

**APPLICATION OF PROTEOLYTIC ENZYMES IN SUPPRESSING
IMMUNOREACTIVITY AND DEACTIVATION OF ANTIGENIC
EPITOPES IN THE WHEY PROTEIN ISOLATE**

By

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Abstract:

Whey protein is a by-product of cheese manufacturing and a rich source of bioactive peptides. However, it is an important cause of food allergy in many patients that are allergic to bovine milk. Enzymatic hydrolysis is an approach that has been used for the production of hypoallergenic products. Many researchers have explored the application of various enzymes for the suppression of whey antigenicity but there are still many reports on antigenic reaction after consumption of hypoallergenic products. The impact of hydrolysis with various enzymes on immunochemical properties, hydrophobicity and particle size of whey protein isolate was studied. Twelve different enzymes were used of which papain and pancreatin hydrolysate showed the highest immunoglobulin (Ig) E inhibition of 47% and 45% among all the hydrolysates. The electrophoretic pattern of the whey isolate treated with papain and pancreatin did not show traces of α -lactalbumin or β -lactoglobulin after hydrolysis. The degree of hydrolysis had a weakly positive correlation with immunoreactivity, which indicated that extensive hydrolysis is not always accompanied with antigenicity suppression. A strong negative correlation between the degree of hydrolysis and hydrophobicity was observed. The peptidomics result of papain hydrolysate was analyzed and enzymatic cleavage sites were determined. The *in silico* predicted cleavage pattern of enzymes did not match the actual cleavage that occurred in the the papain-derived whey protein hydrolysate. However, a comparison of *in silico* and actual results indicated that antigenic epitopes were mostly degraded. This demonstrates antigenic inhibition in the papain whey hydrolysate obtained *in vitro*. These findings will provide information on the application of three enzyme groups and their specificity on lowering whey protein antigenicity and production of hypoallergenic whey products for food applications.

List of Abbreviation Used

CMA	Cow's milk allergy
β -Lg	Beta- lactoglobulin
α -La	Alfa-lactalbumin
BSA	Bovine serum albumin
IGS	Immunoglobulins
BLF	Bovine lactoferrin
TNBS	2,4,6-Trinitrobenzene Sulfonic Acid bovine lactoferrin
WP	Whey proteins
OPA	O-phthaldialdehyde
ELISA	Enzyme immune sorbent assay
HPLC	High-performance chromatography
BMA	Bovine milk allergy
FPLC	Fast Performance Liquid Chromatography
ANS	8-anilino-1-naphthalenesulphonic acid
DH	Degree of hydrolysis
PBSGT	Phosphate buffered saline PBS containing bovine serum albumin BSA & Tween

PBS Phosphate buffered saline

OPD O-phenylene-diamine dihydrochloride

PDI The polydispersity index

PTM Post translational modifications

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Chapter 1

Introduction

Milk proteins have a key role in the human diet as a rich source of essential amino acids and peptides (Kim *et al.* 2007). Since the eighth millennium BC, perhaps after the domestication of cattle, bovine milk has been considered to have a crucial impact on the human diet (Evershed *et al.* 2008). Whey protein is a by-product of cheese production, which was neglected for a long time since it was considered as low-value material. Recently, there have been increasing demands on whey protein due to several nutritional and health promoting properties of this dairy protein (Madureira *et al.* 2010). The European Union and the USA have the highest contribution in whey proteins manufacturing, contributing 70 % of the globe production of whey protein (Mollea *et al.* 2013). However, bovine milk, specifically the whey protein, has a high ratio of allergenicity that can pose limitation towards its use in food formulation (Huth *et al.* 2006). Cow's milk allergy (CMA) is the most common food allergy developed during early infancy, which can persist lifelong in some individuals (Wood *et al.* 2013). Cow milk allergy reactions are provoked by milk proteins, either caseins or whey (Wal 2002).

Beta-lactoglobulin and alpha-lactalbumin are the two major allergenic whey proteins that cause 50% of milk allergies (Wal 2004). Bovine serum albumin (BSA), immunoglobulins, lactoferrin, and phospho-lipoproteins are minor whey proteins with lower cause of allergic reaction. The immune response to milk antigenic proteins is roughly divided into IgE-mediated and non-IgE-mediated (Knipping *et al.* 2012). The IgE-mediated immune response to CMA can cause an allergic reaction in many body organs (Sicherer & Sampson 2006)

The molecular structures of beta-lactoglobulin (β -Lg) and alfa-lactalbumin (α -La) are associated with the allergenicity of these proteins. α -La has 123 amino acid residues in its monomeric globular protein structure. It has 14.4 kDa molecular weight and contains four disulfide bridges and it has 74% homology with α -La available in the human milk (Wal 2002). β -Lg exists in the form of 36-kDa dimers with no similar protein in the human milk (Brownlow *et al.* 1997). Each subunit of β -Lg contains 162-residues of amino acids, two disulfide bonds, and one free cysteine residue in the structure (Wal 2002). The physicochemical properties of this protein can cause resistance to digestion with acid hydrolysis and proteases. This globular protein will remain intact even after digestion in the gut and is reportedly responsible for allergic reaction in patients (Jakobsson *et al.* 1985). Crystallography on the tertiary structure of β -Lg indicated that this protein has a β -barrel structure with a similar 8 or 10 antiparallel β -sheets, which made it a major cause of allergenicity in the whey protein (Flower 1996).

Epitopes are defined as some proteins segments, which are mostly water-soluble glycoproteins (10-70 kDa), that cause food allergenic reactions. The epitopes are classified into linear and conformational categories based on their structure conformation and interaction with the antibodies. An IgE-mediated food allergy can lead to the production of IgE antibodies, which are specific to those antigenic food proteins, and capable of binding to basophils and mast cells components of the human immune system that initiate allergenic symptoms. Therefore, after consumption of an immunoreactive food protein, the stimulated antibodies can trigger the immune response and release the mediators as well as histamine, prostaglandins, and leukotrienes, thereby causing allergic reactions (Sicherer 2002; Sampson 2004; Sicherer & Sampson 2010).

CMA causes predominantly gastrointestinal symptoms and atopic dermatitis (Sporik *et al.* 2000; Magazzù & Scoglio 2002). Several epitopes in whey protein have been recognized within the β -Lg sequence. Some of these epitopes are short linear sequences, whereas the others are conformational epitopes. The following sequences have been recognized as the antigenic epitope in β -Lg: 97-108, 124-134, 149-162; they can also trigger IgE-mediated food allergy reaction (Ball *et al.* 1994; Selo *et al.* 1999). Also, α -La has been reported to pose IgE-binding at its 17-58 and 59-94 sequences (Maynard *et al.* 1997).

Enzymatic hydrolysis is a technique that has been used in the production of whey protein hydrolysates with reduced antigenicity (Ena *et al.* 1995; Guadix *et al.* 2006; Peñas *et al.* 2006; Izquierdo *et al.* 2008). A whey hydrolysate with the least immunoreactivity would be ideal for food industry use (Penas *et al.* 2004). A wide range of proteases including gastrointestinal (pepsin and chymotrypsin), plant enzymes (such as papain and bromelain), microbial (such as Alcalase and neutrase), or their combinations, have been used for the production of bovine whey protein with reduced antigenicity (Nakamura *et al.* 1993; Lakshman *et al.* 2011). There are many studies that have used various enzymes to deactivate the antigenic epitopes presents in the whey proteins. However, there is a scarcity of information regarding ability of many enzymes on suppressing immunoreactivity in whey protein with specific emphasis on the deactivation of linear antigenic epitopes. Also, enzyme activity can impact the whey protein hydrolysate matrix and protein structure, which can lead to the production of intermediate peptide and smaller peptides as hydrolysis progresses (Adler-Nissen 1976).

Peptidomics can be used to identify food peptide composition and the interaction of peptides in the food matrix (Gagnaire *et al.* 2009). Moreover, bioinformatics software can

be used to predict the possible cleavage site based on enzyme structure; also, it can be used to interpret the peptidomics results on specification of enzymes for deactivation of the antigenic epitopes.

The purpose of this project was to develop hypoallergenic whey protein *via* enzymatic hydrolysis using proteases of different specificities, and to explore the effect of hydrolysis on the physicochemical properties of the whey protein hydrolysates. Also, the present study aimed to verify the enzyme activity on deactivation of antigenic epitopes by investigating into the protein sequence after hydrolysis and comparison with the *in silico* outcome for virtual enzymatic cleavage and deactivation of predicted antigenic epitopes to investigate specificity of selected enzyme.

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Chapter 2

Application of Enzymatic Hydrolysis for Suppressing Whey Protein Antigenicity

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Abstract

Bovine whey protein is a rich source of bioactive peptides with several health promoting approaches which is used in many food product formulations. There has been a growing demand for whey protein; however, consumption of whey protein can trigger allergic immune response in some people. Various techniques have been used to produce whey protein with reduced antigenicity. Enzymatic hydrolysis is one of the approaches applied for manufacturing hypoallergenic whey proteins. Several factors can influence the nature of the hydrolysate, namely enzyme type and specificity as well as pretreatment of the protein prior to hydrolysis. In addition, enzymatic hydrolysis can affect the functional properties of the hydrolysate such as emulsifying, foaming properties and palatability. Achieving a hypoallergenic whey protein hydrolysate with improved functionality demands further studies.

Keywords: Whey protein, Food allergy, Enzymatic hydrolysis, Hypoallergenic whey, Functional properties

1.0. Introduction

Food allergy is an adverse immune response to the consumption of certain kinds of food (Sampson 2004). A food allergy occurs due to the presence of protein segments (epitope) available in allergenic food ingredients, and the epitopes are mostly water soluble glycoproteins (Huang and Honda 2006). Cow's milk is the most prevalent food allergy in neonates and 1-2% of newborns are dealing with this allergy (Svenning et al. 2000). (Some infants, outgrow this allergy by the age of three, while the condition persists lifelong in some cases (Wood *et al.* 2013).

Milk is composed of two main protein fractions: casein and whey proteins. The casein fraction makes up 80% of bovine milk while the whey fraction is 20% (Krissansen 2007). Whey proteins have been explored as a valuable source of bioactive peptides (Huth *et al.* 2006). Beta-lactoglobulin (β -Lg) and alpha-lactalbumin (α -La) are two major allergenic proteins present in whey protein and are reportedly responsible for 50% of milk allergy incidence (Wal 2002). The immune response to milk antigenic proteins is roughly divided into IgE-mediated and non-IgE-mediated (Knipping *et al.* 2012). The IgE-mediated immune response to CMA can cause a systemic allergic reaction (Sicherer & Sampson 2006).

Enzymatic hydrolysis is an approach used to reduce food immunoreactivity (Bahna, 2008). Enzymatic hydrolysis of protein can degrade the antigenic epitopes (Sicherer & Sampson 2006; Cabanillas *et al.* 2012). Moreover, peptides with a molecular mass less than 3400 Da have a lower probability of triggering the immune response (Ena *et al.* 1995). Therefore, the risk of provoking an allergenic response is lower with protein hydrolysates than with the original intact protein. A hydrolysate product with the highest degree of

hydrolysis and the least amount of immunoreactivity is appealing to the food industry in food product formulation (Penas *et al.* 2004). Various proteases including gastrointestinal, plant (such as papain and bromelain), microbial (such as Alcalase and neutrase), or their combination, have been used to produce low antigenic bovine whey protein hydrolysates (Nakamura *et al.* 1993b; Lakshman *et al.* 2011). This is a review on previous research reports on the use of enzymatic hydrolysis for suppressing whey protein allergenicity.

2.0. Whey proteins allergenicity

CMA can cause systemic allergic response in which severity of the symptom varies from mild to serious allergic reactions (Wood *et al.* 2013). Patients could be allergic to various combinations of proteins or one specific protein allergen. Casein, β -Lg and α -La (whey proteins) are the predominant allergenic milk proteins. Whey proteins are responsible for 50% of CMA incidence in allergic patients (Wal 2002). Other proteins such as bovine serum albumin (BSA), immunoglobulins (IGS) and bovine lactoferrin (BLF), are considered as a minor cause of milk allergy (Wal *et al.* 1994) (Figure 1). In the case of β -Lg, several epitopes have been recognized. Some of these epitopes are short linear sequences, whereas the others are conformational epitopes. In β -Lg, a linear epitope from a sequence of 97-108, has been identified to cause IgE sensitivity for a significant number of patients (Ball *et al.* 1994; Selo *et al.* 1999). In addition, IgE-binding properties of fragments 124-134 and 149-162 of β -Lg have been reported (Mizumachi *et al.* 1990). Based on the studies of many other groups, coupled with the use of synthetic peptides, the major β -Lg epitopes are recognized to be capable of triggering an immune response by IgE binding. These epitopes have been distinguished in amino acids sequences of 41-60, 102-124 and 149-162 (Tokita 1985; Takahashi *et al.* 1990; Sélo *et al.* 1998). The antigenic

epitopes recognized in α -La are 17-58 and 59-94 sequences of amino acids (Maynard *et al.* 1997).

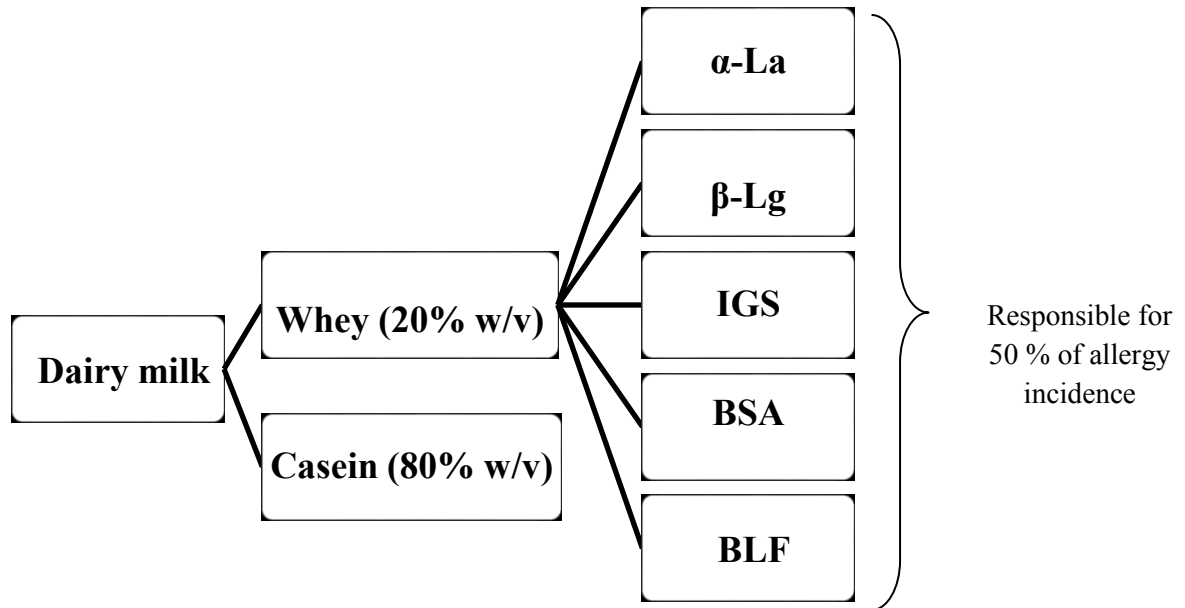


Figure 1: Components of bovine milk protein. Legend: α -La, alpha-lactalbumin; β -Lg, beta-lactoglobulin; BSA, bovine serum albumin; IGS, immunoglobulins; BLF, bovine lactoferrin.

3.0. Hydrolysis approach and antigenicity reduction

Enzymatic hydrolysis can alter the protein structure and has a broad potential to improve protein functionality and aid in the design of hypoallergic food products with enhanced functionality and reduced allergenicity (Foegeding *et al.* 2002). Several studies indicated the positive effect of enzymatic hydrolysis on allergenicity as well as digestibility of antigenic food proteins (Merritt *et al.* 1990; Terracciano *et al.* 2002; Beyer 2007; Gomes-Santos *et al.* 2015). Several factors play a key role in determining the quality of the final hydrolysate product: enzyme specificity, extent of protein denaturation, concentration of

substrate and enzyme, pH, temperature, presence of inhibitors, and ionic strength (Kilara 1985). Enzyme specificity is a crucial factor that can affect the number and location of peptides that are created during hydrolysis (Adler-Nissen 1976). Proteolysis can cleave one peptide bond at a time sequentially to generate intermediate peptides, which are subsequently hydrolyzed to smaller peptides as the hydrolysis is continued (Adler-Nissen 1976). Hydrolysates are categorized into slightly, moderately and extensively hydrolyzed products based on the molecular weight of final hydrolysate (Mahmoud 1994). A lower degree of hydrolysis is used for the production of protein supplements while a higher degree of hydrolysis is used in the production of hypoallergenic formulas in which 90% of the resulting peptides have molecular weights less than 500 Da (Mahmoud 1994). However, extensive hydrolysis generates low molecular weight peptides with exposed hydrophobic amino acid residues that can cause bitterness in the hydrolysate, which is not favorable in terms of palatability (Panyam & Kilara 1996). Hydrolysis can lead to three different effects on the protein structure: increase the development of peptides with low molecular weight, increase the number of ionizable groups and the exposure of hydrophobic groups (Panyam and Kilara 1996).

Enzymatic hydrolysis has been shown to be an effective process for inactivating the antigenic sequential and conformational epitopes of proteins (Fritsché 2009). Thus, protein hydrolysates could be good candidates for use in the production of hypoallergenic formulas. However, the choice of proteases is crucial since it can affect the degradation of allergenic epitopes and release of bitter peptides in the hydrolysates (Spellman et al. 2003). There are many studies on the application of various enzymes for the production of milk hydrolysate with reduced allergenicity (Nakamura *et al.* 1993b; Ena *et al.* 1995; Wróblewska *et al.*

2004; Kim *et al.* 2007). Digestive proteinases (pepsin, chymotrypsin and trypsin) have been used for the commercial production of milk protein hydrolysates with low immunoreactivity (Jost *et al.* 1987; Ena *et al.* 1995; Svenning *et al.* 2000; Kim *et al.* 2007). In addition, as shown in Table 1, plant proteases (papain and bromelain), bacterial proteinase (Alcalase and neutrase), (Nakamura *et al.* 1993a; Nakamura *et al.* 1993b) and fungal proteases have also been used in hydrolysis-based suppression of immunoreactivity (Ena *et al.* 1995) (Table).

Table 1: Summary of the application of digestive, plant and bacterial enzymes in the hydrolysis of bovine milk proteins for reducing immunoreactivity.

Enzyme type	Name of the enzyme	Hydrolysis optimum condition	Treatment after or before hydrolysis	Reduction of immunogenicity	References
Digestive enzymes	Pepsin, chymotrypsin & trypsin	Trypsin & chymotrypsin the optimum pH 8 at 37°C, Pepsin pH 2.5 at 37 °C	pressures of 100, 200 or 300 MPa for 15 min	Trypsin & pepsin lower the antigenicity. No antigenicity reduction observed for chymotrypsin. They indicated that product could be used as a source of peptides in the hypoallergenic infant formulae.	(Peñas <i>et al.</i> 2006)
	Pepsin & chymotrypsin	pepsin at pH 2.5 & 37°C chymotrypsin at pH 6. 8	High pressure of 200 and 400 MPa with the rate of 2.5 MPa	β -Lg was hydrolyzed very efficiently with pepsin under high pressure. β -Lg and α -La were almost completely proteolyzed after 8 h of treatment with chymotrypsin in an atmospheric pressure.	(Chicón <i>et al.</i> 2009)
Bacterial enzymes	Alcalase, Neutrase & corolase	The optimum pH of three enzymes have been considered	Pressure 100, 200 and 300MPa for 15 min at 40 °C and 50 °C.	Corolase decreased the antigenicity of whey protein with pressure treatment of 100, 200 & 300 MPa as follow: 78%,64%,40%. Neutrase with application pressure of 100, 200 & 300 MPa showed a various degree of antigenic inhibition of 36%,32%, 31%.	(Peñas <i>et al.</i> 2006)
	Protex 6L	50° C and pH 8.5	NA	The hydrolysate could pose 30% of antigenic inhibition. Antigenicity was reduced 99.97%. The product obtained is suitable for infant formula	(Guadix <i>et al.</i> 2006)
	Corolase & pepsin	40°C and pH 7.5.	NA	The whey proteins BSA and B-IgG were eliminated by a combination of pepsin and Corolase.	(Ena <i>et al.</i> 1995)
Plant enzymes	Papain & alcalase	50° C, pH 8.0	NA	The enzymes used could significantly lower the antigenicity, however, separated fractions of hydrolysate were still reactive with specific antibodies.	(Wróblewska <i>et al.</i> 2004)
	Papain and proleather, Alcalase, neutrase (Combination of enzymes)	50°C & 70°C	Pre-heating of whey protein for 11 minutes	The combination of papain and proleather showed the highest degree in lowering immunoreactivity.	(Nakamura <i>et al.</i> 1993)
	Papain along with other enzymes as well as Corolases Alcalase, Neutras, Pronase, Chymotrypsin	50°C & pH 8.0	Temperature was 40 or 50°C during 5 min of microwave for 30 seconds	The β -Lg was hydrolyzed by papain under all conditions, whereas this protein was only completely hydrolyzed by pronase, Alcalase and chymotrypsin under MWI.	(Izquierdo <i>et al.</i> 2008)

3.1. Gastric enzymes

In several studies, digestive enzymes from the mammalian digestive system have been used in hydrolysis procedures to reduce the allergenicity of whey proteins. Peñas et al. (2006a) reported the use of a combination of pepsin, trypsin and α -chymotrypsin for whey protein hydrolysis after high pressure treatment (100, 200 or 300 MPa) for 15 minutes at 37°C. They found that trypsin and α -chymotrypsin hydrolyzed β -Lg at both atmospheric and high pressure, but pepsin was only effective under high pressure. Pepsin and trypsin hydrolyzed α -La with or without high pressure treatments, whereas chymotrypsin did not hydrolyze α -La under these experimental conditions. Based on immunoreactivity assessment using a pool of seven sera from patients, the resultant hydrolysates of pepsin and trypsin under high pressure had the potential to be used for infant formula (Peñas *et al.* 2006a). In a similar study, Chicón et al. (2009) applied pepsin and chymotrypsin with high pressure treatments, at various rates and durations, to reduce whey protein immunoreactivity. They demonstrated that pepsin hydrolyzed allergenic β -Lg and α -La very efficiently at pH 2.5 and under high-pressure treatments. In contrast, chymotrypsin was reported to reduce immunoreactivity after treatment at atmospheric pressure.

3.2. Plant enzymes

Enzymes of plant origin such as papain and bromelain have been used to reduce the antigenicity of whey protein. Nakamura et al. (1993) reported that a combination of papain and bacterial enzymes from *Bacillus sp.*, Alcalase from *Bacillus licheniformis* and neutrase from *Bacillus sp.* resulted in the reduction of antigenicity of milk protein in an *in vitro* inhibition ELISA test with rabbit antiserum. They demonstrated that by combining exo- and endo-proteases with extensive hydrolysis, immunoreactivity was reduced

significantly (Nakamura *et al.* 1993a). In another study, papain was used along with other five food-grade proteases (Izquierdo *et al.* 2008). In this study, papain hydrolysed β -Lg under both conditions. The authors suggested that microwave irradiation can increase the hydrolysis of whey protein, which can give various results based on the type of enzyme.

3.3. Bacterial enzymes

Enzymes of bacterial origin have been used for hydrolysis in reducing bovine whey protein allergenicity. Guadix *et al.* (2006) used Protex 6L, a bacterial alkaline subtilisin (E.C. 3.4.21.62) from *Bacillus licheniformis* for the hydrolysis of whey protein in a membrane reactor in order to reduce antigenicity. They reported that the resultant hydrolysate showed 30% inhibition of immunoreactivity of the final hydrolysates, which corresponded to 99.97% antigenicity reduction. Penas *et al.* (2006) used neutrase a metalloendopeptidase from *Bacillus amyloliquefaciens* Corolase 7089 and Corolase PN-L with cleavage specificity for hydrophobic amino acid residues. They used these three enzymes under pressure treatments of 100, 200 and 300 Pa for hydrolysis of whey proteins. They reported that Corolase hydrolysate showed 78% of the reduction in immunoreactivity under 300 Pa in the best condition, whereas, neutrase exhibited 36% of inhibition at 100 Pa. Similarly, Alcalase and papain (in a one-step process) or in combination (in a two-step process) were used to hydrolyze whey proteins (Wróblewska *et al.* 2004). Alcalase and papain in one-step hydrolysis gave different degrees of hydrolysis, 15.3 and 8.9 %, respectively, whereas the two steps hydrolysis gave 15.9% degree of hydrolysis. They reported that Alcalase hydrolysate had a bitter taste, however, by the addition of papain to Alcalase hydrolysate, the product palatability was improved. Alcalase-papain partially hydrolyzed the proteins and the process has the potential for use in manufacturing partial hydrolysate

formulas (Wróblewska *et al.* 2004). In general, bacterial enzymes have been extensively used in suppressing whey protein antigenicity.

4.0. Effect of heat and elevated pressure on whey protein immunoreactivity

Heat treatment and the use of elevated pressure are two major methods that can be used along with hydrolysis to suppress the antigenicity of bovine whey proteins (WP) (Peñas *et al.* 2006b; Belloque *et al.* 2007; Kim *et al.* 2007; Pescuma *et al.* 2011).

Antigenicity of whey protein could be suppressed by physical removal of antigenic epitopes through denaturation by heating (O'Connell & Fox 2001). Low immunoreactivity was observed in the whey protein heated prior to hydrolysis in contrast to the native unheated whey protein (Kim *et al.* 2007). However, heat treatment, resulting in denaturation of proteins, can change the secondary and tertiary structure of protein, which can influence the protein functionality (Kananen *et al.* 2000). It can also be accompanied by the formation of bitter peptides (Heyman 1999).

High temperature (80-100°C) can influence the emulsifying property of proteins (Voutsinas *et al.* 1983). It can increase the collision energy among molecules and cause the reaction of side chain groups that induce the cross-linkages within and between peptide chains, which can influence the secondary and tertiary structure of proteins. This can consequently affect the role of proteins in emulsion formation. These newly formed cross-linkages under thermal conditions can decrease protein solubility (Walstra *et al.* 2005; Cheison *et al.* 2010). High temperature can increase the viscosity of resultant hydrolysate, which may enhance the formation of the enzyme-substrate complex and hence the enzyme

activity. Also, alteration in the surface viscosity of proteins can also affect their emulsifying capacity (Walstra et al. 2005).

Several studies indicate that high pressure can improve enzymatic proteolysis (Hayashi *et al.* 1987; Bonomi *et al.* 2003; Chicón *et al.* 2006; Blayo *et al.* 2016). In previous reports, whey protein immunoreactivity and IgE-binding properties was reduced after enzymatic hydrolysis and high-pressure treatments (Bu *et al.* 2013; Bonomi *et al.* 2003; Peñas *et al.* 2006). High pressure and hydrolysis also have been used to enhance milk digestibility, particularly focused on the degradation of β -Lg, which has a highly stable structure (Aertsen *et al.* 2009). High hydrostatic pressure can change the conformational state of the protein and induce structure flexibility at pressures around 200 MPa. This can lead to enhanced enzyme activity and proteolysis efficiency (Belloque *et al.* 2007). Hence, pressurizing and heating are two practical approaches along with hydrolysis for suppression of allergenicity.

5.0. Assessment of degree of hydrolysis and hydrolysate antigenicity

Hydrolysis often alters both protein structures and functionality (Spellman *et al.* 2003). The number of cleaved sites in the protein resulting from hydrolysis can be calculated and expressed as the degree of hydrolysis (Adler-Nissen & Olsen 1979). Many approaches have been applied to determine the degree of hydrolysis (Spellman *et al.* 2003). Among various techniques, three methods frequently used for measurement of the degree of hydrolysis include: TNBS, OPA and pH stat (Spellman et al. 2003). 2,4,6-Trinitrobenzene Sulfonic Acid (TNBS) can react to primary amino acid residues to yield a chromophore with a maximum absorbance at wavelength 340 nm (Adler-Nissen 1979; Quist *et al.* 2009). The O-phthaldialdehyde (OPA) method measures the reaction between OPA and primary

amino groups in the presence of thiol. The reaction product, 1-alkylthio-2-alkyl-substituted isoinodoles can be measured by spectrophotometry at 340 nm (Medinaá *et al.* 1990). The pH method is based on the liberation of protons from the hydrolyzed protein into the medium thereby decreasing the pH of the solution. In this method, the number of peptide bonds cleaved during hydrolysis is determined by amount of base required to keep a constant pH during the reaction (Adler-Nissen 1986).

Enzyme-linked immunosorbent assay (ELISA) is a technique commonly used for determination of immunoreactivity in whey protein hydrolysates. The ELISA test determines the hydrolysates' antigenicity, based on the reaction of the whey hydrolysate with whey serum antibody. Inhibition ELISA (Ena *et al.* 1995), direct ELISA, indirect ELISA (Kim *et al.* 2007) and competitive ELISA (Babij *et al.* 2015) are different ELISA methods that can be applied for antigenicity assessment. Competitive ELISA and sandwich ELISA are two commonly used approaches for quantification of antigenic proteins in food allergenicity detection. Sandwich ELISA is based on the immobilization of a captured antibody on the solid phase in the microplate. Antigenic proteins are captured by the first antibody, which could be an antibody isolated from patients' sera with CMA or an isolated antibody from an animal such as rabbit and goat on encountering the antigenic protein, and then recognized by a second protein-specific, enzyme-labelled antibody (Koppelman *et al.* 2001; Torp *et al.* 2006). The competitive ELISA is preferred for detection of small molecules. The first antibody is immobilized on the solid surface and the sera and diluted sample (inhibitors) are pre-incubated and added to the solid phase (Koppelman *et al.* 2001; Roux *et al.* 2001). In addition, high-performance chromatography (HPLC) or the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) can be used to observe

the extent of hydrolysis of whey protein to determine the intact α -La, β -Lg and BSA concentrations (Peñas et al 2006).

6.0. Influence of hydrolysis on whey protein functionality

Protein hydrolysis is a technique used for the production of peptides with derivable functional, immunological and bioactive attributes (Cheison *et al.* 2010). Food functionality is defined by the chemical and physical properties of food proteins that can control the behavior and performance of food during processing, preparation, storage and consumption (Kinsella & Whitehead 1989). The functionality of protein hydrolysate is linked to the extent of hydrolysis. Controlled or limited hydrolysis can lead to a hydrolysate protein with better functionality in comparison to extensive hydrolysis (Panyam & Kilara 1996). Hydrolysis can influence the protein and final hydrolysate product functionality in different ways. Solubility, water-holding capacity, gelation and coagulation, emulsification, foaming and surface hydrophobicity are food characteristics that can be influenced by hydrolysis (Panyam and Kilara 1996). Partial hydrolysis can effectively improve the protein solubility (Panyam and Kilara 1996). whey protein treated with trypsin caused 3% degree of hydrolysis and showed a reduction in the solubility of the hydrolysate (Mutilangi *et al.* 1995). Whey protein hydrolysate with lower molecular weight (<10,000 Da) exhibited improvement in foaming and interfacial properties (Althouse *et al.* 1995).

Moreover, one of the most problematic issues in the consumption of hydrolyzed milk formula is a rejection of the formula by infants due to bitter taste (Høst *et al.* 1999). The resultant of hydrolysis generates a bitter taste due to the size of the peptides and their hydrophobicity (Lalasis 1977). Another consideration in the final taste of hydrolysate is

the conformational factor in the hydrolysate, as only some parts of the protein can interact with the gustatory receptors (Bumberger & Belitz 1993). Many techniques in the food industry have been applied for debittering various hydrolysates.

7.0. Bitter peptides available in the milk hydrolysate

The bitter taste associated with hydrolysates is mostly due to the presence of low molecular weight peptides and hydrophobic residues (Saha & Hayashi 2001). The hydrolysis procedure can generate bitter peptides based on the type of enzyme. Gulgoz and Solms (1974), isolated Leu-Trp peptides from Alpkase, a Swiss mountain cheese with a bitter taste (Guigoz & Solms 1974). In another study, Huber and Klostermeyer (1974), isolated peptide Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser from a bitter cheese, Butterkaese (Guigoz & Solms 1974). To date, there is still no consensus on the choice of debittering method for protein hydrolysates. There has been several approaches for the reduction, masking or removal of the bitter peptides from hydrolysates, which all have advantages and disadvantages. Activated carbon has been used during alcohol extraction for the precipitation of bitter peptides from hydrolysates, but it can lead to the loss of tryptophan (Trp) during treatment, which was not nutritionally favorable (Murray & Baker 1952). Another technique that was used is chromatography for separation of the bitter peptides (Visser *et al.* 1975). A study evaluated the use of immune-specific adsorbent chromatography for selective adsorption of peptides, but this was not successful due to difficulty in separating the antigen-antibody complex at the end of the procedure (van Leeuwen 1978). Moreover, there has been many other attempts at using various methods, as well as treatment with alkaline/neutral proteases (Kanekanian *et al.* 2000) and treatment with carboxypeptidases, which could significantly decrease the bitterness (Kanekanian *et*

al. 2000). Kawabata *et al.* (1996) reported serine carboxypeptidase can eliminate the bitter peptides generated from soy protein hydrolyzed with trypsin and pepsin. Moreover, a study demonstrated a method for masking the bitter taste by using monosodium glutamate and several glutamyl oligopeptides (Noguchi *et al.* 1975). Also, by adding acid phospholipids, the bitter taste of amino acids and peptides has been controlled in food (Sugiura, 1996). However, it is always a challenge to choose an adequate debittering method compatible with the needs of the commercial food industry.

8.0. Discussions and Conclusions

Many approaches have been used for the reduction of whey protein antigenicity. The specificity of the enzymes used for cleavage of antigenic epitopes have made it one of the most common methods used in immunoreactivity reduction. There are different kinds of hydrolysis, as well as non-enzymatic acid and alkaline hydrolysis. Although, hydrolysis approaches could be practical in suppressing antigenicity, there are complications and challenges in controlling the process. Alkaline and acid hydrolysis can affect the product's nutritional values with the generation of potentially toxic substances (Sinha *et al.* 2007). Moreover, enzymatic hydrolysis mostly occurs at moderate temperatures (40-60°C) and pH (6-8) conditions, which can also lead to the formation of health-promoting bioactive compounds. Composition and functionality of the hydrolysate are important factors to consider when using the hydrolysate in the food industry. The emulsification property of some of the hydrolysates may decrease in some hydrolysate products due to the interfacial relationship between the hydrophobic and hydrophilic peptide and amino acids in the hydrolysate. Also, water absorption capacity increases after hydrolysis with papain and fungal peptidase, which occurs due to cleavage of protein into smaller peptide after

hydrolysis (Sinha *et al.* 2007). Moreover, Sinha *et al.* (2007) indicated that after hydrolysis with papain and a fungal enzyme, a significant increase was observed in the methionine amino acids hydrophobic amino acids in the final hydrolysate. Since the functionality of the hydrolysate would be varied based on the enzyme choice, further research could be conducted focusing on the functionality of hydrolysate with different enzymes.

Various methods have been used for masking or removal of bitter peptides after hydrolysis. However, all the methods can cause a decrease in some essential amino acids or the use of additives in the final product. Thus, it is necessary to explore novel approaches for solving this issue. Nanoencapsulation is a delivery technique that can mask the bitter taste of protein hydrolysates and improve their functional properties (Wróblewska., *et al.*, 2004; Ortiz, Mauri *et al.*, 2009). There are not many published research on the application of nanoencapsulation for masking the bitter taste of whey protein hydrolysate; therefore, pursuing research on the application of encapsulation and its possible pros and cons for hypoallergenic whey hydrolysate could be an appropriate future research direction.

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Chapter 3

Influence of Proteolytic Enzymes on Antigenicity Reduction and Physicochemical Properties of Bovine Whey Protein Isolate

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Abstract:

The effect of 12 different enzymes on the degradation of antigenic epitopes present in bovine whey protein isolate was investigated. The physicochemical property of each hydrolysate was characterized by surface hydrophobicity, mean particle size and polydispersity index. The effect of each enzyme on the allergenic proteins of bovine whey was determined using inhibition ELISA. The degree of hydrolysis and cleavage intensity were determined by SDS-PAGE. Two gastric enzymes (pancreatin, pepsin), three plant enzymes (bromelain, ficin, papain) and seven bacterial enzymes Alcalase, everlase, esperase, flavourzyme, neutrase, protamex and savinase were used. Pancreatin gave the highest level of degree of hydrolysis and neutrase, bromelain and pepsin gave the lowest. The electrophoretic pattern of hydrolysates generated with papain and pancreatin did not show any discernable evidence that α -lactalbumin or β -lactoglobulin survived the hydrolytic procedure. Furthermore, IgE immunoreactivity of whey protein was inhibited by 47% and 45% with papain and pancreatin, respectively. On the other hand, neutrase and flavourzyme hydrolysate products showed the lowest level of inhibition for IgE

immunoreactivity. These results suggest that one or more of these enzymes may be useful in the formulation of the novel hypoallergenic dairy products.

Keywords: protein hydrolysis; cleavage specificity; IgE inhibited; whey protein isolate; hypoallergenic dairy product.

1.0. Introduction:

According to recent self-reported data on food allergies in Canada, 2.23% of children and 1.89 % of adults are allergic to bovine's milk (Soller *et al.* 2012). Bovine milk allergy (BMA) is the most prevalent food allergy in early infancy up to the age of three and can also persist lifelong in some cases (Wood *et al.* 2013). Whey proteins constitute 20% of milk proteins but are responsible for 50% of the BMA incidence (Wal 2002; Madureira *et al.* 2007). The two major allergenic proteins in whey are α -lactalbumin (α -La) and β -lactoglobulin (β -Lg) (Wal 2002).

Enzymatic hydrolysis is a technique that has been used in the production of hypoallergenic milk formula (Merritt *et al.* 1990; Terracciano *et al.* 2002; Beyer 2007; Gomes-Santos *et al.* 2015). Enzymatic hydrolysis has been successfully used in the deactivation and degradation of the sequential and conformational epitopes in allergenic food proteins (Fritsché 2009). Enzyme specificity is a crucial factor that can influence the location and extent of hydrolysis and, hence, success in reducing the allergenic nature of food proteins (Adler-Nissen 1976). Enzyme specificity affects the site of hydrolysis and the deactivation of epitopes as well as the physiochemical properties of the hydrolysates, such as hydrophobicity (Korhonen & Pihlanto 2006).

Various enzymes possess different types of specificities; some enzymes have broad specificities which could be effective for use in particular reactions while others are narrowly specific for certain chemical bonds or functional groups (Nielsen 2009). Enzyme specificity also can influence the peptide functionality such as hydrophobicity and characteristics such as the molecular size of the resultant peptides (Turgeon *et al.* 1992).

Wróblewska *et al.* (2004) used Alcalase and papain for hydrolysis of whey protein for the deactivation of antigenic epitopes. They used enzymatic hydrolysis with each enzyme individually or in combination with each other. The hydrolysis condition was maintained during the procedure at 50°C and pH 8.0 for 120 minutes. The digested peptide after hydrolysis was separated by fast performance liquid chromatography (FPLC) and the immunoreactivity was determined by enzyme-linked immunosorbent assay (ELISA). They reported that the antigenicity was significantly decreased but some antigenic epitopes were still reactive; in this case, the hydrolysate is suitable for use in the formulation of a product for tolerogenic therapy.

Moreover, protein hydrolysates can possess different immunoreactivities to a specific substrate after treatment with pressure or heat. Penas *et al.* (2006) used three digestive enzymes under high pressure prior to, or during, the hydrolysis. They treated the samples to 100, 200 or 300 MPa for 15 min at 37°C. Pepsin, trypsin, and chymotrypsin were used to lower the antigenicity of the whey protein isolate. They found that trypsin and α -chymotrypsin could hydrolyze β -Lg at both atmospheric and high pressure, but pepsin hydrolyzed this protein only under high pressure. They reported that following hydrolysis by pepsin and chymotrypsin, the hydrolysate products could be used as a source of peptides for formulating hypoallergenic infant formulas (Peñas *et al.* 2006).

However, there is an uncertainty concerning the application of each enzyme and the adequacy of the final enzymatic hydrolysate in hypoallergenic formulas. There are several reports describing immunoreactivity of various hypoallergenic formulas after consumption (Adel-Patient *et al.* 2012; Lowe *et al.* 2013; Vandenplas *et al.* 2014). Furthermore, the proteolytic specificity of some commercially available enzymes is not well documented. Therefore, we conducted a study to follow proteolytic activity and cleavage specificity of 12 food-grade enzymes from three different groups: digestive enzymes, plant enzymes, and bacterial enzymes, with narrow and broad specificities. Resultant hydrolysates were tested with ELISA to assess the deactivation of immunoreactive epitopes present on α -La and β -Lg from whey proteins. This study also investigated the influence of enzyme hydrolysis on physiochemical properties, such as surface hydrophobicity, particle size diameter and polydispersity index of the hydrolysates.

2. Materials & Methods

2.0. Materials

Whey protein isolate was purchased from Bulk Barn Foods Ltd. (Truro, NS, Canada). Enzymes were obtained from Sigma-Aldrich (Oakville, ON, Canada). The detail information about the enzymes is provided in Table. 2.0

2.1. Whey protein hydrolysis:

An aqueous suspension (5% w/v) of bovine whey proteins was prepared and hydrolyzed with plant enzymes: papain (E.C. 3.4.22.2, from *Carica papaya*), bromelain (E.C. 3.4.22.32 from Pineapple, *Ananas comosus*) and ficin (E.C. 3.4.4.12, from latex of fig, *Ficus glabrata*); microbial enzymes: Alcalase (from *Bacillus licheniformis*), flavourzyme

(from *Aspergillus oryzae*), neutrase (from *Bacillus amyloliquefaciens*), esperase (from *Bacillus* sp.), everlase (from *Bacillus* sp.), protamex (E.C. 3.4.21.14, from *Bacillus* sp.), and savinase (E.C. 3.4.21.62, from *Bacillus lentus*); and digestive enzymes: pepsin (E.C. 3.4.23.1, from porcine gastric mucosa) and pancreatin (from porcine pancreas). Each hydrolysis was done separately at enzyme-substrate ratio of 1:100 (w/w). Hydrolysis was performed for 5 hours at the optimum pH and temperature conditions of each enzyme: papain at 65°C, pH 7.0; bromelain at 37°C, pH 6.5; ficin at 37°C, pH 7.0; Alcalase and savinase at 55°C, pH 8.0; flavourzyme and neutrase at 50°C, pH 7.0; esperase and everlase at 60°C, pH 8.0; pancreatin at 40°C, pH 7; protamex at 50°C, pH 6.5; pepsin at 37°C, pH 4.0. The optimum pH was maintained with adding 1 M NaOH or 1 M HCl to the mixture during hydrolysis. Hydrolysis was terminated by heat treatment at 90-95°C to inactivate the proteases and the hydrolysates were collected, lyophilized and stored at -20°C for further analysis.

Enzyme	Enzyme Commission number	Organism name	Type of Enzyme	Site of Action	Peptide Family	Enzyme specificity	Molecular function	Reference
Alcalase	NA	Protease from <i>Bacillus licheniformis</i>	Bacterial enzyme	A Serin endopeptidase	Serin endopeptidase	Suitable for hydrolysis of protein	NA	http://www.sigmaaldrich.com/catalog/product/mm/126741?lang=en&region=CA
Bromelain	E.C. 3.4.22.32	<i>Ananas comosus</i> (Pineapple)	Plant enzyme	Strong preference for Z-Arg-Arg- -NHMeC among small molecule substrates	C1,Cysteine-type peptidases	Broad specificity for protein hydrolysis,	Narrow Cysteine type protease activity,Exact serin family	http://www.brenda-enzymes.org/ http://www.ebi.ac.uk/ http://www.uniprot.org/
Esperase	NA	Protease from <i>Bacillus</i> sp.	Bacterial enzyme	NA	NA	NA	NA	http://www.sigmaaldrich.com/catalog/product/sigma/p5860?lang=en&region=CA
Everlase	NA	Protease from <i>Bacillus</i> sp.	Bacterial enzyme	A serin type protease	NA	NA	NA	http://www.sigmaaldrich.com/catalog/product/sigma/p5985?lang=en&region=CA
Ficin	E.C. 3.4.4.12.	Latex of fig, <i>Ficus glabrata</i> .	Plant enzyme	Amino acid bearing a large hydrophobic side chain at the P2 position. Does not accept Val in P1'.	C1,Cysteine-type peptidases	Broad specificity for protein hydrolysis,	Narrow Cysteine type protease activity, Exact serpin family binding	http://www.brenda-enzymes.org/ http://www.ebi.ac.uk/ http://www.uniprot.org/ http://enzyme.expasy.or
Flavourzyme	NA	Protease from <i>Aspergillus oryzae</i>	Bacterial enzyme	A fungal protease peptidase	Contains endopeptidase & exopeptidase	NA	NA	http://www.sigmaaldrich.com/catalog/product/sigma/p6110?lang=en&region=CA
Neutrase	NA	Protease from <i>Bacillus amyloliquefaciens</i>	Bacterial enzyme	NA	NA	NA	NA	http://www.sigmaaldrich.com/catalog/product/sigma/p1236?lang=en&region=CA
Papain (PPI)	E.C. 3.4.22.2	<i>Carica papaya</i> (Papaya)	Plant enzyme	Amino acid bearing a large hydrophobic side chain at the P2 position. Does not accept Val in P1'.	C1,Cysteine-type peptidases	Broad specificity for protein hydrolysis,	Narrow Cysteine type protease activity, Thiol protease activity	http://www.brenda-enzymes.org/ http://www.uniprot.org/ http://enzyme.expasy.org/
pepsin	E.C. 3.4.23.1	Gastric juice of vertebrates	Digestive enzyme	: hydrophobic, preferably aromatic, residues in P1 and P1' positions. Cleaves Phe1- -Val, Gln4- -His, Glu13- -Ala, Ala14- -Leu, Leu15- -Tyr, Tyr16- -Leu, Gly23- -Phe, Phe24- -Phe	Peptidase family 1 Aspartic type peptidase	Preferential cleavage	Endopeptidase	http://www.brenda-enzymes.org/ http://www.ebi.ac.uk/ http://www.uniprot.org/ http://enzyme.expasy.org/

Table 2.0 Enzymes: Information and specificities

Enzyme	Enzyme Commission number	Organism name	Type of Enzyme	Site of Action	Peptide Family	Enzyme specificity	Molecular function	References	
Pancreatin ¹	NA	Pancreatin from porcine pancreas contained	Digestive enzyme	NA	NA	NA	NA	http://www.sigmaaldrich.com/ http://www.brenda-enzymes.org/ http://www.ebi.ac.uk/ http://www.uniprot.org/ http://enzyme.expasy.org	
	E.C. 3.4.21.34	Kallikreins		Cleaves selectively Arg- -Xaa and Lys- -Xaa bonds, including Lys- -Arg and Arg- -Ser bonds in (human) kininogen to release bradykinin					
	E.C. 3.4.21.4	Trypsin		Preferential cleavage: Arg- -Xaa, Lys- -Xaa	Peptidase family S1	Arg- -Xaa, Lys- -Xaa	Serine protease		
	E.C. 3.4.4.5	Chymotrypsin,		Preferential cleavage: Tyr- -Xaa, Trp- -Xaa, Phe- -Xaa, Leu- -Xaa	Peptidase family S1	Tyr- -Xaa, Trp- -Xaa, Phe- -Xaa, Leu- -Xaa	Serine-type endopeptidase activity		
	E.C. 3.4.21.7	Elastase,		Preferential cleavage: Leu- -Xaa, Met- -Xaa and Phe- -Xaa. Hydrolyzes elastin.					
	E.C. 3.4.17.1	Carboxypeptidase A		Release of C-terminal amino acids but little or no action with -Asp, -Glu, -Arg, -Lys or -Pro			Hydrolysis of peptide bond		Metallo-carboxypeptidase activity, zinc ion binding
	E.C. 3.4.2.2	Carboxypeptidase B,		Preferential release of a C-terminal lysine or arginine amino acid					Metallo-carboxypeptidase activity, zinc ion binding
Protamex	E.C. 3.4.21.14	Protease from <i>Bacillus</i> sp	Bacterial enzyme	NA	NA	NA	NA	http://www.sigmaaldrich.com/catalog/product/sigma/p0029?lang=en&region=CA	
Savinase	NA	Protease from <i>Bacillus species</i> cross-linked enzyme aggregate	Bacterial enzyme	NA	NA	NA	NA	http://www.sigmaaldrich.com/catalog/product/sigma/41493?lang=en&region=CA	

¹Pancreatin included 6 various enzymes in the pancreatic mucosa Kallikreins Trypsin Chymotrypsin, Elastase, Carboxy-peptidase A,

Carboxypeptidase B

2.2. Determination of degree of hydrolysis

The degree of hydrolysis in the hydrolysate samples was determined using the O-phthaldialdehyde (OPA) method (Udenigwe et al., 2014). The hydrolysate sample was mixed with OPA reagent. Briefly, 33 μL of 1 mg/mL hydrolysate was mixed with 250 μL of OPA reagent. The reaction mixtures were loaded in a 96-well microplate. Serine was used as a standard, the absorbance was determined at 340 nm and the free amino nitrogen was expressed as milliequivalent serine NH_2/g . Degree of hydrolysis was calculated as previously reported.

2.3. Determination of surface hydrophobicity

The surface hydrophobicity of the whey protein hydrolysates was determined by fluorescence spectroscopy using a hydrophobic probe, 8-anilino-1-naphthalenesulphonic acid (ANS) (Horax *et al.* 2004). The aqueous solution of hydrolysate was prepared at 0.15%-0.009% concentration of the peptide. The solution was loaded in a 96-well black microplate, followed by the addition of ANS. Fluorescence was then measured at 390 nm (excitation) and 470 nm (emission). The slope of the plot of the fluorescence vs. concentration plot was taken to be the surface hydrophobicity (Horax *et al.* 2004).

2.4. Particle size characteristics

Particle size diameter and polydispersity index were measured with the light scattering/particle electrophoresis instrument (Horiba SZ-100 Nanoparticle series instruments, Kyoto, Japan). All samples were prepared in 200 mM sodium phosphate buffer, pH 7.2 at 30 mg/mL, then diluted 20 fold in nanopore water and then analyzed in disposable capillary cells.

2.5. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 12% precast mini-PROTEAN TGX from Bio-Rad to determine the extent of hydrolysis of whey proteins and the concentrations of intact α -La and β -Lg, according to the procedure described by Laemmli (1970). The samples were loaded at total amount of 25 μ g protein and separation was performed at 75 mV for 30 minutes followed by 150 mV for 1 h. Coomassie Brilliant Blue R-250 (0.1%) was used for staining the gel. The gel was visualized with the the Bio-Rad ChemiDocTM MP instrument.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The antigenicity of the hydrolysates was determined based on a previously described procedure (Beresteijn, Meijer et al., 1995; Schmidt, Meijer et al., 1995), with some modifications. The microplates were coated with 100 μ L of whey protein solution (10 mg/mL, in sodium bicarbonate buffer 100 mM, pH 9.6) and kept overnight at 4°C. Residual-free binding sites were blocked with 150 μ L of 10 mM phosphate buffered saline (PBS) (pH 7.4) containing 0.1% bovine serum albumin and 0.05% Tween 20 (PBSGT), and then incubated for 30 minutes at 37°C. The wells were subsequently washed three times with PBSGT. Afterward, 100 μ L of hydrolysates or whey protein control, at two concentrations, was mixed with 100 μ L of rabbit anti-bovine whey protein (Sigma Aldrich, 1:1000 in 0.05 M PBS) followed by incubation at 37°C for 1 hour. After incubation, the mix of hydrolysate and antibody was transferred to the microplate and incubated at 37°C for another 1 hour. The solution was discarded and the plate was washed three times with PBSGT. To determine the antibodies that have bound to the plate, peroxidase-conjugated goat anti-rabbit IgG (Sigma Aldrich) was used. Samples were diluted in PBS at the ratio of 1:5000 and incubated at 37°C for 2 hours. Afterwards, the solution was removed and

the wells washed three times using PBSGT. This was followed by the addition of 200 μ L of freshly prepared substrate solution containing O-phenylene-diamine dihydrochloride to visualize the substrate interaction with enzyme-conjugated with the secondary antibodies [FAST OPD peroxidase substrate table set, Sigma, added to the wells in the dark for 30 minutes. The absorption was measured at 450 nm using a microplate reader. The experiment was carried out in duplicate. The inhibition percentage was calculated by the following equation: $[(Abs_0 - Abs_x) / (Abs_0 - Abs_m)] * 100$ where:

Abs_0 - absorbance of blank microplate wells without any antigens (to minimize the background)

Abs_x - absorbance obtained from selected two concentration of hydrolysate

Abs_m - minimum detected absorbance obtained from the maximum antigenic proteins specifically β -Lg concentration.

2.7. Statistical analysis

All assays were performed in triplicate with the exception of the ELISA procedure, which was carried out in duplicate. All statistical analysis was carried out with Minitab 17.3.1. A one-way analysis of variance followed by Tukey multiple comparison tests were used to test significance level between the treatments with $\alpha=0.05$. Correlation of results was done by Spearman's rank order and rho was determined using Minitab 17.

3. Results and Discussion

3.1. Physiochemical characteristics of the whey protein hydrolysates

3.1.1. SDS-PAGE profiles

The results of the SDS-PAGE profile are shown in Fig.3.1.1. The results in each electrophoretogram include an untreated whey protein isolate as the control. The result for the untreated whey protein shows two substantial bands corresponding to the two main whey proteins: α -La (molecular weight 14 kDa) and β -Lg (molecular weight 18 kDa) as well as a lighter band for BSA at 66.5 kDa. As presented in the figure, different patterns of peptides fractions were observed in the SDS-PAGE after hydrolysis of the whey proteins. After papain, pancreatin, and esperase treatments, there were no visible bands still present corresponding to α -La, β -Lg or BSA. Flavorzyme had only a faint trace of proteinaceous material at the corresponding molecular weights. After being hydrolyzed with bromelain, ficin, neutrase, and protamex, there was no visual evidence of β -Lg or BSA remaining in the hydrolysate although there was a band corresponding to α -La in each of these hydrolysate samples. On the other hand, the SDS-PAGE results suggested that Alcalase effectively hydrolyzed whey α -La while β -Lg and BSA were still detectable. Hydrolysates prepared with savinase exhibited a trace of α -La and obvious BSA fraction remaining after hydrolysis.

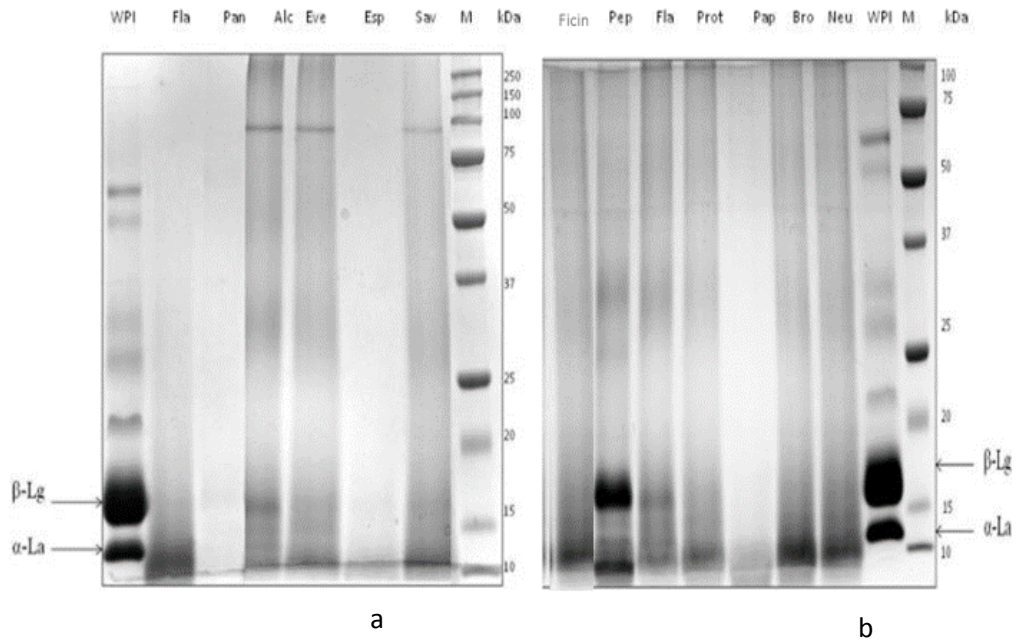


Figure. 3.1.1. a: From the left to the right untreated whey protein, flavourzyme, pancreatin, Alcalase, everlase, esperase, savinase, Marker. b: From the left to the right: ficin, pepsin, flavourzyme, protamex, papain bromelain, neutrase, untreated whey protein, Marker.

3.1.2. The degree of hydrolysis

The degree of hydrolysis was calculated based on the number of peptide bonds cleaved in whey protein after hydrolysis. Free amino groups were determined and expressed as milliequivalent serine NH_2/g . All results are presented in Fig. 3.1.2. Pancreatin hydrolysate gave the highest degree of hydrolysis of 27.15%, which indicated the most extensive peptide bond cleavage of all the enzymes (Mohan *et al.* 2015). On the other hand, pepsin, neutrase, and bromelain gave the lowest DH of 7.23%, 9.76% and 9.82%, respectively. Microbial enzymes have been reported to demonstrate broader specificity for proteolytic activity as they constitute a mixture of enzymes (Oh *et al.* 2013). Hydrolysates produced with enzymes of bacterial sources (savinase, Alcalase, esperase, everlase, flavourzyme, protamex and neutrase) demonstrated various DH ranging from 9% to 19%. Furthermore, pancreatin and pepsin were gastrointestinal enzymes that have been used for the production of hydrolysates. As presented in Table. 2.0, pancreatin contains six different enzymes with broad specificity and various cleavage preferences. Pancreatin includes kallikreins, carboxypeptidase A, carboxypeptidase B, trypsin, chymotrypsin and elastase. Pepsin showed DH of 7.23% which is considered as a low degree of hydrolysis. The results are in agreement with the observation of Penas *et al.* (2006), who reported that degree of hydrolysis with pepsin was negligible except with pressure treatment prior or during hydrolysis, which led to higher degrees of hydrolysis with this enzyme. The SDS-PAGE results in Fig. 3.1.1 also represents the lowest degradation of whey proteins with pepsin at atmospheric pressure. A moderate and insignificant correlation ($r_s = 0.42$, $P = 0.167$) was observed between degree of hydrolysis and deactivation of immunoreactivity of the whey protein hydrolysates, which is in agreement with previous findings (Svenning *et al.* 2000).

It can be concluded that enzyme activity alone does not guarantee the lowering of immunoreactivity and cleavage of desire antigenic epitopes in the proteins. However, different studies have reported conflicting observations. One study reported that as the degree of hydrolysis increased, the immunoreactivity level decreased in the hydrolysate products treated with pepsin and then trypsin proteases (Kim *et al.* 2007). This may attributed to the usage of a combination of enzymes, which can facilitate the epitope deactivation during hydrolysis.

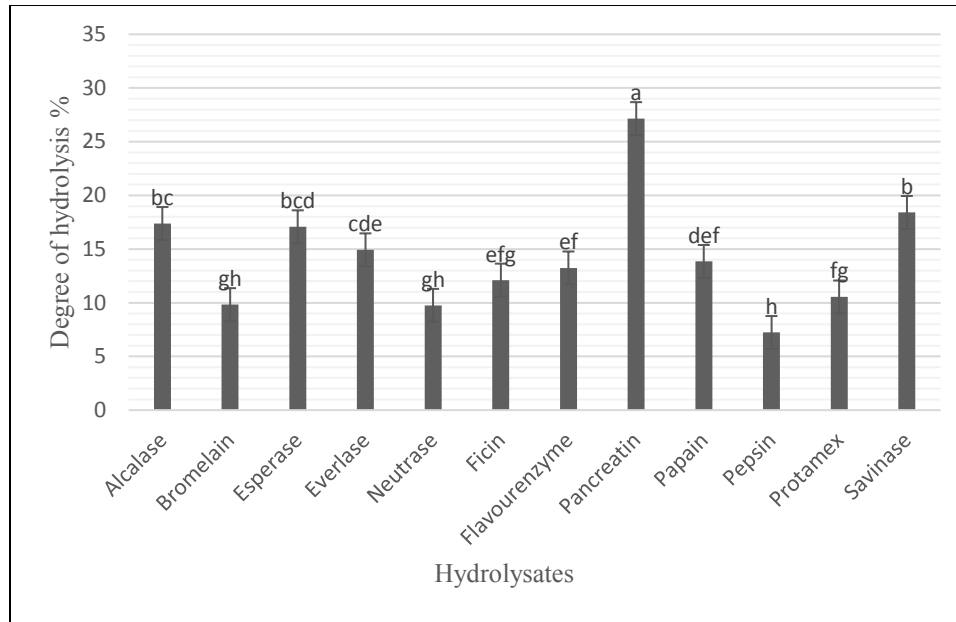


Figure. 3.1.2. Degree of hydrolysis determined by O-Phthaldialdehyde method after hydrolysis of whey protein isolate treated with different enzymes. The difference between mean values was statistically significant which is presented with various letters in bars ($P < 0.05$).

3.1.3. Surface hydrophobicity:

Enzyme type and degree of hydrolysis can influence the surface hydrophobicity of each hydrolysate. Surface hydrophobicity (S_0) is mostly increased after protein hydrolysis due to expose of hydrophobic pockets of the resulting peptides (Mutilangi *et al.* 1996). The presence and exposure of hydrophobic amino acid residues in protein hydrolysate can reflect their bitterness (Cho *et al.* 2004). High levels of hydrophobic groups in the hydrolysate can also reduce the protein solubility and emulsifying properties in the final product. The hydrolysis procedure can unfold the hydrophobic residue during the hydrolysis step and it can also promote aggregation of hydrolysed proteins as well as decrease the solubility and emulsifying properties of hydrolysates (Paraman *et al.* 2007).

The hydrolysate from protamex reaction showed the highest surface hydrophobicity among all the hydrolysates. Flavourzyme hydrolysate had the second highest S_0 after the hydrolysate from protamex treatment. Papain, pancreatin, everalse, Alcalase and savinase had very similar surface hydrophobicity values as shown in Fig. 3.3.1. These results are in agreement with previous reports of rice protein treated with Alcalase and pepsin (Mutilangi *et al.* 1996).

In addition, Spearman rho correlation indicated that there was no apparent correlation between mean particle size and surface hydrophobicity; however, it showed a negative correlation ($r_s = -0.736$) between degree of hydrolysis and surface hydrophobicity. This corresponds to the fact that as the degree of hydrolysis is higher, surface hydrophobicity is lower. This can be attributed to the decrease in hydrophobicity as result of the cleavage of hydrophobic pockets of the proteins and solubilization of the peptides. The hydrophobic residue in hydrolysate can reflect the bitterness of hydrolystae peptides (Cho *et al.* 2004);

Therefore, choosing enzymes with high activity in inhibiting IgE immunoreactivity and leading to moderate surface hydrophobicity of whey protein may result in a hypoallergenic whey protein with improved palatability. Also, many antigenic epitopes identified in previous studies contain hydrophobic amino acid residues such as Gly, Ala, Leu and Pro, which are mostly buried inside the protein structure before hydrolysis; the epitopes include: (41-60), (92-100), (149-162), (84-91) and (95-113) of β -LG sequences (Ball *et al.* 1994; Selo *et al.* 1999).

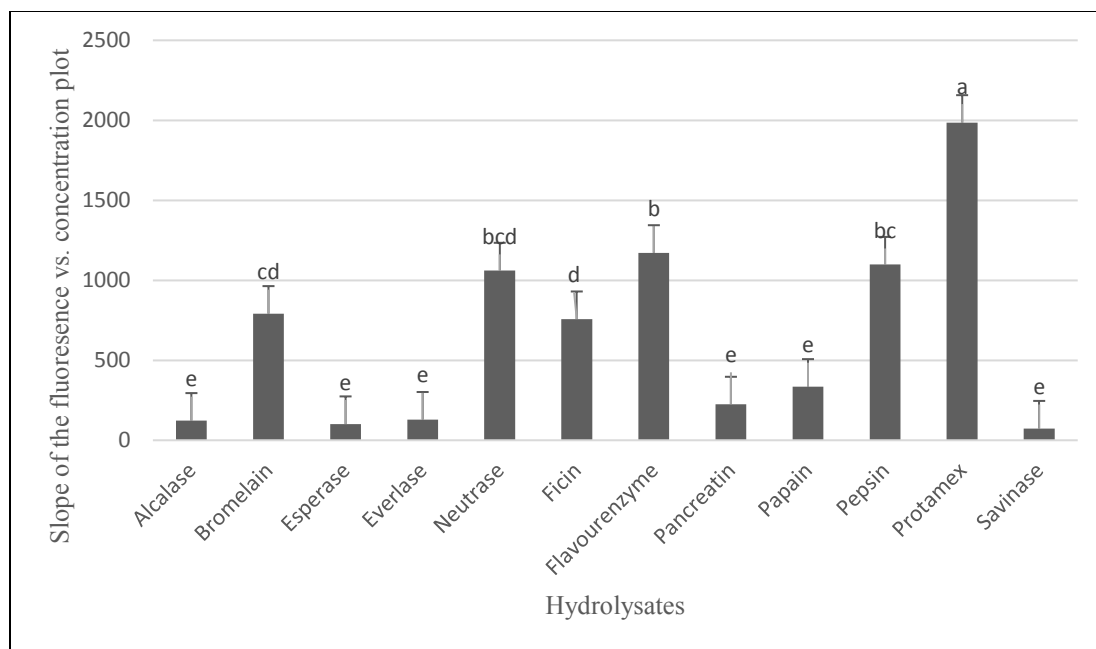


Figure. 3.1.3. Surface hydrophobicity S_0 , which is the slope of the plot of the fluorescence vs. concentration plot. Bars with different letters indicate results (mean values) that were statistically significant ($P < 0.05$).

3.1.4. Particle size analysis

Based on the data presented in Table 3.1.5, Alcalase, pancreatin and papain-generated whey protein hydrolysates had the highest mean particle diameters of all the hydrolysates, and this can be related to their extents of hydrolysis. Also, surface hydrophobicity can influence the particle size. Flavourzyme and neutrase have shown the lowest mean particle diameters. This does not reflect the degree of hydrolysis and can be due to variation in the solubility of the hydrolysates in sodium phosphate buffer. Differences in mean particle size diameter of the hydrolysates could not be statistically determined. Moreover, there was no apparent correlation between the mean particle size and surface hydrophobicity or degree of hydrolysis.

3.1.5. Polydispersity index

The polydispersity index (PDI) is a measure of the breadth of molecular mass distribution which can impact particle suspension in the sample solution (Rane & Choi 2005). PDI is calculated based on the division of square variance of size distribution by average particle size which calculates the square of relative standard deviation of molecular distribution which is known as PDI (Santos & Castanho 1996). As it is presented in the Table 3.1.5, papain hydrolysate showed the highest PDI. It indicated that papain hydrolysate showed the less uniform suspension of particles. Flavourzyme and neutrase hydrolysate had the lowest PDI, which indicate that they formed a more uniform suspension of particles, which can improved emulsifying property (Mohan *et al.* 2016). The rest of the hydrolysates followed the same patterns in the distribution of particles. There is insignificant correlation ($r_s=0.312$) between mean particle size and PDI results. Enzyme degradation and optimum temperature required during hydrolysis can influence the results of the PDI as well.

Table. 3.1.5. Mean particle diameter and polydispersity index of the hydrolysate resultants of 12 different enzymes, numbers with different letters shows the significant statistical difference between mean values

Whey hydrolysates	Alcalase	Bromelain	Esperase	Everlase	Neutrase	Ficin	Flavourzyme	Pancreatin	Papain	Pepsin	Protamex	Savinase
Polydispersity index	2.38 ^{ab}	1.64 ^{ab}	3.406 ^{ab}	5.12 ^{ab}	0.377 ^b	4.77 ^{ab}	0.537 ^b	3.08 ^{ab}	6.53 ^a	3.84 ^{ab}	4.09 ^{ab}	3.36 ^{ab}
Mean particle ¹ diameter (nm)	7141	1620	4003	5250	140.5	3039	110.2	6196	5160	3409	2284	3736

¹The results of conducted statistical test, with $\alpha=0.05$, was under the acceptable power to indicate significant difference

3.2. Immunochemical properties of the whey protein hydrolysates

Hydrolysis has been used successfully for deactivation of antigenic epitopes based on the type of enzyme and the condition (Fritsché 2009). ELISA was used in assessment of IgE binding inhibition and immunoreactivity of the 12 whey protein hydrolysates.

A preliminary study was used to identify the appropriate concentration range in which the maximum IgE inhibition was observed. Afterwards, based on the selected concentration range, 1 mg/mL and 0.00001 mg/mL, that show the lowest and highest IgE binding inhibition in the hydrolysates, the optical density was obtained and IgE binding inhibition calculated accordingly. The obtained optical density of hydrolysates and standard whey protein isolate are presented in the Fig.3.4.. Papain and pancreatin-generated hydrolysates showed the highest IgE binding inhibition of 47% and 45%, respectively. As enzyme information detail is provided in Table 2.0, papain is a plant enzyme with broad specificity for proteins. The ELISA results illustrated that papain hydrolysate has a broad specificity for cleavage of the antigenic epitopes in the whey proteins. These results are in the agreement with other studies that demonstrated the effectiveness of this enzyme in deactivation of antigenic epitopes in the hydrolysates. Izquierdo *et al.* (2008) reported that papain hydrolysate degraded β -Lg effectively regardless of treatment with microwave irradiation. In another study, papain was used as a secondary enzyme in combination with Alcalase in generating hydrolysates that can be used in the production of tolerogenic formulas. Also, the authors reported the improvement of palatability in the papain-generated whey protein hydrolysates (Wróblewska *et al.* 2004).

Pancreatin from the porcine pancreas is a mixture of various enzymes; it showed the second best activity in lowering antigenicity with 45 % of IgE binding inhibition. This result is in

support of previous studies which used different extracted enzyme from pancreatic juice such as chymotrypsin for cleaving the antigenic proteins, α -La and β -Lg (Chicón *et al.* 2009). Another study reported the effective action of this pancreatic enzyme on the complete degradation of β -Lg with microwave radiation of hydrolysates at 50°C for 5 minutes (Izquierdo *et al.* 2008).

As previously stated, pancreatin used in this study was extracted from porcine pancreatic secretion. So, a question arises as to why this mixture of enzyme is capable of deactivation of antigenic epitopes *in vitro* but not in patients, especially infants, with allergic reactions to whey proteins. It is possible that the undeveloped structure of gut organ in neonates, for instance the pH or activity of available enzymes, does not allow for complete protein cleavage, although this can be enhanced as they grow older (Sampson 1999). It takes two years for the newborn's intestinal proteolytic activity to mature (Lebenthal & Lee 1980). The electrophoresis result also supports the ELISA results. As presented in Fig. 3.1.1, in sample treated with papain, β -Lg was hydrolyzed and degraded completely. The electrophoresis results of pancreatin showed the effective degradation of α -La and β -Lg, Esperase showed the third highest IgE binding inhibition of 42% among all the other enzymes. The SDS-PAGE result also supports the ELISA results. Moreover, Alcalase hydrolysate showed 40% of IgE binding inhibition. Another study reported a similar activity for Alcalase but they demonstrated a complete degradation of β -Lg after microwave irradiation prior to hydrolysis (Izquierdo *et al.* 2008).

Protamex, ficin, everlase and bromelain had similar effects in lowering antigenicity of the whey protein hydrolysate from 36-38%. Among these enzymes, bromelain and ficin are

two plant enzymes belonging to the cysteine protease family. Protamex and everlase are both bacterial enzymes from *Bacillus sp.* and *Bacillus licheniformis*, respectively.

Pepsin and savinase treatment of whey proteins resulted in hydrolysates with almost the same IgE binding inhibitory activity (Table 3.2.). The SDS-PAGE results for savinase are in agreement with the ELISA results, unlike the pepsin-produced hydrolysate. In the SDS result, pepsin did not effectively hydrolyze α -La and β -Lg, however, ELISA results indicated a 30% decrease in antigenicity of the whey protein. This could be explained by the minimal specificity of pepsin for antigenic epitopes without degrading the complete β -Lg; β -Lg has a stable structure at acidic pH 2.0, which is the optimum pH for pepsin activity (Dalgarrondo *et al.* 1995). These results are in agreement with the previous study that reported a minimal antigenic reduction in pepsin hydrolysate (López-Expósito *et al.* 2012). Antigenicity reduction with lower extent of protein degradation is favorable. It has been indicated that extensive hydrolysis can negatively influence the aroma, foaming and emulsifying properties of the final product (Sinha *et al.* 2007).

Neutrased gave 25% of IgE binding inhibition in the resultant of hydrolysis. Izquierdo reported the weak activity of neutrased in lowering antigenicity of whey protein; they indicated that β -Lg remained intact even after microwave irradiation prior to hydrolysis (Izquierdo *et al.* 2008). Flavourzyme, a bacterial complex enzyme extracted from *Aspergillus oryzae*, had the lowest IgE binding inhibition of all the hydrolysates. Furthermore, there is moderate negative correlation ($r_s = -0.643$, $P = 0.024$) between surface hydrophobicity and inhibition ELISA data, which shows that as surface hydrophobicity decreases, the IgE binding by the epitopes was inhibited. β -Lg contains the major antigenic epitopes in the whey proteins. These antigenic epitopes with high probability of binding

are located at fragments 25-40, 41-60, 84-91, 97-108, and 95-113 (Ball *et al.* 1994; Sélo *et al.* 1998; Chicón *et al.* 2008). Among the epitopes, fragments 25-40, 41-60 and 95-113 start with hydrophobic amino acids, Gly, Ala, Leu and Pro, respectively. An increase in the surface hydrophobicity of the whey protein hydrolysates can be due to the revealing of the buried (possibly antigenic) hydrophobic residues of the protein, which can lead to enhanced interaction of the antigenic epitopes with IgE. Several major antigenic epitopes have been identified in whey proteins using liquid chromatography-mass spectrometry. Formulation of hypoallergenic products is still a topic on interest since there are still reports of immunoreactivity of hypoallergenic products after oral consumption (Hays & Wood 2005). Moreover, Ena *et al.* (1995) reported that peptides with molecular size less than 3400 Da have a lower probability of triggering immune response. However, a lack of correlation between degree of hydrolysis and ELISA results was observed in this and other studies (Svenning *et al.* 2000). This could be post-hydrolysis processing used in some studies. For instance, membrane ultrafiltration seems to play an essential role in the reduction of antigenicity of protein hydrolysate (Ena *et al.* 1995) as the fractionated low molecular size peptides likely lack of structurally intact antigenic epitopes. Furthermore, Millard reaction can occur during heat treatment causing glycation, reaction of sugar and amino acids of the peptides (Mohan *et al.* 2015). Lactosylated β -Lg is produced by Millard reaction and can change the epitopic areas and reduce antigenic response in of the whey protein in ELISA (Morgan *et al.* 1998). This factor may have contributed in deactivation of antigenic epitopes and lowering *in vitro* immunoreactivity of the whey protein hydrolysates.

Table 3.2. Antigenicity reduction in hydrolysate whey protein isolate with 12 different enzymes

Hydrolysis enzyme	Alcalase	Bromelain	Esperase	Everlase	Neutrase	Ficin	Flavourzyme	Pancreatin	Papain	Pepsin	Protamex	Savinase
Antigenicity reduction (%) in whey hydrolysate	40	36	42	36	25	38	27	45	47	30	38	38

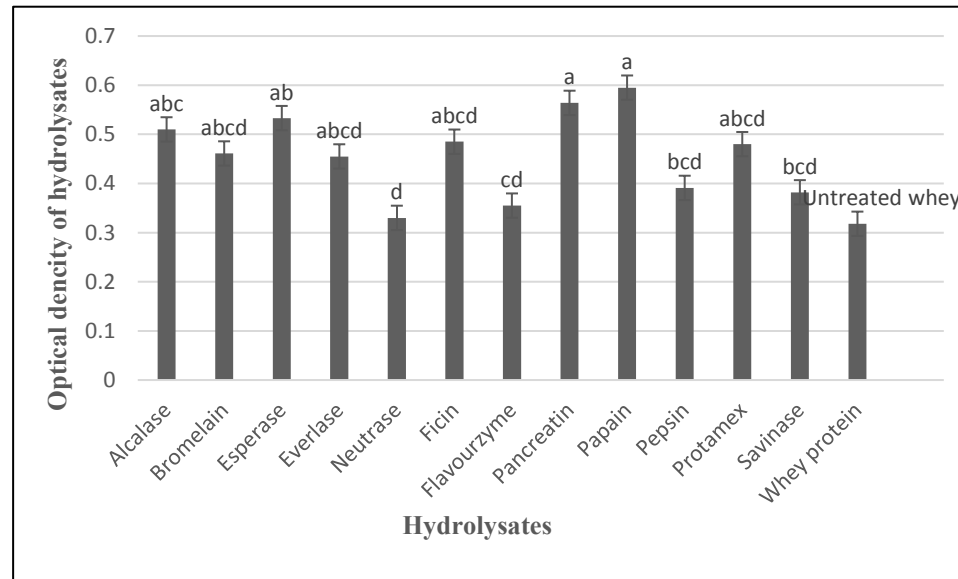


Figure 3.4. The absorption measured at 450 nm for selected inhibitor concentration in ELISA microplate. Bars with different letters indicate results (mean values) that are statistically significant ($P < 0.05$).

4. Conclusions

Enzymatic hydrolysis can be used for the production of hypoallergenic dairy products. Hydrolysis has been previously demonstrated to successfully lower the antigenicity of the whey protein. However, research on improving the hypoallergenic products with adequate palatability and functionality is still in progress. This study focused on using 12 enzymes of different specificities for whey protein hydrolysis and inhibition of immunoreactivity *in vitro*. Papain resulted in a whey hydrolysate with the highest level of IgE binding inhibition followed by pancreatin. In addition, the degree of hydrolysis does not reflect the extent of deactivation of the antigenic epitopes. Moreover, flavourzyme and neutrase have modified the whey isolate to result in products with the lowest mean particle diameters. Based on the findings, extensive degradation of whey proteins was not always accompanied by a reduction of antigenicity and it can be concluded that the extent of hydrolysis and ability of enzyme in protein degradation do not reflect the inhibition of antigenicity. Furthermore, flavourzyme and neutrase hydrolysate formed more uniform suspension, which indicate their potential use in improving the protein emulsifying property. To determine the actual specificity of enzymes on deactivation of antigenic epitopes, peptidomic analysis of the hydrolysate with the highest IgE inhibition needs to be conducted.

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Chapter 4

Peptidomics of Papain-digested Whey Protein Isolate Revealed the Deactivation of Antigenic Epitopes

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Abstract

Peptidomics of papain hydrolyzed whey protein isolate was conducted to evaluate the cleavage of antigenic epitopes. The cleavage pattern of papain was also simulated *in silico* using SitePrediction website and possible antigenic epitopes were predicted using SVMTrip tools. The LC-MS/MS analysis results demonstrated 52 peptides fractioned in the beta-lactoglobulin in hydrolysate. The *in silico* prediction of enzymatic cleavage sites were matched with the actual hydrolysis that occurred in bovine β -lactoglobulin. The matched cleavage site sequence predicted *in silico* had 99% of probability of occurrence. The comparison of *in silico* predicted epitopes within the actual results indicated a significant degradation of amino acid sequences presented in the antigenic epitopes by 35%, 75%, 90% and 100%. In addition, the comparison results of the LC-MS/MS analysis cleavage site with the known epitopes from the literature showed significant breakage in the antigenic epitopes as follow: 53%, 55%, 65%, 77% and 100%. Most of the epitopes predicted *in silico* and those obtained from the literature started with hydrophobic amino acids, which likely enhanced their susceptibility to cleavage by papain as it has specificity for breakage of peptide bonds with hydrophobic amino acid residues. Therefore, the papain

hydrolysate with 47% of IgE binding inhibition demonstrated an acceptable level of degradation.

Keywords: Papain, whey protein, hydrolysate, peptidomics, β -lactoglobulin, antigenic epitopes

1.0. Introduction

The bovine whey protein is a rich source of bioactive peptides and responsible for occurrence of 50% of bovine milk allergy (Wal 2002). Enzymatic hydrolysis is a technique that has been used in the food industry for the production of hypoallergenic products with reduced antigenicity (Ena *et al.* 1995; Guadix *et al.* 2006; Peñas *et al.* 2006; Izquierdo *et al.* 2008). Enzyme specificity can influence the number and location of peptide bonds cleavage during protein hydrolysis (Adler-Nissen 1976).

Apart from suppressing immunoreactivity of antigenic proteins, enzymatic hydrolysis can lead to protein cleavage and generation of oligopeptides and smaller peptides as hydrolysis continues (Adler-Nissen 1976). Peptidomics can be used to determine the composition and sequence of peptides in food protein hydrolysates (Gagnaire *et al.* 2009). In addition, peptidomics can reveal the origin and alteration of peptides released from parent food proteins during hydrolysis (Minkiewicz *et al.* 2008). Also, *in silico* analysis can be used for prediction of protein antigenicity (Stadler & Stadler 2003). BIOPEP enzyme action tool and PeptideCutter from ExPASy can be used for simulation of proteolytic specificities of enzymes (Gasteiger *et al.* 2005). In addition, SitePrediction website can be used for cleavage site prediction. Databases such as BIOPEP can be used for exploring the possible presence of allergenic proteins and epitopes in protein sequences (Dziuba & Dąbek 2013).

The previous chapter reported the application of 12 different proteolytic enzymes in the production of whey protein hydrolysate and *in vitro* assessment of antigenicity reduction in the hydrolysates. Among the hydrolysates, papain-derived hydrolysate was found to have the best antigenicity inhibition of 45% attributable to the broad cleavage specificity of papain for the antigenic epitopes. In the present study, we conducted peptidomics analysis for the papain-derived whey protein hydrolysate to define the sequence of the liberated peptides after hydrolysis. β -Lactoglobulin (β -Lg) has the highest level of antigenicity of the whey proteins, hence, its peptide sequences released after hydrolysis were selected for further analysis. The *in silico* analysis and simulation of papain cleavage of β -Lg was also carried out. The aim of this study was to use *in silico* tools and peptidomics to evaluate the specificity of papain in deactivation of antigenic epitopes of β -Lg after hydrolysis of whey protein isolate.

2. Material & Methods

2.0. Materials:

Whey protein isolate (WPI) was purchased from Bulk Barn Foods Ltd. (Truro, NS, Canada). Papain from *Carica papaya* (papaya latex) was purchased from Sigma-Aldrich (Oakville, ON, Canada). The other reagents were purchased from Fisher Scientific Co. (Ottawa, ON, Canada).

2.1. Whey protein hydrolysis

WPI was hydrolyzed with papain as described in chapter 3. This hydrolysate was selected based on the high percentage of antigenicity reduction due to papain treatment when compared to 11 other enzymes. The ratio of enzyme–substrate was 1:100 (w/w). The optimum pH and temperature condition for papain (65°C, pH 7.0) were maintained during

hydrolysis for 5 hours. Termination of hydrolysis was done by heat treatment at 90-95°C, for 15 minutes, to inactivate the proteases. The hydrolysate sample was then collected, lyophilized and stored at -20°C until further analysis.

Table 2.1. Enzyme information and specificity ¹

¹The provided information was obtained from <http://www.brenda-enzymes.org/enzyme.php?ecno=3.4.22.2>.

Enzyme Name	EC	Enzyme type	Enzyme Origin	Cleavage specificity	Enzyme inhibitors
Papain	3.4.22.2	Plant enzyme	latex from papaya (Carcia papaya)	Amino acid with a large hydrophobic side chain at the P2 position. A narrow Cysteine-type proteinase	Inhibited by compound E_64 and proteins of cystatin family.

2.2. LC–MS/MS Analysis

Q-Exactive Orbitrap analyzer outfitted with a nanospray source and EASY-nLC nano-LC system (Thermo Fisher, San Jose, CA, USA) was used to carry out the LC–MS/MS analysis at the Mass Spectrometry Facility at SPARC BioCentre, The Hospital for Sick Children (Toronto, ON, Canada), according to a previously reported method (Udenigwe *et al.* 2016).

2.3. Peptidomics

PEAKS software (Bioinformatic Solutions Inc., Waterloo, ON, Canada) was used for analysis of the LC–MS/MS data (Han *et al.* 2011). The analysis was carried out according

to a methodology reported by CK Rajendran *et al.* (2016). The peptides were identified with the application of database search module in PEAKS software based on homology search among protein database or *de novo* sequencing which was performed for identification of novel identified peptides according to the reported approach described by (Ma & Johnson 2012). The following parameters were used in association with DB (UniProt, *Bos taurus*) and *de novo* sequencing: a precursor mass tolerance of 10 ppm using monoisotopic mass, and a fragment mass tolerance of 0.02 Da.

2.4. *In silico* analysis

The FASTA sequence of the selected whey protein, β -lactoglobulin, with high antigenic epitopes was obtained from National Centre for Biotechnology Information (NCBI). The sequence was entered in SitePrediction available at:

<http://www.dnbr.ugent.be/prx/bioit2-public/SitePrediction/index.php>

to simulate the proteolytic cleavage with papain. Afterward, the predicted cleaved peptides were compared to peptides identified from peptidomics. Certain criteria were taken into consideration including cleavage specificity, peptide size, and antigenicity. In addition, the potential antigenic epitopes were predicted virtually using SVMTrip, a tool for the prediction of linear antigenic epitopes (Yao *et al.* 2012).

3. Results and Discussion

3.0. Peptidomic data analysis

PEAKS workflow DB was used for analyzing the identified peptides from β -Lg hydrolysis by papain. One hundred and thirty (130) peptides from 19 proteins were recognized in the papain whey protein hydrolysate samples based on the molecular mass derived from the LC-MS/MS spectra. It should be mentioned that the hydrolyzed whey protein isolate

contained some other proteins such as different kinds of caseins. Since β -Lg has the highest level of antigenicity of the whey proteins, it was selected for further analysis.

Table 3.0. Three important antigenic whey proteins identified by peptidomics in the whey protein hydrolysate produced with papain

Protein	-log P	Coverage	Total no. of peptide identified	Unique peptide
β-Lactoglobulin	293.09	52%	49	46
α-Lactalbumin	184.54	37%	13	12
Bovine serum albumin	158.83	7%	7	1

The peptidomics data are presented in Table 3.0. A total of 49 peptides were identified in the hydrolyzed β -Lg of which 46 peptides were unique peptides. β -Lg has the highest -log P value and highest coverage of 52% among other identified whey proteins. In addition, α -lactalbumin and bovine serum albumin have 37% and 7% coverage of protein in the sample, respectively.

3.1. Site cleavage prediction of papain: comparison of *in silico* and peptidomics data

As shown in Table 2.1, papain has a cleavage specificity for amino acids with a large hydrophobic side chain at the P2 position (Placzek *et al.* 2017). Apart from the influence of papain on the primary structure of β -Lg, several factors such as pH can cause several protein conformational changes. For instance, β -Lg is highly stable at acidic pH and, at pH 3.0, small alterations can change the protein structure. Between pH 4.5-6.0, some transitional changes might occur that can influence the compactness of the protein (Timasheff *et al.* 1966). At pH 7.0, some conformational changes in EF loop of protein structure in β -Lg may appear because of some cleavage of hydrogen bond in the G and F strands (Sakurai & Goto 2006). It can be interpreted that papain with optimum enzymatic activity at pH 7.0 which was maintained during hydrolysis for 5 hours during hydrolysis, might have caused conformational changes in the β -Lg structure.

Based on the comparison between the peptidomics and *in silico* results in Table 3.1.1, three peptide sequence were recognized in both actual and *in silico* hydrolysates as follows: Sequence 53-60 (APLR.VYVE), 71-78 (EILL.QKVE) and 46-53 (SLLD.AQSA). Based on the *de novo* peptides fully matched and protein alignment, the following sequences have been identified to reflect the cleavage sites for papain: (21-29), (41-77), (99-121), (139-164). As shown in Table 3.1, there are some predicted cleavage sites with a probability of 99% in the *in silico* results based on the presumption of SitePrediction, which is matched with the actual cleavage site in the β -Lg. Total of 11 site prediction were obtained from virtual treatment of β -lactoglobulin with papain *in silico*. The match cleaved sites of enzymatic activity recognized by both *in silico* and the actual results are sequences: 53-60 (APLR.VYVE), 71-78 (EILL.QKVE) and 46-53 (SLLD.AQSA) which were predicted to occur with 99% probability *in silico*. The rest of the cleavage predicted *in silico* have 95% probability of occurrence and some of them partially matched

the actual results. These findings demonstrate that papain cleavage of β -Lg predicted by SitePrediction with 99% probability was accurate in terms of matching with the papain cleavage patterns that occurred in the actual peptidomics results.

However, in the actual LC-MS/MS analysis, 52 peptides were identified (Table 3.1.2). These peptides mostly showed a high proportion of tyrosine and leucine residues, which are polar and hydrophobic amino acids, respectively, at the point of cleavage in the sequences. Leucine and proline are hydrophobic amino acids present in the carboxyl-terminal of most of the released peptides. Papain has a broad specificity for amino acids with a large hydrophobic side chain at the P2 position is in the carboxyl terminal (Placzek *et al.* 2017), which shows the successful cleavage at these amino acid residues of the protein.

Table. 3.1.1 Site cleavage prediction of enzyme papain, result comparison *in silico* and peptidomics¹

Rank	Position	Site	ASimilarity ^b maxscore	Specificity ^c	Actual Results	<i>In silico</i> Results
1	53-60	APLR.VYVE	100	>99%	YES	YES
2	71-78	EILL.QKVE	86.667	>99%	YES	YES
3	46-53	SLLD.AQSA	79.412	>99%	YES	YES
4	131-138	QSLA.CQCL	86.667	>95%	NO, 140-150	YES
5	170-177	TQLE.EQCH	89.130	>95%	NO, 164-171	YES
6	24-31	KGLD.IQKV	81.579	>95%	NO, 22-29	YES
7	3-10	CLLL.ALAL	84.615	>95%	NO	YES
8	17-24	LIVT.QTMK	83.784	>95%	NO,22-29	YES
9	107-114	KVLV.LDTD	79.487	>95%	NO, 99-107	YES
10	79-86	NGEC.AQKK	80.488	>95%	NO	YES
11	10-17	LTCG.AQLA	82.500	>95%	NO	YES

^{1.a}, The provided data were obtained from SitePrediction software online; ^{1.b}, The similarity maxscore contributes to the calculated score by the software *in silico* which represents the presumption of the software for the enzyme cleavage occurrence; ^{1.c}, It reflects the predicted probability of specificity of the enzyme for cleavage of the β -Lg.

Table. 3.1.2. Peptides characterized from LC-MS/MS analysis of papain hydrolysate

Peak	Sequence	Protein fragment
1	R.TPEVDDEALEKFDKAL	141-156
2	R.TPEVDDEALEKFDKALK	141-157
3	R.TPEVDDEALEKFD	141-153
4	P.EVDDEALEKFDKAL	143-156
5	R.TPEVDDEALEKF	141-152
6	R.TPEVDDEALEK	141-151
7	F.KIDALNENKVL	99-109
8	V.RTPEVDDEALEKFD	140-153
9	P.EVDDEALEKFD	143-153
10	F.KIDALNENKVLVL	99-111
11	F.KIDALN(+98)ENKVL	99-109
12	L.DTDYKKYLLF	112-121
13	D.DEALEKFDKAL	146-156
13	R.TPEVDDEALE	141-150
15	R.TPEVDDEAL	141-149
16	Y.VEELKPTPEGD	59-69
17	K.IDALNENKVLVL	100-111
18	L.VLDTDYKKY	110-118
19	L.DTDYKKYLL	112-120
20	L.KPTPEGDLEIL	63-73
21	P.EVDDEALEKFDKALK	143-157
22	L.DTDYKKYL	112-119
23	M.AASDISLLDAQSAPLRVY	41-58
24	Y.VEELKPTPEGDLEIL	59-73
25	Y.VEELKPTPE	59-67
26	V.EELKPTPEGDLEIL	60-73
27	D.DEALEKFD	146-153
28	Y.VEELKPTPEGDLE	59-71
29	L.LDAQSAPLR	48-56
30	V.RTPEVDDEALE	140-150
31	D,DEALEKFDKALK	146-157
32	Q.TMKGLDIQ	22-29
33	E.VDDEALEKFD	144-153
34	K.IDALNENKVL	100-109
35	K.ALPMHIR	158-164
36	L.VLDTDYKKYL	110-119
37	E.ELKPTPEGDLEILLQ	61-75
38	E.ELKPTPEGDLEIL	61-73
39	K.IDALN(+98)ENKVL	100-109
40	P.TPEGDLEIL	65-73
41	L.VLDTDYK	110-116
42	Y.VEELKPTPEGDLEILLQ	59-75
43	VLDTDYKKYLLF	110-121
44	L.RTPEVDDEAL	140-149
45	V.NENKVLVL	104-111
6	L.KPTPEGDLEILL	63-74
47	L.LVLDTDYKKYLL	110-120
48	P.EVDDEALEK	143-151
49	L.DTDYKKY	112-118
50	Y.VEELKPTPEGDLEILLQ(+98)KW	59-77
51	E.ELKPTPEGDLEILLQ(+98)	61-75
52	E.ELKPTPEGDLEILL	61-74

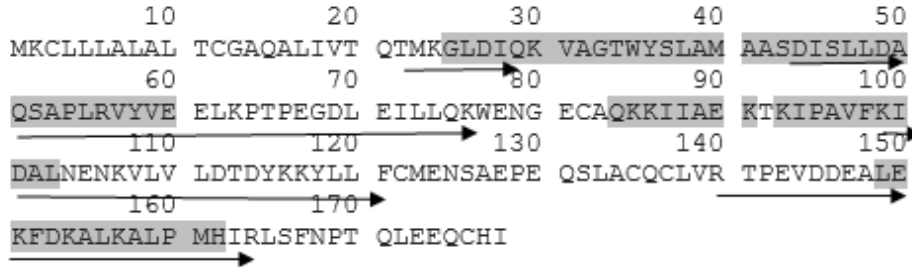
3.2. Whey β -Lg epitope prediction and cleavage

Many antigenic epitopes have been identified in β -Lg. In addition to the known epitopes, an antigenic epitope prediction tool (SVMTrip) was used to predict potential epitopes in the β -Lg. The obtained results are presented in the Table 3.2.1 and show five antigenic epitopes with various probability scores. The first predicted epitope has a high score of occurrence 1.00 among all other predicted epitopes and is predicted to be located in the sequence of 158-177 (LPMHIRLSFNPTQLEEQCHI). The second recognized epitope has the 0.998 score in the sequence 11-30 (TCGAQALIVTQTMKGLDIQK). The results of actual papain cleavage and the predicted epitopes are reported in Fig. 2.3. The percent of deactivated antigenic epitopes *in silico* and in the actual hydrolysis is also presented in Table. 3.2.2. Based on the calculated data in Table 3.2.3, the *in silico* results show degradation of 60% of antigenic amino acids in epitope 1 located in the sequence (158-177). The actual result from LC-MS/MS analysis for % degradation of the same epitope 1 was 75%, which is a considerable cleavage of the antigenic epitope. The second predicted epitopes located in sequence (11-30) presented 30% peptide bond cleavage in epitope 2. However, this predicted epitope was 100% degraded by the enzymatic activity of papain, which can lead to inhibition of β -Lg immunoreactivity. The results for epitopes 3, 4 and 5 in the Table 3.2.2 show 75%, 90% and 35% of degradation in the actual results, respectively. These are higher values in comparison to the predicted *in silico* results. The difference between predicted *in silico* results and actual results for degradation of antigenic epitopes can be due to unspecific cleavages occurring during papain hydrolysis and preparation of the hydrolysates.

The identified peptide from LC-MS/MS analysis also were used to examine percentage degradation of antigenic epitopes reported in the previous literature. The sequences of β -Lg (25-40), (41-60), (92-100), (149-162), (84-91) and (95-113) previously identified as antigenic epitopes are listed in Table 3.2.1 (Ball *et al.* 1994; Selo *et al.* 1999). The comparison results of amino acids characterized from peptidomics with known reported epitopes in the literature showed the various percent of degradation. Selo *et al.* (1999) described the sequence (25-40) in β -Lg as an antigenic epitope that had 72% of positive allergenic reaction toward patients. The peptidomics results in Fig. 3.2.1 show that these epitopes have been partially degraded by papain hydrolysis. The number of peptide bonds degraded in the epitope reflects the 53% breakage within these antigenic epitopes in the actual resultant of peptidomics. The β -Lg (41-60) peptide sequence was previously reported to cause an allergic reaction in 100% of patients (Selo *et al.* 1999). Based on the peptidomics results, 65% of this epitope was degraded by papain. Another identified epitope located in β -Lg (92-100) peptide sequence was reported to cause an allergenic reaction in 52% of patients (Selo *et al.* 1999). The peptide characterized by the LC-MS/MS analysis demonstrate 77% of degradation for this epitope. Another antigenic epitope located in β -Lg (95-113) peptide sequence with a high level of antigenic reactivity towards IgE was found to have 100% of positive antigenic reaction in patients. The percent hydrolysis of the antigenic epitopes in β -Lg (92-100) represents 75% of antigenic degradation. Ball *et al.* (1994) identified some antigenic epitopes such as peptide sequence (84-91) with 44% of antigenic reaction in patients. The peptidomics matching results with this particular epitope represent a 100% degradation after hydrolysis with papain.

However, calculation of epitope degradation in a particular antigenic epitope does not demonstrate if antigenicity would be actually reduced. In other words, if the partial sequence of a particular epitope is located in the released peptides after hydrolysis, should it be considered degraded? Therefore, it is important to identify the shortest peptide sequence in the specific antigenic epitope that can trigger immunoreactivity towards allergic patients. In addition, the comparison of actual cleavage pattern and antigenic epitope matching shows a high level of epitope degradation for β -Lg hydrolyzed by papain. Some of the *in silico* predicted antigenic epitopes begin with hydrophobic amino acids such as Ala, Val or Pro. In addition, the epitopes reported in the literature also start with mostly hydrophobic amino acids such as Leu or Ile, which make them susceptible to cleavage during hydrolysis with papain. As provided in Figure 3.2.1, the epitopes (25-40) (41-60) (149-162) and (95-113) started with hydrophobic amino acids Gly, Ala, Leu, and Pro respectively. Moreover, conformational epitopes are likely degraded earlier than sequential epitopes. The epitopes that contain hydrophobic amino acids within the tertiary structure of the protein may be revealed after hydrolysis, thus interacting more with the IgE receptors (Ena *et al.* 1995). On the other hand, the choice of selected hydrolysate generated with papain was made based on the % inhibition of antigenicity obtained from ELISA in the previous chapter. Based on the previous findings, papain hydrolysate gave the highest IgE inhibition binding of 47% compared to 11 whey protein hydrolysates obtained using other proteases.

Figure 3.2.1. The previously identified epitopes in the β -lactoglobulin and % degradation in the actual results



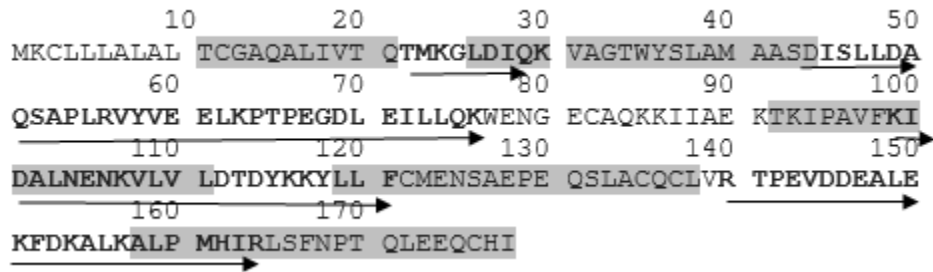
1a. The highlighted sequences are known epitopes identified in the literature: (25-40) 53% degraded, (41-60) 65% degraded, (92-100) 77% degraded, (149-162) preserved 100%, (84-91) 100% degraded and (95-113) 55% degraded, b. The arrows show the identified peptides throughout the hydrolysate which convey the actual cleavage (Ball *et al.* 1994; Selo *et al.* 1999).

Table 3.2.1: The Epitope prediction¹ *in silico*

Rank	Location	Epitope	Score
1	158-177	LPMHIRLSFNPTQLEEQCHI	1.000
2	11-30	TCGAQALIVTQTMKGLDIQK	0.998
3	36-55	YSLAMAASDISLLDAQSAPL	0.991
4	119-138	LFCMENSAEPEQSLACQCLV	0.914
5	92-111	TKIPAVFKIDALNENKVLVL	0.425

¹The *in silico* results was obtained from <http://sysbio.unl.edu/SVMTriP/index.php> (Yao *et al.* 2012).

Figure 3.2.2 The predicted epitopes and cleavage occurrence in the actual results



a. The highlighted amino acid sequences show the potential predicted epitopes; b. The arrows show the identified peptides recognized in the sample.

Table 3.2.3: Comparison between *in silico* and the *in vitro* results of degradation in antigenic epitopes

Epitopes	Degradation of epitopes in <i>silico</i> % ¹	Degradation of epitopes actual results % ²
E1 aa sequence (158-177)	LPMHIRLSFNPTQLEEQCHI ↓ 2/20=60%	75%
E2 aa sequence (11-30)	TCGAQUALIVTQTMKGLDIQK ↓ 6/20= 30%	100%
E3 aa sequence (36-55)	YSLAMAASDISLLDAQSAPL ↓ 1/20= 5%	75%
E4 aa sequence(119-138)	LCMENSAEPEQSLACQCLV ↓ 8/20= 40%	90%
E5 aa sequence(92-111)	TKIPAVFKIDALNENKVLVL 0/20= 0%	35%

Note: ¹ The highlighted parts of the sequences obtained from predicted cleavage pattern *in silico* for the predicted epitopes, ² The epitope degradation % was calculated based on the numbers of amino acids degraded from recognized epitopes divided by the total numbers of amino acids in the epitopes multiply by hundred.

4. Conclusion:

This study evaluated the matching of the predicted cleavage pattern *in silico* and the actual cleavage during hydrolysis of bovine β -Lg with papain with emphasis on the cleavage of the antigenic epitopes. The results show that the first three predicted breakage *in silico* completely match the cleavage that occurred in the actual results. The other *in silico* cleavage sites predictions partially matched the actual results. A total of 52 peptides were identified to originate from β -Lg after papain hydrolysis. The comparison of LC-MS/MS and *in silico* results indicated substantial deactivation of predicted antigenic epitopes. Furthermore, established antigenic epitopes of the whey protein were also effectively degraded by papain treatment. Some of the *in silico* predicted antigenic epitopes begin with hydrophobic amino acids, which may have made them more susceptible to cleavage by papain due to its specificity for cleavage at hydrophobic amino acid residues. However, based on the presented results, some parts of antigenic epitopes are still present in the peptides identified in the papain-hydrolyzed β -Lg. Therefore, immunoreactivity in the resulting product may be lowered but it still may cause allergic reactions. With such epitope deactivation, the whey protein hydrolysate can be considered for application as partially hydrolyzed hypoallergenic or tolerogenic products.

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Chapter 5

Discussion and Conclusions

Whey protein is a dietary source of amino acids and bioactive peptides but is also responsible for 50% of allergic incidence in patients with bovine milk allergy. Enzymatic hydrolysis can be used to lower food allergenicity. Several studies have reported the application of enzymatic hydrolysis for suppression of immune reactivity of proteins. The present research was focused on the influence of 12 different enzymes on suppressing whey protein antigenicity and the physicochemical properties of the hydrolysates. The specificity of one selected hydrolysate was determined by peptidomics. The enzymes from main three enzyme groups were used to examine the enzymatic impact on the immunochemical and physicochemical properties of hydrolysate. ELISA was used to determine the IgE binding inhibition in the hydrolysates. The results of ELISA demonstrated that papain and pancreatin had the highest inhibition of 47% and 45%, respectively when compared to the other hydrolysates. In agreement with our study, Izquierdo *et al.* 2008, reported that papain was able to degrade β -Lg in whey protein with or without microwave irradiation treatment. In another study, papain was used as a secondary enzyme in combination with Alcalase in the production of tolerogenic hydrolysates with improved palatability (Wróblewska *et al.* 2004). In addition, the degree of hydrolysis and immunoreactivity were weakly correlated with each other, as previously reported (Svenning *et al.* 2000). In other words, high degree of hydrolysis does not guarantee the reduction in antigenicity, although it depends on the specificity of the enzyme in the degradation of specific epitopes. In contrast, the degree of hydrolysis and surface hydrophobicity were negatively correlated, which indicates degradation of the hydrophobic pockets of the protein during the hydrolysis.

The peptidomics result for selected papain hydrolysate illustrated that papain with 47% IgE binding inhibition was able to degrade β -lactoglobulin antigenic epitopes. The predicted enzymatic cleavage site and the peptidomics result were matched in the *in silico* predicted results. The results represent the effective degradation of the β -lactoglobulin's epitopes in the predicted antigenic epitopes *in silico* and known previously identified epitopes in the literature. The comparison of LC-MS/MS analysis and *in silico* results indicated significant degradation of antigenic epitopes. In addition, papain degraded some other previously known antigenic epitopes. The study on epitope degradation reveals the effectiveness of papain on the degradation of β -lactoglobulin at hydrophobic residues considering the number of hydrophobic amino acid residues present in the antigenic epitopes. The findings from this study has illustrated the enzymatic specificities on deactivation of antigenic epitopes and influence of enzymatic hydrolysis on the physiochemical properties of the hydrolysate. Papain-derived whey protein hydrolysate is considered to be a product with improved sensory attributes (Wróblewska *et al.* 2004). Therefore, this research can encourage the industry to consider the production of tolerogenic whey protein hydrolysate using papain at industrial scale. In addition, the manufacturing cost on a commercial basis for this particular enzyme is considerably moderate in comparison to the cost of some of the other enzymes used in this research.

The future direction of the study will focus on the influence of enzymatic hydrolysis for selected enzymes on the functionality of the hydrolysate and application of the novel techniques such as encapsulation for palatability improvement. Furthermore, *in vivo* study is needed to substantiate the *in vitro* findings and explore the practical application of the hypoallergenic whey protein hydrolysate.

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APPENDIX

A. 1 Statistical Analysis of Experimental Data

Degree of hydrolysis

Synopsis: The optical density of the hydrolysate was obtained and free amino nitrogen and degree of hydrolysis was calculated accordingly. The degree of hydrolysate was used in statistical analysis.

Statistical design: CRD; triplicate analysis;

Factors: Whey protein isolates hydrolyzed by one of 12 different enzymes (Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase)

Statistical printout from One-way ANOVA: Degree of hydrolysis

* NOTE * Cannot draw the interval plot for the Tukey procedure. Interval plots for comparisons are illegible with more than 45 intervals.

Method

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C2	12	Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C2	11	958.81	87.165	66.78	0.000
Error	24	31.32	1.305		

Total 35 990.13

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.14244	96.84%	95.39%	92.88%

Means

C2	N	Mean	StDev	95% CI
Alcalase	3	17.386	0.256	(16.024, 18.747)
Bromelain	3	9.597	0.503	(8.236, 10.959)
Esperase	3	17.079	1.226	(15.718, 18.441)
Everlase	3	14.934	0.764	(13.573, 16.296)
Ficin	3	12.117	0.767	(10.756, 13.479)
Flavourzyme	3	13.24	2.18	(11.88, 14.60)
Neutrase	3	9.7624	0.1607	(8.4011, 11.1237)
Pancreatin	3	27.157	1.595	(25.796, 28.518)
Pancreatin	3	13.857	1.668	(12.496, 15.218)
Pepsin	3	7.231	0.512	(5.870, 8.592)
Protamex	3	10.967	0.399	(9.606, 12.328)
Savinase	3	19.487	1.468	(18.126, 20.848)

Pooled StDev = 1.14244

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C2	N	Mean	Grouping
Pancreatin	3	27.157	A
Savinase	3	19.487	B
Alcalase	3	17.386	B C
Esperase	3	17.079	B C D
Everlase	3	14.934	C D E
Papain	3	13.857	D E F
Flavourzyme	3	13.24	E F
Ficin	3	12.117	E F G
Protamex	3	10.967	F G
Neutrase	3	9.7624	G H

A. 2 Surface hydrophobicity

Synopsis: The surface hydrophobicity (S_0) of the whey hydrolysates was obtained by fluorescence spectroscopy and the slope of the plot of optical density against concentration is surface hydrophobicity.

Statistical design: CRD; triplicate analysis;

Factors: Whey protein isolates hydrolyzed by one of 12 different enzymes (Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase)

Statistical printout from One-way ANOVA: Surface hydrophobicity

* NOTE * Cannot draw the interval plot for the Tukey procedure. Interval plots for comparisons are illegible with more than 45 intervals.

Method

Null hypothesis All means are equal
 Alternative hypothesis At least one mean is different
 Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor Levels Values
 C3 12 Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C3	12	11766150	980512	83.01	0.000
Error	23	271668	11812		
Total	35	12037817			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
108.681	97.74%	96.57%	*

Means

C3	N	Mean	StDev	95% CI
Alcalase	3	122.6	37.7	(-7.2, 252.4)
Bromelain	3	791.5	149.4	(661.7, 921.3)
Esperase	3	102.0	23.8	(-27.8, 231.8)
Everlase	3	129.59	2.50	(-0.21, 259.39)
Ficin	3	757.8	88.2	(628.0, 887.6)
Flavourzyme3	3	1172.2	79.3	(1042.4, 1302.0)
Neutrase	3	1062.8	71.6	(933.0, 1192.6)
Pancreatin	3	224.7	38.4	(94.9, 354.5)
Papain	3	360.0	55.7	(201.0, 519.0)
Pepsin	3	1099.7	38.8	(969.9, 1229.5)
Protamex	3	1985	295	(1855, 2115)
Savinase	3	73.7	31.7	(-56.1, 203.5)

Pooled StDev = 108.681

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C3	N	Mean	Grouping
Protamex	3	1985	A
Flavourzyme	3	1172.2	B
Pepsin	3	1099.7	B C
Neutrase	3	1062.8	B C D
Bromelain	3	791.5	C D
Ficin	3	757.8	D
Papain	3	360.0	E
Pancreatin	3	224.7	E
Everlase	3	129.59	E
Alcalase	3	122.6	E
Esperase	3	102.0	E
Savinase	3	73.7	E

Means that do not share a letter are significantly different.

A.3 Particle size analysis

Synopsis: Particle size diameter and polydispersity index was measured with the light scattering/particle electrophoresis instrument

Statistical design: CRD; triplicate analysis;

Factors: Whey protein isolates hydrolyzed by one of 12 different enzymes (Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase)

Statistical printout from One-way ANOVA: Particle size

Null hypothesis	All means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C2	12	Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase

Note: No significant difference was observed; Hence, the results were not included.

A. 4 Polydispersity index

Synopsis: Polydispersity index was measured with the light scattering/particle electrophoresis instrument

Statistical design: CRD; triplicate analysis;

Factors: Whey protein isolates hydrolyzed by one of 12 different enzymes (Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase)

Statistical printout from One-way ANOVA: Polydispersity index

* NOTE * Cannot draw the interval plot for the Tukey procedure. Interval plots for comparisons are illegible with more than 45 intervals.

Method

Null hypothesis	All means are equal
Alternative hypothesis	At least one mean is different
Significance level	$\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C2	12	Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C2	11	109.80	9.981	2.85	0.017
Error	23	80.64	3.506		
Total	34	190.44			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
1.87251	57.65%	37.40%	0.00%

Means

C2	N	Mean	StDev	95% CI
Alcalase	3	2.387	0.295	(0.150, 4.623)
Bromelain	3	1.641	0.461	(-0.596, 3.877)
Esperase	3	3.406	0.788	(1.170, 5.643)
Everlase	3	5.12	3.25	(2.88, 7.36)
Ficin	3	4.77	2.20	(2.54, 7.01)
Flavourzyme	3	0.5373	0.1519	(-1.6991, 2.7737)
Neutrase	3	0.3773	0.0408	(-1.8591, 2.6137)
Pancreatin	3	3.08	2.61	(0.34, 5.82)
Papain	3	6.53	2.37	(4.29, 8.76)
Pepsin	3	3.849	1.632	(1.612, 6.085)
Protamex	3	4.09	3.37	(1.85, 6.32)
Savinase	3	3.368	0.976	(1.131, 5.604)

Pooled StDev = 1.87251

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C2	N	Mean	Grouping
Papain	3	6.53	A
Everlase	3	5.12	A B
Ficin	3	4.77	A B
Protamex	3	4.09	A B
Pepsin	3	3.849	A B
Esperase	3	3.406	A B
Savinase	3	3.368	A B
Pancreatin	3	3.08	A B
Alcalase	3	2.387	A B
Bromelain	3	1.641	A B
Flavourzyme	3	0.5373	B
Neutrase	3	0.3773	B

Means that do not share a letter are significantly different.

A. 5 Enzyme-linked immuno-sorbent assay (ELISA):

Synopsis: The ELISA plate result read by a microplate reader at absorbance of 495 nm.

The experiment was carried out in duplicates.

Statistical design: CRD

Factors: Whey protein isolates hydrolyzed by one of 12 different enzymes (Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase)

Statistical printout from One-way ANOVA: Optical density obtained from ELISA microplate

* NOTE * Cannot draw the interval plot for the Tukey procedure. Interval plots for comparisons are illegible with more than 45 intervals.

Method

Null hypothesis All means are equal
Alternative hypothesis At least one mean is different
Significance level $\alpha = 0.05$

Equal variances were assumed for the analysis.

Factor Information

Factor	Levels	Values
C1	12	Alcalase, Bromelain, Esperase, Everlase, Ficin, Flavourzyme, Neutrase, Pancreatin, Papain, Pepsin, Protamex, Savinase

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-Value	P-Value
C1	11	0.15010	0.013645	7.32	0.001
Error	12	0.02237	0.001864		
Total	23	0.17247			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0431794	87.03%	75.14%	48.11%

Means

C1	N	Mean	StDev	95% CI
Alcalase	2	0.5105	0.0516	(0.4440, 0.5770)
Bromelain	2	0.46100	0.00707	(0.39448, 0.52752)
Esperase	2	0.5330	0.0467	(0.4665, 0.5995)
Everlase	2	0.4550	0.0212	(0.3885, 0.5215)
Ficin	2	0.4850	0.0382	(0.4185, 0.5515)
Flavourzyme	2	0.35567	0.00471	(0.28914, 0.42219)
Neutrase	2	0.3308	0.0417	(0.2643, 0.3974)
Pancreatin	2	0.5640	0.0354	(0.4975, 0.6305)
Papain	2	0.5905	0.0148	(0.5240, 0.6570)
Pepsin	2	0.3912	0.0605	(0.3247, 0.4578)
Protamex	2	0.4800	0.0212	(0.4135, 0.5465)
Savinase	2	0.3825	0.0907	(0.3160, 0.4490)

Pooled StDev = 0.0431794

Tukey Pairwise Comparisons

Grouping Information Using the Tukey Method and 95% Confidence

C1	N	Mean	Grouping
Papain	2	0.5905	A
Pancreatin	2	0.5640	A
Esperase	2	0.5330	A B
Alcalase	2	0.5105	A B C
Ficin	2	0.4850	A B C D
Protamex	2	0.4800	A B C D
Bromelain	2	0.46100	A B C D
Everlase	2	0.4550	A B C D
Pepsin	2	0.3912	B C D
Savinase	2	0.3825	B C D
Flavourzyme	2	0.35567	C D
Neutrase	2	0.3308	D

Means that do not share a letter are significantly different.

A. 6 Correlation of various physiochemical characteristics analyzed by calculating the Spearman rho using Minitab 17.

A 6.1 Correlation between surface hydrophobicity and degree of hydrolysis

Spearman Rho: C3 - surface hydrophobicity, C4 - degree of hydrolysis

Spearman rho for C3 and C4 = -0.736
P-Value = 0.000

A. 6.2 Correlation between ELISA results and surface hydrophobicity

Spearman Rho: C3 - ELISA, C4 - surface hydrophobicity

Spearman rho for C2 and C3 = 0.427 ,P-Value = 0.167