Expression and purification of recombinant extracellular proteases originating from non-*Saccharomyces* yeasts

by

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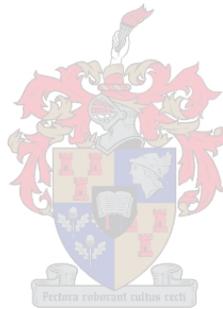
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Declaration

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Summary

During wine fermentation, yeasts release extracellular enzymes that significantly impact wine properties. While the extracellular proteins of *Saccharomyces cerevisiae* have been characterised, those of non-*Saccharomyces* yeasts remain largely unknown. Most of these enzymes break down sugar polymers or catalyse the liberation of glycosidically-bound molecules. Another category of enzymes of oenological interest is represented by acid proteases that are able to prevent or reduce protein haze, as reported in literature, while simultaneously increasing the assimilable nitrogen content of wine. The liberation of amino acids from peptides and proteins that serve as aroma precursors may also have an indirect effect on wine aroma. In a recent study performed at the Institute for Wine Biotechnology (IWBT), the sequences of two aspartic proteases were retrieved from non-*Saccharomyces* yeast species isolated from South African wines. The genes, *MpAPr1* and *CaAPr1*, were isolated from two non-*Saccharomyces* species, *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384, respectively. However, no further characterization was undertaken. This study aimed to clone these two genes into a recombinant bacterial host for expression and purify the corresponding enzymes as a first step toward characterizing their kinetic properties. Considering that some non-*Saccharomyces* species have been shown to produce more than one acid protease, an additional aim was to identify novel acid proteases within *M. pulcherrima* IWBT Y1123.

Cloning of the genes and transformation of the expression vectors into *E. coli* were achieved. Optimal conditions for induced expression were established following extensive optimization. Furthermore, while native extraction of the recombinant proteins was unsuccessful, denaturing conditions allowed their recovery, suggesting that the recombinant proteins are encapsulated into inclusion bodies. Recombinant MpAPr1 was purified by using a nickel based column system and mass fingerprinting of the purified enzyme (MpAPr1) confirmed its identity. Purification was followed by refolding experiments, but yielded poor recovery of active enzymes. Unfortunately, recombinant expression of CaAPr1 could not be observed for reasons yet to be elucidated that may include the large sequence dissimilarities between CaAPr1 and MpAPr1. Finally, Southern blot analysis on the genomes of *M. pulcherrima* IWBT Y1123 and *C. apicola* IWBT Y1384 revealed that both possess at least one additional protease other than those previously described. Further analysis of the extracellular proteome of *M. pulcherrima* IWBT Y1123 also confirmed the presence of at least one enzyme able to hydrolyze BSA at a low pH. Unfortunately, mass fingerprinting performed on the entire extracellular proteome and on small groups of proteins thereof did not allow the identification of these enzymes.

Opsomming

Gedurende fermentasie van druiwe sap skei gis ekstrasellulêre ensieme af wat 'n aanmerklike impak op wyn eienskappe het. Terwyl die ekstrasellulêre proteïene vanaf *Saccharomyces cerevisiae* al gekarakteriseer is, bly die van nie-*Saccharomyces* spesies grootliks onbekend. Meeste van hierdie ensieme breek suiker polimere af of kataliseer die vrystelling van glikosiediese verbonde molekules. 'n Ander klas van ensieme wat van belang is vir oenologie word voorgestel deur proteases wat in staat is daartoe om proteïenewaas te verminder, soos voorheen geraporteer is in literatuur, terwyl dit terselfde tyd die assimileerbare stikstof inhoud kan vermeerder. Die vrystelling van aminosure vanaf peptiede en/of proteïene wat as aroma voorlopers dien mag ook 'n indirekte effek op die wyn se aroma profiel hê. In 'n onlangse studie wat uitgevoer is by die Instituut vir Wynbiotegnologie (IWBT) was die volgordes van twee aspartiese proteases bepaal vanaf twee nie-*Saccharomyces* gis spesies wat geisoleer was uit Suid-Afrikaanse wyne. Die gene *MpAPr1* en *CaAPr1,* was afsonderlik geisoleer vanuit twee nie-*Saccharomyces* giste*, Metschnikowia pulcherrima* IWBT Y1123 en *Candida apicola* IWBT Y1384. Egter was daar geen verder karakterisering van hierdie ensieme nie. Die doel van hierdie studie is om die bogenoemde gene in 'n rekombinante bakteriese gasheer te kloneer vir uitdrukking en suiwering as 'n eerste stap tot karakterisering van hul kinetiese eienskappe. Om in ag te neem dat sommige nie-*Saccharomyces* spesies meer as een protease produseer was 'n aditionele mikpunt om vir nuwe suur proteases te soek binne *M. pulcherrima* IWBT Y1123.

Klonering van hierdie gene en transformasie van die uitdrukkings vektore in *E. coli* was suksesvol. Optimale kondisies vir die induksie van ekspressie was bevestig na omvattende optimalisering. Verder, terwyl inheemse ekstraksie van die rekombinante proteïene onsuksesvol was, het denatureerende kondisies toegelaat vir suksesvolle ekstraksie, wat voorgestel het dat die rekombinante proteïene geinkapsileer word in inklusie liggame. Rekombinante MpAPr1 was gesuiwer deur gebruik te maak van 'n niekel gebaseerde kolom sisteem en massa petied fingerafdrukke van die gesuiwerde ensiem (MpAPr1) het die identiteit bevestig. Suiwering was gevolg deur hervouing eksperimente, maar het swak opbrengste gelewer van die aktiewe ensiem. Ongelukkig kon die rekombinante ekspressie van CaAPr1 nie gevisualiseer word nie vir redes wat nog bevestig moet word, maar wat mag behels dat daar groot volgorde veskille tussen MpAPr1 en CaAPr1 kan wees. Uiteindelik was Southern blot hibridiseering analises uitgevoer op die genome van albei *M. pulcherrima* IWBT Y1123 en *C. apicola* IWBT Y1384 wat voorgestel het dat albei ten minste een addisionele protease, anders as die wat voorheen geidentifiseer was, bevat. Verder analiese van die ekstrasellulêre proteoom van *M. pulcherrima* IWBT Y1123 het ook die teenwoordigheid van ten minste een ensiem bevestig wat die vermoë het om BSA te hidroliseer by 'n lae pH. Ongelukkig het massa peptied vingerafdrukbepaling wat

uitgevoer was op die hele ekstrasellulêre proteoom en op klein groepe protein nie identifikasie van hierdie ensieme bevestig nie.

Biographical sketch

Louwrens W Theron was born in Cape Town, South Africa on 17 November 1988 and was raised in Paarl. He matriculated at Paarl Boys' High School in 2006 and commenced his studies at the University of Stellenbosch in 2008 where he enrolled for a BSc-degree in Molecular Biology and Biotechnology. After graduating in 2010, he pursued postgraduate studies, obtaining a HonsBSc-degree in Wine Biotechnology in 2011.

Acknowledgements

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

Preface

This thesis is presented as a compilation of 5 chapters.

Chapter 1   **General Introduction and project aims**

Chapter 2   **Literature review**
Proteases in industry and wine biotechnology

Chapter 3   **Research results**
Cloning followed by optimizing expression and purification of two aspartic proteases from non-*Saccharomyces* in *E. coli*

Chapter 4   **Research results**
The quest for novel proteases active under wine making conditions

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Chapter 1

General introduction and project aims

Chapter 1 - General introduction and project aims

1.1 Introduction

The role that yeasts have during the bio-conversion of grape must to wine was first elucidated by Louis Pasteur in 1866. Several different species form part of the microbial ecology of grape must and wine, but the yeasts (mainly *Saccharomyces* species) dominate the population due their ability to rapidly adapt and survive in this environment. Furthermore, although the flavour of the wine is largely determined by the grape variety, the yeasts also contribute to the wine flavour and quality through the production of metabolites and extracellular enzymes. Nevertheless, after more than 140 years of research, many aspects are still not well understood especially the actual contribution ofthe non-*Saccharomyces* yeasts have during alcoholic fermentation. These yeasts were initially thought to be detrimental to wine quality and mostly categorized as spoilage organisms (Du Toit and Pretorius, 2000; Loureiro and Malfeito-Ferreira, 2003).

Non-*Saccharomyces* yeasts are naturally present in all fermentations and some have been shown to be good secretors of extracellular enzymes that can have a positive impact on wine quality. These include yeast of the genera *Candida*, *Metschnikowia, Kluyveromyces, Kloeckera* and *Zygosaccharomyces* (Fleet et al. 1984; Heard and Fleet, 1987). Extracellular enzymes include pectinases, glycosidases and proteases that may have a positive impact on wine clarification, filtration, aroma extraction and juice yield for the former two (Maturano et al. 2012; Rogerson et al. 2000). The actual role of proteases has not been investigated as thoroughly as the other enzymes. They could nevertheless fulfil relevant functions with regards to protein instability and release of yeast assimilable nitrogen. Protein instability is characterized by the formation of haze that occurs when pathogenesis related (PR) proteins (in particular chitinases and thaumatin-like proteins) become unstable and aggregate making them visible to the naked eye.

In order to precipitate proteins before bottling to prevent protein instability, winemakers currently add fining agents such as bentonite to their wines (Pocock and Waters 2006; Sauvage et al. 2010). Bentonite is a type of natural clay that has the ability of absorbing high amounts of proteins. The disadvantages to such treatment are that it is expensive and also reduces product yield when removing the precipitated lees. Moreover, it can also affect the quality and flavour of the wine by removing aroma compounds being carried along with the precipitate. Therefore, there is a growing market for proteases in the wine industry. The

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enzymatic treatment of grape proteins also has the added potential of releasing assimilable nitrogen that could be used by the yeast during alcoholic fermentation.

In literature, it has already been reported that the addition of acid proteases reduces protein haze formation without being detrimental to wine quality (Lagace and Bisson 1990; Pocock et al., 2003). Aspartic proteases, also known as acid proteases, are known to be secreted by a range of organisms including retroviruses, filamentous fungi, insects, bacteria and of more importance to the wine making industry some non-*Saccharomyces* yeasts. Unlike other proteases, the activity of this group of enzymes is dependent on low pH conditions (pH 2–5) making it ideal for use in wine making.

In a recent study performed at the Institute for Wine Biotechnology (IWBT), Stellenbosch University, the sequences of two genes encoding extracellular aspartic proteases have been retrieved from non-*Saccharomyces* yeast species isolated from South African wines (Reid et al. 2012). The genes, *MpAPr1* and *CaAPr1*, were isolated from two non-*Saccharomyces* species, *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384, respectively. The latter study also showed that MpAPr1 was actively secreted in the presence of BSA, casein and grape proteins and that it was able to partially degrade these proteins in a medium buffered at pH 3.5. However, no further characterization was undertaken.

1.2 Rational and scope of the study

The aim of this study was to clone the two aspartic genes (*MpAPr1* and *CaApr1*) isolated from two wine related non-*Saccharomyces* yeasts (*M. pulcherrima* IWBT Y1123 and *C. apicola* IWBT Y1384) into *E. coli* for heterologous over expression. This was followed by purification of the acid proteases by use of immobilized metal ion affinity chromatography (IMAC) as a first step toward characterizing their kinetic properties and determining their suitability for winemaking. Additionally, considering that some non-*Saccharomyces* yeasts are known to produce more than one acid protease (Naglik et al. 2003, Aoki et al. 2012), an exploratory goal was set to identify (using techniques such as 2D SDS-PAGE and LC-MS/MS) novel acid proteases within *M. pulcherrima* IWBT Y1123.

Specific objectives for this study

1. To clone *MpAPr1* and *CaAPr1* into suitable expression vectors and transform them into a bacterial host for heterologous expression
2. To optimize expression and purify the recombinant enzymes
3. To seek novel proteases within the exoproteome of *M. pulcherrima* IWBT Y1213 using 2D SDS-PAGE and LC-MS/MS

1.3 References

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Chapter 2

Literature review

Proteases in industry and wine biotechnology

Chapter 2 - Proteases in industry and wine biotechnology

2.1 Introduction

The history of wine production spans thousands of years with evidence suggesting that the earliest known wine fermentations occurred around 7000BC (Berkowitz 1996; Ellsworth 2012; Spilling and Wong 2008). For millennia, the art of winemaking remained empirical and the experiential savoir-faire was passed on from generation to generation without any proper scientific foundation. It was indeed only in the 19th century that Louis Pasteur first elucidated the bio-conversion of grape juice into wine and the role that yeasts play in this complex biological process. However, Pasteur believed that living organisms and not the enzymes produced by these microorganisms were responsible for the process of fermentation. This concept was also referred to as vitalism. Finally in 1897, Eduard Buchner, a German chemist, uncovered that fermentation was the result of a chemical process both inside and outside the cell.

Before the advances of modern microbiology and the subsequent isolation and selection of starter cultures, fermentations were performed by the indigenous yeast population present within the grape must. Today, this phenomenon is referred to as spontaneous fermentation. Over the years, *Saccharomyces cerevisiae* has been identified as the lead fermenter and has thus received much attention from the scientific community in contrast to the other yeast species that has been ignored by large, when not considered as spoilage yeasts. Consequently, after more than 100 years of research on wine microbiology, many areas are still not well understood, especially the role that non-*Saccharomyces* yeasts play during the course of alcoholic fermentation.

These yeasts, naturally present in grape juice, are metabolically active and their metabolites can to a greater or lesser extent have an impact on wine quality. A number of researchers have also reported that some non-*Saccharomyces* yeasts are good secretors of extracellular enzymes that could be of great interest and have a positive impact on the organoleptic properties of wine (Fernandez et al. 2000; Jolly et al. 2006; Strauss et al. 2001). These secreted enzymes from yeast as well as those contributed by the other microorganisms found in grape must are however, not the only enzymes found in wine. Grape enzymes are also an important ingredient of grape must and determining factor of final wine quality. Finally, wine makers also contribute to the enzymes found in must and wine by addition of external enzymes mostly from fungal origin. The enzymatic treatment of grapes, must and wine has multiple purposes, for example improving wine clarification, filtration, aroma

extraction and increasing juice yield (Maturano et al. 2012; Rogerson et al. 2000). These enzymes include pectinases, amylases, xylanases, β-glucosidases and proteases.

Proteases are enzymes that cleave other proteins and surprisingly make up the largest single family of enzymes. They are mainly classified into six groups based on the mechanistic features consistent within each group. Their application in industry is widespread and include the use of pancreatic proteases in the leather industry, alkaline protease in order to remove hair from hides, proteases in chymosin (rennet) to coagulate milk, papain from papayas to tenderize meat and proteases for the recovery of silver from used X-ray films (Gupta et al. 2002; Sumantha et al. 2006; Ward et al. 2009). In literature, some reports have indicated that the addition of proteases to wine is an efficient way of reducing protein haze formation without being detrimental to wine quality. Moreover, protease action can also lead to production of several nitrogen containing compounds, some of which being important aroma compounds (Bell and Henschke 2005; Fleet 2003; Lagace and Bisson 1990; Pocock et al. 2003). However, because of the low pH of wine, only acid proteases, such as aspartic proteases are active in this environment. These proteolytic enzymes are known to be secreted by a range of organisms including retroviruses, filamentous fungi, insects, bacteria and of more relevance for winemaking, some non-*Saccharomyces* yeasts.

This review consists of two main sections. The first part will focus on proteases and their application in industry with a more detailed discussion on acid proteases. The second section will focus on yeast-derived acid proteases and the growing interest in the wine industry on this subject.

2.2 Proteases: a large and diverse family of enzymes

2.2.1 General description, classification and mechanism of action

Proteases can be termed as enzymes which catalyze the cleavage of hydrolytic bonds within proteins, thereby releasing peptides and/or amino acids. Generally, the term proteases can be used interchangeably with the terms proteinases and/or proteolytic enzymes, but the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) and the Enzyme Commission (EC) recommends the term peptidases be used for all enzymes that hydrolyze peptide bonds (subclass E.C.3.4). Proteases have a major function in the global recycling of carbon and nitrogen from proteins. Proteins from diseased organisms are hydrolyzed by microorganisms (decomposition) into peptides and amino acids. These products can be assimilated by the microorganisms that produced the

proteases or by other organisms in the near vicinity. Protease producing microorganisms present in soil have been shown to regulate protease expression in response to carbon and nitrogen limitation (Sims and Wander 2002). Thus proteases can be helpful in nitrogen limiting environments, a fact that is discussed in more detail later on. Proteases are also known to carry out a vast array of functions ranging from blood pressure regulation, viral protein synthesis, degradation of incorrectly folded proteins, protection against harmful peptides and enzymes amongst others (Barrett et al. 2004; Sandhya et al. 2005; Tyndall et al. 2005). Inhibition of various proteases has also become a valuable approach in pharmaceutical application for neurodegenerative diseases, infections and various parasitic diseases (Rao et al. 1998). The most essential property of protease action resides in their ability to control and limit cleavage to intended substrates.

Microorganisms are known to produce intracellular and extracellular proteases. Intracellular proteases intervene in various cellular and metabolic processes, such as differentiation, sporulation, processing of hormones and scavenging of damaged proteins or proteins that are no longer required. These proteases also play a pivotal role in apoptosis and autolysis. Extracellular proteases are more important in the hydrolysis and the adsorption of proteinaceous nutrients (Kalisz 1988). Extracellular proteases especially are of commercial importance and protein extracts prepared from the growth cultures of protease producing microorganisms are commonly used as protein degrading tools during various industrial processes (Kumar and Takagi 1999). Proteases can further be subdivided into either exopeptidases cleaving one or a few amino acids from the N- or C-terminus, or endopeptidases which act on the internal polypeptide chain. Exopeptidases that act on the free C-terminus liberate single amino acid residues (carboxypeptidases) or dipeptides (peptidyldipeptidases). Those acting on the N-terminus liberate single amino acid residues, dipeptides or tripeptides and are commonly known as aminopeptidases, dipeptidyl-peptidases and tripeptidyl-peptidases, respectively. Another group, known as omega-peptidases, also acts close to one or the other terminus, but has no requirement for a charged terminal group. Instead, they are specific in removing terminal residues that are cyclized or linked by isopeptide bonds (Table 2.1).

Endopeptidases are industrially more important than exopeptidases and are classified according to their molecular size, charge, substrate specificity, catalytic mechanism, three dimensional structures and the amino acid residues present in the catalytic site (Beynon and Bond 1990; Sumantha et al. 2006). Each type of protease exhibits a set of amino acid residues arranged in a specific configuration to produce its catalytic site. This gives them the characteristic ability to break certain peptide bonds (Barrett et al. 2004; Tyndall et al. 2005).

Furthermore there is also a specific group of endoproteases, termed oligopeptidases that act only on substrates smaller than proteins.

Table 2.1 Classification of protease according to mode of action

Proteases	Mode of action	Active site
Exopeptidases		Free N-terminus
Aminopeptidases		
Pipetidyl peptidases		
Tripeptidyl peptidases		
Carboxypeptidases		Free C-terminus
Peptidyldipeptidases		
Dipeptidases		
Omega peptidases		Blocked N- or C-terminus
Endopeptidases		Non-terminal
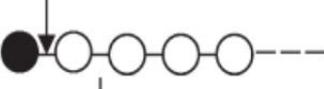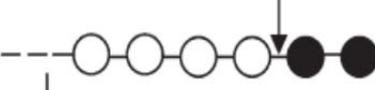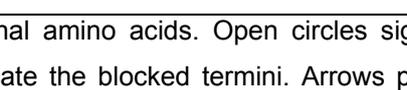
Solid circles represent the terminal amino acids. Open circles signify amino acid residues in the polypeptide chain and stars indicate the blocked termini. Arrows point out the site of action of the enzyme (Rao et al. 1998; Ward et al. 2009).

The MEROPS database (merops.sanger.ac.uk), a manually curated database dedicated to peptidases, divides peptidases into protein species which are then further sub-divided into families according to the statistically significant similarities in their amino acid sequences. Protein species include aspartic/glutamate (4%), cysteine (26%), metallo (34%), serine (30%) and the less characterized threonine peptidases (5%) (Madala et al. 2010). Table 2.2 shows the different species of proteases together with some additional information on their characteristics, sources and industrial applications. In the nomenclature of the NC-IUBMB, endopeptidases which include, serine-, cysteine-, aspartic-, metallo- and threonine endopeptidases are given the subclasses EC 3.4.21, EC 3.4.22, EC 3.4.23, EC 3.4.24 and EC 3.4.25, respectively.

Briefly, serine proteases, which play an important role in digestion, have a catalytic triad in their active site consisting of a serine, histidine and aspartic acid residues. They fall into two categories based on their structure, the chymotrypsin-like (Serine protease I) and subtilisin-like (Serine protease II) proteases (Madala et al. 2010). Cysteine proteases, commonly used in meat tenderizers, have similar folds as the serine proteases but with a catalytic dyad in their active site consisting of cysteine and histidine residues. The metalloproteases, as the name suggests, are classified as any proteases whose catalytic mechanism involves a metal (usually divalent zinc ions) (Rao et al. 1998). Threonine proteases are one of the newer classes of proteases described and harbor a threonine residue in their catalytic domain. The aspartic proteases, which will be discussed in more detail in the following paragraphs, have a tertiary structure consisting of two symmetrical lobes to form the catalytic site, each lobe harboring an aspartic acid residue.

Table 2.2: The different families of proteases and their properties

Family	Example of proteases	Cofactors	Characteristic active site	Optimal pH	Inhibitors	Source
Serine proteases	Trypsin	Ca^{2+}	Asp^{102}, Ser^{105}, His^{87}	7 - 11	PMSF, EDTA, phenol, triamino acetic acid	*Bacillus, Aspergillus*, animal tissue (gut)
Metallo proteases	Thermolysin	Zn^{2+}, Ca^{2+}	Glu^{270}, Try^{248}	7 – 9	Chelating agents such as EDTA, EGTA	*Bacillus, Aspergillus, Penicillium, Pseudomonas, Streptomyces*
Cysteine proteases	Papain	N.d.	Cys^{25}, His^{159}, Asp^{158}	2 - 3	Indoacetamide, *p*-CMB	*Aspergillus, Streptomyces, Clostridium*
Aspartic proteases	Chymosin	Ca^{2+}	Asp^{11}, Asp^{213}	2.5 – 7	Pepstatin, EPNP, DAN	*Aspergillus, Mucor, Rhizopus, Penicillium*, animal tissue (stomach)
Threonine proteases	Polycystin-1	N.d.	Thr	Neutral	DON	*Thermoplasma, Escherichia, Saccharomyces*

DAN, diazoacetylnorleucinemethyl; DON, 5-diazo-4-oxonorvaline; PMSF, phenylmethylsulfonyl fluoride; PCMB, (*p*chloromercuribenzoic acid; EDTA, Ethylenediaminetetraacetic acid; EGTA,

ethylene glycol tetraacetic acid; EPNP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane), Nd., Not determined. (Rao et al. 1998; Sumantha et al. 2006)

2.2.2 Aspartic proteases

Aspartic proteases, commonly known as acid proteases, are distributed across all forms of life including vertebrates, plants, fungi and also viruses (Cooper 2002; Fairlie et al. 2000). This relatively small group of enzymes has received much attention from the scientific community because of their involvement in human diseases. Examples of these proteases are the plasmepsins in malaria, HIV-1 peptidase in acquired immune deficiency syndrome (AIDS) and the secreted aspartic peptidase in *Candida* infections (Madala et al. 2010). From as early as 1989, crystal structures of aspartic proteases from retroviruses such as HIV and *Rous Sarcoma* have been extensively studied and determined (Navia et al. 1989). Aspartic proteases from yeast and fungi have also been studied extensively and several have been purified and cloned (De Viragh et al. 1993; Gomi et al. 1993; Horiuchi et al. 1988; Jarai et al. 1994; Kakimori et al. 1996; Li et al. 2009; Li et al. 2010; Radha et al. 2011; Shivakumar 2012; Togni et al. 1991; Tonovchi et al. 1986; van Kuyk et al. 2000; Young et al. 1996). Also several species of *Aspergillus* are known producers of aspartic proteases: *A. oryzae* (Vishwanatha et al. 2009), *A. fumigatus* (Reichard et al. 1994), *A. saitoi* (Tello-Solis and Hernandez-Arana 1995), *A. awamori* (Moralejo et al. 2002), *A. niger* (O'Donnel et al. 2001; Radha et al. 2012; Siala et al. 2009).

2.2.2.1 Description, classification and properties of microbial aspartic proteases

Most of these enzymes' molecular weights range between 35 and 50 kDa, consisting of 320 to 340 amino acid residues and have isoelectric points in the range of 3 to 4.5. Analysis of various aspartic proteases by X-ray crystallography shows that they are mostly composed of β-strand secondary structures and interestingly they represent some of the largest β-strand structures observed in globular proteins (Claverie-Martin and Vega-Hernandez 2007). The majority of aspartic proteases are also known to have at least one flap made up of a β-hairpin that completes their active site (Madala et al. 2010). The flaps serve as a mechanism that upon closing squeezes all the components into the correct geometry and holds the substrate in place enabling the catalytic process to begin. Well known examples of aspartic proteases include rennet, cathepsin D, cathepsin E and pepsin. The industrial application of some of these will be discussed in more detail in the following section. The Protein Data Bank (PDB) and MEROPS database classify eight sub-families within the aspartic proteases with the sequence Asp-Thr(Ser)-Gly in their active site. Subfamilies differ according to the

position of their catalytic site, the specific residues in their active site, the number of disulphide bridges present within the structure and optimal pH at which the enzyme functions (Cascella et al. 2005; Rawlings et al. 2009; Rawlings and Bateman 2009).

The aspartic proteases are typically inhibited by pepstatin, a hexa-peptide containing the rare amino acid statine. This molecule, which was originally isolated from various species of *Actinomyces,* has the remarkable ability to inhibit pepsin at picomolar concentrations (Marciniszyn et al. 1976; Umezawa et al. 1970). There have however been reports of pestatin-insensitive acid proteases isolated from bacteria including *Xanthomonas* sp., *Pseudomonas* sp., *Bacillus* sp. (Oda et al. 1987; Prescott et al. 1995) and more recently from *Thermoplasma volcanium* (Kocabiyik and Ozel 2007). Roa et al. (1998) reported that aspartic proteases are also sensitive to diazoketone compounds such as 1,2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) and diazoacetyl-DL-norleucine methyl ester (DAN) in the presence of copper. Interestingly in 1990, Fusek et al. purified and cloned a thermophilic acid protease from *Sulfolobus acidocaldarius* (an archaebacteria) which does not have an aspartyl residue in its active site nor does it show any apparent sequence homology to other acid proteases, and therefore represents a new class.

The pepstatin-sensitive aspartic proteases are divided into two families: the retroviral and eukaryotic pepsin-like type proteases. The retroviral types consist out of β-homodimers possessing aspartic residues located within the two loops at the monomer interface with two β-hairpins covering the active site (Sielecki et al. 1991). The eukaryotic pepsin-like protease has a tertiary structure consisting of two approximately symmetrical lobes (α/β monomers) with each lobe carrying an aspartic acid residue in order to form the catalytic site. In the N-terminal domain, the characteristic sequence Asp^{32}-Thr-Gly-Ser can be found with a corresponding Asp^{215}-Thr-Gly–Ser/Thr in the C-terminal domain (De Viragh et al. 1993). Because of their two fold symmetry, it is the general consensus that these domains possibly arose through ancestral gene duplication. A flap made of a β-hairpin covers the catalytic site constituting the active site cleft. This cleft is located perpendicular to the largest diameter of the molecule and can accommodate seven to eight amino acid residues, equally divided on both sides of the catalytic aspartic residues (Dunn 2002; Szecsi 1992). The number and position of disulfide bonds throughout the protein has been suggested to have a strong impact on the native state stability of the enzyme (Cascella et al. 2005; Friedman and Caflisch 2010). Members of the aspartic proteases family generally have one to three disulfide bridges and the one located at the position between 251 – 286 is conserved across all members of the family (Machalinski et al. 2006). In general, most aspartic proteases from

microbial origin exhibit a broad based specificity towards regions in peptides that contain six hydrophobic residues at specific substrate positions (Dash et al. 2003).

2.2.2.2 Mechanism of action

The accepted mechanism of action is a general acid-base catalysis where the one aspartic residue (Asp[32]) acts as a base, accepting a proton, while the other (Asp[215]) acts as an acid, donating a proton. In other terms, the former residue has a relatively low pKa value and the latter a relatively high pKa value, which is crucial to their mechanism of action. Following exposure to low pH, cleavage events occur that lead to conformational rearrangement. Firstly a water molecule is bound to the two aspartic residues through hydrogen bonds and acts as a nucleophile that attacks the carbonyl carbon of the peptide scissile bond. The Asp that acts as a general base removes one proton from the water molecule which is followed by a nucleophilic attack of the water molecule to the carbonyl carbon of the substrate scissile bond. At the same time, the Asp acting as a general acid donates a proton to the carbonyl oxygen atom of the peptide scissile bond. This leads to the formation of a tetrahedral intermediate with the Asp (general base) being hydrogen bonded to the attacking oxygen atom while the hydrogen remaining on that oxygen is hydrogen bonded to the oxygen of Asp (general acid). During the final steps, a reversal of the configuration occurs around the nitrogen atom of the scissile bond with the transfer of a hydrogen from Asp (general base) to the nitrogen atom. In parallel, a proton is transferred from the oxygen atom of Asp (general acid) to the carbonyl oxygen on the peptide bond being cleaved. This leads to the C-N bond breaking and releasing the two peptide products. Consequently the Asp (general base) is negatively charged at this stage and is therefore ready for the next round of catalysis (Coates et al. 2001; Dunn 2002).

Northdrop (2001) proposed an alternative mechanism based on the same principle as described above, but in which a low barrier hydrogen bond (not present in the former proposed mechanism) is formed between the two aspartic residues present in the catalytic site. Another major difference is that the final step involves the binding of a water molecule and the re-formation of the low barrier hydrogen bond. However, some authors disagree with this proposal based on the angle between the two inner oxygen's of the Asp residues being too wide for hydrogen bond formation (Andreeva and Rumsh 2001; Dunn 2002). Nevertheless, all agree on the occurrence of a covalent intermediate.

2.2.2.3 Secretion pathway of acid proteases found in yeast

The most predominant microbial family found on grapes and wine fermentation is the *Ascomycota* and for the purpose of this review only the secretion pathways of aspartic proteases relevant to this family will be discussed further. In yeast, the secretion pathway and processing of aspartic proteases have been extensively studied in *Candida* species because of their involvement in human diseases. It is believed that most fungal aspartic proteases are synthesized as inactive zymogens (preproenzymes), which has an additional N-terminal segment found to be approximately 45 amino acids long that gets cleaved upon activation (Davies 1990). The pro-segments are important for correct folding and control the activation of enzyme zymogens (Koelsch et al. 1994). The pro-segment which is comprised of one β-strand and three helices interacts with the enzyme resulting in blockage of the active site (Dunn 2002). After the cleavage of the signal peptide autocatalytic activation (self-cleavage) happens upon exposure to an acidic environment which causes the acidic residues to get protonated leading to the disruption in electrostatic interactions. Activation reactions are dependent on pH, temperature and salt concentrations (Chitpinityol and Crabbe 1998). Cleavage can occur by intermolecular activation, which dominates between pH 4 and 5, or intramolecular which dominates at pH lower than 4 (Campos and Sancho 2003). Some members of the aspartic proteases family might be glycosylated. This has been suggested to play an important role in stabilizing protein conformation (Machalinski et al. 2006). Some proteases from *Ascomycetes* do not harbor a secretion signal and are believed to be secreted via a non-conventional pathway. An acid protease from *Yarrowia lipolytica* (Axp) does not have a lysine-arginine signal site (as in the case with *Candida* species) and is thus believed to follow a different maturation pathway yet to be elucidated (Beckerich et al. 1998; McEwen and Young 1998).

Species such as *C. albicans*, *C. parapsilosis* and *C. tropicalis* are known to cause oral and vaginal candidiasis. The acid proteases released (classified as Saps) facilitate penetration and invasion of the pathogen and provide nutrients to the cell (Naglik et al. 2003). Processing of the Sap enzymes starts with the transcription of mRNA in the nucleus which is shortly followed by translation into a pre-pro-peptide on the endoplasmic reticulum (ER). A signal peptide found on the N-terminus is recognized by receptors on the ER membrane which directs the protein into the secretion pathway (Cheng et al. 2008; Naglik et al. 2003). The signal peptide is cleaved in the rough ER lumen by a signal peptidase complex. After cleavage, the signal peptide is degraded and once the enzyme enters the ER refolding occurs. At this stage modifications such as glycosylation might also occur. Subsequently, the pro-enzyme is transferred via vesicles to the Golgi apparatus. The propeptide region, found

to be approximately 20 amino acids long (Conesa et al. 2001), is cleaved by the Kex2 subtilisin-like endoproteinase which cleaves peptides after a conserved lysine-arginine sequence (Newport and Agabian 1997; Punt et al. 2003). Finally, the enzyme is packaged into secretory vesicles and transported to the plasma membrane where it is either released into the surrounding medium or remains attached to the cell membrane (depending on the nature of the enzyme).

2.3 Proteases in the industry

Enzymes have been utilised by mankind for many centuries, knowingly or not. Some of the earlier applications of proteolytic enzymes were as milk-clotting agents for the manufacturing of cheese. These were probably first discovered when animal skins and inflated organs were used as storage containers for a range of foodstuffs. Therefore when milk was stored in the stomach of calves it resulted in the formation of curd and whey because of the rennet present in the stomach (which contains several enzymes including chymosin). In Asian countries, proteases were used in the early production of natto, which is made from soy beans fermented with *Bacillus* species. Proteases involved in this process are important for the development of the main flavours associated with natto through the hydrolysis of the soy bean proteins. Proteases from microbes are the most abundant source of enzymes and extensively studied for their application in industry. One of the first reports on this is from 1894, by Jhokichi Takamine who pioneered the industrial production of digestive enzymes prepared from *Aspergillus oryzae* for the treatment of digestive disorders. In 1914, proteases were for the first time used as additives to detergents and since then this industry has seen tremendous growth and development. For the purpose of this review, only proteases of microbial origin will be subsequently discussed in more detail.

2.3.1 Historical overview on microbial proteases of industrial relevance

Plant and animal protease are unable to meet the current world demand because their proteases are not diverse enough to meet industrial requirements thus there is a consistently growing interest in microbial proteases. Microbes can also be easily be manipulated into producing enzymes at high amounts. Because of the large biodiversity amongst microbes they represent an unparalleled source of enzymes with a wide spectrum of characteristics. Extensive reviews on this topic have been published over the last thirty years and thus only some of the main focuses will be discussed. The reader is referred to the reviews cited in the following paragraph for further information.

Some of the first reviews were written by Aunstrup (1980). This author focused on the selection of microbes for proteases of industrial interest. A few years later, Ward (1985) reviewed the sources of microbial proteases and their possible roles in nature. In 1988, Kalisz compiled a detailed description on the types of proteases available and their commercial applications. Shortly after, Outtrup and Boyce (1990) reviewed the proteases of industrial importance with the main focus on their applications and the potential role that molecular biology has in protease research. In 1998, Roa et al. extensively reviewed the genetic, molecular and biochemical aspects of animal, plant and microbial proteases.

In 2005, Tyndall et al. published an excellent review on proteases from various sources followed by a comprehensive summary on protease inhibitors and protease bound structures. Subsequently in 2010, this review was updated by Mandala et al. in order to keep up with more recent findings and techniques for protease ligand interaction. In 2009, Ward et al. published an updated summary on microbial protease production, classification and listed some of the more recent applications in industry. Kasana et al. (2011) published a review in which they attempted to summarize the various methods and techniques for the screening, detection and quantification of proteases ranging from plate assays to nanoparticle based assays. Recently, Rani et al. (2012) provided a broad overview on the latest information available focusing on the sources, types and mode of action of several proteases. Another excellent review was published very recently by Li et al. (2013) in which they summarized the general categories of commercially used proteases and described the strategies currently used in order to improve the properties of proteases for commercial application. They also included the recent progress in the field of proteases engineering. To date, protease form one of the largest groups of industrial enzymes and account for more than 60% of the total worldwide enzyme sales (Rani et al. 2012).

For the purpose of this review, only some of the main points and most recent findings on microbial proteases will be discussed. Proteases will be divided into two groups: alkaline/neutral proteases and acid proteases, with the latter being the main focus.

2.3.2 Alkaline/neutral proteases

Alkaline proteases are active at basic pH range and make up the largest share of the enzyme market because of their use in household detergents. Most of the proteases used in the detergent industry are alkaline or neutral proteases from *Bacillus* species. Some of the most important are the serine alkaline proteases. Highly alkaline detergents use proteases from alkalophilic species such as *B. halodurans* and *B. clausii* whereas proteases from

B. licheniformis are used in low pH detergents. Three main product categories exist: (1) the low pH (7.5 – 9.0), low ionic strength liquid detergents containing no bleach; (2) the high pH (9.5 – 10.5), high ionic strength powders which contains bleach and finally (3) the high pH (9.5 – 10.5) compact powders that contain sodium sulfate (Ward et al. 2009). The use of alkaline/neutral proteases has also received much attention in terms of replacing of harsh or harmful chemicals.

Major components of leather are proteins, including elastin, keratin and collagen. The principal steps in the processing of leather include soaking, dehairing, bating and tanning. The purpose of the soaking step is to swell the hide and this is usually achieved by use of an alkaline reagent. Conventional methods for dehairing include treatment with extremely alkaline chemicals followed by treatment with hydrogen sulfate. This solubilizes and removes the proteins from the hair root. These conventional methods used in the leather industry thus involve the use of harsh chemicals which creates safety risks, disposal problems and chemical pollution (Khan 2013). Collagen exists in hides and skin in association with various globular proteins such as albumin, globulin, mucoids and fibrous proteins such as elastin, keratin and reticulin. The extent to which the non-collagenous constituents are removed determines the characteristics of the final leather such as durability and softness. The success of detergent enzymes has led to them being used in a number of other applications including pest control (Kim et al. 1999), degumming of silk (Kanehisa 2000; Puri 2001), isolation of nucleic acid (Kyon et al. 1994), lens cleaning (Nakagawa 1994), delignification of hemp (Dorado et al. 2001), cleaning of surgical instruments (Gupta et al. 2002), production of peptides (Cheng et al. 1995) and silver recovery from X-ray films (Fujiwara et al. 1991). Because of their popularity and wide spread use across industries, extensive reviews on this topic have been published and thus only some of the main focuses will be discussed. The reader is referred to the reviews cited in the following paragraphs for further information.

In 1998, Anwar and Saleemuddin published one of the first extensive reviews on alkaline proteases that focused on their sources and applications in industries at that time. One year later, Horikoshi et al. (1999) published an excellent review on alkaliphiles (microorganisms that grow at basic pH) and listed some of their applications in biotechnology and various industries. Interestingly, these authors reported that the main industrial application of alkaliphilic enzymes was the detergent industry and accounted for about 30% of total worldwide sales at that time. In parallel, Kumar and Takagi (1999) reviewed different isolation and strain improvement methods by using recombinant DNA technologies together with purification methods and explored the properties of microbial alkaline proteases. They also highlighted and discussed several diverse industrial applications of that time. With the

development of improved molecular techniques, Gupta et al. (2002) compiled a review in which they discussed yield improvement techniques and the use of advanced methods such as protein engineering, site directed and/or random mutagenesis for the development of novel proteases. They also listed several applications for alkaline proteases in industrial sectors with the main focus being on the detergent industry. With the rise in popularity of detergent alkaline proteases, Saeki et al. (2007) published a review focusing solely on this group of enzymes in which they described gene sequences, enzymatic properties and crystal structures. Then in 2010, Fujinami and Fujisawa published an extensive review on industrial applications of alkaliphile organisms as well as their enzymes and proceeded to discuss their continued use in industry over the past few decades and as well as their future within related industries. More recently, Jaouadi et al. (2011) provided an overview of the quest for novel natural bacterial alkaline protease. These authors placed special emphasis on the purification and characterization of two enzymes, namely SAPB (*Bacillus pumilus* CBS alkaline protease) and KERAB (keratinolytic alkaline proteinase), and discussed their applications and potential uses in several related industries. Finally, Khan (2013) recently published a review discussing new microbial proteases being isolated and discovered with special emphasis on the leather and detergents industries and how proteases can potentially replace certain harsh chemicals for a more environmentally friendly approach.

2.3.3 Acid proteases

This section focuses on acid proteases of microbial origin, excluding acid proteases from wine yeast which will be discussed in more detail in the next section. Acid proteases are active at acidic pH range and although they are not as popular as the alkaline/neutral proteases, they are still used in a number of industrial applications. The significant ability of acid proteases to coagulate proteins, especially milk proteins, is the main reason for their high demand in the dairy industry. The major application of acid protease in this industry is the manufacturing of cheese where milk proteins is coagulated forming solid masses, or curds, from which cheese is prepared after the removal of whey (Neelakantan and Mohanty 1999). Basically four categories of milk-coagulating enzymes exist. They include: animal rennets, microbial milk coagulates, genetically engineered chymosin and vegetable rennet (Ward et al. 2009). As the human population increased and the demand for cheese increased, the cheese making industry was hindered by a worldwide shortage of calf rennet which became even scarcer because of resistance from animal rights lobbies. This triggered a search for alternative milk coagulation proteins and proteins of microbial origin started to receive more attention. A primary characteristic of enzymes involved in cheese production is the ability to hydrolyze the specific peptide bond (Phe[105]-Met[106]) to generate para – casein

and macromolecules (Rani et al. 2012). In the 1980's, Genecor International expressed recombinant calf chymosin (rennin) on a large scale using *Aspergillus niger* var. *awamori* as host. Commercially, the most important native enzyme for cheese making was isolated from the mold *Rhizomucor miehei* (Ward et al. 2009).

Fungal derived acid proteases have also been extensively applied in the production of food seasonings and the improvement of protein rich foods. In the making of fruit juices and certain alcoholic liquors acid proteases from *Aspergillus saitoi* (Aspergillopepsin I) are used to degrade the proteins that cause turbidity (Sumantha et al. 2006). Acid proteases are also frequently used in the production of soy sauce and to improve the texture of flour. In the pharmaceutical industry, they are utilized as digestive aids, commercially available as Nortase and Luizym, for treating of certain lytic enzyme deficiency syndromes (Rao et al. 1998). With the fermentation of sake, an alcoholic beverage of Japanese origin, acid proteases determine the taste of the final product because of the manner they hydrolyze the proteins from the steamed rice in order to liberate peptides and amino acids (Shindo et al. 1998). In the beer industry acid proteases have been investigated as tools to degrade proteins that can form haze during storage. However, certain hydrophobic polypeptides originating from the wort, are crucial for the stability of the foam head of the beer. Therefore, proteases used for haze protection should not hydrolyze the foam stabilizing polypeptides. In a study by Lopez and Edens (2005) it was found that addition of proline-specific proteases from *Aspergillus niger* effectively prevented chill-haze formation in beer. Furthermore, foam stability measurements indicated that the enzyme had only slight effects on beer foam formation.

2.4 Acid proteases and the wine industry

2.4.1 Protein haze in white wines

Similarly to the beer industry, protein haze is also a very challenging problem during the production of white wine. The presence of haze is usually perceived as microbial spoilage and results in a reduction of the commercial value of the wine (Waters et al. 2005). Although the problems associated with haze reduction are similar in beer and wine, there are important differences that include the nature of the specific proteins involved, the presence of sulfur dioxide (SO_2) and the difference in alcohol concentrations. In white wine, this phenomenon occurs when proteins of grape origin become unstable under certain conditions and aggregates into light dispersing particles hence making the wine hazy (Hsu et al. 1987; Marangon et al. 2012; Waters et al. 1992). The proteins involved have been identified as pathogenesis-related (PR) proteins, more specifically β-glucanases, chitinases and

thaumatin-like proteins (TLP) which exhibit a molecular weight ranging from 15 to 30 kDa (Le Bourse et al. 2011; Marangon et al. 2011; Van Sluyter et al. 2009; Waters et al. 1996; Waters et al. 1998). They have been shown to be stable at acidic pH and resistant to proteolytic hydrolysis because of their compact globular structure preventing access to the protease enzymes (Conterno and Delfini 1994). Other wine components such as pH, metal ions, polysaccharides and phenolic compound may be used to modulate the haze forming potential of the wine (Batista et al. 2009; Marangon et al. 2011; Pocock et al. 2007; Waters et al. 2005). The role of sulfate ions in protein aggregation has also been confirmed (Marangon et al. 2011; Pocock et al. 2007). In literature, some studies indicated that TLP are the major wine haze proteins (Esteruelas et al. 2009; Vincenzi et al. 2010) whereas other authors indicate that chitinases are the major proteins responsible for haze formation (Sauvage et al. 2010; Vincenzi et al. 2005). It has recently been demonstrated that the two classes of proteins have different unfolding transition temperatures, 55°C and 62°C for chitinases and TLP respectively. The unfolding behaviour of the proteins were also found to differ in that once heated TLP refolds upon cooling while chitinases remain unfolded (irreversible refolding) (Falconer et al. 2010). This finding revealed that chitinases are thus more prone to cause haze formation in wine.

Currently the most effective tool that winemakers have to eliminate haze is treatment with bentonite. This montmorillonite clay has a net negative charge and serves as a cation exchanger adsorbing proteins (Ferreira et al. 2002). Bentonite has been widely used in oenology as a fining agent since as early as the 1930's (Saywell 1934). Despite its widespread use, the application of bentonite has several negative attributions; some of which include the removal of desirable flavour compounds, high handling costs, loss of colour and disposal issues leading to environmental concerns associated with sustainability (Lagace and Bisson 1990; Waters et al. 2005). Because of these negative impacts several alternatives to bentonite treatment have been investigated, including the use of flash pasteurization (Pocock et al. 2003), ultrafiltration (Hsu et al. 1987) and the use of other absorbents (Cabello-Pasini et al. 2005; de Bruijn et al. 2009; Vincenzi et al. 2005).

An ideal solution to this issue would be to use enzymes able to degrade haze forming proteins. Potential haze reduction enzymes would have to meet several criteria for optimum haze reduction in wine. These properties include: (1) activity at a low pH (3.0 – 4.0), (2) activity in the presence of SO_2, (3) activity at wine alcohol concentrations, (4) activity at wine making temperatures, (5) ability to degrade chitinases and TLP and finally (6) activity must be strong enough under these conditions to replace bentonite (at least partially). In 1990, Lagace and Bisson reported that extracellular proteolytic activities produced by *C. olea*,

C. lipolytica, *C. flavous* and *C. pulcherrima* could be correlated to protein haze reduction. A thorough review was published by Ogrydziak (1993) in which he listed all the proteases from *Saccharomyces* and non-*Saccharomyces* yeast known at the time of publication and their properties. In this review, this author described proteases of all classes and briefly surveyed the potential use of acid proteases regarding haze reduction in beer and wine. The author concluded that although in some cases protease activity was found in the extracellular medium, there was no clear evidence indicating whether this activity originated from secreted protease or intracellular proteases found in the extracellular medium as a result of cell lysis. In 2000, Dizy and Bisson demonstrated that certain strains of *Kloeckera* and *Hanseniaspora* produced protease enzymes active in grape juice able to affect the protein profile of the finished wine. During the same year, van Rensburg and Pretorius (2000) also found that a protease from *K. apiculata* caused some degradation of wine proteins. However, the enzymes from each study were not able to efficiently degrade haze forming proteins. Some authors concluded that this is due to their high resistance to proteolysis but more importantly because of wine making conditions that are unfavourable for enzyme activity (Waters et al. 1992; Waters et al. 1995).

Some of the first suggestions for removing wine haze were the use of pasteurization (Ferenczy 1966), but it was demonstrated that this treatment has a detrimental effect on wine quality. Some years later, research performed by Francis et al. (1994) showed that heating wine for a brief period at 90°C, a treatment known as flash-pasteurization, did not have negative effects on the organoleptic properties of the wine. Then in 2003, Pocock et al. demonstrated that combining heat treatment and proteolytic enzymes reduced the requirement for bentonite by 50% - 70% without affecting the sensory profile of final wine. Treatment consisted out of exposing the wine for 1 min at 90°C and adding Trenolion blank which is a commercially available Aspergillopepsin. The idea behind this kind of treatments is that exposure to heat denatures the haze forming proteins allowing access for proteolytic enzymes to hydrolyse the proteins into smaller peptides. Nevertheless, despite these encouraging results, it was concluded that a more efficient protease was needed. Recently in 2012, Marangon et al. investigated the use of acid protease isolated from *A. niger* var. *macrosporus* (Koaze et al. 1964), namely Aspergillopepsin I and II (AGP), together with flash pasteurization to degrade haze proteins in white wine. The sole addition of AGP directly to the fermentation resulted in a 20% reduction in proteins. However, maximum effects were obtained when combining AGP treatment with flash pasteurization (75°C for 1 min). It was found that under the conditions tested the chitinases and TLP were almost completely eliminated in chardonnay and sauvignon blanc wines thereby replacing the need for bentonite. Very recently, Van Sluyter et al. (2013) also demonstrated that an acid protease

from *Botrytis cinerea,* named BcAP8, was able to effectively reduce haze at winemaking temperatures and to remain active after fermentation was completed. Although it was found that the enzyme was not able to remove all the PR proteins, showing more activity against chitinases than the TLP, it was shown that it could still benefit winemakers by reducing bentonite requirements. The success of the previously mentioned reports encourages further investigations into proteases of grape derived non-*Saccharomyces* yeasts and assessment of their potential use in the wine making environment.

2.4.2 Other potential roles in vinification

Proteases present and active during the wine making process also have the added benefit that they may potentially increase the assimilable nitrogen important for microbial growth during fermentations. *S. cerevisiae* is unable to utilize proteins as a nitrogen source. However, proteases are able to liberate peptides and amino acids through the hydrolysis of proteins contributing to the yeast's assimilable nitrogen pool required for coordinating amino acids, purine and pyrimidine synthesis (Bell and Henschke, 2005) needed for cell growth, flavour-active metabolites and also fermentation activity. Proteins present in wine have been found to account for up to 2% of the total nitrogen content (Feuillat 2005). Insufficient nitrogen sources may lead to fermentations that become slow or stop also referred to as sluggish and/or stuck fermentations respectively. A shortage in nitrogen sources also leads to the production of hydrogen sulphide which is known to have a negative effect on sensory attributes. In order to ensure an adequate amount of nitrogen, wine makers supplement the juice with diammonium phosphate (DAP) or ammonium sulphate (Hernandez-Orte et al. 2006). Supplementation with selected amino acids has also been shown to positively affect fermentation kinetics (Crépin et al. 2012). Nitrogen utilization by yeast has been shown to be strain dependent and influenced by fermentation conditions (Valero et al. 1999). It has also been reported that ethanol inhibits nitrogen uptake of some amino acids (Bisson 1991) and that at low temperatures the yeast consumes less nitrogen (Beltran et al. 2007).

The metabolism of nitrogen containing compounds leads to production of several important aroma compounds that contribute to the fermentation bouquet (Fleet 2003). Such compounds include higher alcohols which are produced via the Ehrlich pathway (Bell and Henschke 2005). Organic acids present together with these alcohols provide substrates for ester formation which are known to positively influence the wine quality (Lambrechts and Pretorius 2000). Protein and peptide utilization as sources of nitrogen has been reported in both *S. cerevisiae* and non-*Saccharomyces* species (Milewski et al. 1988; Shallow et al. 1991). Proteases active at wine making conditions naturally present or added as external

enzymes could liberate peptides and amino acids, thus contributing to the overall nitrogen content needed for cell growth and the formation of flavour active compounds.

2.5 Conclusion

As reviewed above, proteases of all families are applied in a vast variety of industries. The food industry has especially shown a large interest in acid proteases. The significant ability of this specific class of proteases to coagulate proteins, especially milk proteins, is the main reason for their high demand in the dairy industry. Other industries including the wine industry are also showing a growing interest in these proteases because of their stability at low pH range. Some reports have shown that these proteases have the ability to reduce wine haze under wine making conditions, therefore reducing the need for bentonite treatment. The activity of such enzymes could also impact more globally on wine physicochemical and organoleptic properties, although this aspect has not been a research focus so far. There is thus a growing interest in finding acid proteases from different sources able to function under wine making conditions, with non-*Saccharomyces* yeast being a strong candidate. In a recent study, two aspartic acid proteases-encoding genes from two non-*Saccharomyces* yeasts*,* isolated from South African grapes, have been isolated (Reid et al. 2012). Further characterization of such enzymes is essential in order to determine their potential use within the wine industry. The global impact that such enzymes have on the final product, when added directly to the wine or via co-inoculation of a natural producer, will also have to be determined.

2.6 References

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Chapter 3

Research results

Optimizing heterologous expression in *E. coli* and purification of two aspartic proteases from non-*Saccharomyces* yeasts

Chapter 3 - Research results

Optimizing heterologous expression in *E. coli* and purification of two aspartic proteases from non-*Saccharomyces* yeasts

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3.1 Introduction

The removal of heat unstable grape proteins is an essential step in the production of white, sparkling and rosé wines. These proteins mostly comprise grape pathogenesis related (PR) proteins, such as chitinases and thaumatin-like proteins (TLP) and have been shown to be responsible for haze formation (Marangon et al. 2011; Waters et al. 1996; Waters et al. 1998). They become unstable under certain conditions (eg. high temperatures, long storage) and aggregate into light dispersing particles which render a wine turbid resulting in consumers rejecting the wine as faulty (Hsu et al. 1987; Waters et al. 1992). Currently, the most effective tool that winemakers have to eliminate haze forming proteins is treatment with bentonite. However, the use of bentonite has several negative impacts on the final product the most severe being the removal of important aroma compounds. Several alternatives for benonite treatments have been investigated including the use of flash pasteurization (Pocock et al. 2003), ultrafiltration (Hsu et al. 1987) and the use of other absorbents (Cabello-Pasini et al. 2005; de Bruijn et al. 2009; Vincenzi et al. 2005). However, the use of acid proteases to prevent haze formation by degrading PR proteins (without being detrimental to wine quality) remains the most ideal solution.

Escherichia coli are one of the most widely used hosts for expression of recombinant proteins because of the vast knowledge available on their genetics and molecular biology. Some advantages also include low cost, fast growth and high levels of heterologous gene expression (Cornelis 2000). Furthermore, techniques for overexpression are well developed and for example a gene sequence cloned into a high copy-number plasmid containing a *lac* promoter can be induced by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG). Despite these encouraging aspects and reports of successful protein expression of cloned genes, each new gene still presents its own unique expression problems. Subsequently, expression of eukaryotic genes is often problematic because of codon bias and proteins being encapsulated into insoluble inclusion bodies.

In a recent study performed at the Institute for Wine Biotechnology (IWBT), two genes encoding extracellular proteases have been retrieved from non-*Saccharomyces* yeast isolated from South African wine (Reid et al. 2012). The genes, *MpAPr1* and *CaAPr1*, were isolated from *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384, respectively. These authors also demonstrated that MpAPr1 was actively secreted in the presence of BSA, casein and proteins extracted from grape juice and that the enzyme was able to degrade these proteins (at least partially). However, the kinetic properties of these enzymes have not been determined. The aim of this study was to over express these novel extracellular aspartic proteases in a bacterial host and to purify them as a first step toward characterizing their properties and evaluating their potential application in the wine industry.

3.2 Materials and methods

3.2.1 Strains, Plasmids and Culture Conditions

The strains used in this study together with their sources are listed in Table 3.1. The grape-derived yeast strains form part of the IWBT culture collection and were kept at -80°C in 25% glycerol prior to experimental use. Selected strains were grown at 30°C on yeast peptone dextrose (YPD) agar (Biolab diagnostics, Wadenville, South Africa) and freshly cultured for use in downstream experiments, after which they were grown at 30°C in YPD broth (Biolab diagnostics). *Escherichia coli* DH5α was used to propagate the plasmids carrying the genes cloned and *E. coli* strain BL21 (Rosetta-gami pLysS) DE3 (Novagen, Madison, USA) was used as host strain for gene expression. Plasmids pGEM®- T Easy (Promega, Whitehead Scientific, Cape Town, South Africa) and pET14b (Novagen) were used as cloning and expression vectors, respectively. The genotypes of the *E. coli* strains and of the plasmids are provided in Table 3.1. Bacterial strains were cultured at 37°C on Luria Bertani (LB) agar (Biolab diagnostics) prior to use in further experiments. Chemicals and antibiotics were supplemented at the following concentrations where appropriate: 100 μg/ml ampicillin (Sigma-Aldrich, Aston Manor, South Africa), 34 μg/ml chloramphenicol (Sigma-Aldrich), 15 μg/ml kanamycin (Roche, Mannheim, Germany), 12.5 μg/ml tetracycline (Fluka, Sigma-Aldrich), 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Sigma-Aldrich) and 100mM Isopropyl β-D-1-thiogalactopyranoside (IPTG; Thermo Scientific, Separations, Johannesburg, South Africa).

3.2.2 Nucleic acid extraction

Genomic DNA was isolated from overnight yeast cultures grown in 10 ml YPD broth (Biolab diagnostics) at 30°C on a spinning wheel, using the method described in Current Protocols in Molecular Biology (2008) according to Hoffman and Winston (1987). Plasmid DNA was

recovered from bacterial cultures using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instructions. Genomic DNA was quantified using the NanoDrop® ND-1000 spectrophotometer (Wilmington, USA).

3.2.3 PCR Methods and DNA Sequencing

The primers used in this study are listed in Table 3.2. All primers were synthesized by Integrated DNA Technologies (Leuven, Belgium). Primers were designed in order to amplify the *MpApr1* gene without its native secretion signal as *E. coli* would not be able to process it. Amplification of the full *CaAPr1* gene was performed as no native secretion signal could be identified (Reid et al. 2012).

PCR reactions were performed using the Expand High Fidelity PCR System (Roche) and the following program: initial denaturation at 94°C for 2 min, 10 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 45 s, elongation at 72°C for 1 min, followed by 20 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 45 s and elongation at 72°C for 1 min with a 5 s increment per cycle. A final elongation at 72°C for 2 min was performed. PCR programs were run using an Applied Biosystems 2720 Thermo Cycler (Life Technologies, Johannesburg, South Africa). The PCR products were resolved in 0.8% agarose gel using TAE buffer. Gels were stained with 0.2 µg/ml ethidium bromide (Sigma-Aldrich) after electrophoresis and visualized through UV transillumination. Gels were documented using a G: Box (Syngene, Cambridge, United Kingdom) using the software provided by the manufacturer (Genesnap Syngene, version 7.09). DNA fragments were purified using the QIAquick Gel Extraction Kit (Qiagen, Whitehead Scientific) according to the manufacturer's instructions.

3.2.4 Cloning and construction of expression vectors

E. coli competent cells were prepared as follows: cells were grown in 5 ml 2X LB broth (Biolab diagnostics) supplemented with 0.2% glucose at 37ºC overnight. The cells were then transferred into 100 ml 2X LB containing 0.2% glucose and incubated at 37ºC on a rotary shaker (135 rpm) until an optical density (OD) of 0.5 - 0.6 at 600 nm was reached. All OD readings were performed using a Lambda 25 UV/Vis spectrophotometer (Perkin Elmer). Thereafter, the cells were placed on ice for 2 h and transferred into two 50 ml sterile Falcon tubes. The tubes were centrifuged at 3000 rpm for 5 min at 4ºC. The supernatant was discarded and the cells resuspended in 25 ml competency buffer (0.1 M $CaCl_2$, 0.07 M $MnCl_2$, 0.04 M NaOAc) followed by an incubation step for 30 min on ice. Again the cells were centrifuged at 3000 rpm for 5 min at 4ºC and the supernatant discarded. Finally, the cells

were resuspended in 2.5 ml competency buffer and added together after which 1.15 ml 80% glycerol was added. Competent cells were stored at -80ºC until further use.

After amplification and purification of the protease-encoding genes from the respective isolated yeast genomic DNA, the PCR products were cloned using the pGEM®-T Easy Vector System, according to the manufacturer's instructions, and transformed into chemically competent *E. coli* DH5α using the heat shock method. Briefly, 0.1-5 µg DNA was added to 200 µl competent cell suspensions and incubated on ice for 30 min after which it was placed in a water bath at 42°C for 90 s. Heat shocked cells were then incubated on ice for 2 min after which 1 ml LB broth was added. Cells were regenerated at 37°C for 60 min and plated on LB agar supplemented with the appropriate chemicals and antibiotics followed by overnight incubation at 37°C. Positive transformants were selected and grown in 5 ml LB broth, supplemented with ampicillin, for 24 h at 37°C on spinning wheel. Plasmids were purified using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) as mentioned above and sequenced using the T7 promoter and SP6 universal primers at the Central Analytical Facility (Stellenbosch University, South Africa) using an 3130 XL Genetic Analyzer (Applied Biosystems).

Following analysis of the sequences obtained, the inserts were excised from the pGEM®-T Easy cloning vector using the restriction enzymes *Bam*HI (Roche, Johannesburg, South Africa) and *Xho*I (Roche) for 60 min at 37°C. In parallel, the pET14b expression vector was digested under the same conditions. After restriction digest, the genes (together with the pET14b vector) were individually excised from an agarose gel, as mentioned above, and cloned into the pET14b expression vector at the *Bam*HI and *Xho*I sites using the T4 DNA ligase (Promega), according to the manufacturer's specifications. The resulting plasmids were transformed into *E. coli* DH5α competent cells (using the heat shock method as described above). Plasmids were sent for sequencing as mentioned above using the T7 promoter and T7 terminator primers (Central Analytical Facility, Stellenbosch University). After confirming the sequence, the plasmids were transformed into *E. coli* strain BL21 (Rosetta-gami pLysS) DE3 competent cells using the heat shock method (as described above) and grown on LB agar supplemented with the appropriate antibiotics. Figure 3.1 illustrates the cassette and the plasmid.

3.2.5 Induction and heterologous expression

In order to induce expression of the recombinant proteins, positive transformants were selected and inoculated into 5 ml LB broth supplemented with the appropriate antibiotics and grown overnight at 37°C. Initially, 5 ml of the preculture were added to 45 ml LB broth

supplemented with the appropriate antibiotics and grown to an OD_{600nm} of 0.5 - 0.6. Thereafter, IPTG was added to a final concentration ranging between 0 - 1 mM and the cultures incubated at 37°C for 4 h. Following induction, proteins were extracted using a native extraction protocol which is described in further detail below.

Subsequently, two sets of cultures were grown to an OD_{600nm} of 1.0 – 1.2 at 37°C and induced with 0.4 mM IPTG. The first set was incubated at room temperature and the second at 14°C for 48 h, respectively. Another set of cultures were grown to an OD_{600nm} of 0.6 after which they were induced with 0.4 mM IPTG and incubated for 48 h at 14°C. Following induction, proteins were extracted under native and denaturing conditions.

Finally, cultures were grown to an OD of 1.0 - 1.2 at 600 nm and supplemented with 0.4 mM IPTG after which proteins were extracted under semi-native conditions.

3.2.6 Extraction of proteins

Cells from 50 ml cultures were harvested at 9,000 rpm and the pellet frozen at -20°C until extraction was carried out. Three different extraction conditions were tested: native, denaturing and semi-native.

Under native conditions, the pellet was resuspended in 1.5 ml native lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl) adjusted to pH 8.0 together with 10 mg/ml lysozyme (Sigma) and incubated on ice for 30 min. Thereafter, the cells were transferred to 2 ml microcentrifuge tubes and flash-frozen using liquid nitrogen after which they were immediately thawed at 37°C. This operation was repeated six times. After the final freeze-thaw cycle, the mixture was centrifuged at 12,500 rpm for 20 min at 4°C. The supernatant was collected and stored at 4°C until further use.

Denaturing conditions involved resuspending the harvested pellet in a denaturing lysis buffer (100 mM NaH_2PO_4, 10 mM Tris, 8 M urea) adjusted to pH 8.0 and incubated for 15 min at room temperature on a rotary shaker. Following incubation the mixture was centrifuged at 12,500 rpm for 20 min at 4°C. The supernatant was collected and stored at 4°C until further use.

For the extraction under semi-native conditions, the pellet was resuspended and treated similarly to what was described for the native conditions but with a few alterations. After the final freeze-thaw cycle, the sample was centrifuged at 15,000 rpm for 20 min at 4°C. Subsequently, protein extraction was performed as described by Singh et al. (2012) with slight modifications. Briefly, the pellet was resuspended in 1 ml lysis buffer containing 2% (w/v) sodium deoxycholate (Sigma-Aldrich) and centrifuged at 12,500 rpm for 10 min, this

was repeated twice. The pellet was resuspended in a 300 µl solubilisation buffer (50 mM Na$_2$PO$_4$, 300 mMNaCl, 2 M urea, 6 M propan-2-ol) and stored at 4°C until further use in downstream experiments.

3.2.7 Purification and refolding of Acid Protease

3.2.7.1 Purification under native and denaturing conditions

The proteins extracted under native conditions, as described above, were purified using the Ni-NTA spin column kit (QIAGEN). Purification was performed using the manufacturer's protocol under native conditions, with slight modifications. Briefly, to equilibrate the Ni-NTA spin column, 600 µl lysis buffer was loaded and centrifuged for 2 min at 2900 rpm. 1 ml lysate (approximately 9,000 µg/ml proteins) from induced cultures was subsequently loaded and centrifuged at 1600 rpm for 4 min. The flow-through was kept and stored at 4°C after which the column was washed twice with 600 µl wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole, pH 8.0). An additional wash step was performed which involved washing twice with a second wash buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 100 mM imidazole, pH 8.0). Wash steps were performed at 2,900 rpm for 2 min and the flow-through solutions were kept and stored at 4°C. Subsequently, the columns were eluted three times with 200 µl elution buffer (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 500 mM imidazole, pH 8.0). All samples were kept at 4°C until further analysis.

Purification under denaturing conditions using the Ni-NTA spin column (QIAGEN) was carried out according to the manufacturer's protocol. Briefly, to equilibrate the column 600 µl of Buffer B (7 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl, pH 8.0) was loaded and centrifuged for 2 min at 2,900 rpm. Subsequently, 1 ml lysate (approximately 10,000 µg/ml total protein) from induced cultures extracted under denaturing conditions, as described above, was loaded onto the column and centrifuged at 1,600 rpm for 4 min. The flow-through solution was kept and stored at 4°C after which the column was washed twice with 600 µl Buffer C (8 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl, pH 6.3) at 2,900 rpm for 2 min. Subsequently, proteins were eluted from the column three times using 200 µl Buffer E (8 M urea, 0.1 M NaH$_2$PO$_4$, 0.01 M Tris-HCl, pH 4.5). All samples were kept at 4°C until further use in downstream experiments.

Purification under semi-native conditions involved equilibrating the column with the same solubilisation buffer as proposed by Singh et al. (2012). Thereafter, purification commenced as for native purification.

3.2.7.2 Protein refolding

Following purification, the proteins extracted under denaturing conditions were refolded using serial dilution with a refolding buffer at pH 8.0 which contained 100 mM NaH_2PO_4, 300 mM NaCl, 0.5 M arginine and 10 mM DTT. Briefly, 500 µl were diluted to a final volume of 4 ml (500 µl refolding buffer per hour) after which the sample was concentrated to 500 µl using Amicon Ultra 2 ml centrifugal filters with a 10 kDa membrane. Subsequently, diafiltration was performed on the samples using the same centrifugal filters as mentioned above together with 0.1 M Mcilvaine's buffer pH 3.5.

Proteins were also refolded using the pulsatile dilution technique. Briefly, 500 µl of sample was added at a rate of 100 µl/h to 3.5 ml refolding buffer. Subsequently, the sample was concentrated to 500 µl using Amicon Ultra 2 ml centrifugal filters with a 10 kDa membrane, after which diafiltration was performed on samples using the same centrifugal filters as mentioned above together with 0.1 M Mcilvaine's buffer pH 3.5.

3.2.8 Determination of protein concentration and SDS-PAGE

Protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's specifications. Reactions were performed in 96-well microtiter plates incubated at 37°C after which they were allowed to cool down to room temperature for 10 min. The OD was then read at 562 nm using a Bio-Tek PowerWave X Microplate Reader and the data analysed using the KC4 software provided with the instrument.

The presence of the recombinant protein was visually confirmed by use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described by Laemmli (1970) using 12% bis-acrylamide gel cast and run on a Bio-Rad Mini-Protean® Tetra Cell System (Bio-Rad Laboratories., Hercules, CA). Proteins were visualized by staining gels with Coomassie blue R-250 (Merck, Darmstadt, Germany). Selected protein bands were excised and sent for sequencing by LC-MS/MS at the Central Analytical Facility, Stellenbosch University.

3.2.9 Determination of Enzyme Activity

Acid protease activity was assayed using BSA (Roche) as substrate. The reaction mixture contained 500 µl 1% BSA solution dissolved in a 0.1 M Mcilvaine's buffer at pH 3.5 and 500 µl sample solution containing the purified enzyme (in the same buffer). Following incubation at 30°C, 200 µl samples were taken at different time points (0, 12, 24 and 48 h) and the reaction was stopped by the addition of 200 µl 20% TCA solution. The mixture was

allowed to stand at room temperature for 15 min and then centrifuged at 10,000 rpm for 15 min. The absorbance of the supernatant was measured at 280 nm using a Lambda 25 UV/Vis spectrophotometer. Controls were included which contained all the reaction components except the enzyme. An acid protease from *Aspergillus saitoi* (Sigma Aldrich) dissolved in 0.1 M Mcilvaines buffer pH 3.5 was also included at different concentrations (1 mg/ml, 0.1 mg/ml and 0.01 mg/ml) to serve as a positive control. One unit of enzyme activity was defined as the amount of enzyme required to hydrolyze BSA and release the equivalent to 1.0 µg of tyrosine in 1 min at 30°C.

3.3 Results and discussion

3.3.1 Production and extraction of recombinant acid protease

3.3.1.1 Cloning of *MpAPr1* and *CaAPr1*

The vast knowledge available on the genetics and molecular biology of *E. coli* makes it the first choice for the expression of heterologous proteins. In order to produce the acid proteases MpAPr1 and CaAPr1, it was decided to use the *E. coli* strain BL21 (Rosetta-gami pLysS) DE3. This strain expresses rare tRNA's necessary for the expression of these eukaryotic genes and also has a *trx/gor* mutation which facilitates disulphide bond formation. The pET14b plasmid was chosen as the expression vector as it fuses a 6x His tag to the N-terminal of the expressed protein which facilitates purification in downstream experiments.

As no introns were predicted according to the Intron Site Finder (http://dna.med.monash.edu.au/~torsten/intron_site_finder) of the Victorian Bioinformatics Consortium (Monash University, Clayton, Australia), genomic DNA was used as a template to amplify *MpAPr1* and *CaAPr1*. However, for *MpAPr1,* primers were designed in order not amplify the secretion signal as *E. coli* would not be able to process it. For *CaAPr1* the full open reading frame (ORF) was amplified as no secretion signal was identified (Reid et al. 2012). The *MpAPr1* and *CaAPr1* putative gene sequences of *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384 were successfully cloned into the expression vector pET14b and named pET-MpApr1 and pET-CaAPr1, respectively (Figure 3.1). The cassettes were sequenced using gene specific primers and *in silico* analysis showed that the sequences were 100% identical to those expected (Accession numbers JQ677912 and JQ677913 for *M. pulcherrima* and *C. apicola,* respectively). These vectors were transformed into *E. coli* strain BL21 (Rosetta-gami pLysS) DE3 for over-expression. This specific strain also harbours a pRARE2 plasmid which provides rare tRNA codons. The genes sequences were analysed for the presence of rare codons using the Rare Codon Calculator (http://nihserver.mbi.ucla.edu/RACC/, UCLA, Los Angeles, CA) and it was indeed found that

the codons provided by the pRARE2 plasmid were necesary for the correct transcription of *MpAPr1* and *CaAPr1*. The bacterium was also transformed with the vector lacking any recombinant gene to serve as a control in downstream experiments. Successful transformation of the correct plasmid was confirmed by PCR with gene specific primers (Table 3.2).

3.3.1.2 Initial induction of *MpAPr1* and *CaApr1* expression

Initially, in order to produce the recombinant acid proteases the protocol proposed by the manufacturer of the host strain and the vector (Novagen) was followed. Briefly, two separate *E. coli* cultures harbouring the pET-MpAPr1 and the pET-CaAPr1 plasmids (respectively) were grown at 37°C to an OD_{600nm} of 0.6. Subsequently, the cultures were induced with 0.4 mM IPTG and incubated for 4 h at 37°C. Controls consisted of an *E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b plasmid and an untransformed bacterium. Extraction of proteins was performed following native conditions (as described above) and visualized using SDS-PAGE. From the results obtained, over-expression of the recombinant acid proteases in both cultures could not be observed (data not shown). It was thus clear that the expression, extraction and purification of an acid protease in *E.coli* would require extensive optimization and therefore only MpAPr1 was used in the subsequent development of this optimization procedure.

One of the most important factors determining expression of a recombinant protein in a bacterial host is the concentration of the inducing agent. In order to reduce the metabolic burden imposed on the cell, the inducing agent (in this case IPTG) can be adjusted for optimal expression (Hansen et al. 1998). The concentration of IPTG as an inducing agent usually ranges between 0.005 to 2 mM IPTG. However, 1 mM is widely used (Donovan et al. 1996). Subsequently, *E. coli* cultures harbouring the pET-MpAPr1 were grown to OD_{600nm} of 0.6 and induced with between 0 - 1 mM IPTG (0.2 mM intervals) after which they were incubated for 4 h at 37°C. Untransformed *E. coli* cultures and cultures transformed with the pET14b plasmid were used as negative expression controls. Proteins were extracted under native conditions following the manufacturer's protocol (Qiagen) and visualised using SDS-PAGE. From the results obtained, over-expression of the recombinant protein could not be observed when compared to the controls (Figure 3.2).

It was hypothesized that the protein was either not being expressed or that following expression, the recombinant proteins were encapsulated into inclusion bodies. The latter phenomenon is sometimes observed when expressing recombinant proteins in *E. coli* (Kapust and Waugh 1999; Mukhopadhyay 1997). This hypothesis was also strengthened by the fact that the enzyme being produced is a protease which might cause adverse effects to

the cell. Furthermore, several reports have shown the expression of different proteins in *E. coli* using a IPTG inducible expression vector and from these reports, it is clear that the efficiency at which the recombinant protein is produced (if at all), depends on several parameters some of which include the expression vector used, the concentration of IPTG added to induce expression, the growth phase that the cells are in upon the time of induction, the amount of time which is allowed for induction to occur and more importantly the temperature at which expression occurs (Barbero et al. 1986; Bronikowski et al. 2001; Peng et al. 2004; Sandén et al. 2003; Srivastava et al. 2005; Valente et al. 2006; Vásquez-Bahena et al. 2006; Yildir et al. 1998).These parameters were therefore also considered in the following optimisation procedure.

Sumby et al. (2009) expressed an esterase enzyme from *Oenococcus oeni* in *E. coli* BL21 (DE3) using pET14b as expression vector and found optimal expression when inducing with 0.4 mM IPTG followed by incubation at 14°C for 48 h. Induction was performed when cultures were at mid-exponential phase. Other reports also found that incubation at lower temperatures after induction resulted in better expression (Bronikowski et al. 2001; Donovan et al. 1996; Mergulhao et al. 2004; Mergulhao et al. 2005; Mergulhao and Monteiro 2007). Considering this, as a starting point to optimizing expression of MpAPr1, it was decided to follow the protocol proposed by Sumby et al. (2009) as the same expression vector and a closely related host were used in our study. Moreover, extraction under conditions using strong denaturing substances (such as urea) has been shown to denature inclusion bodies as well as the proteins encapsulated therein (Tsumoto et al. 2002) and was therefore considered in our study, keeping in mind that when using a strong denaturing substance during extraction, a subsequent step to refold the proteins to their active state is required. Extraction of proteins in their native conformation is usually favoured because it results in fewer steps in further downstream purification experiments.

3.3.1.3 Optimization of induction and extraction

Two sets of cultures harbouring the pET-MpAPr1 plasmid were grown to an OD_{600nm} of 1.0 – 1.2 (mid exponential phase) at 37°C and induced with 0.4 mM IPTG. The first set was incubated at room temperature and the second at 14°C (respectively) for 48 h. Controls included *E. coli* BL21 (Rosetta-gami pLysS) DE3 cultures harbouring an empty pET14b vector and an untransformed strain, induced and incubated under the same conditions. Following induction, the proteins of both sets of culture were extracted under native and denaturing conditions after which SDS-PAGE was conducted in order to visualise the proteins (Figure 3.3 and Figure 3.4). Figure 3.3 shows the results obtained from cultures

extracted under native conditions. Again, the presence of the MpAPr1 enzyme could not be observed when compared to the controls. However, results obtained from extraction under denaturing conditions revealed a thick band at 42 kDa from cultures that were induced at 14°C for 48 h (Figure 3.4), thus confirming the over expression of MpAPr1. This provided evidence for the hypothesis that the recombinant protein expressed is being encapsulated into inclusion bodies. Furthermore, cultures induced at room temperature and extracted under denaturing conditions revealed only a faint band when compared to cultures induced at 14°C. Induction at 14°C was thus more optimal than when preformed at room temperature.

The influence of the physiological state that the cells are in at the time of induction on the expression of *MpAPr1* was then evaluated. An OD_{600nm} of 0.6 represents early exponential phase and an OD_{600nm} of 1.0 mid exponential phase (data not shown). Two sets of cultures were grown to an OD_{600nm} of 0.6 and 1.0, respectively. Subsequently, expression of *MpAPr1* was induced with 0.4 mM IPTG and the cultures incubated for 48 h at 14°C. Following induction, proteins were extracted under native and denaturing conditions as described above. SDS-PAGE (Figures 3.5 and 3.6) revealed that extraction of the recombinant protein was again only successful under denaturing conditions. Thus induction of *MpAPr1* only occurred at mid exponential phase and no expression could be observed with cultures induced at early exponential phase. Induction is commonly performed at mid exponential phase (Peng et al. 2004; Vásquez-Bahena et al. 2006), but similar results were obtained by Yilder et al. (1998) who performed induction at different stages. This phenomenon might be dependent on the nature of the enzyme being produced or by the use of a specific vector with a specific *E. coli* strain.

At this stage of our optimisation process, it was concluded that the optimal conditions for the expression of recombinant MpAPr1 were the following: induction of the culture at mid exponential phase (OD_{600nm} of 1.0) with 0.4 mM IPTG followed by incubation for 48 h at 14°C. Native extraction of the recombinant remained unsuccessful and thus would require further extensive optimization.

In order to determine if the expression levels could be increased, different concentrations of IPTG ranging between 0 - 1 mM (0.2 mM intervals) were used to induce cultures grown and incubated at optimal conditions as determined above. Following induction, the cultures were extracted under denaturing conditions and visualised by SDS-PAGE. The results revealed that IPTG addition at any concentration resulted in higher expression of MpAPr1 compared to samples with no IPTG addition, but a drop in expression level was observed for the 1 mM

IPTG concentration (Figure 3.7). Azaman et al. (2010) reported the same phenomenon when they found that an IPTG concentration higher than 1 mM negatively influenced their specific product yield. However, when altering the IPTG concentrations between 0.2 and 0.8 mM, no observable effect on expression levels was noticed (Figure 3.7).

After optimizing the expression of recombinant *MpAPr1*, *E. coli* BL21 (Rosetta-gami pLysS) DE3 cultures harbouring the pET-CaAPr1 plasmid were induced and incubated under the optimal conditions described above. Proteins were extracted under denaturing conditions and visualised by SDS-PAGE. From the results obtained, over-expression could not be observed (Figure 3.8). Although the active sites are conserved between the enzymes CaAPr1 and MpAPr1, there are differences in their sequences. The MpAPr1 also has a native secretion signal in the original gene which is lacking in the sequence of CaAPr1 indicating that there might be a difference in their processing. Although the actual reasons for an absence of expression in *E. coli* remain unclear, these factors might play a role; the conditions tested were optimized for expression of *MpAPr1* and thus might not be optimal for *CaAPr1*. Therefore further investigation and optimization is required for the recombinant expression of *CaAPr1*.

3.3.1.4 Optimization of extraction under semi-native conditions

Although extraction under denaturing conditions was successful, recombinant proteins would require uncertain refolding. Since extraction under native conditions proved unsuccessful, semi-native conditions were tested in order to obtain functional proteins thus eliminating the need for refolding. Extraction of proteins at different time points were investigated in an attempt to find a time point at which the percentage of recombinant proteins encapsulated was at a minimum. In other words, a point at which the protein is expressed but the concentration is not yet high enough to trigger encapsulation by inclusion bodies produced by the host. After addition of 0.4 mM IPTG, the cultures were incubated at 14°C and proteins were extracted at different time points (4, 8, 15, 24, 39 and 48 h) using a protocol proposed by Singh et al. (2012) with a few minor modifications as described above. Controls consisted of proteins extracted at 48 h under denaturing conditions. The results revealed that extraction was successful under the conditions tested (Figure 3.8) and that incubation for 39 h to 48 h after induction was slightly more efficient for over expression of *MpAPr1* to occur. This might be due to the nature of the enzyme expressed, meaning that the acid protease are degrading proteins within the host cell leading and as a defensive response the host encapsulates them in inclusion bodies rendering them inactive.

3.3.2 Purification and refolding

Purification of samples was performed using the Ni-NTA spin columns. The expression of a protein that contains a histidine tag allows it to bind to a chelated divalent metal ion (such as the nickel present in the Ni-NTA spin columns). These proteins are then eluted by competing with imidazole (native conditions) or through the reduction in pH leading the protonation of histidine residues (denaturing conditions). At first, purification was performed on cultures extracted under native conditions. Although no level of over-expression could be observed on SDS-PAGE gels (Figure 3.3), the aim was to investigate if small amounts of recombinant enzyme could be obtained in its native conformation. Purification proceeded under native conditions as described above. However, despite several attempts, purification following native conditions was unsuccessful (data not shown). This confirmed once again that these conditions of extraction were not appropriate. On the other hand, extraction under denaturing conditions revealed encouraging results (Figures 3.9 and 3.10). Figure 3.9 and 3.10 show the different stages of purification of MpAPr1 and CaAPr1 respectively.

The results for the purification of MpAPr1 following denaturation (Figure 3.9) showed a single band at the expected size of 42 kDa, confirming purification was successful. This band was excised and sent for protein sequencing using LC-MS/MS at the Central Analytical Facility (Stellenbosch University). The results confirmed that this band was indeed MpAPr1 with a score of 473.12 and 9 unique peptides matching the MpAPr1 protein sequence (data not shown). At this stage, although the protein was pure, it was in denaturing conditions, which necessitated refolding to its native state in order for it to be active. The success of this purification after extraction under denaturing conditions further strengthened the idea that all the recombinant enzymes being produced are being encapsulated into inclusion bodies.

After a few attempts at purifying the CaAPr1 enzyme, it was concluded that it was unsuccessful. The results (Figure 3.10) confirmed that the recombinant enzyme was not expressed. Rather, other native proteins of the host loosely bound to the column and were eventually retrieved in the elution fraction. *E. coli* is indeed known to produce native proteins that exhibit a high affinity for divalent cations and are thus able to bind to nickel based columns. This binding is mostly because these proteins have a cluster of histidine residues somewhere on their surfaces. Most of these proteins have been shown to be expressed in response to stress conditions and remain a problem during the purification of a recombinant protein using immobilised metal affinity chromatography (Bolanos-Garcia and Davies 2006). The over-expression of the *CaAPr1* gene in the bacterial host and the purification thereof was thus not successful and would require further investigation.

Proteins from cultures harbouring the pET-MpAPr1 plasmid were extracted under semi-native conditions were also run through the Ni-NTA spin columns as an attempt to purify under native conditions and thus avoiding the need for refolding. However, after several attempts, the results revealed that purification was unsuccessful (data not shown). It was hypothesized that the buffer used interferes with the binding of the proteins to the column. Despite the encouraging results obtained from extraction under semi-native conditions, further optimization of purification could not be pursued because of time constraints. Potential areas that may require optimization include performing a buffer exchange procedure followed by purification and/or performing purification using a reduction in pH.

3.3.3 Determination of acid protease activity

The protein that was successfully purified in denaturing conditions was refolded through serial or pulsatile dilution as indicated in the Materials and Methods section. In order to assess whether any activity had been regained, an enzymatic assay using BSA as substrate was performed. It was decided to perform the assay at 30°C, as it was previously shown that the enzyme is active at this temperature (Reid et al. 2012). From the results obtained (Figure 3.11), no significant activity could be observed from neither samples of MpAPr1 that underwent serial dilution (SD-Mp) nor from those that underwent pulsatile dilution (PD-Mp). However, even though not significant, a slight increase of activity similar to that of the positive control (0.01 mg/ml) could be observed in samples that were refolded by the latter technique. It was determined that after refolding, the protein concentration of both samples was approximately 0.05 mg/ml. Strong activity was observed from positive control samples, which consisted of an acid protease from *A. saitoi* dissolved at different concentrations in 0.1 M Mcilvaine's buffer (pH3.5). Positive controls included at a concentration of 0.01 mg/ml showed extremely low activity under the conditions tested, which might explain why activity could not be observed for MpAPr1 recovered following refolding. In other words, the concentration of enzymes was most probably too low to have a significant effect under the conditions tested. Thus refolding also requires optimization either in terms of increasing the yield obtained or by altering the conditions used.

The conditions used in this enzyme assay might also not be optimal enough for MpAPr1 to show significant activity at such low concentrations, as its kinetic properties are yet to be determined. It has also been shown that the His tag fused to the enzyme might have an effect on the tertiary structure and biological activity (Bucher et al. 2002; Terpe 2003). However, this hypothesis is unlikely as the His tag was fused to the N-terminal of the protein. Using SWISS-MODEL (Arnold et al. 2006; Kiefer et al. 2009; Peitsch 1995), the deduced protein sequence of MpAPr1 was modelled based on an aspartic acid protease from *C.*

tropicalis (1j71A – PDB number) which is the second closest relative to MpAPr1 with 39% identity (Reid et al. 2012), and visualised using Swiss-Pdb Viewer (version 4.0.4). It was shown that the N-terminal of the protein is on the ''tail-end'' (far from the active site). An artificial His tag was built onto the N-terminal (Figure 3.12 in green) and it was hypothesized that the tag would most likely not have any effect on the enzymes activity (Figure 3.12). Nevertheless, the His tag could be removed from the enzyme and the activity evaluated. The pET14b vector indeed fuses a thrombin site between the His tag and the protein (Figures 3.1 and 3.12 in yellow) which can subsequently be cleaved (by a thrombinase), thereby removing the His tag. Moreover, the degree of glycosylation can have a major effect on the kinetics and thermodynamics of protein refolding (Shental-Becher and Levy 2008). The MpAPr1 protein only shows one potential N-glycosylation site as detected by *in silico* analysis (Reid et al. 2012), and it was hypothesized that expression in *E. coli* would not impact severely on MpAPr1 activity considering the potentially low native glycosylation level. However, six potential O-glycosylation sites have also been detected at amino acids 36, 44, 55, 110, 111 and 113, after submitting the sequence to NetOGlyc4.0 (Steentoft et al. 2013). Finally, although acid activation has been reported to be the most effective method to minimize autodigestion (Lin et al. 1993), this phenomenon could partially explain the low yield and activity observed. Thus, if increasing the yield obtained and removing the His tag do not have an impact on the enzyme activity, a eukaryotic host organism would have to be used for recombinant over-expression (eg. *S. cerevisiae, Kluyveromyces lactis, Pichia pastoris*) of the aspartic protease with the correct post-translational modifications. Other strategies would involve large scale culturing of *E. coli* cells in order to increase protein yield followed by large scale purification.

3.4 Conclusion

Non-*Saccharomyces* yeasts are a valuable source for acid proteases able to combat haze formation in white wine. Most proteases that have been investigated in the past have been shown to be ineffective against haze forming proteins because of the high resistance to enzymatic degradation of the haze forming proteins as well as the conditions of wine making being unfavourable for enzymatic activity. Acid proteases isolated from two non-*Saccharomyces* species were previously shown to have high potential to degrade haze forming proteins and therefore reduce and/or replace bentonite treatments. The aim of this study was to express and purify these acid proteases before they could be further characterised. These steps required extensive optimisation.

The genes encoding two aspartic proteases termed MpAPr1 and CaAPr1 were successfully isolated and cloned into a bacterial host for over expression. Optimal expression was found

when cultures were grown to late exponential phase and then induced using 0.4 mM IPTG after which they were incubated for 48 h at 14°C. However, expression resulted in the recombinant acid protease being encapsulated into inclusion bodies. Consequently, extraction and purification under native conditions remained unsuccessful. Extraction and purification following denaturing conditions were only successful for MpAPr1 and it was concluded that CaAPr1 was not expressed, for reasons yet to be elucidated. After optimisation of the protocol, purified samples of recombinant MpAPr1 in denaturing conditions were obtained and refolded using two different methods (both based on the same principle of dilution). However, it was found that recovery was too low to observe an effect under the conditions tested.

Overall, expression of the recombinant MpAPr1 acid protease was indeed very strong even though expression resulted in the enzyme being encapsulated in inclusion bodies. This phenomenon could prove to have some advantages in that the inclusion bodies can be isolated apart from the cell debris resulting in a pre-purification step. Future work will thus have to focus on optimizing extraction and refolding strategies in order to improve yield and/or on performing experiments on a larger scale. Expression of recombinant CaAPr1 also requires extensive investigation and optimization as the results revealed that expression was unsuccessful under the conditions optimised for the extraction of MpAPr1. Optimization of strategies involving extraction under native conditions and/or semi-native conditions is also an alternative to be considered as this would reduce and/or eliminate the need for refolding. Furthermore, once the recombinant acid proteases are extracted and shown to be active they will require to be biochemically characterized and tested under winemaking conditions in order to evaluate their actual potential to reduce protein haze. Other future work will also have to focus on producing the enzyme in its native host and thus also evaluate the potential use of these non-*Saccharomyces* yeasts in co-inoculation and/or sequential inoculation with *S. cerevisiae.* Finally, a global study investigating the impact of such acid protease on wine parameters (fermentation kinetics, chemical composition etc.) and organoleptic properties will also have to be performed.

3.5 Acknowledgement

3.6 References

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Table 3.1: List of microbes and plasmids used in this study

Microbes/ Plasmid	Strain	Description/Genotype	Collection/Reference
Candida apicola	Y1384	Wine yeast	IWBT*
Metschnikowia pulcherrima	Y1123	Wine yeast	IWBT*
Escherichia coli	DH5α	*[F-j80lacZΔM15Δ(lacZYA-argF) U169 deoR recA1 endA1 hsdR17(rk_, mk1) phoA supE44 thi-1 gyrA96 relA1]*	GIBCO-Invitrogen Life Technologies, Mowbray, South Africa
Escherichia coli	Rosetta-gami pLysS (DE3)	*Δ(ara-leu)7697 ΔlacX74 ΔphoA PvuII phoR araD139 ahpC galE galK rpsL (DE3) F'[lac⁺ lacI�q pro] gor522::Tn10 trxB pLysSRARE (CamR, StrR, TetR)*	Novagen
pGEM®-T Easy	-	*ApR LacZ*	Promega
pET14b	-	*ApR*	Novagen

*IWBT: Institute for Wine Biotechnology, Stellenbosch University, South Africa

Table 3.2: List of primers used in this study

Primers	Sequence 5'-3'
MpAPr1-F(*Xho*I)	ACGT<u>CTCGAG</u>ATGGCCATCCCTGGGC
MpAPr1-R(*Bam*HI)	ACGT<u>GGATCC</u>TTAAGCACTTATGATGTTTGACGAGC
CaAPr1-F(*Xho*I)	ACGT<u>CTCGAG</u>GTACTAGCTAAGAACTATGTTCATTTAGG
CaAPr1-R(*Bam*HI)	ACGT<u>GGATCC</u>TTAGTTGACAGATCCGGGA
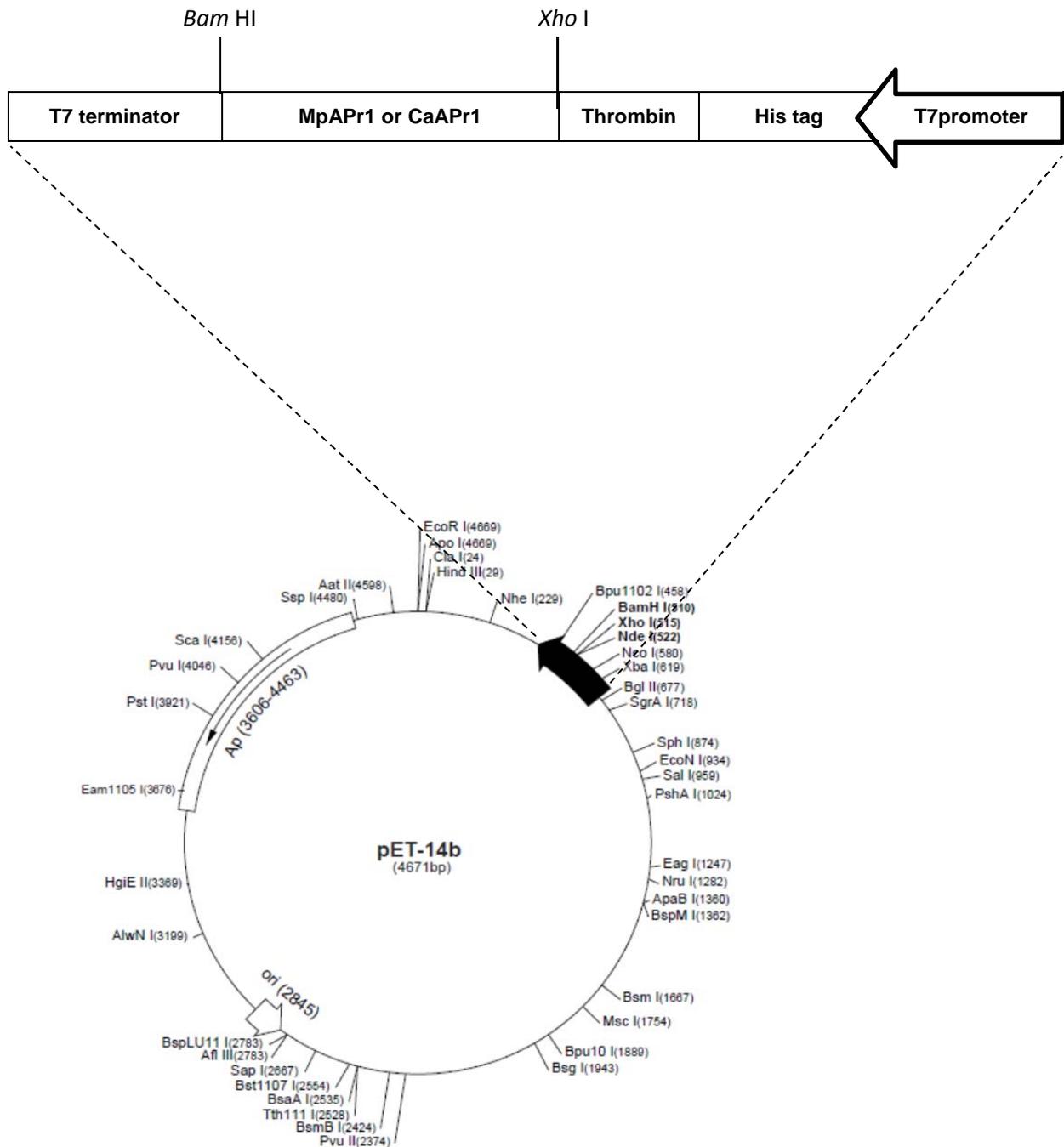
Figure 3.1 Plasmid pET14b and cassette. pET14b with MpAPr1 inserted =pET-MpAPr1 (Aspartic protease from *M. pulcherrima* Y1123), pET14b with CaAPr1 inserted = pET-CaAPr1 (Aspartic protease from *C. apicola* Y1384)
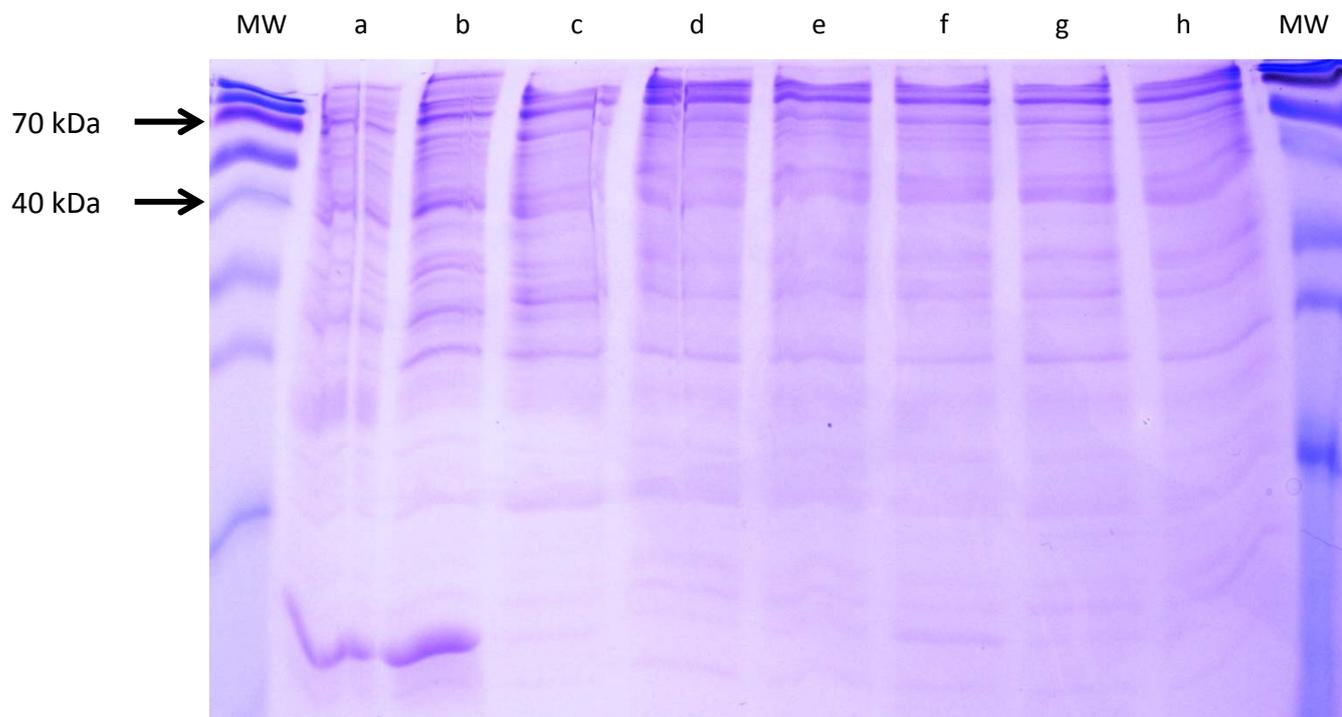
Figure 3.2 SDS-PAGE assessment of proteins extracted from *E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring the pET-MpAPr1 under native conditions using different concentrations of IPTG:(a) 1 mM, (b) 0.8 mM, (c) 0.6 mM, (d) 0.4 mM, (e) 0.2 mM, (f) 0 mM. Lanes: (g) control (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector) and (h) control (untransformed *E. coli* BL21 (Rosetta-gami pLysS) DE3). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
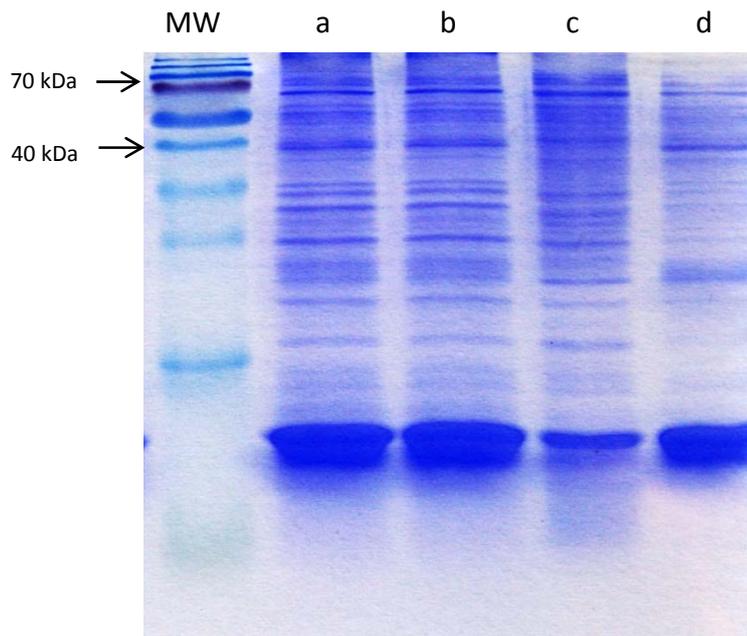
Figure 3.3 SDS-PAGE assessment of proteins extracted from *E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring the pET-MpAPr1 under native conditions following induction at 14°C and room temperature: (a) 14°C, (b) room temperature, (c) 14°C control (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector), (d) room temperature control (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
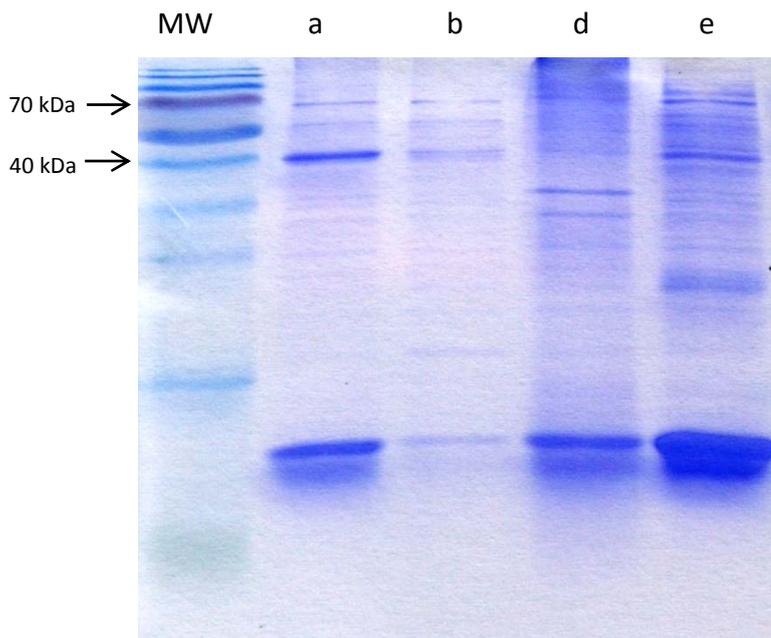
Figure 3.4 SDS-PAGE assessment of proteins extracted from *E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring the pET-MpAPr1 under denaturing conditions following induction at 14°C and room temperature: (a) 14°C, (b) room temperature, (c) 14°C control (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector), (d) room temperature control (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
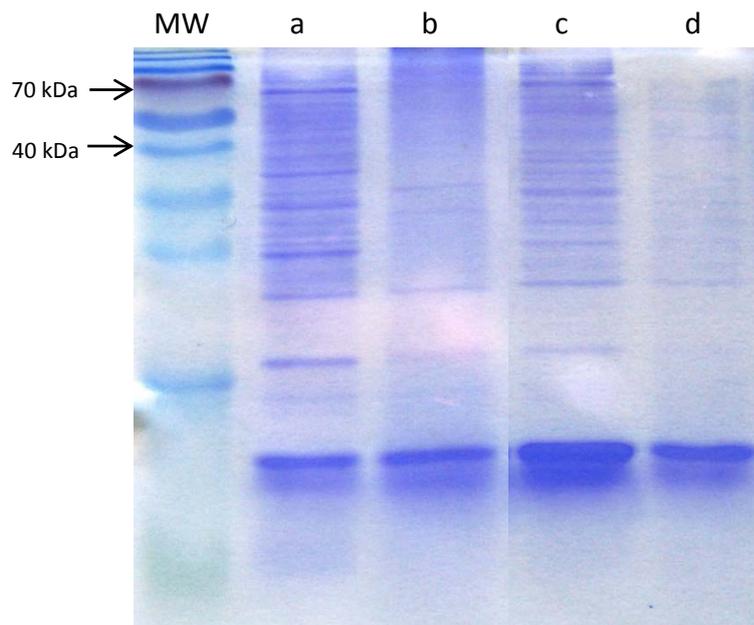
Figure 3.5 SDS-PAGE assessment of proteins extracted from *E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring the pET-MpAPr1 under native conditions and denaturing conditions following induction at an OD of 0.6 at 600nm: (a) native conditions, (b) denaturing conditions, (c) control native conditions (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector), (d) control denaturing conditions (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
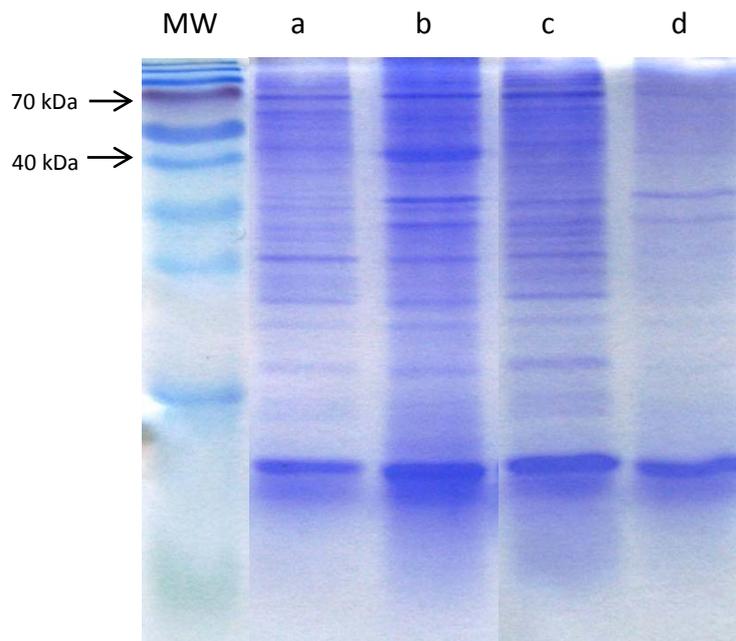
Figure 3.6 Assessment of proteins extracted from *E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring the pET-MpAPr1 under native conditions and denaturing conditions following induction at an OD of 1.0 at 600nm: (a) native conditions, (b) denaturing conditions, (c) control native conditions (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector), (d) control denaturing conditions (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
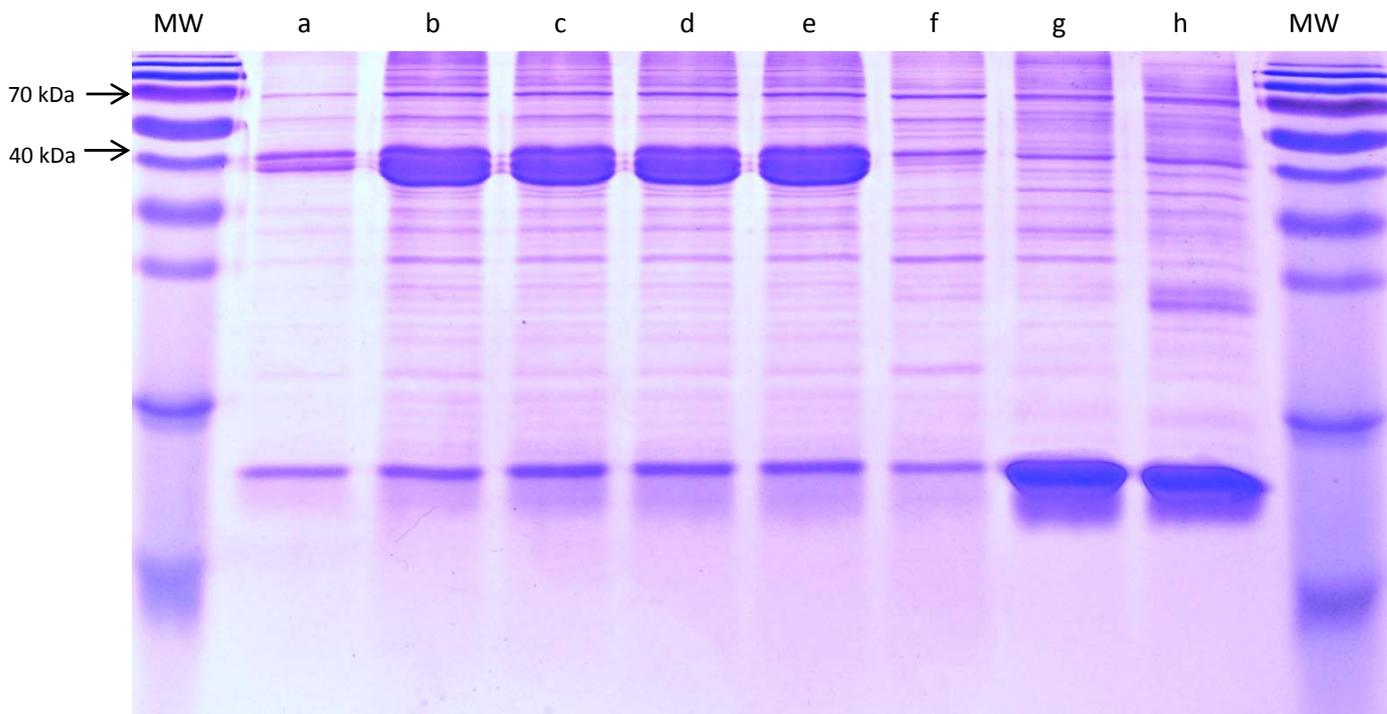
Figure 3.7 Assessment of proteins extracted from *E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring the pET-MpAPr1 under denaturing conditions following induction using different concentrations of IPTG:(a) 1 mM, (b) 0.8 mM, (c) 0.6 mM, (d) 0.4 mM, (e) 0.2 mM, (f) 0 mM. Lanes: (g) control (*E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring an empty pET14b vector) and (h) control (untransformed *E. coli* BL21 (Rosetta-gami pLysS) DE3). Controls were induced with 0.4 mM IPTG. MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
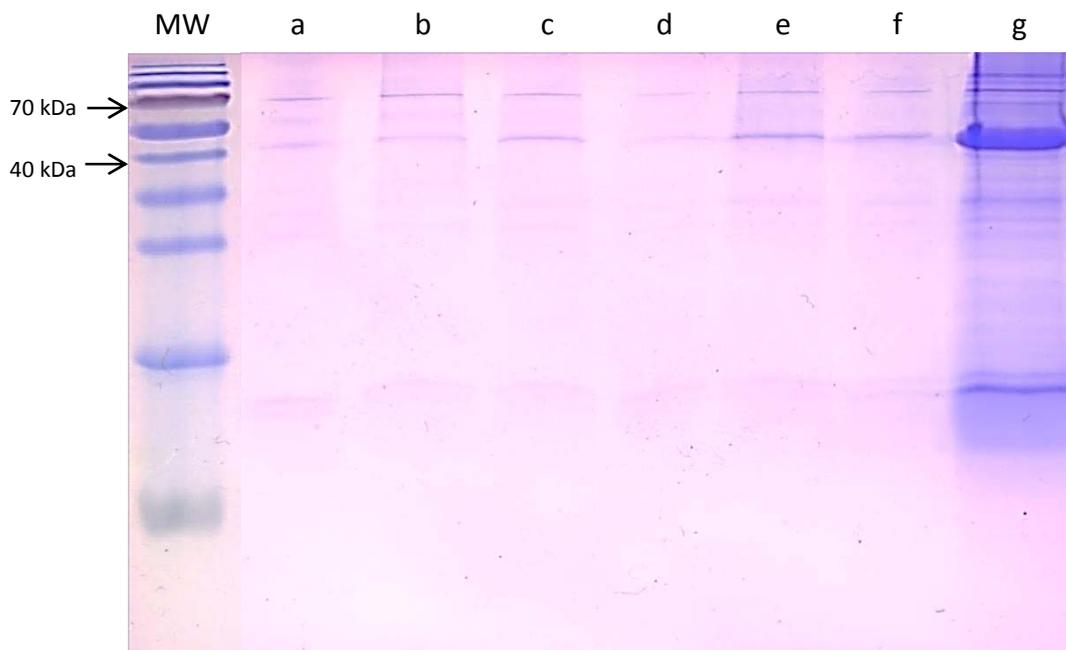
Figure 3.8 SDS-PAGE assessment of proteins extracted from *E. coli* BL21 (Rosetta-gami pLysS) DE3 harbouring the pET-MpAPr1 under semi-native conditions following induction using 0.4 mM IPTG. Extraction was performed at different times after induction: (a) 4 h, (b) 8 h, (c) 15 h, (d) 24 h, (e) 39 h, (f) 48 h. Lane (g) control (proteins extracted under denaturing conditions at 48 h). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
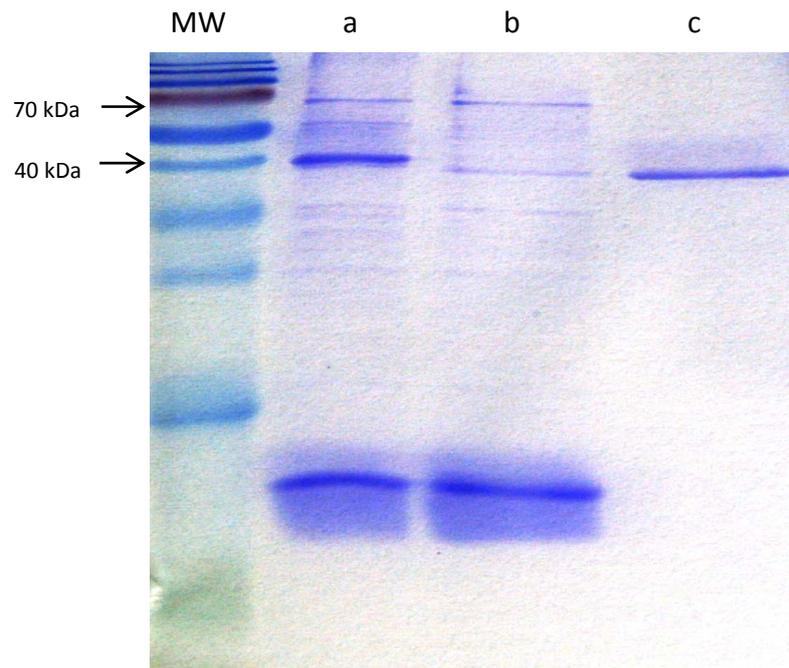
Figure 3.9 Analysis of proteins purified under denaturing conditions from *E. coli* BL21 (Rosetta-gami pLysS) DE3 cultures harbouring the pET-MpAPr1 plasmid. Lanes: (a) lysate (b) flow through (after lysate was run through Ni-NTA spin column), (c) elution sample (pure MpAPr1). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
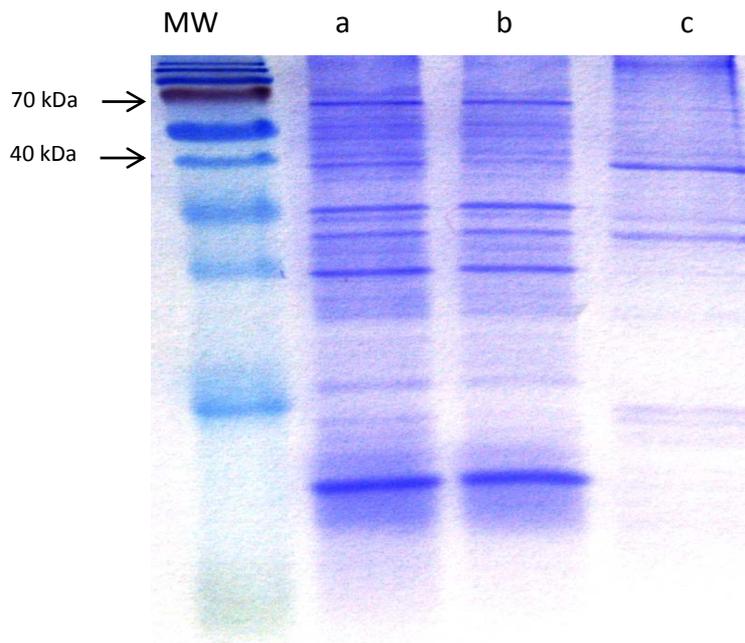
Figure 3.10 Analysis of proteins purified under denaturing conditions from *E. coli* BL21 (Rosetta-gami pLysS) DE3 cultures harbouring the pET-CaAPr1. Lanes: (a) lysate (b) flow through (after lysate was run through Ni-NTA spin column), (c) elution sample. MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
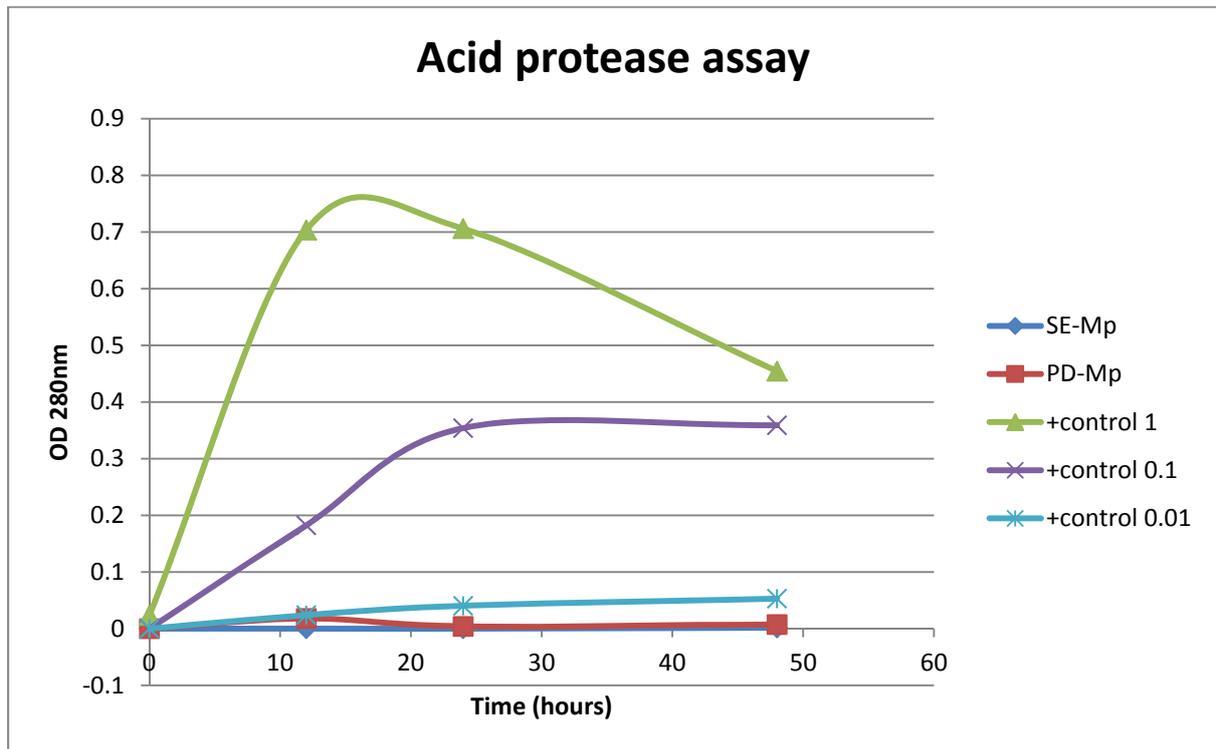
Figure 3.11 Acid protease assay using BSA as substrate. Samples were measured at an OD of 280nm at different time points. (SE-Mp): MpAPr1 proteins refolded using serial dilution, (PD-Mp): MpAPr1 proteins refolded using pulsatile dilution. Positive (+) controls (acid protease from *A. saitoi*) were added as follows: (+ control 1): 1 mg/ml, (+ control 0.1): 0.1 mg/ml, (+ control 0.01): 0.01 mg/ml.
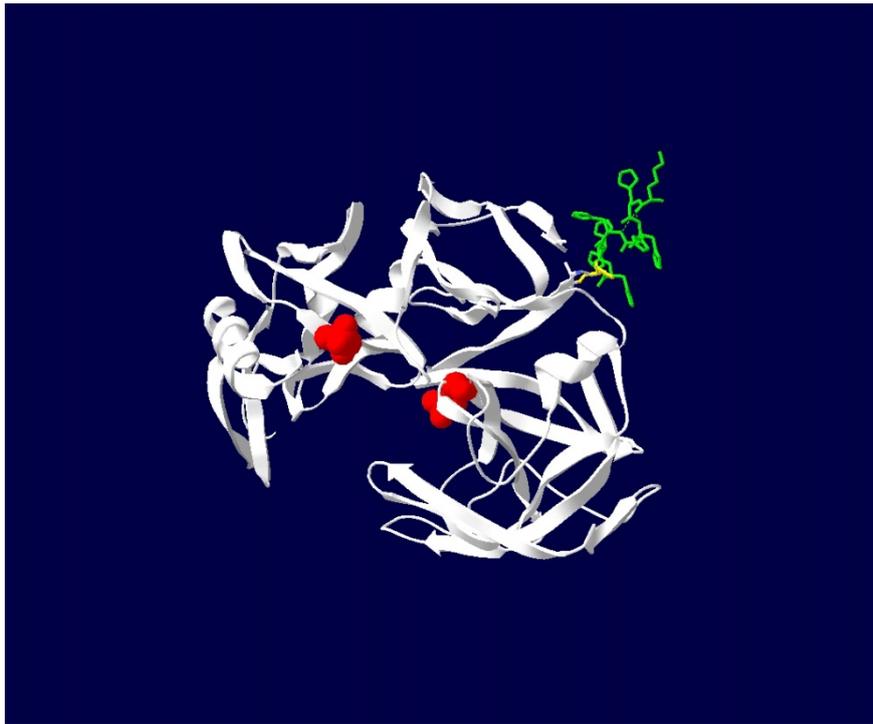
Figure 3.12 Screenshot of an aspartic protease from *C. tropicalis* (1J71A) visualised through Swiss-PbdViewer (v 4.0.4). Red indicates the active site residues, green the added hypothetical 6x His tag and white the protein backbone (ribbon).

Chapter 4

Research results

The quest for novel proteases active under wine making conditions: a preliminary investigation

Chapter 4 - Research results:

The quest for novel proteases active under wine making conditions: a preliminary investigation

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4.1 Introduction

In recent years numerous studies have focused on the contribution that yeast makes towards the protein content of wine. In the past, non-*Saccharomyces* species present on the surface of the grape berries have widely been regarded as spoilage microorganisms during winemaking. However, over the past decade, new attention has been directed towards these species as research indicates that some of them are beneficial in winemaking, especially when used in combination with *Saccharomyces cerevisiae* (Andorrà et al. 2010; Strauss et al. 2001). Furthermore, some non-*Saccharomyces* species have also been shown to produce various extracellular enzymes that can be of oenological importance (Charoenchai et al. 1997; Fernandez et al. 2000; Rosi et al. 1994; Strauss et al. 2001). Among these enzymes are proteases that are able to hydrolyse grape proteins under wine making conditions.

During the making of wine pathogenesis related (PR) proteins, in particular chitinases and thaumatin-like proteins, become unstable under certain conditions and aggregate in into light dispersing particles. This phenomenon causes the wine to appear hazy which is often interpreted by consumers as microbial spoilage. Bentonite has widely been used as a fining agent to remove haze forming (PR-proteins). Because of its negative charge it serves as a cation exchanger adsorbing proteins, however the application of bentonite also has several negative implications (Ferreira et al. 2002; Lagace and Bisson 1990; Waters et al. 2005). The main issues involved are the removal of important aroma compounds and disposal problems leading to environmental concerns associated with sustainability. These negative impacts have caused an investigation into alternative treatments. Out of all the alternatives investigated (use of different fining agents, ultra filtration, and flash pasteurization), the use of acid proteases seems to be the most efficient treatment without being detrimental to wine quality. However in the past several authors have indicated that they are not able to effectively degrade haze forming proteins due to the unfavourable conditions present during wine making (Waters et al. 1992; Waters et al. 1995).

In recent years several studies have also focused on using acid proteases in combination with heat treatment to eliminate haze forming proteins. Treatment at high temperatures causes PR proteins to denaturate, making it easier for the proteases to hydrolyse them. In 2012, Marangon et al. investigated using acid protease isolated from *Aspergillus niger* in combination with flash pasteurisation to eliminate haze in chardonnay and sauvignon blanc wines. The authors found that sole treatment of wines with Aspegillopepsin I and II was able to cause a 20% reduction in protein content. Maximum results were obtained when combining protease treatment with heat treatment at 75°C for 1 min, thereby reducing the need for bentonite. Very recently, an acid protease (BcAP8) isolated from *Botrytis cinerea* has been shown to effectively reduce haze without the need for heat treatment (Van Sluyter et al. 2013). Although not all the PR proteins were degraded it was shown to still be beneficial to winemakers by reducing bentonite requirements. These results encourage the need to search for novel acid protease able to effectively degrade haze forming proteins under wine making conditions.

In the past years several studies have reported that some non-*Saccharomyces* yeast secrete extracellular acid proteases. Many studies have focused on species such as *Kluyveromyces lactis*, *Pichia pastoris* and *Candida albicans* becuase of their importance in the medical industry (Buerth et al. 2011; Mattanovich et al. 2009; Sorgo et al. 2010; Stead et al. 2010). Furthermore, non-*Saccharomyces* species have also been found to secrete more than one acid protease and in some cases as many as ten, as in the case of *C. albicans* (Aoki et al. 2012, Naglik et al. 2003, Monod et al. 1994; Pichova et al. 2001; Zaugg et al. 2001).

Recently two genes (*MpAPr1* and *CaAPr1)* encoding acid protease has been isolated from non-*Saccharomyces* yeast in a study performed at the Institute for Wine Biotechnology. These natural occurring wine yeast *Metschnikowia pulcherrima* IWBT Y1123 and *Candida apicola* IWBT Y1384 was shown to produce extracellular acid proteases able to degrade grape proteins. The aim of this study was to potentially identify other acid protease encoding genes from these non-*Saccharomyces* species and furthermore, identify and isolate proteins released under conditions favouring the production of such produces.

4.2 Materials and methods

4.2.1 Strains and culture condition

The wine yeast strains used in this study all form part of the Institute for Wine Biotechnology (IWBT), Stellenbosch University culture collection. Cultures were kept at -80°C in 25%

glycerol prior to experimental use. Selected strains were grown on yeast peptone dextrose (YPD) agar (Biolab diagnostics, Wadeville, South Africa) at 30°C and freshly cultured in YPD broth (Biolab diagnostics) at 30°C prior to use in downstream experiments. Strains used in this study include: *Candida apicola* IWBT Y1384, *Clavispora lusitanae* IWBT Y833 and *Metschnikowia pulcherrima* IWBT Y1123.

4.2.2 Nucleic acid extraction and restriction enzyme digest

Yeast cultures were grown overnight in 10 ml YPD broth (Biolab diagnostics) at 30°C on a rotating wheel after which genomic DNA (gDNA) was extracted using the method described in Current Protocols in Molecular Biology (2008) according to Hoffman and Winston (1987). Isolated gDNA was treated with 2 µl (10 U/µl) RNase from Thermo Scientific (Inqaba Biotechnologies, Johannesburg, South Africa) for 60 min at 37°C and stored at -20°C prior to use in downstream experiments. Approximately 2 µg gDNA form *C. apicola* IWBT Y1384 and *M. pulcherrima* IWBT Y1123 were digested with *Hin*dIII (Roche, Mannheim, Germany) and *Kpn*I (Roche) in separate reactions, respectively. These enzymes where chosen as they do not cut within the sequences of either *MpAPr1* or *CaAPr1*. Enzymatic reactions were preformed overnight at 37°C. Digested DNA was resolved in 0.8% agarose gel using TAE buffer (0.04 M Tris-acetate, 1 mM EDTA). Gels were stained with 0.2 µg/ml ethidium bromide (Sigma-Aldrich, Aston Manor, South Africa) after electrophoresis and visualized through UV transillumination. Gels were documented using a G:Box (Syngene, Cambridge, United Kingdom) and the software used was provided by the manufacturer (Genesnap Syngene, version 7.09).

4.2.3 PCR methods

The primers used in this study are listed in Table 4.1. All primers were synthesized by Integrated DNA Technologies (Leuven, Belgium). Probes were designed to cover the full lenght of the *MpAPr1* and *CaAPr1* genes. Digoxigenin-labelled DNA probes were synthesized according to the manufacturers protocol (PCR DIG Probe Synthesis Kit, Roche, Johannesburg, South Africa) which incorporates DIG-dNTP's during PCR amplification. Therefore these probes could detect gene sequences similar to that of *MpAPr1* and *CaAPr1.*

PCR reactions were performed using the Expand High Fidelity PCR System (Roche) and the following program: 94°C for 2 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 45 s, elongation at 72°C for 1.5 min. A final elongation at 72°C for 2 min was performed. PCR programmes were run using an Applied Biosystems 2720 Thermo Cycler

(Life Technologies, Johannesburg, South Africa). PCR products were resolved on a 0.8% agarose gel, stained and visualized as previously described.

4.2.4 Southern Blot hybridization

Digested DNA from the agarose gel (as described in section 4.2.3) was transferred onto a positively charged nylon membrane (Roche). DNA was transferred by means of stack assembly using 20X SSC (transfer buffer). Blotted DNA was crossed onto the membrane by exposure to UV irradiation. The membrane was pre-hybridized for 30 min in 20 ml DIG Easy Hyb buffer (Roche) at 42°C in a hybridization oven and hybridized overnight at 42°C with 20 ml DIG Easy Hyb buffer containing 25 ng DNA probe, which had been previously denatured by boiling for 5 min. The membrane with hybridized DNA and probe was washed twice for 5 min at room temperature in a low stringency solution (2X SSC, 0.1% SDS) and twice in a high stringency buffer solution (0.5X SSC, 0.1% SDS) for 15 min at 55°C.

Hybridization bands were visualised by chemiluminescent method (DIG luminescent detection kit, Roche) according to the manufacturer's specifications. The hybridized probes were immunodetected with anti-digoxigenin conjugated to alkaline phosphatase and visualized with a chemiluminescent substrate. Enzymatic dephosphorylation of this substrate by alkaline phosphatase led to the emission of light that was recorded on X-ray film after 4 min.

4.2.5 Protein extraction and determination

Single colonies from plates of *M. pulcherrima* IWBT Y1123 and *C.lusitaniae* IWBT Y833 were inoculated into 10 ml YPD (Bio-lab diagnostics) and incubated overnight at 30°C on a rotating wheel. In order to induce expression of protease activity, the protocol proposed by Reid et al. (2012) was followed with slight modifications. Briefly, 10 ml of the preculture was transferred into 1 l base medium (1% glucose, 0.1% YNB without amino acids and ammonium sulphate and 0.066% ammonium sulphate, pH 3.5). Subsequently, the cultures were incubated for 24 h at 30°C on a rotary shaker (160 rpm) after which 250 mg/l bovine serum albumin (BSA Fraction V, Roche) were added in order to induce expression of the yeast acid protease(s). Cultures were incubated for an additional 48 h at 30°C on a rotary shaker (160 rpm). Proteins were extracted from the culture supernatant using the acetone precipitation method. Briefly, extracellular proteins were collected by adding ice-cold acetone to the cell free supernatant until a protein aggregate appeared. Proteins were precipitated overnight at -20 °C and collected by centrifugation for 30 min at 7,000 rpm and 4°C. The protein pellet was dried overnight and resuspended in 10 ml 0.5 M Tris-HCl (pH 6.8).

Protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (Thermo Scientific) according to the manufacturer's specifications. Reactions were performed in 96-well microtiter plates incubated at 37°C for 30 min after which they were allowed to cool down to room temperature for 10 min. Subsequently, the optical density (OD) was measured at 562 nm using a Bio-Tek PowerWave X Microplate Reader and the data analysed using the KC4 software provided with the instrument.

4.2.6 One dimensional protein separation and sequencing

Proteins extracted from extracellular medium were loaded onto sodium dodecyl sulphate bis-acrylamide gels. Proteins were loaded at a concentration of 1000 µg/ml and 100 µg/ml. The SDS-PAGE gel consisted of a 4% polyacrylamide stacking gel (125 mM Tris-HCl, pH 3.8, 0.1% SDS) casted over a 12% resolving polyacrylamide gel (375 mM Tris-HCl, pH 8.8, 0.1% SDS). Briefly, 3 µl Laemmli buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromophenol blue and 0.125 M Tris-HCl) was added to 12 µl protein samples (1000 µg/ml and 100 µg/ml, respectively) and boiled for 5 min at 95°C after which electrophoresis was conducted at 150 V until the dye reached the bottom of the gels using the Mini-Protean® Tetra Cell System (Bio-Rad Laboratories., Hercules, CA).The electrode chambers were filled with Tris-Glycine buffer (50 mM Tris, 200 mM glycine, 0.2% SDS). Staining was carried out in the microwave with Coomassie blue R-250 (Merck, Darmstadt, Germany) in 50% (v/v) ethanol, 10% (v/v) acetic acid and destained with 12.5% (v/v) isopropanol and 12% (v/v) acetic acid according to the protocol described by de Beer et al. (2008). Entire lanes were excised and sent for protein mass fingerprinting by LC-MS/MS at the Central Analytical Facility (Stellenbosch, South Africa). Proteins were tentatively identified using Thermo Proteome Discoverer 1.3 (Thermo Scientific, Bremen, Germany). The Uniprot Clavispora, Swissprot and NCBI databases were searched using the Mascot server (Mascot, Matrix Science, London, UK).

4.2.7 Two-dimensional protein separation and sequencing

2D-PAGE was performed as follows: 300 µl protein samples (5 mg/ml) were diluted in 300 µl 2D rehydration buffer (8 M urea, 2% CHAPS, 50 mM DTT, 0.2% (w/v) BioLite® 3/10 ampholyte, 0.002% bromophenol blue; Bio-Rad Laboratories), and incubated at room temperature for 1 h for complete protein solubilisation. Subsequently, 600 µl samples were loaded onto a focusing tray and overlaid with a 17 cm (non-linear) Readystrip™ IPG strip pH 3 - 10 (Bio-Rad Laboratories) with the gel side down after which it was covered with mineral oil and allowed to passively rehydrate at room temperature overnight. For the first dimension, isoelectric focusing was performed according to the manufacturer's

recommended protocol using the PROTEAN IEF Cell (Bio-Rad Laboratories). The focusing was carried out automatically with the following program: 250 V for 15 min, linearly increasing the voltage from 250 to 8000 V for 2.5 h, and a final focusing step at 10 000 V for 60 000 Vh. Immobilised focused IPG strips were briefly washed in equilibration buffer (6 M urea, 50 mMTris-HCl, pH 6.8, 20% glycerol, 2% SDS).

In the second dimension, proteins were separated by use of SDS-PAGE as previously described using 12% bis-acrylamide gel. The PROTEAN II XL cell (Bio-Rad Laboratories) was used to allow separation of proteins according to their molecular weights and the gels documented using a Molecular Imager® Gel Doc™ System (Bio-Rad Laboratories) using the software provided with the instrument. The gels were partitioned into grids and selected fractions were sent for protein sequencing by LC-MS/MS at the Central Analytical Facility (Stellenbosch, South Africa). Fractions from *C. lusitaniae* IWBT Y833 were labelled C1 - C11 and that of *M. pulcherrima* IWBT Y1123, M1 - M10.

4.3 Results and discussion

4.3.1 Southern blot hybridization

In order to investigate if the genome of *M. pulcherrima* IWBT Y1123 contains more than one gene sequence similar to that of the aspartic protease *MpAPr1*, southern blot hybridization was performed with designed probes as described in materials and methods. This same procedure was followed with specific probes for *CaAPr1* on genomic DNA isolated from *C. apicola* IWBT Y1384. The method was performed using non-stringent conditions in an attempt to identify similar sequences. The results obtained from Southern blot analysis performed using genomic DNA from *M. pulcherrima* IWBT Y1123 are illustrated in Figure 4.1 and that of *C. apicola* IWBT Y1384 in Figure 4.2. As expected, in both cases a band could be observed in the uncut gDNA (no restriction enzyme digestion) (lanes c).

From Figure 4.1, two bands can be observed and thus it was concluded that there are at least one sequences present within the genome of *M. pulcherrima* IWBT Y1123 that is similar to that of the *MpAPr1*. Results illustrated in Figure 4.2 also indicate the presence of at least one (or more) sequences in the genome of *C. apicola* IWBT Y1384 similar to that of *CaAPr1*. In both cases the results encourage the quest for finding other proteases within the genomes of these yeasts. Further analysis is thus required in order to identify and isolate these different proteases.

The genome of these yeasts has not been annotated and therefore it was decided to explore the secretome in order to observe whether the organisms secreted other proteases

thanthose that are known. Furthermore, it was decided to focus on *M. pulcherrima* IWBT Y1123, as discussed in Chapter 3.

4.3.2 One dimensional protein analysis of induced cultures

Proteins extracted from the extracellular medium of cultures induced with BSA (66.5 kDa) were visualized on a one dimensional SDS-PAGE gel. The addition of BSA has been shown to induce extracellular acid protease production in species of *C. albicans*, *C.tropicalis* and *C. parapsilosis* (de Viragh et al. 1993). Furthermore, it was decided to include cultures from *C. lusitaniae* IWBT Y833 to serve as a comparison as its proteome has been annotated (NCBI accession number: PRJNA41079) and also considering that *Clavispora* and *Metschnikowia* are taxonomically recognized as sister genera (Kurtzman et al. 2011). From the results differences between the samples could be observed (Figure 4.3). Samples obtained from *C. lusitaniae* IWBT Y833 only showed proteins above 25 kDa. However, samples from *M. pulcherrima* IWBT Y1123 showed proteins across the whole range of molecular weights, with thicker bands being visible in the lower range (<15 kDa). Furthermore, several bands from the latter sample appeared to be fainter than when compared to the former, indicating that proteins might have been degraded considering that the bands present at low molecular weights represent peptides. Interestingly, a band between 55 and 70 kDa could be observed in both samples indicating the presence of BSA, but in samples from *M. pulcherrima* IWBT Y1123 this band was much fainter. It was therefore hypothesized that the BSA added to the samples during incubation at 30°C had been hydrolysed by yeast proteases (active under the conditions tested) present within the secretome of *M. pulcherrima* IWBT Y1123. Results obtained by Reid et al. (2012) also demonstrated that a crude extract obtained from of *M. pulcherrima* IWBT Y1123 was able to degrade BSA at 30°C. Thus it was speculated that *C. lusitaniae* IWBT Y833 does not secrete an extracellular acid protease that is active enough to degrade BSA under the conditions tested.

Subsequently, entire lanes were excised and proteins identified by LC-MS/MS as described in the materials and methods section. The results revealed that only peptides from BSA could be identified in both samples (data not shown) and it was concluded that the presence of BSA at the concentration tested overshadowed all other peptides obtained. In order to better visualise the secretome and differences between samples, it was therefore decided to analyzethe protein samples using 2D SDS-PAGE.

4.3.3 Two-dimensional analysis coupled with LC-MS/MS

The results from the secretome of *C. lusitaniae* IWBT Y833 revealed proteins across the whole range of pI values (Figure 4.4). In contrast to 1D SDS-PAGE analysis, proteins could also be observed over the whole range of molecular weights. This might be because proteins have degraded during storage leading to the formation of smaller peptides or because staining was more efficient. A thick spot could be observed in the gel corresponding to a protein with a molecular weight between 55 and 70 kDa and a pI around 4 and 6. Since BSA has a molecular weight of 66.5 kDa and a pI of 4.6, it was hypothesized that this protein spot corresponded to BSA added to the samples during incubation. However, the spot was smeared and not precisely at the expected position on the gel, which could be explained by the high concentration of this protein preventing accurate focusing. In an attempt to identify the proteins present on the gel, certain sections were excised (C1 - C11) and sent for mass fingerprinting by LC-MS/MS (Figure 4.4).

The results obtained from 2D SDS-PAGE of the secretome of *M. pulcherrima* IWBT Y1123 are shown in Figure 4.5. Proteins across the whole range of molecular weights could be visualized but mostly in the vicinity of pI values 3 to 7. When comparing the differences between Figures 4.4 and 4.5, the spot hypothesized to be BSA was not present in the secretome of *M. pulcherrima* IWBT Y1123, but large spots at low molecule weights and pI values could be observed. It was concluded that this might correspond to peptides originating from degraded BSA. In an attempt to identify the proteins present in the gel, certain sections were excised (M1 - M10) and sent for mass fingerprinting by LC-MS/MS (Figure 4.5).

Unfortunately, BSA was once again the only protein identified in most fractions and overshadowed all other peptides (data not shown). Nevertheless, a small number of proteins were tentatively identified for 3 protein fractions, namely M1, M2 and M4. When peptides obtained in fraction M2 were BLASTed against *Clavispora lusitaniae*'s protein database, two proteins of potential interest could be tentatively identified as a glucosidase and an aminopeptidase, with scores of 99.1 and 52.0, respectively. The speculated presence of MpAPr1 (42 kDa and pI 4.2) in sample M2 could not be confirmed and no other extracellular protein of oenological interest could be retrieved from the other fractions. The low scores and poor matches with proteome online databases could be a result of the absence of specific database for *M. pulcherrima* and the overwhelming presence of BSA. The identification of BSA in all fractions was not expected since proteins were well separated on the 2D-SDS-PAGE and could be attributed to the high concentration of BSA in the samples.

This experiment thus requires further optimization and should be repeated with a lower concentration of BSA. Alternatively, other inducing proteins could be tested as well as the absence thereof. Furthermore, since the genomes of these specific organisms are not known degenerate primers (such as those designed by Reid et al. 2012) could be used to amplify novel protease encoding genes.

4.4 Conclusion

Over the years, an abundant knowledge has been generated on non-*Saccharomyces* yeast and their contribution towards various aspects of wine quality. Some of the major impacts are due to the activity of extracellular enzymes produced by the yeasts. Protease enzymes that are produced and secreted by yeasts, either during or after fermentation are especially important with regards to protein haze prevention, foam stability in sparkling wines and the release of aroma compounds from grape precursors. Acid proteases, mainly from fungal sources, have been shown to effectively reduce protein haze formation in white wines without being detrimental to the quality. Non-*Saccharomyces* yeast, present on the surface of the grape berries therefore represents a very good source for the identification and isolation of such proteases. Furthermore, some non-*Saccharomyces* species have also been shown to produce more than one acid protease, some of which are inducible using BSA.

In this study, results suggested that the genomes of *M. pulcherrima* IWBT Y1123 and *C. apicola* IWBT Y1384 contain at least one additional sequence similar to that of *MpAPr1* and *CaAPr1*, respectively. This result also encourages the retrieval and identification of these unknown sequences from these genomes. Following protein analysis after 1D SDS-PAGE it was concluded that the high concentration of BSA added during induction, interfered with protein identification leading to inconclusive results. Future work will thus have to focus on optimizing protein extraction and analysis procedures, however sequencing the genomes of these organisms might be a faster approach. Moreover screening and identifying different acid proteases within non-*Saccharomyces* strains and species of wine origin remain an invaluable source for obtaining enzymes that might be of interest to the wine industry and/or other related industries.

4.5 Acknowledgements

4.6 References

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Table 4.1: List of primers used during this study

Primers	Sequence 5'-3'
MpAPr1-F(*Xho*I)	ACGT<u>CTCGAG</u>ATGGCCATCCCTGGGC
MpAPr1-R(*Bam*HI)	ACGT<u>GGATCC</u>TTAAGCACTTATGATGTTTGACGAGC
CaAPr1-F(*Xho*I)	ACGT<u>CTCGAG</u>GTACTAGCTAAGAACTATGTTCATTTAGG
CaAPr1-R(*Bam*HI)	ACGT<u>GGATCC</u>TTAGTTGACAGATCCGGGA
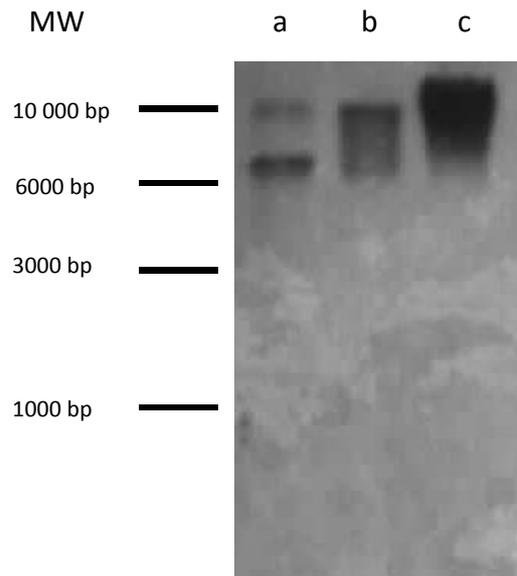
Figure 4.1 Southern blot analysis of genomic DNA isolated from *M pulcherrima*Y1123. Lanes: (a) *Hin*dIII, (b) *Kpn*I and (c) control (untreated gDNA)
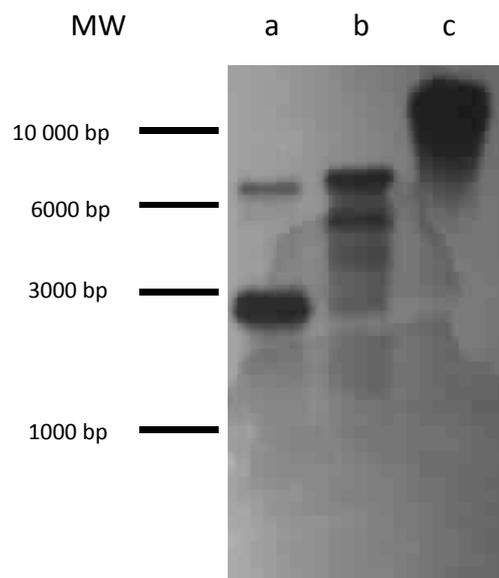
Figure 4.2 Southern blot analysis of genomic DNA isolated from *C. apicola*Y1384. Lanes: (a) *Hin*dIII, (b) *Kpn*I and (c) control (untreated gDNA)
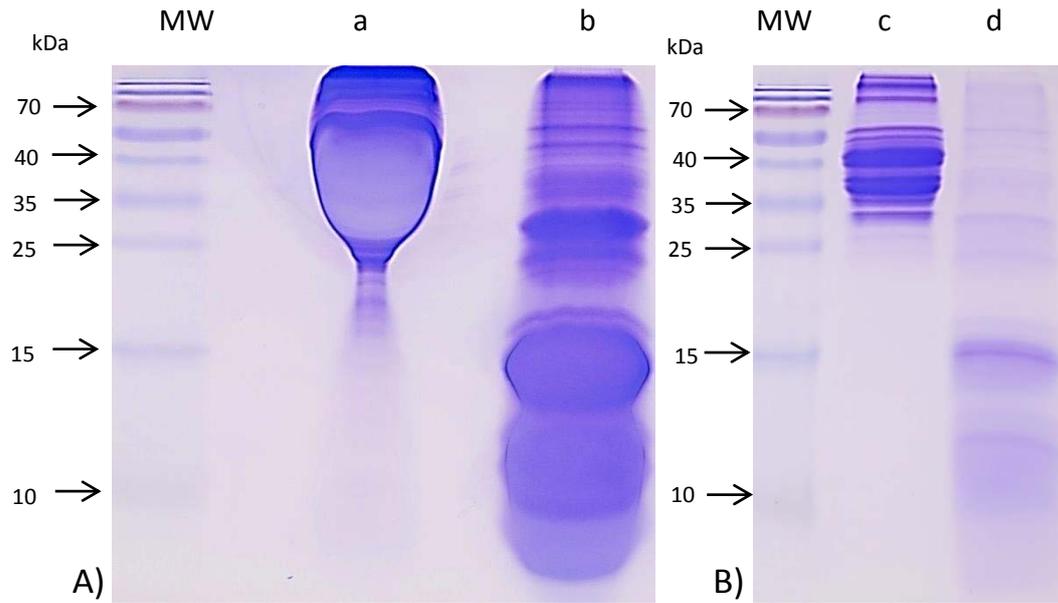
Figure 4.3 SDS-PAGE visualization of proteins extracted from *C. lusitaniae* IWBT Y833 (lanes a and c) and *M. pulcherrima* IWBT Y1123 (lanes b and d). A) proteins loaded at a concentration of 1000 µg/ml and B) 100 µg/ml. MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
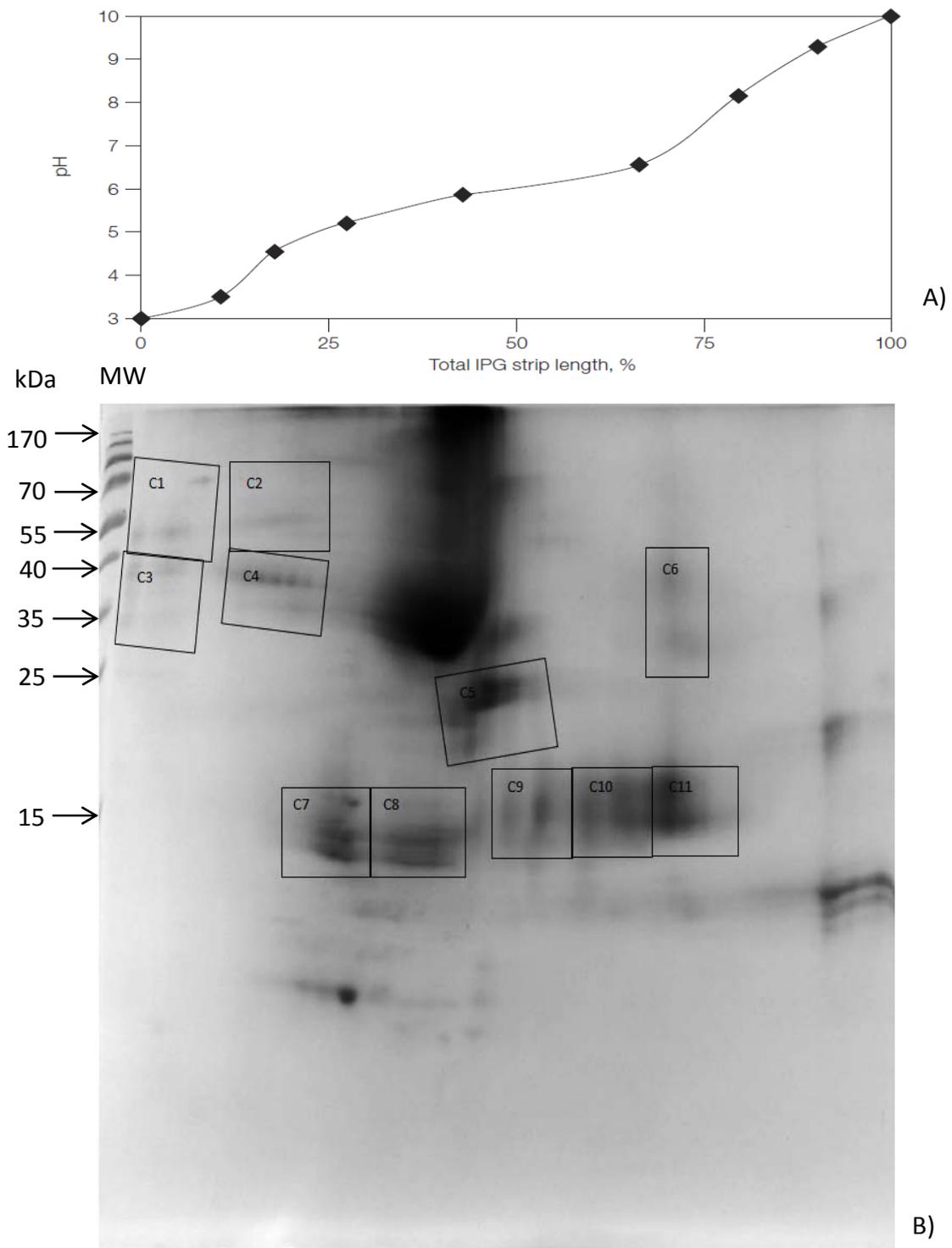
Figure 4.4 2D SDS-PAGE image of proteins extracted from the secretome of *C. lusitaniae* IWBT Y833. A) Graph showing pH vs. length relationship (Bio-Rad Laboratories). B) *C. lusitaniae* IWBT Y833 secretome (C1 to C11 illustrates the grids that was excised and sent for protein sequencing). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).
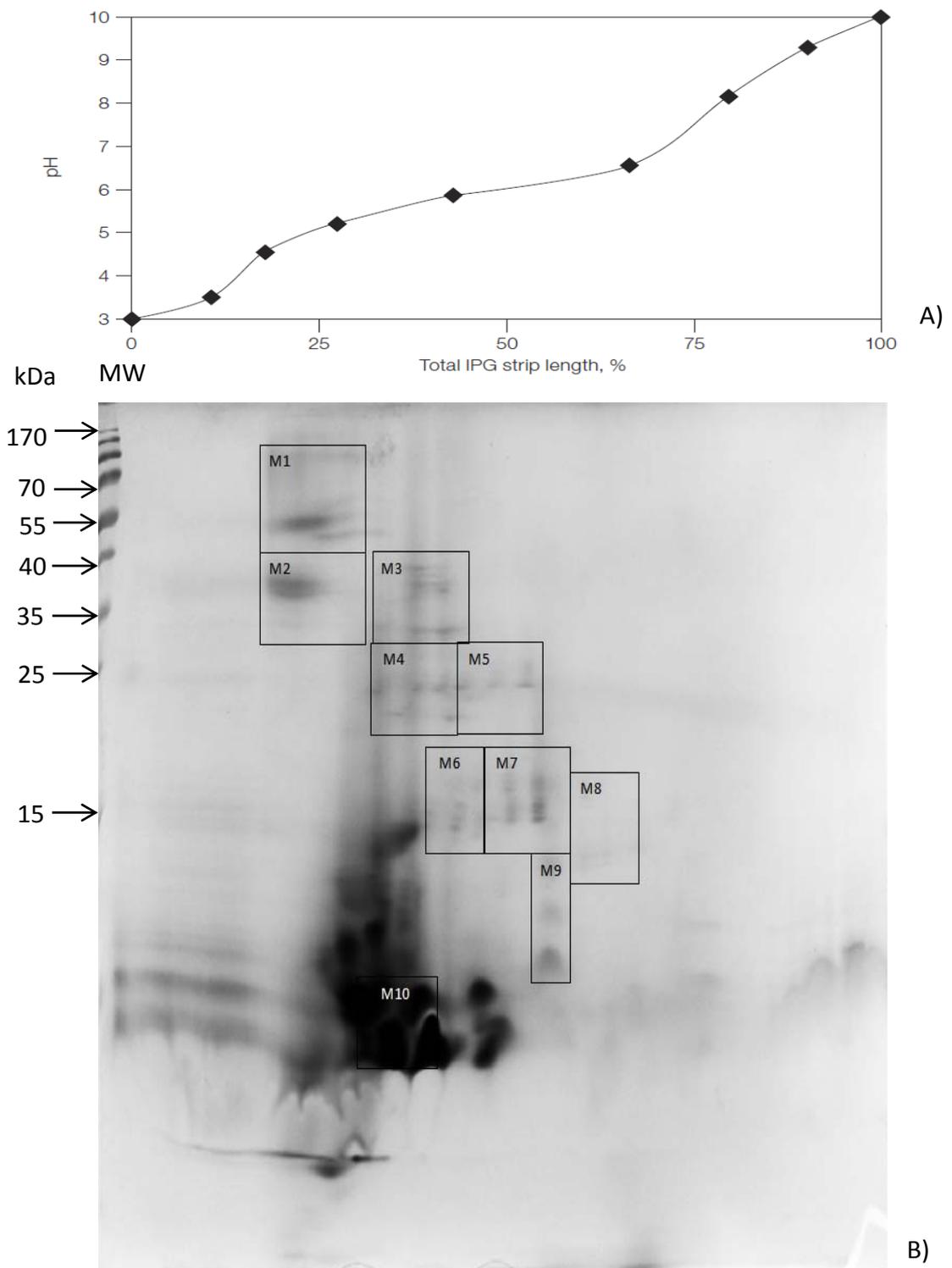
Figure 4.5 2D SDS-PAGE image of proteins extracted from the secretome of *M. pulcherrima* IWBT Y1123. A) Graph showing pH vs. length relationship (Bio-Rad Laboratories). B) *M. pulcherrima* IWBT Y1123 secretome (M1 to M10 illustrates the grids that was excised and sent for protein sequencing). MW is the molecular weight standard, PageRuler™ Prestained Protein Ladder (Thermo Scientific).

Chapter 5

General discussion and conclusions

Chapter 5 - General discussion and conclusions

5.1 General discussion and conclusions

Grape derived proteins (mostly pathogenesis-related proteins) represent the majority of proteins in wine (Marangon et al. 2009). Under poor storage conditions, mainly related to temperature variations, these proteins can become unstable and aggregate leading to the formation of haze. This visually unappealing phenomenon is often incorrectly interpreted as spoilage by the consumer and therefore unacceptable. Protein haze is a specific issue in white wines in which the absence of tannins prevent the precipitation of these proteins during the winemaking process. Bentonite treatment is currently the most effective tool for removing these haze forming proteins, but despite its widespread use it has several negative implications. The most concerning is the removal of important aroma and flavour compounds along with other proteins.

Several non-*Saccharomyces* yeasts present in grape juice and during alcoholic fermentation have been shown to secrete extracellular enzymes in variable amounts depending on genera, species and strains. Acid proteases are of particular interest because of their activity at low pH similar to that of wine. Recent studies have indeed generated encouraging results using acid proteases of fungal origin (Marangon et al. 2012; Van Sluyter et al. 2013) suitable for the hydrolysis of haze forming proteins. Although the combination of heat treatment has sometimes been described as a requirement to ensure initial denaturation of the targeted proteins, these enzymes are able to degrade haze forming proteins without the negative effects following bentonite treatment. Furthermore, amino acids and peptides liberated from the action of proteases could also potentially be used as sources of nitrogen by fermenting organisms and some of these amino acids can be metabolised by the yeast to produce aromatic compounds such as higher alcohols and esters (Swiegers and Pretorius 2005).

Acid proteases form part of the aspartic endoprotease family and are defined by the presence of two aspartic residues in their catalytic site (Coates et al. 2001; Dunn 2002). In some instances, it has also been shown that some non-*Saccharomyces* yeasts are able to produce and secrete more than one acid protease, depending on the environmental conditions (Aoki et al. 2012; Naglik et al. 2003; Zaugg et al. 2001). Recently, in a study performed at the Institute for Wine Biotechnology (IWBT), two aspartic protease-encoding genes have been identified in non-*Saccharomyces* yeasts isolated from grape juice collected in South Africa (Reid et al. 2012). The genes, *MpAPr1* and *CaAPr1,* were retrieved from

Metschnikowia pulcherrima IWBT Y1123 and *Candida apicola* IWBT Y1384, respectively. Furthermore, MpAPr1 was shown to be secreted in the presence of BSA, casein and grape proteins and to partially degrade these proteins in a medium buffered at pH 3.5.

The main aim of this study was to optimize the expression of these two aspartic protease-encoding genes (*MpAPr1* and *CaAPr1*) in *Escherichia coli* and thereafter to purify the recombinant enzymes through the use of metal ion affinity chromatography (IMAC). The optimization of these techniques is regarded as a first step toward characterizing their kinetic properties and determining their suitability for use during winemaking. In parallel, the ability of *M. pulcherrima* IWBT Y1123 to produce and secrete extracellular acid proteases other than MpAPr1 was investigated as an additional aim arisen out of curiosity during the course of this study.

The genes *MpAPr1* and *CaAPr1* were cloned into the expression vector pET14b in order to induce protein expression (under the *lac* promoter) and fuse a 6X His tag to the recombinant proteins, facilitating downstream purification experiments. Subsequently, the new vectors (named pET-MpAPr1 and pET-CaAPr1, respectively) were transformedinto *E. coli* BL21 (Rosetta-gami pLysS) DE3 for over-expression using theinducing agent (IPTG). After extensive optimization, the following optimal expression conditions were established: induction of cultures with 0.4 mM IPTG at mid exponential phase followed by incubation for 48 h at 14°C. Successful extraction of recombinant proteins was only obtained under denaturing conditions. It was concluded that expression resulted in the recombinant acid proteases being encapsulated into inclusion bodies thereby preventing their extraction under native conditions. Furthermore, recombinant expression of CaAPr1 could not be observed for reasons yet to be elucidated that may include the large sequence dissimilarities between CaAPr1 and MpAPr1. Consequently, only MpAPr1 could be purified under denaturing conditions resulting in the protein being in an inactive state. Mass fingerprinting of the purified protein confirmed its identity with a score of 473.12 and 9 unique peptides matching the sequence of MpAPr1. Preliminary refolding experiments using dilution based methods yielded poor recovery of active enzymes. However, a slightly higher level of activity could be observed from proteins recovered using the pulsatile dilution method. Finally, protein extraction under semi-native conditions was attempted and provided encouraging preliminary results. Low amounts of recombinant proteins were indeed retrieved, but downstream purification still requires further optimization.

Additionally, Southern blot analysis of the two non-*Saccharomyces* yeasts, *M. pulcherrima* IWBT Y1123 and *C. apicola* IWBT Y1384, suggested that both potentially possess at least

one additional protease other than those isolated by Reid et al. (2012). Further analysis of the extracellular proteome of *M. pulcherrima* IWBT Y1123 also confirmed the presence of at least one enzyme able to actively hydrolyze BSA at a low pH. Unfortunately, mass fingerprinting performed on the entire extracellular proteome and on small groups of proteins thereof did not allow the identification of these enzymes.

5.2 Potential future prospects

Although in this study recombinant aspartic protease MpAPr1 could be extracted under denaturing conditions, refolding of the enzyme to its active state was unsuccessful and requires further attention. Increasing the yield of recombinant enzyme after extraction remains a priority. Furthermore, extraction under semi-native conditions could be further attempted in order to avoid an unreliable refolding procedure. Regardless of the extraction conditions, it also remains to be verified that the protein refolds properly despite the absence of glycosylation and that the presence of the 6X His tag does not interfere with activity. Considering that our results confirmed that an extracellular protease of *M. pulcherrima* IWBT Y1123 is actively secreted and is able to hydrolyse BSA at a pH similar to that found in wine, the potential of MpAPr1 to prevent protein haze formation in wine requires further investigation. This could be achieved through the direct addition of the purified enzyme at different stages of the winemaking process. Finally, the impact that such a protease has on the technological and organoleptic properties of wine should be investigated holistically. This could also be evaluated when co-inoculating an acid protease producing non-*Saccharomyces* strain (of oenological origin) with *S. cerevisiae* to conduct alcoholic fermentation. Furthermore, the encouraging results obtained from Southern blot analysis revealed more than one possible gene similar to that of MpAPr1 and CaAPr1 in the genomes of *M. pulcherrima* IWBT Y1123 and *C. apicola* IWBT Y1384, respectively. Future investigation is thus required in order to isolate these genes and assess their expression and impact during winemaking. Sequencing the genomes of these organisms and mining thereof might be the most efficient route to isolating these genes.

5.3 References

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