Optimisation of enzymatic hydrolysis of monkfish heads for preparing protein hydrolysates as animal feed ingredient

by

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Thesis presented in partial fulfilment
of the requirements for the Degree

of

MASTER OF ENGINEERING
(CHEMICAL ENGINEERING)

in the Faculty of Engineering
at Stellenbosch University

Supervisor
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March 2017

Declaration

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ABSTRACT

The monkfish *Lophius vomerinus* is found between the coasts of Namibia and KwaZulu-Natal in Southern Africa, and the fillet is used for human consumption. The head is the largest byproduct from the monkfish catch and is currently discarded. Due to increasing demand on animal derived protein, the animal feed industry requires additional sources of protein-rich raw materials to meet the industry needs.

In this study, the potential of the head of the monkfish *Lophius vomerinus* as protein-rich raw material was evaluated. Characterisation of the raw material was conducted, and included a proximate analysis, fatty acid profile, amino acid profile and mineral content analyses. Further aims in this study were to (i) optimise the enzymatic hydrolysis of monkfish head by varying reaction temperature and pH, and using two proteolytic enzymes: alcalase and bromelain, (ii) determine the pK value of the alcalase/monkfish and bromelain/monkfish systems, to use in the equation for the degree of hydrolysis (DH), as described in the pH-stat technique, and (iii) to evaluate the hydrolysate products for functional food application and as animal feed ingredient.

The characterisation data showed that an average amount of 8.19 % (wet basis) protein was found per raw monkfish head, and 43.77 % of the protein was found to be made of essential amino acids. More than 31% of the fat in the monkfish head contained valuable long chain polyunsaturated fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The mineral content showed a large quantity of calcium, and quantities of the toxic elements, Hg, Cd and Pb, present, were well below the maximum allowable values for food applications.

Optimal enzymatic hydrolysis conditions were found for each enzyme/substrate combination used in this investigation. The values of the optimal reaction temperature and pH were significantly influenced by the enzyme used for hydrolysis. The pH range investigated in this study for the bromelain/monkfish system was limited to neutral and alkaline reaction conditions by the pH-stat method used to determine the DH. The value of pK was successfully determined for alcalase/monkfish system. However, the results from the pK investigation for the bromelain/monkfish system showed possible catalyst inhibition of the enzyme, or pH conditions non-conducive to the enzyme/substrate combination used in this study. The pK of the bromelain/monkfish system could not be determined and the literature value for general fish species protein was used subsequently. The functional properties of the sediment and fish protein hydrolysate (FPH) liquid were both tested in this study, as the focus was on valorising the byproducts and the sediment formed a

large proportion of the hydrolysate product. The sediment and FPH showed good antioxidant activity, emulsion stability and fat absorption capacity. The values of the functional properties of the sediment were lower than those observed for FPH, and the enzyme used affected the functional food properties significantly.

The study concludes that the enzymatic hydrolysis of monkfish heads can provide a protein rich FPH and hydrolysis sediment product, suitable for use as animal feed ingredient. The results can contribute to improved resource utilisation in the fisheries and animal feed industry.

OPSOMMING

Die visspesie *Lophius vomerinus* kom voor tussen die kus van Namibië tot en met KwaZulu-Natal in Suid Afrika en die vleis in die stert van die vis word gebruik vir menslike voedseltoepassings. Die kop van die vis maak die grootste deel van die afvalprodukte uit, maar word tans weggegooi. Daar is 'n toenemende aanvraag vir vee- en pluimvee gebasseerde proteïen, en as gevolg daarvan het die dierevoerindustrie nuwe bronne van proteïene nodig om die aanvraag te kan voorsien.

In hierdie studie word die potensiaal van die *Lophius vomerinus* se kop, om te dien as 'n proteïenryke visverwerkingsafvalproduk wat verwerk kan word vir dierevoer, ondersoek. Die viskop word ook gekarakteriseer in terme van die algemene samestelling, vetsuurprofiel, aminosuurprofiel en die mineraalinhoud. Addisionele doelwitte van hierdie studie was (i) die optimering van die ensiem hidroliese reaksie van die roumateriaal deur die reaksietemperatuur en pH te varieer, en twee proteolitiese ensieme naamlik alcalase en bromelain te gebruik, (ii) om die pK waarde van die alcalase/viskop en bromelain/viskop kombinasies te bepaal, sodat dit gebruik kan word in die vergelyking om die mate tot waartoe die viskop gehidroliseer is, soos uiteengesit in die pH-stat metode, te bereken, en (iii) om die hidrolisaatproduk te evalueer vir potensiële toepassings as funksionele kosbestanddeel en dierevoerbestanddeel.

Daar is bevind dat die rou viskop 'n goeie bron van addisionele proteïene en noodsaaklike vetsure is en sal dien as goeie dierevoerbestanddeel. Resultate het ook daarop gedui dat daar heelwat proteïene verryk met noodsaaklike aminosure in die viskop is. 'n Groot porsie van die vet in die viskop het bestaan uit die waardevolle onversadigde vetsure, DHA en EPA. Hoë vlakke van kalsium is in die viskop gevind, en die toksiese elemente wat opgemerk is, se vlakke was alles onder die maksimum toelaatbare waardes vir voedseltoepassings.

Daar is vir elke ensiem/substraat kombinasie wat geëvalueer is in hierdie studie 'n stel optimale ensiem hidroliese parameters gevind. Die ensiem wat gebruik is tydens hidroliese het 'n beduidende invloed op die optimale reaksietemperatuur en pH gehad. Die waarde van die optimale pH vir hidroliese met bromelain was beperk tot 'n alkaliese of neutrale pH, omdat die pH-stat metode slegs toepaslik is onder hierdie spesifieke pH toestande. Die pK waarde vir die alcalase/viskop kombinasie kon suksesvol bepaal word, maar dit was nie die geval vir die bromelain/viskop sisteem nie, moontlik weens die inhibisie van die ensiem

as katalis, of die reaksie-pH wat nie toepaslik was vir die spesifieke ensiem/viskop kombinasie nie. In hierdie studie is die funksionele eienskappe van beide die vis proteïen hidrolisaat (VPH) vloeistof en sediment getoets omdat die studie gebasseer is op die opgradering van visafvalprodukte, en die sediment 'n groot porsie van die hidrolisaatproduk opgemaak het. Goeie waardes vir antioksidatiewe aktiwiteit is waargeneem vir beide die sediment en VPH, sowel as goeie emulsiestabiliteit, en vetabsorpsie waardes. Die waardes van die funksionele eienskappe van die sediment was laer as die van die VPH vloeistof, en in beide gevalle het die ensiem wat gebruik is tydens hidroliese 'n beduidende invloed op die funksionele eienskappe gehad.

Hierdie studie het tot die gevolgtrekking gelei dat ensiem hidroliese van die *Lophius vomerinus* viskop 'n hidrolisaatproduk wat ryk in proteïene is, kan lewer, en dat die produk geskik is om te gebruik as dierevoerbestanddeel. Die resultate wat uit die studie gelewer is, kan 'n bydra lewer tot die optimale verbruiking van hulpbronne in die visverwerkingsindustrie sowel as die dierevoerindustrie.

ACKNOWLEDGEMENTS

I would like to express my appreciation and gratitude to the following people who have contributed to the completion of my work:

- I would like to thank my Lord Jesus Christ for it is only by His grace that I have come so far.
- My supervisor, Dr Neill Goosen for your guidance, patience and support. I truly appreciate your time and understanding of me as an individual, it has made all the difference to me and my successful work completion.
- To my family; my husband, Chris, for your encouraging pep talks when I was down, and making me smile even when I didn't want to, you are my rock and my light. To my little angel, Leja, for giving me a reason, or sometimes being the reason I get up very early in the morning! To my mother, Louise, you have helped me become the strong and independent person I am today, and I am forever grateful for your love and support.
- To the friends I have made, Jasmin and Logan, thank you for listening to all my complaints and helping by making me laugh.
- The technical and laboratory personnel within Process Engineering, Mr. Alvin Petersen and Mr. Jos Weerdenburg.
- The Protein Research Foundation, Stellenbosch University, the NRF and CSIR for provision of project and personal funding.
- Irvin & Johnson for the provision of the raw substrate.
- The personnel from the Department of Aquaculture at Welgevallen for their facilities.
- I would like to give special acknowledgement to Stellenbosch University and the Department of Process Engineering, for doing their best to help us students graduate, during tumultuous academic times in our country.

DEDICATION

I would like to dedicate this work to my father, Brian, and both my grandmothers, Ansie and Louise. You are forever in my heart and prayers. I have learnt so much from you, and the wisdom, encouragement and love you have brought into my life has made me the person I am proud to be today.

NOMENCLATURE & ABBREVIATIONS

Symbol	Description	Units
A_0	Absorbance of control	
A_{500}	Absorbance at 500 nm	
A_f	Absorbance of final product	
AU	Anson unit	
B	Base added	ml
b	Equivalent base consumption	M
b_s	Base consumed by raw substrate	mol
b_x	Base consumed by substrate hydrolysed to x %	mol
c	Concentration of FPH in aqueous solution	g/m^3
C_B	Concentration of base	M
DH	Degree of hydrolysis	%
DHA	Docosahexaenoic acid	
DMPD	*N*,*N*-dimethyl-*p*-phenylenediamine	
E/S	Enzyme to substrate ratio	
EAA	Essential amino acids	
EAI	Emulsification activity index	m^2/g
EPA	Eicosapentaenoic acid	
ES	Emulsion stability	%
FA	Fat absorption activity	g fat/g FPH
FC	Foaming capacity	ml foam/g protein
FPH	Fish protein hydrolysate	
GDU	Gelatin digestion units	
h_{tot}	Equivalent millimoles of peptide bonds per gram protein	meq/g
L	Path length	m
M_P	Mass of protein in substrate	g
MUFA	Monounsaturated fatty acid	

N_B	Normality of base	
NEAA	Non-essential amino acid	
OPA	Ortho-phtalaldehyde	
pI	Ionic product of water at 50 °C	
pK	Logarithmic value of the equilibrium constant for the deprotonisation of an amide group	
PUFA	Polyunsaturated fatty acid	
SD	Standard deviation	
SE	Standard error of the mean	
SFA	Saturated fatty acid	
TAA	Total amino acids	
TFA	Total fatty acids	
TNBS	Trinitrobenzenesulfonic acid	
TU	Tyrosine unit	
V_0	Initial volume	l
V_B	Volume of base	l
α	Average degree of dissociation of α-amino groups	
Φ	Volume fraction of oil phase in emulsion	

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Chapter 1: Introduction

Investigations into the optimal use of fish processing byproducts have become a topic of interest in the past few years. The global amount of fish byproducts produced in 2014 was 21 million tonnes (FAO, 2016e) which is a substantial fraction of the total catch (Gildberg, 2002). These large amounts of byproducts contain protein rich material as well as essential fatty acids, minerals, collagen and gelatin (Ghaly, Brooks, Ramakrishnan, Budge, & Dave, 2013; Halim, Yusof, & Sarbon, 2016). Byproducts are currently either discarded or used as relatively low value fish silage or fishmeal.

Studies in recent years have shown that the fish byproducts can be processed into fish protein hydrolysates (FPH) which have considerable potential as value added ingredients in food products for both animals and humans (Halim et al., 2016). The demand on livestock derived protein is continuously increasing and it is estimated that by 2050 the projected demand for livestock meat will be 463.8 million tonnes (FAO, 2011). The increased demand on animal protein means will lead to an increased demand on animal feed, and the protein component in the feed, which could be supplemented by FPH. Fish protein hydrolysates can also find application as supplement in food products due to the favourable functional food properties such as emulsion properties, water holding capacity, foaming ability, oil binding capacity and antioxidative ability these FPH have been found to have (Ghaly et al., 2013).

Enzymatic hydrolysis is the most commonly used method to produce FPH from fish byproducts(Ghaly et al., 2013; Halim et al., 2016; Kristinsson & Rasco, 2000c). Enzymatic hydrolysis takes place at mild reaction conditions to allow the conservation of nutritional and functional quality of the substrate (Foegeding, Davis, Doucet, & McGuffey, 2002). The important reaction factors for enzymatic hydrolysis include time, temperature, pH, the degree of hydrolysis (DH) and enzyme to substrate ratio (Bhaskar, Benila, Radha, & Lalitha, 2008). The DH is defined as the number of peptide bonds cleaved in the substrate protein during hydrolysis (Rutherfurd, 2010). Proteolytic enzymes such as alcalase, papain, pepsin, trypsin, flavourzyme, neutrase and bromelain are commonly used to catalyse the enzymatic hydrolysis reaction (Halim et al., 2016) and according to Kristinsson and Rasco (2000c), enzymes from micro-organisms and plants are most suitable for fish protein hydrolysis due to FPH yields higher than 50 % and the lower cost of these enzymes. According to Adler-Nissen (1979), it is important to control the DH and limit the value to below 20 % to ensure the nutritional quality of the FPH. A lower DH value corresponds to an FPH with

minimal free amino acids, and good functional properties including emulsifying properties, water holding capacity, fat absorption and foaming ability (Adler-Nissen, 1979).

The pH-stat method is a technique which is commonly used to monitor the degree of hydrolysis of protein substrates in real time (Kristinsson & Rasco, 2000c). This method is advantageous for monitoring the DH when the products of hydrolysis are intended for use in feed ingredients as it is non-denaturing and requires no derivatisation (Spellman, McEvoy, O'Cuinn, & FitzGerald, 2013). The pH-stat method is used under alkaline pH conditions, where base consumption data are used to monitor the DH in real time (Rutherfurd, 2010). The dissociation of free amino-groups as a result of hydrolysis reduces the pH of the mixture and a base is added to the mixture continuously to maintain a constant pH, and the base consumption is correlated to the degree of hydrolysis (Adler-Nissen, 1986, p. 91). In order to use the correlation of base consumption to DH, it is necessary to know the value of the mean degree of dissociation of the liberated α-amino groups (Navarrete del Toro & Garcia-Carreño, 2002). The value of the mean degree of dissociation of the α-amino groups released during hydrolysis is dependent on the pK value of the specific enzyme/substrate system and can be determined by the method described by Camacho, González-Tello, Páez-Dueñas, Guadix, and Guadix (2001).

The monkfish (*Lophius vomerinus*), a white fillet fish with a low oil content, is caught in South African and Namibian waters (Maartens & Booth, 2005). The tail of the monkfish is used for human consumption while the head is currently discarded. The head is an important byproduct from the monkfish catch as it accounts for between 27.2 - 37.9 % of the total length of the fish (Caruso, 1983). The specific species of *Lophius vomerinus* is found only in South African and Namibian waters (Caruso, 1983; FAO, 2016a) and the total catch of this species in 2014 amounted to 9 489 tonnes according to the fishstat of the FAO (2016a). To evaluate the potential value of the monkfish head as a protein source, the nutrient profile should be known. Important parameters for a complete nutrient description for use in an animal feed application include; protein content, amino acid profile of the protein, fatty acid content and the mineral content. Limited literature is available on monkfish head characterisation and processing. Therefore it would be imperative in this study to characterise the monkfish head as a first step into the investigation of the monkfish head valorisation.

In this study, the aim was to optimise the enzymatic hydrolysis of the monkfish head by varying reaction pH and temperature, and using two different proteolytic enzymes namely bromelain and alcalase. The hydrolysis progression was monitored by the pH-stat method. In order to use the pH-stat method, the pK values for the two different enzyme/substrate systems were determined. Prior to enzymatic hydrolysis, the

monkfish head was characterised in terms of protein, fat and moisture content, as well as mineral and fatty acid composition and amino acid profiling of the protein. The functional food properties of the resulting FPH and hydrolysate sediment products were evaluated.

This document reports the findings of the investigation, and follows the following structure:

In Chapter 2, a detailed literature study is conducted covering the process of enzymatic hydrolysis, variable reaction parameters, methods used to determine the degree of hydrolysis as well as how to determine the pK value. Additionally, available data of the monkfish (*Lophius vomerinus*) is reviewed and the potential valuable components contained therein are identified. Finally, the nutritional potential of the hydrolysate is investigated by determining the value of specific functional food properties namely fat absorption, foaming capacity, emulsion stability, emulsification activity index, and antioxidant activity. . The aims and objectives of this study are highlighted in Chapter 3. The research results of this study are presented in the form of two separate articles in Chapter 4 and Chapter 5. The first article details the characterisation of the raw monkfish head, while the second article reports the findings of the optimisation of enzymatic hydrolysis and functional properties of the monkfish head. The conclusion drawn from the research and recommendations are presented in Chapter 6.

Chapter 2: Literature survey

2.1. Introduction

The valorisation and upgrading of fish processing byproducts and waste has gained increased attention in numerous studies recently (Aspmo, Horn, & Eijsink, 2005; Bhaskar et al., 2008; Ghaly et al., 2013; Halim et al., 2016; Kristinsson & Rasco, 2000c)). For the fisheries industry the utilisation of byproducts is important in terms of economic viability and according to Gildberg (2002) more important than most other sectors of industry, because fishery byproducts normally make up a substantial portion of the total catch. Gildberg (2002) also states that, if properly processed in terms of extraction and utilisation, the byproducts could be of even greater economic value than the main product. The amount of world fish byproducts generated in 2011 amounted to 23.2 % of the total fish supply (Ghaly et al., 2013).

The byproducts generated by the fishery industry can prove to be a valuable source of additional protein for the animal feed industry and this is the premise of this study. Byproducts from fisheries are an untapped source for valuable products such as proteins, omega-3 rich oils, minerals and collagen (Ghaly et al., 2013). Proteins are one of the main components targeted for recovery from fish processing byproducts, as it could be used as functional ingredients in human food as well as in animal feed, and have desirable properties including, water holding capacity, oil absorption, foaming properties and emulsion properties (Ghaly et al., 2013). Essential amino acids found in proteins are important in the diets of humans and animals, and the protein found in fish byproducts is an excellent source of essential amino acids (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012). Motivated by an increase in global population and emerging economies, the demand on animal protein for human consumption is steadily on the rise as shown in Figure 2. 1. With a higher demand for animal protein, which is estimated to be more than 463.8 million tonnes in 2050 (FAO, 2011), comes a higher demand for animal feed. Additional sources of protein to supplement the animal feed industry are therefore very important.

Proteins can be extracted from different parts of fish byproducts, such as the muscle, skin, meat, head and viscera (Chalamaiah et al., 2012). For application as a feed ingredient, the proteins need to be extracted under conditions that do not have negative effects on nutritional value or functional properties. Traditional chemical hydrolysis methods using acids or bases destroy some of the desirable properties of proteins

(Kristinsson & Rasco, 2000c). To this extent most protein extractions from fish byproducts are done using enzymatic hydrolysis (Chalamaiah et al., 2012; Ghaly et al., 2013).

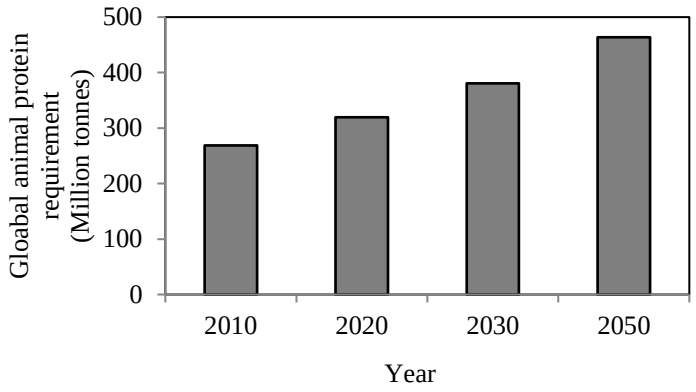

Figure 2. 1: World requirement (2010) and estimated world requirement (>2020) of animal protein (adapted from FAO (2011))

When fish byproducts are subjected to enzymatic hydrolysis a fish protein hydrolysate (FPH) is formed. Fish protein hydrolysates can be produced from many different sections of a fish such as the skin, head, muscle, viscera, liver, bones, frame and eggs (Chalamaiah et al., 2012). The FPH quality will be dependent on the starting substrate for the FPH, the fish species, age, season of catch, health and sex of fish, enzyme used, hydrolysis conditions; many of these factors are not controllable (Chalamaiah et al., 2012; Kristinsson & Rasco, 2000c).

The components of fish byproducts are distributed non-homogeneously throughout the fish and therefore a thorough chemical and physical characterisation of the starting substrate is important, and should be conducted prior to any hydrolysis procedure (Prego, Pazos, Medina, & Aubourg, 2012). The most typical and important qualities of the fish to be characterised are proximate composition to determine protein, fat, water and ash content. The protein can be further analysed to determine the amino acid profile which is important in terms of food ingredient requirements and nutritional value in animal feed ingredients. Byproducts from marine species are well known sources of essential fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) (Hathwar, Bijnu, Rai, & Narayan, 2011). The lipids should be analysed to determine fatty acid profiles so that the mono- and polyunsaturated fatty acids, as well as the ratio of omega-3 to omega-6 fatty acids can be evaluated. Fish byproducts are a good source of minerals that are required for the growth and development of animals (Prego et al., 2012).

Subsequent to substrate characterization as described in the previous paragraph, the planned hydrolysis reaction should be established and characterised in terms of reaction parameters and methods to determine the extent of the reaction. Typical considerations which need to be considered include the enzymes used, reaction conditions and the quality of the hydrolysate produced. Further, the method of determining the extent of reaction (referred to as degree of hydrolysis (DH)), is important to investigate, even though there is not yet a standard protocol set out for DH determination (Rutherfurd, 2010). The functional food properties of the hydrolysate product is an important aspect of the hydrolysate product as it will contribute to the final market value (Klompong, Benjakul, Kantachote, & Shahidi, 2007).

The literature survey will discuss in detail the aspects of the monkfish such as biological characterisation and available data about monkfish processing. The enzymatic hydrolysis process, the reaction parameters and enzymes used will be examined, and the methods used to determine the extent of the hydrolysis reaction will be studied. The pK value determination for use in the DH determination will be discussed. Finally, the quality and potential value of the hydrolysate products in terms of functional properties will be evaluated.

2.2. Monkfish byproducts

In this study, the monkfish head was used as raw substrate. When subsequently referred to in the rest of the document, monkfish head will include the following components:

- skin
- frame
- eyes
- teeth
- flesh (meat and fat)
- gills
- fins
- anterior tentacles

2.2.1. Monkfish

The monkfish is a saltwater fish from the genus *Lophius*. There are seven species of *Lophius* ,the most common of which are the *L. piscatorius* and *L. americanus*. Six of the seven species are found in the

Atlantic Ocean and one is found in the Pacific Ocean. According to Maartens and Booth (2005) the species *Lophius vomerinus* is found from the waters of northern Namibia to Durban on the east coast of South Africa.

Specific *Lophius* characteristics are a wide, gaping mouth, thin, slimy light to dark brown skin on the top and white on the belly, without scales and being dorso-ventrally compressed (Farina et al., 2008), meaning that the passage from the back to the stomach of the fish is flat and appears compressed. The head is very large in proportion to the whole fish, accounting for from 27.7 to 37.9 % of the total length of the fish (Caruso, 1983). The eyes of the *Lophius* are close together on the top of the flat head and the mouth is slightly tilted toward the top and filled with a lot of sharp, long teeth. The *Lophius* has anterior tentacles on the upper jaw which acts as a lure to catch other fish (Gudger, 1945). Figure 2. 2 is an illustration of a *Lophius* genus which shows the relative size of the head of the fish to the rest of the body.

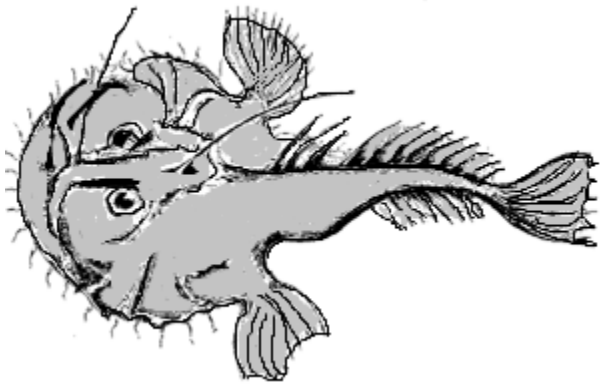

Figure 2. 2: The *Lophius* genus (redrawn from Gudger (1945))

The size of the monkfish found around the South African coastline, *Lophius vomerinus* was investigated by Maartens and Booth (2005) and the results were obtained as illustrated in Figure 2. 3. From the figure it can be seen that in both male and female frequency the average fish caught was between 17 and 32 cm in length and more males were caught than females.

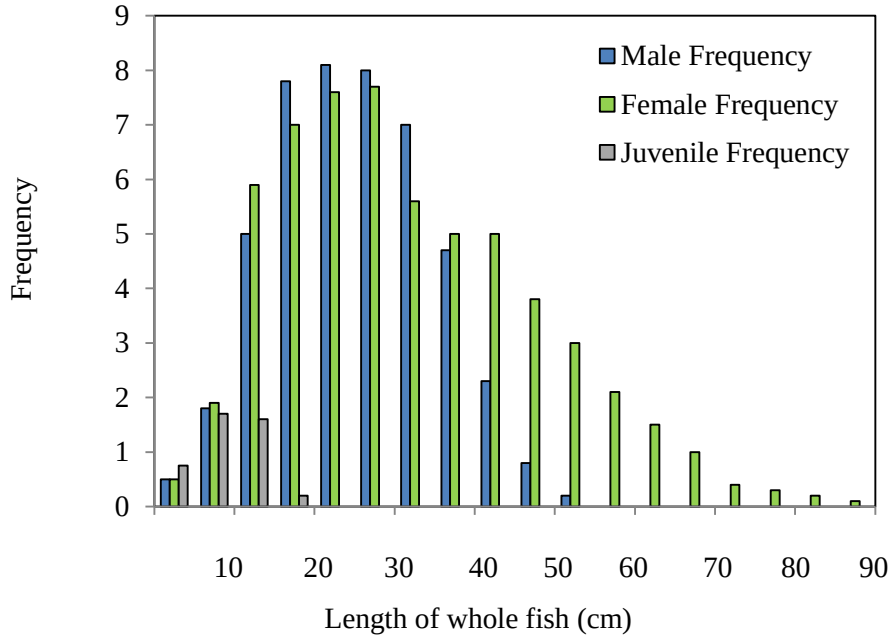

Figure 2. 3: The length frequency of *Lophius vomerinus* sampled from 1997 - 2000 (redrawn from Maartens and Booth (2005))

2.2.2. Literature available about monkfish characterisation and processing

To the best knowledge of the author there is limited literature available on monkfish in terms of characterisation and processing. The available data found included studies on the catch frequency, life strategies, growth patterns and biomass assessment, and reproductive biology (Farina et al., 2008; Maartens & Booth, 2005; Perez, Pezzuto, & Andrade, 2005). There have been studies into the proximate composition, fatty acid composition and mineral analysis of the raw monkfish fillet, muscle and waste, of which the exact contents i.e. viscera, bones, skin etc. were not specified (Batista, 1999; Prego et al., 2012; Sirot, Oseredczuk, Bemrah-Aouachria, Volatier, & Leblanc, 2008). These investigations studied monkfish species found in Brazil (Batista, 1999), France (Sirot et al., 2008) and the *Lophius piscatorius* (Prego et al., 2012). The enzymatic hydrolysis of monkfish with trypsin was investigated in order to determine antioxidant pentapeptide properties of the resulting FPH (Chi et al., 2014) where the main focus was on antioxidant activity of the resulting FPH. It is clear that additional information about monkfish heads, in particular *Lophius vomerinus* found in South Africa, is necessary, particularly in terms of raw substrate characterisation, enzymatic hydrolysis and optimisation, analytical testing of the hydrolysate and evaluation of the functional and nutritional properties of the hydrolysates will be useful. If values for the mentioned parameters can be found, the information will contribute to efforts to valorise monkfish heads as a

byproduct of the monkfish catch for applications such as protein sources for animal feed or use in food products.

2.2.3. Proteins and amino acids in fish waste

The main focus in this study was the preparation of protein rich FPH using enzymatic hydrolysis. All living organisms require protein to live, grow and stay healthy by ingesting the protein through diet and re-synthesise amino acids obtained from the digestion process into specific proteins required for the organism to sustain life (Erasmus, 2009). Proteins from fish byproducts have been found to be very nutritious, easy to digest, and are superior when compared to plant and other mammal protein sources, as fish protein generally contain high proportions of essential amino acids (Ghaly et al., 2013). Amino acids are molecules with one or more carboxyl groups (COOH) and amino groups (NH_2) in its chemical structure. Proteins are macromolecules consisting of amino acids bonded together with peptide bonds (Creighton, 2010, p. 227). Essential amino acids are important for biological functions but are not naturally synthesised by the body and needs to be supplemented in the diet (Tahergorabi, 2011, p. 121). The amount of protein and amino acids present in the monkfish head are important in this study for the indication of the potential for valorisation, and the processing techniques required to extract the maximum amount of proteins,

The amount of crude protein in a sample is determined by measuring the total amount of elemental nitrogen using elemental analysers (Šližyte, Daukšas, Falch, Storrø, & Rustad, 2005) or colorimetrically after Kjeldahl digestion (Liaset, Lied, & Espe, 2000). The Kjeldahl method is a technique used to determine nitrogen content and has been used frequently (Adler Nissen, 1986: 110) but has disadvantages such as long analysis time and many steps during analysis as well as requiring corrosive or toxic chemicals for analysis (Jung et al., 2003). An alternative to the Kjeldahl digestion technique is the Dumas method (Jung et al., 2003). The Dumas method is based on the principle of conversion of all the nitrogen content in the sample to nitrogen oxides using combustion at 800 - 1000 °C, then reducing the nitrogen oxides to nitrogen gas, N_2, and determining the nitrogen gas by thermal conductivity measurement (Jung et al., 2003). In order to determine the amino acid composition of a protein, the peptide bonds of the polypeptide chain are completely hydrolysed and the quantity of each amino acid is measured, usually with an automated amino acid analyser (Creighton, 2010, p. 282). Each amino acid is separated by column chromatography and measured quantitatively for example. by ion-exchange chromatography followed by detection with ninhydrin (Creighton, 2010, p. 282).

2.2.4. Oils in fish waste

The oils found in marine species contain valuable n-3 long chain polyunsaturated fatty acids (LC-PUFA) like DHA and EPA (Ghaly et al., 2013). The LC-PUFA are very important in the human diet and have bioactivities such as improved functions of the nervous system, reduction in cardiovascular disease, and reduced blood pressure (Ghaly et al., 2013; Prego et al., 2012). The n-3 and n-6 fatty acids are also essential in aquaculture feed to maintain cellular metabolism and for maintaining cell membrane structure in fish,(Miller, Nichols, & Carter, 2008), to help with neural development in aquaculture and for enhancing eicosanoid functions like inflammatory responses and blood clotting (Sargent, Bell, McEvoy, Tocher, & Estevez, 1999). The amount of oil found in fish byproducts depend on the fat content of the fish. Lipid content can be determined using solvent extraction method such as the Goldfisch method, the chloroform, methanol method, Bligh and Dyer method or acid digestion (Ghaly et al., 2013). Lipid classes can be determined using thin layer chromatography with a flame ionisation detector (Holler, Skoog, & Crouch, 2007, p. 281).

2.2.5. Mineral composition of fish waste

Minerals are found in the fish bones and the bones together with the skin account for roughly a third of the collagen produced from fish waste processing(Ghaly et al., 2013). Major minerals found in the bones include calcium and phosphorous (Ghaly et al., 2013), while trace minerals consist of cobalt, manganese, nickel, iron, copper, vanadium, zinc and selenium (Ikem & Egilla, 2008). If these elements are too high in concentration in a product intended as food ingredient, it can become toxic if consumed. Other compounds which are important to identify due to known toxicity include lead, cadmium, mercury and arsenic (Ikem & Egilla, 2008). It is therefore of high importance to identify whether any of these elements are present in the fish and the quantity thereof. The regulatory maximum amount of trace element acceptable for nutritional consumption can be found in literature such as the European Community regulation for animal products considered for food ingredients (Ikem & Egilla, 2008).

2.3. Enzymatic hydrolysis of fish processing byproducts

2.3.1. Hydrolysis and factors influencing hydrolysis

Hydrolysis is defined as the breaking of a bond in a molecule so that the broken molecules will go into ionic solution with water (Noyes, 1994, p. 85). Hydrolysis is currently used in the processing of fish byproducts to extract proteins, oils and minerals (Kristinsson & Rasco, 2000c). Šližytė, Rustad, and Storrø (2005a) state that enzymatic hydrolysis recovers protein from fish by products very effectively and that this method is used to improve functional and nutritional properties of underutilised protein. Enzymatic hydrolysis reactions take place under mild conditions and the fact that no acid is required for hydrolysis means that the hydrolysate is much better suited to be used in further processing for food ingredients as the nutritional and functional properties are preserved (Kristinsson & Rasco, 2000c).

The enzymatic hydrolysis process contains many steps which need to be considered in order to optimise the hydrolysis reaction. These steps are set out in Figure 2. 4.

From the flow sheet in Figure 2. 4 it can be seen that factors which influence the hydrolysis process are:

- water addition
- initial heating
- enzyme choice and enzyme to substrate ratio (E/S)
- reaction conditions such as temperature, time and pH

There are many advantages of adding water to the raw substrate prior to hydrolysis in terms of functional properties and product protein yield. When water was added prior to hydrolysis, an increase was found in the fat absorption ability of the FPH (Šližytė et al., 2005a), the highest oil yield in FPH was obtained (Daukšas, Falch, Šližyte, & Rustad, 2005b) and protein recovery in FPH was more than twice the amount found when no water was added (Šližyte et al., 2005b). Without the prior addition of water, the emulsification capacity of FPH was the highest (Šližyte et al., 2005b) but the lowest amount of emulsion was formed (Šližytė et al., 2005a).

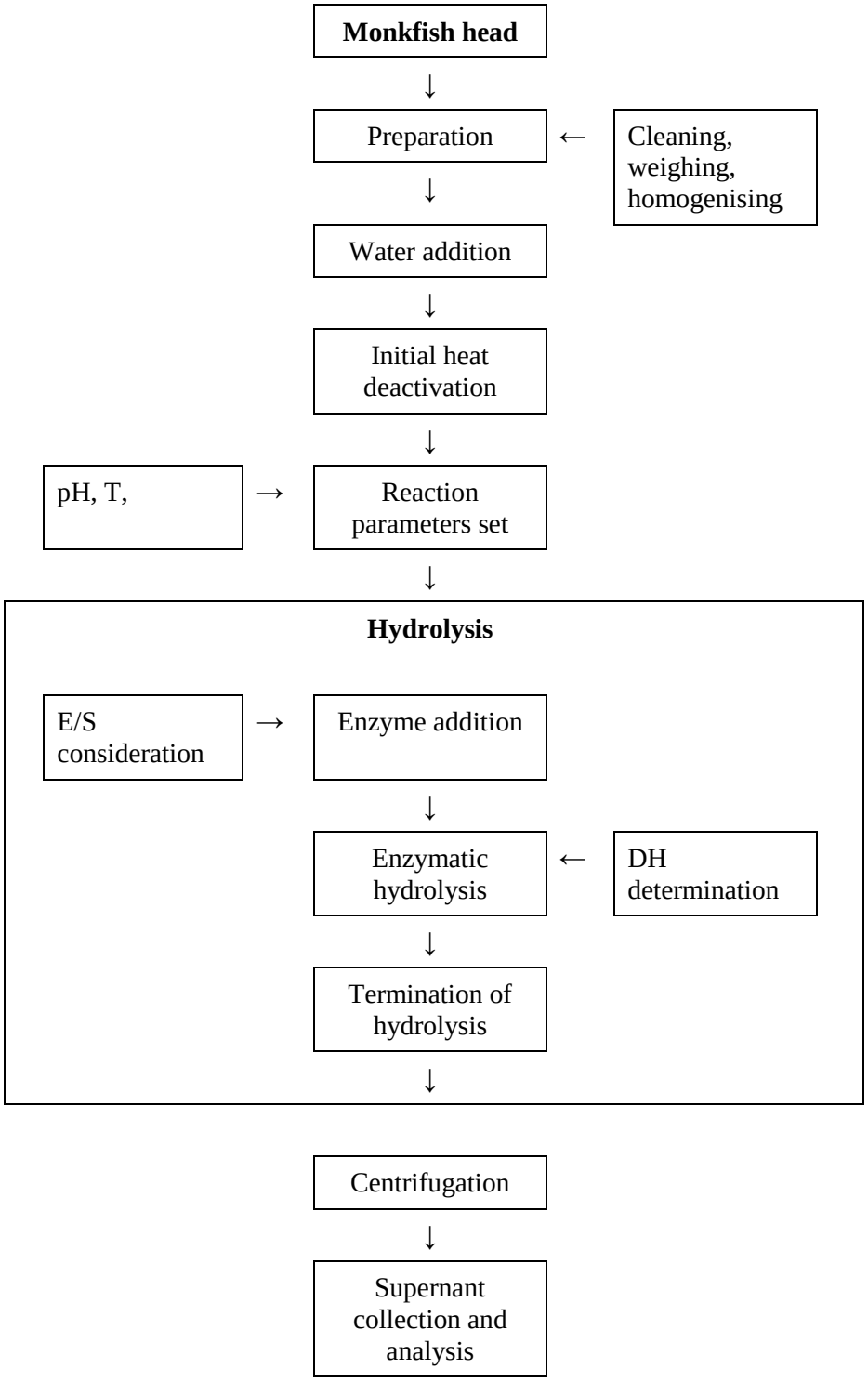

Figure 2. 4: Flow sheet of enzymatic hydrolysis (adapted from Kristinsson and Rasco (2000c))

Initial heating is performed on the raw substrate-water mixture before hydrolysis, when the endogenous enzymes contained in the substrate need to be deactivated (Ghaly et al., 2013). In many studies fish waste includes fish viscera (Ghaly et al., 2013) and the viscera contains many endogenous enzymes which may take part in hydrolysis. Šližytė et al (2005b) found that initial heating decreased the FPH yield and that protein recovery was higher for samples that were not heated initially. Although a decreased FPH yield and lower protein recovery are undesirable when protein extraction is the outcome for enzymatic hydrolysis, the presence of endogenous enzymes means that uncontrolled hydrolysis can take place before the addition of enzymes which influences the true DH (Opheim et al., 2015). As DH was determined using the pH-stat method in this study, uncontrolled hydrolysis prior to enzyme addition was undesirable, as the DH measurement commences once the enzyme is added to the reaction mixture. Any prior hydrolysis would not be measured as a consequence and results would not reflect the true DH.

The optimal reaction parameters and responses for hydrolysis have been investigated in a number of studies (Bhaskar et al., 2008; Chalamaiah et al., 2012; Gbogouri, Linder, Fanni, and Parmentier (2004); Halim et al., 2016; Ovissipour, Abedian Kenari, Motamedzadegan, & Nazari, 2012l; Šližytė et al., 2005a). Optimisation parameters include enzyme to substrate ratio, pH, time, and temperature, while the response variables were DH and protein yield (Chalamaiah et al., 2012; Halim et al., 2016). Kechaou, Bergé, Jaouen, and Amar (2013) investigated reaction time, temperature and enzyme activity as parameters to find an optimum DH. Reaction time and temperature was found to have a more significant effect on the DH than the enzyme activity. Adler-Nissen (1986) states that the enzyme to substrate ratio has a direct relationship to the degree of hydrolysis, meaning that a higher enzyme to substrate ratio will result in a faster rate and decreased reaction time.

2.3.2. Proteolytic enzymes used for hydrolysis

An enzyme is a protein molecule, formed from long folded amino acid chains, which functions as a catalyst for biochemical reactions (Schaschke, 2014, p. 129). A proteolytic enzyme works by catalysing the cleavage of long chains of protein molecules into shorter peptide chains and amino acids. The catalyst function of the enzyme is very specific, which means that different enzymes each have a specific biochemical reaction it will take part in, and an optimum temperature and pH range (Creighton, 2010, p. 628). Hydrolases are protease enzymes which catalyse the cleavage of peptide bonds in proteins (Novozymes, *sa*).

There are two groups of proteolytic enzymes, classified according to the site at which the enzyme acts as catalyst for the cleavage of proteins (Santos, Branquinha, & D'Avila-Levy, 2006). The two major groups are the exopeptidases and endopeptidases, which is an indication of the specificity of an enzyme. An exopeptidase enzyme cleaves a peptide bond adjacent to an amino or carboxyl terminal whereas an endopeptidase cleaves a peptide bond within a protein chain (Santos et al., 2006).

Apart from enzyme specificity, enzyme activity as a further important consideration when selecting enzymes for hydrolysis reactions. Enzyme activity is an indication of the catalytic activity of an enzyme, and describes the rate of action of the enzyme on the substrate (Anson, 1963). The activity of an enzyme is a quantifiable parameter which is determined by using methods such as the Wohlgemuth method, the Warburg method (Campbell & King, 1961) and the method used by Anson (Anson, 1938) to name only a few. It is important to know the enzyme activity in order to optimise the reaction of enzymatic hydrolysis in terms of enzyme to substrate ratio. The different methods of determining enzyme activity yield results in different dimensional units. These different values depend on which of the formed products concentration or converted substrate concentration was measured (Anson, 1963). Each method has a different approach and does not consistently measure only one specific product or starting substrate thus enzyme activities are reported in different units depending on the measurement technique.

The enzyme to substrate ratio (E/S) is a reaction parameter which can be varied to find optimum hydrolysis reaction conditions. Enzymes are the single most costly parameter in the enzymatic hydrolysis process (Aspmo et al., 2005) and the amount of enzymes used is important. Higher nitrogen recovery after hydrolysis with a higher enzyme to substrate ratio was reported by Aspmo et al. (2005), indicative that more nitrogen in the form of amino acids were produced in the fish protein hydrolysate. The addition of more enzyme to increase E/S was only effective up to an E/S value of 2% (w/w, dry basis) and once this value was achieved the initial hydrolysis rate did not change significantly and only a slight increase in nitrogen recovery was observed (Aspmo et al., 2005).

2.3.2.1. Enzymes for hydrolysis

The two enzymes used for hydrolysis in this study were bromelain (EC 3.4.22.32) from pineapple stem and alcalase (EC 3.4.21.62) from *Bacillus lichenformis*. These enzymes were chosen based how successful the enzyme could hydrolyse fish byproducts based on literature and the quality of the fish protein hydrolysate product in terms of FPH yield and soluble protein present in the FPH. (Ghaly et al., 2013; Opheim et al., 2015; Šližyte et al., 2005b).

2.3.2.2. Bromelain

Bromelain (EC 3.4.22.32) is a cysteine protease made from pineapple stem or from the fruit and leaves (Bock, 2015, p. 191) . According to Amid, Ismail, Yusof, and Salleh (2011) and Bock (2015, p. 191) bromelain requires a nucleophilic cysteine side chain, from a free sulfhydryl group, as active site for catalytic activity. The activity of the bromelain used in in this study was 2500 gelatin digestion units per gram protein (GDU/g). Fellows (2009, p. 259) stated that the optimal operating conditions for bromelain are pH between 4 and 9, depending on the specific substrate used, and a temperature between 20 and 65 °C.

2.3.2.3. Alcalase

Alcalase (EC 3.4.21.62) is a proteolytic enzyme (a proteases from the hydrolases enzyme class). This enzyme is produced from a specific strain of *Bacillus licheniformis* which is submerge fermented. Alcalase food grade is an endopeptidase enzyme with an activity of 2.4 AU/g, where 1 AU relates the quantity of enzyme which releases an equivalent of 0.55 µmol tyrosine from haemoglobin. Alcalase is a highly efficient bacterial protease especially developed for the hydrolysis of all kinds of proteins (Bhaskar et al., 2008; Šližytė et al., 2005a). According to Bhaskar et al. (2008) and Šližytė et al. (2005a) the optimal reaction conditions for alcalase are temperatures between 55 and 70 °C, depending on the specific substrate, and pH values between 6.5 and 8.5.

Enzymes were compared in tabular format by (Aspmo et al., 2005) and values from their study for alcalase and bromelain are shown below in Table 2. 1 :

Table 2. 1: Comparison of enzymes (adapted from (Aspmo et al., 2005))

Enzyme name	Source	pH range	Temperature range (°C)	Activity*	Price (€/kg)
Alcalase	*Bacillus licheniformis*	6 - 10	55 - 70	2.4 AU/mg	25
Bromelain	Pineapple stem	5 - 8	20 - 65	~100 TU/mg	20

*1 Anson unit (AU) is the amount of enzyme that will release an equivalent of 0.55 µmol of tyrosine per minute from denatured haemoglobin at 25°C and pH of 7.5 (Anson, 1938)

1 Tyrosine unit (TU) is the amount of enzyme that will release an equivalent of 1 µg of tyrosine per minute from a casein substrate under assay conditions (Aspmo et al., 2005)

2.4. Methods used to determine degree of hydrolysis

The degree of hydrolysis (DH) is an indication of the percentage of peptide bonds in the native protein that have been hydrolysed by enzyme activity, and is therefore an important parameter that quantifies the extent of reaction during hydrolysis. The average length of the peptide chains influence the nutritional, functional and sensory properties of the final FPH (Nguyen et al., 2011). The degree to which the fish byproducts are hydrolysed also influences how the fish protein hydrolysate can be used (Kristinsson & Rasco, 2000c).

The degree of hydrolysis (DH) is defined as the percentage of cleaved peptide bonds to the total amount of peptide bonds (Navarrete del Toro & Garcia-Carreño, 2002) and according to Rutherfurd (2010) there is yet a standard protocol to be developed for determining the DH. Without a standardised protocol, authors use different methods based on aspects such as availability of laboratory equipment, time confinements and budget constraints. Common methods for determining the DH include the trinitrobenzenesulfonic acid (TNBS) method, the ortho-phtaladehyde (OPA) method, pH-stat technique, osmometric method, soluble nitrogen after trichloroacetic acid precipitation and formol titration methods (Rutherfurd, 2010). Some of the methods named are analytical methods where samples are taken from the reaction mixture periodically and the free amino groups in the product treated with a chromogen or fluorescent agent and then tested by spectrophotometry (Navarrete del Toro & Garcia-Carreño, 2002). The methods requiring this disadvantageous and time consuming derivatisation step for spectrophotometric preparation include the TNBS, OPA and formol titration methods. The pH-stat technique is a real-time monitoring technique which doesn't require a derivatisation step and is not a denaturing method (Rutherfurd, 2010). The disadvantage of the pH-stat technique is that the relationship between DH and base consumption is complex and the method can only be used in alkaline conditions (Rutherfurd, 2010).

Spellman et al. (2013) compared the OPA, TNBS and pH-stat method to determine how well each methods results correlated with the others. These methods were compared as they were the most commonly used for the determination of food protein hydrolysis (Spellman et al., 2013). The available data on different methods used to determined DH was reviewed by Rutherfurd (2010), who found that values of DH found by using different methods generally did not agree well. The presence of exopeptidases in enzymes, and protein peptides acting as buffers, were two possibilities for the miscorrelations found in the review article. However, a general consensus could not be found for the miscorrelations, and the best method to determine DH could not be determined by Rutherfurd (2010). The OPA, TNBS and pH-stat methods are described shortly for clarity.

The detection of amino acids using a fluorometric method was described in 1971 where ortho-phtalaldehyde (OPA) and 2-mercaptoethanol was used (Church, Porter, Catignani, & Swaisgood, 1985). The principle of the OPA method is that upon reaction with the amine in the presence of a thiol group, an increase in fluorescence will be observed, and the increase will be proportional to the amount of protein present (Noble & Bailey, 2009; Spellman et al., 2013). The OPA technique requires the presence of a thiol group because it does not react directly with the amine functional group but is simple and fast to use, where an OPA-amino acid derivative fluoresces and the unreacted species do not fluoresce (Rutherford 2010). According to Spellman et al. (2013) the OPA technique is not suitable for proteins rich in proline and cysteine due to poor reactivity. In many instances 2-mercaptoethanol (Noble & Bailey, 2009: 90) is used but authors have reported using ethanethiol (Church et al., 1985) and dithiothreitol (Nielsen, Petersen, & Dambmann, 2001) instead as these substances were considered more environmentally acceptable (Nielsen et al., 2001).

The TNBS method has been used by many researchers since being introduced in 1960 as it is a very reproducible method (Navarrete del Toro & Garcia-Carreño, 2002) (Adler-Nissen, 1979). The reaction of TNBS with N-terminal amino groups is the basis for the TNBS method. The TNBS reacts with primary amines under slight alkaline conditions and the chromophore formed is then analysed spectrophotometrically at 340 nm (Adler-Nissen, 1979). According to (Nielsen et al., 2001) the TNBS method is laborious and make use of unstable and toxic compounds. Like in the case of the OPA method, the TNBS method does not react with proline and also overreacts with ε-amino groups of lysine which causes an overestimation of the degree of hydrolysis (Rutherford, 2010).

The pH-stat technique is based on the principle that when a protein dissociates under neutral or alkaline conditions, the liberation of protons from free amino groups lead to a decrease in pH. The amount of cleaved peptide bonds can then be correlated to the amount of base necessary to keep the reaction at a constant pH (Camacho et al., 2001; Spellman et al., 2013). The relationship depends on complex variables including the pK of the liberated α-amino group, temperature of reaction mixture and peptide chain length (Rutherford, 2010). During hydrolysis in a neutral or alkaline mixture, protons from free amino groups released from hydrolysis are dissociated, which reduces the pH of the mixture (Spellman et al, 2003). This means that the number of peptide bonds broken during hydrolysis can be estimated by base consumption to keep a constant pH throughout the reaction. The conclusion of the comparative study by Spellman et al. (2013) found that the three methods had its own advantages and disadvantages, similar to the comparison by (Rutherfurd, 2010) and no method was stated by either author to be superior to the rest. The condensed summary of the results with regard to the OPA, TNBS and pH-stat methods only, are shown in Table 2. 2.

From Table 2. 2 it can be seen that each method has its own negative and positive aspects and the selection should be done objectively. In this current study of monkfish head hydrolysis the pH-stat method was used for the reason that a non-denaturing and real-time monitoring technique was deemed most suitable for a product to be used as food ingredient.

Table 2. 2: Comparison of methods to determine DH (adapted from Rutherfurd (2010))

Method	Advantages	Disadvantages
OPA	• Rapid derivatisation for spectrophotometric measurement • Real-time monitoring	• Inaccurate for proteins that are cysteine/proline -rich • Works on soluble material only • Lysine side chains interfere • Requires derivatisation
TNBS	• Reproducible results • Rapid derivatization for spectrophotometric measurement	• Not real-time monitoring • Works on soluble material only • Toxic compound • Lysine side chains interfere • Requires derivatisation • Inaccurate for proteins that are proline rich
pH-stat	• Real-time monitoring • Non-denaturing method • Rapid method • No derivatisation	• Accuracy depends on type of enzyme • Exogenous proteases cause underestimation of DH • Complex relationship between DH and base consumption, which may not be accurate for all proteins • Only applicable under alkaline conditions

2.4.1. pH-stat

As stated in the preceding paragraphs, the pH-stat method is a technique that evaluates the progress of a hydrolysis reaction whereby base consumption is measured and correlated with the degree of hydrolysis. As stated by Camacho et al. (2001) as an amide bond is hydrolysed as illustrated by reaction equation 2.1, a carboxyl group and an amino group forms. The carboxyl group dissociates completely as shown in reaction equation 2.2 and the protons formed are distributed in equilibrium with the amino group as shown in reaction equation 2.3:

$$R\text{-}CO\text{-}NH\text{-}R' + H_2O \rightarrow R\text{-}COOH + R'\text{-}NH_2 \quad (2.1)$$

$$R\text{-}COOH \rightarrow R\text{-}COO^- + H^+ \quad (2.2)$$

$$R'\text{-}NH_3^+ \rightleftarrows R'\text{-}NH_2 + H^+ \quad (2.3)$$

If base is added to the reaction to keep the pH constant, the protons generated by hydrolysis is neutralised and the mols of the base added are equivalent to the protons formed by hydrolysis (Camacho et al., 2001).

The degree of hydrolysis is calculated by Equation 2.4 (Adler-Nissen, 1986, p. 91):

$$DH = \frac{B \times N_B}{\alpha \times M_p \times h_{tot}} \times 100\ \% \quad (2.4)$$

where B is the amount of base added (mℓ), N_B is the normality of the base, α the average degree of dissociation of α-amino groups, M_p is the mass of protein in the substrate (g) and h_{tot} is the sum of the millimoles of peptide bonds per unit mass of protein (meq/g).

The equivalent number of peptide bonds per unit mass of protein (h_{tot}) was determined by Adler-Nissen (1986) for many substrates such as casein, meat, haemoglobin, fish protein and wheat gluten. The value of h_{tot} for fish protein of 8.6 meq/g was found by Adler-Nissen (1986) by multiplying the amount of nitrogen present in the substrate by a factor of 6.25.

The average degree of dissociation (α) is related to the pK of the amino groups, which is greatly dependent on reaction temperature and pH. According to Rutherfurd (2010) although the α-value is estimated based on the average pK value for the cleaved amino groups, the actual value is not known and depends on the amino acid profile of the specific substrate and the specificity of the enzyme.

2.4.2. pK determination to use in pH-stat technique

The method to determine the pK is thoroughly discussed in Appendix A, and only a brief explanation is given in this section. The pK determination method proposed by (Camacho et al., 2001) is based on the

fact that when a solution of protein is titrated with an alkali, the equivalent base consumption is related to the protons dissociated due to hydrolysis but also due to the other protonisable functional groups which are released but not from hydrolysis. If the protein solution is first hydrolysed and then titrated with an alkali, the equivalent base consumption (b_x) will reflect both the protonisable groups released from hydrolysis as well as the protonisable groups released not due to hydrolysis. If a protein solution is titrated without being hydrolysed, only the protonisable groups which are released not due to hydrolysis will be the reason for the base consumption (b_s). So now there are two experiments, one determining base consumption of unhydrolysed substrate (b_s) and the other determining the base consumption after hydrolysis (b_x). So if b_s is subtracted from b_x, the equivalent amount of base used due to the hydrolytic process can be determined.

In the equation that is used for pH-stat technique to determine base consumption one needs to know the α value specific to the substrate-enzyme system as shown in Equation 2.4. The α value is calculated by using Equation 2.5:

$$\alpha = \frac{10^{(pH - pK)}}{1 + 10^{(pH - pK)}} \tag{2.5}$$

The pK value is the mean pK of the α-amino acids released during hydrolysis (Rutherfurd, 2010). According to Camacho et al. (2001) the pK value is dependent on pH and the substrate and these authors have proposed a method of determining the specific pK for a system by correlating base consumption to the degree of hydrolysis.

2.5. Fish protein hydrolysis products

Products resulting from the hydrolysis of fish protein have found applications in food ingredients due to good physiochemical properties, as well as protein supplements in animal feed (Chalamaiah et al., 2012). The amino acid profile is important for animal feed and food ingredient considerations, as essential amino acids are important to be supplemented in the diet as it is not synthesised naturally in the body (Tahergorabi, 2011, p. 121). Functional food properties of the hydrolysate product, as a result of the physiochemical properties are important for food ingredient considerations (Ghaly et al., 2013).

Fish protein hydrolysates have been studied for their functional properties for a while and it has been established that FPH have properties that are advantageous if used in food ingredients (Pires, Clemente, & Batista, 2013). Examples of such physiochemical properties, or functional properties, are solubility, oil

binding or fat absorption capacity, emulsification properties, foaming ability, water holding capacity, as well as antioxidant activity (Pires et al., 2013). According to He, Franco, and Zhang (2013) the physiochemical properties of FPH are enhanced during controlled hydrolysis.

FPH are considered a valuable source of protein and have been used as animal- and aqua-feed ingredient (Opheim et al., 2015). It has been found that FPH and hydrolysate sediment are a good source of protein with a balanced amino acid profile, and essential polyunsaturated fatty acids (Chalamaiah et al., 2012; Kristinsson & Rasco, 2000c). Liaset et al, (2000) state that the nutritional value of fish protein hydrolysates is determined by the utilisation of the FPH nitrogen, which in turn have a dependence on the composition of the mixture of amino acids and the digestibility.

2.5.1. Functional properties of products from fish protein hydrolysis

The value of the emulsifying activity index (EAI) indicates how well a protein can contribute to form an emulsion and how well that protein can help keep the emulsion stable (Liceaga-Gesualdo & Li-Chan, 1999). Hydrolysates are surface active agents or surfactants due to the hydrophilic and hydrophobic groups in the protein chains and can therefore aid in formation and stabilisation of emulsions containing oil and water (Gbogouri et al., 2004). According to Gbogouri et al. (2004) the hydrolysates adsorbs to the surface of new oil droplets formed during homogenisation and creates a membrane that prevents droplets from coalescing. The emulsion stability refers to how well an emulsion resists changes in its properties over time (Kristinsson & Rasco, 2000c). Emulsion stability was determined by Pires et al. (2013) and is expressed as amount of emulsified oil (ml) per 1 gram of FPH.

Foaming capacity is important for baking products and depends on the ability of a protein to diffuse to the water-air interface, unfold and reorganise the molecules at the interface (Klompong et al., 2007). It has been found that the hydrophobicity of the proteins affect foaming properties (Klompong et al., 2007).

Fat absorption is a characteristic that influences the taste of the final food product and is an important functional property required in the food industries like meat and confectionary (Kristinsson & Rasco, 2000c). According to Kristinsson and Rasco (2000c), fat absorption is mainly due to physical entrapment of the oil and that a higher fat absorption is therefore expected when the FPH has a higher bulk density.

Free radicals and free oxygen reagents are formed during cellular respirations and takes place not only in humans but also other aerobic organisms (Chalamaiah et al., 2012). These are unstable compounds and

react readily with other molecules and causes tissue or cell damage(Chalamaiah et al., 2012). Antioxidants are used to negate the harmful radicals. The *N,N*-dimethyl-*p*-phenylenediamine (DMPD) method makes use of the chromatic properties of a stable radical cation (Fogliano, Verde, Randazzo, & Ritieni, 1999). When DMPD is in the presence of an oxidant solution, $DMPD^{\bullet+}$ which is a purple radical cation, will be formed which can be discoloured when a hydrogen atom from an antioxidant is transferred to the cation (Fogliano et al., 1999). This reaction scheme is shown by Equations 2.6 and 2.7:

$$DMPD_{(uncoloured)} + oxidant + H^{+} \rightarrow DMPD^{\bullet+}_{(purple)} \quad (2.6)$$

$$DMPD^{\bullet+}_{(purple)} + AOH \rightarrow DMPD^{+}_{(uncoloured)} + AO \quad (2.7)$$

2.6. Conclusion

The monkfish *Lophius vomerinus* is found off the coast of Namibia with the meat of the fish used as food product for humans. The head is important it constitutes a large proportion of the monkfish catch with values up to 37.9 % of the total length of the fish, and is therefore readily available for valorisation, and can be utilised as a fish protein hydrolysate which can find application in animal feed. It is important to characterise the head of the fish in terms of protein, amino acid content, minerals and oils before further processing. Enzymatic hydrolysis is an effective and mild process in which the protein contained in the monkfish head can be extracted. When hydrolysis is complete, the protein can be found in the fish protein hydrolysate layer of the whole product. There are several important factors influencing the optimum enzymatic hydrolysis process. These factors include reaction time, temperature, pH, enzyme, enzyme to substrate ratio, initial heating of the substrate as well as the addition of water prior to hydrolysis. The degree of hydrolysis is used as a measure of the reaction progression and there are several methods used in practice to determine the degree of hydrolysis. The pH-stat technique is a real-time monitoring and non-denaturing technique that is based on the principle that the amount of alkali necessary to maintain a constant pH can be correlated to the degree of hydrolysis. This correlation is complex and dependent on the enzyme and substrate used, and a methodical approach to determine the mean pK value before using the pH-stat method is necessary. The amount of amino acids, specifically the essential amino acid profile, is important for the consideration of fish hydrolysate products as animal feed ingredient. Important functional properties of the hydrolysate in terms of food quality include emulsifying activity index, emulsion stability, fat absorption capacity and antioxidative ability.

Chapter 3: Problem Statement

There is an increasing demand on animal derived protein, and the animal feed industry would benefit from protein-rich products from fish byproduct processing. The byproducts from the monkfish, *Lophius vomerinus*, is currently discarded while valuable nutrients such as protein, fatty acids, minerals, and essential amino acids can be derived from the byproducts, if properly processed. The monkfish head will be the byproducts investigated in this study, as the head accounts for more than 30 % of the total length of the fish. The head will be made up out of skin, frame, teeth, flesh, gills, fins, anterior tentacles, and eyes. As this substrate is not a standard protein, the characterisation of the monkfish head will be very important to conduct before further processing can take place. Enzymatic hydrolysis processes used currently is the best way of producing fish protein hydrolysate as the reaction conditions are mild enough to ensure the conservation of quality and functional, and nutritional properties of the substrate.

The objectives in this study are described below:

- Characterise the contents of the monkfish heads: proximate analysis, fatty acid profile, mineral content and amino acid profile to assess suitability for use in animal feed
- Determine the value of pK for alcalase/monkfish and bromelain/monkfish systems, and use these pK values in the equation to determine the DH in the pH-stat method of monitoring the progression of DH
- Optimise the enzymatic hydrolysis reaction time by varying reaction pH, temperature and two enzymes: alcalase and bromelain
- Determine if the hydrolysis products have functional food properties

Chapter 4: Research results in article format

This chapter is written in article format that describes the research results found in this study.

The first article describes the characterisation of the raw monkfish substrate to assess the potential for the *Lophius vomerinus* head for valorisation. Analyses that were conducted were the determination of protein, amino acid profile, fatty acid profile, and mineral content. The values of EAA found in the monkfish substrate were compared to values of white fishmeal, which is currently used as a protein source in animal feed, to assess the potential of the processed monkfish byproducts to be used as animal feed ingredient.

The characterisation of monkfish (*Lophius vomerinus*) heads to assess nutritional potential for animal feed application

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Abstract

The monkfish *Lophius vomerinus* head makes up approximately 30% of the total fish and is a byproduct of the South African fisheries industry, which is currently processed into relatively low-value fish meal or even discarded. In this study, the monkfish was characterised for nutritional components by analysing the proximate composition, which included crude protein, crude fat, ash and moisture content; amino acid profile, fatty acid profile and the mineral content. The amount of protein found in the monkfish head amounted to 8.19 % (wet basis), and 43.77 % of the amino acids within the protein were essential amino acids. The crude fat in the monkfish head amounted to 2.61 % (wet basis) and the essential n-3 polyunsaturated fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) was found in amounts of 20.48 % and 8.05 % of the crude fat respectively. Mineral analysis results had high variability, due to the physical sample, which were attributed to the stage of maturity of the fish, season the fish was spawned, spawn conditions and geographical location the fish was caught in. Subsequently, it is recommended that the sampling method should allow for all raw material to be prepared and homogenised at once, and the facilities required be adapted to meet this criteria. To allow for extraction of the maximum amount of raw protein, minerals and oils, a tightly controlled process, operating under mild reaction conditions ($T < 70$ °C; $5 < pH < 9$) would be required to ensure the preservation of the quality, functionality and quantity of the proteins in the raw material. The most appropriate method of extraction of protein from fish byproducts is enzymatic hydrolysis.

Key words: Characterisation, monkfish, proximate composition, fatty acids, amino acids, minerals, animal feed

Abbreviations

DHA - docosahexaenoic acid; EAA - essential amino acid; EPA - eicosapentaenoic acid; ICP-MS – inductively coupled plasma-mass spectrometry; LC-MS-MS – liquid chromatography mass spectrometry; MUFA - monounsaturated fatty acids; NEAA - non-essential amino acids; PUFA - polyunsaturated fatty acids; SD - standard deviation; SFA - saturated fatty acids; TFA - total fatty acids

Introduction

The demand on animal derived protein is steadily on the rise and the projected global requirement for the year 2050 is estimated to be 463.8 million tonnes (FAO, 2011). The increased demand on animal protein means that the requirement of protein supplements in animal feed will increase too, as discussed in Chapter 2 of this study. Therefore it has become very important to find additional sources of protein for the animal feed industry. Monkfish processing byproducts could be a potential source of supplementary protein, the details of which are provided in Chapter 2. Whether the raw monkfish head is suitable to be utilised for animal feed ingredient, will be indicated by the nutritional profile. The aim of this study was to characterise monkfish processing byproducts from the South African fish processing industry, in order to determine the values and profile of the main nutritional components found in the byproducts. The objectives were therefore:

- Determine the proximate composition of the monkfish head, using the standard protocol of the AOAC (1997), where the crude protein, crude fat, ash, and moisture content is analysed.
- Establish the amino acid profile of the crude protein using liquid chromatography mass spectrometry (LC-MS-MS).
- Evaluate the crude fat in terms of fatty acid content using chloroform-methanol extraction.
- Determine the mineral content profile of the monkfish head, specifically Fe, Ca, K, Zn, Hg, Cd, Pb, Cu, Cr, and Ni with inductively coupled plasma mass spectrometry (ICP-MS).
- Physical characterisation of the monkfish head in terms of mass, length and specific appearance.

Materials and Methods

Materials

Fresh monkfish heads from the fish processing facility of Irvin & Johnson (I&J) at V&A Waterfront, (Cape Town, South Africa) were collected and transported on ice to the University of Stellenbosch in November 2014, May 2015 and May 2016.

Sample preparation

The monkfish heads were prepared for further analysis by cleaning and homogenising as shown in Figure 4. 1.

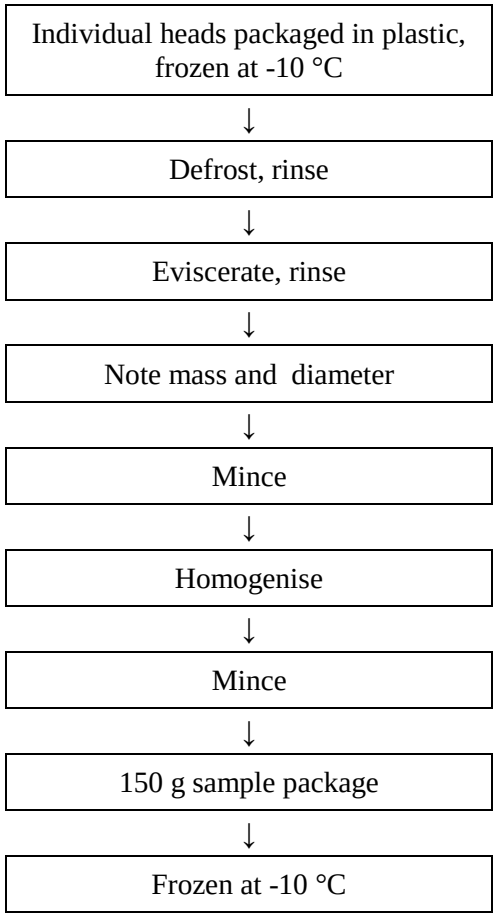

Figure 4. 1: Flow diagram of monkfish head sample preparation

Analyses

The substrate was analysed in terms of total nitrogen, moisture content, dry matter, ash content, crude fat content and fatty acid composition for proximate composition. The substrate was further characterised for specific elemental composition (Fe, Ca, K, Zn, Hg, Cd, Pb, Cu, Cr, and Ni.) and an amino acid profile was evaluated. The proximate composition analysis and fatty acid analysis was conducted by the Department of Animal Science of the Stellenbosch University. Elemental analysis and amino acid analysis was conducted by the Stellenbosch University Central Analytical Facility, in the ICP-MS and MS departments, respectively.

Proximate analysis

A raw minced sample of 30 g (wet basis) was used for proximate analysis. Total nitrogen content was determined by using the Dumas method where the substrate is combusted at a temperature between 800 - 1 000 °C which facilitates the conversion of all the nitrogen in the sample to nitrogen oxide (Jung et al., 2003) with a Leco 528 elemental analyser. After combustion, the nitrogen oxide is reduced to nitrogen gas, N_2, and the amount of nitrogen gas is determined via thermal conductivity measurement (Jung et al., 2003). The total crude protein was calculated by multiplying the amount of nitrogen in the substrate by 6.25. The value of 6.25 is based on the average value of nitrogen content in proteins (Opheim et al., 2015). The rest of the proximate analysis (moisture content, dry matter, ash and crude fat content) was performed according to the standard protocol as described by the AOAC (1997). Moisture content was determined by drying the sample in an oven at 100 °C and expressed by determining the amount of mass lost during drying. Dry matter was the percentage substrate left after drying. The ash content was quantified by heating the substrate in a furnace at 500 °C for 6 hours and measuring the remaining mass. The chloroform/methanol method described by (Folch, Lees, & Sloane Stanley, 1957) was used to extract the crude fat from the substrate.

Major and minor elemental analysis

Element analysis was conducted after the substrate was dried in a furnace at 40 °C for 48 hours and the dried sample had been digested. Digestion is the process of making the acid extractable elemental matter of the sample soluble and was carried out on a MARS microwave digester. Ultra-pure nitric acid (HNO_3) or HNO_3 and hydrochloric acid (HCl) was used and added to 0.5 g of dried sample. The extractants were made up to a volume of 50 ml using deionised water. After digestion the major elements were analysed on

a Thermo ICap 6200 inductively coupled plasma-atomic emission spectrometer. Trace elements were analysed on an Agilent 7700 quadrupole inductively coupled plasma-mass spectrometer. Both analysers were calibrated using the traceable standards of the National Institute of Standards and Technology. A quality control standard from a different supplier than the main calibration standards was used as well to verify the precision of the calibration before substrate analysis. The results were amended for dilution from the digestion process.

Fatty acid analysis

Fatty acid analysis was determined according to a solvent extraction method for animal fat as described by (Folch et al., 1957). A 2:1 (v/v) chloroform:methanol solution containing 0.01% butylated hydroxytoluene (BHT) as an antioxidant was used as the extraction solution and 0.5 g of minced fish was added to the solvent. The mixture was homogenised for 30 seconds using a WiggenHauser D-500 Homogeniser on speed setting D. The internal standard used was heptadecaenoic acid (C17:0). From the homogenised mixture a 250 µl sample was taken and transmethylated at 70 °C for 2 hours with 2 ml of a 19:1 (v/v) methanol:sulphuric acid solution. The fatty acid methyl ethers (FAME) were extracted with water and hexane after cooling to room temperature. The water was separated from the hexane-FAME mixture. An amount of 1 µl of the FAME components was injected into a Thermo TRACE 1300 series gas chromatograph with a flame ionisation detector, using a 30 m TR-FAME capillary column with an internal diameter of 0.25 mm and 0.25 µm film and a run time of 40 minutes. The samples were run from an initial temperature of 50 °C for 1 minute, increased at a rate of 25 °C/min until 175 °C was reached, then a temperature increase at a rate of 1.5 °C/min until 200 °C was reached and maintained for 6 minutes and finally a temperature increase at a rate of 10 °C/min until the temperature reached 240 °C and then maintained for 2 minutes. The hydrogen gas flow rate was 40 ml/min and the FAME was determined by comparing retention times with a sample of standard FAME mixture.

Amino acid analysis

Amino acid profiles were analysed by firstly acid hydrolysing a 0.1 g defatted and dried fish mince sample. The amino acids were analysed with the Waters API Quattro Micro LC-MS-MS and Waters AccQ Tag Ultra Derivatisation kit. The samples were prepared by taking 10 µl of undiluted sample to the Waters AccQ Tag kit constituents and heated to 55 °C and kept at this temperature for 10 minutes. The operating conditions of the instrument were a solvent flow rate of 0.7 ml/min through a AccQ Tag C18, 1.7 µm, 2.1 x 100 mm column.

Statistical analysis

T-tests were performed (Microsoft Excel) to determine if there were statistical differences between samples.

Results and Discussion

Physical description

The monkfish heads were caught during the month of November 2014, May 2015 and May 2016. The raw material as received from the fish processing facility, I & J, were the heads of the fish, with viscera attached, therefore the total length of the fish could not be measured. As reported in literature, (Caruso, 1983), the monkfish head was found to account for between 27.7 to 37.9 % of the total length of the fish. This value could not be verified in this study. The head of the raw monkfish was flat and round (Figure 4. **2**), and the physical size measurements were made in terms of diameter.

Figure 4. 2: Physical appearance of monkfish heads

The head was characterised in terms of mass and diameter as shown in Table 4. 1. The appearance of the fish head was flat and circular in shape, with dark brown skin above and white on the belly, as described by Caruso (1983).

Table 4. 1: Physical measurements of fish heads

Variable	Value
Average mass	1480 ± 260 g
Average diameter	230 ± 40 mm

Value mean ± SD; n = 15

Proximate analysis

The proximate composition of minced monkfish head is set out in Table 4. 2. The monkfish head contained 8.19 ± 2.08 % protein (wet basis), which originates from flesh, muscle and skin within the monkfish head. This value represents a value of 121 grams of protein per fish head, using an average mass of 1480 g per fish head (Table 4.1). The amount of *Lophius vomerinus* caught in 2014 in South African and Namibian waters was 9 489 tonnes (FAO, 2016a). If the head is considered to account for 30 % of the total length of the fish (Caruso, 1983), and the mass of the head is approximated as 30 % of the total fish mass, the amount of protein that could be extracted from the monkfish head, based on catch data from 2014 (FAO, 2016a), is in the order of 230 tonnes. The value of the protein found in the monkfish head was significantly ($\alpha = 0.05$) lower than the value found by Prego et al., (2012) for the muscle in monkfish head, but the difference is expected as the raw head in this study was not limited to only the muscle of the monkfish head.

Although the total amount of protein available is in the order of 230 tonnes, as calculated based on annual catch data, each fish head contributes less than 10 % (wet mass) to this total amount. This means that the process of protein extraction should be tightly controlled to ensure maximum protein yield, and furthermore, the reaction conditions used should ensure preservation of the functionality and quality of the proteins. Temperatures below 70 °C, and reaction pH close to the natural pH of the raw material ($5 < pH < 9$) would be required in order for the proteins not to denature (Kristinsson & Rasco, 2000c). The most appropriate method used currently for extraction of protein from fish byproducts, meeting these requirements, is enzymatic hydrolysis (Kristinsson & Rasco, 2000c). Enzymatic hydrolysis has been proven successful by a number of different authors for the byproducts of many different fish species (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012; Daukšas, Falch, Šližyte, & Rustad, 2005; Ghaly et al., 2013; Guerard, Dufosse, Broise, & Binet, 2001).

Table 4. 2: Proximate composition of monkfish head (% wet basis)

	Monkfish	Ref 1*
Crude protein	8.19 ± 2.08	14.02 ± 5.1
Crude fat	2.61 ± 1.13	3.22 ± 0.29
Moisture	86.86 ± 2.01	84.09 ± 4.4
Dry matter	13.14 ± 2.01	
Ash	2.58 ± 0.68	

* Values for muscle in monkfish head (Prego et al., 2012) (g/kg muscle)
Mean ± SD; n = 6

The crude fat was found to be 2.61 ± 1.13 % of the total wet weight of minced fish, equating to 38 grams per average fish head. This value is comparable to referenced value of the monkfish heads (3.22 ± 0.29%) and indicates a low-fat content fish. The average value of the moisture in the fish head in this study was 86.86 ± 2.01 and the value is correlates well to that of the muscle found in the monkfish head as reported by Prego et al. (2012). The ash component is mostly made up out of bones and teeth, which the monkfish has a lot of as can be seen from Figure 4. 2.

Mineral content

As stated in the previous paragraph, the monkfish has many bones and teeth in the head and the mineral analysis is shown in Table 4. 3 below. The most abundant element found was calcium (51577 ± 13646 mg/kg dry sample) as expected as the whole head was analysed for minerals. The higher values of Fe (875 ± 577 mg/kg dry sample) and Zn (77 ± 10 mg/kg dry sample) observed in this study as compared to the values found be Prego et al. (2012) of 17.5 ± 7.4 and 29.5 ± 6.0 mg/kg dry muscle respectively, can be attributed to the fact that only the muscle in the monkfish head was analysed by Prego et al. (2012), while the whole head was analysed in this study.

The large variability of the results from the mineral analysis are pronounced, specifically in the case of Ca, K and Fe. The variability in results in this study is attributed to the sample and not the measuring technique, as the analysis was conducted in a laboratory where the accuracy and repeatability of the results are controlled on a regular basis and performed by experienced personnel. Possible sources of the sample variation include:

- Stage of maturity of fish,
- Season fish was spawned in,

- Spawn conditions such as food availability,
- The sex of the fish,
- Geographical location where fish was caught in

The sources of variation could influence the diet of the fish, the nutrient intake and development of the fish. If all the raw material were minced and homogenised at once, and a sample collected from the total batch of minced fish heads, the variability of the results might be reduced. Facility and equipment availability should be assessed prior to sample preparation, as room temperatures below 10 °C are required to ensure that the raw material is fresh. An industrial bowl cutter is recommended for mincing as the fish heads have large diameters and each weigh more than 1 kg (Table 4.1).

Table 4. 3: Values for mineral composition of monkfish (mg/kg dry sample)

Mineral	Value	Ref 1*
Macro elements		
Ca	51577 ± 13646	
K	9362 ± 668	
Fe	875 ± 577	17.5 ± 7.4
Zn	77 ± 10	29.5 ± 6.0
Micro elements		
Cu	6.08 ± 1.97	2.34 ± 0.73
Cr	2.38 ± 0.56	0.75 ± 0.11
Ni	0.98 ± 0.18	0.16 ± 0.03
Hg	0.95 ± 0.48	0.43 ± 0.03
Pb	0.40 ± 0.12	0.08 ± 0.05
Cd	0.92 ± 0.27	0.01 ± 0.003

*Values for muscle found in monkfish head (Prego et al., 2012)(mg/kg dry muscle)

Mean ± SD; n = 5

The values of the toxic elements, Pb, Cd and Hg were all higher than those found by Prego et al., (2012), but all still lower than the maximum as prescribed by the European Community Regulation (ECR) 466/2001 for lead (0.2 mg/kg wet mass), cadmium (0.05 mg/kg wet mass), and mercury (1.0 mg/kg wet mass). The toxic minerals present in the monkfish are dependent on the diet of the fish, and could be different for the same species caught but in a different geographical location. The analysis of heavy toxic elements in fish

is important when the raw material is considered for use in a feed application and should always be tested for prior to further processing.

Fatty acid analysis

The fatty acid analysis is shown in Table 4. 4. The order of most abundant fatty acid in terms of decreasing content in this study was: C18:1n9 > C22:6n3 > C16:0 > C20:5n3 > C16:1. Docosahexaenoic acid, DHA, (C22:6n3), and eicosapentaenoic acid, EPA (C20:5n3), both polyunsaturated omega-3 fatty acids and considered healthy fatty acids, were present in the top 5 most abundant fatty acids.

Table 4. 4: Fatty acid content of monkfish head

Fatty acid abbreviation	Fatty Acid composition (% of total FA))	Fatty Acid in sample (mg FA/g sample)
C11:0	0.23 ± 0.09	0.06 ± 0.01
C13:0	0.1 ± 0.06	0.02 ± 0.0003
C14:0	1.23 ± 0.28	0.37 ± 0.26
C15:0	0.12 ± 0.10	0.04 ± 0.05
C16:0	16.28 ± 0.38	4.58 ± 2.56
C18:0	5.74 ± 2.11	1.45 ± 0.28
C21:0	1.32 ± 0.75	0.31 ± 0.01
C22:0	0.49 ± 0.07	0.13 ± 0.05
C24:0	1.26 ± 0.68	0.30 ± 0.0001
C14:1	0.24 ± 0.14	0.06 ± 0.002
C16:1	6.77 ± 2.19	2.06 ± 1.63
C18:1n9c	21.32 ± 1.40	6.06 ± 3.6
C18:1n9t	0.31 ± 0.01	0.09 ± 0.05
C20:1	3.87 ± 0.65	1.03 ± 0.4
C24:1	1.93 ± 0.81	0.48 ± 0.06
C18:2n6c	3.68 ± 2.03	0.87 ± 0.01
C18:2n6t	0.40 ± 0.22	0.10 ± 0.0003
C18:3n3	1.49 ± 0.50	0.38 ± 0.08
C20:2n6	1.90 ±0.02	0.05 ± 0.02

C20:3n3	1.44 ± 0.75	0.35 ± 0.01
C20:4n6	3.06 ± 0.69	0.8 ± 0.27
C20:5n3	8.05 ± 3.14	2.49 ± 2.09
C22:6n3	20.48 ± 2.08	5.88 ± 3.66
SFA	26.78 ± 3.00	7.26 ±3.20
MUFA	34.43 ± 1.99	9.77 ± 5.74
PUFA	38.79 ± 1.01	10.92 ± 6.12
PUFA:SFA	1.46 ± 0.20	1.46 ± 0.20
n-6	7.33 ± 2.95	1.83 ± 0.28
n-3	31.46 ± 3.96	9.09 ± 5.85
(n-6)/(n-3)	0.24 ± 0.12	0.24 ± 0.12
TFA	100	2.80 ± 1.50

Mean ± SD, n = 2

The monkfish head only contains about 2.61% (wet basis) of crude fat as indicated in Table 4.2 but the fat is of good nutritional quality. A high proportion of the oil consists of polyunsaturated fatty acids (38.79 % of total FA). In terms of nutritional quality, the polyunsaturated fatty acids, which include the n-3 fatty acids, are most important. There has recently been more interest in two particular n-3 fatty acids, namely DHA and eicosapentaenoic acid, EPA (C20:5n3) as these n-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) have been reported to be beneficial to human health and essential in aquaculture diets. The benefits of a human diet enriched with DHA and EPA include a reduced the risk of cardiovascular disease, reduced blood pressure and the FA contributes to functions within the nervous system (Ghaly et al., 2013; Prego et al., 2012). In aquaculture the n-3 and n-6 FA are essential for maintaining cell membrane structure, and cell metabolism (Miller et al., 2008), and contributes to eicosanoid functions such as inflammatory responses, blood clotting, and aids in neural development (Sargent et al., 1999).

Amino acid composition

The amino acid composition of monkfish head and that of white fish meal is shown in Table 4. 5. The amount of EAA in the raw substrate was 43.77 ± 3.49 % of the total amino acid. All essential amino acids (EAA) were present in the raw substrate, except for tryptophan. Tryptophan is an acid labile amino acid (MacKenzie, 2009, p. 30), which is destroyed during acid hydrolysis for sample preparation. If additional analysis was to be conducted to determine the tryptophan content, sample preparation could be done by

using an alkaline solution instead of an acid solution for hydrolysis. Tryptophan would be expected to be present in the amino acid profile of the monkfish, in amounts lower than 1 % of the total amino acid (Chalamaiah et al., 2012).

Table 4. 5: Amino acid composition of monkfish heads

Amino acid name	% of total amino acid content	Ref 2* % of total amino acid content
Essential		
Arginine	8.17 ± 0.29	7.0
Histidine	2.03 ± 0.22	2.2
Isoleucine	3.78 ± 0.43	4.0
Leucine	7.07 ± 0.69	7.0
Lysine	6.18 ± 0.51	7.5
Methionine	3.01 ± 0.29	2.8
Phenylalanine	4.35 ± 0.57	3.6
Threonine	4.44 ± 0.26	4.2
Valine	4.74 ± 0.23	4.9
Non-essential		
Alanine	6.72 ± 0.46	6.9
Aspartic acid	8.01 ± 0.40	9.3
Cysteine	0.42 ± 0.05	1.0
Glutamic acid	12.1 ± 0.45	13.9
Glycine	13.8 ± 1.86	10.8
Proline	6.54 ± 0.64	5.8
Serine	5.36 ± 0.13	5.2
Tyrosine	3.34 ± 0.63	2.8
Σ EAA	43.77 ± 3.49	
Σ NEAA	56.29 ± 4.62	

*Values for white fishmeal (Windsor, 2001)

Mean ± SD; n = 5

The amino acid composition is a factor influencing the properties of fish protein hydrolysate when fish byproducts are hydrolysed with enzymes (Halldorsdottir, Sveinsdottir, Gudmundsdottir, Thorkelsson, & Kristinsson, 2014). White fishmeal is currently used as fish protein source in animal feeds (Ghaly et al., 2013). The values of the amino acids for raw monkfish head compared well to that of white fishmeal. The presence of all the EAA shows that the products from monkfish head processing could be a potentially viable source of protein for animal feed application, if the functionality and quality of the amino acids within the protein could be preserved. The processing of the raw material, such as protein extraction through enzymatic hydrolysis, could cause a decrease in the quantity of amino acids in the final product, hence in order to preserve the quantity and quality of the amino acids, the processing procedure should be fixedly controlled, specifically in terms of temperature. During processing, excessive heating would be disadvantageous to the protein, as unfolding of the protein takes place when heated above 70 °C, which leads to the hydrophobic cores of the proteins to be uncovered (Opheim et al., 2015). The hydrophobic cores pack together inside the protein molecule which causes a reduction in the amount of soluble amino acids, which attribute to the nutritional quality of the fish protein hydrolysate product produced from enzymatic hydrolysis (Opheim et al., 2015).

Conclusion

This study has shown that the heads of the monkfish species *Lophius vomerinus*, found in South African and Namibian waters, has the potential to be used in animal feed applications. Nutrients found in the monkfish head were essential amino acid rich-proteins, n-3 fatty acids, and Ca and K. The proteins found in the monkfish head contained more than 40 % essential amino acids, the important fatty acids DHA and EPA were abundant in the monkfish oil, and toxic minerals (Pb, Cd, Hg) were present in low concentrations in the raw material, which are motivating factors in considering the nutritional quality of the raw monkfish head. It was estimated that if the protein could be extracted carefully in a controlled process, a possible amount of 230 tonnes of protein could be valorised from the monkfish head. Enzymatic hydrolysis could be used to extract the protein without compromising the quality and functional properties thereof, as proteins are denatured at excessive temperatures and alkaline or acidic pH values.

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Chapter 5: Research results in article format

This chapter is written in article format that describes the research results found in this study.

This is the second article in this investigation and details the optimisation of enzymatic hydrolysis of the raw monkfish head substrate by varying reaction pH, temperature and using two enzymes: bromelain and alcalase. The determination of pK for use in the equation to calculate the DH for the pH-stat method is investigated for the enzyme/substrate combinations used in this study. Lastly, the products from hydrolysis are tested for functional properties.

Optimisation of enzymatic hydrolysis of monkfish (*Lophius vomerinus*) heads using pH-stat method for preparing fish hydrolysate products

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Abstract

The monkfish head makes up approximately 30% of the total fish and is currently processed into relatively low-value fish meal or even discarded. In this study, the extraction of protein from the monkfish head was achieved using enzymatic hydrolysis. The reaction conditions for enzymatic hydrolysis were optimised, using two industrial proteases: alcalase 2.4L (EC 3.4.21.62), a strain of *Bacillus licheniformis*, and bromelain 2500 (EC 3.4.22.32) from pineapple stem. Temperature and pH were varied using a mixed 2 and 3 level factorial design to reach a controlled degree of hydrolysis of 16% in the shortest reaction time. Optimum reaction conditions were found to be 62 °C and a pH of 8.2 for alcalase – achieved in a reaction time of 7.2 minutes, and 65 °C and a pH of 7 for bromelain – achieved in 12.7 minutes. In this study the degree of hydrolysis (DH) was determined using the pH-stat method, which required values for the pK of the substrate/enzyme system. The relationship between pK and pH for monkfish/alcalase and bromelain/alcalase was investigated by comparing base consumption of raw and hydrolysed samples, and was successfully determined for the alcalase/monkfish system. The pK value could not be determined for the bromelain/monkfish system and literature values were used for hydrolysis optimisation experiments. The hydrolysate product was found to have functional food properties in terms of emulsion stability, fat absorption, antioxidant activity and foaming capacity. All essential amino acids were found in the hydrolysate product, amounting to 49.5% of the total amount of amino acids, indicative that the hydrolysate product has potential for application as animal feed ingredient.

Keywords: Enzymatic hydrolysis, pH-stat, pK determination, monkfish, animal feed

Abbreviations:

DH - degree of hydrolysis; EAA - essential amino acids; EAI - emulsion activity index; ES - emulsion stability; FA - fat absorption; FC - foaming capacity; FPH - fish protein hydrolysate; NEAA - non-essential amino acids; SD - standard deviation; SE - standard error of the mean; TAA - total amino acids;

Introduction

The monkfish head represents a large proportion of the total catch, as the head accounts for 27.2 - 37.9 % of the total length of the fish (Caruso, 1983). In order for the monkfish head to be a viable source of protein for animal feed application, the proteins should be extracted from the raw material so the functional and nutritional properties of the protein are preserved. To this end, enzymatic hydrolysis has been shown to be an effective method of protein extraction from fish byproducts (Foegeding et al., 2002; Ghaly et al., 2013; Kristinsson & Rasco, 2000c). A thorough discussion of the literature is found in Chapter 2.

The aim of this study was to optimise the enzymatic hydrolysis reaction time of monkfish heads to a controlled degree of hydrolysis (DH) of 16 %. The objectives of this study were:

- Determine the pK dependence of the alcalase/monkfish and bromelain/monkfish systems in order to use the pH-stat technique for monitoring the DH.
- Varying reaction temperature between 45 °C and 65 °C, and reaction pH between 7 and 8.5 using alcalase 2.4L (EC 3.4.21.62) and bromelain 2500 (EC 3.4.22.32) in order to obtain a set of reaction parameters in which the shortest reaction time is achieved.
- Determine if the hydrolysate products could be used as animal feed ingredient by analysing the amino acid content using liquid chromatography mass spectrometry (LC-MS-MS).
- Determine if the hydrolysate products could be used in other food products by conducting a functional food property analysis where emulsion stability, fat absorption, foaming capacity, antioxidant activity, and emulsification activity index were evaluated.

Materials and Methods

Materials

Fresh monkfish heads from the fish processing facility of Irvin & Johnson (I&J) at V&A Waterfront, (Cape Town, South Africa) were collected and transported on ice to the University of Stellenbosch in November 2014, May 2015 and May 2016. The chemicals used in this study and the suppliers, are listed in Table 5.1.

Table 5. 1: List of chemicals used in experimental work and the suppliers

Component	Concentration	Supplier
Alcalase (EC 3.4.21.62)	2.4L	Novozymes
Bromelain	2500	Bromelain Enzyme, PT
Canola oil		
Deionised water		
Ferric chloride	0.05 M	Sigma Aldrich
Hydrochloric acid	1 M	Merck
N,N-dimethyl-p-phenylenediamine		Sigma Aldrich
Salt		
Sodium acetate	0.1 M	Sigma Aldrich
Sodium dodecyl sulfate	0.1% (w)	Sigma Aldrich
Sodium hydroxide	1 M	Sigma Aldrich

Raw material preparation

The monkfish heads were prepared for further analysis by cleaning and homogenising as shown in Figure 5.1.

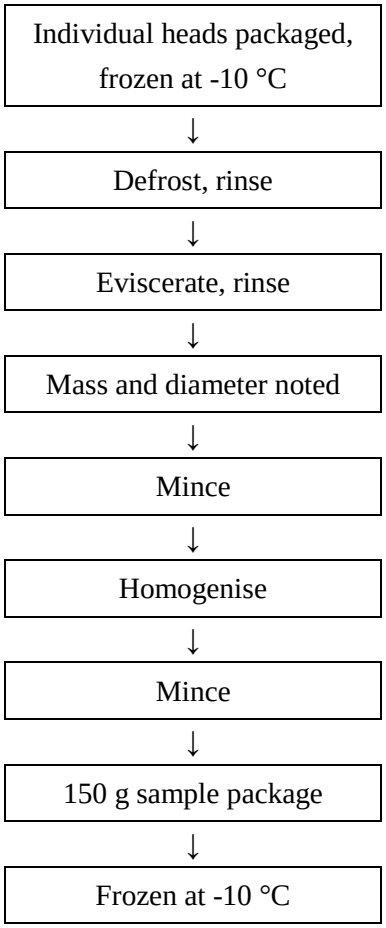

Figure 5. 1: Flow diagram of raw monkfish preparation

pK-determination

pK is the logarithmic value of the equilibrium constant for the deprotonisation of the amide group (Camacho et al., 2001). The pK value needs to be determined in order to calculate the α-value, or mean degree of dissociation, in the Equation 5.1 (Adler Nissen, 1986: p 91) for DH as described in the pH-stat method.

$$DH\ (\%) = \frac{B\ N_B}{\alpha\ M_p\ h_{tot}} \times 100\ \% \quad (5.1)$$

In Equation 5.1, B is the volume of base added (ml), α the average degree of α-amino group dissociation, M_p the mass protein contained in substrate (g), N_B the normality of the base and h_{tot} the equivalent moles of peptide bonds per gram of protein (meq/g). The equivalent number of peptide bonds per unit mass of protein (h_{tot}) was determined by Adler-Nissen (1986) for many substrates such as casein, meat, haemoglobin, fish protein and wheat gluten. The value of h_{tot} for fish protein of 8.6 meq/g was found by Adler-Nissen (1986) by using data from amino acid analyses and determining the sum of millimols of individual amino acids per gram of protein.

The α value is dependent on the pK of the reaction (Kristinsson & Rasco, 2000c) as described by Equation 5.2 (Spellman et al., 2013):

$$\alpha = \frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} \quad (5.2)$$

The value of the pK is dependent on the specific enzyme/substrate combination (Camacho et al., 2001). The method of determining the pK for a specific substrate and enzyme combination as proposed by (Camacho et al., 2001) was used. With this technique the amount of base required to titrate a sample to a pH of 10 is determined experimentally. Samples were subjected to 20% DH (b_{x20}), 10% DH (b_{x10}) or unhydrolysed (b_s) prior to titration. The DH of the hydrolysed samples were monitored by the pH-stat method, where literature values for pK, as described by Adler Nissen (1986: p 91) were used for fist approximation. Hydrolysis conditions for the pK-determination experiments were at a pH of 8 and a temperature of 50 °C, based on the conditions used by Camacho et al., (2001) and operating conditions recommended by the enzyme suppliers.

Figure 5.2 shows the process followed to determine the pK value in flow diagram format.

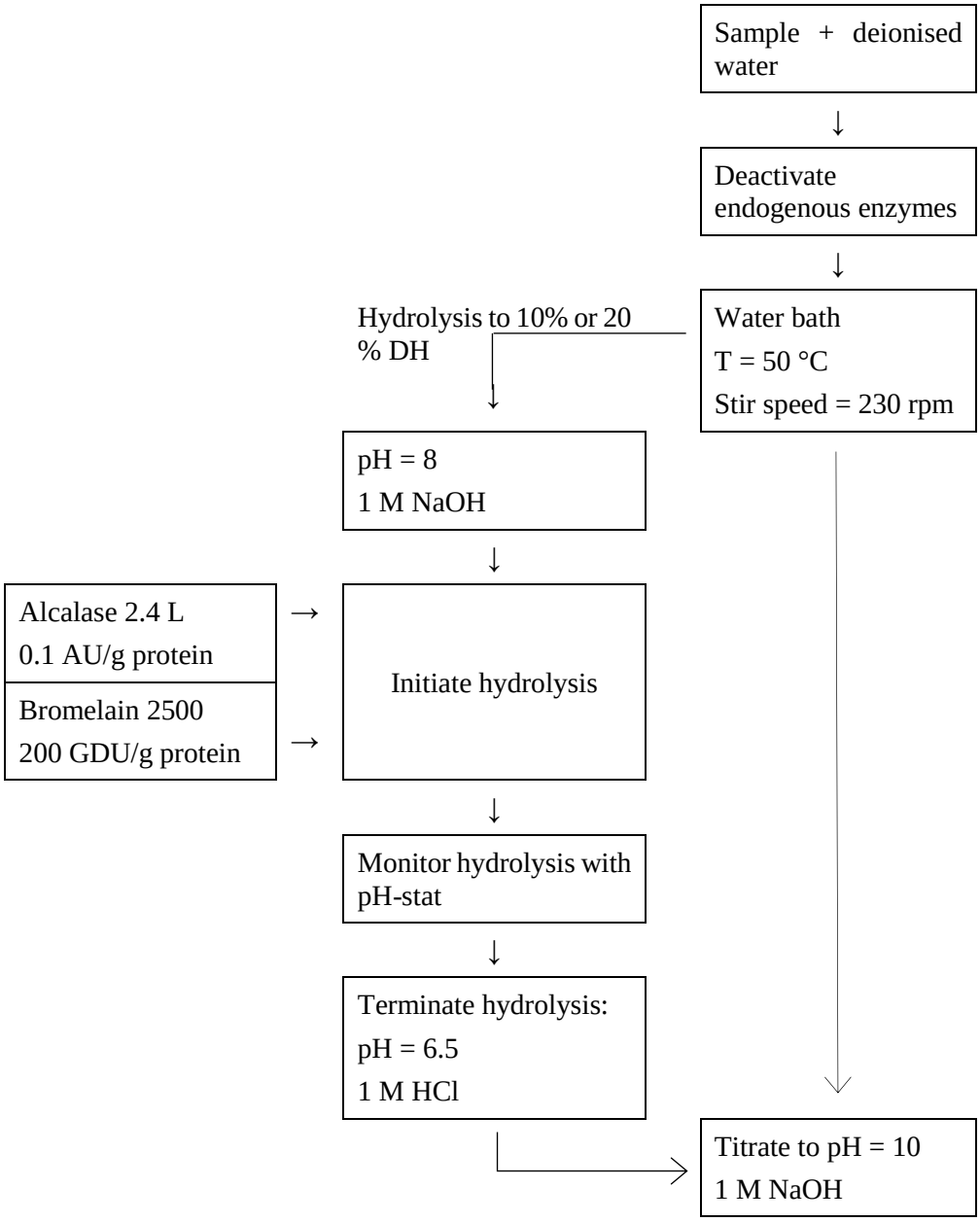

Figure 5. 2: Flow diagram of pK determination method

Raw material of mass 30.5 ± 1.5 g (wet basis) was weighed into a 500 ml glass beaker and 30 ± 1 ml deionised water was added to the fish for a 1:1 (w:v) substrate to water ratio (Šližytė et al., 2005). The beaker with the mixture was immersed into a 1200 ml beaker filled with 600 ml tap water and both beakers were heated using a hot plate. The mixture was heated to 90 °C and kept at this temperature for 15 minutes to deactivate endogenous enzymes (Guerard, Dufosse, Broise, & Binet, 2001; Nguyen et al., 2011; Šližyte

et al., 2005). The raw material-water mixture was placed in a water bath at 50 °C and continuously agitated using a Heidolph RZR overhead mixer at 230 rpm. The pH of the mixture was adjusted to 8 with 1 M NaOH. When the temperature and pH were stable, the enzyme was added to the reaction mixture to initiate hydrolysis. Prior screening experiments (data not shown) indicated that the rate of initial hydrolysis were independent of the enzyme concentration at enzyme to substrate ratio (E/S) above 0.1 Anson unit (AU) per gram protein for alcalase and 200 gelatin digestion units (GDU) per gram protein for bromelain. These values for E/S were used for all subsequent hydrolysis reactions. The raw material-water mixture was hydrolysed to either to 10% DH or 20% DH. The DH was monitored using the pH-stat method, using a pK value as recommended by Adler-Nissen (1986, p. 91).

After hydrolysis, the hydrolysed sample was titrated to a pH of 10. When the required DH had been reached (10% or 20%), the pH of the reaction mixture was lowered to 6.5 with 1 M HCl. When the pH was stable at 6.5, the reaction mixture was titrated to a pH of 10 using 1 M NaOH while recording the volume of NaOH used, and the pH of the mixture as titration progressed.

The base consumption of a non-hydrolysed sample was also determined by titrating the raw material-water mixture at its natural pH, (6.8 ± 0.2) immediately after the mixture reached a temperature of 50 °C. Equivalent base consumption was calculated using Equation 5.3 where b-equivalent base consumption (mol), V_B-volume of base (l), C_B-concentration of base (M), V_0-initial volume of mixture, pI-ionic product of water (Camacho et al., 2001). A complete explanation and mathematical description of the procedure is outlined in Appendix A. Each experiment was replicated twice and experiments were run in random order.

$$b = \frac{V_B C_B}{V_0} - \left(\left[1 + \frac{V_B}{V_0}\right] \times 10^{pH - pI} - 10^{pH_0 - pI}\right) \quad (5.3)$$

Enzymatic hydrolysis

The enzymatic hydrolysis reactions were performed as described in Figure 5.2 with the following exceptions: A sample of 50.5 ± 1.5 g (wet basis) raw material and 50 ± 1 ml deionised water was used. The reaction progression was monitored using the pH-stat technique, with the calculated pK value for the alcalase/monkfish system and the literature value (Adler Nissen, 1986: p91) for the bromelain/monkfish system. All hydrolysis runs were performed until a predetermined DH of 16% was reached. Hydrolysis was terminated by heating the hydrolysed mixture to 95 °C for 5 minutes on a hot plate to deactivate the exogenous enzyme. The sample was left to cool, centrifuged at 3 000 x g for 20 minutes and the water

phase FPH was separated from the sediment. The FPH and sediment were poured into separate 50 mℓ plastic containers with screw caps and frozen at -10 °C until used for further analysis.

Optimisation of hydrolysis conditions

The process parameters are indicated in Table 5.2 below. Hydrolysis parameters were investigated and optimised using a mixed 2 and 3 level factorial design and experiments were replicated 4 times. Experiments were performed in a random order, as is standard experimental practice.

Reaction parameters were chosen based on available literature values and method constraints. The recommended reaction conditions, based on literature, for alcalase were temperatures between 55 - 70 °C and pH values between 6.5 and 8.5 depending on the substrate used (Bhaskar et al., 2008; Šližytė et al., 2005). The literature recommended reaction parameters for bromelain were pH values between 4 and 9 and temperatures between 20 - 65 °C (Fellows, 2009, p. 259). The pH-stat method is restricted to operating only at alkaline pH, as amino groups within the protein are deionised under alkaline pH, and protons resulting from the amino group deionisation causes pH to decrease (Rutherfurd, 2010). The values chosen for the independent factors are shown in Table 5. 2, based on the literature recommended values and the pH limitations if the pH-stat method is used. The optimum conditions were defined as those that resulted in the predetermined DH of 16% in the shortest hydrolysis time. A second set of experiments were conducted for the bromelain/monkfish system at pH 7 and 55 and 65 °C as literature indicated that bromelain had a large recommended pH range down to values of 4, but due to the pH limitations in this study, the lowest pH that could be investigated was pH 7.

Table 5. 2: Independent factors used for enzymatic hydrolysis investigation

Factor	Alcalase/monkfish system Exp. set 1	Bromelain/monkfish system Exp. set 1	Bromelain/monkfish system Exp. set 2
Temperature (°C)	45 55 65	45 55 65	55 65
pH	7.5 8 8.5	7.5 8 8.5	7
Enzyme	Alcalase 2.4L EC 3.4.21.62 0.1 AU/g protein	Bromelain 2500 EC 3.4.22.32 200 GDU/g protein	Bromelain 2500 EC 3.4.22.32 200 GDU/g protein

Functional property testing of FPH

Fish protein hydrolysate was prepared by centrifuging the hydrolysate product at 3 000 x g for 20 minutes and separating the top light yellow liquid layer of FPH and the dark, viscous sediment. The functional properties for the FPH tested were emulsion stability, fat absorption, antioxidant activity and foaming capacity and each for the FPH was replicated once (Table 5.3). Three bromelain/monkfish FPH and three alcalase/monkfish FPH samples were chosen for functional property tests. Based on sample availability, each sample was chosen so that treatments at different temperatures and pH could be investigated.

Table 5. 3: Reaction conditions under which FPH was produced

Sample	Enzyme	Temperature (°C)	pH
FPH 1	A	45	8.5
FPH 2	A	65	7.5
FPH 3	B	65	8
FPH 4	B	45	8
FPH 5	A	55	7.5
FPH 6	B	55	7.5

Emulsion stability (ES)

The procedure as described by Pires et al. (2013) was used to determine emulsion stability. 5 ml of canola oil, and 5 ml of 0.1 M NaCl solution was added to 5 ml of FPH in a graduated cylinder. The mixture was vortexed for 2 minutes. The volume fraction of each phase present was noted directly after shaking and again 15 minutes after it was left to stand at ambient conditions. The emulsion stability was determined by using Equation 5.4 (Pires et al., 2013):

$$ES\ (\%) = \frac{\text{Total volume (ml)- water phase volume (ml)}}{\text{Total volume (ml)}} \times 100 \qquad (5.4)$$

Fat absorption (FA)

The technique used to determine FA was described by Pires et al. (2013). 1 gram of liquid FPH was weighed into a centrifuge tube and 30 ml of canola oil was added to the centrifuge tube thereafter. The

contents of the centrifuge tube was mixed with a vortex shaker and left to stand at ambient conditions for 30 minutes. The mixture was then centrifuged at 3 000 x g for 25 minutes and free oil was immediately decanted after centrifugation. The mass of the final sample was weighed after decanting the oil to calculate the mass of oil that a sample of 1 gram of FPH absorbed.

Foaming capacity (FC)

Foaming capacity was measured according to the method by Pires et al. (2013), where the volume of foam formed immediately after 10 ml FPH was vortexed for 3 minutes was measured. FC was expressed as the foam volume per gram of protein in the 10 ml FPH.

Antioxidant activity

The antioxidant activity was determined using N,N-dimethyl-p-phenylenediamine (DMPD) as reagent. The method as described by Fogliano et al. (1999) was used. The DMPD reagent was prepared by dissolving 209 mg of DMPD in 10 ml of demineralised water. 1 ml of the DMPD reagent and 0.2 ml of 0.05 M ferric chloride solution was added to 100 ml of a 0.1 M sodium acetate solution. The absorbance of this final solution was measured at 505 nm in a plastic cuvette and with an AE-S60-4U UV VIS spectrophotometer for the value of A_0. Samples were prepared by diluting 500 mg of liquid FPH in 10 ml demineralised water. 50 µl of sample was added to 2 ml of DMPD reagent mixture and shaken. After 10 minutes the absorbance of the mixture (A_f) was measured at 505 nm with an AE-S60-4U UV VIS spectrophotometer and plastic cuvette. The antioxidant activity was determined by Equation 5.5 (Fogliano et al., 1999):

$$\text{antioxidant activity (\%)} = \left(1 - \frac{A_f}{A_0}\right) \times 100 \qquad (5.5)$$

Functional food property testing of hydrolysis sediment

The sediment, or sludge fraction, was analysed prior to functional property testing to determine if the sediment had nutritive value. A proximate analysis and amino acid analyses was used to this end. After the analysis, the functional food properties of the sediment was evaluated. Properties that were tested include emulsion stability, fat absorption, antioxidant activity and emulsion activity index (Table 5.4). The sediment in this study did not exhibit foaming properties when tested (data not shown), and therefore an

additional emulsion property, in this case the emulsion activity index (EAI), was tested on the sediment. The ES, FA and AA were determined as described for the FPH, and the method for determining the EAI is described in the paragraph below. Three alcalase/monkfish sediment and three bromelain/monkfish sediment samples were chosen for functional property tests. Based on sample availability, each sample was chosen so that treatments at different temperatures and pH could be investigated.

Table 5. 4: Reaction conditions under which sediment was produced

Sample	Enzyme	Temperature (°C)	pH
S 1	A	45	8
S 2	B	55	8.5
S 3	B	45	8
S 4	A	55	8.5
S 5	A	65	7.5
S 6	B	65	7.5

Emulsion activity index (EAI)

The EAI is reported in units of interface area stabilised per unit mass of protein (m^2/g) and is determined by Equation 5.6 (Pearce & Kinsella, 1978):

$$\text{EAI}\left(\frac{m^2}{g}\right) = \frac{2 \times 2.303 \times A_{500}}{\Phi \times c \times L} \tag{5.6}$$

The A500 term is the absorbance of the sample measured at 500 nm, Φ is the volume fraction of the oil phase, c (g/m^3) the concentration of fish protein hydrolysate in the aqueous solution and L (m) is the path length through the cuvette. The method as described by Pires et al. (2013) was used to determine EAI. To 10 ml of sediment, 3.5 ml of canola oil was added and the mixture was homogenised with a vortex shaker for 2 minutes. 1 ml of a 0.1% (by mass) solution of sodium dodecyl sulfate was added to 10 ml of FPH-oil emulsion and shaken. The absorbance of this mixture at 500 nm was measured in a plastic cuvette with an AE-S60-4U UV VIS spectrophotometer and the emulsifying activity index was calculated by Equation 5.6.

Analytical testing of sediment

Proximate analysis

A sample of 50 g (wet basis) raw material was used for the proximate analysis. The proximate analysis was conducted by the Department of Animal Science of the University of Stellenbosch. Total nitrogen, moisture content, dry matter, ash content, crude fat content and fatty acid composition was determined using the standard protocol of the AOAC (1997). Total nitrogen content was determined by using the Dumas method (Jung et al., 2003) with a Leco 528 elemental analyser. The total crude protein was calculated by multiplying the percentage of nitrogen in the substrate by 6.25. Moisture content was determined by drying the sample in an oven at 100 °C and the expressed as the loss in mass in the sample after drying. Dry matter was the percentage substrate left after drying. The ash content was quantified by heating the substrate in a furnace at 500 °C for 6 hours and measuring the remaining mass. A chloroform/methanol mixture was used to extract the crude fat out of the substrate in order to determine crude fat content.

Amino acid profile

Amino acid analysis was performed by the Central Analytical Facility, Department of Mass Spectrometry at the University of Stellenbosch. A volume 10 µl of a fully hydrolysed FPH sample was added to the constituents in the AccQ Tag Ultra Derivatisation Kit (Waters Corporation, Milford, USA), the sample was placed in a heating block at a temperature of 55 °C for 10 minutes. The amino acid profile was measured with a Waters API Quattro Micro mass spectrometer.

Statistical analysis

Analysis of variance (ANOVA) were determined in Statistica v12 software (StatSoft, Inc.) for hydrolysis optimisation data and functional food property data. A distance weighted least squares fit model in Statistica was used for identifying optimum hydrolysis conditions. Differences in means were considered significant when $p < 0.05$.

Results and Discussion

pK determination

Results for the equivalent base consumption for raw substrate (b_s), and samples hydrolysed to 20% (b_{x20}) and 10% (b_{x10}) for the alcalase/monkfish system are shown in Figure 5. 3 and for bromelain monkfish in Figure 5.4. The graph of b_s as a function of pH starts at the natural pH of the monkfish substrate (6.8 ± 0.2), as the method requires the titration of the raw substrate to start at an unaltered pH (Camacho et al., 2001).

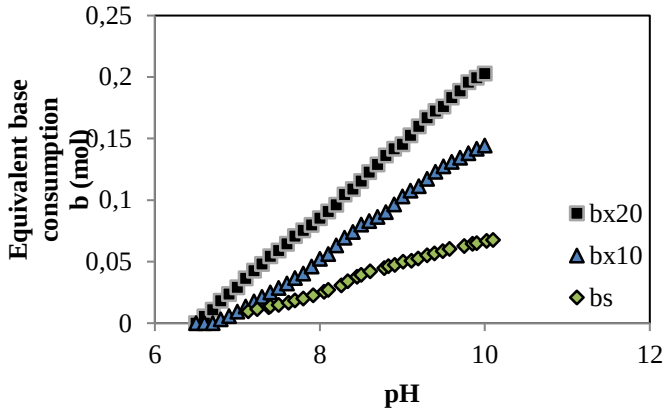

Figure 5. 3: Equivalent base consumption (b) during titration from pH 6.5 to pH 10 of raw substrate (bs), sample hydrolysed to 10 % DH (bx10) and sample hydrolysed to 20 % DH (bx20) for alcalase/monkfish system

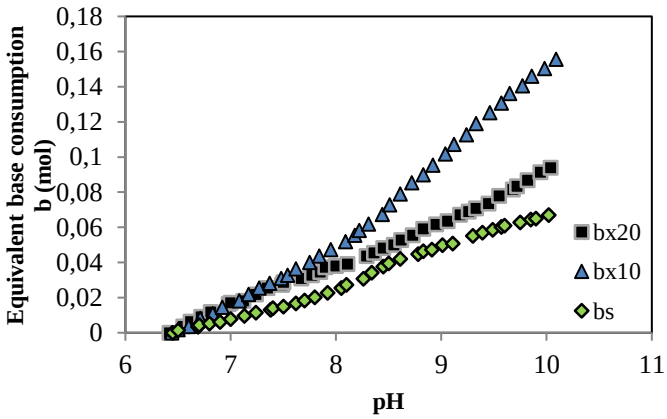

Figure 5. 4: Equivalent base consumption (b) during titration from pH 6.5 to pH 10 of raw substrate (bs), sample hydrolysed to 10 % DH (bx10) and sample hydrolysed to 20 % DH (bx20) for bromelain/monkfish system

The cumulative base consumption for the alcalase/monkfish sample hydrolysed to 20 % (b_{x20}) was 0.174 ± 0.031 mol and 0.123 ± 0.022 mol for the sample hydrolysed to 10%. The amount of base required for the raw substrate (b_s) to reach a pH of 10 was 0.056 ± 0.008 mol. The results of the base consumption of the substrate hydrolysed with bromelain is presented in Figure 5. 4. The base consumption of the sample hydrolysed to 20 % DH in the bromelain/monkfish system was low (0.094 ± 0.006 mol) in comparison to the bromelain/monkfish sample hydrolysed to 10 % DH (0.155 ± 0.009 mol).

The dimensionless base consumption in the form of $\frac{b_x - b_s}{(b_x - b_s)_{max}}$ (Camacho et al., 2001)(Appendix A) was determined for b_{x20} and b_{x10} and is presented in Figure 5. 5 for the alcalase/monkfish system. The values were used to determine the dependence of pK on pH as described in detail in Appendix A. The dimensionless base consumption for b_{x10} and b_{x20} for alcalase as depicted in Figure 5. 5 showed that the two graphs followed the same trend but do not coincide to form a single curve until a pH value of 9 is reached. According to Camacho et al. (2001), if the curves do not coincide it could imply that the mean pK value depends on the degree of hydrolysis in addition to depending on pH. The difference between the two graphs in Figure 5. 5 could possibly be due to minor deviations between substrates in terms of amino acid sequences or specific amino acid concentration differences. The relationship between pK and pH for the alcalase/monkfish system was determined as

$$pK = 7.02 + 0.668\ (pH - 7.02) \qquad (5.7)$$

The dimensionless base consumption for the bromelain/monkfish system is given in Figure 5. 6. The curve for the 20 % hydrolysed sample b_{x20} shows that the dimensionless base consumption increases with pH initially, becomes constant between pH 7.6 and 7.9, and then decreases from pH 7.9 up to a pH of 8.6 and then finally increases until the pH of 10 is reached. Dimensionless base consumption for the sample hydrolysed to 10 % does not show the same trend as b_{x20}.

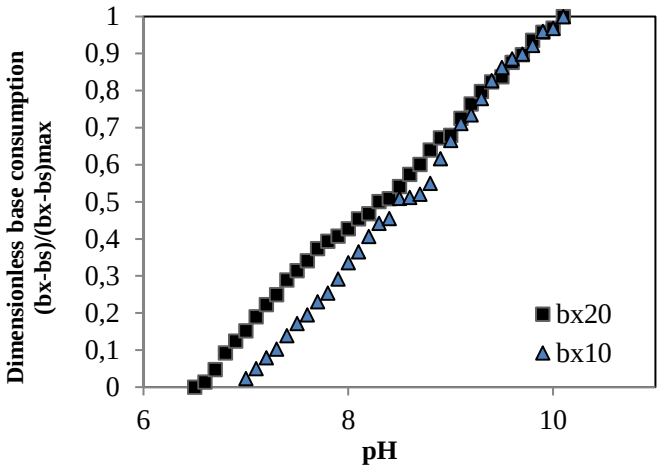

Figure 5. 5: Titration of α-amino groups released at 10 % DH (bx10) and 20 % DH (bx20) for the alcalase/monkfish system. Difference between base consumption for hydrolysed and raw sample (bx – bs)

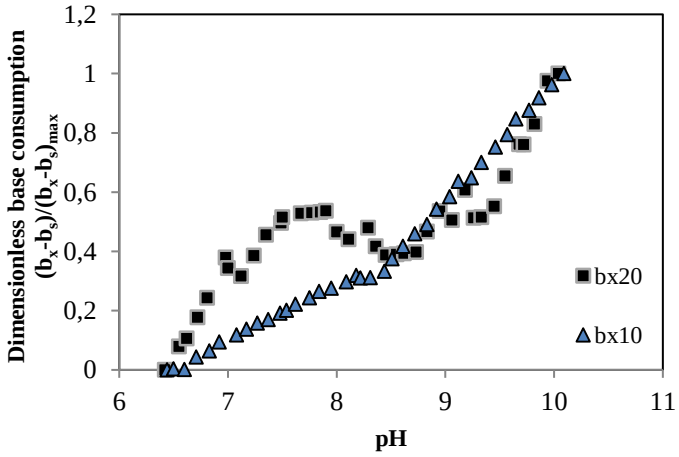

Figure 5. 6: Titration of α-amino groups released at 10 % DH (bx10) and 20 % DH (bx20) for the bromelain/monkfish system. Difference between base consumption for hydrolysed and raw sample (bx – bs)

The base consumption for the bromelain/monkfish system did not give typical results expected from literature by Camacho et al. (2001) or from the experimental results of alcalase/monkfish in this study. Firstly, the base consumption for the sample hydrolysed to 20% (b_{x20}) is lower than the base consumption for the sample hydrolysed to 10% (b_{x10}) for the bromelain system, and this occurred for all the replicates measured. A protein sample hydrolysed to a higher DH should have produced more protonisable groups than a sample hydrolysed to a lesser extent, which would mean that more base would be needed to titrate the b_{x20} sample to a pH of 10. This was observed during hydrolysis using the alcalase/monkfish system of the current study, and in the study of Camacho et al. (2001) and a deviation from this could indicate that

pK is dependent on the DH as well (Camacho et al., 2001). Secondly, when inspecting the graph of dimensionless base consumption $\frac{b_x - b_s}{(b_x - b_s)_{max}}$ against pH in Figure 5. 6, it is apparent that b_{x20} exhibits a different dependence on pH than b_{x10} does, especially from a pH of 7.6 to 8.6 where the dimensionless base consumption for b_{x20} decreases. Thus from the points highlighted here, there is an indication that monkfish samples hydrolysed to 20% have unexpected values of base consumption, or amount of protonisable groups formed during hydrolysis, and that this observation is very pronounced at pH values between 7.6 and 8.6. Hydrolysis of the samples in this study took place at a pH of 8. Even though values of optimum pH between 4 and 9 (Aspmo et al., 2005; Fellows, 2009, p. 259) are reported, it could be considered that the combination of bromelain/monkfish system has an optimum pH which isn't conducive to the pH used for hydrolysis in this investigation (pH 8) to determine pK. Protein hydrolysis with bromelain requires the presence of a nucleophilic cysteine side chain from a free sulfydryl group to act as an active site for catalytic reaction (Amid et al., 2011; Bock, 2015, p. 191). Studies on addition of fresh enzyme during the course of hydrolysis (Guerard et al., 2001) suggested that as hydrolysis progressed, the amount of available peptide bonds for hydrolysis decreased and that enzymes partially deactivated during the course of the reaction. It could be possible that the reaction pH in this study, in combination with a DH of 20%, influenced the catalytic reaction negatively so that a sample hydrolysed to a value greater than 10% DH could inhibit the catalytic activity of bromelain. The pK experiments were not repeated at different pH values in this study and consequently the value as recommended by Adler-Nissen (1986, p. 91) for pK was used for bromelain for the α-value in Equation 5.1 for DH determination by the pH-stat technique.

Enzymatic hydrolysis

The summary of the enzymatic hydrolysis reaction parameters to reach the set value of a DH of 16 % is shown in Table 5. 5. All investigated parameters namely temperature and pH, were found to have a significant effect ($p < 0.05$) on the enzymatic hydrolysis reaction time and DH. The optimum conditions for protein hydrolysis using alcalase was achieved at 65 °C and a pH of 8, and resulted in a total hydrolysis time of 13.26 ± 0.63 minutes. The longest reaction time when alcalase was used was found at a pH of 7.5 and a temperature of 45 °C and was 82.49 ± 10.83 minutes.

Table 5. 5: The influence of enzyme, temperature and pH on hydrolysis reaction time

Enzyme*	Temperature (°C)	pH	Time (min)
	Experimental set 1		
A	45	7.5	82.49 ± 10.83
A	55	7.5	36.15 ± 3.19
A	65	7.5	19.30 ± 1.75
A	45	8	58.27 ± 8.01
A	55	8	21.32 ± 3.19
A	65	8	13.26 ± 0.63
A	45	8.5	34.57 ± 2.93
A	55	8.5	17.96 ± 2.57
A	65	8.5	18.86 ± 3.08
B	45	7.5	182.9 ± 2.90
B	55	7.5	75.4 ± 12.34
B	65	7.5	61.7 ± 10.06
B	45	8	138.37 ± 18.33
B	55	8	75.68 ± 15.59
B	65	8	98.73 ± 28.80
B	45	8.5	114.2 ± 9.55
B	55	8.5	86.79 ± 16.09
B	65	8.5	82.80 ± 9.49
	Experimental set 2		
B	55	7	31.67 ± 9.21
B	65	7	13.18 ± 1.20

*A - Alcalase; B – Bromelain

Values are mean ± SE; n = 5

The shortest reaction time achieved in experimental set 1 for the bromelain/monkfish system was 61.7 ± 10.06 minutes at 65 °C and a pH of 7.5. The longest reaction time found in experimental set 1 that the bromelain/monkfish system yielded was 182.9 ± 2.90 minutes at 45 °C and a pH of 7.5. The data from experimental set 2 for bromelain/monkfish shows that the reaction time is significantly decreased at a pH 7. The shortest reaction time found for experimental set 2 was 13. 18 ± 1.20 minutes at 65 °C and a pH of 7.

The results of the enzymatic hydrolysis showed that the independent variables used in this study, namely temperature, pH and enzyme choice are all parameters that influence the hydrolysis reaction significantly ($p < 0.05$). This is consistent with the literature where other studies (Bhaskar et al., 2008; Gbogouri et al., 2004; Ovissipour et al., 2009) all found that hydrolysis was dependent on temperature, time, enzyme to substrate ratio and pH. After the pK determination experiments in this study, it was evident that samples hydrolysed with bromelain to 20% DH could possibly present enzyme activity decrease during the course of hydrolysis as is consistent with results found by the study by Aspmo et al. (2005) and consequently a lower value of 16 % DH was chosen as target DH. All the samples investigated in the bromelain/monkfish system could be successfully hydrolysed to 16 % DH, leading to the conclusion that if the bromelain/monkfish system would present partial enzyme deactivation or inhibited catalytic activity, it occurred at a DH greater than 16 %.

Although the enzymatic hydrolysis reaction parameter data is presented in tabular format as in Table 5. 5, with explicit values for reaction time, the graphs show the trends of reaction time better, with less explicit reaction time values. For this reason, the contour plots as well as the tabular data is presented in this paper.

A contour plot to show the trends of enzymatic hydrolysis reaction time in terms of temperature and pH, and was used to predict the optimal reaction parameters for the alcalase/monkfish system (Figure 5.7) and the bromelain/monkfish system (Figure 5.8). Figure 5.7 shows that the shortest reaction time for the alcalase/monkfish system would be found in a window between a pH of 8.5 and pH 7.7 and a temperature higher than 58 °C. The model predicts a reaction time shorter than 16 minutes within this optimum window. Figure 5.8 for the bromelain/monkfish system shows a saddle point between a pH of 8.2 and 8.5 and temperature between 58 and 61 °C. The plot indicates that there should be an optimum, where the reaction time would be shortest, at a temperature between 60 and 65 °C and a pH lower than 7.5, and another optimum between 61 and 64 °C and a pH higher than 8.6.

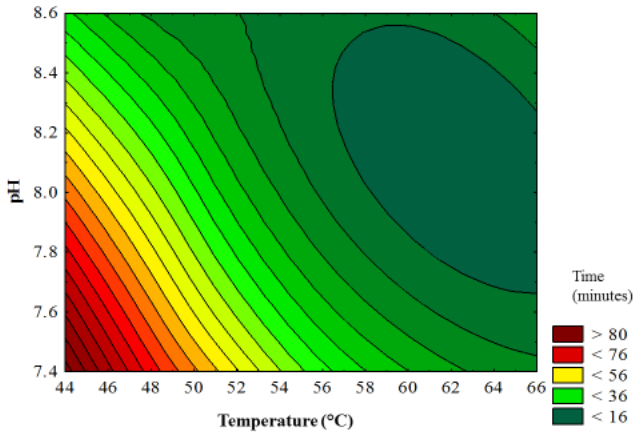

Figure 5. 7: Influence of temperature and pH on the enzymatic hydrolysis reaction time in alcalase/monkfish system

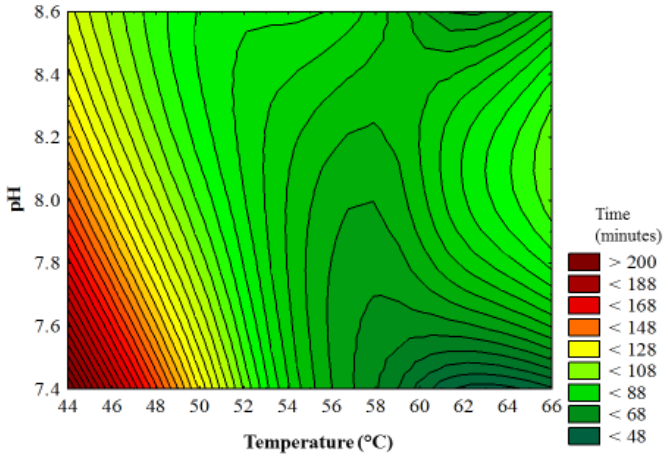

Figure 5. 8: Results of the influence of temperature and pH on the enzymatic hydrolysis reaction time based on experimental set 1 in bromelain/monkfish system

The results from experimental set 2 for the bromelain/monkfish system, with data points for pH 7.5 and temperatures of 55 and 65 °C used from experimental set 1 for bromelain/monkfish, is shown in Figure 5.9. The plot indicates a linear trend that reaction time decreases as pH decreases. Due to the limitations on the considered pH range in this study, the optimum reaction time could only be investigated to a minimum value of pH 7.

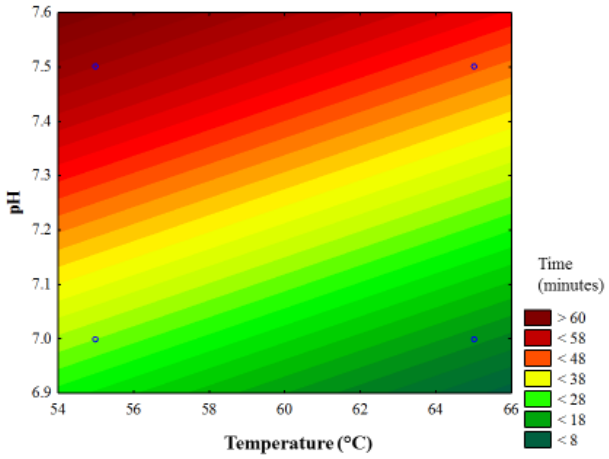

Figure 5. 9: Results of the effect of temperature and pH on enzymatic hydrolysis time based on experimental set 2 in bromelain/monkfish system

In this study the pH range that could be investigated was limited between a pH 7 and pH 8.5. As the pH-stat method, used in this study to determine DH, is limited to operating under alkaline pH conditions (Rutherfurd, 2010), the lowest pH value that could be investigated in this study was a pH value of 7. The highest investigated pH in this study was pH 8.5, as the hydrolysate was considered for potential use in food applications, and the solubility of the proteins, which plays an important role in the nutritional functionality of a food product, in monkfish has been reported to be at a maximum between a pH 7 and pH 8 (Batista, 1999).

The exact values of the optimum reaction conditions for the alcalase/monkfish system predicted by a distance weighted least squares model (Figure 5.7) was a pH of 8.2 and a temperature of 62 °C, and 65 °C and pH 7 for the bromelain/monkfish system. A set of 2 experiments were run to validate this predicted optimum and the DH as a function of time for the optimised enzymatic hydrolysis with alcalase and bromelain is given in Figure 5. 10. The enzymatic hydrolysis under optimal conditions using the different enzymes took 7.2 minutes with alcalase and 12.7 minutes with bromelain, to reach the specified DH of 16 %.

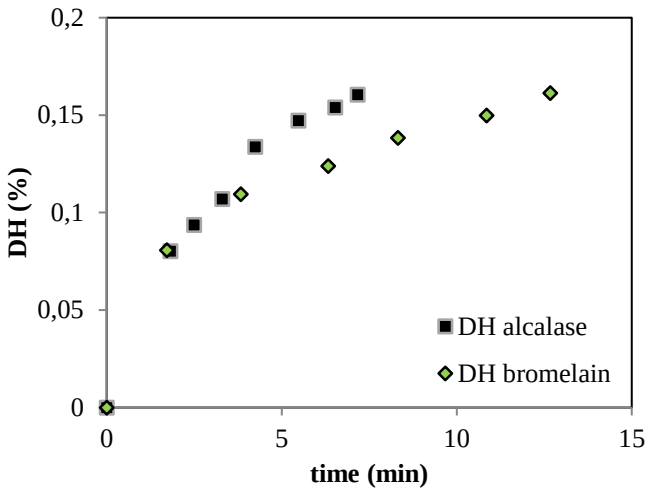

Figure 5. 10: DH vs hydrolysis time under optimal reaction conditions, for alcalase/monkfish and bromelain/monkfish systems

The enzymatic hydrolysis was monitored with the pH-stat method, and typical behaviour of the progression of DH as a function of time was observed for both enzymes (Figure 5.10). Similar curves of progression of DH against time as found in this study were also achieved by Kristinsson and Rasco (2000a) for salmon, and (Guerard et al., 2001) for yellowfin tuna. As the pH-stat method requires titration to stabilise the pH, the pH could be overshot or take too long to be stabilised. This obstacle can be overcome by using an automatic, controlled titration system, or manually by being proficiently acquainted with the experimental setup, and exercising experienced control over the experimental system. Although the optimum reaction time for the alcalase/monkfish system was reached successfully, the results from the study (Figures 5.9 and 5.10) indicated that optimum reaction parameters for the bromelain/monkfish system was not obtained in this study. This was due to the pH constraints dictated by the method used to determine the DH. An alternative method, the ortho-phtaladehyde (OPA) method which has been used for many years for determining the DH in sheep milk whey, casein and fish protein (Rutherfurd, 2010) is recommended to determine the DH in the future when bromelain/monkfish systems are investigated. This method allows for real time monitoring of the DH with a derivatisation step for reagents to be analysed spectrophotometrically (Rutherfurd, 2010).

Functional properties

The results of the FPH food property tests are shown in Table 5. 6. The highest values of functional properties tested were ES of 48.33 ± 1.36 %; FA value of 2.0 ± 0.16 g oil/g protein; antioxidant activity of 52.52 ± 1.60 %; and foaming capacity of 22.15 ± 1.85 ml foam/g protein. The choice of enzyme and

the temperature at which the FPH was treated during hydrolysis had a significant ($p < 0.05$; data shown in Appendix B) influence on all the functional food properties tested. The values for ES, FA, antioxidant activity and FC observed for samples treated with bromelain were higher in all cases compared to samples treated with alcalase.

Table 5. 6: Functional food properties of FPH

Sample*	ES (%)	FA $\left(\frac{\text{g oil}}{\text{g FPH}}\right)$	Antioxidant activity (%)	FC $\left(\frac{\text{ml foam}}{\text{g protein}}\right)$
FPH 1	25 ± 1.36	1.05 ± 0.04	20.62 ± 7.26	9 ± 1.5
FPH 2	36.67 ± 2.72	0.8 ± 0.16	37.12 ± 2.37	19.75 ± 2.75
FPH 3	31.67 ± 1.36	1.35 ± 0.20	44.43 ± 2.71	21.09 ± 1.41
FPH 4	35 ± 1.36	0.5 ± 0.16	52.52 ± 1.60	22.15 ± 1.85
FPH 5	36.67 ± 2.72	1.25 ± 0.12	49.08 ± 1.36	10.04 ± 1.96
FPH 6	48.33 ± 1.36	2.0 ± 0.16	50.19 ± 1.21	16.08 ± 2.67

Values mean ± SE; n = 2

* Sample conditions defined in Table 5.3

The results of the functional food property tests on the sediment are shown in Table 5. 7. The emulsion activity index (EAI) values were between 3.44 m^2/g - 5.53 m^2/g, emulsion stability (ES) values between 32.22% - 43.80%, fat absorption (FA) values were 0.2 g oil/g FPH - 0.55 g oil/g FPH and antioxidant activity values between 20.74% - 41. 25%. The values of ES and antioxidant activity were observed to be dependent on the hydrolysis temperature, pH and enzyme used to treat the sample. The FA values were influenced by the temperature and pH.

Table 5. 7: Functional food properties of sediment

Sample*	ES (%)	FA $\left(\frac{\text{g oil}}{\text{g FPH}}\right)$	Antioxidant Activity (%)	EAI $\left(\frac{\text{m}^2}{\text{g}}\right)$
S 1	32.22 ± 0.26	0.55 ± 0.02	20.74 ± 1.70	3.44 ± 0.11
S 2	33.03 ± 0.30	0.20 ± 0.04	28.91 ± 2.10	4.61 ± 0.24
S 3	35.82 ± 0.86	0.52 ± 0.03	37.36 ± 1.39	4.25 ± 0.30
S 4	40.85 ± 2.01	0.34 ± 0.05	35.32 ± 0.75	3.96 ± 0.40
S 5	43.80 ± 0.72	0.43 ± 0.03	38.69 ± 1.23	5.53 ± .011
S 6	32.27 ± 1.12	0.48 ± 0.02	41.25 ± 0.91	3.47 ± 0.18

Values mean ± SE; n = 3

* Sample conditions defined in Table 5.4

Literature values for monkfish functional food properties are not readily available but monkfish is comparable to hake as both are white fillet fish with a low fat content (Batista, 1999). For this reason, values for functional properties found in this study were compared to values found for hake by Pires et al. (2013).

Foaming Capacity

According to (Klompong et al., 2007) the foaming capacity of a product depends on how well a protein can be transported to the water-air interface, unfold and reorganise the molecules at the interface. The hydrophobicity of the proteins have also been show to affect foaming properties (Klompong et al., 2007). In this study FPH treated with bromelain at high temperatures yielded the highest foaming capacity values and were similar to those values observed for hake hydrolysed to values between 11% and 21 % by Pires et al. (2013). The specificity of bromelain could possibly cause the release of more hydrophobic peptides during hydrolysis than alcalase does for the same DH value. Foaming properties of the sediment were not tested, but an additional EAI experiment was performed on the sediment and is discussed in the following paragraph.

Emulsion activity index and emulsion stability

The values of EAI for monkfish hydrolysate sediment found in this study were lower than that of hake as reported by (Pires et al., 2013) in all cases, but in the same order of magnitude. The DH influences the emulsion activity and stability of the hydrolysate sediment, and lower DH values is correlated to strong emulsification properties (Klompong et al., 2007); however, even taking this into consideration, the EAI for monkfish hydrolysis sediment was still lower than the hake hydrolysed to the highest DH by Pires et al. (2013). An emulsion is formed when the adsorption of peptides on new oil droplets takes place (Klompong et al., 2007) but the peptides present in the sediment may not have been flexible enough, or have low solubility which would mean peptides could not diffuse fast enough to the interface of the emulsion, causing low values of EAI (Klompong et al., 2007).

Emulsion stability is an indication of a products' ability to resist changes in its properties over time (Kristinsson & Rasco, 2000c) and influences the shelf life and processing ability of a food product. Treatment with different enzymes affected the ES, as significantly higher values ($p < 0.05$) were observed for sediment after treatment with alcalase than sediment samples after treatment with bromelain. The opposite observation was found for FPH, as higher ES values were found for FPH treated with bromelain.

This observation could possibly be an indication of the specificity of the enzymes, and that bromelain produces larger soluble peptide molecules that are found in the FPH than alcalase. The addition of water to the substrate prior to hydrolysis, which was the case in this study, has been found to be the most significant factor contributing to the decrease in emulsification properties (Šližyte et al., 2005). The values of ES in this study for both FPH and sediment were found to be lower than those observed by Pires et al. (2013) for hake, but in the same order of magnitude. Proteins hydrolysed to a DH greater than 5% contains peptides which are small in size, and these small sized peptides have been shown to be inefficient in decreasing interface tension between new oils droplets (Klompong et al., 2007), which would cause the coalescence of the new oil droplets and low values in ES.

Fat absorption

Fat absorption in food properties is an important parameter as it influences the flavour retention, improves palatability and extends the shelf life of meat or bakery products (Acuna, Gonzalez, & Torres, 2012). The interaction of hydrophobic groups with the oil is mainly responsible for fat absorption (Gbogouri et al., 2004) but DH and substrate specificity of enzymes are also factors which influence the fat absorption capacity (Kristinsson & Rasco, 2000c). FA values of the FPH were higher when treated with bromelain, but sediment FA values were lower when bromelain was used compared to when alcalase was used. As stated in the discussion on foaming capacity, this observation could be explained by the possibility that bromelain releases more hydrophobic peptides into the soluble FPH phase than alcalase does during the hydrolysis reaction. The values of monkfish sediment fat absorption were lower, but in the same order of magnitude, than those found for hake, but the FPH FA values were comparable to the values found for hake (Pires et al., 2013).

Antioxidant activity

Antioxidant activity was tested using DMPD. An antioxidant activity value is indicative of the extent of the reaction of the radical in the product which is tested, to react with an oxidant (Apak et al., 2013). Fish protein contains inactive antioxidative peptides which can be released after hydrolysis (Chalamaiah et al., 2012). The antioxidant activity of FPH as well as hydrolysis sediment was higher when treated with bromelain when compared to FPH samples treated with alcalase. During hydrolysis, small peptides and free amino acids are formed, the size, level and composition of which is dependent on the enzyme specificity (Klompong et al., 2007). These peptides and free amino acids affect the antioxidant activity (Klompong et al., 2007). From the results in this study, it can be deduced that bromelain produces

hydrolysis products with peptides and free amino acids which demonstrates good antioxidant activity. The antioxidant activity values of monkfish FPH and sediment were comparable to values of 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity observed by Pires et al. (2013) for hake hydrolysed to 19%.

The values of the functional food properties of monkfish sediment and FPH were compared to hake (Pires et al., 2013) and found to be comparable or lower. However, the reaction parameters, such as % DH, enzyme, temperature and pH, which produced the monkfish and the hake were not the same. Although some the values of the monkfish FPH and sediment functional food properties were lower than that of hake, the results show that the monkfish hydrolysate products do indeed have functional food characteristics and the values are in the same order of magnitude of that of hake. For this reason, the monkfish hydrolysate products are recognised as having useful functional food properties and could be used as ingredient in animal feed.

Analytical results of hydrolysis sediment

The results for the proximate composition of hydrolysis sediment are given in Table 5. 8. The crude protein was 9.82 ± 2.75% of the total sample (mean ± SD), moisture value was 85.58 ± 1.75% and there was 2.01 ± 1.5% crude fat in the sediment. The two samples were both produced under a pH 8 and temperature of 55 °C, and alcalase as enzyme for the first sample and bromelain as enzyme for the second sample.

Table 5. 8: Proximate composition of sediment (% of total sample)

Parameter	Value
Crude protein	9. 82 ± 2.75
Moisture	85.58 ± 1.75
Dry matter	14.43 ± 1.75
Ash	3.05 ± 0.50
Crude fat	2.01 ± 1.5

Values mean ± SD; n = 2

The amino acid composition of the sediment is presented in Table 5. 9. The monkfish sediment contained all the essential amino acids (EAA) with the exception of tryptophan. Tryptophan was not detected as it is known to be acid labile (MacKenzie, 2009, p. 30), and during acid hydrolysis of the sample prior to amino acid analysis it would have been destroyed. The ratio of EAA to total amino acids (TAA) was 0.495. The

least common essential amino acid was histidine, and amounted to 1.40 ± 0.82% (mean ± SD) of the total amino acids. The most prevalent essential amino acid was lysine, and comprised 12.2 ± 3.45% of the total amino acids. Of the non-essential amino acids (NEAA), glutamic acid had the highest concentration, containing 13.98 ± 0.11% of the total amino acids, while cysteine was the least prevalent NEAA at 0.77 ± 0.08%.

Table 5. 9: Amino acid composition of sediment

Amino acid name	% of total amino acid	mg/g sample
Essential		
Arginine	5.22 ± 0.73	7.45 ± 2.04
Histidine	1.40 ± 0.82	1.99 ± 1.55
Isoleucine	5.39 ± 0.21	7.10 ± 0.68
Leucine	8.47 ± 0.40	11.10 ± 0.85
Lysine	12.2 ± 3.45	13.97 ± 3.78
Methionine	3.42 ± 0.15	4.64 ± 0.74
Phenylalanine	4.15 ± 1.81	6.80 ± 3.73
Threonine	4.49 ± 0.10	5.95 ± 0.67
Valine	5.50 ± 0.26	7.19 ± 0.55
Non-essential		
Alanine	5.92 ± 0.64	7.51 ± 0.50
Aspartic acid	10.13 ± 0.99	12.91 ± 0.57
Cysteine	0.77 ± 0.08	1.07 ± 0.26
Glutamic acid	13.98 ± 0.11	18.72 ± 2.41
Glycine	6.45 ± 0.79	9.13 ± 2.37
Proline	4.37 ± 0.09	5.83 ± 0.72
Serine	4.98 ± 0.14	6.68 ± 0.933
Tyrosine	3.49 ± 1.77	5.88 ± 3.56
Σ EEA	49.53 ± 1.04	66.18 ± 3.47
Σ NEAA	50.47 ± 1.04	67.74 ± 5.32
Σ EAA/Σ TAA	0.495	0.495

Values mean ±SD; n = 6

Analytical results of hydrolysis sediment

Studies on the enzymatic hydrolysis of protein often only consider the FPH, or water soluble fraction of the hydrolysis product, as the main product of hydrolysis. The sediment however, may contain protein and could have good functional properties as the study by Šližyte et al. (2005) showed. As the focus in the current study was the optimal utilisation of byproducts, it was important that all the fractions of the hydrolysis product is taken into consideration and tested for functional properties to assess potential applications.

Proximate analysis showed that 9.82 ± 2.75 % of the total sample was crude protein, which means that if the protein also contained a large proportion of EAA, it could be used as protein supplement.

Essential amino acids are not synthesised by the body naturally (Tahergorabi, 2011, p. 121) and need to be supplemented with food ingredients containing the EAA. The nutritive value of an ingredient depends on the ability of the protein to meet feed requirements in terms of EAA (Bhaskar et al., 2008). All the essential amino acids, except tryptophan which was not tested for due to reasons set out in the Materials and Methods section of this paper, was found in the sediment of the monkfish hydrolysis product and 49.53 ± 1.04 % of the total amino acid profile was made up out of EAA. The presence of all the EAA, except tryptophan, indicate that the sediment contains valuable protein that could be a possible dietary protein supplement. These findings are consistent with those of Šližyte et al. (2005) and Liaset and Espe (2007) where EAA were found in the insoluble fraction of enzymatic hydrolysis products of cod, saithe, salmon and soy protein isolate.

Conclusions

It was determined that protein can be extracted from monkfish heads using enzymatic hydrolysis, so that underutilised byproducts of the monkfish need not go to waste. Enzymatic hydrolysis proved to be a process that successfully hydrolysed the monkfish head using the enzymes alcalase and bromelain. The pH-stat method, which is a real-time monitoring technique used to determine the DH of the reaction proved successful in the alcalase/monkfish system, but limited the pH investigated for the bromelain/monkfish system to pH 7. The OPA method, which is also a real-time monitoring technique for DH determine, was recommended for future investigations of bromelain/monkfish systems, as this method does not constrain the reaction pH.

It was important in this study to investigate the functional properties of all fractions of the hydrolysate product as the focus in the current study was the optimal utilisation of byproducts. Studies often only consider the FPH the main product of hydrolysis when investigating the enzymatic hydrolysis of protein. In this study, the sediment as well as the FPH was shown to contain protein and have functional properties useful in the food and animal feed industries. The FPH and hydrolysis sediment was found to have the potential to be used as feed ingredient for animal nutrition as all the essential amino acids was found in monkfish hydrolysis product. The relative amount of EAA to TAA was 0.495 which corroborates that the FPH would have nutritional value. The antioxidant activity values found in this study indicate that monkfish FPH can be used to delay or inhibit oxidation of a substance. Overall, monkfish FPH does have favourable functional food properties and could be used as animal feed ingredient.

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Chapter 6: Conclusions and Recommendations

This study has illustrated the successful enzymatic hydrolysis of the heads of the monkfish (*Lophius vomerinus*). This is important for monkfish valorisation as the head accounts for roughly 30% of the total length of the monkfish. To date and to the best knowledge of the author the available literature on monkfish is limited and the raw substrate characterisation, enzymatic parameter investigation and optimisation, and evaluation of product functionality will contribute to efforts to valorise monkfish heads. Furthermore the information is indicative of the potential of the monkfish head as protein sources for animal feed application.

The characterisation of the raw monkfish heads included a proximate analysis, determination of the fatty acid profile, amino acid profile and mineral profile. The monkfish contained high proportions of n-3 long chain polyunsaturated fatty acids, DHA and EPA, which can contribute to bioactivities such as reduced blood pressure, reduction in cardiovascular disease, and improved functions of the nervous system. The oil can also be put to use in aquaculture feed where DHA and EPA are important for cellular metabolism, maintaining cell membrane structure in fish, and enhancing eicosanoid functions like blood clotting and inflammatory responses and neural development. The protein found in the monkfish head was rich in essential amino acids. Minerals such as Ca and K were present in large proportions in the monkfish head. The characterisation of the monkfish head indicated that the monkfish could potentially be used as raw material to produce animal feed ingredients. For the successful extraction of the nutrients found in the monkfish heads, the extraction would need to be tightly controlled, and the process of enzymatic hydrolysis, where mild reaction conditions are used, was found to be the best viable option for nutrient extraction. Results of the mineral analysis showed variations in data that could possibly be due to stage of maturity of the fish spawn conditions and season, sex of the fish and the geographical locations the fish was caught in.

The dependence of pK on pH was determined in this study in order to determine the mean degree of dissociation of α-amino groups during hydrolysis, so that the pH-stat method for DH determination could be used. It was confirmed in this study that the pK depends strongly on the enzyme/substrate combination. The pK was successfully determined for the alcalase/monkfish system in this study, but could not be determined for the bromelain/monkfish system. This was due to possible enzyme deactivation or catalytic inhibition taking place between a DH of 16 % and 20 %. The pH-stat method allowed for instantaneous calculation of the DH at any time interval but required the mean pK value to be known. Another limitation

of the pH-stat method is that the technique is restricted to operating only between neutral and alkaline pH conditions. It was found that the optimum reaction time for the bromelain/monkfish system could not be attained in this study due to the pH constraints, and it is recommended that the OPA method be used in future studies on the enzymatic hydrolysis of bromelain/monkfish systems. The monkfish heads were treated with two different enzymes, alcalase and bromelain. Alcalase was found to be the best proteolytic enzyme for fish hydrolysis in literature and was confirmed in this study. Bromelain was more sensitive to reaction pH and substrate than alcalase. The reaction pH and temperature were both found to have a significant influence on the hydrolysis reaction.

The values of the functional food properties of monkfish FPH and sediment in this study were found to be comparable or in some cases, lower than the values found in literature for hake. Emulsion properties, foaming ability, fat absorption and antioxidant activity values were determined in this study. The values indicated that the hydrolysate products had useful functional food properties and could be used as value added ingredient in food products. The analytical analyses of the sediment showed that the protein was rich in essential amino acids, and would be suitable as a protein ingredient for animal feed applications.

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Appendix A: Description of pK determination

In the equation that is used for pH-stat technique to determine base consumption one needs to know the α value specific to the substrate-enzyme system as shown in Equation 1. The α value is calculated by using Equation 1:

$$\alpha = \frac{10^{(pH-pK)}}{1+10^{(pH-pK)}} \quad (1)$$

The pK value is the mean pK of the α-amino acids released during hydrolysis (Rutherfurd, 2010). According to Camacho et al. (2001) the pK value is dependent on pH and the substrate and these authors have proposed a method of determining the specific pK for a system by correlating base consumption to the degree of hydrolysis. This method entails titrating a raw protein solution from pH 7 up to 10, titrating a partially hydrolysed protein solution form pH 7 up to 10 and comparing base consumption of the two. Both the raw protein and partially hydrolysed protein should be of equal protein concentration i.e. the starting amount of protein in the raw protein should be the same as that of the starting amount of hydrolysed protein before it undergoes hydrolysis (Camacho et al., 2001). The degree of hydrolysis of the partially hydrolysed protein substrate is initially determined using the pH-stat technique and a pK of 7.1, as used by Adler-Nissen (1986, p. 91). The value of the degree of hydrolysis will be recalculated at the end of the procedure using the new pK value currently being determined. During titration, the volume of titrating agent against pH is recorded so that equivalent titrating agent volume can be calculated with Equation 2:

$$b = \frac{V_B C_B}{V_0} - \left(\left[1+\frac{V_B}{V_0}\right] \times 10^{pH-pI} - 10^{pH_0-pI}\right) \quad (2)$$

The equivalent titrating agent volume for the raw protein and hydrolysed protein is calculated so that the maximum difference between titrating agent volume required for raw and partially hydrolysed protein substrates can be calculated and used in Equation 3 as derived by Camacho et al. (2001):

$$\frac{b_x - b_s}{(b_x - b_s)_{max}} = \frac{\frac{1}{1+10^{pK-pH}} - \beta}{1-\beta} \quad (3)$$

where β is determined by Equation 4 when titration begins:

$$\beta = \frac{1}{1 + 10^{pK_0 - pH_0}} \quad (4)$$

Equations 3 and 4 both contain the unknown pK parameter and Camacho et al. (2001) explained that by plotting $(b_x-b_s)/(b_x-b_s)_{max}$ against pH and using non-linear regression to fit Equation 3 to the plotted values, the mean pK value can be found where the pK value is described by the following statistical function:

$$pK = \mu + a(pH - \mu) \quad (5)$$

where a is a function of the standard deviation and μ is the centre of the distribution.

Appendix B: Statistical supplement

Table B. 1: T-test values for results of proximate composition in Chapter 4

Parameter	DF	$t_{0.025,DF}$	t0
Moisture	5	4.773	2.77
Crude fat	5	4.773	-0.61
Crude protein	5	4.773	-5.83

Abbreviations for Table B2:

n - number of observations

F value - test statistic

P - value - probability value

FC - foaming capacity

FA - fat absorption

ES - emulsion stability

EAI - emulsification activity index

Table B. 2: Additional statistical data for results reported in Chapter 5

Parameter	n	F - value	P - value
Statistical supplement for data in Table 5. ; Figure 5. ; Figure 5. Influence of parameters on reaction time			
Enzyme	5	161.27	0.00
Temperature	5	40.34	0.00
pH	5	3.38	0.04

Statistical supplement for data in Table 5. 6;
Results of FPH functional food properties

Influence of parameters on FC

Parameter	n	F - value	P - value
Parameter			
Enzyme	2	18.55	0.005
Temperature	2	7.39	0.024

Influence of parameters on FA

Parameter	n	F - value	P - value
Enzyme	2	8.53	0.027
Temperature	2	27.23	0.001

Influence of parameters on ES

Parameter	n	F - value	P - value
Enzyme	2	21.17	0.003
Temperature	2	14.44	0.005

Influence of parameters on antioxidant activity

Parameter	n	F - value	P - value
Enzyme	2	143.7	0.000
Temperature	2	70.36	0.000

Statistical supplement for data in Table 5.

Results of sediment functional food properties

Influence of parameters on EAI

Parameter	n	F - value	P - value
Enzyme	3	0.94	0.352
Temperature	3	3.60	0.059
pH	3	3.60	0.059

Influence of parameters on FA

Parameter	n	F - value	P - value
Enzyme	3	2.14	0.169
Temperature	3	36.5	0.000
pH	3	4.90	0.028

Influence of parameters on ES

Parameter	n	F - value	P - value
Enzyme	3	36.96	0.000
Temperature	3	7.74	0.007
pH	3	27.84	0.000

Influence of parameters on antioxidant activity

Parameter	n	F - value	P - value
Enzyme	3	13.42	0.003
Temperature	3	31.33	0.000
pH	3	33.27	0.000