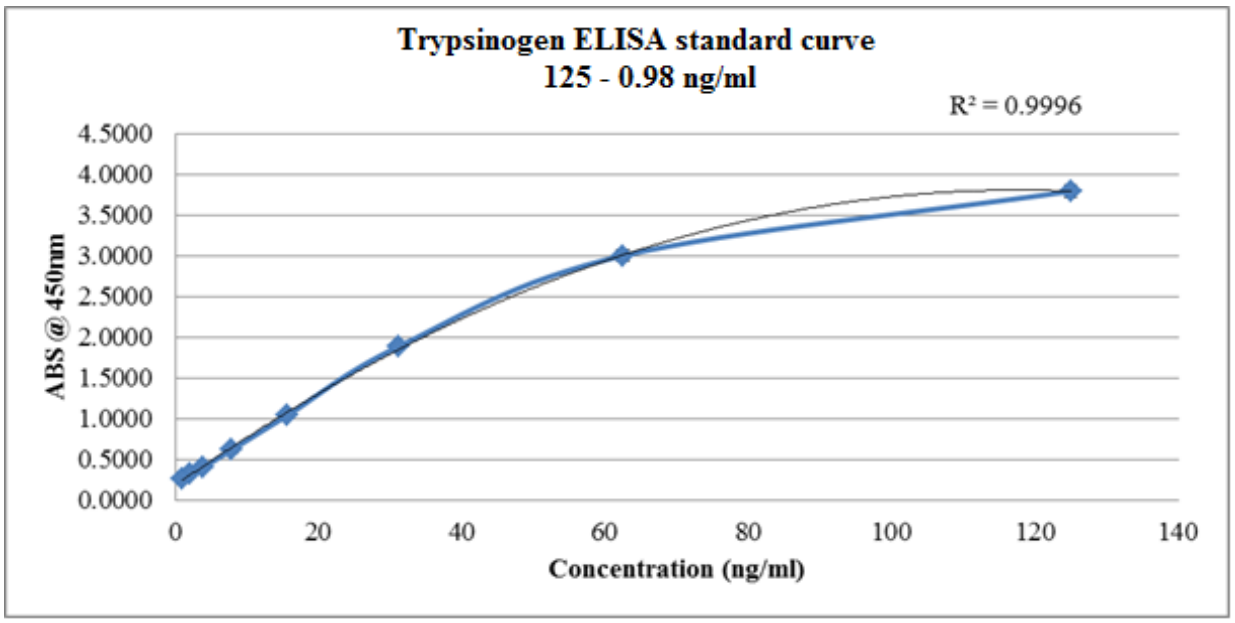


Figure 54. The standard curve obtained with standards ranging from 125 – 0.98 ng/ml. The running conditions were the same as the final defined protocol. n = 3, error bars not visible as the assay repeatability was too high to insert error bars.

6.5.3 INVESTIGATION OF CROSS-REACTIVITY WITH CONTAMINATING ENZYMES

After a standard curve for the assay had been established, the robustness of the assay was assessed since the cross reactivity of the assay with other proteins present needed to be established. During the initial stages of the purification, several other proteins were present in the pancreas extraction mixture which included: CTG, chymotrypsin, deoxyribonuclease, ribonuclease and trypsin. To ensure that there was no cross reactivity of the anti-trypsinogen antibodies with any of these proteins, lyophilized samples of each of these proteins were prepared and incubated with the anti-trypsinogen antibodies to see if any of them would bind to the antibodies under the reaction conditions. If it was found that the antibodies bound to many of the other proteins in the mixture, indicating that the assay was not specific enough to be considered as an in-process assay, and new (more specific anti-trypsinogen antibodies) needed to be prepared.

Lyophilized samples (obtained from BBI Enzymes) were prepared in 10 ml dH_2O in the following concentrations (these concentrations are roughly the concentrations of the enzymes during the initial stages of the process): Chymotrypsin (1 mg/ml), CTG (1 mg/ml), Ribonuclease (0.2 mg/ml), Deoxyribonuclease (0.2 mg/ml) and trypsin (0.6 mg/ml). These

values were obtained based on the work of Keller *et al.* (1958) and the theoretical yield targets for this project.

All subsequent dilutions were prepared in Assay buffer to obtain a starting dilution of 1:10000 for all the samples (as this was the starting dilution for the assay). Figure 55 illustrates the results of the cross reactivity experiments. Each of these samples was assayed under the same assay conditions as described above.

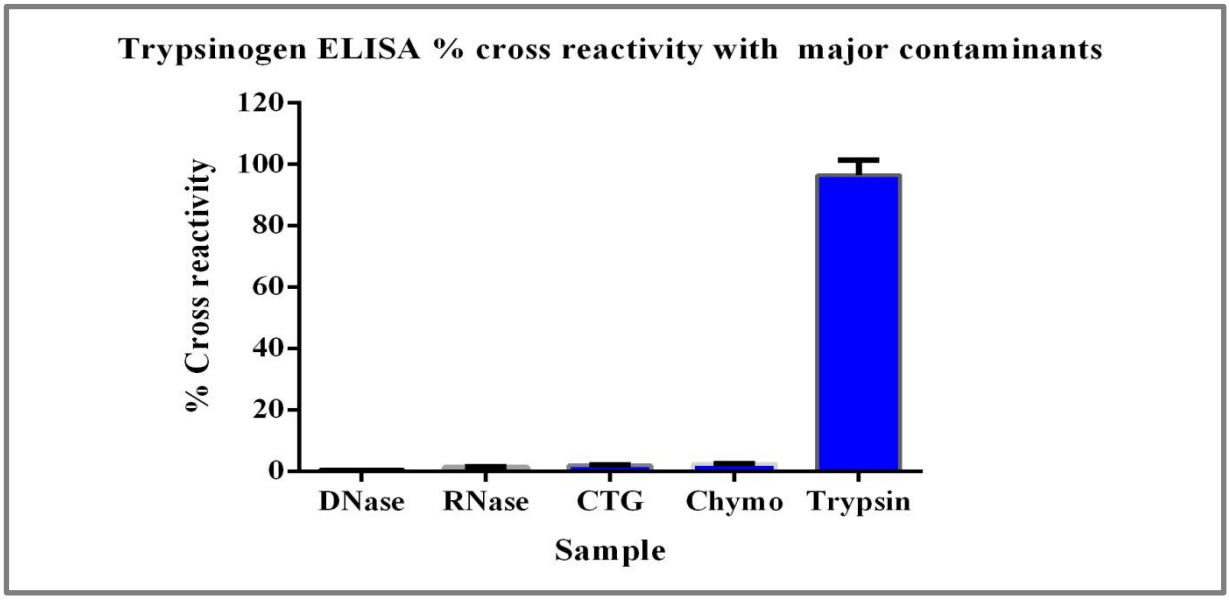


Figure 55. Anti-trypsinogen ELISA cross reactivity with the major contaminants in the primary processing stages. There appeared to be 100% cross reactivity with trypsin. The activity observed in both the chymotrypsin and CTG samples were as a result of the intrinsic trypsin present in both these samples. (n = 3)

There appeared to be 100% cross reactivity with trypsin and the assay could not distinguish the zymogen from the active enzyme. This ELISA assay result for the trypsin sample that was prepared for this experiment corresponded 100% with the actual concentration of the sample that was prepared. During the activation of trypsinogen to trypsin, only 6 amino acids are cleaved off (V-18/D-19/D-20/D-21/D-22/K-23) (Uniprot, 2002). This causes a conformational change in the active site of trypsinogen (Bringer, 1986), and according to the results presented here, this does not alter the exposure of the epitope on the trypsin and the epitope of trypsinogen was still visible to the antibodies.

Trypsin was thus used as 100% binding and all the other contaminants were expressed as a percentage of trypsin binding.

During the chymotrypsin purification process, lyophilized trypsin was added to the liquid to initiate the activation of CTG (see section 3.2.2). The 2.6% cross reactivity observed in the chymotrypsin sample was largely due to the trypsin present in the chymotrypsin sample (The native trypsin in this specific batch was 26.7 U/mg). During the secondary purification stages, the CTG was crystallized out of the solution and removed via centrifugation. The crystals were washed to remove any entrained contaminating proteins (trypsinogen being one of them), but all the trypsinogen is not removed as there are still small traces present. The trypsin(ogen) observed in the CTG sample was largely as a result of the trypsin(ogen) which could not be washed out of the CTG.

Little or no cross reactivity was observed with the DNase sample.

Ribonuclease indicated 1.5% cross reactivity. This was potentially from the fragments observed in the final product. During the ribonuclease final purification stages, there was a protease denaturing stage where the proteases are denatured by heating. These small fragments are clearly visible when analysing lyophilized ribonuclease product on SDS PAGE.

There was thus little cross reactivity observed with the major contaminants in the mixture during the primary processing. The cross reactivity observed with the major contaminants was mainly as a result of the portion of trypsin still present as a contaminant in the preparations that was used.

Future development of this assay to make it more specific would be to specifically raise polyclonal antibodies against the 6 amino acid activation peptide of trypsinogen in order to make the assay specific for trypsinogen. High purity preparations of each of the contaminants needed to be analysed to confirm the findings.

6.5.4 CONCLUSION

Two testing methods were developed to test for the presence of trypsinogen during the primary processing, and to test for the presence of trypsin during the secondary processing. Both these methods were microtitre plate based assays, and were proven to be specific for the enzyme of interest. In the case of the ELISA assay for trypsinogen, further development is recommended to develop an assay that is completely specific for trypsinogen as cross reactivity was observed with a trypsin sample. Both these assays include a control sample for every plate assayed, and every sample is assayed in duplicate at at-least three different

dilutions. This made these assays very reliable and accurate. The advantage these assays gave was improved process control. For the first time the primary processing could be monitored and with an increased amount of samples being submitted during the process, the kinetic assay is capable of handling multiple samples at once, allowing for a bigger capacity.

By implementing these assays into the process, the level of control exercised on the process was greatly improved, and the chances of unexpected and unexplainable yield losses were reduced.

CHAPTER 7

7. PROCESS OVERVIEW OF THE NEW PURIFICATION PROCESS DEVELOPED FOR TRYPSIN AND CHYMOTRYPSIN

Following the method development described in section 4.0 and 5.0 above, a superior method was devised for the purification of trypsin, CTG and chymotrypsin. Improvements were made in extraction, clarification, concentration, crystallization and assay development. This section will describe the new processing method for the purification of the enzymes (see figure 56).

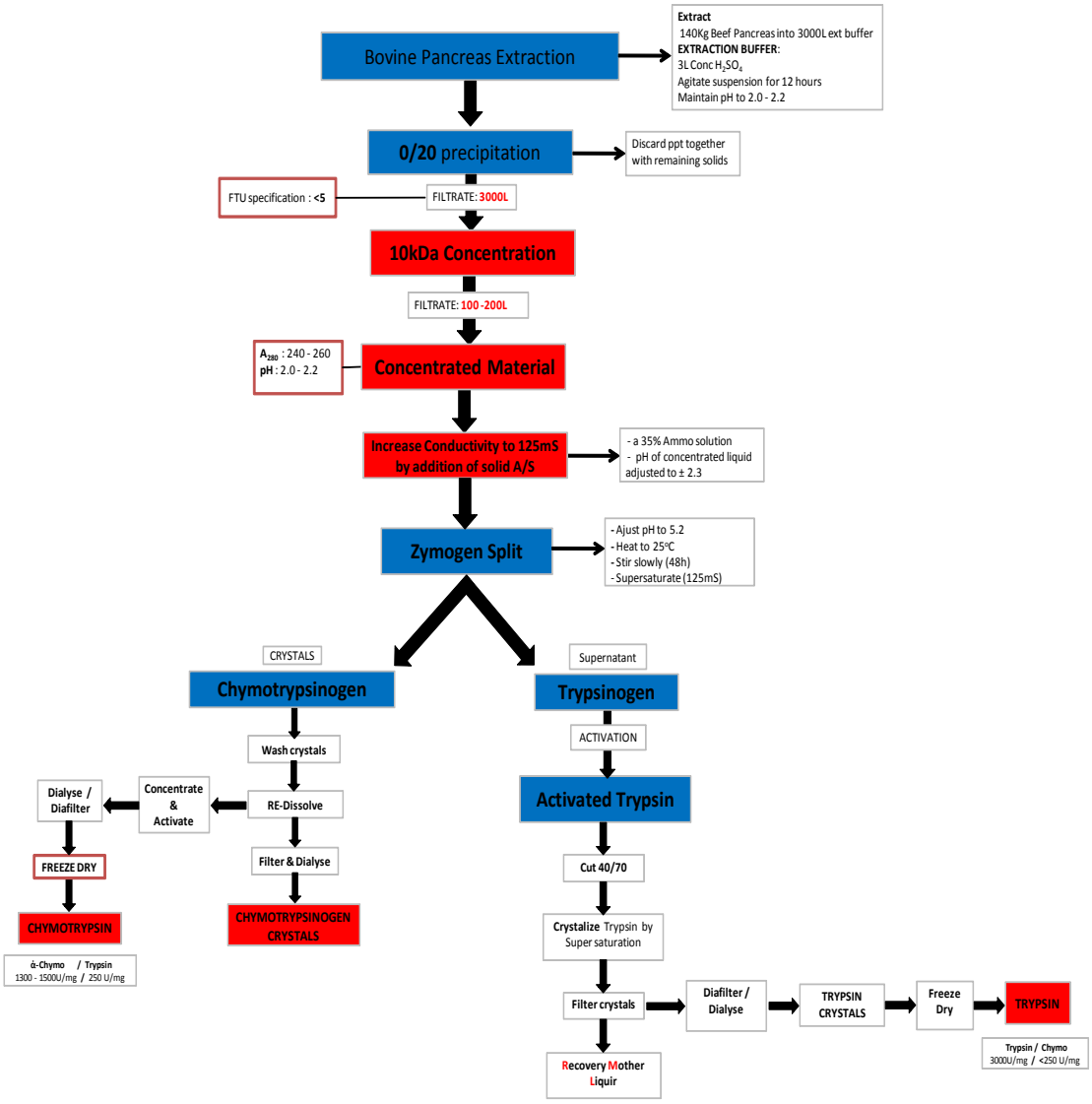


Figure 56. Flowchart of the newly developed production process for pancreas derived factors including chymotrypsin, CTG and trypsin.

Extraction

Frozen bovine pancreas are minced through a frozen tissue grinder which houses a 10 mm and 8 mm hole plate into a fine pulp which is mixed with the extraction medium in a hopper. From the hopper, the tissue / extract is pumped into extraction tanks where it is continuously stirred via a top entry agitator. As the tissue is transferred to the extraction tanks, the pH of the extract is adjusted to 2.0 – 2.2 using 2.5 M H_2SO_4.

A total of 1.4 ton of pancreas is mixed with 3000 L of extraction medium. The 3000 L of water is measured through a calibrated flow meter, ensuring a total of 3000 L is added to the extraction mixture. The extract is agitated for 16hours to allow maximal extraction of the proteases. The mincing process was superior to the flaking method, and better yields were achieved as a result of proper extraction.

The use of a mincer – hopper configuration increased the process time with 3.5 hours, and the extraction efficiency with 20% compared to the traditional processing methodologies.

After 16 hours, the tissue is separated from the liquid extract by means of centrifugation. A continuous flow centrifuge (decanter) was installed to remove the tissue. The new centrifuge had a throughput of approx. 1200 L/h that reduced the processing time with another hour compared to the traditional process where the tissue was removed with a batch centrifuge. The tissue debris of this new decanter had a very low liquid content, and as a result, there was not a major loss of product into the tissue debris (See section 4.2.1).

Clarification

The decanted liquid still contained some insoluble particles and fine tissue debris that could not be removed by the decanter. A new high-speed centrifuge was installed to remove any excess insoluble particles and fats. This unit had a maximum rpm of 12000 rpm, and maximum G-force of 23,000. This machine was able to reduce the solid content with 80%, and the output solid content was approximately 0.8%. The solid content was measured with calibrated centrifuge tubes (See section 4.2.2).

After the additional solids and fats have been removed by centrifugation, the material was precipitated with 20% A/S (114 g/L) to remove non-specific proteins. Because the A/S precipitation is volume dependant, the tanks used to perform the precipitation were calibrated by an external calibration specialist to ensure the volume measured for the A/S precipitation was as accurate as possible. The addition of A/S was regulated by a specially designed hopper

configuration that allowed solid A/S to be added into the tank at a constant (controlled feeding rate) to prevent that excessive solid A/S accumulates at the bottom of the tank.

The precipitated material is clarified through a cloth filter press. The clarity of the liquid after clarification had to be < 5 Formazin Turbidity Units (FTU's) to allow it to progress to the next step in the process. If the material did not meet the specification, it had to be re-worked to ensure a clear liquid.

Ultrafiltration

The clarified 0/20 material was concentrated with a 10 kDa PALL ultrafiltration system. The end of the concentration step was marked by a final protein concentration (A_{280}) of 240 – 260. The volume of the extract was typically reduced 14 – 20 fold to achieve this protein concentration. The filtrate was routinely tested for any potential trypsin / chymotrypsin activity to ensure there was no leakage of the enzymes through the membranes. The filtrate was discarded as liquid waste. The concentration step was performed at a TMP of 1.5 with 2 bar inlet pressure and 1 bar outlet pressure (See section 5.1).

Zymogen Separation

The % A/S saturation of the concentrated 0/20 material was increased to 35% by the addition of solid A/S to the liquid. The 35% extract was clarified through a diatomaceous earth filter bed to remove any insoluble particles that formed during the 20/35 precipitation (Deoxyribonuclease precipitates at 35% A/S).

The clarified liquid is transferred into a temperature-controlled vessel where it will undergo crystallization of CTG for 48 hours. The temperature of the tank was maintained at 25^{o}C by circulating hot water through the jacketed vessel. The pH of the liquid was raised to 5.1 – 5.2 using 1 M NaOH. The conductivity of the liquid is measured and slowly to 108mS by the addition of saturated A/S solution after 16 hours of crystallization. Once the conductivity had reached 108 mS/cm, the conductivity was further increased to 125 mS/cm by the addition of saturated A/S (refer to section 4.3.3 for more details on CTG crystallization).

After 48 hours, the crystals were removed from the supernatant by centrifugation in a vertical axes batch centrifuge. The crystals were washed with a 40% A/S solution, pH 5.2 to remove any entrained trypsinogen from the crystals. The washings were combined with the supernatant recovered from the centrifugation step, and used to further purify trypsin. All the

crystals were combined and used to purify CTG or chymotrypsin from, depending on the demand for product.

7.1 PREPARATION OF CHYMOTRYPSINOGEN

The harvested crystals were washed with 40% A/S to remove any entrained trypsinogen. Washed crystals were dissolved in water at a pH of 2 – 3. The dissolved crystals were clarified and diafiltered until salt-free in preparation for freeze dry.

7.2 PREPARATION OF CHYMOTRYPSIN

The harvested CTG crystals were washed thoroughly with 40% A/S remove all entrained supernatant containing trypsinogen and other non-specific proteins. The crystals were dissolved in water and clarified using diatomaceous earth.

CTG was activated by adding 26.1 g/L K_2HPO_4 to the clarified liquid, and raising the pH to 7.6. The activation of CTG was reliant on native trypsin. If the native trypsin activity in the solution was < 300 U/ml, lyophilized trypsin was added to the solution to initialize the chymotrypsin activation cascade. The chymotrypsin activity was monitored over a 4 – 6 hour period, and was assayed every hour. The end of the chymotrypsin activation was marked by a plateau or decline in the specific activity of chymotrypsin (>750 U/A_{280}). The pH of the solution was lowered to 3.0 by the addition of H_2SO_4 to terminate the activation.

The solution is immediately precipitated with 70% A/S by the addition of 205 g/L solid A/S. All chymotrypsin is precipitated at 70% A/S. The precipitate is collected on a coffin filter under a vacuum.

The precipitate is dissolved in water and dialysed against acidified tap water for 3 days until salt free, or the product is diafiltered until salt free. The salt free solution is clarified using diatomaceous earth and prepared for freeze dry.

7.3 PREPARATION OF TRYPSIN

After the CTG crystals are harvested and washed, the supernatant and the washings of the crystals are combined and further processed to purify trypsin. 2.42 g/L Tris (0.02 M) and 2.94 g/L $CaCl_2$ (0.02 M) was added to the trypsinogen containing solution in preparation for trypsin activation.

While stirring, the pH of the trypsinogen liquid is slowly raised to 8.0 with 5 M NaOH to initialize the trypsin activation at 5°C. The liquid is continually assayed for trypsin activity to monitor the activation sequence. If the starting trypsin activity is <100 U/ml, 100 g lyophilized trypsin is added to the stirring liquid. The activation is monitored hourly (assayed for trypsin activity (U/ml) and protein content (A_{280}). The activation is completed when the trypsin activity (U/ml) has reached a plateau and the specific activity (U/A_{280}) of trypsin is between 900 to 1000.

The activation is terminated by lowering the pH to 3.0 with H_2SO_4 and immediately precipitated with 75% solid A/S by adding 245 g/L solid A/S to the liquid. The precipitate is removed by filtration using a coffin filter.

In preparation for trypsin crystallization, the 40/75 precipitate is dissolved in 0.4 M borate buffer at a pH of 9.0 in a 2 : 1 (w/v) ratio in a cold room (8°C)

The % A/S saturation of the solutions was reduced to 35% using 0.4 M borate buffer followed by addition of 1 M calcium chloride solution (20 ml/L). The pH was adjusted to 7.0 using 5 M NaOH. The crystallization was started by seeding (90g/L) with trypsin crystals from previous bathes.

Overnight, when crystallization was obvious, saturated A/S was added slowly (0.8L/h) to increase the % A/S saturation in the solutions from 35% to 45% saturation and the crystallization was allowed to continue for 7 days. After the 7th day, the trypsin crystals were harvested by filtration on a coffin filter. (See section 4.3.4).

The crystals are washed with 0.4 M borate buffer, 45% A/S, pH 9 to remove any entrained proteins. After the crystals are thoroughly washed, they are dissolved in RO water at pH 3 (acidified with H_2SO_4). The liquid is either diafiltered or dialyzed until salt free and prepared for freeze dry.

The supernatant of the trypsin crystallization and the washings of the crystals are combined and referred to as recovery mother liquor (RML). This liquid contains any un-crystallized trypsin and any chymotrypsin that carried through from the CTG crystallization. The RML is precipitated with 70% A/S and stored in the freezer.

7.4 CHARACTERIZATION OF PRODUCT PRODUCED BY THE NEW PROCESS

To ensure that the products (trypsin and chymotrypsin) produced by the new process are equivalent to, or better than those produced by the traditional process, a series of characterization assays were conducted. SDS PAGE analysis (figure 57 and 58) followed by a series of assays that were performed according to the customer specification. The requirement for this process to be successful was that all the product specifications as specified in table 23 and 24 below were to be met.

7.4.1 SDS PAGE ANALYSIS OF FINAL LYOPHILIZED TRYPSIN PRODUCTS

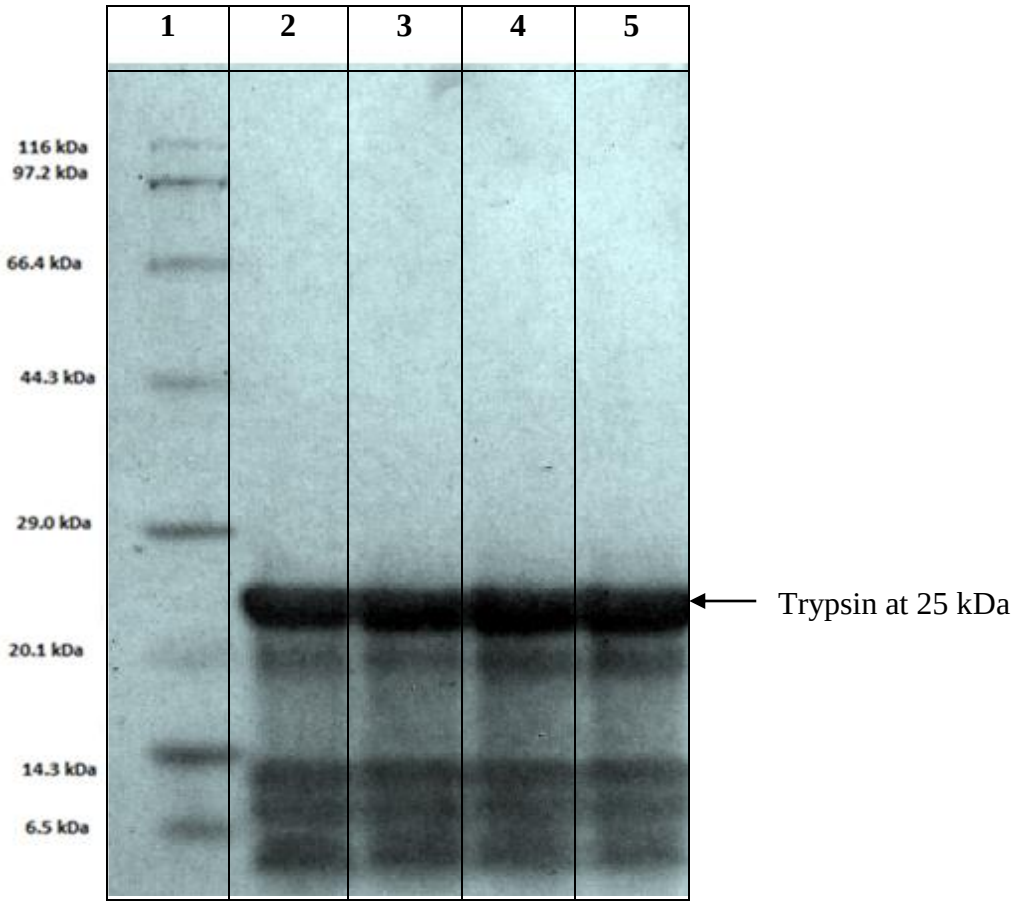


Figure 57. SDS PAGE analysis of three consecutive representative batches of trypsin produced by the new process compared to a control samples from the traditional process. *Lane 1*: Molecular Weight Marker, *Lane 2:* Control batch of traditional processing methodologies (L1230T), *Lane 3:* Batch 1031 processed by new process, *Lane 4:* batch 1041 processed by new processing method, and *lane 5*: batch 1051 processed by new process.

SDS PAGE analysis confirmed the presence of trypsin in all the batches. All the trypsin bands were visible at 25,78 kDa, confirming the molecular weight of trypsin (Graf, 2003, Keller, 1958) . The SDS PAGE analysis indicates no marked difference between trypsin produced by the new process compared to product produced by the traditional process. There were no additional protein bands visible in any of the three validation batches, and the same proteins present in the control sample (15 and 6.5 kDa) are visible in the three validation batches. This 4 – 20% SDS PAGE was run at 50 µg/well at 20 mA for 3.5 hours.

7.4.2 SDS PAGE ANALYSIS OF FINAL LYOPHILIZED CHYMOTRYPSIN PRODUCTS COMPARING TRADITIONAL VERSUS NEW PROCESSING METHODOLOGIES

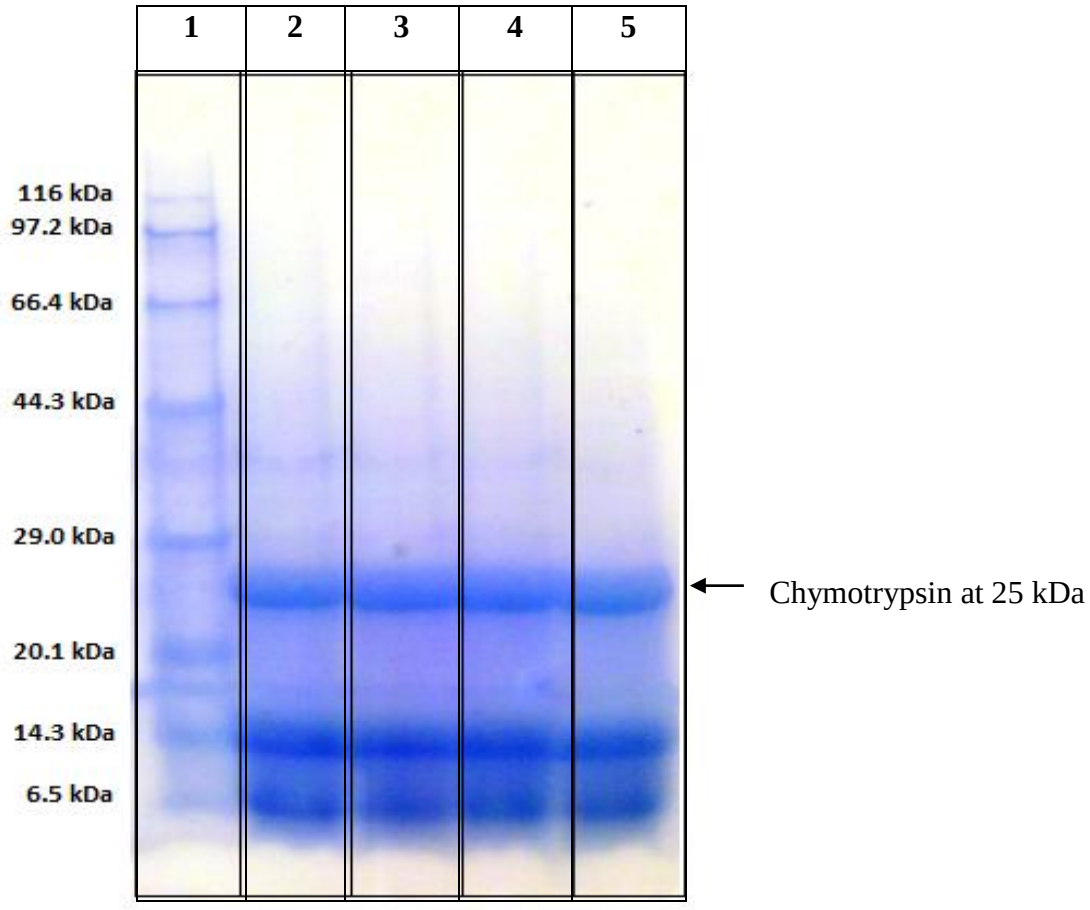


Figure 58. SDS PAGE analysis of three consecutive representative batches of chymotrypsin produced by the new process compared to a control samples from the traditional process. *Lane 1*: Molecular Weight Marker, *Lane 2:* Control batch of traditional processing methodologies (L12260AC), *Lane 3:* Batch 38810 processed by new process, *Lane 4:* batch 38910 processed by new processing method, and *lane 5*: batch 39010 processed by new process.

SDS PAGE analysis confirms the presence of chymotrypsin in all the batches. All the chymotrypsin bands were visible at 25 kDa, confirming the molecular weight of chymotrypsin (Graf, 2003). The SDS PAGE analysis indicated no marked difference between chymotrypsin produced by the new process compared to product produced by the traditional process. There were no additional protein bands visible in any of the three validation batches, and the same contaminating proteins present in the control sample (15 and 6.5 kDa) were visible in the three validation batches. This 4 – 20% SDS PAGE was run at 50 ug/well at 20 mA for 3.5 hours.

To determine the success of the process development, the final lyophilized products needed to be fully compliant with the specification s set out in table 23 and 24 respectively.

Table 23. Summary of the chymotrypsin Quality Control results of the final lyophilized material of the validation batches. These batches were all tested at the same conditions according to methods indicated in the last column. All assay methods are attached as appendices to this document.

Aspect	Description	Units	Limits	L12260AC (Control)	38810	38910	39010	Method
Description	Physical form as per specification	N/A	**As per specification**	White buff powder	White buff powder	White buff powder	White buff powder	Visual
Activity	Chymotrypsin	μkatal/mg	**>5.4**	6.2502	6.2053	6.2468	6.2273	APPENDIX 2
	Chymotrypsin	NFU/mg	**≤1307**	1691.5	1770.3	1942.4	1730.5	APPENDIX 3
	Trypsin BP	%	**<1**	< 1	< 1	< 1	< 1	APPENDIX 4
Additional Data	Moisture (4hours @ 60 °C)	%	**≤5**	0.296	1.095	0.579	0.297%	APPENDIX 5
	Sulphated Ash	%	**≤2.5**	0.438	0.948	0.542	0.742%	APPENDIX 6
	pH in distilled H_2O (10 mg/ml)	N/A	**3.0-5.0**	3.1	3.15	3.16	3.19	APPENDIX 7
	Absorbance	281 nm	**18.5-22.5**	18.791	18.52	18.717	18.62	APPENDIX 8
	Absorbance	250 nm	**<8**	6.83	6.678	6.875	6.698	APPENDIX 8
	Opalescence	N/A	**≤Soln 11**	between I & II	between I & II	between I & II	between I & II	APPENDIX 9
	Enzymatic Activity A	%	**Reddish**	red colour	red colour	red colour	red colour	APPENDIX 10
	Enzymatic Activity B	%	**No colour**	no red colour	no red colour	no red colour	no red colour	APPENDIX 10
	Trypsin Identification	N/A	**No colour**	no colour	no colour	no colour	no colour	APPENDIX 11
	Trypsin	NFU/mg	**Record**	22.57	41.34	36.14	23.1	APPENDIX 12
Solubility	Distilled H_2O	10 mg/ml	**Soluble**	Soluble	Soluble	Soluble	Soluble	APPENDIX 13
Microbiological Data	Total Aerobic Microbial Count	cfu/g	**≤1000**	85	145	40	5	APPENDIX 14
	Total Combined Yeast & Mould	cfu/g	**<100**	<10	<10	<10	<10	APPENDIX 15
	Salmonella	cfu/10g	**0**	Not detected	Not detected	Not detected	Not detected	APPENDIX 16
	Pseudomonas aeruginosa	cfu/g	**0**	Not detected	Not detected	Not detected	Not detected	APPENDIX 17
	Staphylococcus aureus	cfu/g	**0**	Not detected	Not detected	Not detected	Not detected	APPENDIX 18

Table 24. Summary of the trypsin Quality Control results of the final lyophilized material of the validation batches. These batches were all tested at the same conditions according to methods indicated in the last column. All assay methods are attached as appendices to this document.

Aspect	Description	Units	Limits	Control (L1230T)	Batch 1	Batch 2	Batch 3	Method
Description	Physical form as per specification	N/A	**As per specification**	Buff coloured powder	Buff coloured powder	Buff coloured powder	Buff coloured powder	Visual
Activity	Trypsin	u/mg	**>3000**	3946	4198.7	4379	4167	APPENDIX 12
	Trypsin	µkatal/mg	**≥0.5**	1.03	1.004	1.024	0.98	APPENDIX 19
Additional Data	Chymotrypsin / Trypsin	N/A	**≤50/2500**	31	24.7	22	25	
	Trypsin Identification A	N/A	**Purple**	Purple	Purple	Purple	Purple	APPENDIX 20
	Trypsin Identification B	N/A	**No colour**	No Colour	No Colour	No Colour	No Colour	APPENDIX 20
	Chymotrypsin	pH	**>Reference**	Complies	Complies	Complies	Complies	APPENDIX 21
	Loss on drying (0.5g for 2hrs @60°C)	%m/m	**≤5.0**	1.00	0.39	1.00	1.00	APPENDIX 23
	pH (10 mg/ml)	N/A	**3.0 – 6.0**	3.3	3.43	3.3	3.3	APPENDIX 7
	Absorption 280nm	N/A	**13.5-16.5**	15.2	15.7	14.7	14.9	APPENDIX 22
	Absorption 250nm	N/A	**≤7.0**	5.2	5.5	5.4	5.4	APPENDIX 22
	Opalescence (0.10 g in 10ml water)	N/A	**≥ Ref Sol II**	Similar to Ref I	Similar to Ref I	Similar to Ref I	Similar to Ref I	APPENDIX 9
Microbiological Data	*Salmonella*	Count/10g	**0**	0	0	0	0	APPENDIX 16
	E.coli	Count/g	**0**	0	0	0	0	APPENDIX 24
	Total Aerobic Microbial Count	cfu/g	**≤10000**	5	<10	10	10	APPENDIX 14
Solubility	Distilled water	10 mg/ml	**Sparingly soluble**	Soluble	Soluble	Soluble	Soluble	APPENDIX 13

CHAPTER 8

8. CONCLUSION

Following the implementation of the newly designed process (as described in chapter 7), and the changes brought about to the manufacturing plant, the overall yield of the process increased with up to 100% compared to the process yields prior to the study. The total amount of enzyme produced is expressed as Billion units per tonne of starting material produced (BU/ton). The processing time and overall production costs of the process were dramatically reduced. The BU/ton target is obtained when the yield target (kg/ton) was multiplied with the required specific activity of each product.

The total amount of enzyme (expressed as BU/ton) were trended over a 15-month period from September 2010 to December 2011, and summarized in figure 59 and 60 below.

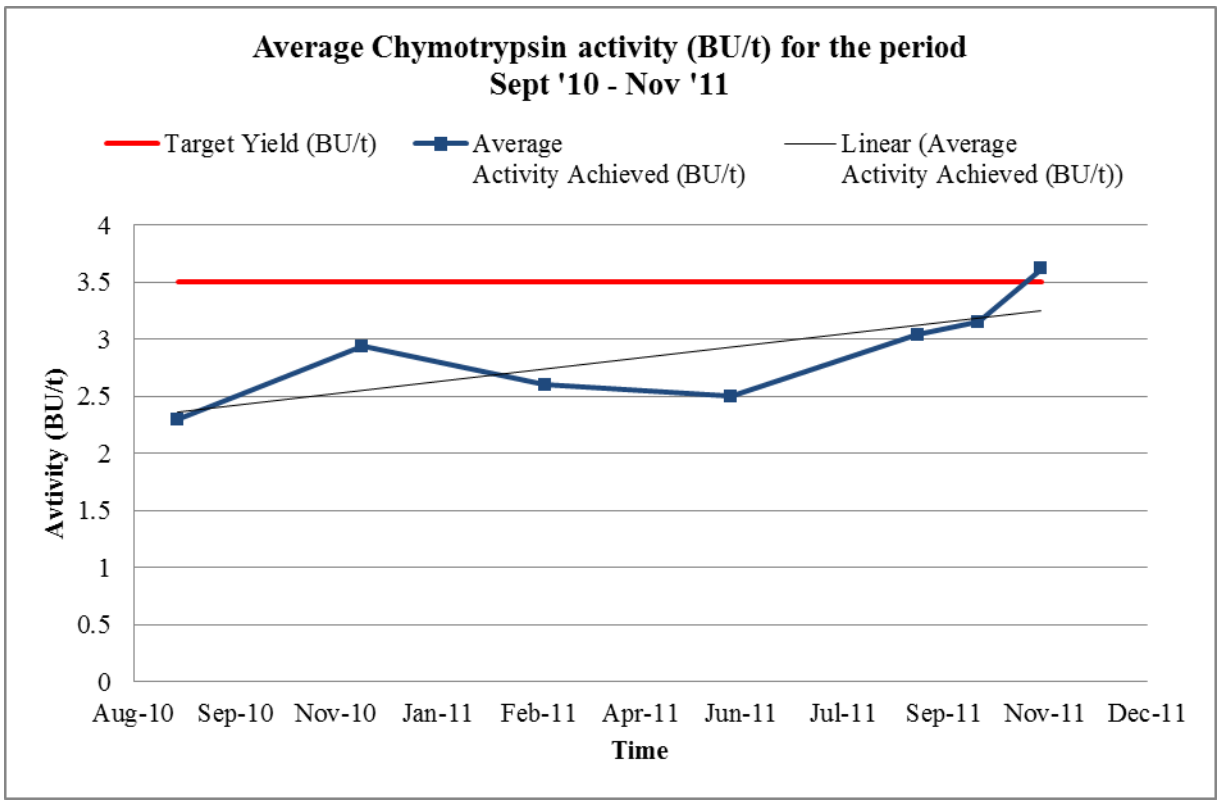


Figure 59. The chymotrypsin yield (Expressed as BU/ton) over the 15-month period from Sept 2010 until Dec 2011. This dataset did not include the implementation of A/S saturation of the zymogen separation stage.

The increase in the chymotrypsin yield over time was not as rapid when compared to trypsin. This was due to regulatory challenges faced with the implementation of the optimized zymogen separation and the change in the secondary process of chymotrypsin. The reduction in total activity per batch from November 2010 to June 2011 could be ascribed to the usage of poor raw material, and a change in the management of the production line, resulting in poor control exerted over the process. Poor pancreases were not acid treated according to the prescribed method enforced by BBI Enzymes, and the fat content of the pancreas was extremely high, complicating process steps such as centrifugation and clarification. As described in section 5.3, when pancreases are not acid treated, the native trypsin content was high, which resulted in CTG activation. When CTG is converted to chymotrypsin prior to the zymogen separation, the CTG crystallization process is ineffective. Process control was aggressively implemented during June 2011, which saw the incline in the final product yields, especially chymotrypsin yields. The full implementation of the optimized zymogen separation was implemented in September 2011.

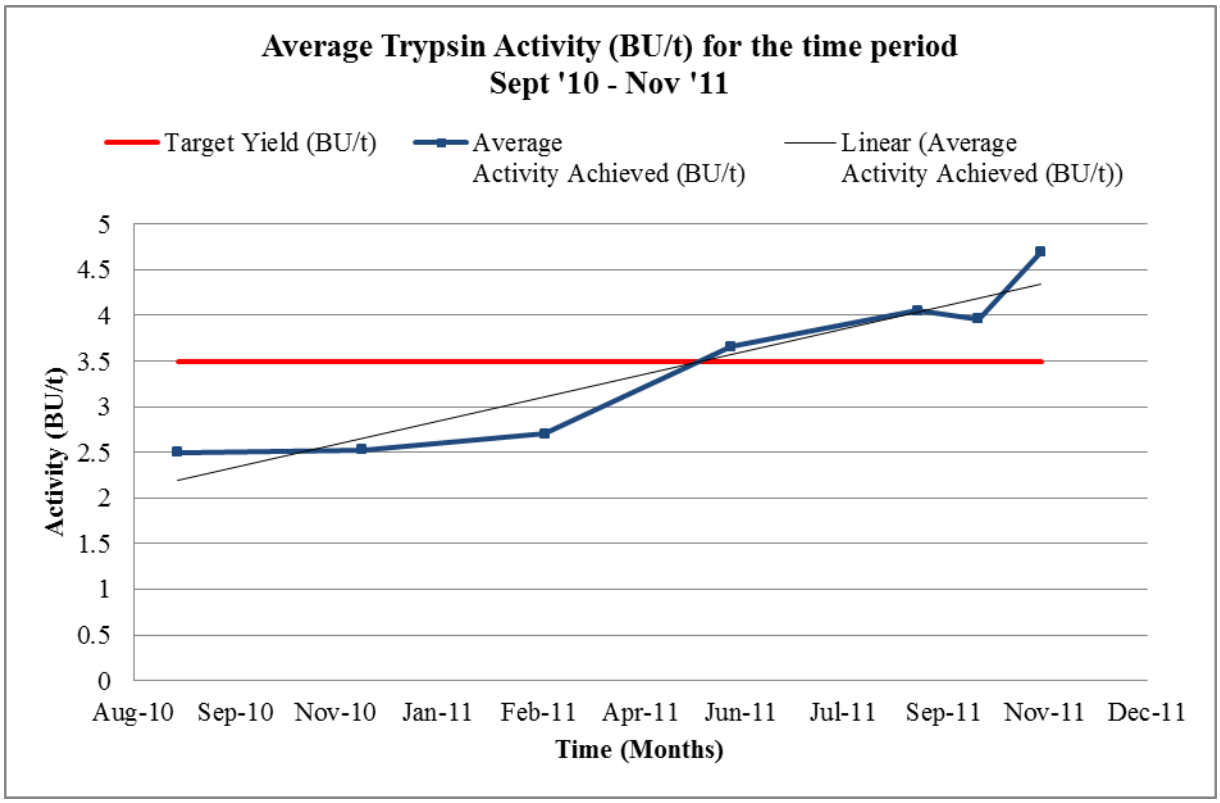


Figure 60. The trypsin yield (Expressed as BU/ton) over the 15-month period from Sept 2010 until Dec 2011, indicating steady positive growth. The marked increase in the yield from February 2011 was brought about by the implementation of the A/S saturation of the trypsin crystallization.

The specific activity of both products improved as a result of process improvements. The average chymotrypsin activity increased from 1300 to ± 1900 U/mg lyophilized product, and trypsin specific activity increased from 3500 to ± 4300 U/mg.

The overall production time was reduced by 4 days, and the total production costs was reduced with R 15 000 per batch.

The overall impact of the process changes had a major impact on the performance of the business. The installation of additional equipment and equipment with higher throughput capabilities ensured that the overall throughput through the factory was increased. The total amount of pancreas processed per month increased from 56 ton to 72 tons.

In addition to the process changes and improvements made to the production processes, the importance of establishing control in a production process was also addressed.

Before the process changes were brought about, the production processes were not properly controlled, and losses during the process could not be established. In the production environment, the methods used to purify proteins should be simple, and instructions should be clear. It should be difficult for production operators to make mistakes. As part of the investigation into the optimization of the purification a process, process control was addressed and measures were put into place to improve the overall control exerted over the production processes. Additional control measures include the following:

- Tighter pH controls. The pH range for the primary process was 1.8 – 2.2. This was changed to 2.0 – 2.2, as 1.8 was too close to the lower stability of trypsin and chymotrypsin (Outzen, 1996).
- Adjustment of pH with 2.5 M H_2SO_4 instead of concentrated H_2SO_4.
- Better control over the addition of solid A/S into the fractionation tanks by the modification of the hopper used to add the A/S. This prevented operators from adding the A/S too fast, and prevented excessive build-up of solid A/S in the bottom of the tanks.
- Strict pH control (pH adjusted to 2.0 – 2.2) across the entire primary processing prevented the activation of trypsin and/or chymotrypsin.
- Accurate calibration of A/S fractionation tanks. The tanks were calibrated using an extremely sensitive and accurate flow meter. This allowed for better volume

determination when performing A/S fractionation. All the tanks were externally calibrated with sight glasses to simplify volume measurements.

- Liquid clarity specifications were established for the clarified 0/20 liquid (< 5FTU) to allow for optimal performance of the ultrafiltration unit.
- Conductivity measurements when performing crystallization
- A/S saturation of CTG and trypsin crystallization

Additional QC analyses were introduced (see figure 61) into the process to quantify and characterise the production process.

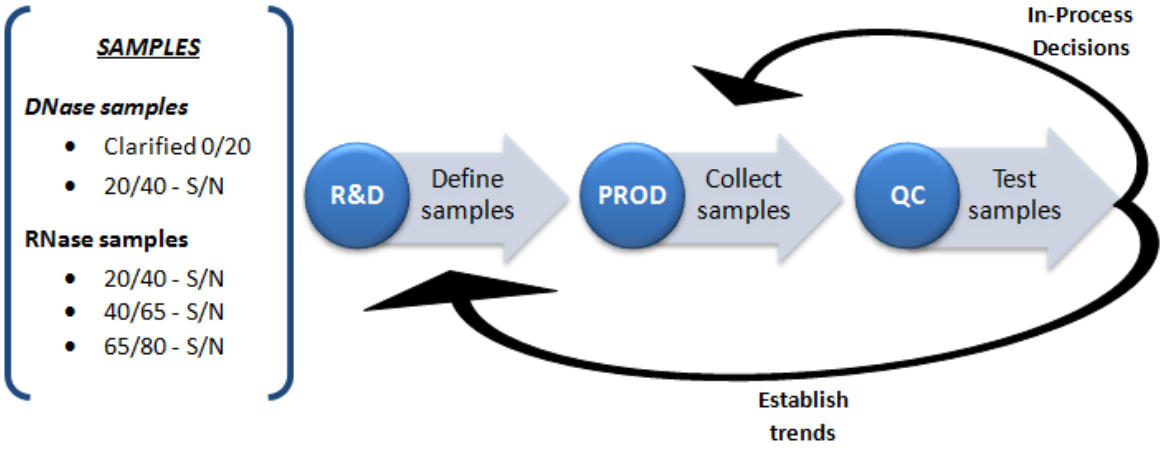


Figure 61. Definition of in-process QC samples to characterize the processing steps. Additional ELISA assays were conducted during the primary processing stages to quantify the total amount of trypsin present in a batch.

This document provides documented evidence that the improvements made to the trypsin and chymotrypsin manufacturing processes did not change the quality of the final product when compared to that of the traditional processing method. All the batches produced as validation batches (incorporating all the changes discussed within this report) did comply with the specification set out in Table 2 and 3.

All the process operators within the PDF business unit have received formal training on all newly installed equipment, and are acquainted with the new processing methodologies. Competency evaluations of each operator proved that the staff working with the new equipment are fully capable / competent to operate the equipment. The master batch records

were updated to reflect the process as described within this report. All the new equipment used in the new process was incorporated in these documents.

BBI Enzymes strive to continually improve all their processes and the practices applied to produce their products better and faster, any future improvements to the process will be managed via the site change control in accordance to their quality management system. BBI Enzymes can implement the changes and equipment described in this report with a measure of confidence in the production plant. Furthermore, the process proved to be flexible and robust. The final product did not differ from the material previously supplied to our customers.

Table 25. Comparison of the major differences between the traditional and the new processing methods.

TRADITIONALPROCESSING METHODS	NEW DEVISED METHOD
Low yields achieved	Target yield (100% improvement on old) achieved.
Time consuming due to a series of precipitation and crystallization stages.	5 days shorter than the traditional processing method, and did not require a series of A/S precipitation steps
Large amounts of A/S used which increased production costs.	5 times less A/S used for precipitation
No in-process assays during primary and limited number of assays during secondary.	In-process control applied in both primary and secondary processing
± 55% crystallization efficiency	Up to 90% crystallization efficiency
Poor process control	Improved process control
	Very high specific activities achieved at final product stage

Future work to be investigated leading on from the work presented in this document include:

1. Optimization of Chromatographic conditions for the purification of trypsin and chymotrypsin and as a replacement for the Zymogen separation.

2. Recovery of trypsin from the TRML that was precipitated and stored in the freezers. This remained a rich source of trypsin and chymotrypsin, and can be further investigated.

3. Crystallization of proteins (CTG and trypsin) at an oil – water interface. This was discovered as a production non-conformance when oil leaked into the product whilst crystallizing, when increased yields were observed. The work of Chayen *et al*. (2004) indicates this is still a developing science, and can be further developed for commercial application.

4. The ELISA assay method for determining trypsinogen content during the primary processing can be further optimized by obtaining new antibodies against a specific Amino acid sequence unique to trypsinogen (a 6 amino acid sequence called the Activation peptide) which is cleaved during trypsinogen activation. This would ensure a higher level of specificity of the assay for trypsinogen, and would not cross react with trypsin.

CHAPTER 9

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10. APPENDICIES

APPENDIX 1

Primary amino acid sequence of Bovine Trypsin. (Uniprot, 2002)

```
        10         20         30         40         50         60
MKTFIFLALL GAAVAFPVDD DDKIVGGYTC GANTVPYQVS LNSGYHFCGG SLINSQWVVS
        70         80         90        100        110        120
AAHCYKSGIQ VRLGEDNINV VEGNEQFISA SKSIVHPSYN SNTLNNDIML IKLKSAASLN
       130        140        150        160        170        180
SRVASISLPT SCASAGTQCL ISGWGNTKSS GTSYPDVLKC LKAPILSDSS CKSAYPGQIT
       190        200        210        220        230        240
SNMFCAGYLE GGKDSCQGDS GGPVVCSGKL QGIVSWGSGC AQKNKPGVYT KVCNYVSWIK


QTIASN
```

Primary amino acid sequence of Bovine Chymotrypsin. (Uniprot, 2002)

```
       10         20         30         40         50         60
CGVPAIQPVL SGLSRIVNGE EAVPGSWPWQ VSLQDKTGFH FCGGSLINEN WVVTAAHCGV

        70         80         90        100        110        120
TTSDVVVAGE FDQGSSSEKI QKLKIAKVFK NSKYNSLTIN NDITLLKLST AASFSQTVSA

       130        140        150        160        170        180
VCLPSASDDF AAGTTCVTTG WGLTRYTNAN TPDRLQQASL PLLSNTNCKK YWGTKIKDAM

       190        200        210        220        230        240
ICAGASGVSS CMGDSGGPLV CKKNGAWTLV GIVSWGSSTC STSTPGVYAR VTALVNWVQQ
TLAAN
```

APPENDIX 2

PROCESS: Assay Methods	**ISSUE DATE:** 26.11.2009	**DOC NO:** BP6.2.2-WI145
TITLE: µKatal - Chymotrypsin	**REVISION:** 1	**PAGE:** 1 of 4
AUTHORISED: Senior Technical Officer	**APPROVED: QA & Compliance Manager**	

1. Purpose

To determine the activity of the samples using the Crison Compact Titrator.

2. Scope

All samples requiring katal results.

3. Responsibility

All assayists determining the katal activity.

4. Safety

Protective clothing to be worn.
Refer to labels of the chemicals regarding safe handling.

5. Reagents

5.1 0,01M $CaCl_2$
Dissolve 147 mg $CaCl_2$ dihydrate (MM147.02) in 100 ml distilled water.

5.2 0,1M NaOH
0,1M NaOH standardised solution.

5.3 0.02M NaOH
Dilute 100 ml 0,1M NaOH (1.2) to 500ml with distilled H_2O.

5.4 0,001M HCl
Dilute 0,089 ml concentrated HCl to 1 ℓ with distilled H_2O

5.5 Substrate
0,2M ATEE
Dissolve 539 mg ATEE monohydrate (MM 269,3) in 96% absolute ethanol and dilute to 10 ml with 96% ethanol.

6. Equipment

Compact Titrator D with 2x syringes
Combined pH electrode 5212
Water bath with a heater circulator
Jacketed vessel
Small magnetic stirrer bar
Test tubes, 5ml & 10 ml glass pipettes, volumetric flasks
Computer and printer
ComCom 2000 software
Analytical balance.

7. Procedure.

7.1 Sample Preparation
Dissolve 25 mg Chymotrypsin sample in 0,001M HCl and dilute to 250 ml with 0,001M HCl. Store solution on ice.

7.2 Reference Standard Preparation:
Dissolve 10mg reference standard in 0,001M HCl and dilute to 100 ml with 0,001M HCl. Store solution on ice.

7.3 **pH Calibration**

The pH calibration is to be completed each day before use. The speed of the stirrer should be set to approx 40%.

ComCom 2000 Software
Open the ComCom 2000 Software
Open the "Titrator" screen before starting any calibration. Instruments→Titrator→Open→Titrator 1.
The software is now ready to received information.

1. Press **3** for the calibration option.
2. Select the correct user number.
3. Press **1** for electrode nr1.
4. Place buffer pH 7 in the electrode holder and press **RUN.**
5. The mV readings should be 0 mV ± 20 mV.
6. Rinse the electrode and place buffer pH 4 in the electrode holder and press **RUN.**
7. The mV reading should be 168 mV ± 20 mV.
8. Press Stop.
9. The Sensitivity allowed is to between 85 – 110%.
10. Press C to clear.

7.4 **Priming of burettes:**

Place the inlet tubes in the reagent bottle containing the NaOH – solution and do the following:
Press **6** for the "More" option.
Press **6** for the "Burette" option.
Press **1** for the "Move burette option.
Select the burette nr and press **enter.**
Use 1&2 to move the burette up & down.
Press "Stop" after 3 times.
Press O to quit

Or

Press **1** for the "Execute" option
Press **5** and **enter** to select Prg 5 for the Priming program.
"**Place waste vessel**" press **Run.**
Place H_2O bottle for priming" Press Run
The syringe will be rinsed 3x times with distilled water
"Place reagent bottle for priming" press Run
The burette will be rinsed with the reagents 3x times
Press **0** to Quit

7.5 **Determination of Chymotrypsin Activity**

Execution
ComCom 2000 Software
Open the ComCom 2000 Software
Ensure that the correct Database is open (i.e. Trypsin, Chymotrypsin etc)
Open the "Tritrator" screen before starting any titrations.
Instruments→Tritrator→Open→Tritrator1.
The software is now ready to receive information

7.6 **Sample Preparation**

1. Accurately add 10 ml buffer solution (5.1) to the jacketed vessel.
2. Accurately add 0,35 ml of 0,2M ATEE (5.5)
3. Add a magnetic stirrer bar.
4. Place the jacketed vessel onto the magnetic stirrer and equilibrate the solution to 25ºC ± 0,1ºC.
5. Press **1** for the "**Execute**" option.
6. Select the correct user number.
7. Press 2 and **enter** to select Prg 2 for the Chymotrypsin determination.
8. "**Code Sample**" - Key in the sample code via the PC keyboard (i.e. HST B113) or display keyboard and press **enter.**
9. The syringe will fill up
10. "**Place sample vessel**", Ensure that the sample vessel is inserted into the magnetic stirrer and ensure that electrode height is correctly positioned. **Pre-titration**". The pre-titration will be carried out to adjust the medium to a pre-defined pH. It will only be executed if it has been selected in the Programming.
11. Press **Run** to start the pre-titration
12. The magnetic stirrer will automatically be activated. The stirring speed maybe adjusted using the + and – key.
13. The titrator will complete the pre-titrator with 0,02M NaOH up to pH 7,90
14. <1. Start stat><2. Reagent Add>
    Press **2** to add 5 µl increments of reagent to adjust the final pH to pH 8,00.
    Press **1** to start the pH Stat titration
15. The syringe will fill with the reagent
16. Accurately add 50 µl solution and press **Run** to start the reaction.
17. The pH will be maintained at pH 8,00 for 4 minutes via the addition of 0.02M NaOH. Every 30 seconds the volume added will be recorded.
18. Calculate the volume of 0.02M sodium hydroxide used per second between 30 s and 210 s.
19. Press **C** to clear the results from the titrator screen. The results will be stored in the database.
20. The same procedure in the same conditions with the reference solution must be preformed.
21. Press **0** to Quit or press1 to repeat the process. (Steps 8 to 19 will be repeated)

7.7 **Cleaning of burettes:**

To rinse the burettes with distilled water do the following:

1 Remove the inlet tubes for the reagents bottles and place the inlet tube in a beaker containing distilled water.
2 Press **6** for the "Move" option
3 Press **6** for the "Burette" option
4 Press **1** for the "Move burette option
5 Select the burette nr and press **enter**
6 Use 1&2 to move the burette up & down

or

1 Press **1** for the "Execute" option.
2 Press 4 and **enter** to select Prg 4 for the cleaning program
3 "**Place waste vessel**" Press **Run**
4 "**Return reagent to bottle**" Press **2**
The burette will be empty into the waste container
5 "Place H_2O bottle for cleaning" Press **Run**
6 Press **0** to Quit

7.8 **Protocol to follow**

a) Prepare two weighings of the reference standard.
b) Assay each weighing in duplicate.
c) Prepare one weighing of the control sample.
d) Assay in duplicate.
e) For the sample, at least three weightings must be prepared.
f) Assay each weighing in duplicate.
g) After completion of the above, another weighing of the reference standard should be tested in duplicate.

Note: For the reference standard, the results obtained must be within 3% of each other.
For the control and the samples, the results obtained must be within 5% of each other respectively.

8. **Calculation**

$$\text{ACTIVITY (micro katals/mg)} = \frac{m' \times V \times A}{m \times V'}$$

Where m = mass in milligrams of test sample.
m' = mass in milligrams of reference sample.
V = volume of 0,02M NaOH used per second by the test solution.
V' = volume of 0,02M NaOH used per second by the reference solution.
A = activity of reference sample in microkatals per milligram.

APPENDIX 3

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI11
TITLE: Chymotrypsin	**REVISION:** 0	**PAGE:** 1 of 2
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. Purpose

The method is utilised to determine the hydrolysis of N-acetyl-L-tyrosine ethyl ester (ATEE) which causes a decrease in absorbance.

2. Scope

Applies to all freeze dried as well as liquid products.

3. Responsibility

All assayists performing hydrolysis analysis.

4. Safety precautions

Protective eyewear to be worn.
Practice extreme caution when handling chromic acid for cleaning purposes.
Practice caution when handling the quartz cuvette.

5. Reaction

As suggested by Schwert and Takenaka and modified to conform to that laid down by the National Formulary X11 edition in which N-acetyl-L-tyrosine ethyl ester (ATEE) is hydrolyzed at the ester linkage causing a decrease of absorbance measured at 237nm and 25°C.

$$ATEE + H_2O \text{ ---------[CT]-------> N-acetyl-L-tyrosine + ethanol}$$

Unit Definition

That amount of enzyme causing a decrease in absorbance at 237nm of 0,0075 per minute at 25°C.

6. Reagents

6.1 0,067 M Potassium Phosphate Buffer pH 7,0

Dissolve 3,53 g KH_2PO_4 anhydrous (MM 136,09) and 7,07 g K_2HPO_4 anhydrous (MM 174,18) in water, check pH to 7,0 and dilute to 1 l. Store at R/T.

6.2 0,001 M HCl

Dilute 0,089 ml concentrated HCl to 1 l with water. Store on ice.

6.3 Substrate

0,000934 M ATEE in buffer
Dissolve 25,2 mg ATEE monohydrate (MM 269,3) in 100 ml buffer at 70°C, cool rapidly and adjust absorbance 237nm to 1,2. Equilibrate in water bath at 25°C or;

For frozen sub-packed aliquots, allow substrate to thaw and adjust absorbance 237nm to 1,2 using buffer (2.1). Equilibrate in water bath at 25°C.

7. Sample Preparation/Enzyme

Prepare 5 mg enzyme ml ice-cold 0,001M HCl, Weigh approximately 20 mg – 25 mg enzyme. Immediately before assay dilute in ice cold HCl to yield approximately 10-20 units/ml (0,015 - 0,030 ΔA/min).

8. Procedure

Into a 10 mm quartz cell, pipette the following:

Substrate	3,0 ml
Equilibrate at 25°C and monitor ΔA/min	
Enzyme at zero time	0,2 ml 3,2 ml
Record rate of decrease in absorbance at 237 nm for approximately 5 minutes.	

9. Calculation

9.1 Dilution $= \dfrac{\text{Expected activity x concentration of sample (mg/ml)}}{\text{15 u / ml}}$

9.2 Units/mg material $= \dfrac{\Delta A237/\text{min X dilution}}{\text{0,0075 X 0,2 X mg enzyme/ml original solution}}$

Due to variations in substrate batches it is necessary to determine the suitability of the substrate by carrying out a reference assay using a NF Chymotrypsin reference standard. The quoted and actual activity obtained for the standard is then used to correct the activity obtained for the sample as follows.

$$\frac{\text{Units/mg material x quoted standard activity}}{\text{Standard activity obtained}} = \text{corrected sample activity}$$

10. Reference

Schwert G.W. and Takenaka Y.: (1955) Biochim. Biophys. Acta. 16 570.

APPENDIX 4

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI90
TITLE: Determination of Trypsin in Chymotrypsin	**REVISION:** 0	**PAGE:** 1 of 1
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. **REACTION**

The spot plate test is used to determine the level of trypsin in a chymotrypsin sample.

2. **REAGENTS**

2.1 Methyl Red T.S.

Dissolve 10 mg methyl red in 10 ml alcohol and filter if neccessary. Store in an amber bottle at ambient temperature.

2.2 Methylene Blue T.S.

Dissolve 5 mg methylene blue in 4 ml alcohol and dilute with alcohol to 10 ml. Store in an amber bottle at ambient temperature.

2.3 Methyl red/Methylene Blue T.S.

Add 1 ml methyl red T.S. to 1 ml methylene blue T.S. and mix.

2.4 0,20 M Tris(hydroxymethyl)aminomethane

Dissolve 1,2114 g Tris (MM 121,14) in 30 ml water. Adjust volume to 50 ml with water.

2.5 0,08 M Tris(hydroxymethyl)aminomethane Buffer pH 8,1

Dissolve 294 mg $CaCl_2$ (MM 110,99) in 40 ml of solution (2.4) and adjust to pH 8,1 with HCl then dilute to 100 ml.

2.6 **Substrate**

Transfer 98,5 mg p-toluenesulphonyl-L-arginine methyl ester.HCl (TAME) to a 25 ml volumetric flask. Add 5 ml 0,08M Tris buffer pH 8,1 and mix. Add 0,2 ml (2.3). Adjust volume to 25 ml with water.

3. **SAMPLE PREPARATION/ENZYME**

Prepare 10 mg chymotrypsin/ml distilled water.

4. **PROCEDURE**

Pipette 50 µl sample to a depression on a white spot plate.
Add 0,2 ml substrate solution.

Note:

If no purple colour develops within 3 minutes then not more than 1% trypsin is present in the sample.

APPENDIX 5

PROCESS: Assay Methods	**ISSUE DATE:** 19.02.2010	**DOC NO:** BP6.2.2-WI28
TITLE: Loss on drying	**REVISION:** 1	**PAGE:** 1 of 2
AUTHORISED: Senior Technical Officer	**APPROVED: QA Compliance Manager**	

1. Purpose

Method to determine the Loss on Drying of samples submitted to the QC Laboratory.

2. Scope

Applies to all products which require loss on drying analysis as per the product specification submitted to the QC laboratory.

3. Responsibility

All Assayists who perform loss on drying analysis.

4. Safety Precautions

Wear appropriate protective clothing and glasses.
Use tongs when handling hot crucibles.
Practice caution when handling the desiccator containing Silica Gel. Do not exert excessive force. Request assistance if additional force is required.
Practice caution when operating the vacuum oven. Release vacuum gradually to allow the pressure differential to equilibrate.

5. Equipment

Vacuum oven
Vacuum pump
Crucible tongs
Porcelain or silica crucibles with lids
Analytical balance
Desiccator containing Silica Gel

6 Procedure

6.2.1 Set the vacuum oven to the 60˚C to equilibrate.

6.2.2 Weigh and record the mass of an empty clean silica or porcelain crucible (A). Handle the crucible with crucible tongs.

Note: Tests on each sample to be prepared in duplicate. Do not handle the crucibles with bare hands to avoid contamination. Take precaution to limit exposure of the sample to the environment to avoid possible moisture pick up from the atmosphere.

6.2.2 Add approximately 0.5g sample to the crucible. Weigh and record the mass (B) accurately.
6.2.3 Place the crucible in the vacuum oven. Ensure that the door is closed securely and apply vacuum.
6.2.3 Allow for drying time of 4 hours and remove the crucible using tongs.
6.2.4 Place the crucible in a desiccator and cover with the crucible lid to cool for approximately 30 minutes.
6.2.5 Weigh and record mass (C) of the dried crucible and sample without the crucible lid.

7. Calculation

$$\% \text{ LOD} = \frac{\text{B (wt. cruc + sample before dry)} - \text{C (wt. cruc + sample after dry)} \times 100}{\text{B (wt. cruc + sample before dry)} - \text{A (wt. cruc)}}$$

8. Cleaning of crucibles

8.1 Inspection of crucibles is required after every use (inspect for any cracks)
8.2 Clean the crucibles in water containing Extran.
8.3 Rinse the crucibles thoroughly with distilled water.
8.4 Place the crucibles in the oven at 70^0C.
8.5 Remove from the oven and allow to cool to ambient temperature.

References:

United States Pharmacopeia, Chapter <731> Loss on Drying.
European Pharmacopeia.

The sample quantity and drying time specified has been standardised for consistency.

APPENDIX 6

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI33
TITLE: Assay - Sulphated Ash	**REVISION:** 0	**PAGE:** 1 of 2
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. Purpose

Method to determine the mass of residual substance not volatised.

2. Scope

Applies to all products which require sulphated ash analysis. The sulphated ash is also known as residue on ignition.

3. Responsibility

All assayists performing sulphated ash analysis.

4. Safety

Wear appropriate protective clothing eg. heat resistant gloves, safety goggles.

Use tongs when handling hot crucibles.

All hot work to be done in the fumehood.

Practise extreme caution when handling sulphuric acid.

Place hot crucibles gauze mesh or suitable surface (asbestos sheet) to cool for at least 5minutes before placing it in the dessicator.

5. Equipment

Furnace preset at 800℃ ± 25℃;
Heat resistant gloves;
Crucible tongs;
Safety goggles;
Porcelain or any other suitable crucibles;
Analytical balance;
Dessicator containing silica gel;
Gauze mesh or asbestos sheet;

6. Procedure

6.1 Tare the analytical balance.

6.2 Place a previously ignited, clean and dry crucible on the balance. Record the weight. Ensure that the weight displayed is stable. If drifting is observed, place the crucible in the dessicator and repeat until constant weight is observed.

6.3 Add approximately 0,2 grams sample to the crucible. Weigh and record the mass.

6.4 Place the crucible and sample into the furnace.

6.5 Allow the furnace to reach 800 °C and leave the sample for 1hour to ignite. Alternatively, preheat the crucible using a bunsen burner and place in the preheated furnace for 1hour.

6.6 After this period, remove the crucible from the furnace and place it on gauze mesh or asbestos sheet for a least 5minutes. Place in a dessicator and allow to cool to ambient temperature for at least 1½hours. DO NOT APPLY VACUUM.

6.6 Once cooled to ambient temperature, record the crucible and sulphated ash weight. If the residue obtained, exceeds the specification limit, repeat the ignition until a constant mass is obtained.

6.7 The sample maybe treated with a few drops of concentrated sulphuric acid (at least 1ml).

Note: Tests on each sample to be prepared in duplicate.

7. Calculation

$$\% \text{ ash} = \frac{(\text{mass cruc. + sample after ash}) - (\text{mass cruc.}) \times 100}{(\text{mass cruc. + sample before ash}) - (\text{mass cruc.})}$$

8. Cleaning of Crucibles

- Inspect the crucibles for any cracks or chips which can compromise the integrity of the crucibles.
- Clean the crucibles with suitable solvent (eg. IN HCl)
- Rinse thoroughly with distilled water.
- Place the crucibles in the furnace at 800 °C and allow to dry (approximately 1hour)
- Remove from the furnace and allow to cool to ambient temperature in a dessicator.

References: EP (2.4.14) Sulphated Ash, USP 31 (281) Residue on Ignition

APPENDIX 7

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI117
TITLE: pH Meter – Mettler Toledo SevenMulti	**REVISION:** 0	**PAGE:** 1 of 3
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. Purpose

Method to calibrate and measure pH using the Mettler Toledo Sevenmulti.

2. Scope

All samples requiring pH measurement.

3. Responsibility

All assayists.

4. Safety

The electrode contains glass parts.

5. Equipment

Mettler Toledo SevenMulti
Calibration buffer (pH 7,00 & pH 4,01)
3M KCl electrolyte
Pepsin/HCl
Conductivity standards (12,88mS.cm^{-1} & 1413 µS.cm $^{-1}$)

5. Procedure

5.1 pH Meter

5.1.2 Calibration

- Switch on the meter.
- Press, 'dual'.
- Press 'left' to select the pH mode.
- Ensure the pH buffer solutions is at 20°±1°C.
- Place the electrode in the first calibration buffer (pH7,00) and press 'Cal' to start calibration.
- Wait until 'A' stops flashing and record the reading on form BP6.2.2-WI117-SF01.
- Rinse the electrode with distilled water, insert the second calibration buffer (pH 4,01) and press 'Cal' to start calibration.
- Wait until 'A' stops flashing and record the readings on form BP6.2.2-WI117-SF01.
- End calibration with 'End' after reading the last calibration buffer, a table with the calibration results will appear on the display.
- Record the slope.

- Press 'save' to use the calibration and to make adjustments for subsequent measurement.
- Rinse the electrode with distilled water.
- Insert the pH 7,00 buffer and press 'read'.
- Wait for 'A' to stop flashing and record the reading on form BP6.2.2-WI117-SF02.

5.1.2 Sample measurement

- Place the pH electrode in the sample and press 'Read'.
- Wait for 'A' to stop flashing and record the pH reading as well as the temperature displayed.
- End the measurement manually by pressing 'Read' again.
- In general measurement using stirred samples is preferred.
- Ensure that the solution is above the cell chamber slot.
- To prevent carry over from solution to solution, rinse the electrode with distilled water between measurements.
- After pH measurements, soak the electrode in 3M KCl electrolyte.

5.1.3 For other models of pH meters

- Standardise pH meter with pH 7,00 and pH 4,00 buffer at 20ºC.
- Prepare samples at specified concentrations using the appropriate diluent (see specification file for details)
- Adjust temperature of samples at 20ºC.
- Insert electrode into sample. Gently swirl sample around electrode and allow to stand.
- Wait for reading to stabilize and record reading as well as temperature of sample.
- Rinse the electrode well with distilled H_2O and re-check pH 7,00 buffer.

NB:

After rinsing do not rub the glass membrane, since this can increase response time or damage the electrode. With high protein concentrations, the protein may precipitate at the junction if they may come into contact with the KCl reference electrode. Immersing the electrode into a pepsin/HCl solution or electrode cleaning solution for several hours or overnight can clean junctions contaminated with proteins.

5.2 Conductivity meter

5.2.1 Calibration

- Press 'right' to select the conductivity mode. Ensure that the temperature of the standard solutions are 25°±1°C.
- Place the conductivity electrode in the first standard solution and press 'cal'.
- Wait for 'A' to stop flashing and record the reading on form BP6.2.2-WI117-SF01.
- Rinse the electrode with distilled water, insert the second standard solution and press 'cal'.
- Wait for 'A' to stop flashing and record the reading on form BP6.2.2-WI117-SF01.
- Press 'end' to stop calibration and press 'save'.

5.2.2 Sample Measurement

- To measure the conductivity of a sample, insert the electrode into the sample and press 'read'.
- Record the results as well as the temperature.
- End the measurement manually by pressing 'read' again.
- Wash the electrode thoroughly with distilled water to prevent carry over between samples.

References

Toledo guide to pH measurement
Mettler Toledo SevenMulti operating instructions

APPENDIX 8

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI138
TITLE: Chymotrypsin Absorbance Test	**REVISION:** 0	**PAGE:** 1 of 1
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. **Reagent**

   0,001 M HCl

   Dilute 0,089 ml concentrated Hydrochloric Acid (MM 36,46) to 1 litre with distilled water.

2. **Procedure**

   2.1 Accurately weigh 30,0 mg Chymotrypsin Code: 031110 in a 100 ml volumetric flask. Add 0,001M HCl to dissolved the material and adjust volume to 100 ml with 0,001M HCl.

   2.2 Determine the absorbance of the solution at 281 nm and 250 nm respectively. Subtract the blank readings.

3. **Calculation**

$$E^{1\%}_{281} = \Delta A_{281} \times \frac{10}{0,3}$$

$$E^{1\%}_{250} = \Delta A_{250} \times \frac{10}{0,3}$$

4. **Reference**

2000 British Pharmacopoeia CD-ROM
British Pharmacopoeia volume 1

APPENDIX 9

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI103
TITLE: Clarity and Degree of Opalescence	**REVISION:** 0	**PAGE:** 1 of 1
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. APPEARANCE OF 1% SOLUTION [10 mg/ml distilled H_2O]

Prepare a 10 mg enzyme/ml in distilled H_2O and check the solubility. Divide this solution into two and store one on ice and the other at room temperature and check their respective solubilities after 1 hour.

2. DEGREE OF OPALESCENCE:

2.1 REAGENTS:

2.1.1 Hexamine soln (0,713M)

Dissolve 2,5 g hexamine (MM 140,19) in 25 ml distilled H_2O

2.1.2 Hydrazine Sulphate Solution

Dissolve 1 g hydrazine sulphate AR (MM 130,12) in H_2O and dilute to 100 ml with distilled H_2O. Allow to stand for 4-6 hours.

2.1.3 Primary Opalescence Suspension

Add 25 ml of hydrazine sulphate solution to the 25 ml of hexamine solution and mix. Allow to stand for 24 hours. This suspension is stable for 2 months, provided it is stored in a glass container free from surface defects. The suspension must not adhere to the glass and must be well mixed before use.

2.1.4 Standard of Opalescence

Dilute 15 ml of the primary opalescence suspension to 1 000 ml with distilled H_2O. This suspension is freshly prepared and may be stored for at most 24 hours.

3. PROCEDURE

Reference Suspensions

Prepare the reference suspensions according to the following table. Mix and shake before use.

	I	II	III	IV
STANDARD OF OPALESCENCE	5 ml	10 ml	30 ml	50 ml
WATER	95 ml	90 ml	70 ml	50 ml
Compare identical volumes of sample and reference suspension in identical comparison tubes and record.				

APPENDIX 10

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI152
TITLE: Identification of Chymotrypsin	**REVISION:** 0	**PAGE:** 1 of 2
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. Purpose

To perform identification tests on the products as prescribed by the European and British pharmacopoeia monographs.

2. Scope

All samples requiring the test as per customer specification.

3. Responsibility

All assayists.

4. Safety Precautions

Refer to the manufacturer's instructions or labels for safe handling.

5. Reagents

5.1 0,067 M Phoshate buffer ph 7,0

Dissolve 3,53 g KH_2PO_4 anhydrous (MM 136,09) and 7,07g KH_2PO_4 anhydrous (MM 174,18) in distilled water, check pH to 7,0 and dilute to 1ℓ with distilled water.

5.2 Methyl red T.S

Dissolve 10 mg methyl red indicator in 10mℓ ethanol.

5.3 0,1M NaOH

0,1M NaOH standardised solution.

5.4 0,02M NaOH

Dilute 10mℓ 0,1M NaOH to 50mℓ with distilled water.

5.5 2% Tosyl-phenalanyl-chloroethane

Dissolve 20 mg N-p-Tosyl-L-phenylalanine chloromethyl ketone Sigma T4376 (MM351,9) / mℓ ethanol.

6. Substrate

Dissolve 24mg N-Acetyl-L-tyrosine ethyl ester monohydrate (ATEE) and 0,2mℓ ethanol to 2mℓ 0,067M phosphate buffer pH 7,0. Add 1mℓ methyl red indicator and adjust the volume to 10mℓ with distilled water.

7. Sample Preparation

Test solution: Chymotrypsin solution at 10mg/mℓ distilled water.

APPENDIX 11

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI147
TITLE: Identification of Trypsin	**REVISION:** 0	**PAGE:** 1 of 2
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. **Reagents**

1.1 2% Tosyl-lysyl – chloromethane hydrochloride:

Dissolve 20 mg Tosyl – L – lysyl-chloromethyl ketone hydrochloride, FLUKA 90182 (MM 369,3)/ ml distilled water.

1.2 0,1N NaOH

0,1N NaOH standardised solution.

1.3 Methyl Red T.S.

Dissolve 10 mg methyl red in 10 ml alcohol and filter if neccessary. Store in an amber bottle at ambient temperature.

1.4 Methylene Blue T.S.

Dissolve 5 mg methylene blue in 4 ml alcohol and dilute with alcohol to 10 ml. Store in an amber bottle at ambient temperature.

1.5 Methyl red/Methylene Blue T.S.

Add 1 ml methyl red T.S. to 1 ml methylene blue T.S. and mix.

1.6 0,20 M Tris(hydroxymethyl)aminomethane

Dissolve 1,2114 g Tris (MM 121,14) in 30 ml water. Adjust volume to 50 ml with water.

1.7 0,08 M Tris(hydroxymethyl)aminomethane Buffer pH 8,1

Dissolve 294 mg $CaCl_2$ (MM 110,99) in 40 ml of solution (1.6) and adjust to pH 8,1 with HCl then dilute to 100 ml.

1.8 Substrate

Transfer 98,5 mg p-toluenesulphonyl-L-arginine methyl ester.HCl (TAME) to a 25 ml volumetric flask. Add 5 ml 0,08M Tris buffer pH 8,1 and mix. Add 0,2 ml (1.5). Adjust volume to 25 ml with water.

2. **Sample Preparation**

Prepare Trypsin at 10 mg/ml with distilled water.

3 **Procedure**

A. Dilute 1 ml sample to 100 ml with distilled water.
Mix 0,1 of this solution with 0,2 ml substrate.
A purple colour develops within 3 minutes

B. Dilute 1 ml sample to 10 ml with distilled water.
Add 0,2 ml 2% Tosyl-lysyl-chloromethane HCl
Adjust pH to 7,0 with 0,1N NaOH and stir for 2 hours.
After 2 hours dilute the solution to 50 ml with distilled water.
Mix 0,1 ml of this solution with 0,2 ml substrate
No purple colour develops

APPENDIX 12

PROCESS: Assay Methods	**ISSUE DATE:** 25.10.2011	**DOC NO:** BP6.2.2-WI20
TITLE: Trypsin	**REVISION:** 1	**PAGE:** 1 of 3
AUTHORISED: Testing Associate	**APPROVED: QA Manager**	

1. Purpose

The method is utilised to determine the hydrolysis of N-benzoyl-L-arginine ethyl ester (BAEE) which causes an increase in absorbance.

2. Scope

Applies to all freeze dried and liquid products.

3. Responsibility

All Testing Associates performing hydrolysis analysis.

4. Safety precautions

Protective eyewear to be worn.
Practice extreme caution when handling chromic acid for cleaning purposes.
Practice caution when handling the quartz cuvette.

5. Reaction

That of Schwert and Takenaka and modified to conform to that laid down by the National Formulary X11 edition in which N-benzoyl-L-arginine ethyl ester (BAEE) is hydrolyzed at the ester linkage causing an increase of absorbance measured at 253nm and 25°C.

$$BAEE + H_2O \text{ ---------[TRYPSIN]----------> } BA + \text{ethanol}$$

Unit Definition

That amount of enzyme causing an increase in absorbance at 253nm of 0.003 per minute at 25°C.

6. Reagents

6.1 0.001 M HCl

Dilute 0.089 ml concentrated HCl with water to 1 L. Store on ice.

6.2 0.067 M Potassium Phosphate Buffer pH 7.6

Dissolve 1.17 g KH_2PO_4 anhydrous (MM 136.09) and 10.08 g K_2HPO_4 anhydrous (MM 174.18) in water, check pH 7.6 and dilute to 1 L.

6.3 **Substrate**

0.00025 M BAEE in buffer

Dissolve 8.6 mg BAEE HCl (MM 342.82)/100 ml buffer, and adjust absorbance to 0.575 @ 253nm. Equilibrate in water bath at 25°C, or;

For Freeze dried substrate, dissolve the powder in distilled H_2O. Adjust absorbance of this solution to 0.575 – 0.585 at 253nm using distilled H_2O. Equilibrate in water bath at 25°C.

7. Sample Preparation/Enzyme

Prepare 5 mg enzyme/ml ice cold HCl and dilute immediately before assay to yield approximately 40 units/ml ice cold HCl. (0.022 – 0.026 ΔA/min).

8. Procedure

Into a 10 mm cell, pipette the following:

Substrate	3.0 ml
Equilibrate at 25°C and monitor ΔA/min.	
Enzyme	0.2 ml
	3.2 ml
Record rate of increase in absorbance at 253nm for approximately 5 minutes. Check temperature in cell.	

9. Calculation

9.1 Dilution = $\dfrac{\text{Expected activity (u/mg) x Concentration of sample (mg/ml)}}{\text{40 u/ml}}$

9.2 Units/mg material = $\dfrac{\Delta A_{253}/\text{min X dilution}}{\text{0.003 X 0.2 X mg enzyme/ml original solution}}$

Due to variations in substrate batches it is necessary to determine the suitability of the substrate by carrying out a reference assay using a NF Trypsin reference standard. The quoted and actual activity obtained for the standard is then used to correct the activity obtained for the sample as follows.

$$\frac{\text{Units/mg material x quoted standard activity}}{\text{Standard activity obtained}} = \text{corrected sample activity}$$

9.3 Alternative Calculation

Calculation:
Reference standard (tested as per sample preparation)

$$\text{Volume activity (U/ml)} = \frac{\Delta A_{253}\ \text{nm/min x dilution faction x 5}}{0.003}$$

Calculation the expected U/ml of the reference material (U/mg x 5 (5mg/ml)) and use this to calculate the system correction factor (F) such that:

$$F = \frac{\text{Known activity of standard (U/ml)}}{\text{Measure activity of standards (U/ml)}}$$

Samples:

$$\text{NF U/ml of sample} = \frac{\Delta A_{253}\text{nm/min x dilution factor x 5 x F}}{0.003}$$

$$\text{Weight activity (U/mg material)} = \frac{\text{U/ml}}{\text{mg material / ml}}$$

10. Reference

Schwert G.W. and Takenaka Y.: (1955) Biochim. Biophys. Acta. 16 570.

APPENDIX 13

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI106
TITLE: Solubility Testing as per BP	**REVISION:** 0	**PAGE:** 1 of 1
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. **Purpose**

To standardise the solubility test procedure for Alpha Chymotrypsin as per the British Pharmacopeia.

2. **Scope**

Applies to in-process and final freeze dried material requiring solubility analysis.

3. **Responsibility**

All assayists.

4. **Procedure**

Prepare samples and reference samples as follows:

- Prepare a solution of 10mg/ml concentration using distilled water as solvent. e.g. 40mg sample + 4ml H_2O or 50mg sample + 5ml H_2O.
- Weigh the sample quantity into a clean particulate-matter free glass test tube.
- Add the water slowly along the walls of the test tube.

NB: Temperature of water and test conditions must be in the range of 15ºC - 25ºC

- Immediately mix the solution with a vortex mixer at maximum speed for exactly 1 minute.
- Compare the sample solution relative to the reference solution.

Reference sample:
The reference sample is a previously approved lot accepted by the customer.

Result interpretation:
Results which do not comply with the specification or the reference, must be investigated.

Reference:

APPENDIX 14

PROCESS: Micro Assay Methods	**ISSUE DATE:** 01.07.2011	**DOC NO:** BP6.2.3-WI49
TITLE: Total Aerobic Microbial Count	**REVISION:** 1	**PAGE:** 1 of 2
AUTHORISED: Microbiologist	**APPROVED: QA Manager**	

1. Purpose

To standardise the procedure for the Total Aerobic Microbial Count (TAMC) using the pour plate method.

2. Scope

All total aerobic microbial counts (TAMC) performed on all samples.

3. Responsibility

3.1 Microbiologist.

4. Safety Precautions

4.1 Personal protective clothing as set out in BP6.2.1-WI131 shall be worn.
4.2 Heat resistant gloves to be worn when removing media from microwave/ autoclave. Alternatively, allow goods to cool down before handling.
4.3 Reference cultures should be handled as per recommended guidelines or Material safety Data Sheets (MSDS).

5. Procedure

5.1 Equipment and Materials

Tryptone Soya Agar (TSA) – CM0131 Oxoid
Peptone water (diluent) – CM0009 Oxoid
Automated pipette (1ml)
Petri dishes (65mm and 90mm)
Disposable culture loops
Staphylococcus aureus ATCC 25923
Incubator at 30^0 - 35°C

5.2 Sample Preparation

5.2.1 Two dilutions are prepared; 1 in 10 (1g of sample into 9ml peptone water) and 1 in 100(1ml of 1 in 10 dilution into 9m1 peptone water). Homogenised by means of agitation (vortex).

5.2.2 Prepare two 90mm Petri dishes per dilution. 1 ml of each dilution is pipetted into the 90mm Petri dishes which are appropriately marked and coded.

5.2.3 Pour 15 – 20mℓ of molten TSA into each plate. The agar should not be more than 45^0C.

5.2.4 Allow agar to solidify.

5.2.5 Invert and incubate the plates at 30 – 35°C for 3 – 5 days.

5.3 Positive and Negative control

5.3.1 For positive controls streak a 65mm TSA Petri dish with *Staphylococcus aureus* ATCC 25923.

5.3.2 For a negative control streak a 65mm TSA Petri dish with diluent only.

5.3.3 Incubate together with samples.

5.4 Evaluation

The Total Aerobic Microbial Count (TAMC) is considered to be equal to the average number of colony forming units found on TSA.

Select the plate corresponding to the dilution and showing the highest number of colonies less than 250.

Calculation:

$$\text{TAMC} = \frac{\text{Total number of colonies counted (add colonies on both Petri dishes)}}{\text{Number of plates counted (2)}} \text{ X dilution factor}$$

5.4.1 For solid samples results are reported as cfu/g (Colony Forming Units/gram).

5.4.2 For liquid samples results are reported as cfu/ml (Colony Forming Units per millilitre)

5.4.3 If no growth is detected on the agar plates results are reported as <10cfu/g or <10cfu/ml.

Reference

Harmonized pharmacopoeia (European pharmacopoeia) 6.0 Volume 1. Pages: 167 – 174

APPENDIX 15

PROCESS: Micro Assay Methods	**ISSUE DATE:** 01.07.2011	**DOC NO:** BP6.2.3-WI70
TITLE: Total Combined Yeast & Mould Count	**REVISION:** 1	**PAGE:** 1 of 2
AUTHORISED: Microbiologist	**APPROVED: QA Manager**	

1. Purpose

To standardise the procedure for the Total Combined Yeast and Mould Count (TCYM) using the pour plate method.

2. Scope

All Total Combined Yeast and Mould Counts (TCYM) performed on all samples.

3. Responsibility

3.1 Microbiologist.

4. Safety Precautions

4.1 Personal protective clothing shall be worn as in BP 6.2.1-WI131
4.2 Heat resistant gloves must be worn when removing media from microwave/autoclave. Alternatively, allow goods to cool down before handling.
4.3 Reference cultures should be handled as per recommended guidelines or Material Safety Data Sheets (MSDS).

5. Procedure

5.1 Equipment and Materials
Sabouraud dextrose agar (SDA) – CM 0041 Oxoid
Peptone water (diluent) - CM0009 Oxoid
Automated pipette (1ml)
Petri dishes(65mm and 90mm)
Disposable culture loops
Candida albicans ATCC 10231
Aspergillus brasiliensis ATCC 16404
Incubator at 20^0 – 25^0C

5.2 Sample preparation

5.2.1 Two dilutions are prepared:
- 1 in 10 (1g of sample into 9 ml peptone water). Dissolve by agitation (vortex).
- 1 in 100 (1 ml of 1 in 10 dilution) into 9 ml peptone water. Dissolve by agitation (vortex).

5.2.2 Prepare two 90mm Petri dishes per dilution. 1 ml of each dilution is pipetted into the 90mm Petri dishes which are appropriately marked and coded.
5.2.3 Pour 15 to 20 ml of molten SDA into each Petri dish. The agar should not be more than 45^0C.
5.2.4 Allow the agar to solidify.
5.2.5 Invert and incubate the Petri dishes at 20 – 25^oC for 5 to 7 days.

5.3 Positive and negative control

5.3.1 For positive controls streak a 65mm SDA Petri dish with *Candida albicans* ATCC 10231 and *Aspergillus brasiliensis* ATCC 16404.
5.3.2 For a negative control streak a 65 mm SDA Petri dish with diluent only.
5.3.3 Incubate together with samples.

5.4 Evaluation

5.4.1 The total yeast and mould count is considered to be equal to the average number of colony forming units found on SDA.
Select the plate corresponding to the dilution and showing the highest number of colonies less than 25.

Calculation

$$\text{TCYM} = \frac{\text{Total number of colonies counted (add colonies counted on both Petri dishes)}}{\text{Number of Petri dishes counted (2)}} \text{ X dilution factor}$$

5.4.2 For solid samples results are reported as cfu/g (Colony Forming Units/gram).
5.4.3 For liquid samples results are reported as cfu/ml (Colony Forming Units per millilitre)
5.4.4 If no growth is detected on the agar plates results are reported as <10cfu/g or <10cfu/ml.

Reference

Harmonized Pharmacopoeia (European pharmacopoeia) 6.0 volume 1 Pages 157 – 174
MSDS for reference (ATCC) culture

APPENDIX 16

PROCESS: Micro Assay Methods	**ISSUE DATE:** 01.07.2011	**DOC NO:** BP6.2.3-WI66
TITLE: Detection of *Salmonella*	**REVISION:** 1	**PAGE:** 1 of 2
AUTHORISED: Microbiologist	**APPROVED: QA Manager**	

1. Purpose

To standardise the procedure for detection of *Salmonella.*

2. Scope

All product samples that require *Salmonella* testing as per the product specifications. Does not apply to in-process samples unless specifically requested.

3. Responsibility

3.1 Microbiologist.

4. Safety Precautions

4.1 Appropriate personal protective clothing as set out in BP6.2.1-WI31 shall be worn.
4.2 Heat resistant gloves must be worn when removing media from autoclave or microwave. Alternatively, allow autoclaved goods to cool off before handling.
4.3 Reference cultures should be handled as per recommended guidelines or Material Safety Data Sheets (MSDS).

5. Procedure

5.1 Equipment and Materials
Tryptone Soya broth (TSB) – CM0131 Oxoid
Peptone water (diluent) - CM0009 Oxoid
Automated pipette (1 ml)
Schott bottles (200 ml)
McCartney bottles (30 ml)
Disposable culture loops
Petri dishes (90 mm and 65 mm)
RVS enrichment broth – CM 0866 Oxoid
XLD agar – CM 0469 Oxoid
Kovacs reagent – MB0209A Oxoid
Incubator at 30 – 35^{0}C
Salmonella typhimurium ATCC 25241.

5.2 Sample preparation
5.2.1 Transfer 10 g or 10 ml of sample to 100 ml of TSB, homogenise and incubate at 30 – 35^{0}C for 18 to 24 hours.
5.2.2 Agitate by swirling. Pipette 1 ml of the first dilution (in 5.2.1 after incubation) into 100 ml of RVS enrichment broth and incubate at 30^0 – 35^{0}C for 18 to 24 hours.
5.2.3 Using a disposable culture loop, transfer (streak) a loopful of the RVS enrichment broth (after incubation in 5.2.2) on plates of XLD agar at 30 – 38^{0}C for 18 to 24 hours.
5.2.4 Possible presence of *Salmonella* is indicated by the growth of well developed, red colonies with or without black centres. This is confirmed by confirmatory tests.
5.2.5 Confirmation is done by indole test (addition of Kovacs reagent)

- Using a disposable culture loop, transfer the presumptory colony to 10ml peptone water and incubate at 37^{0}C for 24 to 48 hours.
- Add 5 drops of Kovacs reagent and gently agitate (swirl).
- Examine the upper layer of the liquid
- Positive result – red colour (occurring within a few seconds)
- Negative result – yellow colour

5.3 Positive and negative control:

5.3.1 For positive controls streak a plate of XLD agar plate with *Salmonella typhimurium* ATCC 25241.

5.3.2 For a negative control inoculate XLD agar plate with diluent only.

5.3.3 Incubate together with samples.

Reference

Harmonized pharmacopoeia (European Pharmacopoeia) 6.0 Volume 1 – Page 180
MSDS for reference (ATCC) culture

APPENDIX 17

PROCESS: Micro Assay Methods	**ISSUE DATE:** 01.07.2011	**DOC NO:** BP6.2.3-WI63
TITLE: Detection of *Pseudomonas aeruginosa*	**REVISION:** 1	**PAGE:** 1 of 2
AUTHORISED: Microbiologist Associate	**APPROVED: Compliance and Validation Associate**	

1. Purpose

To standardise the procedure for detection of *Pseudomonas aeruginosa*.

2. Scope

All product samples that require *Pseudomonas aeruginosa* testing as per the product specifications. Does not apply to in-process samples unless specifically requested.

3. Responsibility

3.1 Microbiologist.

4. Safety Precautions

4.1 Personal protective clothing as set out in BP 6.2.1-WI131shall be worn.

4.2 Heat resistant gloves must be worn when removing media from autoclave or microwave. Alternatively, allow autoclaved/microwaved goods to cool off before handling.

4.3 Reference cultures should be handled as per recommended guidelines or Material safety Data Sheets (MSDS)

5. Procedure

5.1 Equipment and Materials

Tryptone Soya Broth (TSB) – CM0131 Oxoid
Peptone water (diluent) – CM0009
Automated pipette (1ml)
Schott bottles 200ml
McCartney bottles 30ml
Disposable culture loops
Petri dishes (65mm and 90mm)
Pseudomonas Cetrimide Agar (CET) – CM 0597 Oxoid
Kovacs reagent – Oxoid MB0209A
Incubator at 30^0 – 35^0C
Incubator at 42^0 - 44^0C
Pseudomonas aeruginosa ATCC 9027

5.2 Sample preparation

5.2.1 Transfer 1 g of sample to 9 ml peptone water (McCartney bottle). Dissolve by agitation (vortex).

5.2.2 Pipette 1 ml of the first dilution to 100 ml of TSB (Schott bottle), mix and incubate at 30^0 - 35^0C for 18 – 24 hours.

5.2.3 Agitate by swirling and pipette 1 ml of the second dilution to 100 ml of MacConkey broth (Scott bottle) and incubate at 42^0 – 44^0C for 24 – 48 hours.

5.2.4 Using a disposable culture loop, transfer (streak) a loopful of the third dilution (after incubation as in 5.2.3)CET Agar plate and incubate at 30^0 – 35^0C for 18 – 72 hours.

5.2.5 Growth of green colonies indicates the possible presence of *Pseudomonas aeruginosa*.

5.3.6 Confirmation done by transferring colonies to TSB (McCartney bottle) and no growth occurring at 41 – 43^0C after 18 – 24 hours.

5.4 Positive and negative control;

5.4.1 For positive controls streak a CET agarplate with*Pseudomonas aeruginosa*ATCC 9027.

5.4.2 For a negative control inoculate CET agar plate with diluent only.

5.4.3 Incubate together with samples.

Reference

Harmonized Pharmacopoeia (European Pharmacopoeia) 6.0 Volume 1

Page 180

MSDS for reference (ATCC) culture

APPENDIX 18

PROCESS: Micro Assay Methods	**ISSUE DATE:** 25.01.2011	**DOC NO:** BP6.2.3-WI65
TITLE: Detection of *Staphylococcus aureus*	**REVISION: 1**	**PAGE:** 1 of 2
AUTHORISED: Microbiologist Associate	**APPROVED: Compliance & Validation Associate**	

1. Purpose

To standardise the procedure for detection of *Staphylococcus aureus*.

2. Scope

All product samples that require *Staphylococcus aureus* testing as per the product specification. Does not apply to in-process samples unless specifically requested.

3. Responsibility

3.1 Microbiologist Associate.

4. Safety Precautions

4.1 Personal protective clothing as set out in BP 6.2.1-WI131 shall be worn.
4.2 Heat resistant gloves must be worn when removing media from autoclave or microwave. Alternatively, allow autoclaved/microwaved goods to cool off before handling.
4.3 Reference cultures should be handled as per recommended guidelines or Material Safety Data Sheets (MSDS)

5. Procedure

5.1 Equipment and Materials
Tryptone Soya Broth (TSB) – CM0131 Oxoid
Peptone water (diluent) – CM 0009 Oxoid
Automated pipette (1ml)
Schott bottle 200ml
McCartney bottle 30ml
Disposable culture loops
Petri dishes(65mm and 90mm)
Baird Parker agar (BPA)– CM 0275 Oxoid
Mannitol-Salt agar (MSA) – CM 0085 Oxoid
Incubator at 30^0 – 35^0C
Staphylococcus aureus ATCC 25923.

5.2 Sample preparation

5.2.1 Transfer 1 g of sample to 9 ml peptone water (McCartney bottle)
5.2.2 Pipette 1 ml of the first dilution to 100 ml of TSB (Schott bottle), homogenise and incubate at 30 - 35^0C for 18 – 24 hours.
5.2.3 After agitation by swirling, use a disposable culture loop to transfer (streak) a loopful of the second dilution (as in 5.2.3 after incubation) onto a MSA agar plate and incubate at 30 - 35^0C for 18 – 72 hours.
5.2.4 Possible presence of *Staphylococcus aureus* is indicated by the growth of yellow/white colonies surrounded by a yellow zone. This is confirmed by confirmatory tests.
5.2.5 Confirmation can be done by the Catalase test and Baird-Parker agar test.

5.2.6 *Staphylococcus aureus* is catalase positive (meaning that it can produce the enzyme 'catalase') and able to convert hydrogen peroxide to water and oxygen – drops of hydrogen peroxide are dropped onto presumptive *Staphylococcus aureus* colonies on a glass microscope slide and if bubbles are noted it is considered to be *Staphylococcus aureus* positive.

5.2.7 Using a disposable culture loop, transfer (streak) a presumptory colony onto a BPA plate. Incubate at $30^0 - 35^0$C for 24 hours. *Staphylococcus aureus* produces grey-black shiny colonies surrounded by clear zones.

5.3 Positive and negative control

5.3.1 For positive controls streak a MSA plate with *Staphylococcus aureus* ATCC 25923.
5.3.2 For a negative control inoculate MSA with diluent only.
5.3.3 Incubate together with samples.

Reference

Harmonized Pharmacopoeia (European Pharmacopoeia) 6.0 Volume 1- Page 130
MSDS for reference (ATCC) culture

APPENDIX 19

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI131
TITLE: µKatal - Trypsin	**REVISION:** 0	**PAGE:** 1 of 4
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. Purpose

To determine the activity of the samples using the Crison compact titrator.

2. Scope

All samples requiring katal results.

3. Responsibility

All assayists determining the katal activity.

4. Safety

Protective clothing to be worn.
Refer to labels of the chemicals regarding safe handling.

5. Reagents

5.1 0,1M NaOH
0,1M NaOH standardised solution.

5.2 0,001M HCl
Dilute 0,089 ml concentrated HCl to 1 ℓ with distilled H_2O.

5.3 1,0M HCl
Dilute 8,9 ml concentrated HCl to 100ml with distilled H_2O.

5.4 0,0015M Borate Buffer pH 8,0
Dissolve 572 mg borax $Na_2B_4O_7.10H_2O$ (MM 381,36) and 2,94 g $CaCl_2.2H_2O$ (MM 147,02) in distilled water, adjust the to pH 8,0 with 1N HCl and dilute to 1 ℓ with distilled H_2O.

5.5 Crison Buffer solution set, pH 7,0 and pH 4,0.

5.5.1 Substrate
Dissolve 6,86 g BAEE.HCl (MM 342,8) in 1000 ml distilled water.

6. Equipment

Compact Titrator D with 2x syringes
Combined pH electrode 5212
Water bath with a heater circulator
Jacketed vessel
Small magnetic stirrer bar
Test tubes, 5ml & 10 ml glass pipettes, volumetric flasks
Computer and printer
ComCom 2000 software
Analytical balance.

7. Procedure.

7.1 Sample Preparation

Prepare the sample to yield approximately 700 nanokatals per ml 0,001M HCl (± 1mg/1ml 0,001MHCl). Store on ice.

Calculation:

Volume of diluent to be added to sample = $\frac{\text{mass of sample weighed x expected activity}}{\text{Approximately 0.7 µkatal/ml}}$

7.2 Reference Standard Preparation:

Prepare the standard at 1 mg/ml 0,001M HCl and store on ice.

7.3. pH Calibration

The pH calibration is to be completed each day before use. The speed of the stirrer should be set to approx 40%.

7.4 ComCom 2000 Software

Open the ComCom 2000 Software
Open the "Titrator" screen before starting any calibration. Instruments→Titrator→Open→Titrator
The software is now ready to received information.

1. Press **3** for the calibration option
2. Select the correct user number
3. Press **1** for electrode nr1
4. Place buffer pH 7 in the electrode holder and press **RUN**
5. The mV readings should be 0 mV ± 20 mV
6. Rinse the electrode and place buffer pH 4 in the electrode holder and press **RUN.**
7. The mV reading should be 168 mV ± 20 mV
8. Press Stop.
9. The Sensitivity allowed is to between 85 – 110%
10. Press C to clear.

7.5 Priming of burettes

Place the inlet tubes in the reagent bottle containing the NaOH – solution and do the following:

1 Press **6** for the "More" option
2 Press **6** for the "Burette" option
3 Press **1** for the "Move burette option
4 Select the burette nr and press **enter**
5 Use 1&2 to move the burette up & down.
6 Press "Stop" after 3 times.
7 Press O to Quit

or

1 Press **1** for the "Execute" option
2 Press **5** and **enter** to select Prg 5 for the Priming program.
3 "**Place waste vessel**" press **Run.**
4 Place H_2O bottle for priming" Press Run
5 The syringe will be rinsed 3x times with distilled water
6 "Place reagent bottle for priming" press Run
7 The burette will be rinsed with the reagents 3x times
8 Press **0** to Quit

7.6 Determination of Trypsin Activity

ComCom 2000 Software

Open the ComCom 2000 Software

Ensure that the correct Database is open (i.e. Trypsin, Chymotrypsin etc)

Open the "Tritrator" screen before starting any titrations. Instruments→Tritrator→Open→Tritrator1.

The software is now ready to receive information

7.7 Sample Preparation

1 Accurately add 10 ml buffer solution (5.4) to the jacketed vessel
2 Accurately add 1.0 ml substrate (5.6)
3 Add a magnetic stirrer bar
4 Place the jacketed vessel onto the magnetic stirrer and equilibrate the solution to 25ºC ± 0,1ºC
5 Press 1 for the "Execute" option
6 Select the correct user number
7 Press 1 and enter to select Prg 1 for the Trypsin determination
8 "Code Sample"
Key in the sample code via the PC keyboard (i.e. HST B113) or display keyboard and press enter
9 The syringe will fill up
10 "Place sample vessel"
Ensure that the sample vessel is inserted into the magnetic stirrer and ensure that electrode height is correctly positioned.
11 "Pre-titration"
The pre-titration will be carried out to adjust the medium to a pre-defined pH. It will only be executed if it has been selected in the Programming.
12 Press Run to start the pre-titration
The magnetic stirrer will automatically be activated. The stirring speed maybe adjusted using the + and – key.
13 The titrator will complete the pre-titrator with 0,1 N NaOH up to pH 7,90
14 <1. Start stat><2. Reagent Add>
Press 2 to add 5 µl increments of reagent to adjust the final pH to pH 8,00.
Press 1 to start the pH Stat titration
15 The syringe will fill with the reagent
16 Accurately add 50 µl Trypsin solution and press Run to start the reaction.
17 The pH will be maintained at pH 8,00 for 8 minutes via the addition of 0,1 N NaOH. Every 30 seconds the volume added will be recorded.
18 Press C to clear the results from the titrator screen. The results will be stored in the database.
19 The same procedure in the same conditions with the reference solution must be preformed.
20 Press **0** to Quit or press1 to repeat the process. (Steps 8 to 18 will be repeated)

7.8 Cleaning of burettes:

To rinse the burettes with distilled water do the following:

Remove the inlet tubes for the reagents bottles and place the inlet tube in a beaker containing distilled water.

1 Press **6** for the "Move" option
2 Press **6** for the "Burette" option
3 Press **1** for the "Move burette option
4 Select the burette nr and press **enter**
5 Use 1&2 to move the burette up & down

or

1. Press 1 for the "Execute" option.
2. Press 4 and enter to select Prg 4 for the cleaning program
3. "Place waste vessel" Press Run
4. "Return reagent to bottle" Press 2
5. The burette will be empty into the waste container
6. Place H_2O bottle for cleaning" Press Run
7. Press 0 to Quit

7.9 **Protocol to follow**

a) Prepare two weighings of the reference standard.
b) Assay each weighing in duplicate.
c) Prepare one weighing of the control sample.
d) Assay in duplicate.
e) For the sample, at least three weightings must be prepared.
f) Assay each weighing in duplicate.
g) After completion of the above, another weighing of the reference standard should be tested in duplicate.

Note: For the reference standard, the results obtained must be within 3% of each other.
For the control and the samples, the results obtained must be within 5% of each other respectively

8. **Calculation:**

$$\text{ACTIVITY (microkatals/mg)} = \frac{m' \times V \times A}{m \times V'}$$

Where m = mass in milligrams of test sample.
m' = mass in milligrams of reference sample.
V = volume of 0,1 N NaOH used per second by the test solution.
V' = volume of 0,1 N NaOH used per second by the reference solution.
A = activity of reference sample in microkatals per milligram.

APPENDIX 20

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI147
TITLE: Identification of Trypsin	**REVISION:** 0	**PAGE:** 1 of 2
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. **Reagents**

1.1 2% Tosyl-lysyl – chloromethane hydrochloride:

Dissolve 20 mg Tosyl – L – lysyl-chloromethyl ketone hydrochloride, FLUKA 90182 (MM 369,3)/ ml distilled water.

1.2 0,1N NaOH

0,1N NaOH standardised solution.

1.3 Methyl Red T.S.

Dissolve 10 mg methyl red in 10 ml alcohol and filter if neccessary. Store in an amber bottle at ambient temperature.

1.4 Methylene Blue T.S.

Dissolve 5 mg methylene blue in 4 ml alcohol and dilute with alcohol to 10 ml. Store in an amber bottle at ambient temperature.

1.5 Methyl red/Methylene Blue T.S.

Add 1 ml methyl red T.S. to 1 ml methylene blue T.S. and mix.

1.6 0,20 M Tris(hydroxymethyl)aminomethane

Dissolve 1,2114 g Tris (MM 121,14) in 30 ml water. Adjust volume to 50 ml with water.

1.7 0,08 M Tris(hydroxymethyl)aminomethane Buffer pH 8,1

Dissolve 294 mg $CaCl_2$ (MM 110,99) in 40 ml of solution (1.6) and adjust to pH 8,1 with HCl then dilute to 100 ml.

1.8 Substrate

Transfer 98,5 mg p-toluenesulphonyl-L-arginine methyl ester.HCl (TAME) to a 25 ml volumetric flask. Add 5 ml 0,08M Tris buffer pH 8,1 and mix. Add 0,2 ml (1.5). Adjust volume to 25 ml with water.

2. **Sample Preparation**

Prepare Trypsin at 10 mg/ml with distilled water.

3 **Procedure**

A. Dilute 1 ml sample to 100 ml with distilled water.
Mix 0,1 of this solution with 0,2 ml substrate.
A purple colour develops within 3 minutes

B. Dilute 1 ml sample to 10 ml with distilled water.
Add 0,2 ml 2% Tosyl-lysyl-chloromethane HCl
Adjust pH to 7,0 with 0,1N NaOH and stir for 2 hours.
After 2 hours dilute the solution to 50 ml with distilled water.
Mix 0,1 ml of this solution with 0,2 ml substrate
No purple colour develops

APPENDIX 21

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI43
TITLE: Protein (Biuret Method)	**REVISION:** 0	**PAGE:** 1 of 2
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. REACTION

Based on that of Weichselbaum in which the violet colour formed is measured spectrophotometrically at 546 nm and compared to the colour formed by standard bovine serum albumin under the same conditions.

2. REAGENTS

2.1 Biuret Reagent

Dissolve 9 g Na-K-tartrate.$4H_2O$ (MM 282,23) in 400 ml 0,2 N NaOH, add 3 g finely powdered $CuSO_4.5H_2O$ (MM 249,68), dissolve, add 5 g KI (MM 166,00), dissolve, and dilute to 1 l with 0,2 N NaOH. Store in brown glass bottle.

2.2 3 M Trichloroacetic Acid

Dissolve 49 g TCA (MM 163,39)/100 ml water.

2.3 Standard

Factors used to convert absorbance to protein were determined using crystalline bovine albumin code 81-001 as standard.

3. PROCEDURE

3.1 Salt-Free Powder

Dissolve 1 - 6 mg protein in 5 ml biuret reagent. Allow to react for 30 minutes. Centrifuge **TO REMOVE ANY POTENTIAL HAZE**. Measure absorbance at 546 nm in 1 cm cell versus biuret reagent (2.1) blank.

mg protein/original mass of sample = A_{546} X 19,05

3.2 Salt-Free Solution

Dissolve 1 ml sample containing 1 - 6 mg protein in 5 ml biuret reagent. Allow to react for 30 minutes. Measure absorbance at 546nm in 1 cm cell versus blank of 5 ml biuret reagent and 1 ml water.

mg protein/ml sample = A_{546} X 22,85

3.3 Powder or Solution Containing Salt

Dilute sample containing 1 - 6 mg protein with water to 1,7 ml in tube. With mixing add 0,3 ml TCA dropwise. Centrifuge and decant supernatant without disturbing precipitate. Add 2 ml TCA, suspend the pellet by mixing then centrifuge. Repeat 3x. This will remove all traces of salt. Add 5 ml biuret reagent to precipitate. Allow to react for 30 minutes. Centrifuge. Measure absorbance at 546nm in 1 cm cell versus biuret reagent blank.

mg protein/original mass or volume of sample = A_{546} X 19,05

NOTE:

Ammonia interferes by complexing with copper, so ammonium sulphate fractions do not give accurate results.

4. REFERENCE

Weichselbaum, T.E. (1946) Amer. J. Clin. Path. Tech. Sec. 10 40.

APPENDIX 22

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI142
TITLE: Trypsin Absorbance Test	**REVISION:** 0	**PAGE:** 1 of 1
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. **Reagents**

0,001 M HCl

Dilute 0,089ml conc. HCl (MM 36,46) to 1l with distilled water. Store on ice.

2. **Procedure**

2.1 Accurately weigh 30,0mg Trypsin Code 201553 in a 100ml volumetric flask. Add 0,001M HCl to dissolve the material and adjust the volume to a 100ml with 0,001M HCl.

2.2 Determine the absorbance of the solution at 280nm and 250nm respectively. Subtract the blank readings.

3. **Calculation**

$E^{1\%}_{280} = \Delta A_{280} \times 10/0,3$

$E^{1\%}_{250} = \Delta A_{250} \times 10/0,3$

4. **Reference**

2000 British Pharmacopoeia CD ROM.
European Pharmacopoeia 5.0.

APPENDIX 23

PROCESS: Assay Methods	**ISSUE DATE:** 19.08.2009	**DOC NO:** BP6.2.2-WI29
TITLE: Moisture Determination C	**REVISION:** 0	**PAGE:** 1 of 1
AUTHORISED: Senior Technical Officer	**APPROVED: QA Manager**	

1. Purpose

Method to determine the mass of moisture.

2. Scope

Applies to all products which require loss of drying analysis.

3. Responsibility

All assayists performing moisture analysis.

4. Safety Precautions

Wear appropriate protective clothing and glasses.
Use tongs when handling hot crucibles.
Practice caution when handling the desiccator containing silica gel.
Do not use cracked or chipped crucibles.

5. Equipment and material

Vacuum Oven
Crucible tongs
Porcelain crucibles
Analytical balance
Desiccator containing silica gel

6. Procedure

6.1 Weigh and record mass of empty moisture crucible. Handle crucible with crucible tongs.
6.2 Add approximately 0,1 g sample to crucible. Weigh and record mass.
6.3 Allow sample to dry 2 hours under vacuum at 60°C.
6.4 Place crucible in desiccator to cool.
6.5 Weigh and record mass.

Note: Tests on each sample to be prepared in duplicate.

7. Calculation

Calculate using the following formula:

$$\% \text{ moisture} = \frac{(\text{wt. cruc + sample before dry}) - (\text{wt. cruc + sample after dry}) \times 100}{(\text{wt. cruc + sample before dry}) - (\text{wt. cruc})}$$

8. Cleaning of crucibles

8.1 Inspection of crucibles is required after every use (inspect for any cracks).
8.2 Clean the crucibles in water containing Extran.
8.3 Rinse the crucibles thoroughly with distilled water.
8.4 Place the crucibles in the oven at 70°C.
8.5 Remove from the oven and allow to cool to ambient temperature.

9. Reference

European Pharmacopoeia 5,0.

APPENDIX 24

PROCESS: Micro Assay Methods	**ISSUE DATE:** 01.07.2011	**DOC NO:** BP6.2.3-WI64
TITLE: Detection of *Escherichia coli*	**REVISION:** 1	**PAGE:** 1 of 2
AUTHORISED: Microbiologist	**APPROVED: QA Manager**	

1. Purpose

To standardise the procedure for detection of *Escherichia coli.*

2. Scope

All product samples that require *Escherichia coli* testing as per the product specifications. Does not apply to in-process samples unless specifically requested

3. Responsibility

3.1 Microbiologist.

4. Safety Precautions

4.1 Personal protective clothing as set out in BP6.2.1-WI131 shall be worn.
4.2 Heat resistant gloves must be worn when removing media from autoclave or microwave. Alternatively allow autoclaved/microwaved goods to cool off before handling.
4.3 Reference cultures should be handled as per recommended guidelines or Material Safety Data Sheets (MSDS)

5. Procedure

5.1 Equipment and Materials
Tryptone Soya Broth (TSB) – CM0131 Oxoid
Peptone water (diluent) – CM0009 Oxoid
Automated pipette (1 ml)
Schott bottles 200 ml
McCartney bottles 30 ml
Disposable culture loops
Petri dishes (65 mm and 90 mm)
Kovacs reagent – MB0209A Oxoid
MacConkey agar – CM0115 No. 3 Oxoid
MacConkey Broth - CM0505 (CM5a) Oxoid
Incubator at 30 – 35^{0}C
Incubator at 42 - 44^{0}C
Escherichia coli ATCC 8739

5.2 Sample preparation

5.2.1 Transfer 1 g of sample to 9 ml peptone water (McCartney bottle) and dissolve by agitation (vortex).
5.2.2 Pipette 1 ml of the first dilution to 100 ml of TSB (Schott bottle), mix and incubate at 30 - 35^{0}C for 18 – 24 hours.
5.2.3 Agitate by swirling and pipette 1ml of the second dilution into 100ml MacConkey broth (Schott bottle) and incubate at 42 – 44^{0}C for 24 – 48 hours.
5.2.4 Using a disposable culture loop, transfer (streak) a loopful of the third dilution (after incubation as in 5.2.3) on a 90 mm MacConkey agar plate. Incubate at 30 – 35^{0}C for 18 – 72 hours.

5.2.5 Growth of pink colonies indicates possible presence of *Escherichia coli*. No growth indicates the absence of *Escherichia coli.*
Confirmation done by indole test (addition of Kovacs reagent).

- Using a disposable culture loop, transfer the presumptory colony to10ml peptone water and incubate at 37^0C for 24 – 48 hours.
- Add 5 drops of Kovacs reagent and gently agitate (shake)
- Examine the upper layer of the liquid
- Negative result – yellow colour
- Positive result - red colour (occurring within seconds)

5.3 Positive and negative control

5.3.1 For positive controls streak a 65 mm plate of solidified MacConkey agar with *Escherichia coli* ATCC 8739

5.3.2 For a negative control inoculate MacConkey agar plate with diluent only.

5.3.3 Incubate together with samples.

Reference

Harmonized Pharmacopoeia (European Pharmacopoeia) 6.0 Volume 1 – Page 180
MSDS for reference (ATCC) culture