Optimisation for production of fish protein hydrolysate from Nile Perch (*Lates niloticus)* by-products

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DECLARATION

Except where reference is made to the work of others, the work described in this thesis is my own, or was done in collaboration with my advisory committee. This thesis does not include proprietary or classified information.

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DEDICATION

To the poverty-stricken nations of Africa. Here's praying that this work can continue in
further capable hands, to see fruition borne of a simple idea.

To my parents, Rohit, Geeta, and Hadi, without whose support this work would not be
possible. I know it's been a frustrating road, this. You held my hand through it always,
and for that I am forever in your debt.

Om Shanti.

ACKNOWLEDGMENTS

ABSTRACT

The global rising concern for food and environmental security has forced established industries such as fish processingto explore new areas of improvement. In Kenya thus far, no studies have been carried out to explore the use of enzyme technology in the possible recovery of products of high nutritional value from industrial co-streams. Recent advances in the field of enzyme technology have led to the development of new functional products with various properties to address this concern. Fish protein hydrolysate (FPH) is one of the results of such advances, resulting in a product with high quality protein possessing a good amino acid profile.Nile Perch is the predominant species fished from Lake Victoria, and is a major export commodity. The present study was carried out to determine the most suitable enzyme and corresponding concentration (enzyme/substrate ratio) required to hydrolyse Nile Perch (*Lates niloticus)* heads and frames (by-product) within a suitable time, to obtain a final fish protein hydrolysatewith desirable protein recoveries.Heads and frames (tissue-intact by-product) was obtained from W.E. Tilley, an exporter based in Nairobi,and hydrolysed using three commercially available food grade enzymes: Alcalase, Protex 30L, and Bromelain from pineapple stem. For unhydrolysedby-product, the Biuret and Kjeldahl methods were chosen to analyse percentage protein content, determined as 18.42 ± 0.04 and 20.4 ± 0.47 %, respectively. Moisture, ash/mineral, and lipid content were found to be 58.8 ± 1.26, 10.82 ± 1.99, and 7.86 ± 0.63 %, respectively. Optimisation for enzyme concentration involved two-hour reactions using 0.5 and 1.0 % enzyme/substrate ratio for each enzyme. Bromelain gave best recoveries, determined by the Biuret method, with an enzyme/substrate ratio of 1 %, yielding a protein recovery of 68.2 %. Optimisation for hydrolysis time followed four/six-hour reactions with Bromelain, with a change in enzyme batch between the two reactions.

The four-hour reaction yielded a final protein recovery of 97.7 %, while that of six hours yielded a recovery of 68.2 %. The six-hour batch was retained for freeze-drying to obtain powder FPH.The optimal conditions for hydrolysis were determined to be a reaction time of 4 hours with a Bromelain:Substrate concentration of 1%. The moisture, ash/mineral, lipid and protein content of the final FPH where; 14.8 ± 0.67, 5.3 ± 0.4, 9.8 ± 0.31 and $68.2 \pm 1.03\%$, respectively. All analyses were performed in triplicate and subjected to standard deviation calculations for mean results.The high protein content indicated that a nutritious soluble FPH could be obtained from Nile Perch by-products. It maypossibly be used in food fortification, nutraceuticals, pharmaceuticals, and microbial growth media.Further characterization of the FPH is necessary to determine bioactive and nutritionalproperties of the product, before their applications can be taken to market.

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CHAPTER 1

INTRODUCTION

Total world fishery production for the year 2011 was estimated at 154 million tonnes, of which 85% was used for direct human consumption, and the remainder directed to other uses such as animal meal production and non-food purposes(FAO, 2012). Human consumption showed a marked increase from 2004, where the direct consumption was approximately 75%(FAO, 2011).This could be attributed to dramatic population increase and better fishing technology.Processing and filleting in the fish industries generate by-products (e.g. skin, bones, trimmings, frames, heads, viscera and fins) thattogether with fish species with no commercial value (by-catch) are generally discarded.

The marine bioprocess industries convert and utilize these discards or by-products into valuable products such as fish protein hydrolysate, fish oil, fishmeal, fertilizer, pet food and fish silage (Ramirez- Ramirez *et. al.*, 2008). The recovery of proteins from fish by-products for their utilization as food ingredients has gained interest in the food industry, as they may possess good functional and therapeutic properties e.g.emulsifying and antihypertensive abilities. Enzyme technology is being embraced in this area as it preserves essential properties that may be destroyed through acid or alkaline hydrolysis.

During enzymatic hydrolysis of fish by-products, three or four fractions are left after centrifugation: fish protein concentrate (FPC - soluble fraction), a solid by-product (insoluble fraction, mostly bone), emulsion layer, and oil (in case of fatty fish). Peptides constitute the main component in aqueous fish protein concentrates and have

a high nutritional value that may also possess bioactive properties (Kim and Mendis, 2006). Dried FPC, also known as fish protein hydrolysates (FPHs) are currently used in animal feeds, but may in the future be used as functional ingredients in specialized animal feeds, or in drinks and food products (nutraceuticals) for human consumption. For FPH to have a high nutritive value, it has to have a high content of low molecular weight peptides, especially di- and tripeptides, and a low content of free amino acids(Vijayalakshmi *et. al.*, 1986). Bitterness due to exposure of hydrophobic amino acids during peptide chain breakdown is the major problem affecting the sensory acceptability of FPH. This can be minimised by controlling the degree of hydrolysis (DH), use of specific enzymes, or debittering of the productby masking with agents such as fruity flavours(Adler-Nissen, 1984; Dauksas *et. al.*, 2004; Saha and Hayashi, 2001).

1.1. Context of the study

Statistically, Lake Victoria is the most productive freshwater fishery in Africa. Yields are in the order of 800,000 ó 1,000,000 metric tonnes annually, with earnings from export estimated at $ 250 million(LVFO, 2012). There are three predominant fish species: Nile Perch (*Lates niloticus*), Nile Tilapia (*Oreochromis niloticus*), and,*Rastrineobolaargentea*(÷Dagaaø, ÷Omenaø, or ÷Mukeneø). As of the year 2006, estimated total catch was 1,061,107.6 metric tonnes, of which Nile Perch contribution was 254,666 metric tonnes; over 75% of the latter catch is directed to the fish processing factories for the export market (LVFO, 2012).

The Tilley Group of companies is one of East Africaøs largest exporters of fresh and frozen, skinless Nile Perch. In Kenya alone, W.E. Tilley based in Nairobi, processes close to 2700 metric tonnes per annum for filleting. Of this, approximately 48% is by-

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product or co-stream. This portion represents a major source of high quality protein that is not meeting its full market potential, as well as an important source of food industry wastes.

Tilley's statistics for 2011 are given in Table 1:

Species	Amount of fish for fillet, T/Annum	Co-streams in filleting line	Amount of co-stream, %	Use of the co-stream	Amount, T/Annum	Price of fraction, KES/Kg
Nile Perch	2668	Frames (heads, skeleton, fins)	35.92	Food, fishmeal for animal feeds	958	22 - 24
		Trimmings	2.98	Food	79	300
		Roe	0.12	Food	3	42
		Skin	6.12	Fuel, tanned to leather at small scale	163	8
		Fat pads	1.78	Unrefined oil for frying other foods	47	40
		Factory rejects	0.28	Food	7	47 - 50
		Viscera	0.26	Discarded	7	-

Table 1: Tilley processing statistics, 2011 (*Courtesy of W.E. Tilley*)

Within Kenya, enzyme technology has never been utilised to recover value added fractions from these co-streams. If such technology is embraced, high valued products such as FPH, Omega-3 concentrates, gelatine etc. could be recovered. The present study aims to apply practical enzyme technology to by-products from W.E. Tilley, and thereby begin contribution to a promising future for the potential of the bioprocessing sector within the country.

1.2.   General Objective

To explore the enzymatic hydrolysis of by-products from Nile Perch processingusing commercial food grade enzymes and establish the conditions that facilitate the recovery of a soluble proteinfraction with high nutritionalproperties.

1.3.   Specific Objectives

a)   To determine the proximate composition of Nile perch by-products.

b)   To optimise hydrolysis conditions of Nile Perch by-products using three different commercial food grade proteases, for maximum fish protein hydrolysate production and highest possible protein recovery.

c)   To determine the proximate composition of the recovered final fish protein hydrolysate.

1.4.   Justification for the study

Nile Perch by-product from fishing activities around Lake Victoria and subsequent filleting for export has left much room for processing of by-products such as offal and frames, into more functional, value-added products such as FPH. The need for such products is compounded by the constant, and ever increasing global need for alternative protein sources. This study serves a two-fold purpose: to propose a by-product management system based on bioconversion using enzymes, for the Nile Perch filleting industry, and, to yield a possibly nutritional product with economic benefits to the industry as well as the consumer.

By embracing enzyme technology it is possible to perform extraction of bioactive compoundspossessing nutritional and therapeutic properties from fish by-products; this includes collagen and gelatine, peptides, protein hydrolysates (FPH), and omega-3 fatty acids. Food industry wastes are also an important environmental contamination

source. This study aims to provide a possibly viable solution that will see the minimization of huge amounts of protein-rich by-products being discarded by the filleting industry in Kenya.

1.5.   Scope of the study

Due to the need for specialised equipment and chemicals such as Mass Spectrometry and appropriate standards for separation, studies to determine therapeutic properties of the FPH obtained, as well as peptide and amino acid fingerprinting were not performed. The FPH obtained is a crude extract on which we recommend these studies be further carried out in order to determine its suitability, and applicability to value addition of co-streams from the local fish processing marketif it is to meet rigorous food and drug administration regulations.

CHAPTER 2

LITERATURE REVIEW

2.1. Protein hydrolysates and their applications

Protein hydrolysates, also known as peptones or peptides, are produced from the partial digestion of proteins, and used in a vast number of products in the fermentation and biotechnology industries. This digestion can be carried out using acids, alkalis, fermentation methods, and proteolytic enzymes (proteases)(Pasupuleti and Braun, 2010). Produced peptones provide a source of organic nitrogen that is readily available for cellular uptake(Pasupuleti and Braun, 2010). Initially used for growing microbial cultures, peptones have since found application in commercial fermentation as well, supporting growth of animal cells and recombinant microorganism cultures for the production of value added products(Pasupuleti and Braun, 2010). These products include, but are not limited to, hormones, proteins, and vaccines.

Today, the manufacturing of protein hydrolysates has advanced considerably. Researchers are now able to screen for, characterise, separate and purify peptones of interest. Improvements in analytical techniques such as HPLC and Mass Spectrometry (separation and identification of proteins/peptides, respectively), and other semi-automated systems, have provided greater insight into the novel uses of protein hydrolysates.The knowledge attained from these systems also enables the tailoring of products for specific applications e.g. therapeutic proteins(Bougatef *et. al.*, 2008; Enari*et. al.*, 2008; Fitzgerald *et. al.*, 2005; Geirsdottir *et. al.*, 2011; Gu*et. al.*, 2011; Picot *et. al.*, 2006; Sathivel *et. al.*, 2003; Shirai and Ramirez-Ramirez, 2010; Slizyte *et. al.*, 2009). Current applications span from providing nutriment to fermentation systems supporting microorganisms that produce primary and secondary metabolites,

to the rapidly expanding area of industrial biotechnology for their incorporation in biopharmaceuticals (Pasupuleti and Demain, 2010). Thus far, literature on studies involving the optimisation of conditions necessary to produce high-grade nutritional protein hydrolysates with biotechnological applications, are limited.

The history of protein hydrolysate applications are best summarised by Fig. 1(Pasupuleti and Demain, 2010):

2000	**Biotechnology**
1950	**Diagnostic Media**
1900	**Industrial Fermentation**
1850	**Microbiological Media**
1800	**Petri Dishes**

Fig. 1: Applications of protein hydrolysates over the years (*adapted from Pasupuleti, 2010*)

2.2.   Production methods for protein hydrolysates

Over the last few decades, the basic manufacturing procedure for protein hydrolysates has remained relatively the same. With their use in specific applications, great improvements have been made in manufacturing practices, but, the science still remains in its infancy to uncover the specific peptide, peptides, or combination of amino acids that produce an intended effect for each application (Pasupuleti and Braun, 2010, pp. 24-43). Changing this scenario for the better will involve extensive communication between protein hydrolysate manufacturers and their end-users; as specific and newer applications are brought to light, it is expected that target peptones/amino acids of interest could then be produced utilising the latest production techniques.

Chemical and biological methods are the most widely used for production of hydrolysates, with chemical treatment dominating on an industrial scale. This treatment involves hydrolyzing high-protein substrate with either acid or alkali. These agents cause the hydrolytic degradation of the peptide bonds that link together the amino acids that make up the peptides of a protein, releasing them into solution.

2.2.1. Chemical methods

2.2.1.1. Acid hydrolysis

Acid hydrolysis took several decades to commercialise, with some of the older processes still in use today. Most acid hydrolysed proteins are used in the food industry as flavour enhancers, with their use in biotechnology being restricted due to high salt concentration(Loffler, 1986; Webster*et. al.*, 1982). Common hydrolysates include Hydrolysed Vegetable Proteins(HVPs), soy sauce, and beef, chicken or fish extracts. Two acids are principally used to carry out this hydrolysis: Hydrochloric and Sulphuric acid, the former being most common. The shortfalls of this method of hydrolysis include:

i) Neutralisation of the acid, resulting in a high salt concentration in the final product.

ii) Complete protein breakdown may be achieved by this method, yielding a highly soluble product, but the concentrated acid used destroys essential amino acids such as threonine, tryptophan, methionine, cystine and cysteine. In an industry looking to incorporate these hydrolysates in food products, this is understandably an undesirable result.

iii) Acid hydrolysis requires the use of industrial glass-lined stainless steel reactors that are capable of withstanding extreme temperatures and pressures. This escalates cost and also involves the implementation of operational safety control measures, and their careful monitoring.

iv) Acid hydrolysis typically requires longer reaction times, and initial protein concentrations of up to 65% (Pasupuleti and Demain, 2010)- all these

factors, whether individually applied or combined, will have an effect on the final quality of the hydrolysate.

2.2.1.2.Alkali hydrolysis

Alkaline hydrolysis is a simpler and more straightforward method for production of protein hydrolysates. It often makes use of protein concentrate as the initial substrate that is obtained from solubilisation of protein by heating the initial material, and then treating with a strong alkali such as sodium or potassium hydroxide. The reaction is normally carried forward under a steady-state temperature until a desired degree of hydrolysis is reached(Kristinsson and Rasco, 2000a). The product may then be evaporated, pasteurised and spray dried to give the final hydrolysate. Simpler though this method may be, there are still similar design and safety issues involved, as with those of production of acid hydrolysates. Several adverse reactions have also been reported to occur with the use of high pH/temperature reactions. Noteworthy to the context of this study are: racemisation of L-amino acids to D-amino acids which are not absorbed by humans; the splitting of protein disulphide bonds and freeing of amino acids; production of the amino acid lysinoalanine which has been shown to cause toxicity in animals, and, in some cases, addition/elimination reactions leading to toxic by-products that are highly undesirable in food (Lahl and Braun, 1994; Raa*et. al.*, 1982). Furthermore, alkaline hydrolysis conditions also contribute to a high ash content that is attributed to the alkali added during pH adjustment and its subsequent control(Kristinsson and Rasco, 2000a). This naturally proves to be an issue at the industrial level when it comes to downstream processing and the ultimate disposal/secondary use of ash.

2.2.2. Biological methods

In lieu of the arguments previously put forward, it seemed only logical for scientists to explore a more ‑naturalø way of producing protein hydrolysates. The production of gelatine hydrolysate (hydrolysed collagen) by fermentative methods is an excellent example of how far research has come in the field. Olsen *et. al.*(Olsen*et. al.*, 2010) have shown that the methylotrophic yeast, *Pichiapastoris*, a microorganism widely used in the expression of recombinant proteins, is widely-suited to the large scale production of human gelatines.

2.2.2.1. Hydrolysis using endogenous enzymes (Autolysis)

Traditional methods for preparation of hydrolysates by autolysis, like fish silage, exploit the endogenous enzymes, but it is rather difficult to control the autolysis due to several factors including fish species and seasonality, as well as the type and amount of enzyme (Sikorski*et. al.*, 1981).

2.2.2.2. Hydrolysis using exogenous enzymes

The addition of exogenous enzymes, apart from speeding up hydrolysis time and introducing a mechanism of reaction control, generates reproducible results. Controlling conditions like temperature, time, pH, and enzyme/substrate ratio allow for the production of a protein hydrolysate with highly soluble peptides that may exhibit functional nutritional properties (Guerard*et. al.*, 2002). The enzymatic modification of proteins, using commercially available or specificallytailored proteolytic preparations to cleave peptide bonds, has been widely employed in the food and nutraceutical industry.

Examples include manufacture of milk replacers, protein supplements, beverage stabilisers, and flavour enhancers for confectionery products (Kristinsson and Rasco, 2000a). This form of hydrolysis results in less undesirable by-products (Kristinsson and Rasco, 2000b). The main advantages of this hydrolysis technique are the relatively mild conditions applied, and the specificity of the enzymes involved. Enzymes with different specificities for cleaving peptide bonds are currently being used, enabling researchers and manufacturesto customise the end products depending on their intended applications.

Most of the enzymes used are obtained from plant, animal and microbial sources. Recent focus tends towards proteolytic enzymes being extracted from industrial fermentations i.e. microbial sources(Pasupuleti and Braun, 2010). Microbes represent the largest source of a majority of industrial proteolytic enzymes in use today. Proteases such as Alcalase, Flavourzyme, Protex 30L, Neutrase and Protamex, are classic examples of this. Plant sources represent the next largest sector, with cysteine proteases such as Bromelain from pineapple (*Ananuscomosus*) stem, and Papain from papaya (*Carica papaya*) being most common. With respect to this study the three enzymes of main interest were Alcalase, Protex 30L, and Bromelain.

Alcalase and Protex 30L are both obtained from the bacterial species *Bacillus* (*lichenformis*and*subtilis,* respectively). Both sub speciesare generally regarded as thermophilic microorganisms; a property that becomes most useful in performing enzymatic hydrolysis with *Bacillus*-derived proteases at elevated temperatures. It follows naturally that if the microorganism were able to withstand higher temperatures, their enzymes would also be able to do the same, as these proteases are a vital part of the cell machinery.These enzymes are represented by a group of endopeptidases called subtilisins, preferentially cleaving hydrophobic amino acids in

parent protein molecules (Ton, 2002). These hydrophobic amino acids are contributors to the bitterness of the final product so using these proteases to cleave them early in the hydrolysis reaction implies that they can easily be separated out of the final product using Reverse-Phase High Performance Liquid Chromatography (RP-HPLC), which has a preference towards hydrophobic molecules(Lemieux*et. al.*, 1991).

Alcalase appears to be the most common protease applied in enzymatic hydrolysis, with its first reported use dating back to 1963 as a laundry detergent protease (ChiralVision, 2007).Bromelain obtained from pineapple stem is a cysteine endopeptidase with broad specificity(Taussig, 1980); this translates to a higher cleavage of various peptide bonds in protein substrate, resulting in a protein fraction with a greater amount of polypeptides and amino acids.

The chemical and biological methods described for hydrolysing protein from various sources are summarised in Fig. 2 (Pasupuleti and Braun, 2010).
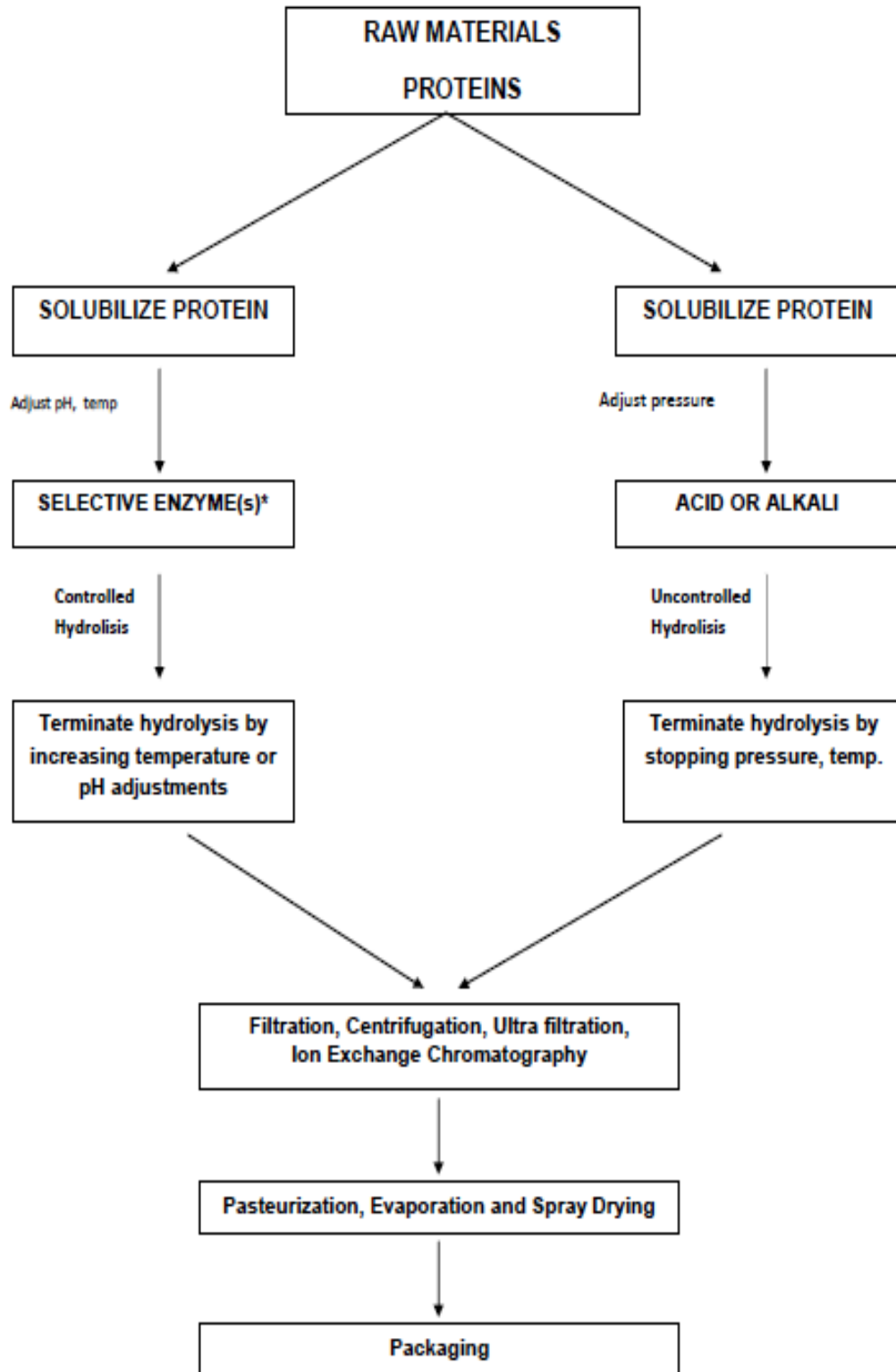
Fig. 2: Generic manufacture of protein hydrolysates (*adapted from* Pasupuleti, 2010)

2.3. Production of Fish Protein Hydrolysate (FPH) from fish by-products

Utilisation of fishery by-products for production of FPH is an important subject because of limited fish resources. Fishery by-products are a valuable source of high quality products such as protein, lipids, minerals, flavourants, carotenoids, enzymes, and chitin, used widely in the pharmaceutical and cosmetic industries, and for production of functional foods (Jonsdottir*et. al.*, 2006). However, their use has been restricted to low-value products such as fishmeal and oils. Enzymatic hydrolysis is one approach that could be used to upgrade these low-value by-products.

2.3.1. Fish protein structure

Fish are supported by a mass of water, and as a result, it is expected that their muscle will comprise of less structural protein when compared to that of land mammals. The protein, like any other structural protein, occurs in a complex, three-dimensional folded structure. Since fish are cold adapted, the proteins will tend to denature more easily at elevated temperatures; temperatures that would not normally cause the denaturation of animal muscle proteins (Kristinsson and Rasco, 2000a). Their muscle protein comprises mainly of ₌whiteø and ₌darkø meat; white meat being the more predominant and reserved for filleting, whereas dark meat (skins, heads, frames) contains a higher content of lipids and is generally discarded. Due to this fact, it is reasonable to expect that FPH obtained from processing by-products would contain a high content of fish oils. Myofibrillar proteins comprise the majority of food protein in fish, accounting for up to 80%. The myofibril structure is further divided into the myosin and actin proteins, with myosin dominating. Myosin is subject to cleavage at different sites by specific endopeptidases such as Papain, and this preferential mechanism of action is similar to that of Bromelain(Ton, 2002).

2.3.2. Enzymatic hydrolysis of fish protein

Enzymatic hydrolysis of fish protein from by-product is a complex process that is generally not fully understood. This is most likely due to the fact that the substrate contains a multitude of peptide bonds that proteolytic enzymes must be accessible to. This is obviously an ideal scenario. In reality, the environmental conditions such as temperature and pH, that affect not only the enzyme kinetics, but also the denaturation rate of proteins, play a vital role.

In order for an endopeptidase to cleave peptide bonds internally found within fish protein, simple denaturation (unfolding) of this stable tertiary conformation must first be effected, thereby exposing these bonds to the enzymeøs active sites. This can be carried out with simple heat treatment and the addition of water, which initiates non-catalytic hydrolysis. With the addition of a specific protease, and under the right environmental conditions that result in catalytic hydrolysis, several large, medium and small peptides having different molecular and functional characteristics can be obtained (Geirsdottir *et. al.*, 2011). For the production of FPH, enzyme selection is done following specific criteria; they must be food grade, and if they are of microbial origin, the responsible microorganism must be non-pathogenic (Bhaskar and Mahendrakar, 2008).

2.3.3. Production of bioactive peptides from fish by-products

Much research has been done on by-products from the fish filleting industry, with the main focus being placed on the possibility that these co-streams, when hydrolysed to produce FPH, will possess peptides with nutritional and functional properties. Researchers have mainly explored the possibility of obtaining biologically active peptides (Benjakul and Morrissey, 1997; Kim and Mendis, 2006).

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These peptides present in enzymatically digested FPH possess varying physicochemical properties and biological activities, dependent on their molecular weights and amino acid sequences. The biological activity of any given peptide is directly related to its molecular weight, so efficient methods of separation need be employed in order to gain this advantage from FPH. Ultrafiltration using molecular cut-offs is a suitable method(Kristinsson and Rasco, 2000a).

Bioactive peptides isolated form various fish protein hydrolysates (FPHs) have shown to possess nutritional, antioxidant, antihypertensive, and antibacterial, among other properties. The main focus will be placed on these, as they are most predominant in literature with respect to FPH.

Looking at peptide antihypertensive activity, it is important to have simple, fundamental understanding of the control of blood pressure in humans. This is regulated by the renin-angiotensin system (Udenigwe and Aluko, 2012)as shown in Fig. 3.

Fundamentally, bioactive peptides that serve as inhibitors of angiotensin-I converting enzyme (ACE) in the right concentration, will mediate the elevation of blood pressure and simultaneously, the degradation of bradykinin, which is responsible for vasodilation(Udenigwe and Aluko, 2012).
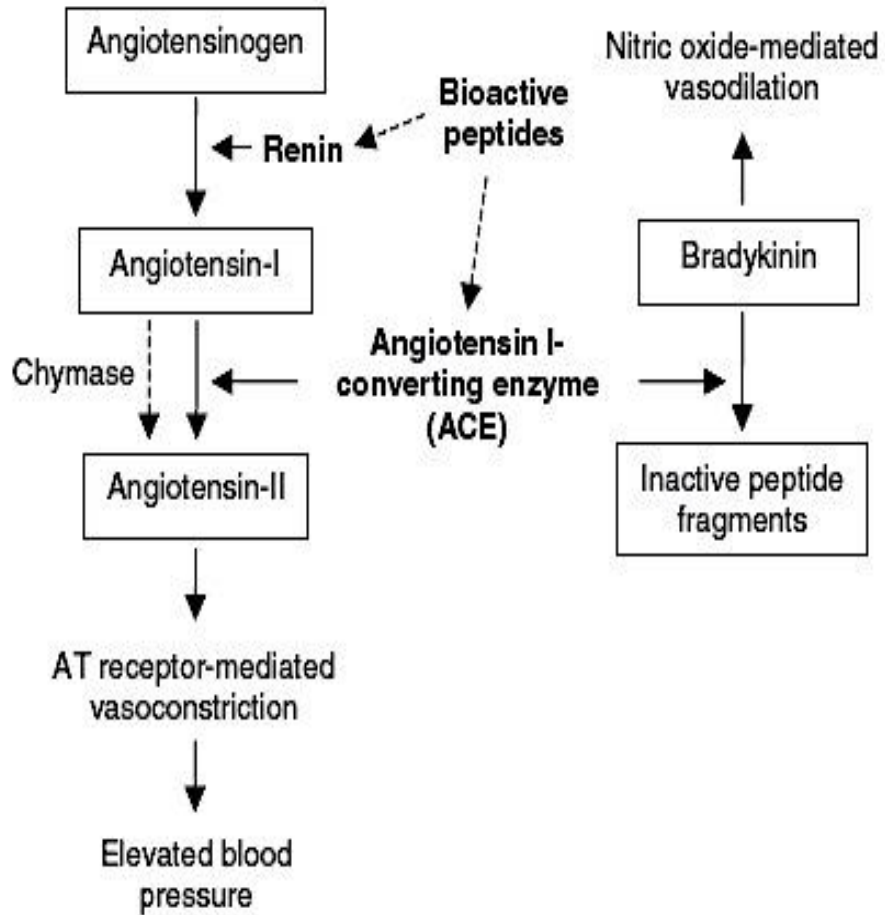
**Fig. 3: Renin-angiotensin system (*adapted from UdenigweandAluko, 2012)*

An excellent schematic to depict the production and processing of bioactive peptides from various food sources is also shown in Fig. 4 (Udenigwe and Aluko, 2012).
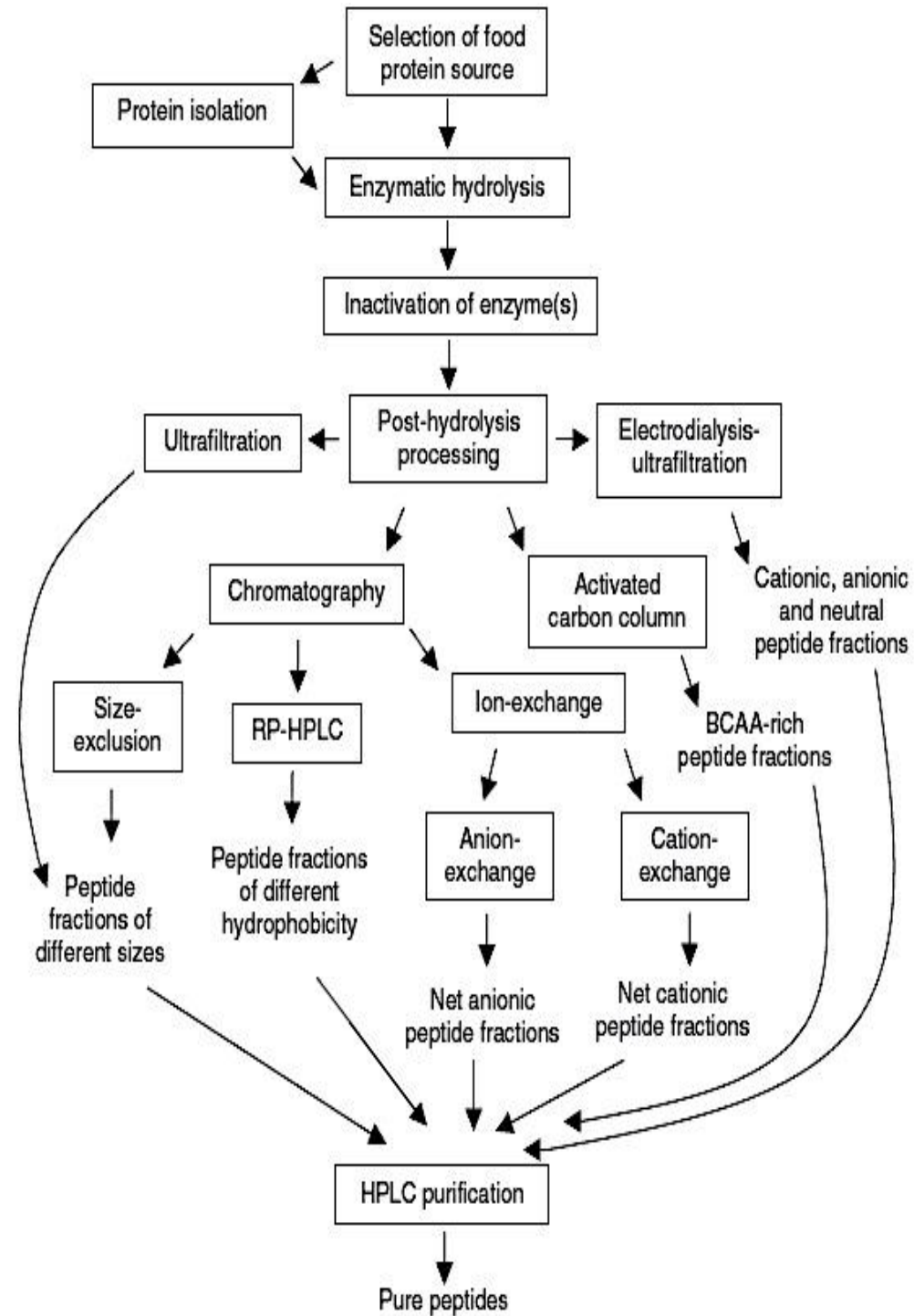
Fig. 4: Production and processing of bioactive peptides from protein source (*adapted from UdenigweandAluko, 2012)*

2.3.4. Hydrolysis of fish by-products using Alcalase

Alcalase is the most widely used protease for hydrolysis of fish muscles, due to the high degree of hydrolysis (DH) that can be achieved in a relatively short time. The DH is a measure of the extent of hydrolysis of a protein (peptide bonds broken) and is a widely used indicator for comparison of different protein hydrolysates. Novozymes AS (Bagsvaerd, Denmark), the manufacturer of Alcalase, recommends heat treatment to arrest enzymatic hydrolysis reactions performed with this enzyme. In fish by-product processing this translates to better preservation of flavours, and also good microbial control (crude pasteurisation) in the final FPH(Novozymes, 2001).

Alcalase has been used in hydrolysis of by-products from tuna processing (Guerard*et. al.*, 2001). The enzyme was added in varying concentrations ranging from 0.2 ó 3% (v/w), with pH maintained at 8 using 2N NaOH, and temperature controlled at 50°C for 5.5 hours. The reaction proceeded until termination by heating the slurry to 95°C for 20 mins.to inactivate the enzyme. Amaximum DH of 23% was achieved with maximum enzyme concentration (E/S of 3%). The hydrolysates were freeze-dried and used in the culture of six microorganisms (bacteria, yeast and fungi) of importance in biotechnology, including *Escherichia coli* and *Saccharomycescerevisiae*that find applications in genetic engineering and food production, respectively. Pacific Whiting (*Merlucciusproductus*), fished in the Gulf of California, has also been hydrolysed using Alcalase 0.6L at different E/S ratios of 1, 1.5, and 3% (v/w), and the reaction was carried forward at pH 8 and at 50°C for 2 hours. The DH attained was 10, 15 and 20%,respectively. Enzyme activity was arrested by heating the reaction contents at 80°C for 10 mins(Pacheco-Aguilar*et. al.*, 2008).

The FPH produced observed a protein recovery as high as 85% (at 20% DH), and the powder exhibited good functional characteristics better than some current emulsifying, foaming, and dispersing agents used in food preservation (Pacheco-Aguilar *et. al.*, 2008). Since Pacific Whiting is a grossly under utilised fish species mainly due to its tendency to succumb to parasitic infection, it has been suggested that this could prove a valuable resource for production of hydrolysates possessing decent functional properties for use in the food industry.

An Atlantic fish of the cod family, Blue Whiting (*Micromesistiuspoutassou*)protein isolates from fillets with skinwere hydrolysed using Alcalase 2.4L in varying enzyme to substrate (E/S) concentrations ranging from, 0.1 to 2% (v/w) to achieve different degrees of hydrolysis(Geirsdottir *et. al.*, 2011). Reaction time was not considered, but instead DH used as a controlling parameter for monitoring progress; arresting reactions once a DH of 15% was achieved. This was done using a combination of shift in pH as well as thermal inactivation at 85°C for 20 mins. These extreme conditions were applied to preserve the functional properties of the final FPH; given Blue Whiting flesh is particularly sensitive to frozen storage.The FPH powder producedpossessed angiotensin-I-converting enzymeinhibitory peptides, whose activity increased with increasing DH(Geirsdottir *et. al.*, 2011).

Comparative studies have used Alcalase and other enzymes (individually) to explore the presence of these novel bioactive peptides in the FPH generated from hydrolysis of the skin from Atlantic Salmon (*Salmosalar L.*), and heads and viscera ofSardinelle (*Sardinellaaurita*). Gu*et. al.*(Gu *et. al.*, 2011) obtained salmon skin by-product containing high amounts of collagen protein, and applied Alcalase and Papain in their hydrolysis experiments.

Before hydrolysis was performed, the skins were mixed with acetic acid in a 1:1 ratio to remove fishy odours, attributed in animals, to trimethylamine(Benja-arporn*et. al.*, 1993). After rinsing in water, the skins were minced then homogenised by addition of alkaline water in the ratio 1:2. Homogenate was centrifuged at 10,000x*g* for 15 mins.to collect supernatant, and initiate a first hydrolysis of this fraction with Alcalase for 3 hours, at a pH of 8.5 and temperature of 50°C (E/S of 1%). After 3 hours, the resulting phase was further hydrolysed using Papain (E/S of 2%), at neutral pH and 60°C, for 2 hours. The final hydrolysed supernatant was heated at 100°C for 10 mins to stop the reaction. The mixture was centrifuged at 3000 x*g* for 15 mins.

Molecular weight cut-off membranes of 10 and 1 kDa were used to filter the aqueous phase in order to separate peptides of interest. Further treatments included dialysis (to remove residual salt), evaporation (to concentrate the peptides), and, decolourisation with activated carbon, before spray drying. A high protein recovery (91%) was achieved in the hydrolysate.

To identify ACE-inhibiting peptides, peptides were separated using reverse-phase high performance liquid chromatography (RP-HPLC), coupled to time-of-flight mass spectrometry (TOF-MS)Several di- and tri-peptide sequences with ACE-inhibiting activity were isolated(Bougatef *et. al.*, 2008; Enari *et. al.*, 2008; Ono*et. al.*, 2006).

2.3.4.1. FPH with antioxidant properties

Oxidation is a vital, natural process in living systems. The generation of free radicals are a by-product of respiratory metabolism, and those that are not eliminated from a biological system are realised to be the cause of various, degradative chain reactions that are implicated in several age-associated diseases (Dong *et. al.*, 2008).

In food, oxidation reactions affect the sensory and structural properties of proteins, carbohydrates and lipids.Synthetic antioxidants have found limited use in food due to their general perception by consumers (preference towards organic/natural ingredients).

FPHs have also been shown to contain antioxidant peptides: Sathivel*et. al.,*(Sathivel *et. al.*, 2003)investigated the possibility of obtaining antioxidant peptides derived from hydrolysis of Herring (*Clupeaharengus*) using Alcalase 2.4L. Whole Herring, body (heads and gonads removed), heads, and gonads were all separately hydrolysed under the same conditions.

A single antioxidant capacity assay indicated that whole Herring hydrolysate and Herring head hydrolysate inhibited lipid oxidation by 48.8 and 44.4%, respectively, when compared to standard food grade antioxidants; -tocopherol (86.6%), butylatedhydroxyanisole (BHA ó 95.6%), and butylatedhydroxytoluene (BHT ó 97.5%). Given that all samples were assayed using standard volumes, it would be safe to assume that pending further investigation, such hydrolysates (with appropriate concentration adjustments) may potentially augment, or even replace, the synthetic ones already in use in the food industry today.

The search for antioxidant peptides from Silver carp (*Hypophthalmichthysmolitrix*), Yellowstripe trevally (*Selaroidesleptolepis*), and Grass carp

(*Ctenopharyngodonidella*) have also been carried out using Alcalase and Flavourzyme, and a panel of different enzymes, respectively. Dong*et. al.*(Dong *et. al.*, 2008) performed enzymatic hydrolysis on homogenised, defatted, Silver carp fillets (muscle)with Alcalase 2.4L and Flavourzyme 500L. Alcalase proved to give higher DH values than Flavourzyme, with a hydrolysis time of between 1.5 ó 2 hours yielding an FPH with optimal antioxidant activity.

An underutilised fish species found in the East Indian Ocean and West Pacific, Yellowstripe trevally has also been used as a low-grade source of producing hydrolysate in order to investigate presence of antioxidant peptides (Klompong*et. al.*, 2007).

Antioxidant assays performed revealed that Alcalase hydrolysate with a low DH of 5% provided the best results, and antioxidative capacity decreased with increasing DH. The antioxidant power of hydrolysates is largely dependent on process conditions and type of protease used, generating different peptides and amino acid profiles (Jun*et. al.*, 2004).

Five different enzymes: Alcalase 2.4L, Neutrase 1.5MG, Bromelain, Papain, and bovine pancreatin 6, were employed in the hydrolytic reaction of homogenised Grass carp fillets (Ren *et. al.*, 2008) for resolving antioxidant peptides. Homogenate to water ratio was 1:1 for all samples, and E/S ratio ranged from 0.1 to 0.2%, with all hydrolyses carried out at 50°C and at neutral pH (except for bovine pancreatin and Alcalase ó pH 8) for 4 hours. Hydrolysates from the Alcalase reaction showed the most promising results for antioxidant peptides from the two assays performed; hydrolysate was subjected to separative chromatography techniques in order to isolate the peptides of interest, and then ionised in an ion trap mass spectrometer for peptide

sequence generation. It must be noted that the manufacturer-reported enzyme activities were used to determine the suitable E/S ratios, and Alcalase was added in excess; given it initially possessed the highest activity, this could possibly explain the reason for generating highly bioactive peptides in a fixed amount of time.

The hydrolysis of male and spent Capelin (*Mallotusvillosus*) initiated with Alcalase, Neutrase and Papain (Shahidi*et. al.,* 1995), Atlantic Cod/Salmon (*Gadusmorhua/Salmosalar*, respectively) frames using Alcalase, Neutrase and Pepsin (Liase*et. al.*, 2000), and, Pacific Whiting solid wastes, utilising Alcalase and Neutrase(Benjakul and Morrissey, 1997), These hydrolysates were analysed for antioxidant power by incorporation in meat model systems, and it was found that oxidation of the substrate was reduced by up to 60% after a specified time.

2.3.4.2. FPH with Anti-tumour properties

Alcalase and Protamex were both compared in their ability to generate anti-tumour peptidesfrom the hydrolysis of enriched aqueous fractions of Atlantic salmon, Atlantic cod, Plaice (*Pleuronectesplatessa*), Blue Whiting, Atlantic emperor (*Lethrinusatlanticus*), Pollack (*Pollachiuspollachius*) and Portuguese dogfish (*Centroscymnuscoelolepis*)(Picot *et. al.*, 2006). By-product was minced and washed before centrifuging at 3000*g* for 10 mins to precipitate proteins. The protein pellet thus obtained was dissolved in water in the ratio 1:6 (w/v), and the resulting solutionøs pH adjusted to 10.8 by titration with 1M NaOH; done to solubilise muscle myofibrillar and sarcoplasmic proteins(Undeland*et. al.*, 2002). Further centrifugation at the same conditions separated the undesirables (lipids, skins, membranes) from the second, aqueous phase containing soluble protein - decanted and pH adjusted to 5.6 using 1M HCl to precipitate a protein pellet, after again centrifuging at the

aforementioned conditions. The pellet was dissolved in two parts water, and hydrolysed at 55°C with Alcalase (2%, v/v), and Protamex (9.8%, w/v) for 106 mins. Reactions were terminated in the prescribed manner (temperature, time).

It has been discussed previously that FPH from different sources most likely contain a medley of peptones, minerals, vitamins and unknown growth factors that support the growth of industrially grown cell cultures, so the authors naturally expected that the addition of a standard amount of hydrolysate from each fish species in this study would enhance the proliferation of human breast carcinoma cell lines.Conversely,Blue Whiting, Cod, Plaice and Salmon hydrolysates all exhibited cytotoxic effect on the cancer cell lines, with the first three predominating in terms of activity.

2.3.5. Hydrolysis of fish by-productsusingBromelain and Protex 30L

Cod frames have been hydrolysed using Bromelain, Pancreatin and Papain. The main focus of the first study were to optimise system parameters using model systems, in order to replicate the reaction on a pilot scale (Himonides*et. al.* 2011). The second study, aimed to identify antimicrobial peptides present in the final FPH (Salampessy*et. al.*, 2010).

In the first of these, Cod frames were blended with warm water (1:2 ratio) in order to raise the temperature of the initial mixture, and then transferred to a reaction vessel for hydrolysis. Bromelain, Pancreatin (porcine pancreas), and Papain were each pre-dissolved in a little water before adding to the reactor contents to initiate hydrolysis. E/S ratios were all held at 1%, and three separate reactions carried out for 20, 60 and

120 mins.Almost complete solubilisation was achieved with Bromelain and Papain in 60 mins, while the same order of recovery in the FPH required more than two hours for Pancreatin.

Bromelain and Papain have also been used to hydrolyseFresh Leatherjacket (*Meuchenia sp.*) fish. It is a common resident of the Gulf of Mexico, South Atlantic and Australian waters usually not consumed as food("Leatherjacket (*Oligoplites saurus)*," 2011). The fish was deboned and skinned, mixed with water and pelletised. The pelletised proteins in suspension and supernatants were hydrolysed in the same ratio Papain was used to hydrolyse the pellet fraction (E/S 1%, w/v), and Bromelain the supernatant (E/S 0.5%, w/v). Bromelain hydrolysate produced after 8 hours (DH approx. 28%) showed antimicrobial activity against *Staphylococcusaureus*and *Bacillus cereus*.

Protex 30L has recently been used in the recovery of omega polyunsaturated fatty acids (-PUFAs), alongside Bromelain, from Salmon and Nile Perch viscera and heads(Mbatia*et. al.*, 2010a; Mbatia*et. al.*, 2010b; Mbatia*et. al.*, 2011); these by-product fractions are naturally rich in lipids.

Literature encompassing the bioactive properties of derived peptides from enzymatic hydrolysis of fish proteins is abundant but suffer a shortfall in the same; describing the value of these peptides in nutraceuticals/food supplements. In Europe, marine bioprocessing companies have explored the potential of by-product from their indigenous fish species contributing to the nutraceutical market, many are geared up towards achieving large scale production within the next few years (Aquapreneur, 2014). The present study addresses that gap in research for Nile Perch by-product from the filleting industry within Kenya.

CHAPTER 3

MATERIALS AND METHODS

3.1. Sample collectionand preparation

20 Kg of Nile Perch heads and frames (including some skin, and bones) were kindly donated by W.E Tilley Ltd, Kenya, who obtain their stock form Lake Victoria. They were acquired fresh off the filleting line, and transported to the laboratory at the University of Nairobi in cooling boxes and preserved at -20°C until use. These were cut into approximately 2-inch cubes, and fed through a Lips Combirex-I RB combined mincer (Rotor Lips AG, Switzerland) at the University of Nairobiøs Kabete campus pilot labs. 5mm grinder plates were used, followed by 4mm plates; all samples were minced to obtain some consistency, before being mixed thoroughly by hand to impart homogeneity. By-product mince was then weighed in approximately 1 Kg zip-lock bags, labeled, and stored at -80°C until further use.

3.2. Enzymes and reagents

Alcalase 2.4L ($\times$ 2.4U/g) was a donation from Novozymes AS, Bagsvaerd, Denmark (P/N: P4860), Bromelain ($\times$ 3.0U/mg) from pineapple stem (Sigma-Aldrich, P/N: B4882), and Protex 30L ($\times$2750 GSU/g) was a donation from Genencor, Danisco, (P/N: A01038). A second batch of Bromelain was necessary and was graciously donated by VTT Technical Research Centre of Finland, Finland. Hexane (P/N: H306-1), Sodium Hydroxide pellets (P/N: S320-10), Rochelle salt (Potassium Sodium Tartaratetetrahydrate, P/N: S25503A), Copper Sulphate crystalline (Cupric Sulphatepentahydrate, P/N: BP346-500),Potassium Iodide crystalline (P/N: BP367-500), 4% Boric Acid (Fluka, P/N: 31144-2.5L), and1 N Hydrochloric Acid

Concentrate (Fluka, P/N: 38283-1EA), and Methyl Red Solution (Fluka, P/N: 32941-100ML)were all purchased from Fisher Scientific East Africa Ltd. Kjeltabs were purchased from Nairobi Medical Stores Ltd.

Distilled water for all experiments was provided by the Department of Biochemistry's water still, at the University of Nairobi, Chiromo campus. Biotechnology grade Chloroform (P/N: 496189-1L), Methanol (34860-1L-R), Albumin from bovine serum (P/N: A7030-10 g), and Whatman filter paper No.3 were all acquired from Sigma-Aldrich (P/N: Z240478-1PAK).

2L of Biuret Reagent was prepared in a volumetric flask by modifying the method described by Layne (Layne, 1957). 16g of NaOH was dissolved in 2L of distilled water to make a 0.2N alkali stock solution. Approximately 800ml of this solution was pipetted into a fresh volumetric flask (2L) in order to completely dissolve the salts: 18g of Rochelle salt, and 10 g of Copper Sulphate and Potassium Iodide each, were individually dissolved in small quantities of distilled water, then added to the volumetric flask in the order above. This ensured that a black or red precipitate (copper complex) is not formed, in which case the reagent is discarded. The final volume was made up to 2L with distilled water; the reagent keeps indefinitely in a brown bottle ("The Alkaline Copper Reagent," 2002).

3.3. Proximate Analyses

3.3.1. Determination of crude protein: raw mince and hydrolysed samples

Crude protein content of raw minced by-product and hydrolysed samples was determined by a modified Biuret method for meat proteins described by Torten and Whitaker (Torten and Whitaker, 1964), and for raw mince and final FPH, by semi-

automated Kjeldahl analysis(Maina) using aTecator preheated digestion block (FOSS, Denmark) and 2200 Kjeltec Auto Distillation unit (FOSS, Denmark).

3.3.1.1.Biuret method for determination of crude protein

Protein standard stock solution was prepared from bovine serum albumin (BSA). 50mg of BSA was dissolved in 5 ml of distilled water with gentle shaking (to prevent foaming), to give a final stock strength of 10mg/ml. To generate a standard curve, aliquots of 100, 200, 400, 600, 800, and 1000 l BSA stock were diluted (except 1000 l sample) to 1 ml, and then 4 ml of Biuret reagent added (total 5 ml as per Biuret test protocol) before standing for 30 mins; this resulted in standard solutions of 1, 2, 4, 6, 8 and 10 mg/ml strength. The absorbance of each sample was read spectrophotometrically at A_{550}, and graph of Absorbance (m) versus Concentration (mg/ml) plotted to obtain an equation of linear regression (for calculating concentrations of unknown protein samples).

To determine protein concentration in samples, 1g of raw mince was placed in a 50ml Erlenmeyer flask, and 20ml of 0.5 N NaOH (10 g dissolved in 250ml distilled water) added to begin solubilisation of bound peptides in the protein matrix (alkaline hydrolysis). This mixture was heated for 10 mins over a boiling water bath, before cooling to room temperature and filtering through Whatman No.3 filter paper to remove residual fat. 15 ml of filtrate was shaken with an equal volume of Hexane before centrifuging at 8,000 rpm for 5 mins.to remove any remaining fat. The clarified aqueous phase was used for Biuret reaction. 970 l of distilled water was added to 30 l of protein extract and volume made up to, 5ml with Biuret reagent.The mixture was allowed to stand at room temperature for 30 mins before absorbance

readings were taken at A_{550} using a spectrophotometer (UVmini-1240, Shimadzu Scientific Instruments).

To measure crude protein recovered in enzymatically hydrolysed samples, the same protocol was used except starting material was fish protein concentrate (FPC) recovered after centrifuging enzymatically hydrolysed aliquots at 10,000 rpm for 15 mins followed by lipid extraction by shaking with equal volume of Hexane. This was done to minimise interferences (Krohn, 2001). Blanks were prepared by using 1ml of distilled water with 4ml of Biuret reagent.

3.3.1.2. Kjeldahl method for determination of crude protein

The Kjeldahl method for crude protein determination to evaluate Total Kjeldahl Nitrogen (TKN) was a modified protocol (AOAC, 1990) and consisted of three main steps: digestion, distillation, and titration. In the first step, 0.5g sample was weighed into Kjeldahl tubes, and 1 Kjeltab Selenium catalyst added, followed by 5 ml of Sulphuric acid before being loaded onto a preheated (420°C) digestion block. The digestion was carried out until a clear solution was obtained (up to 60 mins). For the second step, the tube was removed and allowed to cool to room temperature, before being loaded onto the Kjeltec Auto Distillation unit; 30 ml each of distilled water and 40% NaOH (80 g dissolved in 200ml distilled water), respectively, automatically dilutes the sample. 25 ml of 4% Boric acidwas added to an Erlenmeyer flask, followed by a few drops of Methyl Red solution, and also loaded onto the distillation unit. Ammonia generated from the sample was distilled into the Boric acid solution until it turned from pink to green (approx. 4 mins). In the final step, Boric acid containing Ammonia was titrated against a standard 1N HCl solution until a definitive light pink colour marked the end-point. Amount of titrant used was noted.

Appropriate blank was made using distilled water in place of sample. The analyses were performed in triplicate.

3.3.2. Determination of total moisture

Moisture in the raw mince and final FPH was determined gravimetrically (AOAC, 1990). Crucibles were dried overnight in an oven (Memmert) held at 105°C, before being removed and cooled in a desiccator. Each crucible was then weighed, and a small amount of sample added (approximately 2g), before re-weighing the crucibles. The samples were then transferred to an oven operating at 105°C, and dried for 16 hours. Samples were removed, cooled in a desiccator and re-weighed to determine the residual weight.

3.3.3. Determination of total ash/minerals

Dried samples from the moisture analysis were placed in a pre-heated muffle furnace at 550°C, and held for 2 hours at this temperature (modified protocol as same samples for total moisture were used)(AOAC, 2000).

After the allotted time, samples in crucibles were removed using tongs, and placed in a desiccator to cool to room temperature before being weighed for final mass.

3.3.4. Determination of total lipids

Lipid content was determined for raw mince and final FPH by the modified Bligh and Dyer method (Bligh and Dyer, 1959). As a precaution, all chloroform used (including in samples) was kept on ice during the procedure, to prevent evaporation and make pipetting easier. 10 g of sample was accurately weighed into chloroform resistant tubes, followed by the addition of 10 ml of distilled water, 20ml of chloroform, and

40ml of methanol (cold). The mixture was homogenised for 2 mins. 20ml of chloroform was again added before homogenising for a further 30 sec.

The same was repeated for this mixture using 20ml of distilled water, before centrifugation was carried out at 9,000 rpm for 10 mins. The chloroform phase (containing lipids) was transferred to a glass tube and kept on ice.

A separate, empty glass tube was weighed, and an exact amount of 20ml of the chloroform phase transferred to it before re-weighing. The tube was placed under a fume hood and the chloroform evaporated at 70°C. Samples were tested for complete evaporation of chloroform (by smelling) before taking final mass measurements.

3.4. Enzymatic hydrolysis

Minced by-product was thawed overnight at 4°C before being weighed. All enzymatic reactions were carried out in a jacketed glass reactor, fabricated in the University of Nairobiøs Chiromo campus workshop. A recirculating water bath with thermostat was used to provide temperature control to reactor contents, and the slurry was stirred continuously at low speed (approx. 100 rpm). An independent water bath with heating element was employed in arresting enzyme activity in aliquots withdrawn from the reaction mixture.

Conditions were kept identical in all runs. Substrate to water ratio was always 1:1. Reaction temperature was maintained at 60 ± 2°C for all enzymes(Gilmartin and Jervis, 2002) (chosen temperature based on available literature; limited for Protex 30L), while that of the reaction-arresting water bath was kept at 90 ± 5°C ó exposure to this temperature was kept short to minimise risk of protein denaturation; aliquots

were withdrawn at 30 min intervals up to 2 hours, and every hour thereafter; each one heated in the water bath and occasionally shaken for 10 mins, to arrest hydrolysis.

3.4.1. Optimisation for enzyme concentration

Three enzymes: Alcalase, Bromelain, and Protex 30L, were tested at two different concentrations.

3.4.1.1. Enzyme/Substrate ratio 0.5%

150g of sample was weighed out, and mixed with 150ml of distilled water in the reactor. The slurry was mixed until visual homogeneity and reaction temperature was achieved. After withdrawing the first aliquot and immersing in the arresting-water bath, 750 mg/750 l (w/w and v/w, respectively) of either Bromelain, Alcalase 2.4L, or Protex 30L was added.The reaction was allowed to proceed for two hours and samples collected at 0, 30, 60, 90 and 120, mins. At the end of reaction, reactor contents were discarded.

3.4.1.2. Enzyme/Substrate ratio 1%

150 g of sample was weighed out, and mixed with 150 ml of distilled water in the reactor. The slurry was mixed until visual homogeneity and enzyme-specific temperature was achieved. After withdrawing the first aliquot and immersing in the arresting-water bath, 1.5g/1.5ml (w/w and v/w, respectively) of either Bromelain, Alcalase 2.4L, or Protex 30L was added. The reaction was allowed to proceed for two hours and samples collected at 0, 30, 60, 90 and 120, mins. At the end of reaction, reactor contents were discarded.

3.4.2. Optimisation for hydrolysis time

Based on protein recovery data, Bromelain(1%) was selected to optimise hydrolysis time.

3.4.2.1. 4-hour reaction

150 g of sample was weighed out, and mixed with 150ml of distilled water in the reactor. The slurry was mixed until visual homogeneity and enzyme-specific temperature was achieved. After withdrawing the first aliquot and immersing in the arresting-water bath, 1.5g (w/w) of Bromelain was added.The reaction was allowed to proceed for four hours and samples collected at 0, 30, 60, 90, 120, 180 and 240 mins. At the end of reaction, reactor contents were discarded.

3.4.2.2. 6-hour reaction

200 g of sample was weighed out, and mixed with 200ml of distilled water in the reactor. The slurry was mixed until visual homogeneity and enzyme-specific temperature was achieved. After withdrawing the first aliquot and immersing in the arresting-water bath, 2g (w/w) of Bromelain was added.The reaction was allowed to proceed for six hours and samples collected at 0, 30, 60, 90, 120, 180, 240, 300, and 360 mins. This reaction was performed in triplicate: twice to achieve graphing data for protein recovery, and the third time (no sampling aliquots except at time 0 and 360 mins for confirmation) to collect contents for producing a final FPH from Nile Perch by-product mince.

Two suitable control experiments were performed; 4 hours and 6 hours, under the exact conditions as the respective hydrolyses above, except no enzyme was added.

3.4.3. Final FPH

Reactor contents from the 6-hour run were collected, centrifuged (HeraeusBiofuge Stratos, Thermo Fisher) at 10,000 rpm for 15 mins, and the aqueous phase decanted. The nature of this process led to some lipids overflow into the FPC obtained; no solvent extraction was attempted to separate these lipids because the aim was to produce a crude product, with minimised purification steps involved. FPC was poured into a separating funnel and allowed to stand in the cold room for 30 mins. This ensured a better-defined lipid layer that could then be separated normally using the funnel. The aqueous layer was collected and subjected to LTLT (Low temperature, long time) pasteurisation at 63°C for 30 mins (CODEX, 2004), before freeze-drying overnight (Freezemobile Sentry 2, VirTis) to yield a final FPH.

All analyses detailed were performed in triplicate, with values reported as means and on wet weight basis.

3.5.   Calculations

3.5.1. Line of regression from BSA standards

The equation obtained from standard curve was in the form $Y = mX + C$:

$$Y = 0.0511X - 0.0545 \qquad \text{(Equation 1)}$$

Where:

Y = Absorbance values of BSA standard, protein samples, and controls (mg/ml)

X = Concentration values of BSA standard, and protein samples (mg/ml)

m = Slope of curve (dimensionless)

C = Curve intercept with Y-axis (mg/ml)

3.5.2. Biuret Crude Protein

Rearranging equation 1 previously to calculate concentration of protein in unknown samples:

$$X = [(Y + 0.0545) \times (D)/0.0511] \qquad \textbf{(Equation 2)}$$

$$\% \text{ Recovery} = (X/\text{Biuret crude protein in raw mince}) \times 100 \qquad \textbf{(Equation 3)}$$

Where:

D = Dilution factor from Biuret analysis i.e. (970 l/30 l) = 32.33 (dimensionless)

Here, X values were for raw mince, and FPC aliquots,plotted against time to determine a protein recovery curve as the hydrolysis reaction progressed.

3.5.3. Kjeldahl Crude Protein

$$\% \text{ Nitrogen} = (T-B) \times N \times 1.4007/S \qquad \textbf{(Equation 4)}$$

$$\% \text{ Crude Protein (TKN)} = \% \text{ Nitrogen} \times F \qquad \textbf{(Equation 5)}$$

Where:

T = HCl Titrant volume for sample (ml)

B = HCl Titrant volume for blank = 0ml as Boric acid does not change colour

N = Normality of HCl Titrant = 1N

S = Sample weight (g)

F = Correction factor for protein matrix = 6.25 for fish (Adler-Nissen, 1984)

CHAPTER 4

RESULTS

4.1. BSA standard curve

The Absorbance values obtained using different BSA concentrations are shown in Table 2 below.

Table 2: Absorbance values for different BSA concentrations

Concentration (mg/ml)	Absorbance (ηm) at 550ηm
1	0.020 ± 0.67
2	0.470 ± 0.71
4	0.145 ± 0.66
6	0.250 ± 0.45
8	0.348 ± 0.47
10	0.464 ± 0.34

The BSA standard curve obtained had a regression coefficient, R^2 of 0.99912 as shown in Fig.5.
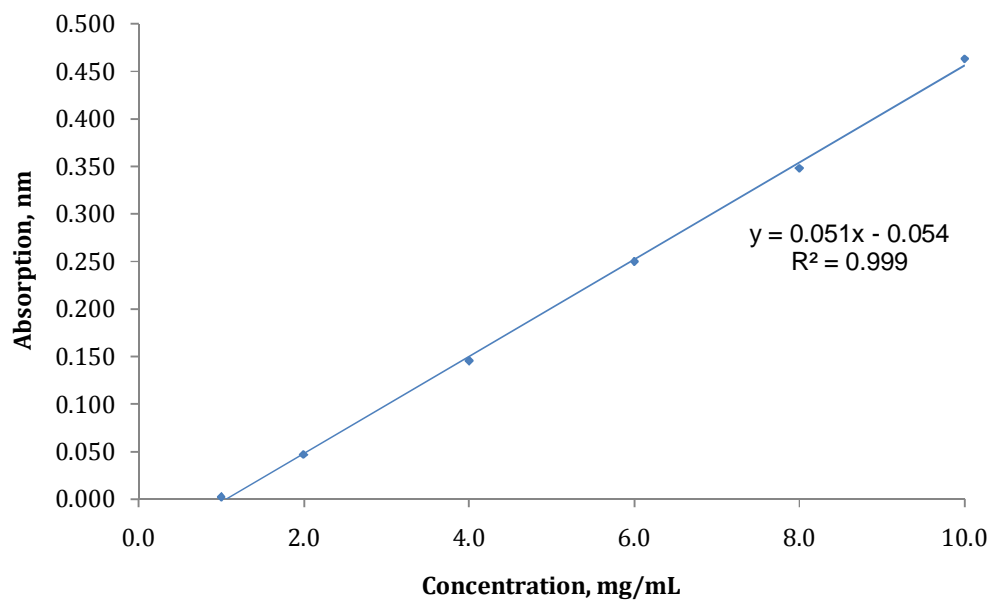
4.2. Crude protein concentration in Nile Perch by-product as determined by the Biuretand Kjeldahl methods

The Biuret crude protein raw mince was calculated by substituting absorbance values obtained at A_{550} (Y-values), in Equation 2, and subsequently in Equation 3. Kjeldahl crude protein was calculated by substituting titration values in equation 4, then subsequently in equation 5.The raw mince was shown to have a protein concentration as per the results shown in Table 3.

Table 3. Crude Protein concentration in Nile Perch by-product by Biuret & Kjeldahl Methods

Raw by-product	Method	% Protein
1	Biuret	18.42 ± 0.04
2	Kjeldahl	20.19 ± 0.47

4.3. Proximate composition of Nile Perch by-product and Final FPH

Total moisture, ash, and lipid concentration in Nile perch by-product and final FPH are shown in Table 4.

Table 4: Proximate composition of Nile Perch by-product& Final FPH

Property	% In by-product	% In Final FPH
Protein	18.4 ± 0.04	68.2 ± 1.03
Moisture	58.8 ± 1.26	14.8 ± 0.67
Ash/Mineral	10.8 ± 1.99	5.3 ± 0.40
Lipid	7.8 ± 0.63	9.8 ± 0.31

4.4. Enzymatic hydrolyses: protein recoveries in FPC and final FPH

4.4.1. Optimisation for enzyme concentration

Protein yields obtained when the by-products were hydrolysed using different enzymes at different concentrations for 2 hours are shown in Table 5. The recoveries were calculated from Equations 2 and 3, respectively

Table 5: % Protein recoveries for 2-hour hydrolysis at different enzyme conc.

	Alcalase		Protex 30L		Bromelain	
Time (min)	0.5%	1%	0.5%	1%	0.5%	1%
0	33.8± 0.93	34.5± 0.90	33.8 ± 0.81	34.9 ± 0.34	34.5 ± 0.79	33.8 ± 0.80
30	42.8± 0.91	49.6 ± 0.83	44.8 ± 0.67	48.3 ± 0.71	49.6 ± 0.77	46.9 ± 0.65
60	44.8± 0.88	50.7 ± 0.34	48.3 ± 1.05	51.7 ± 0.77	52.4 ± 0.65	52.7 ± 0.56
90	46.9 ± 0.73	59.2 ± 0.59	49.6 ± 0.66	53.8 ± 0.92	53.1 ± 0.90	58.6 ± 0.41
120	**50.0 ± 0.61**	**66.1 ± 0.67**	**51.7 ± 0.34**	**59.2 ± 0.88**	**56.8 ± 0.55**	**68.2 ± 0.39**

4.4.2. Optimisation for hydrolysis time

Protein yields obtained when the by-products were hydrolysed using 1 % Bromelain for 4/6 hours are shown in Table 6. Recoveries were calculated from Equations 2 and 3, respectively

Table 6:% Protein recoveries for 4/6-hour hydrolyses at 1% Bromelain conc.

Time (min)	Bromelain		
	4 hour	**6 hour**	**Final FPH**
0	34.5 ± 0.87	35.2 ± 0.78	35.2 ± 0.60
30	55.5 ± 0.69	41.4 ± 0.67	-
60	55.5 ± 0.93	41.7 ± 0.60	-
90	55.5 ± 0.92	42.1 ± 0.84	-
120	58.2 ± 0.78	43.4 ± 0.32	-
180	70.9 ± 0.54	46.2 ± 0.80	-
240	**97.7 ± 0.51**	49.6 ± 0.44	-
300	-	56.5 ± 0.39	-
360	-	**66.5 ± 0.21**	**68.2 ± 0.19**

4.5. Control experiments

Controls for each hydrolysis were carried out for 4 hours and 6 hours, and are recorded in Table 7. Since the initial sample mass was the same for the 2/4 hour reactions, the control for these reactions was shared and represented by the values in Table 8. Recoveries in this case are represented by the dotted line in all graphs.

Table 7: Pct. Protein recoveries for 2/4/6-hour hydrolyses – Controls

Time (min)	Control	
	4 hour	**6 hour**
0	33.8 ± 0.77	33.8 ± 0.96
30	39.7 ± 0.65	39.7 ± 0.88
60	41.0 ± 0.36	40.7 ± 0.37
90	43.8 ± 0.58	40.7 ± 0.22
120	45.5 ± 0.49	41.0 ± 0.45
180	47.9 ± 0.42	42.4 ± 0.53
240	**55.5 ± 0.76**	46.2 ± 0.67
300	-	52.4 ± 0.37
360	-	**58.2 ± 0.29**

4.6. Yield of Final FPH

The yield of the final FPH was calculated based on the initial amount of substrate (200 g) weighed for hydrolysis in the 6-hour reaction, and the weight of final FPH obtained.

Initial weight of substrate ó 200 g

Final weight of FPH obtained after freeze-drying ó 15g

Yield (Y) of final FPH = (15/200)*100 = 7.5%

CHAPTER 5

DISCUSSION

5.1.   Crude protein concentration in Nile Perch by-product as determined by the Biuret and Kjeldahlmethods

The Biuret method of measuring animal protein content is based on the formation of coloured complexes between copper ions in the reagent and proteins in the sample, and became applicable to food protein in the 1940øs ("The Alkaline Copper Reagent," 2002). The cupric ions are thought to react with three or more amino acid residues (tripeptides), but in some cases also dipeptides and amino acids(Krohn, 2001), to form chelated complexes that absorb light at 540 ó 550 m. The method is simple, fast, relatively accurate, and inexpensive. All these properties described made it easier to select this method for measuring crude protein in by-product, corresponding FPC, and, FPH. What is more, the method allows the direct measurement of concentration of dipeptides (some), tripeptides, and larger peptides; a property most useful in estimating the quality of hydrolysate obtained since this range of peptides have been described to be easily assimilated in mammals, and be the lower target range for discovering bioactive properties (Di Bernardini *et. al.*, 2011; Fitzgerald *et. al.*, 2005; Geirsdottir *et. al.*, 2011; Kim and Mendis, 2006; Salampessy *et. al.*, 2010; Schaafsma, 2009; Shirai and Ramirez-Ramirez, 2010; Slizyte *et. al.*, 2009; Udenigwe and Aluko, 2012). This being said, interferences in the method have also been reported ("The Alkaline Copper Reagent," 2002; Hortin and Meilinger, 2005), and include - with respect to this study ó lipids, removed by extraction with a suitable organic solvent such as hexane.

It was expected that the Biuret crude protein value of $18.42 \pm 04\%$ would be slightly less than that obtained by the Kjeldahl method since it only measures proteins with a minimum of two peptide bonds or more, whereas the latter gives values for total nitrogen content (including free/loosely-bound amino acids).

Several authors have also reported a high correlation between the Kjeldahl and Biuret methods for determining proteins in food matrices ("The Alkaline Copper Reagent," 2002; Keller and Neville, 1986; Pomeranz, 1965; Pomeranz *et. al.*, 1977; Torten and Whitaker, 1964), further extending confidence in using the latter method in the present study.

It is widely known in the food research community that the Kjeldahl method for determination of total nitrogen in meat samples is the most validated, accepted analysis used (Moore *et. al.*, 2010).The method indirectly quantifies the protein content of a food matrix by determining total nitrogen in it, then requiring this be multiplied by a conversion factor for that particular matrix. Generally it is assumed that all proteins in a food contain 16% nitrogen, hence the multiplication by a nitrogen-to-protein conversion factor, usually 6.25 for animal proteins (Rhee, 2001), unless otherwise validated and specified for different matrices. It is also assumed that the non-protein nitrogen (nucleic acids, phospholipids etc.) contribution is insignificant: a reason why it is also described as a method for the determination of crude protein in a sample.

The Kjeldahl crude protein value of $20.40 \pm 0.47\%$ for raw mince is more or less in agreement with that found by other authors for research carried out on Nile Perch (Kabahenda *et. al.*, 2011; Muyonga *et. al.*, 2004), though their studies did not combine

heads and frames, but relied rather on individual fractions (heads, frames, bones, skins).

5.2. Proximate composition of Nile Perch by-product

To our knowledge, published literature on results regarding moisture and ash in Nile Perch heads and frames, when homogenised into mince, is limited. Authors conclude slightly varying results because of working on these fractions independently.

Moisture results of $58.80 \pm 1.26\%$ in the present study, differ significantly from those found in literature where values lie in the range 70 ó 75% (Kabahenda *et. al.*, 2011). The samples in this study were also devoid of the muscle fraction, which is ordinarily considered to hold more moisture.

The same kind of differences may be held true for the ash/mineral results. Literature reports values in the order of 6%(skin only) ó 39%(bones only) (Muyonga *et. al.*, 2004), so the result of $10.82 \pm 1.99\%$ obtained in this study could be assumed reasonable considering the homogenate consists of heads and frames i.e. greater bone fraction than skin alone. This value would naturally be higher than that expected if Nile Perch fillets alone were used for hydrolysis (no bone).

Several methods are in existence to determine the quantity of total lipids in fish samples. In this study, a modified Bligh and Dyer (Bligh and Dyer, 1959) protocol was employed. It was originally developed for rapidly determining lipid concentration in fish samples, which mostly contain a high concentration of phospholipids (Iverson*et. al.*, 2001). Heads and frames consist mostly of dark meat - the portion in fish responsible for higher lipid content. The result of $7.86 \pm 0.63\%$ for total lipids, obtained from using this protocol, is not representative of normal lipid levels in whole

Nile Perch, because of the fractions considered. Kabahenda*et. al.* (Kabahenda *et. al.*, 2011) have reported lower values of approximately 2.5% in boiled head fractions, although the method used for the determination of crude fat in their case was Soxhlet extraction. This method has been shown to be inefficient for fish samples/samples containing high amounts of phospholipids (Xiao, 2010), and was hence not considered.

5.3. Enzymatic hydrolyses: protein recoveries in FPC and final FPH

All protein recovery data show an expected trend that follows to some extent that of DH curves(Bougatef *et. al.*, 2008; Di Bernardini *et. al.*, 2011; Kristinsson and Rasco, 2000a; Pacheco-Aguilar *et. al.*, 2008), when plotted against time. The initial steep rise in recovery within the first 30 mins is typical of enzyme hydrolysis, where the enzyme makes contact with the outermost polypeptides first, and proceeds to cleave them at sites directly related to the natural mechanism of each enzyme. As the protein structure unfolds, and time carries forward, the enzyme then complexes with the innermost peptides, before proceeding to cleave these as well. Just as is with DH curves, there is an initial rise, then levelling off, as an increase in degree of hydrolysis no longer corresponds to an increase in protein recovery in the final aqueous fraction. It has also been concluded that the release of soluble peptides from the initial phase of hydrolysis, into the reaction mixture, reduces the further rate of hydrolysis and recovery of soluble proteins (Shahidi *et. al.*, 1995).

5.3.1. Optimisation for enzyme concentration

In the 2-hour reactions with E/S ratio 0.5% and 1% respectively, Bromelain performed best in terms of recovery of soluble proteins in the final fraction. However, for an enzyme concentration of 1%, the initial 30 mins showed that Alcalase and

Protex 30L data gave better recovery of soluble protein, before proceeding to level off and fall into a gentler gradient, compared to that for Bromelain. There could be various reasons for this trend.

Both Alcalase and Protex 30L are subtilisin-like serine proteases that show broad specificity towards hydrophobic amino acid residues(Gupta*et. al.*, 2002; Kumar and Takagi, 1999), and function better under more alkaline conditions; it could be that initial pH in the reaction slurry (not determined during experiments) was slightly alkaline, favouring higher reaction rates for both these enzymes. No enzyme activity studies were carried out to verify manufacturer specifications, giving rise to yet another reason for differing results within the first 30 mins of reaction ó enzymes were added to the reaction mixture on a w/w or v/w (/substrate) basis, and not according to their respective activities.

All factors considered, Bromelain performed best at the end of the reaction and was chosen for further optimisation studies.

5.3.2. Optimisation for hydrolysis time

Increasing hydrolyses intervals by two hours, two more reactions were carried out to find the ideal hydrolysis time for 1 % E/S: 4/6-hour trials. The 4-hour reaction resulted in a very high protein recovery of 97.7 %, indicating breakdown of primary protein structure into mainly di-/tripeptides. This was a desirable outcome, and the 6-hour trial was performed. At this point, it is important to mention that the Bromelain batch previously used (Sigma-Aldrich, P/N: B4882) had been exhausted, and the new batch donated by VTT was put to the test for the final trial. This change in enzyme gave rise to very different results. It was expected that the increase in time would yield a peak in data, up to the point of maximum di-/tripeptides recovery, and then a

gradual drop as the peptides were further hydrolysed into free amino acids, not accurately measurable by the Biuret method. After the 6-hour reaction, the maximum recovery of 66.46% was quite low compared to that achieved with the Bromelain batch from Sigma-Aldrich. In a personal communication from VTT, it was conveyed that the reported activity of the second Bromelain batch was unusually low (0.7 U/mg compared to a standard of $\times$ 3.0U/mg) (Hakala, 2012).This explains why the expected trend in data was not observed. This communication also put into perspective the large difference of 48.9% in maximum recoveries achieved between the 1% 4-hour (97.7%) and 6-hour reactions, at the 4-hour mark (exact same conditions and E/S ratios).

The 6-hour reaction was repeated without sample aliquots being withdrawn at the usual intervals, except at 0 and 360 mins, in order to obtain a final FPH with a protein recovery of 68.18%. To our knowledge, no current literature has been published for pasteurisation of FPC/FPH, so the standards for milk were employed (CODEX, 2004). Current manufacturers of protein hydrolysates do not detail their pasteurisation conditions, but give a general overview of their process. All that is known is that it is carried out before industrial spray drying. In addition, the reaction-arresting temperatureand time of $90 \pm 5°C$ for 10 mins.employed in our studies, was assumed to provide a decent measure of microbial control.

5.3.3. Controls

The control experiments provide some insight into the extent of autolysis taking place. Hydrolysis is a process that will inevitably occur once water is added to the substrate. Our studies show that it is worthwhile employing an externally added protease if it possesses sufficient activity. Considering only Bromelain at 1 %

concentration, the two, four, and six-hour reactions all show an increase in recovery greater than 10 %, with that of four hours showing the highest difference between the control and reaction with enzyme (97.7 ± 0.51 ó 55.5 ± 0.76 = 42.1 ± 0.75 %).

5.4.   Proximate composition of Final FPH

It was observed that the moisture and ash/mineral content of the Nile Perch FPH had been significantly reduced through enzymatic hydrolysis with Bromelain, and subsequent freeze-drying. While moisture was removed through freeze-drying, the ash/mineral content (largely attributed to fish bone) was lessened through decanting of the aqueous layer in the final FPC fraction of hydrolysis; a measure not performed in proximate analysis of by-product mince. The lipid content, however, showed a slight increase in concentration. In both cases, solvent extraction with Hexane was not performed, so the only reasonable explanation would be that as hydrolysis proceeded, phospholipids and lipoproteins present in the muscular fraction of the by-product were enzymatically broken down, and lipids released into solution ó these would be harder to separate by simple decanting as lipoproteins and phospholipids form a very thin layer between the lipid and aqueous (protein-rich) layer following centrifugation, and is indistinguishable by visual inspection (Kristinsson and Rasco, 2000a). Naturally, one would expect to see an increase in lipid content in the freeze-dried FPH thereafter.

5.5.   Yield of Final FPH

The yield of the final FPH was found to be very low at just 7.5%, which is consistent with reports from other authors(Kristinsson and Rasco, 2000a), and constitutes an economic challenge when factoring in the possible scaling up of this technology for the industrial setting. However, such a low yield is commonplace when dealing with

a fish protein concentrate that is mainly moisture ó the protein content of the final FPH, is however, high at 68.2 %, and could further be enriched by efficient methods of separation such as ultrafiltration (Kristinsson and Rasco, 2000a), but not without escalation is cost of the process. The moisture lost during drying may be recovered through condensation, and be used for further hydrolyses reactions ó this could be a possible offset for the cost of ultrafiltration.

5.6. Other considerations and future prospect

Being the first of its kind, this study of the enzymatic hydrolysis of Nile Perch by-products (heads and frames) is bound to be subject to further improvement and research. This being said, we feel that the data gathered here lends a good foundation to the future of this science in Kenya. For manufacturers interested in producing an FPH for purely nutritional purposes, scale-up studies combined with implementation of food safety regulations could potentially lead to enormous value addition activities in this industry.

Several authors report the use of extensive sample preparation/hydrolysis measures in their enzymatic studies; varying from suspension in pH buffers, to extraction of proteins prior to hydrolysis. In our view, this results in clearer data generation, but also complicates the science when it comes to considerations for industrialisation. It is naturally realised that the more steps that are involved in sample treatmentand hydrolysis, the more expensive the final FPH will be. Translating sample pre-treatment to the industrial scale creates huge impacts on capital investments e.g. the use of pH manipulation resulting in downstream salt removal from the FPH, by processes such as evaporation and ion exchange; pre-heating by-product in order to destroy endogenous enzyme activity (strain on utilities) etc. For reasons such as these,

pH manipulation and pre-heating samples was never considered, even though some studies and enzyme manufacturers site the use of such conditions. We felt it best to keep things simple and generate meaningful results that would have less issues during future scale-up studies.

The FPH from this study should ideally also be subjected to further experiments to sequencegenerated peptide fragments and determine their respective sizes, along with amino acid profiling. This would be essential if the FPH is to have any future in the nutraceutical sector, by way of discovering any bioactive peptides and their possible effects on human physiology. A recent patent for FPH filed describes the use of the product in lowering the concentration of cholesterol in plasma and triglycerides in the liver of Zucker rats (Berge, 2012). The author carried out extensive studies on plasma levels of these compounds in the rats, post-FPH production. This is just an example of the many investigations that can and should be carried out on FPH from Nile Perch.

However, something must be said of the safety of such an FPH. For natural/engineered enzymes, there is always the post-hydrolysis digestion to be considered; though the enzyme has been inactivated, its protein structure may change and affect its assimilation in the gut(Nielsen, 2010). Another major area that needs to be looked into before these activities can proceed is the allergenicity of peptides in the final FPH. Though less allergenicity has been associated with low molecular weight peptides (Maebuchi *et. al.*, 2007), and enzyme hydrolysis has been repeatedly used to reduce the allergenicity of baby formula (Knights, 1985), it is still worth considering if these products are to be used in human nutrition.

In conclusion, this work represents a major starting point for further studies in the field of enzymatic hydrolysis of Nile Perch by-products within Kenya, to expand on

the formulation of nutritional supplements and contribute towards better food security. The possibilities of discovering good nutritional and bioactive properties in the resulting FPH may by limitless, as manipulations of reaction conditions to alter final protein hydrolysatescan be performed in order to generate targeted properties. It is our recommendation that for the findings of this study to be industrially relevant, further research must be carried out to assess the amino acid profile of the resulting FPH in order to determine itøs nutritional suitability, and, attempts be made to maximise yields for further scale up studies to achieve economies of scale.

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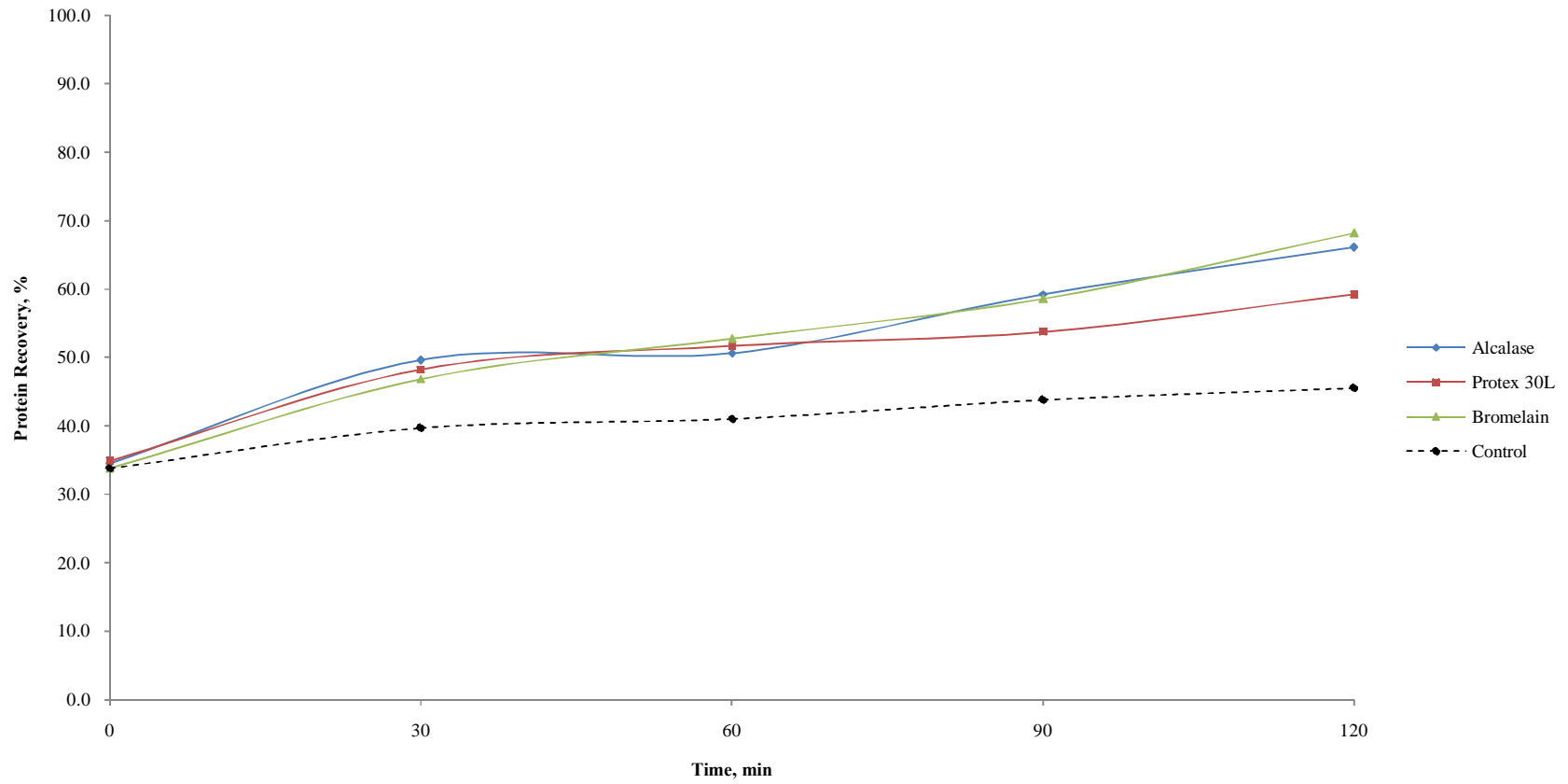
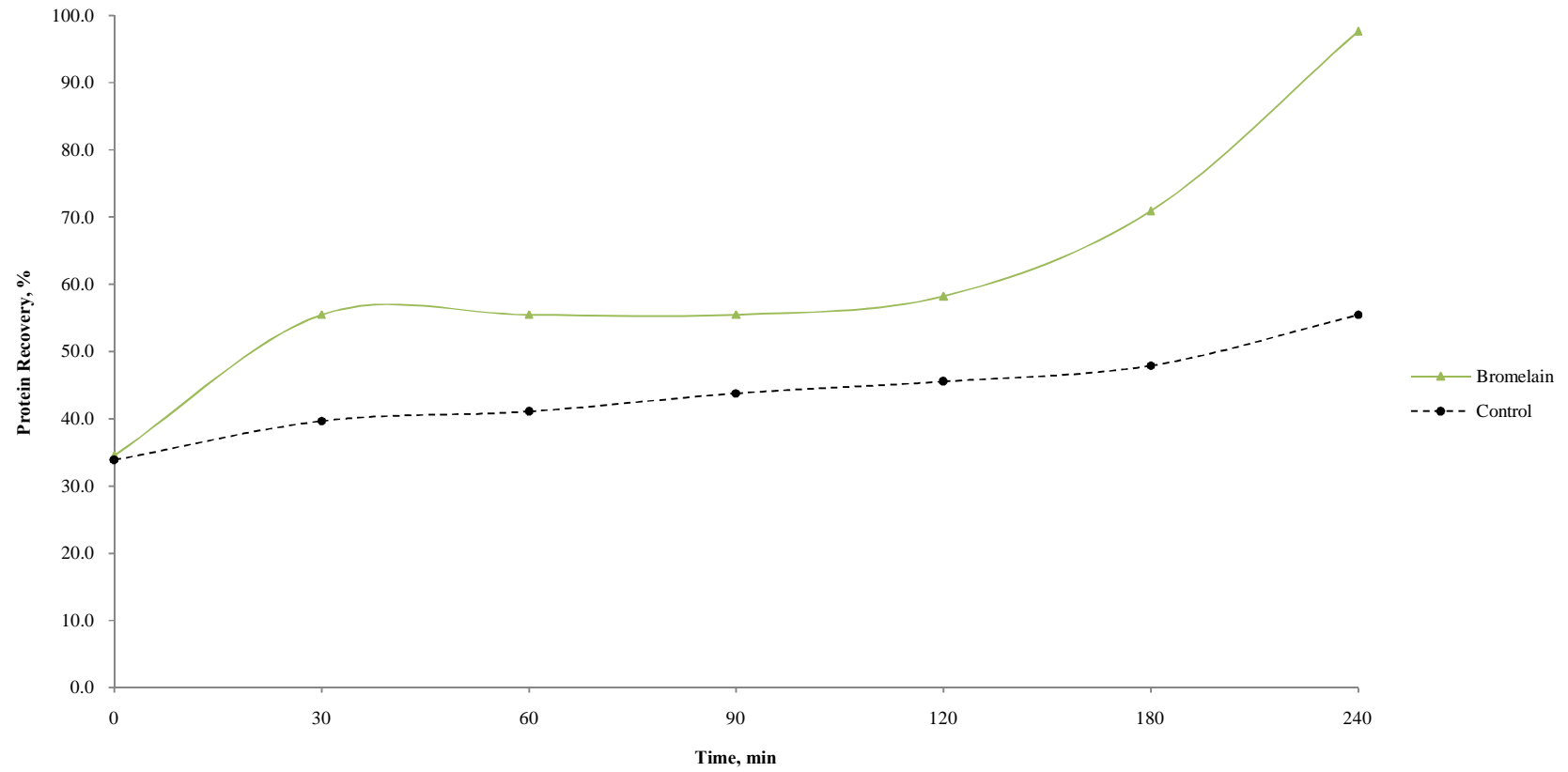**Protein recoveries for 2 hour reaction, 0.5% Enzyme/Substrate ratio**
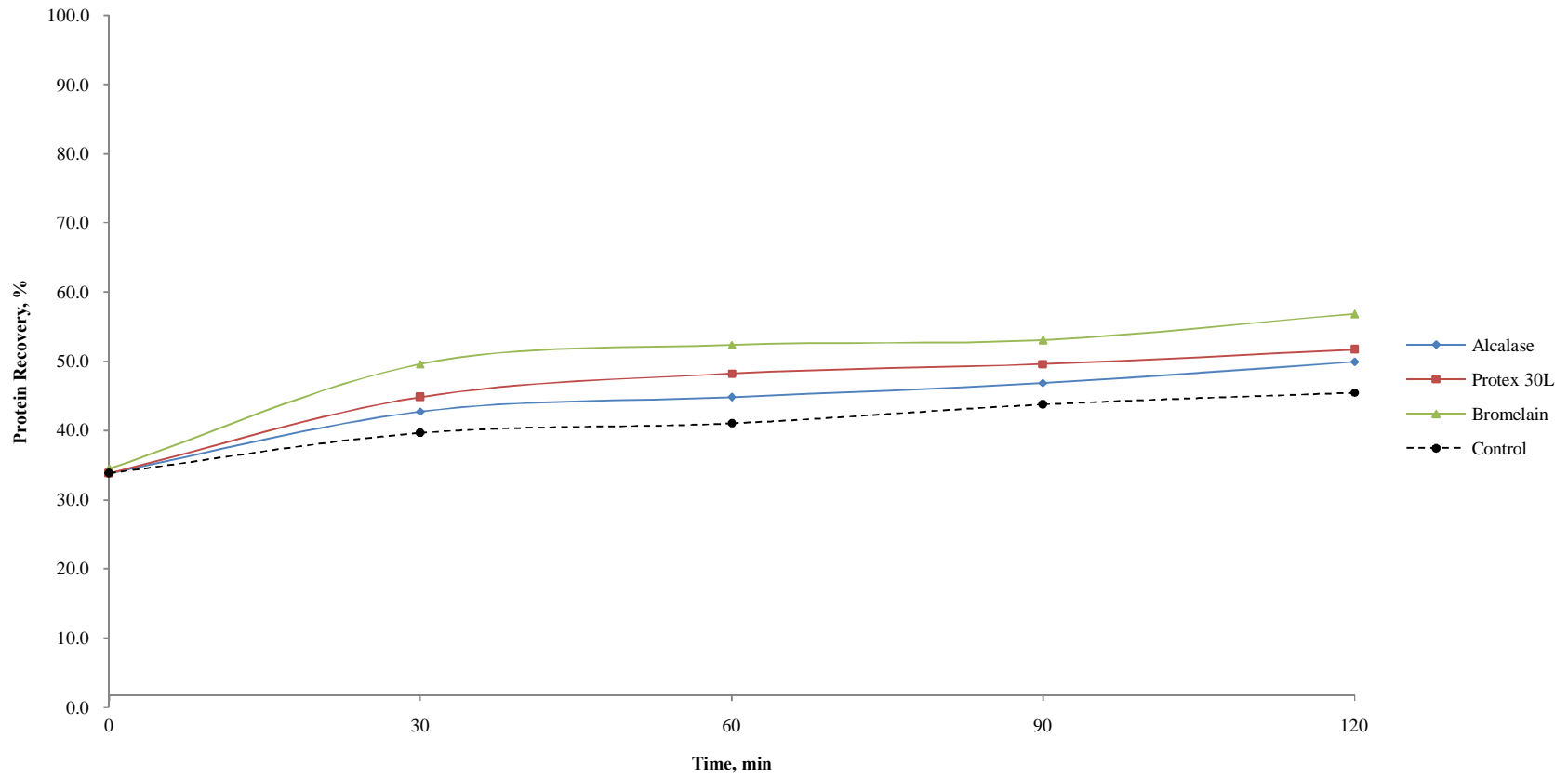
Protein recoveries for 2 hour reaction, 1.0% Enzyme/Substrate ratio

APPENDIX 3

Protein recoveries for 4 hour reaction, 1.0% Enzyme/Substrate ratio

Protein recoveries for 6 hour reaction, 1.0% Enzyme/Substrate ratio