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Allergic Properties of Egg White Proteins in Fermentation

by

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Dedication

With all my love to my family.

Abstract

Eggs are an economical source of nutrient rich food containing essential nutrients but are also one of the eight most common foods causing allergy today. Eggs are present in a wide variety of foods including sauces, bread and pasta. A novel method for the production of hypoallergenic egg ingredients should be developed. Fermentation of egg white proteins in sourdough was studied and revealed that ovotransferrin was degraded. No significant change was noted in ELISA while immunoblot showed elimination of IgE binding to the single protein ovotransferrin.

Fermentation of egg white solutions and extracted ovomucoid was also studied and revealed that *A. oryzae* fermented samples showed a decrease in IgE binding with a minor shift in ovalbumin-related protein Y, characterized by LC ESI-MS and MS/MS. The use of fermentation as a pretreatment in ovomucoids' susceptibility to enzymatic hydrolysis showed that there was no significant difference before and after fermentation.

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My family and my friends have walked beside me this entire length giving me love and support from Winnipeg to Edmonton. I love you all.

Doraemon rocks!

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1.1 Introduction

Hen eggs are one of the most nutritious and versatile food commodities in the market. Canada produced over 641 thousand dozens of eggs in 2011 increasing from just over 600 thousand dozens of eggs in 2007 (Statistics Canada, 2012). They are an excellent source of essential amino acids, proteins, polyunsaturated fats, minerals, and vitamins A, B complexes, D, E, and K as well as containing a low amount of calories. Eggs also exhibit numerous biological activities including antibacterial, anti-inflammatory, antihypertensive and antioxidant capacities (Huang, Majumder, & Wu, 2010), but can also cause harmful allergic reactions in some humans.

The major components of egg white are moisture (88%), proteins (9.7-10.6%, w/w), carbohydrates (0.5-0.6%, w/w), and lipids (less than 0.01%, w/w) (Mine, 2007). Egg whites are extremely important to the food industry for their uses in products such as baked goods, meringues, ice cream and pasta because of their high nutritional value, low cost and excellent functional properties including gelling, emulsifying and foaming properties. There are over 40 different types of proteins found in the egg white including ovalbumin, ovotransferrin, ovomucoid, G2 globulin, G3 globulin, ovomucin, lysozyme, ovoinhibitor, ovoglycoprotein, ovoflavoprotein, ovomacroglobulin, cystatin and avidin (Hirose, Doi, Kitabatake, & Narita, 2006; Mine, 2007). Table 1-1 shows composition of egg white present at more than 0.05% of dry matter (w/w). Ovalbumin is the predominant protein found in egg white contributing to the gelling and foaming properties of egg white (Mine, 1995) but its biological functions in the egg are still unknown.

Ovotransferrin is an iron-binding protein much like lactoferrin with minor antibacterial effects in the absence of iron (Mine, 1995). It consists of 15 disulfide bridges and makes up approximately 12% of egg white protein (Loponen, Konig, Wu, & Ganzle, 2008). Ovomucoid is a highly glycosylated protein with inhibitory effects on trypsin (Mine, 1995). It consists of nine disulfide bonds and no free sulfhydrl groups making up approximately 11% of egg white protein. This protein is also considered to be the major egg white protein responsible for allergic reactions (Mine, 1995). Lysozyme is a low molecular weight egg white protein with antimicrobial activity and constitutes approximately 3.5% of egg white protein (Matsuda, Watanabe, & Sato, 1982). Ovomucin constitutes approximately 1.5-3.5% of egg white protein and is an extremely viscous glycoprotein consisting of two subunits (Itoh, Miyazaki, Sugawara, & Adachi, 1987).

Table 1-1. Protein composition of hen's egg white in percentage of dry matter. Adapted from Mine (2007) and Hirose et al. (2006).

Egg White Protein	**Percent of dry matter (% w/w)**
Ovalbumin	54
Ovotransferrin	12
Ovomucoid	11
G2 globulin	4
G3 globulin	4
Ovomucin	3.5
Lysozyme	3.4
Ovoinhibitor	1.5
Ovoglycoprotein	1
Ovoflavoprotein	0.8
Ovomacroglobulin	0.5
Cystatin	0.05
Avidin	0.05

1.2 Food Allergy

The human body can react to ingested food components in two different ways. The first reaction is known as food intolerance where there is no specific type of mechanism involved but is a reproducible adverse reaction to a food or food component (Vieluf, Besler, Paschke, Steinhart, & Vieluf, 2002). A common example of food intolerance is lactose intolerance where the immune system is no involved but the host experiences symptoms because of improper lactose digestion. The second reaction is known as food allergy which is mediated by the immune system. Food allergies are a specific hypersensitivity reaction in the immune system usually in response to a naturally occurring protein in certain foods once it is ingested (Bush & Hefle, 1996; Mekori, 1996). Allergic reactions caused by food are also known as type I food hypersensitivity reactions or immunoglobulin E (IgE) mediated reactions where IgE is involved in the response to a food allergen (Elgert, 2009).

Once a food allergen enters the human body the immune system will start to respond. The first line of defense or innate immunity is a general barrier consisting of anatomic (skin), physiologic (temperature and pH), phagocytic and inflammatory defences that are not specific to pathogens. The adaptive system consists of antibodies and T lymphocyte cells and becomes activated when a food allergen is present. When eggs are ingested in hypersensitive individuals the allergic proteins are essentially revealed in the gastrointestinal tract upon break down in the mucosal layers. The allergen enters through the mouth and first digested inside the stomach and then further digested and absorbed through the numerous villi in the small intestine. When the allergen crosses the epithelium

upon absorption it enters the blood stream where the hypersensitivity reaction commences. During the first exposure or sensitization phase, the epitope, which consists of a short amino acid sequence on the allergic protein, is recognized as harmful and foreign by the body leads to a coupling with the major histocompatibility complex presented on the antigen presenting cell. This allows for future identification of the same allergen. Once the antigen presenting cell is labeled with the allergen it is recognized by T lymphocytes which leads to production of various cytokines. These cytokines stimulate the production of allergen specific IgE by B lymphocytes. Some of the B lymphocytes will differentiate into memory cells which stay in the body for a long period of time as protection from future exposures. Further exposure to the same allergen causes the allergen specific IgE to crosslink forming a sensitized mast cell leading to degranulation. During degranulation various vasoactive amines are produced including histamine. These vasoactive amines can cause smooth muscle contractions, vasodilation and increased vascular permeability (Goldsby et al., 2003).

Food allergies cannot be cured but there are ways available for diagnosis. Common practices include the double-blind, placebo-controlled food challenge which can evaluate reaction symptoms through different food challenge procedures, and diagnostic tests such as the skin prick test, histamine release and radioallergosorbent test (Nørgaard & Bindslev‐Jensen, 1992).

1.2.1 Food Allergy Prevalence. There are eight common foods causing allergy including milk, eggs, peanuts, shellfish, tree nuts, wheat, soy and fish

(United States Food and Drug Administration). The prevalence of food allergies in the United States is around 6% in children and 3.7% in adults (Sampson, 2004) and children with atopic dermatitis are more likely to have food allergies (Eigenmann, Sicherer, Borkowski, Cohen, & Sampson, 1998). About one third of hypersensitive children with atopic dermatitis show skin symptoms (Burks, Mallory, Williams, & Shirrell, 1988), while 6-16% of hypersensitive children with asthma also show breathing difficulties (Bock, 1992; Novembre, Martino, & Vierucci, 1988).

In young children, usually under the age of three, approximately 1.3% of food allergies are caused by eggs, 2.5% by milk, 0.8% by peanuts, and less than 0.5% by each of tree nuts, soy and wheat (Sicherer & Leung, 2008). Many food allergies tend to relieve as the patient enters adulthood. Strobel (1997) indicates that there is a decrease in food allergy prevalence by approximately 8% from the age of one to the age of six. The prevalence of food allergies decreased to 2% shellfish allergy, 0.6% peanut allergy, 0.5% tree nut allergy and 0.4% fish allergy (Wood, 2003). In adults approximately 1.5-2% still remains reactive to food allergens most likely of IgE levels that were persistently higher (Niestijl Jansen et al., 1994; Young, Stoneham, Petruckevitch, Barton, & Rona, 1994). In a Swedish study adults who are diagnosed with asthma or seasonal rhinitis may have food allergy prevalence up to 24% (Eriksson, 1978). Recent studies indicated that the overall prevalence of food allergies is increasing (Grundy, Matthews, Bateman, Dean, & Arshad, 2002; Sicherer, Muñoz-Furlong, & Sampson, 2003; Sicherer, Muñoz-Furlong, & Sampson, 2004).

The decrease in allergy prevalence in adulthood may be due to the maturing of the gastrointestinal tract from infants to children and to adolescence which decreases the amount of proteins that can pass through the epithelial membrane into blood circulation (Hefle, 1996). There are many pathways for protein uptake in the intestines. Depending on the maturity level of the small intestine allergic proteins may or may not be absorbed most frequently. These factors include endocytosis and exocytosis through specific receptors across the tight junctions of epithelial cells, gastrointestinal secretions such as proteolytic enzymes, and peristaltic activity of the intestine (Metcalfe, Sampson, & Simon, 2009). The acidity in the human stomach along with decreased permeability in the small intestine can also limit the absorption of specific allergens resulting in a decreased prevalence (Wood, 2003; Yamada et al., 2000).

*1.2.2 Egg Allergy Prevalence***.** The prevalence of egg allergy in the population is a major challenge facing the food industry. It is estimated that Ig-E mediated hypersensitivity affects more than 25% of the population (Valenta et al., 2009). Approximately 7% of children up to three years of age are affected by egg allergy (Benhamou et al., 2010). Most children grow out of egg allergies around the age of seven but some still remain severely allergic in adulthood (Heine, Laske, & Hill, 2006). A recent study conducted by Savage, Matsui, Skripak, & Wood (2007) suggests that from infancy to the beginning of the teenage years the prevalence of egg allergy decreases from approximately 4% by the age of four, 12% by the age of six, 37% by the age of 10, and 68% by the age of 16.

Eggs are commonly introduced to the diet in early life during infancy. It is also during this time period where sensitization to eggs occurs. Many studies have been conducted to see the relationship between egg allergy and infantile eczema. The cure for food allergies is basically avoidance. This avoidance after birth has shown a reduction in prevalence of egg allergies and severity of childhood eczema (Arshad, Matthews, Gant, & Hide, 1992; Zeiger et al., 1992). The elimination of egg from the infant diet showed to have positive effect on the severity of eczema while the reintroduction of egg into the diet lead to a relapse of eczema (Atherton, Soothill, Sewell, Wells, & Chilvers, 1978; Lever, MacDonald, Waugh, & Aitchison, 1998; Talbot, 1918). Egg allergy is not only closely tied to severity of eczema but egg allergy and eczema together may also play a role in increased prevalence of asthma as well as other allergens present in the air (Tariq, Matthews, Hakim, & Arshad, 2000).

1.2.3 Food Allergy Symptoms. Once the allergen is absorbed into the blood stream and vasoactive amines are released physical symptoms will start to appear on the patient. For type I hypersensitivities, this process normally occurs within minutes to hours following the ingestion of the food allergen. In most circumstances, the longer it takes for the reaction to occur the less severe and less life threatening the symptoms are. More severe reactions are most often related to higher permeability in the mucosa layers (Joneja, 2007). Some exceptions are reactions that are severe but delayed food hypersensitivities are related to food allergens being trapped in a matrix of fat and other food components which slows the degradation and release allergen into the blood stream (Grimshaw et al.,

2003). Many different factors ranging from the concentration of IgE antibodies and released mediators to the target organs affected contribute to the clinical reactions noticed on patients (Caffarelli, Cavagni, Giordano, Stapane, & Rossi, 1995; Martorell, Bone, Garc á, Nevot, & Plaza, 2001; Sicherer & Leung, 2008). These clinical reactions occur most commonly in the respiratory system, gastrointestinal tract and skin. Approximately 80% of the clinical reactions related to food allergens are cutaneous (Perry et al., 2006). Clinical symptoms in hypersensitive patients may vary from rash, urticaria (hives), angioedema to eczema on the skin, swelling and itching of the throat which may lead to asthma, nasal congestion, sneezing and rhinitis, nausea and abdominal pain causing vomiting and diarrhea, enteropathy, and in worst cases anaphylactic shock (Aas, 1978; May & Block, 1978; May, 1979; Perry et al., 2006). Common symptoms that are often seen in infant hypersensitivites include eczema, nonspecific gastrointestinal discomforts and urticaria (Kuitunen, Rapola, Savilaht, & Visakorp, 1973; May, 1979).

*1.2.4 Egg Allergy Symptoms***.** Egg allergies are most commonly seen in children with atopic dermatitis (Benhamou et al., 2010) and increases the risk for developing respiratory allergic diseases after the age of four (Tariq et al., 2000). In a recent study with over 800 patients from the Johns Hopkins Pediatric Allergy Clinic symptoms occurring from egg allergy reactions were recorded noting that skin related discomforts were the most common including urticaria and non-eczema rash, followed by vomiting, diarrhea, abdominal pain and respiratory discomforts including wheezing, coughing, asthma, rhinitis and nasal congestion

(Savage et al., 2007). Many studies conducted in different places with both adults and children conclude similar symptoms to egg allergic reactions with some symptoms such as eczema being more common among children than adults (Boyano Martinez et al., 2001; Ford & Taylor, 1982; Martorell Aragones et al., 2001; Nørgaard & Bindslev‐Jensen, 1992).

1.3 Food Allergen Proteins

Proteins are present in all foods but it does not mean that all foods are allergenic. Food proteins and allergic food proteins are distinguished based on the fact that one protein can bring a response in the immune system while the other protein is broken down and absorbed into the bloodstream for systemic functions. Allergen proteins normally have a molecular weight between 10 and 70 kDa (Lehrer, Horner, Reese, & Taylor, 1996). Allergic food proteins have unique biochemical characteristics that allow them to survive during harsh conditions in food processing such as baking, stay intact during enzymatic digestion in the stomach, resist changes in pH during digestion as well as interact with the immune system to create an allergic response (Bannon, 2004).

All food all ergies are type 1 hypersensitivity reactions mediated by IgE. Under normal nonallergic circumstances IgE antibodies are produced in response to a pathogen, but in allergic individuals high levels of IgE are produced in response to an otherwise harmless food protein leading to clinical symptoms. There are three main steps to be met for an allergic reaction to be complete: first the patient's immune system must be capable of escalating an abnormal IgE

response to a nonpathogenic substance possibly through genetic predisposition; second the patient must be exposed to a certain allergen under a certain environmental circumstance; and third the patient must be exposed more than once for a clinical reaction to happen (Bannon, 2004).

A study by Aalberse (1997) classified food allergens into two separate categories: either complete or incomplete allergens. A complete food allergen is an allergic protein which is capable of sensitizing an individual as well as evoking an allergic reaction with clinical symptoms. An incomplete food allergen is an allergic protein which can only evoke clinical symptoms based on their homological similarity with another allergen.

Over 700 different allergen sequences have been identified from different food and non-food sources and even with this knowledge only a few characteristics have been confirmed about food allergens. These characteristics include the amount of the allergen present in the food, type of IgE binding epitopes present, resistance to processing and protein structure of the allergen (Bannon, 2004). The abundance of a certain food allergen in the food determines the probability or chance that this protein with interact with the immune system thus leading to a hypersensitivity reaction. Typically food allergen proteins account for >1% of the major protein constituents in the food (Metcalfe et al., 1996). In shrimp the major allergen Pen a 1 accounts for 20% of the shrimp tail protein (Daul, Slattery, Reese, & Lehrer, 1994), the major peanut allergens account for 2% and 6% of total peanut protein (Metcalfe et al., 1996), and in egg ovomucoid, ovotransferrin, ovalbumin and lysozyme account for 11%, 12%, 54%

and 3.4% of total egg white proteins (Mine, 2007). These proteins can all cause severe reactions such as anaphylaxis during the worst cases. The amount of food allergen protein is not a definite criterion in the causes of hypersensitivity reactions but it is certainly a predisposing factor and shows extreme potential when paired with other biochemical factors mentioned above.

1.3.1 IgE Binding Epitopes. There are at least 20 different amino acids that can make up a protein. An epitope is a sequence of approximately six to fifteen amino acids present in food allergen proteins (Bannon, 2004). Each allergic protein may contain more than one epitope capable of evoking an allergic response. These epitopes are needed as an indicator for IgE binding to effector cells during an immune response. There are two categories of epitopes present, conformational epitopes and linear epitopes. Conformational epitopes contain many peptide chains normally resulting in secondary or tertiary structure and are comprised of amino acids from different regions on the protein brought together by folding (Bannon, 2004; Järvinen et al., 2007). This structure is required for successful binding of IgE in some allergens, most commonly aeroallergens which cause respiratory discomfort. Linear epitopes are more commonly related to food allergies since it only requires a primary amino acid sequence. Järvinen et al. (2007) investigated antibody binding in both conformational and linear epitopes presend in ovomucoid and determined that patients with long lasting egg allergy produced increased IgE to linear epitopes.

1.3.2 Resistance to Digestion. Digestion of food starts in the mouth and works its way through the stomach and intestines before excretion out of the

body. When the food allergen protein encounters proteolytic enzymes in the stomach such as pepsin and trypsin, degradation starts to occur. It appears that when a food allergen is extremely resistant to enzymatic degradation the chances of it causing a hypersensitivity reaction increases. Many studies have been conducted to determine the stability between food allergen proteins and nonallergen food proteins during enzymatic breakdown to mimic changes occurring in the mammalian stomach (Astwood, Leach, & Fuchs, 1996; Astwood & Fuchs, 1996; Besler, Steinhart, & Paschke, 2001; Fu, Abbott, & Hatzos, 2002). These studies indicate that many of the food allergen proteins were indeed more stable compared to nonallergen food proteins at varying pH values with enzymatic activity. Many studies conducted have shown the importance of disulfide bonds in stabilizing a protein protecting it from digestion. When food allergen proteins are reduced the disulfide bonds are disrupted resulting with an enzyme sensitive protein extremely prone to pepsin degradation leading to a loss of allergenicity, determined with the skin prick test and animal tests on previously sensitized dogs (Buchanan et al., 1997; del Val et al., 1999).

1.3.3 Food Allergen Protein Structure. The crucial role of disulfide bonds in maintaining protein structure, accessibility to epitopes and resistance to digestion mentioned above suggest that the protein structure is a very important factor in food allergen proteins. These intramolecular as well as intermolecular disulfide bonds aid to form the compactness of proteins which may limit the accessibility of proteases unless the protein is denatured or reduced (Breiteneder & Mills, 2005). In the peanut allergen Ara h 1 the quaternary structure plays an

important role by protecting potential cleavage sites and in turn protecting the epitopes by limiting access until some form of disruption occurs (Maleki et al., 2000).

Disulfide bonds can stabilize protein contributing to its resistantace against heat, chemical and enzymatic treatments (Breiteneder & Mills, 2005). Many studies have been conducted on the presence of disulfide bonds and their effects on enzymatic and chemical treatments. Dom ńguez, Cuevas, Urena, Munoz, & Moneo (1990) showed that soybean storage protein 2S albumins consist of four disulfide bonds which contribute to excellent resistance against heat treatment, protease activity and chemical modification. Likewise, the allergic milk protein beta-lactoglobulin contains two disulfide bonds and when these disulfide bonds are disrupted the structure is also affected. This structural change in beta-lactoglobulin provides better accessibility to the epitopes which increase the sensitivity to pepsin digestion resulting in a decrease in allergenicity (Brownlow et al., 1997; Kaminogawa et al., 1989; Peitsch, 1996). Another study conducted by Sen et al. (2002) on Ara h 2, a peanut allergen with four disulfide bonds showed that when these bonds are disrupted by reduction methods the protein becomes susceptible to pepsin, chymotrypsin, and trypsin digestion resulting in reduced allergenicity.

1.4 Allergic Egg White Proteins.

Egg proteins found in the albumen are the main causative for egg induced allergies in children and adults. Four of the major allergens are located in the albumin with two in the egg yolk. The allergic albumin proteins are all

glycoproteins and their sequences have already been characterized. Ovomucoid (Gal d 1), ovalbumin (Gal d 2) and ovotransferrin (Gal d 3) are the main allergic proteins found in hen egg whites with ovomucoid being the most dominant (Cooke & Sampson, 1997; Hoffman, 1983; Kato, Eri, & Matsuda, 2001; Langeland, 1983) while lysozyme (Gal d 4) contribute to a lesser extent (Holen & Elsayed, 1990). Although ovalbumin is the most abundant protein by far in egg white the allergenicity is much lower because of its sensitivity to heat (Joo & Kato, 2006). In the egg yolk apovitelin I and IV have been identified as allergic proteins but also do not cause hypersensitivity reactions to the extent of ovomucoid (Walsh et al., 1988).

1.4.1 Ovomucoid (Gal d 1). Ovomucoid is a highly glycosylated protein containing approximately 20 – 25 % carbohydrate and has a molecular weight of 28kDa (Mine, Sasaki, & Zhang, 2003). The major carbohydrate components consist of galactose, mannose and N-acetylglucosamine (Yamashita, Kamerling, & Kobata, 1982; Yamashita, Kamerling, & Kobata, 1983). The isoelectric point of ovomucoid is at approximately 4.1 which is acidic, a general characteristic of food allergens (Lehrer et al., 1996). It is composed of 186 amino acids arranged into three sections or domains each containing approximately 60 amino acids (Lin & Feeney, 1972). The first two domains both contain two glycosylation sites while the third domain only consists of one site (Egge et al., 1983). The tertiary structures of these domains are cross linked by disulfide bonds maintaining the integrity of the entire protein contributing to the difficulty in accessing epitopes as well as the resistance against denaturing agents (Mine et al., 2003). Ovomucoid is

considered to be the dominant egg white allergen (Bernhisel-Broadbent, Dintzis, Dintzis, & Sampson, 1994).

The ovomucoid protein has apparent homology to pancreatic secretory trypsin inhibitor which also makes it a trypsin inhibitor protein. Some serine protease inhibitors are known to have effects in innate immune responses (Augustin, Siebert, & Bosch, 2009; Li, Wang, Wang, Zhao, & Wang, 2009; Somprasong, Rimphanitchayakit, & Tassanakajon, 2006). Ovomucoid can only successfully inhibit the trypsin enzyme, it has minimal or no effect on other proteolytic enzymes (Fraenkel-Conrat, Bean, & Lineweaver, 1949). Ovomucoid is a single-headed inhibitor meaning it can only combine with one molecule of a serine protease compared to turkey ovomucoid which can combine with two molecules (Laskowski Jr, 1986). The reactive site for trypsin inhibition is located in the second domain from Arg89 – Ala90 (Kato, Schrode, Kohr, & Laskowski Jr, 1987) and has the ability to form a stable complex with trypsin in an equimolar ratio thus inhibiting trypsin activity (Matsuda, Watanabe, & Nakamura, 1983).

Ovomucoid is extremely heat stable and can resist denaturation and proteolytic degradation (Kato et al., 2001). Gu et al. (1989) showed that an egg boiled for over an hour still contains soluble ovomucoid with antigenicity. Likewise Deutsch & Morton (1956) concluded that boiling purified ovomucoid at 100˚C also retains its ability to cause an allergic reaction in patients. Even the removal of the carbohydrate portions on ovomucoid leaving the protein with a molecular weight of approximately 21 kDa can still retain its entire ability to cause a hypersensitivity reaction (Besler, Steinhart, & Paschke, 1997) although

the deglycosylated form is extremely sensitive to heat treatment (Yamamoto et al., 1998).

1.4.2 Ovalbumin (Gal d 2). Ovalbumin is the major protein present in egg white accounting for approximately 54% of protein (Li-Chan & Nakai, 1989). It was previously thought to be the dominant allergen present in egg white until further studies showed ovomucoid elicited more immune responses (Bernhisel-Broadbent et al., 1994; Urisu et al., 1997). Ovalbumin is a water-soluble glycoprotein with one carbohydrate moiety attached to the Asn293 position and has a molecular weight of 45 kDa. This protein consists of 386 amino acids including six cysteine residues with one disulfide bond between Cys74 and Cys121 making this the only egg white protein with free sulfhydryl groups (McReynolds et al., 1978; Thompson & Fisher, 1978; Woo et al., 1981). Because of the many free sulfhydryl groups not coming together to form strong disulfide bonds, ovalbumin is extremely sensitive to heat denaturation resulting in the loss of allergenicity. Kim et al. (2002) have shown that with heat treatment of ovomucoid at 75˚C the allergenicity starts to decrease. Once the temperature reached 80˚C results showed that patient antibodies could not bind to epitopes anymore. Compared to heat treated ovalbumin, native ovalbumin is more susceptible to IgE antibody binding to create an immune response.

Aside from the allergenic properties of ovalbumin it is also a protein of the serpin family. The serpins consist of more than 300 homologous proteins with various functions in animals, insects, plants as well as viruses (Gettins, Olson, & Patston, 1996; Potempa, Korzus, & Travis, 1994). This family includes major

serine protease inhibitors found in human plasma but also includes proteins such as angiotensinogen and ovalbumin with no known inhibitory properties (Huntington & Stein, 2001). Ovalbumin contains the typical three turn α-helix of the serpin family and was the first model for studying structures of native serpins.

1.4.3 Ovotransferrin (Gal d 3). Ovotransferrin is a transferrin protein consisting of 12 disulfide bonds with a molecular weight of 77 kDa. It is also a glycosylated protein containing 2.6% of carbohydrate moieties. Transferrin proteins are widely distributed in biological life and have the ability to bind and transfer iron (Keung & Azari, 1982). Studies show that the two iron-binding sites in ovotransferrin are functionally identical to those found in human lactoferrin (Keung & Azari, 1982; Line, Sly, & Bezkorovainy, 1976; Williams, 1975).

Ovotransferrin accounts for approximately 12% of the total egg white protein and is made up of 686 amino acids (Williams, Elleman, Kingston, Wilkins, & Kuhn, 1982). The mRNA sequence of ovotransferrin has been determined and studies show that in their free form this protein exhibits antibacterial activity by depriving microorganisms from iron essential for growth (Jeltsch & Chambon, 1982; Valenti et al., 1980). Ibrahim, Sugimoto, & Aoki (2000) have shown that ovotransferrin contains an antimicrobial peptide with the ability to penetrate into the outer membrane of *Escherichia coli* by a self-promoted uptake mechanism. Most studies regarding ovotransferrin relate to its iron binding ability and not to its cause of allergenicity. Aabin et al. (1996) demonstrated that the amount of IgE that could bind to ovomucoid and

ovotransferrin was higher than the amount that could bind to ovalbumin and lysozyme.

1.4.4 Lysozyme (Gal d 4). Lysozyme is a widely known antimicrobial protein found in many places including tears, mucus, saliva and egg white. This protein makes up approximately 3.5% of egg white protein and has a molecular weight of 14.3 kDa. This small protein consisting of 129 amino acids has the ability to lyse gram positive bacteria such as *Micrococcus ssp*. (Salton, 1957). Lysozyme can cleave the β-1, 4 glycosidic linkage between N-acetylmuraminic acid and N-acetylglucosamine in the cell wall of these bacteria causing cell lysis (Mine, 2008).

Because lysozyme has antimicrobial effects it is used in many food products such as cheese and wine as an unlabeled bactericidal additive but the allergenic property of lysozyme poses a huge problem for the food industry as well as the uninformed population. Frémont, Kanny, Nicolas, & Moneret‐Vautrin (1997) conducted a study with 52 patients who are clinically allergic to egg. Results showed that 35% of those patients had the presence of the anti-lysozyme IgE in their blood. Lysozyme is also used in the pharmaceutical industry in applications to increase natural body defenses, contact lens decontamination and in infant formula. An incident involving a patient taking Lizipaina, a throat medication containing lysozyme, caused a series of allergic reactions for a persistent amount of time (Pérez-Calderón, Gonzalo-Garijo, Lamilla-Yerga, Mangas-Santos, & Moreno-Gastón, 2007). Skin prick test results showed positive with raw egg white and yolk but came negative with cooked egg

white and yolk. No specific IgE was found against ovomucoid, egg white or egg yolk but lysozyme specific IgE was detected. These examples show the importance of food labeling especially when there is chance of incorporating allergens into the product even though lysozyme is an affordable, easy to get antimicrobial source.

1.5 Methods to Reduce Allergenicity

Food processing techniques are used to prepare all products before they are put out on the shelf. There are many reasons for food processing including flavour and texture improvement, convenience for the population, shelf life enhancement and most importantly the inactivation of enzymes and harmful microorganisms (Sathe & Sharma, 2009). Processing foods can lead to three different outcomes for the allergic protein: no change in allergenicity, decrease in allergenicity or increase in allergenicity. Limited studies have been done on methods for the reduction of egg allergies and most of these studies focus on the major egg allergen ovomucoid but favourably it has been reported that food processing techniques are more likely to reduce allergenicity by disrupting or modifying epitopes (Sathe, Teuber, & Roux, 2005). Many food processing methods have been applied and evaluated for reducing allergenicity in egg products including thermal treatments and non-thermally treatment methods (Poms & Anklam, 2004). These are the two categories most processing treatments fall under. Thermal treatments include microwaving, moist and dry heating while

non-thermal processing include irradiation, genetic modification, high hydrostatic pressure, enzymatic hydrolysis, and refining and ultrafiltration.

1.5.1 Heat Treatment. Thermal processing operates on the basis of using high heat to denature proteins from its native structural arrangement to destroy the presence of epitopes. It is the most common type of processing technique used in the food industry. Common heat treatments applied in egg processing include boiling, microwaving, frying and baking. The major steps during heat denaturation include loss of tertiary structure, reversible unfolding, loss of secondary structures, disulfide bond disruption, formation and rearrangement of new bonds followed by protein aggregation (Wal, 2003).

In a previous study Kato, Watanabe, & Matsuda (1997) experimented by adding egg white into dough for bread making and evaluated antigenicity with enzyme linked immunosorbent assay (ELISA) and western blotting. Ovomucoid antigenicity in dough was measured at different stages of bread making including kneading and baking. After baking, results showed that antigenicity disappeared in the bread. The same team followed up by another study using pasta containing egg. Likewise after boiling pasta for 15 minutes in salt water, antigenicity of ovomucoid was almost all eliminated (Kato et al., 2001). These two studies however are egg proteins in a food system. Studies with isolated egg white proteins show that even with extensive heat treatments at boiling temperature the allergenicity of ovomucoid still remains intact (Deutsch & Morton, 1956; Gu et al., 1989). Ovalbumin which is the more heat sensitive protein can denature very easily but can also convert into S-ovalbumin which is more heat stable during

food storage creating a potential obstacle for this type of processing method (Gremmel, Paschke, Mills, Wichers, & Hoffmann-Sommergruber, 2007, Gremmel and Paschke, 2007).

1.5.2 Irradiation Treatment. Irradiation treatment with the usage of gamma rays is commonly used in the food industry as a method of preservation by enzyme inactivation and microbe elimination (Poms et al., 2004). Irradiation processing can change the structure of egg proteins causing aggregation, short chain formations or amino acid modifications. Irradiation can cause irreversible changes to conformational epitopes by destruction of covalent bonds linkages but linear epitopes may still remain intact even with high dose irradiation treatment (Kume & Matsuda, 1995; Yang, Kim, Matsuhashi, & Kume, 1996). These changes may affect protein solubility as well as allergenicity by epitope alteration (Kume & Matsuda, 1995; Lee et al., 2002). Protein solubility may decrease after irradiation treatment in some cases and leave linear epitopes buried within the structure causing increased allergenicity after human digestion releases the buried sequence (Lee et al., 2002; Poms & Anklam, 2004).

Lee et al. (2002) conducted a study using irradiation on egg proteins resulting in exposure of linear epitopes that were previously hidden in the protein structure which in turn increased allergenicity (Lee et al., 2002). Yang et al. (1996) have studied the effects of irradiation on ovomucoid and ovalbumin. Results showed that trypsin inhibitory effects of ovomucoid could be inactivated with certain treatments as well a decrease in allergenicity was observed. Similar effects were noted with irradiated ovalbumin.

1.5.3 Genetic Modification. Recombinant DNA technology is becoming an increasingly popular tool in research now and may be possible to apply to the reduction of egg allergy. Investigations of the allergic egg white proteins at a molecular levels allows to the specific determination of allergic epitopes. It is suggested that recombinant molecules can decrease IgE binding by destroying epitopes via disrupting the protein structure (Rupa, Mine, & Shahidi, 2006). The effects of genetic modification on ovomucoid IgE binding was studied by Rupa et al. (2006). An undecane peptide was genetically attached onto the N-terminal of ovomucoid in the third domain and allergenicity was evaluated and compared between native ovomucoid and wild-type ovomucoid. Phenylalanine at position 37 was replaced with methionine and decreased antigenicity drastically. Replacing glycine at position 32 along with the position 37 also decreased antigenicity. This still did not completely eliminate the allergy causing agent in ovomucoid and would be a costly process at large scale.

1.5.4 High Hydrostatic Pressure Processing. High hydrostatic pressure processing is a method where food is subjected to elevated pressure treatments ranging anywhere from 100 MPa to 900 MPa (San Martin, Barbosa-Canovas, & Swanson, 2002; Ludikhuyze, Van Loey, Indrawati, Smout, & Hendrickx, 2003). Minimal nutrient and sensory properties are lost during this type of processing (Ludikhuyze, Van Loey, Indrawati, Smout, & Hendrickx, 2003). High hydrostatic pressure processing alter proteins by non-covalent and disulfide bond modification not affected the primary protein structure (Cheftel, 1992; Schurer, Kieffer, Wieser, & Koehler, 2007).

A recent study conducted by López-Expósito et al. (2008) investigated the potential of high hydrostatic pressure treatment in conjunction with enzymatic hydrolysis on the antigenicity of ovalbumin. Pepsin treatment was used under pressure conditions of 400 MPa to evaluate ovalbumin. Results showed that pressure treated ovalbumin lowered antigenicity compared to atmospheric ovalbumin but only to a certain extent (López-Expósito et al., 2008). Another study looked at the effects of pressure treatments at 800 MPa on allergenicity and trypsin susceptibility. Iametti et al. (1999) combined additives with egg whites to prevent gel formation after hydrostatic pressure treatment and then evaluated changes in characteristics. Results showed that the egg whites increased in viscosity but still retained their functional properties while susceptibility to trypsin increased drastically. The more heat resistant form of ovalbumin S-ovalbumin was not detected in any of the pressure treated samples and the antigenicity was reduced by approximately 40% compared to the control sample (Iametti et al., 1999). On the contrary allergenicity may increase as a result of high hydrostatic pressure treatment shown in a study conducted by Kato, Katayama, Matsubara, Omi, & Matsuda (2000). Different pressure treatments and time periods were used to treat *Indica* rice and results showed that with increasing time and pressure an increased amount of protein was released from the rice. The proteins were then detected with immunoblots as the major rice allergens being released from the rice grains during treatment.

1.5.5 Enzymatic Hydrolysis. Enzymatic hydrolysis has the potential to destroy linear epitopes by removing certain amino acid residues compared to

other treatments such as irradiation or high hydrostatic pressure treatment where conformational epitopes are targeted. The potential disruption of linear epitopes may lead to a reduction in allergenicity. A disadvantage of using enzymatic hydrolysis to reduce allergenicity is that the protein is cut into many fragments during treatment and these peptides may affect smell, taste and functionality of the protein and final food product (Sathe et al., 2005) . A good example of this is with whey proteins from milk which are used to manufacture hypoallergenic infant formula. Through enzymatic hydrolysis of whey allergic proteins are eliminated but whey hydrolysates in infant formula produce a bitter taste (Ena, Beresteijn, Robben, & Schmidt, 1995).

The enzyme pepsin found in the mammalian stomach is a very commonly used enzyme in the food industry. Pepsin digestibility was first used to determine stability and nutritional value of proteins at extreme pH values in the stomach (Marquez & Lajolo, 1981; Zikakis, Rzucidlo, & Biasotto, 1977). Soon after, pepsin and other proteases were used to study proteins and protein fragments and their effects on IgE binding (Budd, Kuo, Cazin Jr, & Yoo, 1983; Lorusso, Moffat, & Ohman, 1986; Astwood & Fuchs, 1996). Many studies were conducted to determine IgE binding of different food allergens from peanuts, soybeans, eggs and milk before and after pepsin digestion with a general result of showing resistance to digestion compared to non-food allergen proteins (Astwood, 1996; Ogawa et al., 1991; Burks et al., 1994; Gonzalez, Menendez-Arias, Monsalve, & Rodriguez, 1991). Van der Plancken, Van Loey, & Hendrickx (2005) conducted a study on the effects of pre-treatments including temperature and pressure

treatments in combination with enzymatic hydrolysis on egg white proteins. Results showed that ovalbumin became more susceptible to enzymatic hydrolysis after heat treatment and the ability of ovomucoid to inhibit trypsin activity decreased after heat treatment.

1.5.6 Fermentation. Fermentation is a food processing method using bacterial starter cultures to produce foods with increased microbial safety, nutrition, organoleptic properties and health advantages (Leroy & De Vuyst, 2004). Starter cultures contain at least one strain of microorganism most commonly lactic acid bacteria added to the raw material to direct the fermentation process through the conversion of carbohydrates to organic acids such as lactic acid and acetic acid as well as ethanol, exopolysaccharides, enzymes and carbon dioxide. Lactic acid bacteria have been used to produce fermented foods and beverages for an extremely long time discovered accidentally by spontaneous fermentation of natural micro flora in the raw material (Caplice & Fitzgerald, 1999). Now on the market many foods are processed fermentation such as yogurt, soy sauce, salami, sausages, coffee and wine. Some yogurt brands are also producing probiotic yogurts with healthy gut bacteria that consumers now recognize and preferentially purchase.

Lactic acid bacteria fermentation of allergic proteins has been reported in literature but there is minimal research on the effect of lactic acid bacteria fermentation on allergic properties of egg white proteins. Many studies have been conducted related to whey protein allergy in milk investigating different ways of allergy reduction. Heat treatment to denature whey proteins, hydrolysis with

different enzymes found in the digestive system, use of oligosaccharide enriched whey food and effects of fermentation on whey proteins have been studied to produce a hypoallergenic milk product for infant formulae (Ena et al., 1995). Fermentation of the main milk allergen β-lactoglobulin in yogurt was noted to have reduced allergenicity compared to unfermented milk (Ehn, Ekstrand, Bengtsson, & Ahlstedt, 2004) and in a similar study with sweet whey and skim milk allergenicity was decreased substantially (Kleber, Weyrich, & Hinrichs, 2006). Current progress has demonstrated that fermentation of egg white by lactic acid bacteria could potentially lead to reduction of IgE binding capacity using patient plasmas. Fermentation may change the structure of proteins in many ways that are distinct from other methods of processing. In a study by Loponen et al. (2008) three different strains of lactic acid bacteria were used in the fermentation of sourdough bread to see effects on egg white protein. Only changes to ovotransferrin were noted by *Lactobacillus sanfranciscensis* which was seen to have the ability to degrade ovotransferrin completely.

The utilization of microorganisms, in particular lactic acid bacteria seems to be a promising direction to reduce food protein allergies in many foods. Studies on the fermentation of antigenic milk proteins with *L. acidophilus* and *S. salivarius* by Kleber et al. (2006) showed that allergenicity could be reduced by over 70% (Kleber et al., 2006). Similarly soybean proteins fermented with *L. plantarum* showed a 96% decrease in allergenicity (Frias, Song, Martínez-Villaluenga, De Mejia, & Vidal-Valverde, 2007). Fermentation by *L. brevis* MS-99 in wheat flour reduced gluten allergy by 80% (Leszczyńska et al., 2009;

Lechevalier, Jeantet, Arhaliass, Legrand, & Nau, 2007). Our previous study of egg white fermentation with *L. delbruekii* and *Aspergillus oryzae* demonstrated a reduction of allergenicity but not total elimination. Further studies are needed to look at the effect of food preparation on allergenicity of eggs in food matrixes.

Desugarization is a process dating back many years using microorganisms to remove glucose from egg to reduce browning, off flavours and odours as well as increasing the shelf life. Processors used to remove sugar from eggs by spontaneous microbial fermentation which in some cases leads to health hazards. Now a controlled bacterial fermentation is used with reduced time and costs with a better quality end product. A study conducted on the influences of industrial processing steps on egg white functionalities suggested that during desugarization the foaming properties improved (Lechevalier, Jeantet, Arhaliass, Legrand, & Nau, 2007). In industry now fermentation of egg white is used to remove glucose to prepare egg white powder to prevent biochemical reactions such as the maillard reaction.

1.6 Egg white proteins in finished foods.

Many foods on the market today contain egg ingredients including cake, cookies, bread, ice cream, pasta, sauces and beverages. Interactions of egg white proteins and other food components such as wheat proteins in bread and pasta products have been studied (Kato et al., 1997; Kato et al., 2001; Loponen et al., 2008; Schurer et al., 2007). The interactions between wheat proteins including gluten proteins and egg white proteins have proved to have disulfide exchange

reactions resulting in protein changes. Kato et al. (2001) used a pasta like product and studied allergenicity before and after boiling with immunoassays. Results showed that one variety of wheat Durham semolina flour finished with the most reduction in patient egg allergy. Previously Kato et al. (1997) also studied the effects of combining egg white into dough to make bread and evaluated allergenicity with immunoassays. Results showed that after baking allergenicty was eliminated in the bread. During the processing steps it was suggested that a disulfide exchange reaction occurred between ovomucoid and wheat proteins gliaden and glutenin followed by heat-induced polymerization of intermolecular disulfide bonds between ovomucoid and wheat proteins reduced allergenicity (Kato et al., 1997). After these studies another group incorporated fermentation with lactic acid bacteria in wheat systems to see the effects on allergy. Sourdough bread is a common fermented food product with improved sensory qualities and extended shelf life (Gänzle, Ehmann, & Hammes, 1998). Loponen et al. (2008) conducted a study using lactic acid bacteria to ferment an egg and wheat mixture to produce sourdough. After a two stage fermentation results showed that the egg white protein ovotransferrin was completely hydrolyzed. This degradation is associated with the thiol metabolism of the bacteria resulting in redox reactions with glutathione reductase resulting in changes to the disulfide linkages within the wheat and egg matrix. Schurer et al. (2007) confirmed that disulfide exchange reactions were necessary for the degradation of proteins in food matrices in a study where additives such as cysteine and N-ethylmaleimide were used to block thiol groups.

1.7 Objectives

The prevalence of egg allergy in the population is another major challenge facing the industry. Eggs are one of the most common foods that cause allergy in our population today. Approximately 7% of children up to three years of age are affected by egg allergy (Benhamou et al., 2010). As mentioned in section 1.4 ovotransferrin is one of the major allergic egg white proteins and based on previous research it was found that ovotransferrin could be degraded in a two step sourdough fermentation (Loponen et al., 2008). We hypothesize that in a one step fermentation ovotransferrin could be altered through thiol exchange reactions in the presence of wheat proteins leading to a change in IgE binding capacity. The objectives of this study are to develop a more industry oriented fermentation method to degrade ovotransferrin in wheat sourdough, evaluate allergenicity with patient plasmas of ovotransferrin depleted sourdough, and to investigate the effects of bread making on allergenicity.

Ovomucoid is the major egg allergen protein and is highly glycosylated. Unpublished data from previous research showed a potential decrease in allergenicity through ELISA with fermentation with *Aspergillus oryzae* in egg white. The second part of this study was to confirm the fermentation results and take a closer look at which proteins may contribute to the decrease with LC ESI-MS and MS/MS. Since ovomucoid is such a tenacious protein another portion of the study was to look at the effects of using fermentation as a pre-treatment. Different enzymes were used to observe differences in susceptibility with different fermentation times with different strains.

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Chapter 2: Effects of Lactic Acid Bacteria Fermentation on Egg White Allergenicity in Sourdough

2.1 Introduction

Eggs are an economical source of nutrient rich food available in all parts of the world containing vitamins, minerals, essential fatty acids and protein. They are important ingredients and additives in many processed foods such as pasta, bread, cakes, sauces and desserts because of their many functional properties such as foaming and gelling, but are limited to some of the population due to the prevalence of food allergies. Eggs are one of the eight most common allergy causing foods. It is estimated that immunoglobulin E (Ig-E) mediated hypersensitivity affects more than 25% of the population (Valenta et al., 2009). Approximately 7% of children up to three years of age are affected by egg allergy (Benhamou et al., 2010). It is suggested that from infancy to the beginning of the teenage years the prevalence of egg allergy decreases from approximately 4% by the age of four, 12% by the age of six, 37% by the age of 10, and 68% by the age of 16 (Savage et al., 2007). Adults who remain allergic generally have relatively severe symptoms.

Egg proteins found in the albumen are the main causative for egg induced allergy in children and adults. There are over 40 different proteins in egg albumen out of which four are allergy causing proteins. The major egg white proteins contributing to allergy include ovomucoid, ovotransferrin, ovalbumin, and lysozyme. Ovomucoid is a highly glycosylated protein with trypsin inhibitory activity and nine disulfide bridges (Mine, 1995), ovotransferrin is an iron-binding

protein consisting of 15 disulfide bridges (Loponen et al., 2008), ovalbumin is the predominant protein found in the egg white contributing to the gelling and foaming properties of egg white with one disulfide bridge (Loponen et al., 2008; Mine, 1995) and lysozyme is a low molecular weight egg white protein with antimicrobial activity consisting of four disulfide bridges and constitutes approximately 3.5% of egg white protein (Matsuda et al., 1982).The main causative protein for egg allergy is ovomucoid (Kato et al., 2001) and because of its nature and structure this protein is very resistant to processing parameters such as heating, microwaving, irradiation and filtration techniques which create a problem to the food industry and limit the variety of foods the allergic population can have.

Many studies have been conducted regarding the antigenic and allergenic properties of egg white proteins (Bock, Sampson, & Atkins, 1988; Bock & Atkins, 1990; Matsuda, Watanabe, & Sato, 1981; Sampson & McCaskill, 1985; Urisu et al., 1997) and show that ovomucoid is extremely resistant and may still remain soluble after processing thus retaining its allergenicity. Ovomucoid was also reported to remain soluble after boiling for one hour and still retaining IgE binding activity (Gu et al., 1989). In a recent study ovomucoid was found to be less allergic to patients with known egg allergies under heat processing with the presence of wheat flour (Kato et al., 2001). A pasta like product was evaluated before and after boiling by immunoassays and results showed that Durham semolina wheat flour proved most reduction in patient egg allergy. The same group also studied the effects of combining egg white into dough for bread

making and evaluated antigenicity with enzyme linked immunosorbent assay and immunoblotting. After baking, results showed that antigenicity disappeared in the bread (Kato et al., 2001). During the processing steps it was suggested that a disulfide exchange reaction occurred between ovomucoid and wheat proteins gliaden and glutenin followed by heat-induced polymerization of intermolecular disulfide bonds between ovomucoid and wheat proteins reduced allergenicity. Current progress in the field has demonstrated that fermentation of egg white by lactic acid bacteria could lead to reduction of IgE binding capacity using patient plasmas. Sourdough fermentation with lactic acid bacteria is a baking process to prolong shelf life and also to improve sensory properties of bread (Kato et al., 1997). Loponen et al. (2008) conducted a study using two strains of lactic acid bacteria in a two stage fermentation procedure. After a two stage fermentation results showed that the egg white protein ovotransferrin was completely degraded. This degradation is associated with the thiol metabolism of the bacteria resulting in redox reactions with glutathione reductase resulting in changes to the disulfide linkages within the wheat and egg matrix.

Fermentation, a natural processing mechanism accepted by consumers, may change the structure of proteins in many ways that are distinct from other methods of processing; in this more industry oriented study, allergic egg white proteins will be subjected to a short one stage sourdough fermentation with three strains of lactic acid bacteria and baking parameters to be finally evaluated by immunoassays for potential allergy reductions. The objective of this study is to

develop a hypoallergenic egg product in a safe and cost efficient manner so that the entire population can benefit from the nutritional qualities.

2.2 Materials and Methods

2.2.1 Materials. Ingredients used for sourdough fermentation and bread making (hen's eggs, wheat flour, yeast, sugar and salt) were obtained from the local supermarket.

2.2.2 Strains and Growth Conditions. The following lactic acid bacteria strains were used in this study: *Lactobacillus delbrueckii*, *Lactobacillus sanfranciscensis* DSM20451, and an isogenic mutant strain lacking the glutathione reductase gene *Lactobacillus sanfranciscensis* DSM20451 Δ*gshR* (Loponen et al., 2008). *Lactobacillus delbrueckii* was grown in de Man, Rogosa, Sharpe (MRS) medium at pH 6.2 and incubated overnight under modified atmospheric conditions (4% O_2, 20% CO_2, 76% N_2) at 37 ℃. For solid MRS media 15 g of agar was added. *Lactobacillus sanfranciscensis* DSM20451 and *Lactobacillus sanfranciscensis* DSM20451 Δ*gshR* were grown in modified de Man, Rogosa, Sharpe (mMRS) medium containing 10 g maltose, 5 g glucose, 5 g fructose, 10 g peptone, 5 g yeast extract, 5 g beef extract, 4 g K_2HPO_4• $3H_2O$, 2.6 g KH_2PO_4, 3 g NH_4Cl, 0.5 g L-Cys HCl•H_2O, 1 g Tween 80, 0.05 g $MnSO_4$•H_2O, 0.2 g $MgSO_4$•$3H_2O$, 10 g malt extract, and 15 g of agar for solid media preparation per litre. Growth of the mutant strain requires the addition of 10 mg of erythromycin (Sigma, Oakville, Canada). Strains are also grown in modified atmospheric conditions and incubated at 30 °overnight.

*2.2.3 Sourdough Fermentation and Bread Making***.** Strains described above were subcultured twice in either MRS or mMRS broth and washed with tap water. A final volume of 1 mL (approximately 10^9 cells) of resuspended culture was obtained from a 10 mL culture and added for every 10 g of flour used. Dough was prepared by combining 65 g of flour, 30 g of acidified egg white, 25 mL of tap water, and 6 mL of inoculum. Sourdough prepared with *Lactobacillus delbruekii* was incubated at 37℃ for 24 hours and sourdough prepared with *Lactobacillus sanfranciscensis* strains was incubated at 30℃ for 24 hours. Sourdough control was prepared the same way without inoculum and acidified chemically with acetic and lactic acid (1:4). Samples were taken at 0 hours, 5 hours, and 24 hours of fermentation. Cell counts and pH were monitored at every stage of fermentation. The mutant strain was enumerated on solid media containing erythromycin to ensure no contamination of plasmid deleted strain as a second control. Samples were freeze dried and prepared for amino nitrogen content determination, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and enzyme linked immunosorbent assay (ELISA). Remaining sourdough was used as a preferment for sourdough bread making. Additional ingredients obtained from the local supermarket was combined with the preferment (180 g wheat flour, 110 mL tap water, and 3.6 g each of yeast, sugar, and salt) and mixed high speed for three minutes followed by a 30 minute rest at room temperature. Before baking at 200℃ for 15 minutes the dough was proofed for 90 minutes at 30℃ and 85% relative humidity. Dough samples were taken after each stage (mixing, resting and proofing). After baking

bread was cooled and samples were freeze dried for SDS-PAGE, immunoblotting and ELISA analyses.

2.2.4 Extraction of Egg White Proteins from Bread. An extraction buffer containing a surfactant and reducing agent modified from (Jänsch, Korakli, Vogel, & Gänzle, 2007) was used for the extraction of egg white proteins from baked bread. 50 mM phosphate buffered saline solution containing 4% sodium dodecyl sulfate (SDS) and 10% 2-mercaptoethanol was used to extract proteins from bread. Then 200 mg of freeze dried bread sample was mixed with 1 mL of extraction buffer and vortexed at high speed for 1 minute and then sonicated at 15 kHz for 3 hours in ice. The samples were kept in the 4℃ overnight and were centrifuged (16000 g for 20 minutes). Commercially available egg protein detection kits were used to confirm successful extraction of egg white proteins for analyses.

2.2.5 Determination of Protein Cross linkage. During fermentation egg proteins may have cross linked with wheat proteins to form higher molecular weight components. To determine if there was crosslinking present a method from Watanabe et al. (2005) was used. First SDS soluble proteins were extracted by vortexing 1:4 (w/v) of dough and 1.5% SDS in 50 mM sodium phosphate buffer, pH 6.9 followed by shaking for 15 minutes with an overhead shaker. After shaking the samples were centrifuged for 10 minutes at 4000 rpm and the precipitate was kept for the second extraction. The precipitate was then solubilized in 1.5% SDS, 4% DTT (dithiothreitol) in 50 mM sodium phosphate

buffer, pH 6.9 followed by overhead shaking for 15 minutes. The samples were used directly for SDS-PAGE analysis.

2.2.6 Amino Nitrogen Content Determination. The ninhydrin method was used to measure the amount of amino nitrogen present in the sourdough (ASBC, 1992). Freeze dried samples were extracted at 1:10 (w/v) with 100 mM sodium phosphate pH 8 for 1 hour shaking at 5℃. Samples were then centrifuged at 10000 g for 10 minutes (Eppendorf Centrifuge 5417C, Eppendorf AG, Hamburg, Germany) and supernatants were used for analysis. Then 200 µL of the sample supernatant was mixed with 100 µL of ninhydrin color reagent pH 6.7 containing 0.94 g $Na_2HPO_4 \cdot 7H_2O$, 0.6 g KH_2PO_4, 0.05 g ninhydrin, and 0.03 g fructose in 10 mL of distilled water. The samples were heated in a boiling water bath for 16 minutes then cooled to room temperature followed by the addition of 500 µL of dilution solution containing 0.2% KIO_3 in 40% ethanol. Absorbance at 570 nm was recorded and analyzed. Glycine was used for the standard curve preparation.

2.2.7 SDS-PAGE and Immunoblotting. Freeze dried sourdough and dough samples were extracted 1:10 (w/v) with 1M NaCl in 200 mM Tris-HCl buffer pH 8 at 250 rpm shaking for 1 hour. Freeze dried bread samples were extracted with 4% SDS and 10% 2-mercaptoethanol. After centrifugation at 10000 g for 10 minutes (Eppendorf Centrifuge 5417C, Eppendorf AG, Hamburg, Germany), 50 µL of the sample supernatant was mixed with 50 µL of Laemmli sample buffer containing 2% dithiothreitol and heated at 95℃ for 5 minutes. After flash centrifugation samples were loaded into two 12% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA) and analyzed by SDS-PAGE (Thiele, Grassl, &

Gänzle, 2004). One gel was stained with Coomassie Brilliant Blue (R-250) and the other was used for immunoblotting. The proteins were transferred to a nitrocellulose membrane electrophoretically for 60 minutes at 100 V and then blocked overnight at 4 ℃ with 5% nonfat dry milk (NFDM) in 0.05% Tween Tris-HCl buffer (TBST) solution pH 7.4. After removing the blocking solution, primary antibody solution containing patient plasma (PlasmaLab International, Everett, WA, USA) diluted 1:1000 in TBST was incubated at room temperature for 1 hour. The membrane is then washed with TBST 5 times at 5 minute intervals to remove excess antibody then incubated at room temperature for 1 hour with 1:2000 dilution of goat anti-human IgE conjugated with horseradish peroxidase (MP Biomedicals). After secondary antibody incubation and washing with TBST again the membrane is detected using ECL+ fluorescent detection kit from GE healthcare.

2.2.8 Indirect ELISA Analysis. Freeze dried samples were extracted at 1:10 (w/v) with 100 mM sodium phosphate pH 8 for 1 hour shaking at 5 ℃. Sample supernatants were diluted with 50 mM sodium carbonate buffer at pH 9.6 then applied onto 96 well flat bottomed microtiter plates and incubated overnight at 4 ℃. The plate is then washed 4 times with 0.1% PBST (phosphate buffered saline tween solution) pH 7.2 washing buffer (SkanWasher 400, Molecular Devices, Sunnyvale, USA). The unoccupied spaces were blocked with 2% bovine albumin serum (BSA) in 50 mM sodium carbonate buffer at pH 9.6 solution for 2 hours at 37 ℃ with gentle shaking. The microtiter plate was washed again with PBST followed by the addition of 100 µL of human plasma (PlasmaLab International,

Everett, WA, USA) diluted 1:25 in 1% BSA in PBST solution and incubated overnight at room temperature with gentle shaking. After washing 100 µL of goat anti-human IgE secondary antibody diluted 1:2000 in 1% BSA in PBST solution was added and incubated overnight at room temperature with gentle shaking. The next day the plate is washed again with PBST before the addition of 100 µL of p-nitrophenyl phosphate (pNPP) for color formation. After 45 minutes the reaction is stopped with 25 µL of 3M sodium hydroxide and absorbance is read at 405 nm.

2.3 Results

2.3.1 Sourdough Fermentation. The sourdough fermentations were all carried out in a one stage 24 hour fermentation with three strains of lactobacilli compared to a chemically acidified control sourdough. Cell counts were taken at 0, 5, and 24 hours of fermentation while pH measurements were also taken to monitor acid production, both confirmed successful growth and fermentation. Cell counts of the lactobacilli by the end of fermentation ranged from 3.5 - 9.3 x 10^8 CFU g^{-1}. Acidified egg white was used in the fermentation to ensure the pH level of the dough was around 6.0 and suitable for the growth of lactobacilli. The pH of the sourdough at 0 hours was 6.0 $\pm$ 0.07, 4.6 $\pm$ 0.4 at 5 hours and then lowered to 3.73 $\pm$ 0.04 by 24 hours. The amino nitrogen content was determined to follow proteolytic degradation. Figure 2-1 shows the amino nitrogen levels in the sourdoughs increasing as the fermentation time increases.

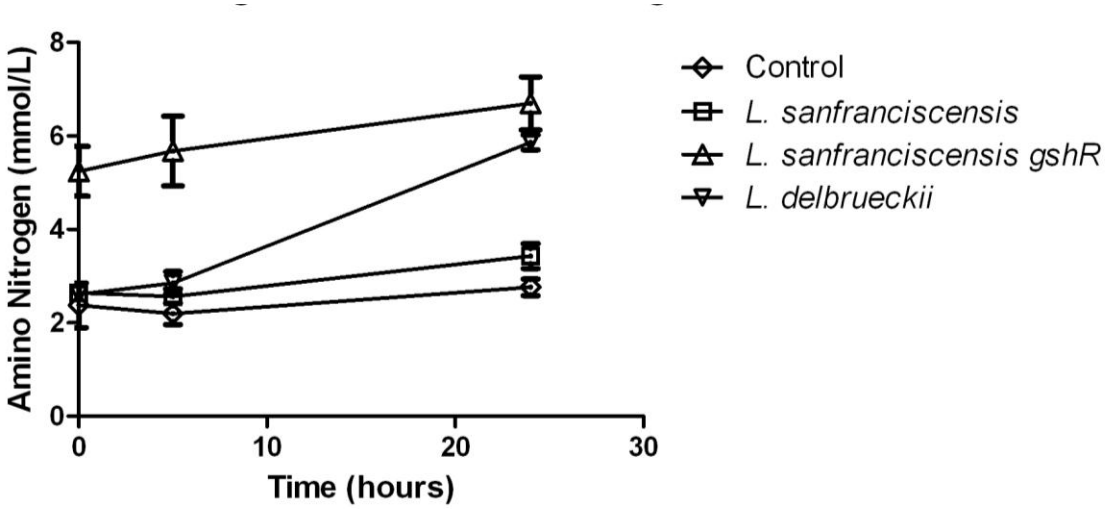

Figure 2-1. Amino nitrogen concentrations in sourdough samples at 0 hour, 5 hours, and 24 hours of fermentation. Measurements were carried out using ninhydrin method (ASBC, 1992) with water soluble fractions of egg white proteins. Results are shown ± standard deviation of triplicate analyses.

2.3.2 SDS-PAGE Analysis of Egg White Proteins during Fermentation. During the course of sourdough fermentation specific egg white proteins were degraded. No changes occurred in ovomucoid or ovalbumin but ovotransferrin was completely degraded after 24 hours of fermentation with *L. sanfranciscensis* and *L. delbrueckii* (Figure 2-2). The ovotransferrin band intensities in the control samples at 0, 5, and 24 hours remained the same as with the bands in the mutant strain.

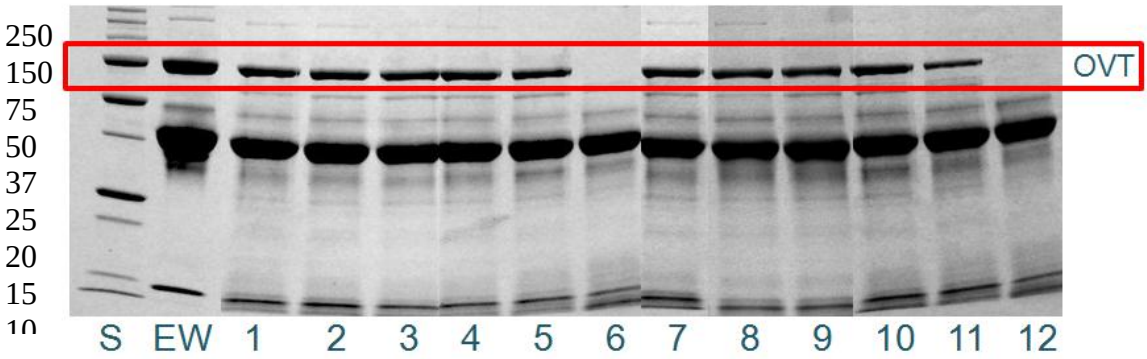

Figure 2-2. SDS-PAGE analysis of soluble egg white proteins extracted from sourdough using 1 M NaCl/200 mM Tris-HCl solution. The red rectangle highlights the ovotransferrin band with a molecular weight of 78 kDa which was degraded by *L. sanfranciscensis* and *L. delbrueckii* after 24 hours of fermentation. S: standard; EW: acidified egg white; 1, 2, 3: Control 0, 5, 24 h; 4, 5, 6: *L. sanfranciscensis* 0, 5, 24 h; 7, 8, 9: *L. sanfranciscensis* Δ *gshR* 0, 5, 24 h; 10, 11, 12: *L. delbrueckii* 0, 5, 24 h.

2.3.3 SDS-PAGE to Determine Protein Cross Linkage. From Figure 2-2 it is apparent that ovotransferrin is the only egg white protein showing a change after fermentation. To confirm that this protein was degraded and not cross linked with other wheat proteins SDS-PAGE was performed to analyze any new high molecular weight compounds. Figure 2-3 shows the first extraction of SDS soluble proteins (A) and the second extraction with SDS and DTT soluble proteins (B). There appears to be no high molecular weight compounds formed during sourdough fermentation as well as during the proofing step in bread making. There is also no indication of cross linkage comparing the dough with no

egg white proteins to the doughs with egg white proteins. This confirms ovotransferrin was successfully degraded during the 24 hour fermentation.

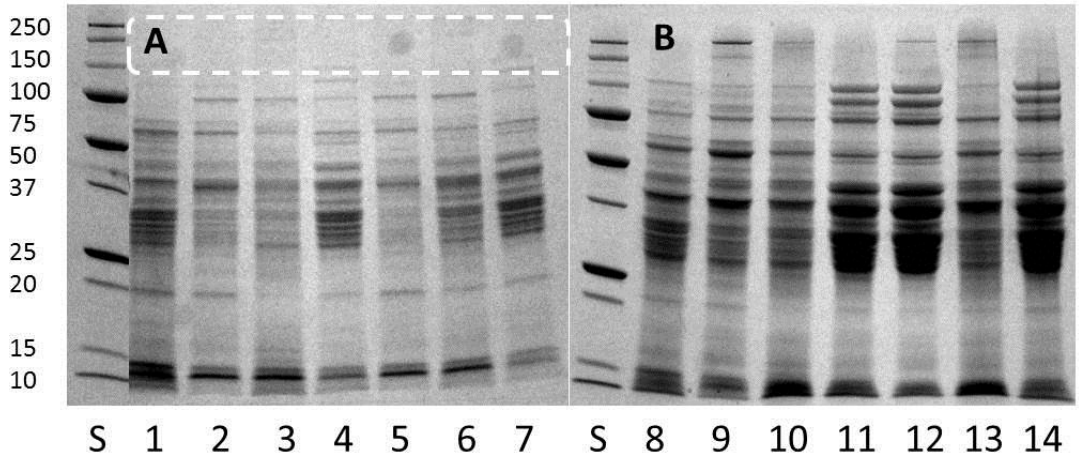

Figure 2-3. SDS-PAGE of SDS soluble proteins (A) and SDS and DTT solubilized proteins (B). Lane abbreviations: S: standard marker; 1, 8: bread dough with no egg white proteins; 2, 9: *L. sanfranciscensis* sourdough at 0 h; 3, 10: *L. sanfranciscensis* sourdough at 24 h; 4, 11: *L. sanfranciscensis* proofed dough; 5, 12: Control sourdough 0 h; 6, 13: Control sourdough 24 h; 7, 14: Control proofed dough.

2.3.4 Change in Allergenicity during Sourdough Fermentation. Sourdough samples from 0, 5, and 24 hours of fermentation were freeze dried and proteins were extracted for analysis of changes in allergenicity with patients with known egg white allergies by western blotting and ELISA methods. Figure 2-4 shows western blot analysis of egg whites after sourdough fermentation and before baking. All patients were allergic to mainly ovotransferrin before fermentation but at 24 hours there is no antibody binding to the allergic patient plasma anymore. Indirect ELISA analysis was also used to evaluate egg allergy in the sourdough. The results in Figure 2-5 show that there is no significant change in the allergenicity in the three different patients before, during and after fermentation with the control, *L. sanfranciscensis*, *L. delbrueckii* and *L. sanfranciscensis ΔgshR* sourdough samples.

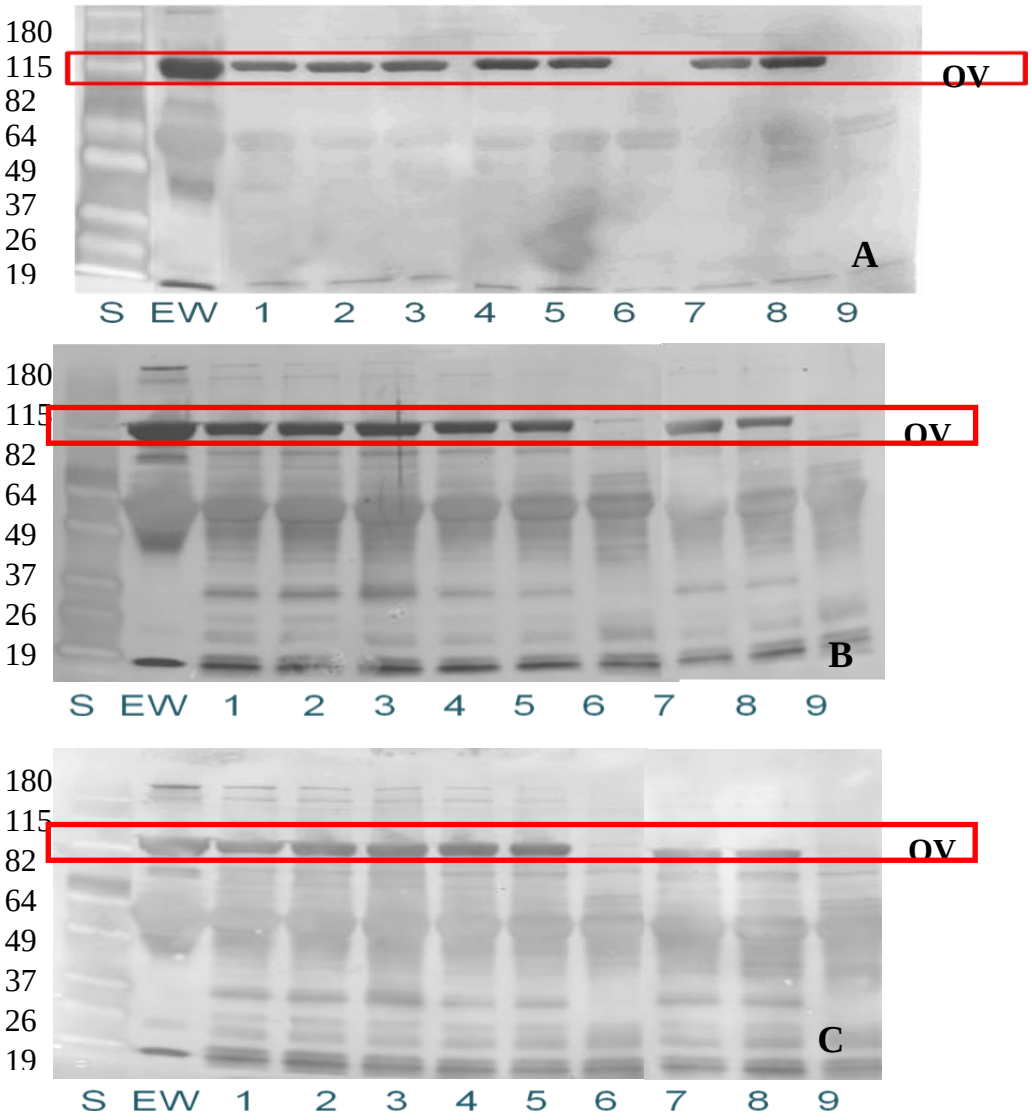

Figure 2-4. Western blot analysis of soluble egg white proteins extracted from sourdough using 1 M NaCl/200 mM Tris-HCl solution. A is patient 12388, B is patient 17912, C is patient 14982. OV is ovotransferrin. The red rectangle highlights the ovotransferrin band with a molecular weight of 78 kDa which was degraded by *L. sanfranciscensis* and *L. delbrueckii* after 24 hours of fermentation. S: standard; EW: acidified egg white; 1, 2, 3: Control 0, 5, 24 h; 4, 5, 6: *L. sanfranciscensis* 0, 5 , 24 h; 7, 8, 9: *L. delbrueckii* 0, 5, 24 h. Experiments were run in triplicates in two separate analyses.

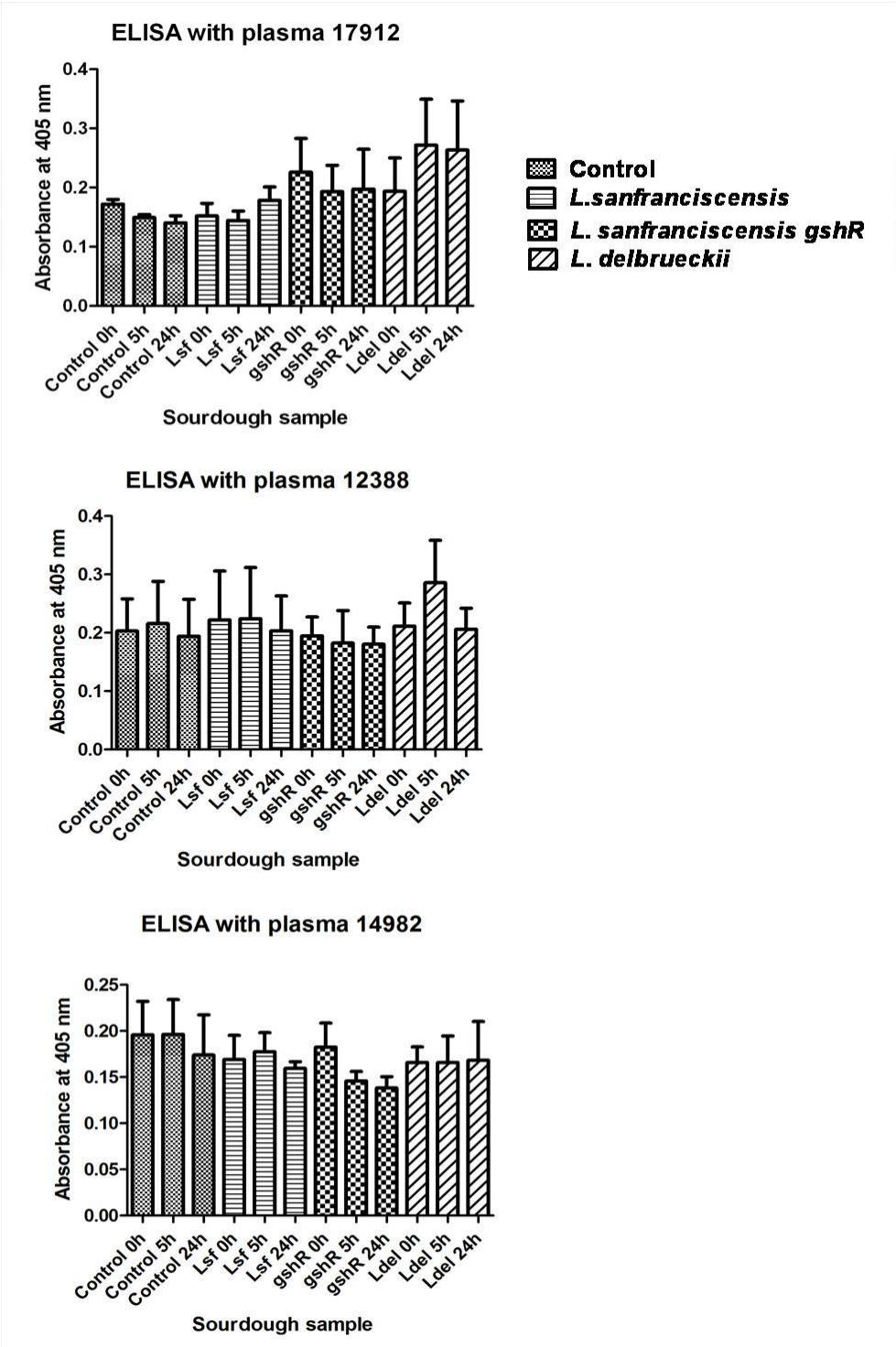

Figure 2-5: Indirect ELISA analysis of the egg white control (fresh), negative sodium carbonate buffer control and sourdoughs fermented with no bacteria (C), *L. sanfranciscensis* (Lsf), *L. sanfranciscensis* Δ*gshR* (gshR), and *L. delbruekii*

(Ldel) sampled at 0, 5, and 24 hours. Samples were extracted with 100 mM sodium phosphate pH 8 for 1 hour shaking at 5 ℃ and experiments were run in triplicates in two separate analyses. Statistical analysis was carried out using ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

2.3.5 SDS-PAGE Analysis of Egg White Proteins after Bread Making. Through the mixing, resting, and proofing stages of baking, the ratio of egg white proteins present is reduced due to the addition of fresh ingredients. Therefore the egg white bands of ovomucoid and ovalbumin are less visible but still present and ovotransferrin is still hydrolyzed since the additional fresh ingredients did not include egg whites. In Figure 2-6 the egg proteins are still extracted with a 1M NaCl in 200 mM Tris-HCl buffer before baking.

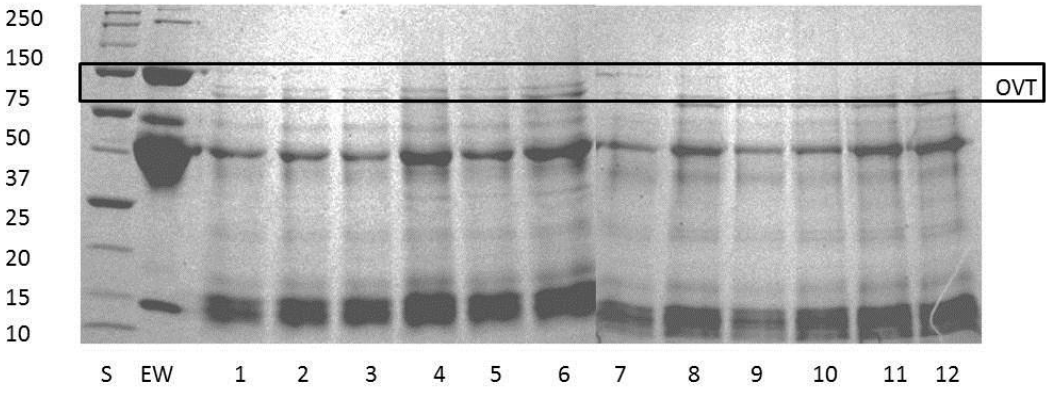

Figure 2-6: SDS-PAGE analysis of sourdough after the addition of fresh ingredients at the end of fermentation sampled after different steps in the breadmaking process (mixing, resting and proofing) for the control, *L. sanfranciscensis*, *L. sanfranciscensis* Δ*gshR*, and *L. delbruekii* sourdoughs. S: standard; EW: acidified egg white; 1, 2, 3: Control mix, rest, proof; 4, 5, 6: *L.*

sanfranciscensis mix, rest, proof; 7, 8, 9: *L. sanfranciscensis* Δ*gshR* mix, rest, proof; 10, 11, 12: *L. delbrueckii* mix, rest, proof. OV is ovotransferrin.

2.3.6 Change of Allergenicity after Bread Making. After baking for 15 minutes at 200℃ the egg white proteins were extracted with denaturing agents and sodium dodecyl sulfate due to the increased hydrophobicity of the bread matrix. Bread samples from each of the control, *L. sanfranciscensis*, *L. sanfranciscensis* Δ*gshR*, and *L. delbruekii* were selected and used to evaluate allergenicity after baking. Figure 2-7 shows the western blot analysis from three patients allergic to egg white. After a baking heat treatment the egg proteins have denatured and do not cause an antibody binding reaction anymore.

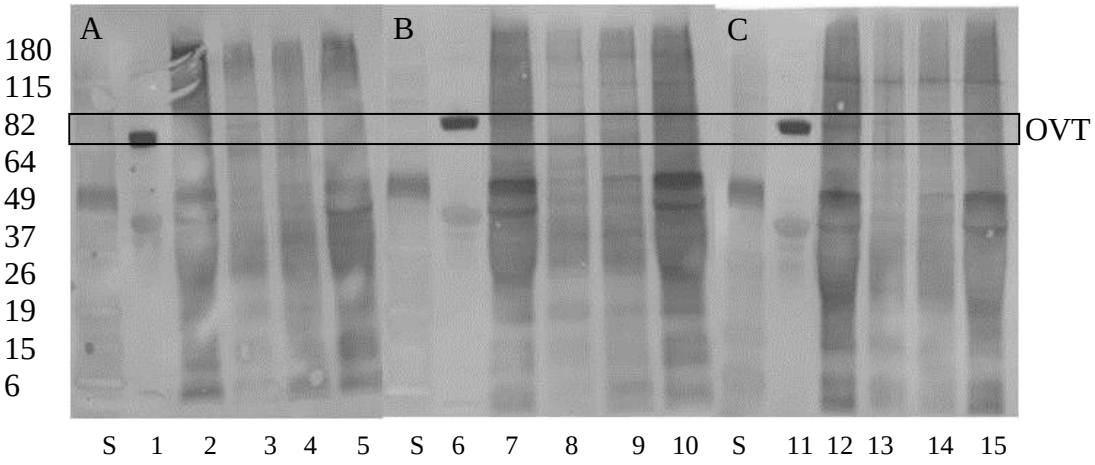

Figure 2-7: Western blot analysis of bread samples after baking. Patient plasma 12388: A; plasma 17912: B; plasma 14982: C. S: standard molecular weight marker; 1, 6, 11: egg white controls (fresh); 2, 7, 12: control bread extracted with 5% SDS and 2% 2-mercaptoethanol; 3, 8, 13: *L. sanfranciscensis* extracted with 3% SDS and 2% 2-mercaptoethanol; 4, 9, 14: *L. sanfranciscensis* Δ *gshR*

extracted with 3% SDS and 2% 2-mercaptoethanol; and *L. delbrueckii* extracted with 5% SDS and 2% 2-mercaptoethanol. Experiments were conducted in triplicates with two separate analyses.

2.4 Discussion

2.4.1 Effects of Lactobacilli on Proteolytic Degradation. All of the sourdough fermentations were incubated for 24 hours with the addition of all ingredients in the first step with three strains of lactobacilli including one isogenic control strain compared to a chemically acidified control. The naturally high pH of egg white as well as the presence of some natural antimicrobial egg white proteins such as lysozyme and avidin may account for inhibitory growth of lactobacilli (Laemmli, 1970) so a more suitable environment was required. The pH of fresh egg white is normally around 7.9 and may increase to approximately pH 9 during storage because of carbon dioxide diffusion out of the albumin. It was necessary to acidify the egg white until approximately pH 6 to create a more suitable environment for the growth of lactic acid bacteria. The sourdough system provided an anaerobic environment for the growth of the lactic acid bacteria. Cell counts and pH measurements were taken to monitor acid production; both confirmed successful growth of bacteria and ongoing fermentation. The main products produced by these lactic acid bacteria are lactic acid, acetic acid and carbon dioxide which contribute to lowering the pH for enzyme functions (Stiles, 1996).

Natural enzymes present in the wheat flour with an optimal pH between 3.5 and 4.0 were mainly responsible for any egg white protein degradation (Vogel & Ehrmann, 2008). By the end of fermentation the pH was decreased to approximately 3.73 ± 0.04 which allowed the natural wheat enzymes to work at their optimum. These wheat aspartic proteases are mainly responsible for the proteolytic degradation in traditional wheat and rye sourdoughs (Gänzle et al., 1998) and can also be seen degrading ovotransferrin in this particular fermentation with the addition of egg white proteins. This was confirmed in a similar sourdough study where an aspartic protease inhibitor was added into the fermentation and ovotransferrin remained unchanged (Loponen et al. 2004; Tuukkanen et al. 2005; Vermeulen et al. 2006).

L. sanfranciscensis DSM20451 has the ability to reduce oxidized glutathione back into its normal form. The increase of this reducing agent drives the depolymerization of gluten increasing its solubility and promoting proteolytic degradation resulting in the increase of amino nitrogen levels and runnier texture (Loponen et al., 2008). The increase in amino nitrogen content came into accordance with a similar study by Loponen et al. (2008) where a two stage sourdough fermentation was performed.

2.4.2 Effect of Disulfide Exchange Reactions on Proteolytic Degradation. In the SDS-PAGE of the sourdough samples between 0 and 24 hours of fermentation it was noted that the egg white protein ovotransferrin was resistant to degradation in the sourdough fermented with the *L. sanfranciscensis* mutant strain because of the lack of the glutathione reductase enzyme activity. Both of the wild

type lactobacilli degraded the ovotransferrin completely by the end of fermentation. Glutathione is present naturally in wheat flour and is the most abundant reducing agent. Glutathione is converted into oxidized glutathione while undergoing disulfide exchange reactions with wheat gluten proteins. At the same time the newly formed oxidized glutathione is converted back by glutathione reductase produced by *L. sanfranciscensis.* This cycle maintains constant glutathione levels in the sourdough matrix which continuously pushes disulfide exchanges between gluten proteins resulting in increased solubility leading to depolymerization (Loponen et al., 2008).

Disulfide bonds are extremely important in the stability of a protein as well as maintaining protein structure and function (Thiele et al., 2004). Ovotransferrin contains 15 disulfide bonds (Breiteneder & Mills, 2005) and during fermentation it can also act as a substrate for this type of enzymatic degradation. This protein is not very glycosylated leaving the disulfide bonds more accessible and prone to degradation compared to a highly glycosylated protein such as ovomucoid which is extremely resistant to many mechanisms. During the exchange reactions wheat gluten becomes depolymerized to form a thinner and runnier texture while ovotransferrin is also broken down. A study on the disulfide exchange reactions in wheat protein systems such as sourdough has been studied with the effects of different additives such as cysteine and N-ethylmaleimide to block the exchange reaction has shown to be successful (Schurer et al. 2007). The presence of free thiol groups is crucial for protein changes to occur thus altering the environment may alter the fate of the protein.

Because the ELISA results (Figure 2-5) did not show any change in allergenicity during and after sourdough fermentation; to confirm that the disappearance of ovotransferrin during fermentation was due to enzymatic degradation but not due to cross linkage between different proteins in the sourdough system another analysis was conducted. Glutenin macropolymer proteins present in the wheat sourdough were thought to have cross linked during fermentation or bread making causing the disappearance of the ovotransferrin band in SDS-PAGE. Cross linking of ovotransferrin to wheat glutenin proteins will form higher molecular weight compounds which could be extracted with an SDS buffer since there is increased hydrophobicity. In Figure 2-3(A) no new high molecular weight compounds were observed indicating no cross link of ovotransferrin to another protein. This was further confirmed in Figure 2-3(B) where a reducing agent was added into the extraction. There is no reappearance of the ovotransferrin band indicating complete degradation by aspartic proteases with glutathione reductase.

2.4.3 Effect of Fermentation on Allergenicity. The degradation of the ovotransferrin protein reflected clearly on the western blot analysis of the sourdough (Figure 2-4). From Figure 2-2 of the SDS-PAGE analysis of the sourdough, the only major egg white allergen protein that changed during the fermentation was ovotransferrin. Western blot analysis will show changes in allergenicity according to each separate protein so the degradation of ovotransferrin which is seen in Figure 2-2 indicates that there is no longer an allergy to ovotransferrin in those patients, but this does not mean that the patient

is no longer allergic to egg white proteins. Shown in Figure 2-5 is the ELISA analysis is the overall effect on allergenicity after fermentation. ELISA takes into account all proteins together and shows their combined effect on allergic patients. In Figure 2-5 there is no significant change in allergenicity in these three patients even though in the same three patients there was elimination in ovotransferrin allergy after fermentation. Ovotransferrin accounts for approximately 10% of the total egg white protein which may be too minimal to have a total overall effect on patients with egg allergies as reflected in the ELISA analysis.

2.4.4 Effect of Bread Making on Allergenicity. After fermentation the sourdough was mixed, rested, and proofed in preparation for baking (15 minutes at 200℃). For bread making fresh ingredients including flour, water, salt and sugar were added to the sourdough preferment and a secondary fermentation with yeast during the proofing step was carried out. In Figure 2-6 the same aqueous extraction used for sourdough protein extraction was used to extract egg white proteins to confirm ovotransferrin is still eliminated after fermentation and before baking. The addition of fresh ingredients increases the amount of wheat proteins present and in Figure 2-3 proofing samples were also evaluated to show that there is also no cross linking happening during bread making.

During baking, because of high heat the egg white proteins become insoluble and aggregated within the food matrix of egg proteins and wheat proteins. Extraction requires denaturing agents and an increased level of sodium dodecyl sulfate due to the increased hydrophobicity of the bread matrix. Different combinations of 2-mercaptoethanol and SDS were used to optimize the amount of

egg protein extracted in comparison to wheat proteins which are present in an overwhelming amount. The samples with highest recovery percentages according to commercially available egg protein detection kits were used for western blot analysis. Combinations with 3 to 5% of SDS seemed to have the highest amount of egg protein recovery with 2% of reducing agent. The control samples have a higher recovery percentage compared to the sourdough samples fermented with lactic acid bacteria. This may be caused by enzymatic egg protein degradation during fermentation resulting in a lesser quantifiable amount of egg protein compared to the non-hydrolyzed control. Bread samples from each of the control, *L. sanfranciscensis*, *L. sanfranciscensis* Δ*gshR*, and *L. delbruekii* were selected and used to evaluate allergenicity after baking. Figure 2-7 shows the western blot analysis from three patients allergic to egg white. After a baking heat treatment the egg proteins have denatured and according to Figure 2-7, do not cause an antibody binding reaction anymore. Because the control bread with no lactic acid fermentation also does not cause the antibody binding reaction this result may be due to heat treatment in combination with the presence of wheat proteins. A previous study conducted by Kato et al. (2001) showed reduced ovomucoid allergy as a result of heat treatment in the presence of wheat flour. These results may be due the intermolecular interactions between different food proteins altering protein bonds and structure with the aid of heat causing a change of the allergic epitope in the original protein thus seeing a reduction of allergenicity.

2.5 Conclusion

Sourdough fermentation is a potential method for egg protein degradation inside a food matrix. Acidified egg white addition is necessary to promote the growth of lactic acid bacteria inside the sourdough system. One of the major allergens in egg white, ovotransferrin was degraded completely during twenty four hours of fermentation with *L. sanfranciscensis* DSM20451 and *L. delbrueckii* and was reflected in the SDS-PAGE analysis. A result from this protein degradation was that patients previously allergic to the ovotransferrin protein showed no more IgE binding after fermentation. The overall allergenicity evaluated with ELISA however did not show a decrease in allergy before baking. After baking through western blot analysis it was determined that there was no further allergy caused by the egg white proteins. This could be due to the combination of heat treatment with the presence of wheat proteins aggregating with the egg white proteins. This study was in continuation of previous egg protein and sourdough fermentation studies to further investigate one stage fermentations, its effects on allergenicity, bread making and allergenicity of the final food product. Future work may involve fermentation with different types of flour which brings in a new assortment of enzymes for possible egg protein degradation.

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Chapter 3: Effects of Fermentation in Egg White and Ovomucoid on Egg White Allergenicity

3.1 Introduction

Egg allergy is one of the leading causes for type 1 hypersensitivity reaction in childhood. It affects approximately 7% of children up to three years of age (Kato et al., 2001; Benhamou et al., 2010) while prevalence in children with atopic dermatitis is higher (Eigenmann, Sicherer, Borkowski, Cohen, & Sampson, 1998; Eigenmann et al., 1998). Likewise approximately 33% of children with atopic dermatitis show skin symptoms during an allergic reaction and 16% with asthma have breathing difficulties during an allergic reaction (Sampson & McCaskill, 1985). From infancy to the beginning of adolescence the prevalence of egg allergy decreases from approximately 4% by the age of four, 12% by the age of six, 37% by the age of 10, and 68% by the age of 16 but some adults still remain severely allergic (Bock, 1992; Burks et al., 1988; Novembre et al., 1988). Common symptoms of egg allergy include urticaria, rash, vomiting, diarrhea, wheezing, coughing, asthma, rhinitis and nasal congestion (Savage et al., 2007).

There are four allergic proteins found in egg white: ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3) and lysozyme (Gal d 4) (Savage et al., 2007). Although ovalbumin makes up 54% of egg white protein, its allergenicity is decreased because of its sensitivity to heat denaturation (Joo & Kato 2006). Ovomucoid is considered as the dominant egg allergen (Cooke & Sampson, 1997; Hoffman, 1983; Holen & Elsayed, 1990; Kato et al., 2001;

Langeland, 1983; Kato et al., 2001); it consists of three tandem domains linked with disulfide bridges creating an extremely heat resistant structure contributing to persistent allergenicity (Bernhisel-Broadbent et al., 1994). Ovomucoid still remains soluble after processing and therefore can cause an allergic reaction after boiling of either an egg or purified ovomucoid for one hour (Mine et al., 2003). The removal of carbohydrate moieties on ovomucoid leaves the protein with a molecular weight of approximately 21 kDa. This increases its sensitivity to thermal denaturation but still retains its entire ability to cause a hypersensitivity reaction (Deutsch & Morton, 1956; Gu et al., 1989; Besler et al., 1997; Yamamoto et al., 1998).

Although food processing is more likely to reduce allergenicity by disrupting or modifying epitopes, there are reports of no change or even increase in allergenicity (Sathe, Teuber, & Roux, 2005; Sathe et al., 2005; Kerr & McLean, 1981). Incorporation of egg whites into bread or pasta preparation was led to reduced egg allergenicity due to interactions between egg white proteins and wheat proteins in the presence of heat creating specific reactions between protein groups (Nakamura, Watanabe, Ojima, Ahn, & Saeki, 2005). Lactic acid fermentation of milk and yogurt products was reported to reduce dairy proteins' allergenicity (Kato et al., 2001). Fermentation may change the structure of proteins in many ways that are distinct from other methods of processing. Fermentation of antigenic milk proteins with *L. acidophilus* and *S. salivarius* led to over 70% reduction in allergenicity (Bu, Luo, Zhang, & Chen, 2010; Ehn et al., 2004; Kleber et al., 2006; Schouten et al., 2009). Similarly soybean proteins

fermented with *L. plantarum* showed a 96% decrease in allergenicity (Kleber et al., 2006). Fermentation by *L. brevis* MS-99 in wheat flour reduced gluten allergy by 80% (Frias et al., 2007). An allergic egg white protein ovotransferrin was completely degraded in sourdough fermentation (Loponen et al., 2008). This shows potential to apply fermentation to egg white proteins to reduce IgE binding. The objectives of this study are to first confirm the reduction seen in egg white fermentation with lactic acid bacteria and fungal strains then investigate using fermentation as a pretreatment of the dominant allergen ovomucoid to assess susceptibility to enzymatic hydrolysis.

3.2 Materials and Methods

3.2.1 Materials. Table eggs used for egg white fermentation and ovomucoid extraction were obtained from the local supermarket. Fungal protease and pepsin from porcine gastric mucosa were purchased from Sigma-Aldrich (St. Louis, MO, USA) while rye malt was ground in the laboratory (Laihan Mallas, Laihia, Finland).

3.2.2 Strains and Growth Conditions. Lactic acid bacteria strain *Lactobacillus delbrueckii* was grown in de Man, Rogosa, Sharpe (MRS) medium at pH 6.2 for overnight under modified atmospheric conditions (4% O_2, 20% CO_2, 76% N_2) at 37℃. For solid MRS media 15 g of agar was added. *Aspergillus oryzae* ATCC 1011 and *Aspergillus oryzae* ATCC 16868 (Microfungus Collection and Herbarium, University of Alberta) were used for the study. *A. oryzae* strains were grown in mould broth containing 20 g/L malt extract, 1 g/L

peptone, and 20 g/L glucose at pH 5.5 and for solid media with the addition of 15 g of agar. Moulds were incubated at 25 ℃ for seven days in atmospheric conditions.

*3.2.3 Egg White Preparation and Fermentation***.** Egg whites were prepared by flaming the egg in 100% ethanol and hand breaking and separating whites and yolks. The pH of the egg white solution was adjusted from 9 to 6 using 3 M HCl. *L. delbrueckii* was grown in solid media and a colony was picked into MRS broth. The strain was subcultured twice in MRS and washed twice with peptone water. A final volume of 1 mL (approximately 10^9 cells) of resuspended culture was obtained from a 10 mL culture and mixed with every 10 mL of egg whites for fermentation. The addition of 10% tryptone solution was necessary to support the growth of *L. delbrueckii*. *A. oryzae* strains were inoculated onto solid media from the stock culture and left to grow in 25 ℃ for seven days. Spores were harvested with peptone water and mycelia were filtered off. After centrifugation, spores were resuspended in 1 mL of peptone water and added for every 10 mL of egg white solution used for fermentation. All fermentations were carried out for a total of 96 hours, sampling every 24 hours.

*3.2.4 Ovomucoid Extraction and Fermentation***.** Ovomucoid was extracted according to a modified protocol (Juliàet al., 2007). Eggs were wiped with 70% ethanol and hand broken to separate whites and yolks. Equal volumes of egg white and 10% trichloroacetic acid were mixed and the pH was adjusted to pH 3.5 with 1 M NaOH. The mixture was left to precipitate overnight at 4 ℃.The following day the mixture was centrifuged at 1400 g for 25 minutes at 10 ℃. The

supernatant was then brought to 90% saturation (w/v) of ammonium sulfate and then adjusted to pH 4.6. The mixture was left to settle again overnight at 4 ℃. The next day the mixture was centrifuged again and the precipitate was recovered and dialyzed against water followed by freeze drying.

Fermentation was carried out with 20 g/L of ovomucoid in a 50 mM phosphate nutrient broth containing 50 mg/L manganese sulfate, 200 mg/L magnesium sulfate, 5 g/L tryptone, 2 g/L glucose and incubated for up to 96 hours. Strains and growth conditions are as mentioned in the previous section. The broth was subjected to heat treatment before fermentation according to Juli à et al. (2007) to minimize contamination. Since the extracted ovomucoid did not dissolve well in the nutrient broth and could not be autoclaved or filter sterilized, a heat treatment was necessary to minimize the microorganisms present in the starting broth. The nutrient broth was first put into a water bath at 75 ℃ until the temperature was brought up to approximately 57 ℃ and then transferred to a second water bath at 57 ℃ and held there for 3.5 minutes. It was cooled to room temperature before the addition of inoculum.

3.2.5 Enzymatic Hydrolysis of Ovomucoid. Ovomucoid before and after fermentation was digested by either pepsin (37 ℃; E/S, 2%, w/w in 50 mM potassium chloride and HCl buffer, pH 2), or fungal protease (50 ℃; E/S: 2%, w/w in 50 mM phosphate buffer, pH 7), or rye malt (37 ℃; E/S: 10%, w/w in 50 mM phosphate buffer, pH 7) for three hours at 1000 rpm in Thermomixer (Eppendorf Thermomixer R, NY, USA). The reaction was stopped at 95 ℃ for 10 minutes.

3.2.6 SDS-PAGE and Immunoblotting. Egg white and ovomucoid samples before and after fermentation were diluted to 4 mg/mL with Laemmli buffer containing 2% dithiothreitol, heated at 95℃ for 5 min, flash centrifuged and 20 uL supernatant was loaded into 12% Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA) (Wong, Herald, & Hachmeister, 1996). Gels were either stained with Coomassie Brilliant Blue (R-250) for electrophoresis or used for immunoblotting. For immunoblotting, the proteins were transferred to a nitrocellulose membrane electrophoretically for 60 min at 100 V and then blocked for 1 hour at room temperature with 5% nonfat dry milk (NFDM) in 0.05% Tween Tris-HCl buffer (TBST) solution pH 7.4. After removing the blocking solution, primary antibody solution containing patient plasma (PlasmaLab International, Everett, WA, USA) diluted 1:1000 in TBST was incubated at room temperature for 1 hour. The membrane was then washed with TBST 5 times at 5 min intervals to remove excess antibody, incubated at room temperature for 1 hour with 1:2000 dilution of goat anti-human IgE conjugated with horseradish peroxidase (MP Biomedicals), washed again with TBST buffer, and was detected using ECL+ fluorescent detection kit from GE healthcare.

3.2.7 Indirect ELISA Analysis. Egg white and ovomucoid samples before and after fermentation were diluted with 50 mM sodium carbonate buffer at pH 9.6 to 5 mg/mL, then 100 µL was applied onto 96 well flat bottomed microtiter plates and incubated overnight at 4℃. The plate was then washed 4 times with 0.1% PBST (phosphate buffered saline tween solution) pH 7.2 washing buffer (SkanWasher 400, Molecular Devices, Sunnyvale, USA). Unoccupied spaces

were blocked with 2% bovine albumin serum (BSA) in 50 mM sodium carbonate buffer at pH 9.6 solution at 37℃ for 2 hours with gentle shaking. The microtiter plate was washed again with PBST followed by the addition of 100 µL of human plasma (PlasmaLab International, Everett, WA, USA) diluted at 1:25 in 1% BSA in PBST solution and incubated overnight at room temperature with gentle shaking. After washing, 100 µL of goat anti-human IgE secondary antibody diluted 1:2000 in 1% BSA in PBST solution was added and incubated overnight at room temperature with gentle shaking. The next day the plate is washed again with PBST before the addition of 100 µL of nitrophenyl phosphate (pNPP) for color formation. After 45 min the reaction was stopped with 25 µL of 3M sodium hydroxide and absorbance was read at 405 nm.

3.2.8 In-gel Digestion. *In-gel* digestion of the 52 kDa band before fermentation and after fermentation (~ 46 kDa) was performed according to the method modified from Laemmli (1970). The bands were cut from the SDS-PAGE gel, transferred into low-retention microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada), and then de-stained by double incubation with 200 µL of 200 mM ammonium bicarbonate with 40% acetonitrile for 30 min followed by washing with 200 µL acetonitrile. After drying, the protein gel bands were reduced with 40 µL of 10 mM dithiothreitol for 30 min followed by alkylation with 40 µL of 50 mM iodoacetamide for another 30 min. After removing the iodoacetamide, the gels were washed with 100 mM ammonium bicarbonate and 100% acetonitrile, then dried in a Vacofuge (Eppendorf, Hamburg, Germany). The dried gel pieces were digested overnight at 37 ^{o}C with 0.8 µg Promega

modified trypsin (Madison, WI, USA) in 50 mM ammonium bicarbonate buffer. Following digestion, the peptide fragments were extracted into microcentrifuge tubes with 30 µL of 100 mM ammonium bicarbonate, and then extracted two more times with 30 µL solution containing 5% formic acid and 50% acetonitrile in water, and finally dried to 15 µL in Eppendorf Vacofuge.

3.2.9 Liquid Chromatography Electron Spray Ionization-Mass Spectrometry (LC ESI-MS) and MS/MS Analysis. The tryptic digest of peptides was analyzed using a hybrid quadruple orthogonal acceleration time-of-flight mass spectrometer (QToF, Premier, Waters, Milford, MA) equipped with ultra high performance liquid chromatography (Waters nanoACQUITY, Milford, MA). First 5 µL of the digest was injected into the nanoAcquity liquid chromatography system consisting of a peptide trap (180 µm x 20 mm, Symmetry® C18 nanoAcquity™ column, Waters, Milford, MA) and nano analytical column (75 µm × 150 mm, Atlantis™ dC18 nanoAcquity™ column, Waters, Milford, MA). The trapped sample was then desalted by flushing trap column with a 2% acetonitrile and 0.1% formic acid solution (solvent A) at a flow rate of 10 µL/min for 2 minutes. Then the peptides were separated with a gradient of 2- 65% solvent B (acetonitrile, 0.1% formic acid) for approximately 35 min with a flow rate of 350 nL/min. The QToF premier (Waters, Milford, MA) was used to analyze the effluent of desired peptides with ESI-MS and MS/MS.

3.3 Results and Discussion

3.3.1 Changes in Egg White Proteins after Fermentation. Egg whites were fermented for up to 96 hours with *L. delbrueckii*, *A. oryzae* ATCC 1011, or *A. oryzae* 16868. Effects of fermentation on protein degradation were evaluated by SDS-PAGE as shown in Figure 3-1. Four major egg allergens, ovomucoid, ovotransferrin, ovalbumin, and lysozyme were not significantly changed and there were no new low molecular weight bands although there was a very faint shift (arrow labeled in Figure 3-1) in lanes 8, 9, 10, 13, 14, and 15 with *A. oryzae* strains corresponding to 48 hours to 96 hours of fermentation. The addition of tryptone as a nitrogen source was necessary for the growth of lactobacilli, but this may have affected its potential to produce its own proteolytic enzymes to break down egg white proteins (Shevchenko, Wilm, Vorm, & Mann, 1996). Also the presence of a natural antimicrobial protein lysozyme in egg white may inhibit the gram-positive lactobacilli growth to a certain extent even though the pH of the egg white solution was lowered to pH 6. Studies have shown that lysozyme does have the ability to inhibit growth of some lactobacilli in milk (Nguyen Thi, Champagne, Lee, & Goulet, 2003).

Aspergillus oryzae is commonly used to ferment foods because of its ability to secrete many enzymes for flavor and texture improvements (Bottazzi, Battistotti, Bosi, Corradini, & Dell'Acqua, 1978; Kovacs-Nolan, Phillips, & Mine, 2005). Many enzymes produced are active against carbohydrates including α-amylase and α –galactosidase (Machida, Yamada, & Gomi, 2008), which theoretically has the potential to cleave many glycoproteins in egg white

including ovomucoid. The very faint shift in molecular weight of an unknown protein directly above the ovalbumin band (arrow labeled in Figure 3-1) during 48 hours of fermentation may be linked to the reduction of egg allergenicity in *A. oryzae* fermented samples. This change was not seen in *L. delbrueckii* fermented egg white thus showing no change in allergenicity. It is suspected that this protein may be ovalbumin-related protein Y (Annunziato, Mahoney, & Mudgett, 1986; Hashimoto et al., 1999; Suganuma, Fujita, & Kitahara, 2007).

The band that showed a decrease in molecular weight in Figure 3-1 were excised from the gel of 0 hour and 72 hour of fermentation with *A. oryzae* ATC 16868 and subjected to *in-gel* tryptic digestion as described in section 3.2.8 and analyzed by LC-MS/MS. The raw data was converted to a pkl file and subjected to database search by Mascot search engine (www.matrixscience.com) for protein identification. The main proteins found by Mascot in the bands were ovalbumin (gi|129293) and ovalbumin-related protein Y (gi|71897377) which confirmed previous assumptions.

Hirose et al. (2006) conducted a study on the allergic properties of ovomucoid carbohydrate moieties and unknown contaminant allergens revealing that specific antibodies could also react and bind to carbohydrate sections and identified the contaminant to be a potentially allergic protein ovalbumin-related protein Y with an approximate molecular weight of 52 kDa. This confirms our observation of the 52 kDa band that shifted during fermentation to be ovalbumin-related protein Y. According to Figure 3-1 the unknown protein was at that

approximate molecular weight and shifted to a lower molecular weight after 48 hours of fermentation. Their study also revealed that ovalbumin-related protein Y is a unique glycoprotein with similar amino acid sequence to ovalbumin and a carbohydrate moiety similar to ovomucoid. This suggests that *A. oryzae* strains could alter the potentially allergy causing ovalbumin-related protein Y to decrease allergy without apparent changes in SDS-PAGE.

After the identities of the proteins were confirmed by Mascot, the predicted tryptic peptides from both proteins were searched in the raw data of both samples for any differences that may have resulted from fermentation. The base peak ion chromatograms are presented in Figure 3-2. There was no difference in the presence of the peptides from ovalbumin (*data not shown*). However, there was a difference in peptides from ovalbumin-related protein Y before and after fermentation: the relative intensity of peptides from the C-terminus of this protein in *A. oryzae* ATC 16868 fermented sample was very low in qualitative comparison to the relative intensity of the peptides before fermentation. Though this is not a quantitative way to compare between samples, this may suggest that a decrease in the molecular weight of the band from Figure 3-1 resulted from degradation of the C-terminus of this protein. It was noted that there was no difference in intesnity in both the N-terminus and central region of the protein. A representative mass spectra showing the peptide ^{363}ADHPFLFFIR^{372} that eluted between 34.5-36.5 min is presented in Figure 3-3. The peptide can be seen in both singly charged form at *m/z* of 1262.7 and doubly charged form at *m/z* of 631.9 in the control sample (a). These peaks show very

low intensity in the *A. oryzae* 16868 fermented sample at 72 hours (b). The MS/MS of peptide ^{363}ADHPFLFFIR^{372} from the 0 hour fermentation sample by *A. oryzae* 16868 is shown in Figure 3-4. The figure gives confirmation that the peptide which shows decrease after 72 hours of fermentation is indeed the peptide matched by MASCOT from the tryptic digest. Other peptides located in the C-terminal portion of ovalbumin-related protein Y also showed significant decrease as a result of fermentation. These peptides are presented in Table 3-1. Although this is not an absolute measure of the amount of ovalbumin-related protein Y before and after fermentation it does indicate qualitatively that there is evidence of degradation. Unfortunately it was impossible to determine the exact amino acid residue at which degradation commenced due to the presence of a large tryptic glycopeptides located towards the N-terminus from peptide ^{354}HSLELEEFR^{362}.

Ovalbumin-related protein Y has an amino acid sequence similar to ovalbumin and four potential glycosylation sites at N^{95}, N^{215}, N^{293} and N^{312} that are similar to the glycans of ovomucoid (Hirose et al., 2006). It is possible that the presence of the glycan at N^{312} prevented the protein from further degradation towards the N-terminus. Sequences at the N-terminal and the center of the protein did not show significant differences between the samples. A representative mass spectrum of peptide ^{125}FYTGGV EEVNFK^{136} from the centre of the protein is shown in Figure 3-5. As can be seen there is no significant difference between the two samples showing no evidence of protein degradation.

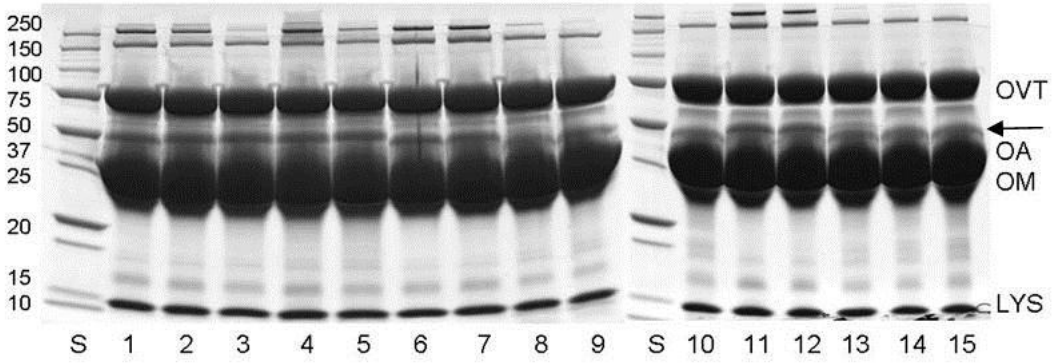

Figure 3-1: SDS-PAGE analysis of egg white proteins during fermentation with either *L. delbrueckii* or *A. oryzae* strains. The arrow represents ovalbumin-related protein Y, OVT ovotransferrin, OA ovalbumin, OM ovomucoid, LYS lysozyme. S: standard marker; 1-5: *L. delbrueckii* 0 - 96 hours, 6-10: *A. oryzae* ATCC 1011 0 – 96 hours, 11-15: *A. oryzae* ATCC 16868 0 – 96 hours.

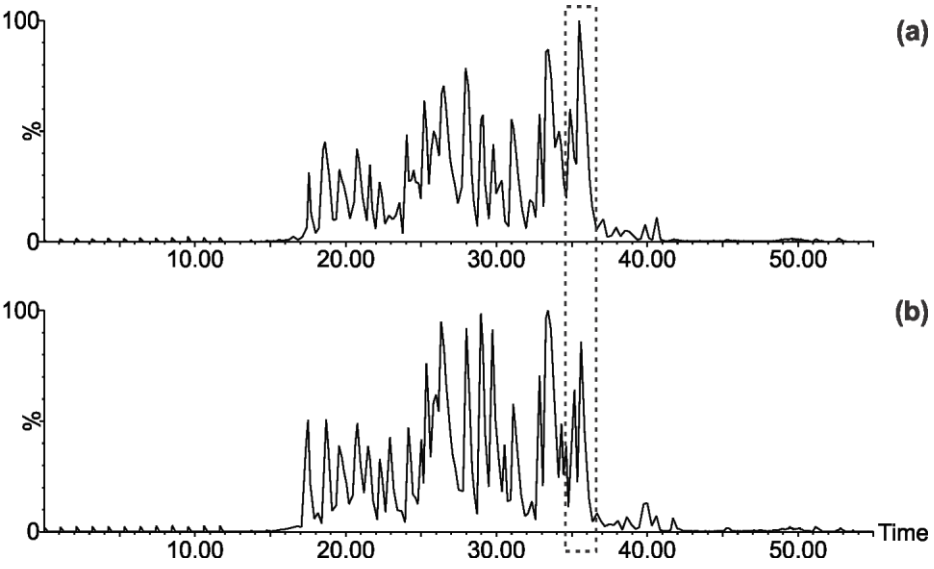

Figure 3-2: Base peak ion chromatograms of the 0 hour fermentation sample (a) and 72 hour fermentation sample (b), showing the region where peptides ^{363}ADHPFLFFIR^{372} and ^{373}YNPTNAILFFGR^{384} eluted.

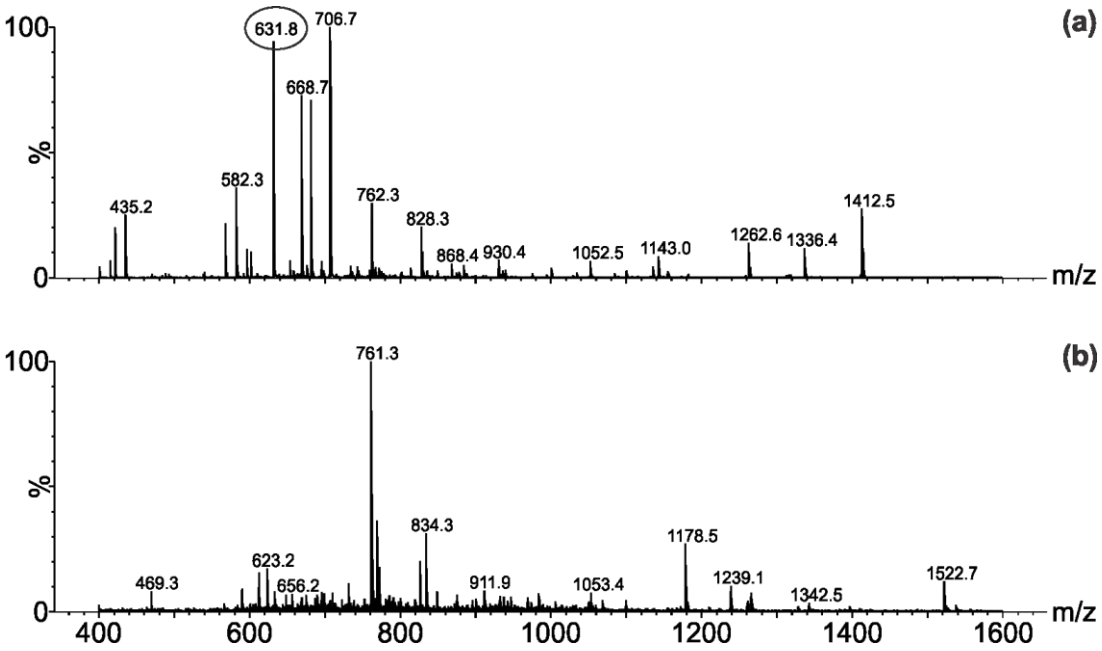

Figure 3-3: MS spectrum of peptides that eluted between 34.5 to 36.5 min in 0 hour fermentation sample (a) and 72 hour fermentation sample (b). The peptide ^{363}ADHPFLFFIR^{372} can be seen in a singly charged form at *m/z* of 1262.6 and doubly charged form at *m/z* of 631.8. These peaks are very small in (b). A decrease in the peptide ^{373}YNPTNAILFFGR^{384} in a singly charged form at *m/*z of 1412.5 and doubly charged form at 706.7 can also be seen.

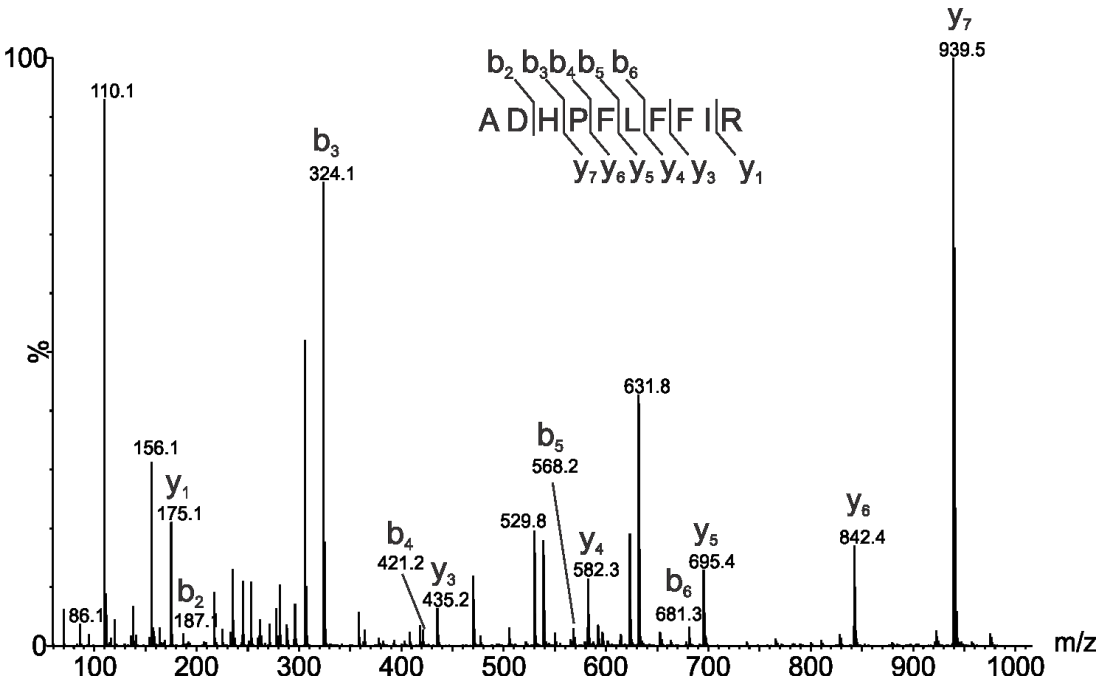

Figure 3-4: MS/MS of peptide ^{363}ADHPFLFFIR^{372} from 0 hour fermentation sample that shows its sequence. The y (positive charge from the C-terminus) and b ions (positive charge from N-terminus) that were identified are shown.

Table 3-1: Peptides located in the C-terminus that decreased as a result of fermentation with *A. oryzae* 16868 after 72 hours.

Peptide Sequence	**Mass (observed)**	**Elution time (min)**
^{354}HSLELEEFR^{362}	1159.6^{+} 580.3^{2+}	24-26
^{363}ADHPFLFFIR^{372}	1262.7^{+} 631.8^{2+}	34.5-36.5
^{373}YNPTNAILFFGR^{384}	1412.7^{+} 706.7^{2+}	35-37

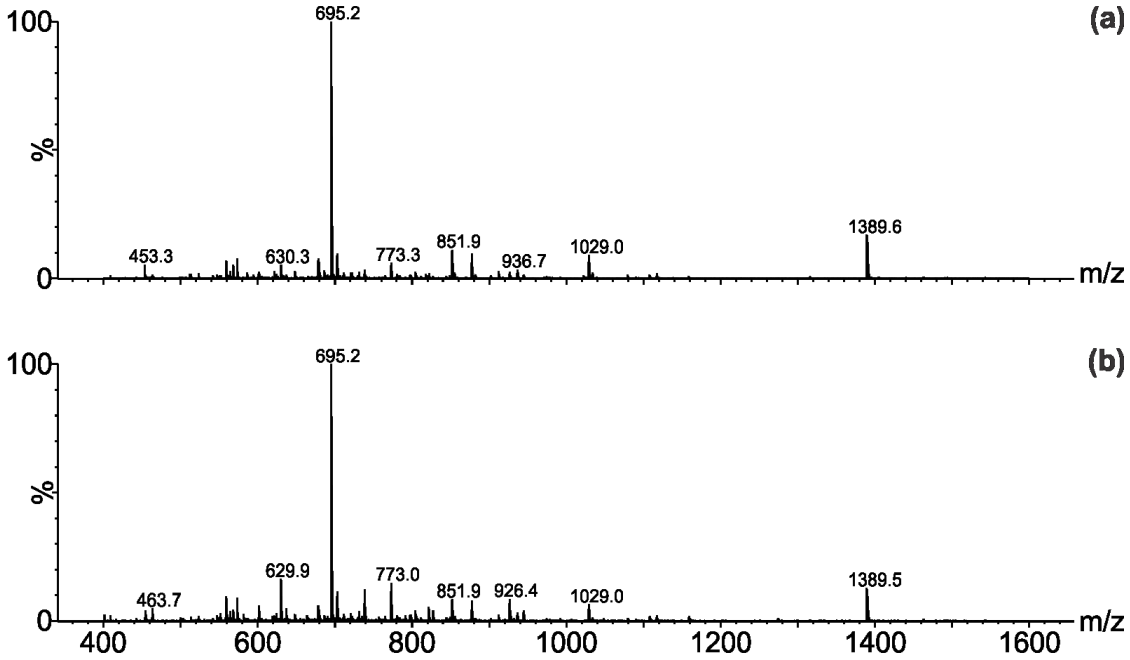

Figure 3-5: Mass spectrum of peptides that eluted between 27.7-28.4 min in (a) 0 hour fermentation and (b) 72 hour fermentation with *A. oryzae* 16868, showing the doubly charged form of the peptide ^{125}FYTGGVEEVNFK^{136} at m/z of 695.2 and its singly charged form at m/z of 1389.5. No difference in the peptide can be seen between the two samples.

3.3.2 Changes in Allergenicity during Egg White Fermentation. Changes of allergenicity after fermentation were analyzed by western blot and ELISA. Western blot results in Figure 3-6 show that there is no change according to individual proteins as seen in the SDS-PAGE (Figure 3-1). However, a decrease in IgE binding activity was seen with the indirect ELISA analysis in *A. oryzae* strains fermented egg whites but not in *L. delbrueckii* fermented one. Figure 3-7 shows a decrease in IgE binding activity at different fermentation times with the greatest reduction at 72 and 96 hours depending on patient plasma for *A. oryzae*

strains. It seems that that shift in the ovalbumin-related protein Y may be responsible for a decrease in IgE binding. Ovalbumin-related protein Y makes up 5% of total egg white protein (w/w) and has not been widely studied (Hirose et al., 2006). Recently it has been identified as a potentially allergenic protein because of its unique characteristics. Ovalbumin-related protein Y has an amino acid sequence highly homologous to ovalbumin and carbohydrate moieties similar to ovomucoid (Hirose et al., 2006). These characteristics suggest that ovalbumin-related protein Y should also exhibit similar antigenic properties similar to both ovalbumin and ovomucoid which are both major egg white allergens.

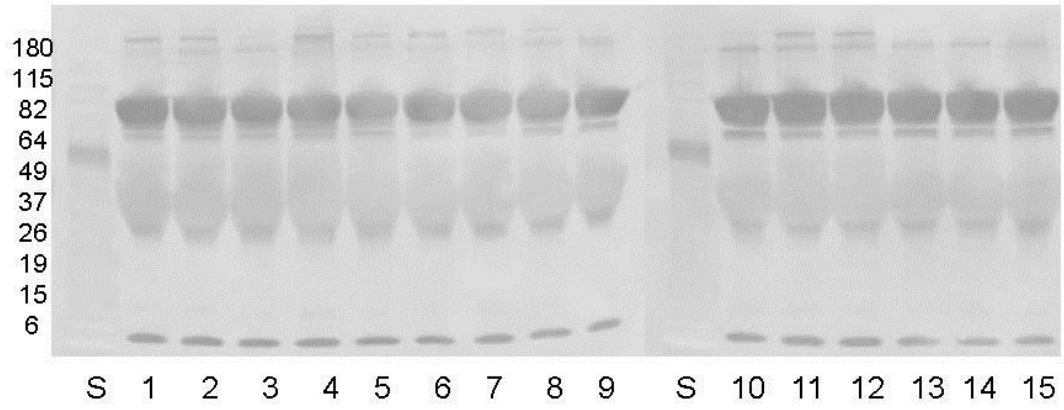

Figure 3-6: Western blot analysis of egg white solutions during fermentation with *L. delbrueckii*, *A. oryzae* 1011 and *A. oryzae* 16868. S: pre-stained protein marker, 1-5: *L. delbrueckii* 0h – 96h, 6-10: *A. oryzae* 1011 0h – 96h, 11-15: *A. oryzae* 16868 0h – 96h. Experiments were fermented in triplicates in two separate analyses.

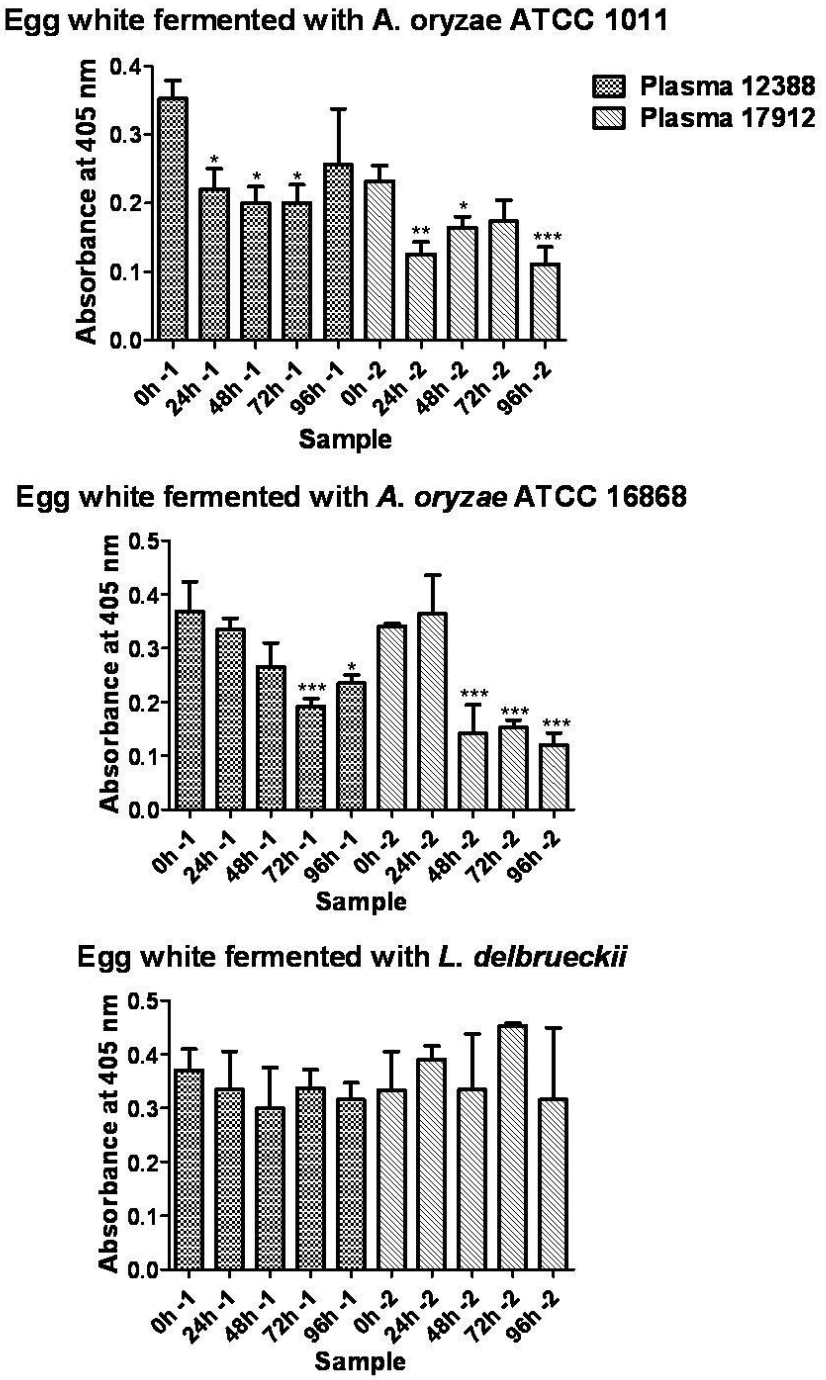

Figure 3-7: Indirect ELISA analysis of egg white solution fermented with *L. delbruekii* and *A. oryzae* strains. Experiments were fermented in triplicates in two

separate analyses. Statistical analysis was carried out using ANOVA followed by Tukey's multiple comparison test ($p<0.05$).

3.3.3 Changes in Ovomucoid after Fermentation. The ovomucoid in nutrient broth was fermented to 96 hours sampling every 24 hours with *L. delbrueckii*, *A. oryzae* ATCC 1011, and *A. oryzae* 16868. Effects of fermentation on protein degradation were evaluated and results are shown in Figure 3-8. Ovomucoid is shown at a molecular weight of 28 kDa with no significant changes in any of the fermentations and no new low molecular weight compounds were noted. These results come to agreement with Figure 3-1 of egg white fermentations. This indicates that there is no apparent protein degradation and no enzyme production by the *A. oryzae* and *L. delbrueckii* strains. The extracted ovomucoid was not very pure therefore other egg white proteins were also incorporated into the fermentation. Figure 3-8 does not show changes in protein degradation with any of the other egg white proteins either.

From previous unpublished work in our laboratory it was suspected that ovomucoid may have been slightly altered to result in a decrease in allergenicity. Since ovomucoid is the dominant egg white allergen further investigation was done. In Figure 3-8 the protein bands from 0 hours to 96 hours are the same in molecular weight and size showing no apparent degradation in any of the three strains. It is not a surprise that the strains were unable to utilize ovomucoid as a substrate for fermentation. A study was conducted using many strains of *Bacteroides* to ferment glycoproteins and mucopolysaccharides including

ovomucoid. The results indicated that some strains were able to ferment certain mucopolysaccharides but none could ferment ovomucoid (Salyers, Vercellotti, West, & Wilkins, 1977). The same group conducted a similar study with 154 strains from 22 species dominating the human gut including *Bifidobacterium* and *Lactobacillus* to ferment 21 different substrates including ovomucoid (Salyers, Vercellotti, West, & Wilkins, 1977; Salyers, West, Vercellotti, & Wilkins, 1977). Results showed that ovomucoid was one of the 11 substrates not fermented by any of the strains.

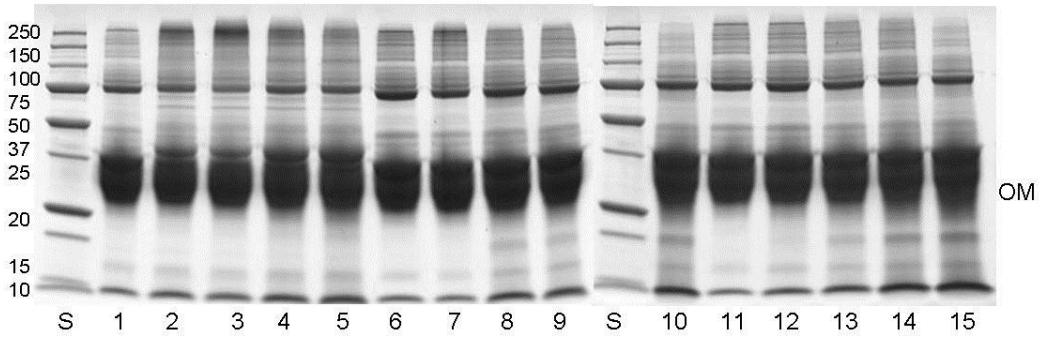

Figure 3-8: SDS-PAGE analysis of ovomucoid fermentation with either *L. delbrueckii* or *A. oryzae* strains. OM ovomucoid. S: standard marker; 1-5: *L. delbrueckii* 0 - 96 hours, 6-10: *A. oryzae* ATCC 1011 0 – 96 hours, 11-15: *A. oryzae* ATCC 16868 0 – 96 hours.

3.3.4 Changes in Allergenicity during Ovomucoid Fermentation. Ovomucoid was sampled at 0, 24, 48, 72, and 96 hours for western blot and ELISA analysis to determine changes in allergenicity. Western blot results in

Figure 3-9 show that there is no change according to individual proteins as seen in the SDS-PAGE in the previous figure. Surprisingly a decrease in IgE binding ability was seen with the indirect ELISA analysis in *A. oryzae* strains fermented ovomucoid. Figure 3-10 shows a decrease in allergenicity at different fermentation times with greatest reduction at 72 and 96 hours depending on patient plasma for *A. oryzae* strains but no significant changes with *L. delbrueckii* fermented ovomucoid. During the egg white fermentation with *L. delbrueckii* it was suspected that no protein degradation and/or decrease in allergenicity occurred because of the presence of lysozyme partially inhibiting the organism to grow and secrete to its full potential. However, during the ovomucoid fermentation, a significant decrease in allergenicity starting at 24 hours of fermentation up to 96 hours of fermentation was observed since most of the lysozyme was removed during ovomucoid extraction. In the two *A. oryzae* strains a significant decrease in allergenicity was also observed although not much as in the egg white fermentation possibly due to the absence of ovalbumin-related protein Y.

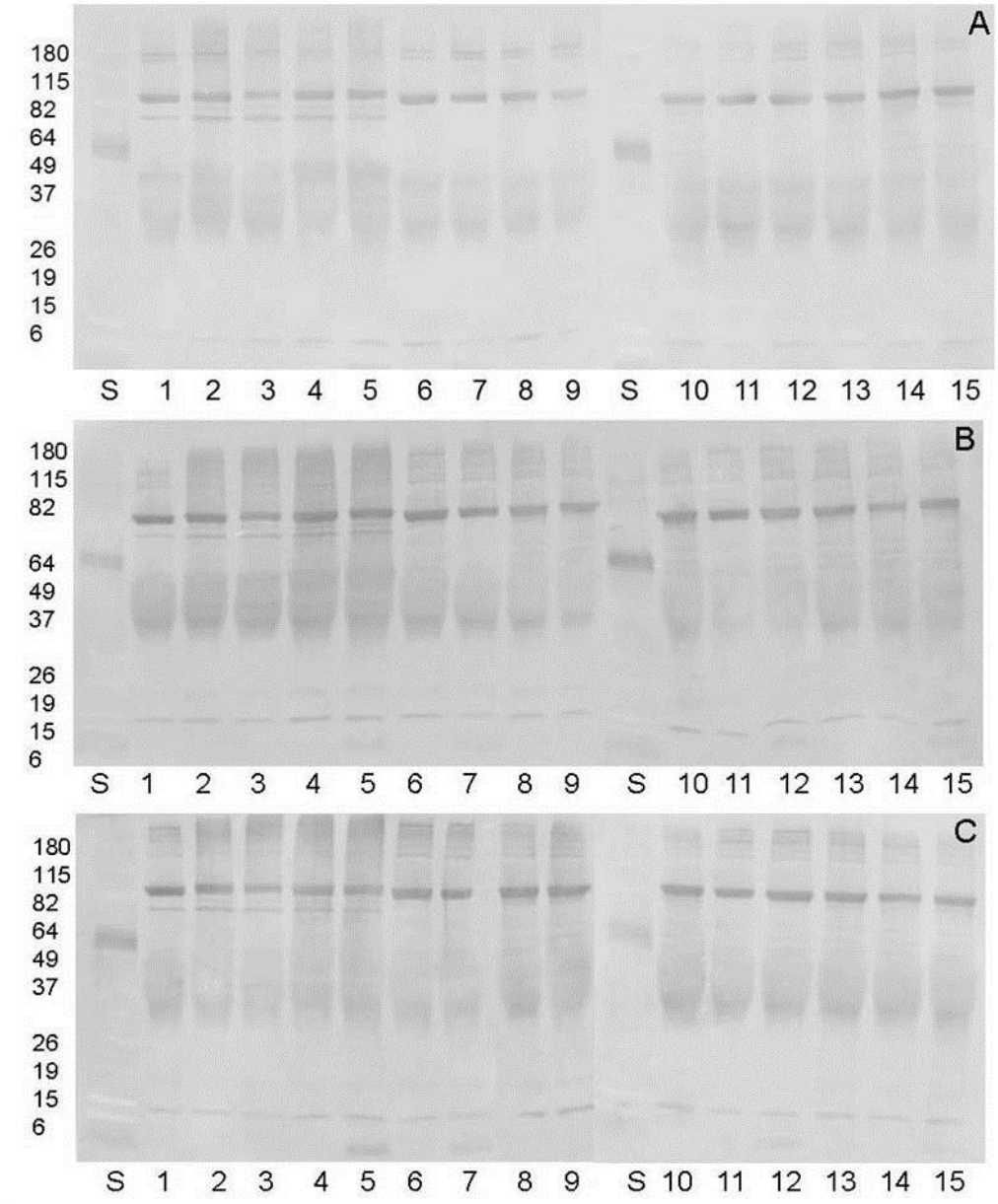

Figure 3-9: Western blot analysis of ovomucoid during fermentation with *L. delbrueckii*, *A. oryzae* 1011 and *A. oryzae* 16868. A: plasma 12388, B: plasma 14982, C: plasma 17912. S: pre-stained protein marker, 1-5: *L. delbrueckii* 0h – 96h, 6-10: *A. oryzae* 1011 0h – 96h, 11-15: *A. oryzae* 16868 0h – 96h. Experiments were fermented in triplicates in two separate analyses.

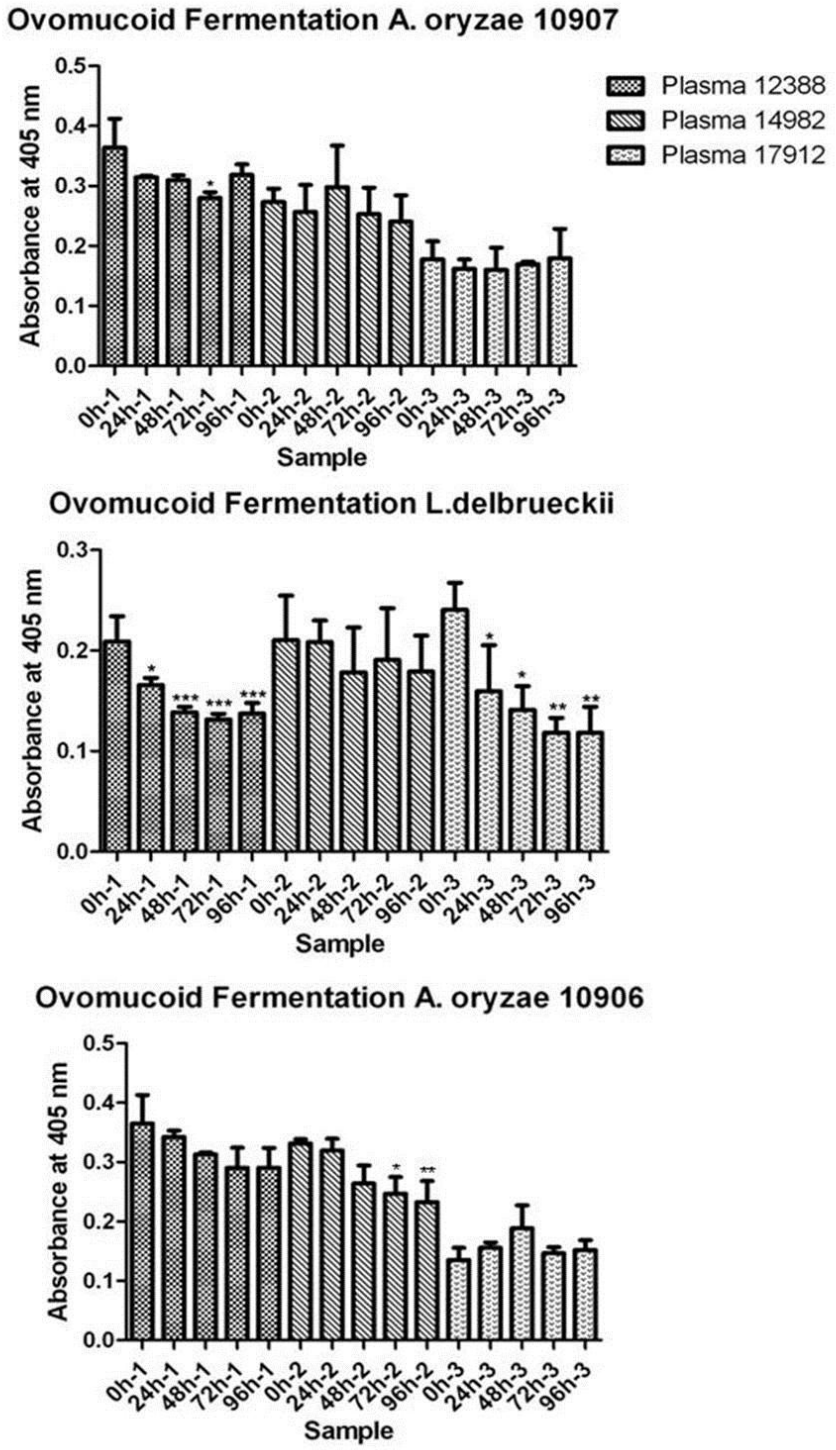

Figure 3-10: Indirect ELISA analysis of the ovomucoid fermented with *L. delbruekii* and *A. oryzae* strains. Experiments were fermented in triplicates in two separate analyses. Statistical analysis was carried out using ANOVA followed by

Tukey's multiple comparison test ($p<0.05$); 0h, 24h, 48h, 72h, and 96h represents sampling time during fermentation; -1, -2, -3 represents different plasmas.

3.3.5 Ovomucoid Hydrolysis Peptides. Three different enzymes were used to perform enzymatic hydrolysis on fermented and unfermented ovomucoid samples. Unfermented ovomucoid extracted according to section 3.2.4 were compared with ovomucoid fermented in nutrient broth with *L. delbrueckii* and *A. oryzae* strains to investigate fermentation as a pretreatment regarding susceptibility to hydrolysis. Figure 3-11 shows SDS-PAGE analysis of protein degradation during hydrolysis. Comparing unfermented to fermented samples with any of the three strains, there is no apparent difference with the effects of fermentation as a pretreatment to the susceptibility of protein digestion. It appears that both pepsin (B) and fungal protease (A) have the ability to digest ovomucoid into small molecular weight compounds where the star is shown. Fungal protease was the most successful at hydrolyzing ovomucoid for unfermented and fermented samples, leaving only low molecular weight compounds. The commercial fungal protease used in this experiment was purchased from Sigma and was produced from *Aspergillus oryzae.* This suggests that potentially the fermentations outlined and conducted should also be able to degrade ovomucoid. In the pepsin digestion of ovomucoid some protein or protein fragments are still present with a lesser amount of new low molecular weight compounds formed. It is suspected that even after pepsin digestion with partial elimination of ovomucoid an allergic response can still be evoked. This can be seen in allergic reactions because natural digestion in the stomach with pepsin is not enough to

destroy the epitope to enter the blood stream and cause symptoms. Rye malt (C) is a flour mixture which did not degrade the proteins; digestion time may have been too short to alter any of the egg white proteins. Rye malt fermentation is most commonly used with sourdough and bread making (Salyers et al., 1977).

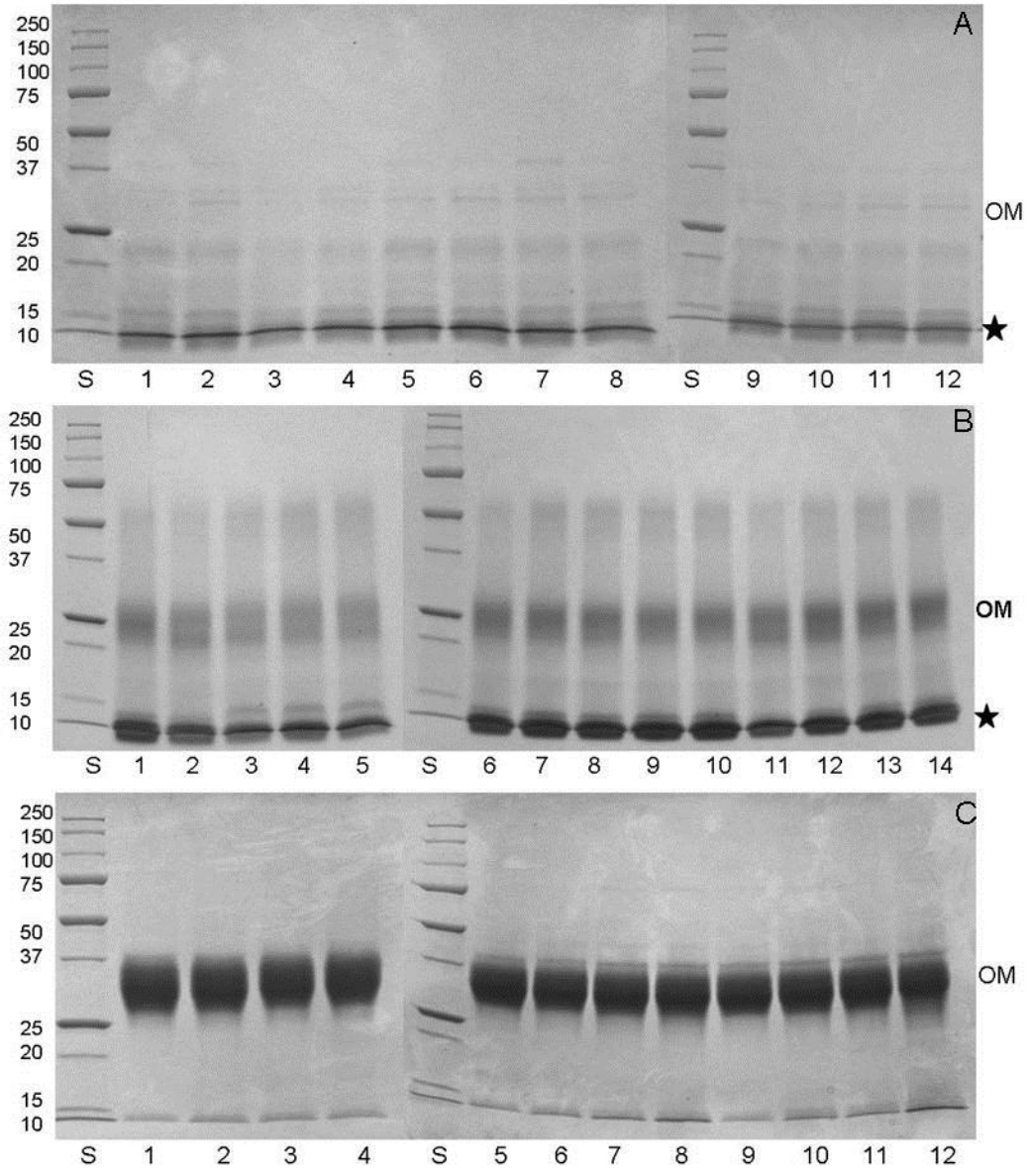

Figure 3-11: SDS-PAGE analysis of ovomucoid fermentation compared with unfermented ovomucoid after hydrolysis with fungal protease (A), pepsin (B), or

rye malt (C). OM: ovomucoid. The star represents new low molecular weight compounds that were formed during hydrolysis. S: standard marker. A1: unfermented ovomucoid; A2-A4: *L. delbrueckii* 0h, 48h, 72h; A5-A8: *A. oryzae* ATCC 1011 0h, 48h, 72h, 96h; A9-A12: *A. oryzae* ATCC 16868 0h, 48h, 72h, 96h. B1: unfermented ovomucoid; B2-B5: *L. delbrueckii* 0h, 48h, 72h, 96h; B6: unfermented ovomucoid; B7-B10: *A. oryzae* ATCC 1011 0h, 48h, 72h, 96h; B11-A14: *A. oryzae* ATCC 16868 0h, 48h, 72h, 96h. C1: unfermented ovomucoid; C2-C4: *L. delbrueckii* 0h, 48h, 72h; C5-C8: *A. oryzae* ATCC 1011 0h, 48h, 72h, 96h; C9-C12: *A. oryzae* ATCC 16868 0h, 48h, 72h, 96h.

3.4 Conclusion

The use of egg white and ovomucoid as a nutrient medium to ferment with different microorganisms to reduce allergy could be an effective method. In this study the two *Aspergillus oryzae* strains showed potential in significantly reducing allergenicity in different patient plasmas with minimal changes seen in SDS-PAGE and western blot but significant differences in ELISA. Different environments (supplementations or extractions) should be explored to create the right environment to support growth but also press the microorganisms to produce and secrete enzymes. Only in this way will there be effective protein degradation to in turn reduce allergenicity. Ovomucoid is an extremely tenacious protein proving very difficult to ferment by many studies including the current one (Heini ö et al., 2003; Rizzello, Cassone, Di Cagno, & Gobbetti, 2008). Perhaps a different approach such as pretreatments with food safe denaturing agents,

sonications or heat treatment may yield a more suitable substrate for fermentation though this study shows that fermentation alone as a pretreatment does not have significant effect on altering the protein. Ovalbumin-related protein Y is not a very widely studied protein although it makes up approximately 5% (w/w) of egg white protein (Salyers et al., 1977; Salyers et al., 1977). The MS results give a good indication of some important properties of this protein and further in depth analysis of peptide sequences where cleavage may occur could help to characterize this type of protein. Allergenicity studies should also be conducted since this protein is highly suspicious of being allergenic. Ovalbumin-related protein Y is one of the major contaminants found in commercial ovalbumin so it is of importance to investigate its allergic potential.

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Chapter 4: Final Remarks

4.1 Summary of Present Research

4.1.1 Effects of Lactic Acid Bacteria Fermentation on Egg White Allergenicity in Sourdough. Sourdough fermentation in a one stage process was conducted with different lactic acid bacteria to observe changes in allergic proteins and IgE binding capacity. *L. sanfranciscensis* and *L. delbreuckii* have the ability to degrade ovotransferrin completely after 24 hours of fermentation. Single protein allergenicity shown by western blot analysis indicates allergy to ovotransferrin alone was eliminated. However overall allergenicity shown by ELISA indicated that there was no significant difference before and after sourdough fermentation. Following fermentation the sourdough was mixed with fresh ingredients to bake bread. Samples were taken at each bread making step. After baking western blot analysis showed that the egg white proteins were no longer allergenic in both the *L. sanfranciscensis* and *L. delreuckii* fermented sourdoughs as well as the control sourdough.

4.1.2 Effects of Fermentation in Egg White and Ovomucoid on Egg White Allergenicity. Fermentation was conducted with *L. delbrueckii* and *A. oryzae* strains in both egg white solutions and ovomucoid. Fermentation was carried out until 96 hours. Egg white fermentation with *L. delbrueckii* showed no significant different while *A. oryzae* showed a decrease in IgE binding after 24 hours. However western blot analysis showed no significant difference in IgE binding. A minor shift of ovalbumin-related protein Y was noted after 48 hours of fermentation in *A. oryzae* fermented strains and LC-MS/MS analysis confirmed

that there was partial degradation of this protein near the C-terminus. Fermented ovomucoid showed a significant difference in IgE binding with all three strains used but showed no difference in SDS-PAGE for reasons that have not been identified. Fermentation did not have any effect on susceptibility to enzymatic hydrolysis with commercial enzymes indicating a poor choice of pre-treatment method.

4.2 Applications and Further Research

Lactic acid bacteria fermentation of allergic proteins has been reported in literature. However, there is no study on the effect of fermentation on allergic egg white proteins. Since egg whites are used in a wide variety of food products there is a need to develop a hypoallergenic egg product, preserving its excellent functional properties to be used in food commodities. Currently there is no cure or treatment for egg allergy besides avoidance of the food all together. Results and further research may lead to an innovative technology in producing hypoallergenic egg products.

Further research from the first portion of the study may include a more in depth analysis of the thiol exchange reactions that are happening during fermentation. Investigation of effects with the addition of chemical additives known to block free sulfhydryl groups such as N-ethylmaleimide could give more information on the reactions during fermentation (Loponen et al., 2008). The use of different types of flours such as rye malt may incorporate a new selection of

enzymes into the sourdough. This may lead to different exchange reactions during fermentation and possibly the degradation of other egg white proteins.

During the egg white solution fermentation in the second portion of the study further in depth study of ovalbumin-related protein Y needs to be conducted. This protein consists of 5% (w/w) of egg white proteins and is highly suspicious of being allergenic (Schurer et al., 2007). ELISA and western blot analysis should be carried out to further characterize the allergic potential of this protein. The use of ovomucoid fermentation as a pre-treatment before protein hydrolysis proved to have no affect during pepsin, rye malt and fungal protease hydrolysis. Other pre-treatment methods such as sonication a high frequency may be enough to potentially lyse the bacterial cells to release enzymes into the solution. However a different nutrient broth could be the key in successfully degrading egg white proteins during fermentation. A more limited nutrient environment may push strains used to produce their own enzymes to be released into the solution.

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