DEVELOPMENT OF BIOACTIVE PEPTIDES FROM WHEY BY ENZYMATIC HYDROLYSIS

By

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A Dissertation Submitted in Fulfilment of the Requirement for the Degree of Master of Science in Food Technology: Quality Assurance of the University of Reading

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CERTIFICATION

The undersigned certifies that she has read and hereby recommends for Examination a Dissertation entitled ***Development of Bioactive Peptides from Whey by Enzymatic Hydrolysis***, in fulfilment of the Requirement of Master of Science in Food Technology: Quality Assurance of the University of Reading.

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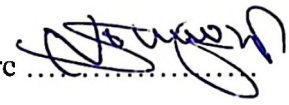
Signature 

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DEDICATION

This Dissertation is dedicated to my beloved parents Mr. Wenaty Ngungulu and Mrs. Elizabeth Ngovano and my brother Ponsiano Ngungulu. I highly acknowledge their day to day prayers to me. These people are really the backbone of my success.

ABSTRACT

The major purpose of the project was to develop and apply an integrative process for the production of bioactive peptides from the waste stream after adsorption of the acid whey by enzymatic hydrolysis, particularly the use of Protease N Amano. The study involved use of anionic resin in the adsorption process and Protease N Amano in the hydrolysis process at an enzyme: substrate ratio of 1/100 (wt/wt) added to the adsorbed protein in a batch membrane reactor at 45^{0}C. Before hydrolysis the adsorbed protein was re - solubilised using 30ml. of a 10mM (0.01M) potassium phosphate buffer at pH 7. The resulting fractions were analysed for total protein by BCA assay and major whey proteins by HPLC. Results of the adsorption of waste stream show that 60.68% of β-Lactoglobulin and 17.27% of α-Lactalbumin were adsorbed by the anionic resin. Moreover, in the hydrolysis process the amount of protein hydrolyzed was found to increase with increase in hydrolysis time.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ACRONYMS

ACE - Angiotensin - converting enzyme

α-LA - Alfa Lactalbumin

BCA - Bicinchninic acid

β-LG - Beta Lactoglobulin

BSA - Bovine Serum Albumin

CMP - Caseinomacropeptide

CPPs - Calcium-binding phosphopeptides

HA - Hyppuric acid

HHL - N- Hyppuryl – L – Hystidyle – L – Leucine

HPLC - High Performance liquid Chromatography

TFA - Trifluoroacetic acid

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Background information

Whey, the serum or liquid part of milk remaining after separation of the curd, results from the coagulation of milk by acid or proteolytic enzymes. Whey proteins have a relevant nutritional value, and several commercial uses have been developed by the industry associated to the dairy. The composition and characteristics of whey depend on the production technology, the end product and the quality of milk used. Liquid whey consists of 93% water and contains 50% of the total solids present in milk, of which lactose is the main constituent. A high nutritional value of bovine milk proteins and in particular to the whey proteins is widely recognized. Also, the multiple functional properties of major milk proteins are well characterized and exploited by various industries. Milk proteins have attracted growing scientific and commercial interest as a source of biologically active molecules having distinct characteristics.

Casein and whey are two important milk proteins found to be rich sources of biologically active peptides (Guo, Pan & Tanokura, 2009) which are studied and revealed to play nutritional and functional role in human health. Foods providing functional and nutritional roles have been gaining much popularity as well as receiving much attention in the society in recent years due to the reason that food need to have a quality that promotes health of the consumer (Wei and Chiang, 2008, Welderufael and Jauregi, 2010) and in this case, different biologically active peptides

have studied in the widest sense (Wei and Chiang, 2008) to explore the functional and nutritional attributes of the food proteins in question.

Whey is a milk protein accounting to about 20% (Madureira, Tavares, Gomes, Pintado & Malcata, 2010 and Korhonen, Pihlanto – Leppala, Rantamaki & Tupasela, 1998) of the total milk protein representing an excellent source of both functional and nutritional proteins (Korhonen *et al.*, 1998). It is clearly understood as that watery portion of milk remaining following the removal of casein protein in the process of cheese making (Welderufael and Jauregi, 2010).

The major components of whey proteins which account to about 70 - 80% of the total whey proteins are β-Lactoglobulin and α-Lactalbumin (Dereck, Geoffrey, Peter & Andre, 2006). Whey proteins are proved to have high amounts of β-Lactoglobulin and α-Lactalbumin (Sylvia, Chung, Paul, Ajay & Hugh, 2009) of which β-Lactoglobulin contributes about 50 – 55%" of overall whey protein in bovine milk (Hernandez – Ledesma, Ramos, Recio & Amigo, 2006). As compared to many bioactive peptides derived from different food proteins, those of bovine milk proteins origins are the most widely researched (Wei and Chiang, 2008). In addition to the mentioned major whey proteins, another major whey protein which is also important is Bovine Serum Albumin (BSA) (Welderufael and Jauregi, 2010). Apart from these three known major whey proteins, there are some minor whey protein components present in minute quantities which are important sources of biologically active peptides; these are immunoglobulins, glycomacropeptide, lactoferrin, proteosepeptones and numerous enzymes (Korhonen *et al.*, 1998). Studies carried out

have shown that hydrolysates of whey proteins have reasonable increased quantities of biologically active peptides (Ferreira *et al.*, 2006) and are increasingly becoming popular and have been widely used throughout the World as ingredients added to special foods to optimize their functional and nutritional value. In this particular respect, enzymatic hydrolysis has been widely employed in the hydrolysis of such milk proteins in order to produce ingredients with improved functional characteristics such as gelling, emulsifying, foaming and solubility (Ferreira *et al.*, 2006) and improve the nutritional properties (Guadix, Camacho & Guadix, 2006) of whey proteins. This is due to the fact that, studies carried out have revealed that the hydrolysates of whey protein are generally observed to posses high solubility, low viscosity and considerable changes in foaming, gelling and emulsifying characteristics in comparison to those of the parent proteins. These changes in functional and nutritional properties of whey proteins are a result of development of bioactive peptides released by enzymatic hydrolysis of the entire proteins (Gauthier and Pouliot, 2003).

Milk and milk proteins, whey and casein in particular are nowadays regarded as main origins of biologically active peptides and very large number of these peptides are being detected in the hydrolysates of milk proteins and also in dairy products which have been fermented as fermentation is an alternative way of generating bioactive peptides (Pihlanto and Korhonen, 2006). Many studies carried out indicate that during the process of milk fermentation and when the cheese matures, the indigenous enzyme available in these products disintegrate the major proteins into a large

number of small fragments known as peptides (Gill, Lopez – Fandino, Jorba & Vulfson, 1996).

Moreover, apart from milk proteins i.e. casein and whey, there are a large number of proteins originating from animals and plants which have been studied and exploited as sources of bioactive peptides. These include eggs, meat muscle proteins, marine proteins including shrimp, fish, salmon, snow crab, oyster and seahorse and plant proteins such as soy, pulses, wheat, oats and hemp seeds (Udenigwe and Aluko, 2012).

The biologically active peptides commonly referred to as bioactive peptides are basically referred to as specific protein fragments that have a positive impact on various functions that the body perform or circumstances and may finally have a significant influence on the health of the consumer (Pihlanto and Korhonen, 2006). According to Belinda, Erdmann & Schroder, 2008; bioactive peptides may impart local effects into the GIT (gastrointestinal tract) or may be absorbed during digestion by the intestinal tract and enter blood streams unchanged where it can exert systemic effects. They are generally peptides derived from food protein which are considered to posses properties that are pharmacologically and nutritionally beneficial to the health of the ultimate consumer (Udenigwe and Aluko, 2012). Studies have shown that, consumption of bioactive peptides produces significant effects on major body systems including the digestive, cardiovascular, immune and nervous systems (Pihlanto and Korhonen, 2006) and this has been found to depend on the amino acid sequence of the protein under consideration. Depending on the sequence of amino acids in a protein under consideration, these biologically active peptides herein

referred to as bioactive peptides may exhibit diverse activities. These activities may include for instance: mineral binding, immunomodulatory, antimicrobial, antioxidant, hypocholesterolemic and antihypertensive functions. They are mainly used as components of functional foods or nutraceuticals due to their safety profiles and health- enhancing potentials (Belinda *et al.*, 2008).

1.2 Research Problem Statement

Whey protein is produced as a result of milk coagulation following the addition of an enzyme for sweet whey or an acid for acid whey in a process of cheese production. It is generally estimated that only about 10 - 20% of the raw milk is utilized in the production of cheese where as the remaining 80 - 90% gives up whey as a waste product (Welderufael and Jauregi, 2010). Furthermore, according to a study carried out by Welderufael and Jauregi, 2010 the quantity of whey produced in a year Worldwide is approximately 115 million metric tons and only about 61 million metric tons which amounts to about 53% of the amount produced annually are potentially utilized as whey products in various foods consumed by human beings while the remaining 54 million metric tons equivalent to about 47% are treated as wastes and discharged off into the environment. This huge amount of the waste stream discarded into the environment is likely to consist of significant quantities of valuable proteins including β-Lactoglobulin, α-Lactalbumin, Serum albumin, Lactoperoxidase and Lactoferrin which are important sources of bioactive peptides having nutritional and functional characteristics of healthy importance to the consumer (Welderufael and Jauregi, 2010; Meisel, 1997). The present study therefore will involve development and application of an integrative process for

production of biologically active (bioactive) peptides from the waste stream (non adsorbed protein) by using enzymatic hydrolysis. According to Welderufael and Jauregi, 2010; the waste stream is mainly composed of α-Lactalbumin and BSA as large percent of β-Lactoglobulin is adsorbed by the anionic resin, therefore the study will be based on finding out whether the waste stream can produce bioactive peptides through hydrolysis using a suitable enzyme.

1.3 Objectives of the Study

1.3.1 General objective

The general objective of this study was to develop and apply an integrative process for the production of bioactive peptides from the waste stream after adsorption of the acid whey by enzymatic hydrolysis using Protease N Amano.

1.3.2 Specific objectives

The specific objectives of this study were:

1. To prepare the acid whey from locally purchased cow's milk
2. To perform an adsorption process of β-lactoglobulin in the acid whey
3. To develop an Integrative process for production of hydrolysate from waste stream (Non adsorbed protein)
4. To perform a BCA assay for total protein of whey and whey fractions and HPLC analysis for peptide profile

CHAPTER TWO

LITERATURE REVIEW

2.1 Enzymatic hydrolysis of whey proteins

The most common way to produce bioactive peptides is through enzymatic hydrolysis of whole protein molecules. Many of the known bioactive peptides have been produced using gastrointestinal enzymes, usually pepsin and trypsin. Angiotensin-converting enzyme (ACE)-inhibitory peptides and calcium-binding phosphopeptides (CPPs), for example, are most commonly produced by trypsin (FitzGerald *et al.*, 2004). Moreover, ACE-inhibitory peptides have recently been identified in the tryptic hydrolysates of bovine as2-casein (Tauzin, Miclo, and Gaillard, 2002) and in bovine, ovine and caprine k-casein macropeptides (Manso and Lopez-Fandino, 2003). Other digestive enzymes and different enzyme combinations of proteinases—including alcalase, chymotrypsin, pancreatin, pepsin and thermolysin as well as enzymes from bacterial and fungal sources —have also been utilized to generate bioactive peptides from various proteins (Kilara and Panyam, 2003).

Production of bioactive peptides from whey and other protein molecules by hydrolysis under the influence of enzyme actions is the most common method which has attracted special attention of different researchers in recent years (Pihlanto & Korhonen, 2006; Korhonen, 2009 and Hernandez – Ledesma, Contreras & Recio, 2011). Due to the fact that many bioactivities of whey and protein in general are latent, enzymatic hydrolysis process is required in order to release the bioactive

peptides from inactive proteins (Schanbacher, Talhouk & Murray, 1997 and Meisel, 1997). Apart from conventional production of peptides from natural protein sources by proteolytic enzymes, recombinant DNA techniques have been experimented for the production of specific peptides or their precursors in microorganisms.

2.2 Nutritional benefits of bioactive peptides

The nutritional and techno-functional properties of whey are studied and advances in whey research have shown that whey is a valuable source for highly prized nutraceutical ingredients.

Whey proteins after ingestion are cleaved by the gut proteases and made available through the uptake by oligopeptide pumps and transporters. Thus, short peptide sequences contained within whey proteins are the supposed effectors of the bioactivity of individual whey proteins. Availability and stability: longer peptides must enter the gut barrier and show some degree of resistance to proteolysis. Many studies on whey peptides have been made using parenteral injection in animals.

In fact, an in vitro study indicated a β-lactoglobulin fragment to be a potent inhibitor of Angiotensin Converting Enzyme (ACE) activity in vitro. However it is probably not sufficiently stable to gastrointestinal and serum proteinases and peptidases to act as an hypotensive agent in humans following oral ingestion (Walsh *et al.*, 2004).

Functional peptides have been studied in milk, whey, enzymatic protein hydrolysates and fermented dairy products (Meisel 2005; Korhonen and Pihlanto 2007; Gobbetti *et al.*, 2002; Gobbetti *et al.*, 2007; Hartmann and Meisel, 2007; Korhonen 2009;

Korhonen 2010). Whey peptides have been studied for anti-clotting, anti-thrombotic and hypotensive activity (Gobbetti *et al.*, 2007), mood regulation and opioid-like activity, antibacterial activity (Clare *et al.*, 2003), immunomodulation, anti-inflammatory activity, anti-caric properties (Martinez *et al.*, 2009), prebiotic activity, mineral binding properties, gastrointestinal health effect, hypocholesterolemic effects, insulinotropic effects, memory and stress effects (Korhonen, 2010).

A study carried out by Wei and Chiang, 2008 based on bioactive peptides production by hydrolysis of porcine blood proteins in a continuous enzymatic reactor revealed that enzymatic hydrolysis is an important process for producing whey protein hydrolysates having low allergenicity and antioxidant activity. It is further observed that, in case of allergenicity of whey proteins, the protein that is responsible for allergic reactions is β-Lactoglobulin and hydrolysis of such proteins using suitable enzymes has been shown to be a better method for avoiding such allergic reactions (Prieto, Guadix, Gonzalez Tello & Guadix, 2007).

The amino acid composition of whey proteins gives them a special role in physiology and functionality. Whey proteins contain a high amount of sulphured amino acids, which contributes to their great nutritional quality. Sulphur containing amino acids seem to enhance the immune function of the organism, due to regulation of glutathione, a sulphured tripeptide which protects the cell and is a substrate for enzymes that reduce oxidised substrates. Whey proteins are rich in branched amino acids isoleucine, leucine and valine, necessary in the muscle cells to promote protein synthesis. These amino acids are metabolized to generate energy in muscles rather than in the liver. Many mass and body-building drinks and snack are enriched in

whey proteins and branched chain peptides. Whey proteins have a high content in sialic acid (N-acetyl-neuraminic acid, NANA).

In addition to be assimilated for the synthesis of neuronal membranes and glycosylated antigens, sialic acid forms glycans with highly restricted expression on human leukocytes, modulating their immune responses (Drake *et al.*, 2008). Several nutraceutical products are marketed today from whey proteins, with specific health claims (Faryabi *et al.*, 2008). Among other products, there are: Cysteine Peptide, produced by DMV International, NL, (whey hydrolysate), claimed to aid to raise energy level and sleep, and Vivinal Alpha, produced by Borculo Domo Ingredients (BDI), NL, (whey hydrolysate), containing a peptide claimed to aid relaxation and sleep.

2.3 Bioactivity of whey proteins and peptides

2.3.1 β-Lactoglobulin (β-LG)

β-Lactoglobulin (β-LG) is the most abundant protein in whey of other mammal species but not present in human and camel milk. It sums up to 3,7 g/L in sheep, 74% of whey proteins, from 3 to 3.2 g/L in goat and cow' whey respectively, 58 to 65% of total whey proteins. Its sequence (bovine, ovine and equine β-LGs) contains several amino acid substitutions most of which conserve the overall charge and isoelectric points, and in some case (equine β-LG) introduces a glycine producing a longer protein. Bovine β-LG is quite resistant to digestion by pepsin. This resistance may contribute to the allergy to cow milk in sensible individuals that develop atopic dermatitis, urticaria, or even anaphylactic reactions. Resistance to digestion is not uniform among species, with ovine β-LG being highly sensible to pepsin proteolysis

(El-Zahar *et al.*, 2005). Some individuals show cross-reactivity to milks of related species (sheep, goat), that is less frequent with equine milk. Patients with negative Skin Prick Tests (SPT) tolerate well camel, donkey and (to a lesser extent) goat milk (Ehlayel *et al.*, 2012). Proteolytic digestion of bovine β-LG by trypsin yields four peptide fragments with bactericidal activity. These peptides, negatively charged, inhibit only Gram-positive bacteria (Pellegrini *et al.*, 2001). Similarly, β-LG originates other opioid-like peptides such as β- lactotensin. β-LG exhibits prebiotic effects on *Bifidobacterium* and *Lactobacillus* species.

2.3.2 α-lactalbumin (α-LA)

α-lactalbumin (α-LA) is the second most abundant whey protein. Its content is 2 g/L in goat, 27% of whey proteins, 1.2 g/L in sheep and cow, 15% of total whey proteins, and 2.4 g/L in equine whey. It binds and transports retinol and fatty acids, and calcium ions. α-LA is rich in branched amino acids isoleucine, leucine and valine, necessary in the muscle cells to promote protein synthesis. Its variants show conserved isoelectric points, ranging between 4.8 and 5.11. α-LA originates peptides with antimicrobial (Pellegrini *et al.*, 1999; Haque and Chand, 2008) and anti-stress properties, partly mediated by a high tryptophan content used in serotonin synthesis. α-LA originates α-lactophorin/ α-lactorphin, which act on opioid receptors (Ijaes *et al.*, 2004). α-lactophorin has been shown to exert a weak opioid activity to smooth muscles. When injected into the bloodstream, these peptides induce an analgesic and sedative effect on the nervous system. When α -lactophorin was given subcutaneously to conscious, unrestrained spontaneously hypertensive (SHR) and normotensive rats, blood pressure decreased in the SHR rats. This response was blocked by opioid receptor antagonist naloxone, suggesting the opioid receptors were mediating the effect. Positive

neurocognitive effects have been associated with bioactive peptides originating from α-LA and other whey components (Camfield *et al.*, 2011).

2.3.3 Caseinomacropeptide (CMP)

Caseinomacropeptide (CMP) consists of 10 to15% of whey proteins and therefore it is the third most abundant peptide in rennet-based cheese whey. It corresponds to the 106-169 C-terminal peptide from κ-casein, containing several phosphorylated serines and glycosylated threonines, and contains sialic acid, *N*-acetylgalactosamine and other mucin-like glucans (Thomä-Worringer *et al.*, 2006). The level of phosphorylation and glycosylation is highly heterogeneous, with CMP variants present in different glycosylated forms (disaccharide, trisaccharide and tetrasaccharide chains). The goat CMP contains 25 μg sialic acid per mg of dry weight, 2-fold less than in bovine CMP (Moreno *et al.*, 2001). CMP was found glycosylated up to 36% in goat, and to a lesser extent, approximately 30% of total CMP, in ovine milk (Moreno *et al.*, 2000). CMP is a hydrophilic, thermostable peptide, with a net negative charge even at low pH. CMP content is higher in cow, sheep, and goat whey, reflecting the higher caseins content. CMP is very low in equine milk, either due to a low caseins/whey proteins ratio, and to the low content of κ-casein (since other caseins support the precipitation of casein coagulum), but contains 68 amino acids, compared to 63 amino acids in bovine CMP and 65 in human CMP. Phenylalanine does not occur in goat CMP and its content is negligible in CMP of other origins. CMP exhibits prebiotic effects on Bifidobacteria and Lactobacilli. CMP, and other phosphorylated peptides derived from casein, have been shown to increase the solubility of calcium and enhance the absorption of

calcium (Martinez *et al.*, 2009). CMP can be used as an antimicrobial peptide to fight caries and displaces *Streptococcus mutans* and *Porphyromonas gingivalis*, two oral opportunistic pathogens (Malkoski *et al.*, 2001; Aimutis, 2004), while it favours the growth of Lactobacilli in the oral cavity. CMP showed to possess intestinal anti-inflammatory activity on monocytes (Requena *et al.*, 2009). CMP may exert an indirect intestinal anti-inflammatory effect through potentiation of host defences against invading microorganisms.

2.3.4 Lactoferrin (Lf)

Lactoferrin (Lf) content in whey varies according to the species of origin: 1.6 g/L in human milk, up to 600 mg/L in equine whey, but only 100 - 200 mg/L in bovine, goat and sheep whey. Cow, buffalo, goat and sheep Lfs share over 90 % sequence identity with each other and form an extremely closely related group. Lf binds iron ions on a wide range of pH (10% of Lf is saturated with Fe^{3+}) and also other essential elements (vanadium, manganese, molybdenum, and zinc ions). Lf structure is bi-lobular, each lobe binding and retaining one Fe^{3+}, even at low pH (except the C-terminal lobe of camel Lf), the crystallographic and three dimensional analysis showed a highly conserved three-dimensional structure in Lfs of different origin (Baker *et al.*, 2000). The lobes are connected by an alpha-helical linker peptide that is cleaved by pepsin in two symmetric lobes (Baker and Baker, 2005). A comparison of native and deglycosylated Lf has shown that Lf binding properties for iron are unaffected, but the loss of carbohydrate increased its sensitivity to proteolysis. The N-terminal lobe includes the lactoferricin (Lfcin) sequence, shown to possess antibacterial properties (Bellamy *et al.*, 1992; Lopez Exposito and Recio, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials and Methods

3.1.1 Materials and Reagents

All chemicals and reagents used in the study were of analytical grade including: Bovine Serum albumin (BSA), β-Lactoglobulin, α-Lactalbumin, bicinchninic acid solution (BCA), DEAE sepharose, Monobasic potassium phosphate, Dibasic potassium phosphate, Sodium chloride (NaCl), Protease N Amano enzyme, microfiltration membranes (0.45 µm), syringe driven PVDF filters (0.45 µm and 0.22 µm), Angiotensin- Converting Enzyme (ACE), N- Hyppuryl - L – Hystidyle - L - Leucine (HHL), methanol, acetonitrile, trifluoroacetic acid (TFA), hydrochloric acid and hyppuric acid (HA) and skimmed milk obtained from a local supermarket.

3.2 Methods

3.2.1 Acid Whey Preparation

Pasteurized milk (2Litres) bought from a local supermarket was heated at 20^0C in a water bath. Then a 5M Hydrochloric acid was continuously added to milk at 20^0C with vigorous stirring to produce a soft cheese. The soft cheese was then incubated at 20^0C in a water bath for 30minutes to allow casein separate from whey. After that the whey was filtered using cheese cloth and centrifuged at 3200RFC and 4^0C for 30 minutes. The whey was then filtered using microfiltration membranes (0.45µm) and stored at -18^0C for use in the subsequent experiments.

3.2.2 Adsorption of β-Lactoglobulin in the acid whey

The adsorption process is illustrated in Appendix 1. The acid whey was mixed with an adsorption resin in a filtration reactor fitted with a microfiltration membrane and stirred for 10 minutes. After the process of protein adsorption, filtration of the mixture was carried out followed by washing the loosely bound proteins and intrinsically bound materials with 25mL of 10mM potassium phosphate buffer at pH 6.5. This was then followed by three times elution steps by using 10mM potassium phosphate buffer (50mL and 10mL twice) at pH 4.6 at which large amount of β-Lactoglobulin was adsorbed and retained and some of the protein was non - adsorbed and passed through the filter with the filtrate.

The acid whey and the resulting fractions (i.e. waste stream, wash and elutes 1, 2 and 3) were analyzed for total protein by BCA assay and peptide profile by HPLC to complete the mass balance.

3.2.3 Development of the Integrative process for the production of hydrolysate from waste stream (non adsorbed protein) mainly α-Lactalbumin and BSA and small amount of β-Lactoglobulin.

The integrative process for the production of hydrolysate is indicated in Appendix 2. This process is the same as that developed by Welderufael and Jauregi, 2010 but with some modifications in order to incorporate the adsorption and hydrolysis processes of the waste stream.

The filtrate after adsorption of the acid whey (non- adsorbed protein) containing mainly α-Lactalbumin and BSA which is in this particular study is referred to as a waste stream was re- adsorbed with an adsorption resin, stirred for 10 minutes in a reactor, microfiltered and washed in the same way as the acid whey. The adsorbed protein was then re - solubilized using 30mL of a 10mM potassium phosphate buffer at a pH and temperature of 7 and 45^0C respectively. Hydrolysis was then initiated by the addition of 0.5mL of protease N Amano corresponding to an enzyme: substrate ratio of 1:100 (wt/wt) and incubated in a water bath at a temperature of 45^0C for different times 2, 4 and 6 hours. After this process of hydrolysis, the mixtures were filtered and the hydrolysates (products) were boiled with water at a temperature of 100^0C for 5 minutes to deactivate the enzyme activity. The resulting products (Figure 2) were stored at -18^0C for further analysis.

3.2.4 BCA assay for total protein of whey and whey fractions

The BCA assay was employed in the determination of total protein of the acid whey and its different fractions after adsorption and hydrolysis. A standard BSA calibration curve (concentrations ranging from 0.0, to 1.0mgmL^{-1} at the interval of 0.2 mgmL^{-1}) was constructed. A 0.1mL of standard and samples was transferred into test tubes and 2.0mL of the BCA working reagent was added into each test tube. The test tubes were then incubated at 37^0C for 30 minutes and cooled to room temperature. The reaction was then transferred into cuvettes and absorbance measured at 562nm with utrospec 1100 pro UV/ visible spectrophotometer. This assay was conducted in duplicate and the protein content of the samples was determined from the calibration curve.

3.2.5 HPLC analysis for peptide profile

The High Performance liquid Chromatography was used in the determination of major whey proteins i.e. β-Lactoglobulin, α-Lactalbumin and BSA of the acid whey and its fractions (i.e. waste stream, wash after adsorption of the acid whey, elutes 1, 2 and 3 after adsorption of the acid whey and Hydrolysates after 2, 4 and 6hrs hydrolysis). In this case, 1 mL of acid whey and whey fractions were filtered using 0.22μm filters and placed into vials for analysis in a Dionex containing P680 HPLC pump, ASI – 100 automated sample injector, thermostatted column compartment TCC100, PDA – 100 photodiode array detector with a C18 column (250 x 4.6 mm). A gradient of solvent A which is ideally containing 0.1% trifluoroacetic acid in HPLC grade water and solvent B which is 0.08% trifluoroacetic acid in acetonitrile was used. Solvent B was 45% over 60 - 65min, 70% over 65 - 70min, and 70% over 75 - 80min. The temperature of the column was kept at 40^0C. The injection volume and flow rate of samples analysed were maintained at 50 μL and 0.8mL/min respectively where as the areas of the peaks were monitored at a wavelength of 214nm. β-Lactoglobulin, α-Lactalbumin and BSA model proteins were used as standards.

3.3 Statistical analysis

Descriptive statistics were used for data analysis and the results were expressed as Mean ± S.E.M. Where; S.E.M stands for standard error of the mean.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results and Discussions

4.1.1 Adsorption of β-Lactoglobulin from the acid whey

The results of the adsorption of β-Lactoglobulin from the acid whey and compositions of the major whey proteins are given in Table 1 to Table 3. The isoelectric point (pI) of β-Lactoglobulin is reported to be in a range of 5.2 to 5.4 (Table 2), it thus bears a chemically negative charge at the pH of whey of 6.5 (Welderufael and Jauregi, 2010) and based on these conditions it means that the protein adsorbs to the anionic resin (positively charged resin). The composition of major whey proteins particularly β-Lactoglobulin in the waste stream (Table 1) indicates preferential large quantities of β-Lactoglobulin adsorbed to anionic resin. The waste stream generated following adsorption of the acid whey contains about 3% of β-Lactoglobulin, 78% of α-Lactalbumin and 79% of BSA. These findings are in agreement with another research carried out by Welderufael and Jauregi, 2010 which indicated adsorption of the majority of β-Lactoglobulin leaving the waste stream with about 2% of β-Lactoglobulin and 75% of α-Lactalbumin. It is further shown in this study that only small amounts of α-Lactalbumin and BSA were washed out in the subsequent washing step. The results obtained are in agreement with other researches carried out by Fuda & Jauregi, 2006 and Goodall, Grandison, Jauregi & Price, 2008 based on Mechanism of protein separation by colloidal gas aphrons

(CGA) generated from ionic surfactants and Selective separation of major whey proteins using exchange membrane respectively.

Sample	Volume (ml)	Total protein (mg)	β-Lactoglobulin (mg)	α-Lactalbumin (mg)	BSA (mg)
Acid whey	50	392.50 ± 2.50	107.25 ± 0.25	64.25 ± 0.75	21.48 ± 0.03
Waste stream	50	217.75 ± 4.60	3.23 ± 0.03	47.19 ± 0.26	15.64 ± 0.01
Wash	25	27.83 ± 1.25	0.34 ± 0.00	3.19 ± 0.02	1.31 ± 0.01
Elute 1	50	103.88 ± 4.99	84.74 ± 0.01	8.36 ± 0.02	2.98 ± 0.05
Elute 2	10	17.85± 0.35	9.74 ± 0.01	1.75 ± 0.04	0.50 ± 0.02
Elute 3	10	2.10 ± 0.99	1.90 ± 0.03	0.12 ± 0.01	0.11 ± 0.01
Deviation %		5.90	6.80	5.70	4.40

Table 1: β-Lactoglobulin, α-Lactalbumin and BSA adsorbed from acid whey using the anionic resin. The deviation % is a mass balance deviation %

Whey Proteins	Concentration ($mgmL^{-1}$)	Isoelectric point (pI)
β-Lactoglobulin	2.0 - 4.0	5.2 – 5.4
α-Lactalbumin	1.2 – 1.5	4.2
BSA	0.3 - 0.6	4.9 – 5.1

Table 2: Some of the important physico-chemical characteristics of major whey proteins (Welderufael and Jauregi, 2010 and Dereck *et al*, 2006)

Whey proteins	Concentration ($mgmL^{-1}$)
β-Lactoglobulin	2.15 ±0.005
α-Lactalbumin	1.30 ± 0.150
BSA	0.43 ± 0.001

Table 3: Concentrations of major whey proteins as determined in acid whey using HPLC

4.1.2 Hydrolysis of adsorbed proteins by Protease N Amano

The results of hydrolysis of adsorbed proteins by Protease N Amano enzyme are indicated in Figures 1 - 3 and Tables 4 - 5. In the process of protein hydrolysis to produce biologically active peptides, it is essential to select a suitable enzyme. According to Welderufael and Jauregi, 2010, "Protease N Amano has a greater superiority in the production of high quality protein hydrolysate". It is further reported that Protease N Amano is capable of producing a "strong ACE inhibitory peptides from a standard protein" of β-Lactoglobulin (Ortiz – Chao *et al*, 2008). In this study Protease N Amano enzyme was used and was found to give better results which are in agreement with previously undertaken researches. The measurement of hydrolysis of the waste stream using Protease N Amano enzyme was done using the chromatograms generated from the HPLC for the 2, 4 and 6hrs hydrolysates (Figures 1 - 3). The major whey proteins were quantified from the peak areas of the chromatograms with reference to the peak areas of standards (i.e. β-Lactoglobulin, α-Lactalbumin and BSA). It is revealed from the study that total protein increases with increase in hydrolysis time. This is basically due to the reason that most proteins are hydrolyzed by the enzyme and filtered out with the hydolysate. Furthermore, it is shown that the quantity of bioactive peptides released from proteins by Protease N

Amano increases with increase in hydrolysis time. This is because the bioactive peptides released from adsorbed proteins after two hours of hydrolysis might have been hydrolyzed after a longer time of hydrolysis resulting into an increased quantity of bioactive peptides released. In this case the bioactive peptides are mainly released from α-Lactalbumin, partly from β-Lactoglobulin and BSA and most probably from minor whey proteins. This is because only small amounts of the major whey proteins were adsorbed in the adsorption process of the waste stream.

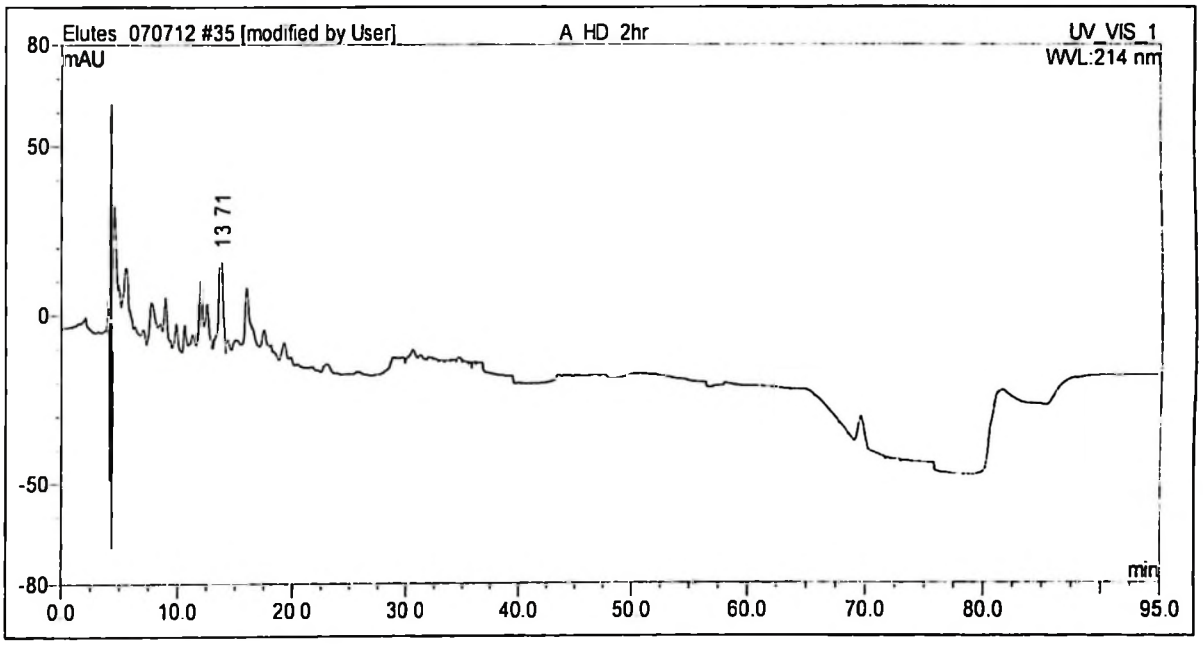

Figure 1: Chromatogram generated from HPLC after 2 hrs of Hydrolysis

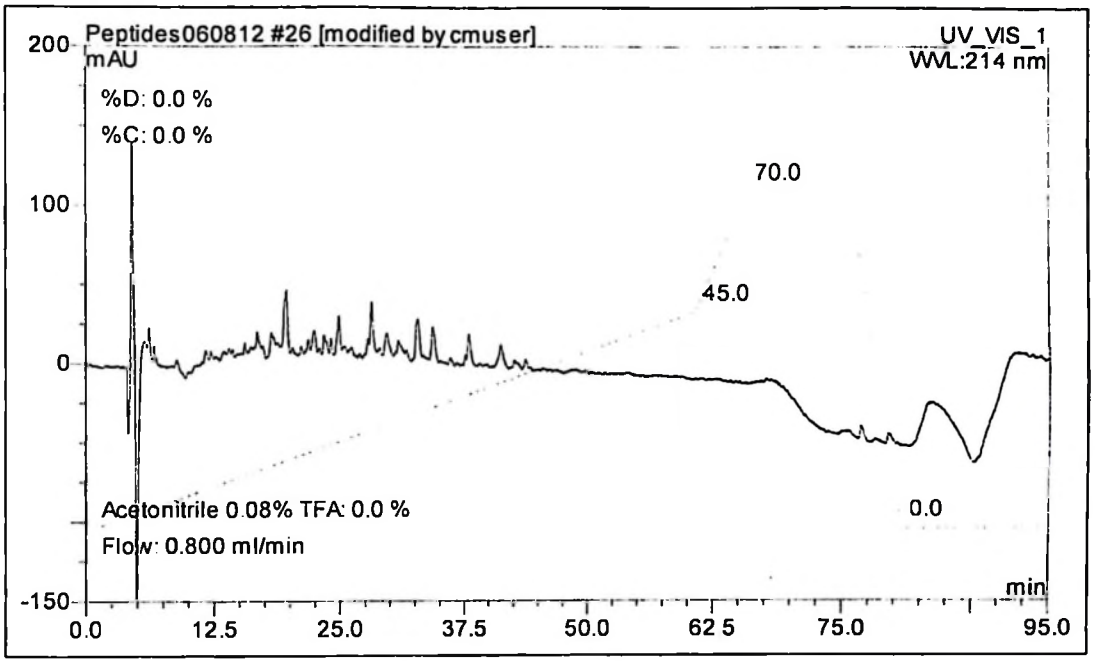

Figure 2: Chromatogram generated from HPLC after 4 hrs of Hydrolysis

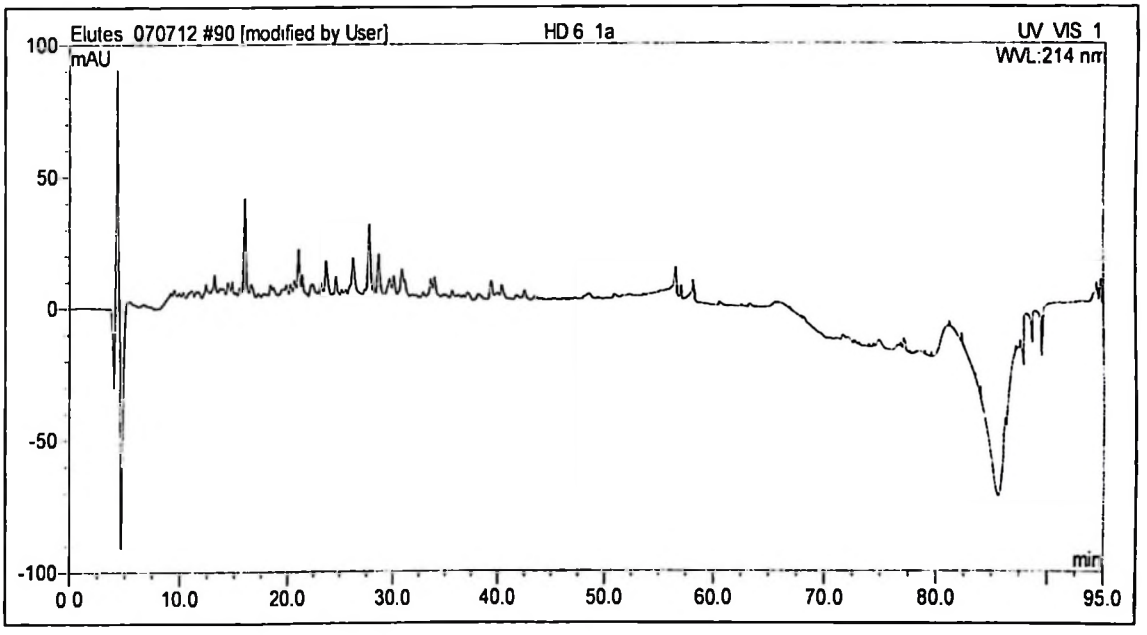

Figure 3: Chromatogram generated from HPLC after 6 hrs of Hydrolysis

Sample	Total protein (mg)	β-Lactoglobulin (mg)	α-Lactalbumin (mg)	BSA (mg)
Waste stream	217.75 ± 4.60	3.23 ± 0.03	47.19 ± 0.26	15.64 ± 0.01
Filtrate after adsorption of the waste stream	120.88 ± 0.12	1.11 ± 0.01	38.95 ± 0.01	14.46 ± 0.095
Wash	3.41 ± 0.01	0.16 ± 0.005	0.09 ± 0.0005	1.12 ± 0.190
Amount adsorbed	**93.46**	**1.96**	**8.15**	**0.06**

Table 4: Total and major whey proteins determined after adsorption of the waste stream

Hydrolysis time (Hrs)	2	4	6
Amount of protein hydrolyzed (mg)	6.53 ± 0.06	15.87± 0.07	56.10 ± 0.09
% hydrolyzed	7.0	17.0	60.0

Table 5: Total proteins quantified in the hydrolysate product at different times of hydrolysis starting with **93.46mg** of adsorbed proteins.

4.1.3 HPLC analysis for peptide profile

The High Performance Liquid Chromatography was employed in the determination of the major whey proteins (β-Lactoglobulin, α-Lactalbumin and BSA). The amounts of these proteins in whey sample (Table 2) are in agreement with the recommended ranges available in literature (Welderufael & Jauregi, 2010 and Dereck *et al*, 2006) (Table 3). Similarly, for the waste stream, the concentrations of major whey proteins were again found to be in agreement with the recommended ranges due to the fact that 97.0% of β-Lactoglobulin was adsorbed by the anionic resin. According to Welderufael, 2010 it was observed that 98% of β-Lactoglobulin was adsorbed in the adsorption step. Furthermore, the results indicate that 22% of α-Lactalbumin was

adsorbed in the adsorption step which is approximately the same as that determined by Welderufael (25%). In addition to that 21% of BSA was also adsorbed.

On the other hand, the major findings of this study indicates further that: in the process of adsorption of the waste stream using the anionic resin 60.68% and 17.27% of β-Lactoglobulin and α-Lactalbumin were respectively adsorbed and a very small amount of BSA was adsorbed.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

Protease N Amano enzyme is a very important enzyme for protein hydrolysis and releasing bioactive peptides from the waste stream as revealed by this study. The study indicates the increasing trend of protein hydrolyzed using this enzyme with increase in hydrolysis time. This implies that to hydrolyze the adsorbed proteins completely, a longer time is required. Similarly; even if very small amounts of major whey proteins were adsorbed during adsorption of the waste stream, it is shown through hydrolysis that peptide production increases with increase in hydrolysis time suggesting that hydrolysis time is an important determinant for the development of bioactive peptides from the waste stream. It is therefore concluded from the findings that; while the waste stream is generally treated as a waste product and discharged into the environment, it can still be hydrolyzed by Protease N Amano or any other suitable enzyme to release bioactive peptides from milk proteins which are proved to have functional and nutritional roles to the healthy and well being of the consumers.

Based on the findings of this study it is recommended that;

- In depth studies should be taken to specifically identify the types of bioactive peptides generated from the waste stream following adsorption of the acid whey.

- Further studies should also be undertaken to assess the bioactivity of the bioactive peptides generated by enzymatic hydrolysis of the waste stream at different hydrolysis time.

- It is further recommended that, specific studies should be carried out to investigate the effects of hydrolysis time on bioactivity i.e. how the bioactivity changes with a change in hydrolysis time on the waste stream using the same amount of the enzyme.

- More researches are needed for the waste streams in order to identify other factors important for the production of bioactive peptides with reasonable bioactivities apart from hydrolysis time.

- Protease N Amano enzyme is proved to be superior in releasing bioactive peptides from milk proteins, more researches are needed to investigate more other superior enzymes which can enable production of bioactive peptides of functional and nutritional importance to the bodies of the consumers.

- Most researches based on production of bioactive peptides from milk proteins for a long period of time have been based on a laboratory scale. It is therefore argued that more studies to be done in order to transform this into industrial scale which will enable production of large quantities of these important bioactive peptides.

- In this present study, only the major whey proteins were quantified from the hydrolysates generated after hydrolysis of the waste stream. More research

activities are recommended to investigate the availability of minor whey proteins such as lactoferrin and lactoperoxidase which are also important sources of bioactive peptides.

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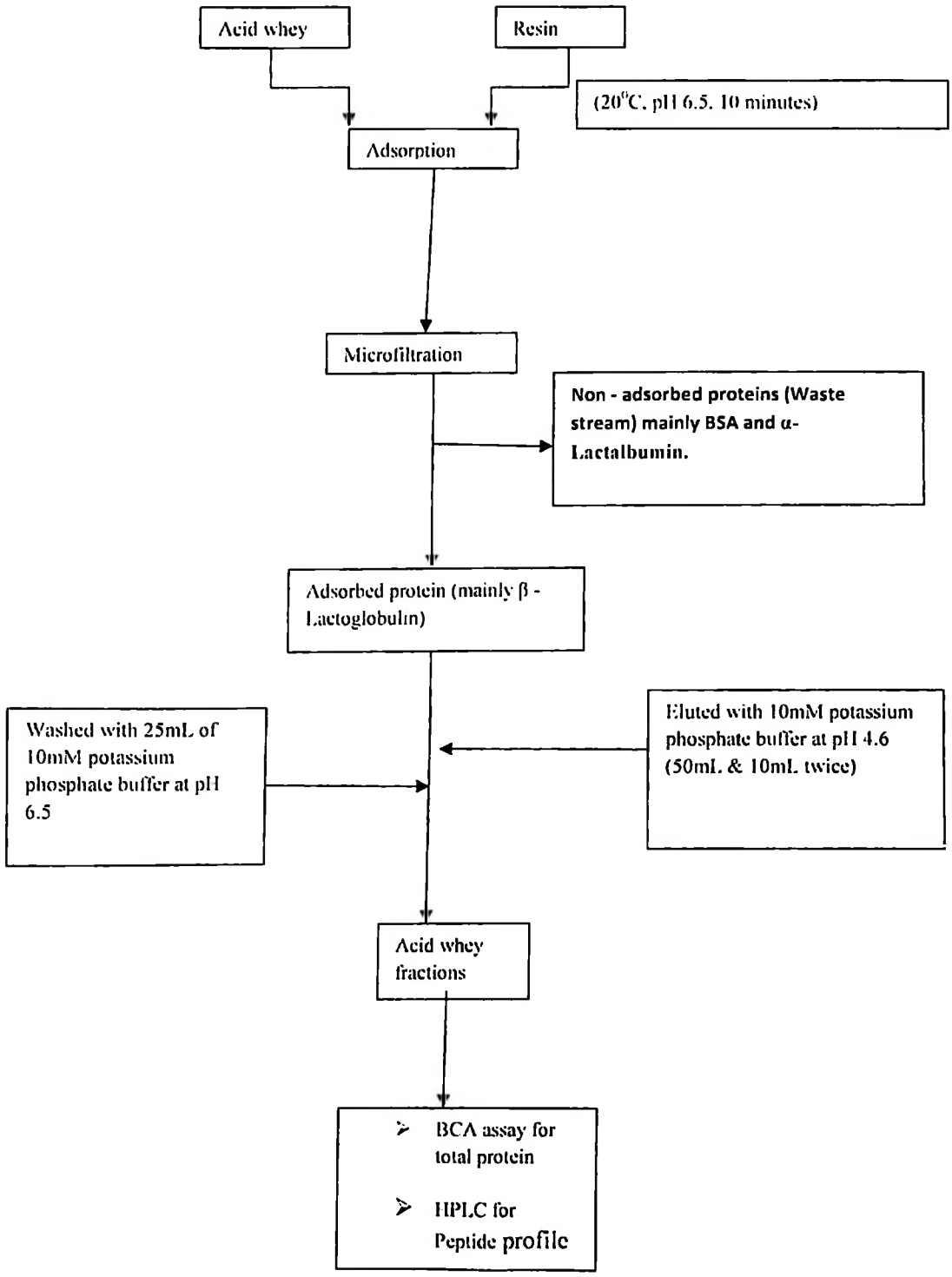
APPENDICES

Appendix 1: A complete process indicating adsorption of β-lactoglobulin from the acid whey.



Appendix 2: A complete process of bioactive peptide production from the waste stream (non adsorbed protein) which contains mainly BSA and α-Lactalbumin and a small amount of β-Lactoglobulin after adsorption of acid whey.