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Reduce the IgE Binding Ability of Egg White Proteins by Fermentation

by

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Abstract

Egg is one of the major food allergens that affects 1.6~3.2% of the infants and young children population. The objective of this study is to reduce the egg white IgE binding ability by lactobacilli or *Aspergillus oryzae* fermentation. Modifications of egg white proteins during fermentation were analyzed by Ninhydrin method, Ellman method, SDS-PAGE, ELISA, and MALDI-TOF-MS. Tryptone supplementation and acidification are necessary to grow lactobacilli in egg white. Egg whites were fermented by *L sanfranciscensis*, *L. sakei*, and *L. delbrueckii* subsp. *delbrueckii* individually for 96 h; and *Aspergillus oryzae* for 120 h. The IgE binding ability of egg white was significantly reduced (~50%) by *L. delbrueckii* subsp. *delbrueckii* after 48 h of incubation and almost eliminated by *Aspergillus oryzae* after 24 h of inoculation. In addition to slight modification of ovomucoid (the dominant egg allergen), no substantial protein degradation was observed during fermentation.

KEY WORDS: Egg white, IgE binding ability, Lactobacilli, *Aspergillus oryzae*, Ovomucoid.

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CHAPTER 1 LITERATURE REVIEW

1.1. FOOD ALLERGY

Food allergies are illnesses that occur in some individuals as a result of abnormal immunological responses to a particular food or food component, which are usually caused by a naturally occurring protein (Mekori, 1996; Bush & Hefle, 1996). There are two broad types of adverse reactions to food. One is induced by the toxic or pharmacological component in food, while the other one is caused by the nontoxic constituents of food (Vieluf et al., 2002). The reactions towards nontoxic components can also be divided into two types based on whether or not immune system is involved. Non-allergic reaction, also known as food intolerance, does not directly involve the immune system. One common example of a non-allergic reaction is lactose intolerance, which results from the inability to digest the lactose. Allergic reaction is based on the function of the immune system, which can result in many clinical symptoms or disorders (Mine, 2008). Food allergic reactions can be broadly divided into two types: immunoglobulin-E (IgE) mediated and non-IgE mediated reactions, which is dependent on whether or not IgE is involved in the immune responses. IgE mediated food allergic reactions are type I food hypersensitivity, which usually happen immediately after an allergen is ingested and are the most widely recognized tissue damage disorders (Elgert, 2009). Normally, Immunoglobulin E is produced upon pathogen invasion. However, it could also be generated by the infection of innocuous substance such as food components in the atopic individuals (Bannon, 2004).

Gastrointestinal ingestion is one of the most important routes of food allergy besides cutaneous contact or mucosal exposure. In this route, food allergens are initially digested in stomach, absorbed into small intestine, and then flow throughout the body via the circulatory system before triggering an immune response. The gastrointestinal tract is responsible for absorbing nutrients and defending the host. The large surface area and thin epithelium of the gut favor the absorbing function (MacDonald & Monteleone, 2005). High permeability of the gut mucosa in atopic patients might lead to the absorption of intact food allergens into the body, which explains why genetically regulated epithelial permeability is critical in arousing the allergic disease (Hilsden, Meddings, & Sutherland, 1996).

Type-I food hypersensitivity involves two phases: the sensitization phase that takes place when a food allergen is ingested (the first time immune response is induced) and the activation phase that occurs in all subsequent ingestions of the same food allergen. When food allergens are transported from the mucosa of the gut barrier to lymphoid tissues, the major histocompatibility complex (MHC) molecules in antigen presenting cell (APC) may identify those allergens as potential detrimental foreign substances and bind and carry them to T-cell. T-cell can only recognize and bind to allergens that are associated with MHC molecules through T cell receptors (TCRs). After binding, the T-cell will produce many types of cytokines, such as interleukins-4 (IL-4), which will stimulate the B-cell to generate allergen-specific IgE. However, B-cell allergenic epitopes may be different from T-cell allergenic epitopes: T-cell epitopes are related to the sensitization while B-cell epitopes are associated with IgE binding and clinical

hypersensitivity. Some of the activated B-cells will become memory cells and stay in the body for many years. Large amounts of the IgE initially produced will bind to the mast cells. Mast cells are present in most tissues; a high concentration of mast cells may be found in skin and the mucosa of lung and digestive tract (Hannigan, Moore, & Quinn, 2009; Elgert, 2009). The binding of allergen-specific IgE with the mast cells completes the sensitization phase. In fact, the number of mast cells in gut mucosa is higher in food allergy patients than in healthy people (Bischoff, 1996). The sensitization phase would not induce any reactions; but severe and harmful reactions occur in the subsequent exposures, the activation phase. When an allergic patient consumes the same food allergen after sensitization, the food allergen will bind to IgE that are already bound to the mast cells and several IgE on one mast cell will bind to the same allergen (cross-linking of IgE). This cross-linking of IgE by allergen can rapidly degranulate the mast cell. During this process, the mast cell can release many inflammatory mediators, such as histamine, which are directly responsible for the clinical symptoms of food allergy by increasing vascular permeability and resulting in transient muscle contraction (Hannigan, Moore, & Quinn, 2009; Elgert, 2009;Wershil et al., 1996; Mills et al., 2003).

The explanation for the increasing numbers of food allergy incidences in developed countries includes the T_H1 versus T_H2 hypothesis (Matricardi & Bonini, 2000) and the theory of anti- inflammatory axis with a powerful regulatory T cell network (Rook et al., 2000). T_H1 versus T_H2 theory explains that: due to the improved hygiene, vaccination, and use of antibiotics in developed

countries; the opportunity of exposure to bacterial and viral pathogens in early childhood is reduced, which leads to the insufficient inducement for T helper 1 cell (T_H1, associated with bacterial and viral infections and autoimmune diseases) that cannot counterbalance the development of T_H2 (associated with helminth infections and allergic diseases) (Matricardi & Bonini, 2000; Yazdanbakhsh, Kremsner, & van Ree, 2002)

Most current researches support the following regulatory T cell theory. Regulatory T cells are stimulated by dendritic cells under the low hygiene environment with a high pathogen burden. Interleukin 10 (IL-10), produced by the regulatory T cells (Maloy & Powrie, 2001), is the essential constitute of a strong anti-inflammatory network because it is capable of inhibiting mast cell degranulation (Royer et al., 2001). The inflammatory T cells (both T_H1 and T_H2) and their downstream effectors are restrained by the anti-inflammatory network that involves the inhibitory cytokines from regulatory T cells (Yazdanbakhsh, Kremsner, & van Ree, 2002). Several studies in developed countries confirmed that anti-inflammatory cytokines play an essential role in allergic diseases: the amount of IL-10 from T cells increased substantially in patients with successful immunotherapy (Akdis et al., 1998) and the IL-10 in atopic children also increased after they receiving probiotic (Pessi et al., 2000). The high hygiene environment accompanying with the low pathogen burden in industrialized countries may limit and/or inhibit the development of regulatory T cells and further stop the generation of inhibitory cytokines, such as IL-10, which is

probably responsible for the increasing numbers of allergy occurrences in these countries.

1.2. FOOD ALLERGY PREVALENCE

In the United States, around 6% of children and 3.7% of adults have food allergies (Sampson, 2004). According to the European Community Respiratory Health Survey, out of 17,280 adults in 15 countries, food intolerance or food allergy occurred up to 12% of all the respondents and their prevalence ranged from 4.6% in Spain to 19.1% in Australia (Woods & Abramson, 2001). In France, 3.5% of 33,110 people claimed to have experienced food allergic symptoms (Kanny et al., 2001). A study in the UK revealed that 1.8% of 22,000 people are allergic towards eight selected foods (Young & Stoneham, 1994).

According to the US Food and Drug Administration (FDA), milk, eggs, fish, shellfish, tree nuts, peanuts, wheat, and soybeans are responsible for more than 90% of the food allergic reactions. Young children and infants are most likely to be sensitive to milk (2.5% allergy prevalence), eggs (1.3%), peanut (0.8%), wheat (approximately 0.4%), soy (approximately 0.4%), tree nuts (0.2%), and fish (0.1%) (Wood, 2003). The most common food allergens for adults are shellfish (2%), peanut (0.6%), tree nuts (0.5%), and fish (0.4%) (Sicherer & Sampson, 2006). Food allergy incidences, mediated in the digestion tract, decrease from 10% at 1 year of age, to 2% at 6 years of age (Strobel, 1997). Most patients outgrow their food allergy by school age. One of the reasons for this phenomenon is that their gastrointestinal tract becomes mature: a more acidic condition in the stomach favors the activity of pepsin and a lower permeability of

small intestinal mucosa may restrict the absorption of certain allergens (Wood, 2003; Yamada et al., 2000). However, an allergy to peanuts is more of a life-long threat to anaphylaxis patients (King et al., 2005). Severe anaphylactic reactions to food rarely occur in infants and young children and mostly occur between 17 to 27 years of age, especially in individuals with asthma (Joneja, 2007).

1.2.1. Egg allergy prevalence

As the determination methods and/or definitions differ, the estimate of egg allergy prevalence ranges from 1.6% to 3.2% (Hill, Hosking, & Heine, 1999; Osterballe et al., 2005; Pereira et al., 2005). Egg allergies typically occur in the first two years of life and two thirds of the infants become tolerant to egg allergens by seven years of age (Heine, Laske, & Hill, 2006). In Spain, 355 children with Immunoglobulin E (IgE)-mediated food allergies were diagnosed for the prevalence of different food allergens. According to the result, the principal allergenic foods were eggs, fish, and cow's milk (Crespo et al., 1995). Some infants can develop allergic symptoms upon their first ingestion of egg, which could happen in breastfed children. Small doses of allergens in the breast milk may be responsible for the egg sensitization. Some infants were born with egg specific IgE in their blood, so sensitization may even occur in uterus (Caffarelli et al., 1995).

Atopic dermatitis (AD) is a chronic, relapsing, and inflammatory disease (Guillet & Guillet, 1992). Among the children who suffer from severe AD, two-thirds of them are sensitized to egg (Sampson, 1997). In Sweden, one study examined 94 children with AD for food sensitization until they were 7 years old.

Among these 94 children, 46 out of 58 food allergic patients showed detectable levels of specific IgE for egg, which is the most common food sensitizer. All patients developed allergic reactions towards eggs before 3 years of age, and approximately 56% of them outgrew the allergy by the age of 7 (Gustafsson, Sjoberg, & Foucard, 2003). In Korea, a study investigated the food allergy prevalence in AD patients. The levels of IgE antibodies specific for eggs, milk, peanut, and soybean in the serum of 266 patients was measured. Eighty seven patients showed a high level of IgE that is specific for egg, 12 for cow's milk, 8 for peanut, and 3 for soybean. The specific IgE antibodies for these four food antigens were higher in infants who are less than one year old and were also higher in moderate to severe AD patients who were infants and young children (Han et al., 2004).

Escudero et al. (2003) studied 4 bakery workers who have occupational respiratory symptoms when egg components are inhaled. In this trial, all of the workers reacted positively to skin prick tests and possessed specific IgE against egg white proteins. They also concluded that people who are exposed to airborne egg proteins, such as bakery and confectionery workers, have the potential to develop egg allergy followed by occupational asthma.

1.3. FOOD ALLERGY SYMPTOMS

The food allergy threshold doses of patients who suffer from severe symptoms are generally lower than patients with mild symptoms (Sicherer & Leung, 2008). Several individual factors can regulate the clinical reactions of immediate food hypersensitivities, such as the amount of specific IgE, the

quantities of released mediators, and the sensitivity of target organs towards the mediators (most important) (Caffarelli et al., 1995; Martorell et al., 2001; Sicherer & Leung, 2008).

IgE mediated immune responses induce the acute symptoms that occur in the skin, the gastrointestinal tract, the respiratory tract, or even the cardiovascular system; those symptoms usually happens within minutes or hours after consuming the food allergens. Skin, gastrointestinal, and respiratory symptoms are more frequent than symptoms of cardiovascular system (Wershil et al., 1996). Cutaneous symptoms occur in more than 80% of food allergic reactions, and acute urticaria and angioedema are the most common skin manifestations. Allergic food reactions may induce rhinorhea, sneezing, nasal congestion, and pruritis to the upper respiratory system. Wheezing, coughing, and laryngospasm that may need medical treatment or have the potential to be life-threatening may occur as well (Perry, Scurlock, & Jones, 2006). The intensity of IgE mediated immune responses may vary from temperate to life-threatening. Non-IgE mediated food allergic reactions can be mediated by T-cells, which can be rapid, sub-acute, or chronic, primarily affect the gastrointestinal tract and cause the profound vomiting and diarrhoea (Wershil et al., 1996; Joneja, 2007).

Both pollen-food allergy syndrome and immediate gastrointestinal hypersensitivity may belong to the IgE mediated food allergy. The former syndrome includes rapid onset of pruritus and angioedema of the lips, tongue, palate, and throat. The latter varies and may include nausea, abdominal pain, cramping, vomiting, and/or diarrhoea (Perry, Scurlock, & Jones, 2006).

The symptoms of type I hypersensitivity occur immediately or within a few minutes after ingesting food. Normally, a weaker allergic reaction takes place if symptoms occur long after the ingestion of food. The intensive responses always involve the high mucosa permeability in patients that are severely allergic to a food source (Joneja, 2007). However, some very serious food allergic responses may not happen immediately after ingestion, but will be delayed when the food allergens are mixed with fat, as antigens are slowly released with the interference of fat. In that case, more antigens would be consumed by the patients. When all of the allergenic substances are finally exposed to the intestinal mucosa after the food is hydrolyzed, more serious clinical symptoms would be induced (Grimshaw et al., 2003).

1.3.1. Egg allergy symptoms

Egg hypersensitivities are possibly cutaneous, respiratory, and gastrointestinal symptoms (Ford & Taylor, 1982). The clinical symptoms of egg allergies involve: urticaria, angioedema and atopic dermatitis on the skin; asthma and rhinocojonctivitis of respiratory system; and vomiting and diarrhoea of gastrointestinal system (Martorell, et al., 2001).

Some children will outgrow their egg allergy and for these patients eggs should be eaten regularly to maintain tolerance once they stop causing allergic reactions (Joneja, 2007). Patients can be classified into either the resolving or the persisting group, based on whether they are likely to outgrow egg allergy. In the resolving group, patients have either cutaneous or gastrointestinal symptoms. The patients in the persisting group are more likely to suffer from multisystem

disorders, among which angioedema and respiratory symptoms are most common. If an allergic patient exhibits angioedema and respiratory symptoms, displays multiple system involvement, and shows consistent positive results to the egg skin-prick test; he/she is probably in persisting group (Ford & Taylor, 1982).

Consuming egg is not the only way to stimulate hypersensitivity reactions, as direct contact with the skin can also trigger clinical symptoms. Among patients who suffer from urticaria when their skin came in contact with an egg, 75% of them developed allergic reactions after oral ingestion (Caffarelli et al., 1995). If IgE antibodies of patients recognize pepsin, chymotrypsin, and trypsin susceptible epitopes of ovomucoid, the dominant egg allergen; they are more likely to have skin contact urticaria, but can eat eggs without allergic reactions (Yamada et al., 2000).

1.4. FOOD ALLERGENS

The identification of allergens is their IgE reactivity (skin test sensitization or diffusing of specific IgE) even without inducing clinical symptoms towards sensitive individuals; although the clinical hypersensitivity (allergic reactions or symptoms) is important in allergic history of patients (Johansson et al., 2001). Food allergens can be classified into two types: complete and incomplete allergens. Complete food allergens have the ability to sensitize allergenic individuals and induce clinical symptoms; while incomplete food allergens can only elicit clinical symptoms of sensitized individuals. The allergenic characteristic of an incomplete food allergen comes from their homology with other complete allergens (Aalberse, 1997). Since only proteins can stimulate T-

cell responses, allergens are usually proteins or the combination of proteins and polysaccharides (Elgert, 2009).

Proteins are made of up to 20 different amino acids. The sites on proteins that can bind to IgE are called allergenic epitopes. There are two types of epitopes: linear and conformational. The binding ability of linear epitopes is based on the integrity of their primary sequences. Conformational epitopes are made up of several separated peptide chains, which may be quite distant in their primary sequences but are linked loosely by three-dimensional structures (Van Regenmortel, 1992). The secondary or tertiary structures of the conformational epitopes are critical for their binding capacity to IgE (Bannon, 2004). Linear epitopes are, to some extent, stable when they are going through heat treatment and partial proteolysis (Dominguez et al., 1990). Most of the aeroallergen-mediated allergic reactions are caused by conformational epitopes. Linear epitopes are prevalent in food allergens, because they can be presented to the immune system after digestion in the gastrointestinal tract (Bannon, 2004).

Essentially, food allergens contain certain epitopes and their integral structures will protect those epitopes from being cleaved or destroyed during food processing and/or human digestion. The side chains of amino acids along the primary sequence of proteins are fundamentally important, as they serve as the constituents of allergenic sequences and dictate the protein to create a compact structure to protect those sequences through numerous interactions based on the components of side chains.

1.4.1. Characteristics of food allergens

1.4.1.1. Homology of allergens to human proteins

The specific protein in an individual organism has a constant and unique amino acids sequence, which is encoded by genes. The proteins that have similar functions in different species usually have related, but not identical amino acid sequences. This is probably due to variation in evolution. Many proteins conserved certain sequences during evolution and these sequences always represent stable functions (Matthews, Freedland, & Miesfeld, 1997). As the immune system of human has the ability to distinguish between self-proteins and non-self proteins, proteins that can trigger the human immune system are recognized as non-self substances and their primary sequence composition is usually different from human proteins. Little similarity to a human's proteome is an attribute that will induce immune reactions (Kanduc, Lucchese, & Mittelman, 2001). The more homology of human's proteome proteins possess, the more likely they would be tolerated by human immune system. There is no α_{S2}-casein proteome in the human gene and only 12% of α_{S2}-casein sequences are similar with human proteins. The α_{S1}-casein and β-casein sequences are more similar to human proteins than α_{S2}-casein; so α_{S2}-casein, α_{S1}-casein, and β-caseins IgE exist in 90%, 55%, and 15% of sera from infants who are allergic to milk, respectively. If the homology of animal and human protein is greater than 63%, these animal proteins will probably not induce immune reactions with the exception of bovine serum albumin, a cow's milk allergen (Natale et al., 2004; Jenkins, Breiteneder, & Mills, 2007). The homology between human proteome and plant proteins is far

less than animal proteins; therefore, plant proteins are more likely to be allergenic to human.

1.4.1.2. Conformational structures of protein

The conformational structures of protein, no matter whether secondary, tertiary, or quaternary structures, are originally controlled by the amino acids composition and the primary sequence. For example, the hydrophobic molecules will aggregate in water to reduce the surface area that is exposed to the water molecules (Matthews, Freedland, & Miesfeld, 1997). Although there are many protease cleaving sites along the primary sequence of protein, the structure of protein may be so compact that it is difficult for the protease to access these sites unless the protein is denatured (Bannon, 2004). The prolamin superfamily includes the prolamin storage proteins of cereals, non specific lipid transfer proteins, α-amylase inhibitors, and 2S albumins; the cupin superfamily contains 11S and 7S globulin storage proteins and cysteine proteases. Proteins in these two families above have been proven to be related to the allergic reactions in human digestion tract (Mills et al., 2003). The stability of cupin and prolamin superfamilies that are caused by the proteins conformational folding can prevent them from been hydrolyzed by enzymes in digestion tract, which helps the cupin and prolamin retain their allergenicity (Jenkins et al., 2005).

1.4.1.3. Disulfide bonds of proteins

Proteins may have one or more than one peptide chains, which are usually linked by non-covalent forces or covalent cystine cross-links (disulfide bounds) (Matthews, Freedland, & Miesfeld, 1997). Both intra-chain and inter-chain

disulfide bonds can strengthen the three-dimensional structure of proteins and make the proteins stable against heat and chemical treatments (Breiteneder & Mills, 2005). For example, 2S albumins which possess 4 disulfide bonds, exhibit very good thermo-stability and are resistant to protease and chemical modifications (Dominguez et al., 1990). Ara h2 is one of the major peanut allergens and its overall structure is greatly changed when the disulfide bonds are cleaved (Sen et al., 2002). The β-sheet and β-turn configuration can help the Ara h2 maintain its structure, keeping its order after the disulfides bonds are reduced. However, an Ara h2 with reduced disulfide bonds is more sensitive to digestion, which suggests that its overall allergenicity has been reduced (Bannon, 2004). The aspartyl protease, a gastrointestinal enzyme, will act on 6 to 8 residues of the polypeptide chain of substrates. It is easier for this enzyme to react with substrates that have flexible structures, while the disulfide bonds will increase the stability and compactness of proteins (Fontana et al., 1986).

1.4.1.4. Ligands binding proteins

Many protein allergens can bind ligands, such as metal ions and lipids. Some metal ions can go inside proteins and become integrated within them. After binding with a ligand, the general mobility of the polypeptide backbone of a protein will be reduced. In that case, proteins are more resistant to heating and proteolysis and their allergenicity will diminish very little. One example of this integration is the nonspecific lipid-transfer in wheat protein (Douliez et al., 2001; Breiteneder & Mills, 2005). When these ions are lost, the folded protein structure will be lost and the polypeptides will become more motile, resulting in the protein

becoming partially folded. It is easier for protease to hydrolyze proteins with high flexibility. The mobility of the polypeptides will be inhibited when proteins are bound with ligands; as a result, they will become more stable to thermal and proteolysis treatment (Breiteneder & Mills, 2005). The ability of binding ligands also relies on the side chain formation of amino acids in the proteins.

1.4.1.5. Rheomorphic proteins

Rheomorphic proteins, such as caseins, are dynamic. In their secondary structure, polypeptide chains are in equilibrium with each other and their structures can be unfolded, denatured, or partially folded (Holt & Sawyer, 1993). Rheomorphic proteins will not change their structure from a conventional state to a denatured state during the heating process, thus the thermo-stable epitopes in these proteins will maintain their activity (Breiteneder & Mills, 2005). The conformational epitopes in rheomorhic proteins can hardly be damaged by protein structural changes.

1.4.2. Egg white allergens

Egg white is composed of 88% water and 9.7~10.6% (w/w) proteins. The major proteins in egg white include: ovalbumin (54%, w/w of dry matter), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), lysozyme (3.4%), G2 globulin (4%), G3 globulin (4%), ovoinhibitor (1.5%), ovoglycoprotein (1%), ovoflavoprotein (0.8%), ovomacroglobulin (0.5%), cystatin (0.05%), and avidin (0.05%) (Mine, 2007). Carbohydrate, lipids, and minerals account for 0.4~0.9%, 0.03%, and 0.5~0.6% of the dry matter of egg white, respectively. Egg white contains 4 of 5 major egg allergens that have been characterized as Gal d 1-5:

ovomucoid (Gal d 1), ovalbumin (Gal d 2), ovotransferrin (Gal d 3), and lysozyme (Gal d 4). Gal d 5, α-livetin, is in egg yolk.

1.4.2.1. Gal d1—Ovomucoid

Ovomucoid, a glycoprotein, consists of 186 amino acids and has a molecular weight (MW) of 28 kDa (Lin & Feeney, 1972; Kato et al., 1987). The three tandem domains of ovomucoid are all homologous to the pancreatic secretary trypsin inhibitor and each of them has either an actual or putative site that is accountable for the inhibition of serine protease. Peptide bound, Arg89~Ala90, in the second domain is the major reactive site for inhibiting the bovine β-trypsin (Kato et al., 1987). Although ovomucoid contains no inter-domain disulfide bonds, each domain is cross-linked by three intra-domain disulfide bonds. Due to intra-domain disulfide bonds, ovomucoid is very resistant to heat denaturation and digestive enzymes, such as pepsin (Matsuda, Watanabe, & Nakamura, 1982; Kato et al., 1987). The intra-domain disulfide bonds in ovomucoid help this protein to maintain its compact structure in each motif, which favours maintaining epitopes of this protein. One may speculate that if certain treatments can break the disulfide bonds in ovomucoid, the allergenicity of this protein may be diminished.

The main saccahrides in ovomucoid, N-acetylglucosamine (GlcNAc), mannose, galactose, and N-acetylneuramic acid (NeuNAc) represent around 14~16, 6.5~8.5, 0.5~4, and 0.04~2.2% of its total molecular weight, respectively. The entire carbohydrate moieties of ovomucoid comprise up to 25-30% of its total molecular weight. In ovomucoid, Asn10, Asn53, Asn69, Asn75, and Asn175 are

glycosylated (Beeley, 1971; Kato et al., 1987). Compared to the natural ovomucoid, the deglycosylated form is very sensitive to heating, but has the ability to retain its trypsin inhibitory activity. This phenomenon indicates that the carbohydrate moiety contributes to the stabilization of ovomucoid against heat (Yamamoto et al., 1998). Chemical deglycosylated ovomucoid with molecular weight between 20.7 and 21.5 kDa possess as strong IgE binding ability as the natural form of this protein (Besler, Steinhart, & Paschke, 1997).

As a heat-stable protein, allergenicity of ovomucoid was not affected by heating at 100^{o}C (Deutsch & Morton, 1956; Urisu et al., 1997). Denaturing ovomucoid at acidic conditions is more difficult than at an alkaline environment (Lineweaver & Murray, 1947). After peptic digestion, the fragments of ovomucoid still preserve their allergenic properties (Kovacs-Nolan et al., 2000). The optimum pH for peptic digestion of ovomucoid in raw egg white and coagulated egg white are 1.5~2.5 and 1.5~2, respectively; therefore pepsin cannot digest ovomucoid at a higher pH. Infants' predisposition to egg allergies may be associated with insufficient pepsin secretion and a relatively high pH in their stomach, pH 3 (Yoshino et al., 2004).

Although some egg allergy patients possess a high concentration of IgE that can bind to egg white proteins, they do not suffer from clinical symptoms with ingestion. The result of an oral challenge test is the standard method in dividing patients into positive and negative symptom group. Patients from the positive group have much more IgE antibodies towards ovomucoid fragments that are digested by pepsin than those from negative group. This IgE can be used as a

diagnostic indicator for patients with symptoms and who are unlikely to outgrow egg allergy (Urisu et al., 1999). A study revealed that patients with persistent egg allergies contain a significantly higher concentration of antibodies against HPLC-purified ovomucoid than those who outgrow clinical symptoms but both groups had similar amount of antibodies against ovalbumin (Bernhiselbroadbent et al., 1994). Antibodies from mice prepared by commercial grade egg ovalbumin can react with commercial grade ovalbumin and ovomucoid, HPLC-purified ovomucoid but not HPLC-purified ovalbumin (Bernhiselbroadbent et al., 1994). Their research again confirmed that the ovomucoid is the dominant egg allergen.

Kato et al. (2001) kneaded dough with eggs and boiled the dough in 1% NaCl solution. They assumed that after kneading for 30 min, ovomucoid was insolublilized in the dough as it did not exist in either boiled dough or the NaCl buffer. They suggest that the kneading/benching process polymerizes the ovomucoid with the wheat proteins and the disulfide exchange reactions between those proteins make the ovomucoid become insoluble during heating. Wheat proteins, gliadin and/or glutenin, may exchange disulfide with ovomucoid (Kato, Ozawa, & Matsuda, 2001).

1.4.2.2. Gal d2—Ovalbumin

Ovalbumin, with a molecular weight (MW) of 45 kDa, is composed of 386 amino acids and constitutes 54% of the total egg white proteins. The single carbohydrate moiety that is linked to Asn293 makes ovalbumin a glycoprotein. As the only egg white protein that contains free SH groups, ovalbumin has 6 cysteine residues and one disulfide bond between Cys74 and Cys121 (Nakai, 1989).

Although ovalbumin has 6 cysteine residues and its amino acids composition has the potential to form a compact structure, its primary sequence does not aid in forming many disulfide bounds, which would strengthen its natural configuration. Even though ovalbumin possesses many linear allergenic sequences, they can hardly sustain their integration during heat treatment and/or digestion. On the other hand, some epitopes in ovalbumin are conformational, as they can denature upon thermal treatment.

Mine & Rupa (2003) discovered the regions of major linear epitopes in ovalbumin and this research indicates that the critical amino acids in these regions are primarily hydrophobic, polar, and charged amino acids. Most linear epitopes in ovalbumin are of β-sheet and β-turn structures, except for the α-helix of Asp95~Ala102.

Kim et al. (2002) has shown that heat diminishes the allergenicity of ovalbumin. When ovalbumin is exposed to 75 ^{o}C, it lost some binding ability to IgG. When the temperature reaches 80 ^{o}C, the IgE from the egg allergic patients can not bind to the ovalbumin properly. The ELISA shows that the binding ability of heated ovalbumin is lower than ovalbumin without a heat treatment.

1.4.2.3. Gal d3—Ovotransferrin

Ovotransferrin, with a molecular weight of 77,000 Da, contains 12 disulfide bonds and 2.6% carbohydrate moieties. One mole ovotransferrin can bind with two moles metal ions (Mine & Rupa, 2004). Ovotransferrin is not considered as the dominant egg allergen except that Aabin et al. (1996) who reported the number of IgE that were bound to ovotransferrin and ovomucoid was

higher than the number bound to ovalbumin and lysozyme. Information on the allergenicity of this protein is limited.

1.4.2.4. Gal d4—Lysozyme

Egg white lysozyme, with a molecular weight of 14,300 Da, contains 129 amino acids and four disulfide bonds (Mine & Rupa, 2004a). Lysozyme possesses antimicrobial activity, especially towards the Gram-positive bacteria. This is due to its cleavage of the β-1, 4-glycosidic linkage between N-acetylmuraminic acid and N-acetylglucosamine in the polysaccharide components in the cell wall of Gram-positive bacteria (Mine, 2008).

Since lysozyme has been used as a food additive; special attention should be paid to prevent egg allergic patients from consuming these products (Martorell et al., 2001). According to WHO-FAO, lysozyme is innocuous and has been used in the cheese industry as an unlabelled additive to prevent the growth of anaerobic bacteria. Yamada et al. (1993) in Japan ascertained the allergenicity of lysozyme against patients with egg allergies. Among 39 egg allergic patients, 30 of them have IgE against lysozyme. Fremont et al. (1997) conducted research to detect the percentage of egg allergic patients who are sensitized to lysozyme and their results showed that 35% of 52 patients possess lysozyme specific IgE. These data suggest that lysozyme maybe an important allergen and should be identified in the label of food product.

1.5. REDUCE PROTEINS' ALLERGENICITY BY FOOD PROCESSING

The purposes of food processing are to inactivate microbes and enzymes to extend the shelf life of a product, to improve the flavor and color of a food product, and to enhance the suitability for specific applications (Sathe & Sharma, 2009; Poms & Anklam, 2004). Food processing can disrupt, modify, and denature the proteins in the food matrix, and therefore can diminish, enhance, or have no impact on their allergenicity (Sathe, Teuber, & Roux, 2005). Some neoallergens might be generated during food processing, as some allergic patients can tolerant un-processed food rather than their corresponding processed products (Sathe, Teuber & Roux, 2005; Leduc et al., 2003). However, food processing is more commonly associated with allergenicity reduction as the disruption, modification, and denaturation of processed proteins causes damage to the conformational epitopes (Sathe, Teuber, & Roux, 2005).

Allergenicity reduction requires food processing to damage both linear and conformational epitopes. Linear and conformational epitopes on allergens are the positions that can be recognized by IgE. Linear epitopes normally contain 8-10 amino acids residues and some of which are named as critical amino acid residues. Those residues may be more important in determining the allergenic properties of the epitope, as they are the binding sites of epitopes against major histocompatibility complex (MHC) in antigen presenting cell (APC). Modifying, substituting, and/or deleting these critical amino acid residues is required in order to reduce or eliminate the allergenicity of linear epitopes. The changes of tertiary

and quaternary structure of proteins are very likely to alter their conformational epitopes. In order to reduce the allergenicity of egg white proteins by food processing, we need to find the most suitable treatment to deal with this liquid food matrix and destroy the linear and conformational epitopes in egg white allergens.

Food processing can be broadly classified into two types: thermal processing and non-thermal processing. Thermal processing includes: moist heating, dry heating, microwave, and pressure heating (autoclave); while non-thermal processing includes: irradiation, high hydrostatic pressure, refining, ultrafiltration, enzyme-catalyzed hydrolysis, and fermentation (Poms & Anklam, 2004; Sathe, Teuber, & Roux, 2005).

1.5.1. Refining and ultrafiltration

The purpose of refining is to extract and/or purify certain compounds from the food matrix. Ideally this process can get rid of the original accompanying components. For example, edible oil will become almost free of carbohydrates and proteins after refining (Poms & Anklam, 2004). Ultrafiltration employs membranes with specific pour sizes that selectively separate food components based on their molecular weight. In order to produce hypo-allergenic peach juice, ultrafiltration is used to remove the thermal stable peach allergens (Poms & Anklam, 2004; Brenna et al., 2000). Out of the 4 major egg allergens, ovalbumin constitutes 54% of the total egg white protein; therefore it is not applicable to reduce egg white allergenicity by removing certain allergens.

1.5.2. Irradiation

Irradiation has been used in food industry to deactivate microbes and enzymes for food preservation (Poms & Anklam, 2004). Although the conformational epitopes in proteins may be destroyed, the linear epitopes can still sustain their integrity even if the dose of irradiation is very high (Kume & Matsuda, 1995). Some buried linear epitopes in proteins may be further exposed during the irradiation treatment, which can enhance the proteins' allergenicity (Lee et al., 2002). After being exposed to irradiation, proteins may become aggregated and their solubility will decline (Poms & Anklam, 2004). The protein precipitate cannot benefit reducing the allergenicity and linear epitpoes in protein aggregate can be released after human digestion.

1.5.3. High hydrostatic pressure

High hydrostatic pressures (HHP) is an isostatic pressing process to treat food (liquid or solid) above 100 MPa and up to 900 MPa, generally ranging from 400 to 700 MPa at commercial uses (Martín et al., 2002). No or almost no nutrients or sensory attributes are lost during this process (Ludikhuyze et al., 2003). Globular proteins are denatured when high pressure is applied without the combination with high temperature (Privalov, 1990). After a high pressure treatment, only the tertiary structure of bovine gamma globulin (BGG) has been changed and at the same time the IgE binding capacity and allergenicity of BGG has been reduced (Yamamoto et al., 2010). Although HHP can damage the conformational epitopes by disrupting and/or denaturing a protein's structure, it

cannot impact the linear epitopes. The allergenicity of proteins may even increase as some buried linear epitopes are exposed during HHP. One example is that when the pressure rises from 200 MPa to 600 MPa, the allergenicity of β-lactoglobulin increases with the enhanced pressure (Kleber, Maier, & Hinrichs, 2007). High hydrostatic pressure can also induce the precipitate of egg white proteins. When the pressure rises from 200 MPa to 600MPa, the solule protein content in egg white declined from more than 80% to less than 40% (Van der Plancken, Van Loey, & Hendrickx, 2005). The functional value of egg white is lost after the proteins are precipitated, making HHP not an ideal food processing technique for egg white proteins.

1.5.4. Thermal processing

Although various thermal processing techniques have their own specific applications, they all essentially modify the allergenicity of proteins by denaturing. During heat processing, the structure of protein is modified considerably. The characteristic and extent of the proteins alteration is affected tremendously by the following factors: the intrinsic features of proteins, the temperature and length of the thermal treatments, and the physical-chemical conditions of the environment around proteins (Poms & Anklam, 2004). Wal (2003) stated that the progressive steps of protein denaturation during the heating process is as follows: loss of tertiary structure, revisable unfolding, loss of secondary structures (55-70 ^{o}C), cleavage of disulfide bounds (70-80 ^{o}C), formation of new intra-/inter-molecular interactions, rearrangements of disulfide bonds (80-90 ^{o}C), and then the formation of aggregates (90-100 ^{o}C). However,

these steps do not apply to every protein as one important property of allergens is heat-resistance (Sathe & Sharma, 2009; Davis, Smales & James, 2001).

1.5.5. Hydrolysis

Even though all of the conformational epitopes may be destroyed during thermal processing, irradiation, or high pressure treatment; linear epitopes are still present in the proteins. Although the denaturation of proteins has the potential to damage the conformational epitopes of proteins, the complete loss of all epitopes through those processes is not realistic (Sathe & Sharma, 2009). However, the enzymatic hydrolysis has the potential to disintegrate and remove the critical amino acid residues in the linear epitopes (Sathe & Sharma, 2009; Hannigan, Moore, & Quinn, 2009).

The modification of epitopes on proteins is an energy demanding process. The energy of activation (Ea) for protein denaturation is 350~700 kJ/mol; and the Ea for enzyme-catalyzed protein hydrolysis is 17~60 kJ/mol (Thijssen et al., 1977). Destroying or modifying the target epitopes on proteins through heating, irradiation, or high pressure, demands significant higher amount of energy to be consumed; compared to other treatments, enzymatic hydrolysis is a more energetically effective method to destroy target epitopes, especially linear epitopes (Sathe & Sharma, 2009). Although enzymatic hydrolysis may be the most efficient and effective way to damage some linear epitopes, it also has tremendous influence on the quality and acceptability of food products. Undesirable and/or unacceptable altered properties, such as structure, functionality, smell, and taste might be generated during the proteolysis (Sathe,

Teuber & Roux, 2005). This is evident in hypo-allergenic infant formulas that may give the unfavorable bitter taste as they contain hydrolyzates of enzyme digested whey proteins (Ena et al., 1995; Vegarud & Langsrud, 1989). The protein hydrolyzates normally provide the premier nutritional value to food products and can be used as amino acids and peptide additives, such as hydrolyzed β-lactoglobulin in infant formula. Small peptides such as di- and tripeptides or amino acids can be absorbed easier and are ready for use, compared to long chain proteins. However, the shorter peptides that are generated from enzyme hydrolysis are usually very small and almost lose all of the original functional properties of the protein. Although the allergenicity of original proteins have been greatly reduced or even eliminated through hydrolysis, the hydrolyzates lose the functional values that are required when they are added into certain food products.

1.5.6. Fermentation reduce proteins' allergenicity

Used as a food processing method, fermentation employs the capacities of specific microorganisms for preserving and giving flavor or color to food products. The main procedure of fermentation is to provide a carbohydrate and nitrogen source for microbes and control growing conditions, such as temperature and pH. Volatile fatty acids, alcohol, organic acids, and certain enzymes are the expected products generated by microorganisms during fermentation (Poms & Anklam, 2004). Lactic acid fermentation has been used for producing numerous types of dairy products. Fermentation of lactobacilli can affect the allergenicity of proteins in two aspects: cleaving the protein by proteases and modifying the

protein structure by decreasing the pH of the solution with lactic acid. The primary cause of milk allergy in infants and young children, β –lactoglobulin, compromises approximately 80% of whey proteins (Lee, 1992). The allergenicity of β-lactoglobulin in yogurt has been substantially reduced compared to that of unfermented milk (Ehn et al., 2004). Another study fermented β –lactoglobulin in sweet whey and skim milk with some lactic acid bacteria. The allergenicity of the sweet whey and skim milk was reduced for more than 70% and 90%, respectively (Kleber, Weyrich, & Hinrichs, 2006). This phenomenon results from the combination of a low pH and proteolytic activity of lactobacilli. The proteolytic system of lactic acid bacteria is critical for them to be able to grow in milk. The enzymes from lactobacilli may include proteases, endopeptidases, dipeptidases, tripeptidases, and aminopeptidases. Since tri- and dipeptides are generated from lactobacilli fermentation of milk proteins, some linear epitopes in milk allergens may possibly be destroyed (Law & Haandrikman, 1997). Electrostatic interaction is involved in dictating the conformational structure of proteins, in addition to hydrogen bond, Van der Waals force, and hydrophobic interactions. A shift of pH will result in the alternation of electrostatic interactions within a protein molecule, allowing its structure to be changed (Belitz, Grosch, & Schieberle, 2009). In that case, both conformational and linear epitopes in proteins have the potential to be modified. Furthermore, the use of microorganism demands relatively low energy input compared to other food processing methods.

Lactic acid fermentation has been used not only in the dairy industry, but also in other food material in an attempt to reduce their allergenicity. Soybean

contains approximately 33 allergenic proteins with a molecular weight ranging from 7.0 to 71 kDa, but only a few of them are responsible for more than 90% of the soybean allergenic reactions (Wilson, Blaschek & de Mejia, 2005). The immune-reactivity of soybean can be dramatically reduced up to 96% by the inoculation of *L.plantarum*, which has the potential to be used for producing hypoallergenic soybean products (Frias et al. 2008). Wheat proteins are one of the major food allergens and can be classified into 4 categories: albumins, globulins, glutenins, and gliadins. The IgE from wheat allergy patients can recognize the epitopes on all of these proteins, especially albumins, globulins, and gliadins (Palosuo et al., 1999). Selected lactic acid bacteria, *Lactobacillus sanfranciscensis* and *Lactobacillus plantarum*, rendered allergenic wheat proteins to be susceptible to digestive enzymes during sourdough fermentation (Di Cagno et al., 2008). In this case, the lactic acid bacteria disable the allergens and prevent an immune response by accelerating the hydrolysis of food allergens in the digestive tract before they are absorbed into intestinal mucosa.

In addition to the proteolytic system, other enzyme production of lactobacilli may also help reduce food allergenicity. Both intra-chain and inter-chain disulfide bonds can strengthen the three-dimensional structure of proteins and make proteins stable to heat and chemical treatments (Breiteneder & Mills, 2005). In other words, proteins are more likely to be denatured through cooking and digestion, and their allergenicity will be lost easier if their disulfide bonds are cleaved. *L. sanfranciscensis* reduces the disulfide bonds of gluten proteins into free thiol groups in sourdough fermentation (Vermeulen et al., 2006). This strain

can also cleave the disulfide bonds in ovotransferrin when egg white is added into sourdough (Loponen et al., 2008). In sourdough fermentation, *L. sanfranciscensis* produces a glutathione-reductase, reducing the extracellular low molecular weight GSSG to GSH, which reduces the disulfide bonds in gluten to free thiols (Vermeulen et al., 2006). Many egg white proteins contain disulfide bonds and the numbers of disulfide bonds found in the proteins of egg white are as follows: 1 in ovalbumin, 9 in ovomucoid, 15 in ovotransferrin, 9 in ovomucin, and 4 in lysozyme (Mine, 2008). It is reasonable to speculate that fermentation may also cleave the disulfide bonds in egg white proteins.

No excessive enzymes will be generated to hydrolyze proteins in food matrix during fermentation. Feedback repression is a widespread phenomenon in microorganism regulating the synthesis of amino acids that are used for building macromolecules such as enzymes. The cell will shut the biosynthetic pathway down and not waste energy making a superfluous pathway or compounds (Wang et al., 1949). Enzymes are generated only when they are critical for the survival of bacteria. In addition to the secretion of enzymes, microorganisms may also render some other stress upon food allergens to aid in the allergenicity reduction. We want to take advantage of the merit of fermentation for being able to provide appropriate amounts of enzymes and some unclear mechanisms to modify proteins. As fermentation has been successfully proven to be capable of reducing the allergenicity of other food components, we hope it may also exert the same effects to egg white proteins. Food allergenicity includes the ability of a food substance to induce an immune response and to react specifically with the

elements of the immune system they induced. In IgE mediated food allergic responses, IgE binding ability of the food substance is essential for its allergenicity. In our research, IgE binding ability of egg white proteins was measured to reflect the change of their allergenicity during fermentation.

1.6. OBJECTIVES

We hypothesize that enzymes produced in fermentation may modify egg white proteins and therefore reduce the IgE binding ability of egg white proteins. The overall objective of this study was to develop methods of preparing hypoallergenic egg products by fermentation. Specific objectives of this thesis were to:

- develop conditions to grow lactic acid bacteria in egg white
- screen lactic acid bacteria that might reduce IgE binding ability of egg white
- determine the effect of lactobacilli fermentation on IgE binding ability and structural changes of egg white proteins
- determine the effect of *Aspergillus oryze* fermentation on IgE binding ability and structural changes of egg white proteins

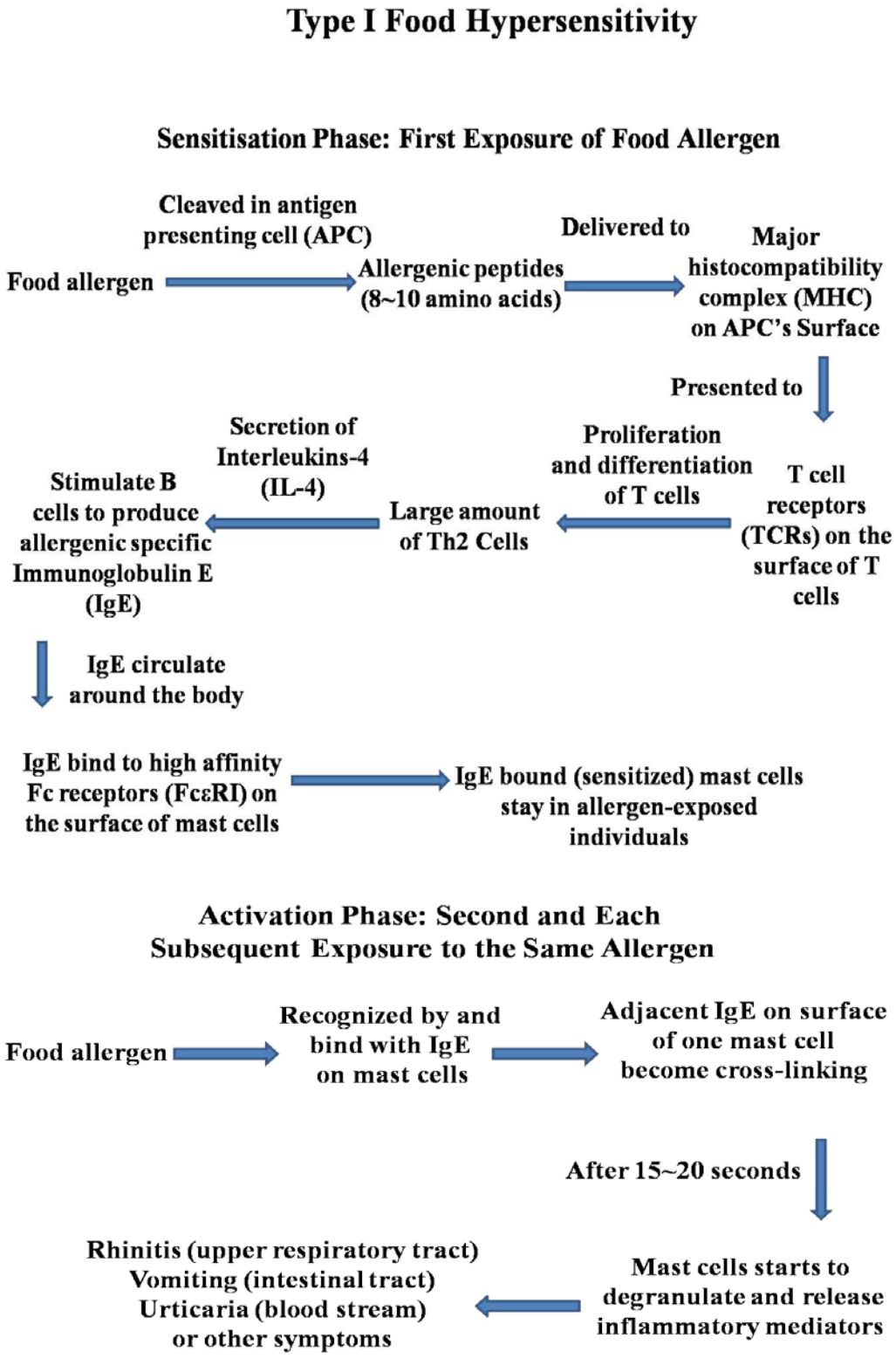

Figure 1. 1 Clinical mechanism of Type I food hypersensitivity

1.7. REFERENCES

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CHAPTER 2 EFFECT OF LACTOBACILLI FERMENTATION ON IGE BINDING ABILITY OF EGG WHITE PROTEINS

2.1. INTRODUCTION

Food allergy is an illness that occurs in some individuals as a result of abnormal immunological response to a particular food or food component, usually a food protein (Mekori, 1996; Bush & Hefle, 1996). IgE-mediated food allergy, also known as type-I food hypersensitivity, is the dominant type of food induced immunological response (King et al., 2005; Kagan, 2003). The occurrence of promoted specific IgE levels in serum is a principal indicator in type-I food hypersensitivity (Perelmutter, 2001). Interactions between food allergens and IgE lead to the release of many inflammatory factors, such as histamine, which are directly responsible for the clinical allergenic symptoms (Bradding et al., 1992; Till et al., 1997). Allergic symptoms, such as urticaria, angioedema, atopic dermatitis, and asthma are more frequent than vomiting and diarrhea. In rare cases, life threatening or fatal anaphylactic reactions can occur (Novembre et al., 1998). The other types, non-IgE mediated food allergies, which are usually sub-acute or chronic and primarily affect the gastrointestinal tract, can be mediated by T-cells (Wershil et al., 1996; Joneja, 2007).

As the determination methods and/or definitions differ, the estimated prevalence of egg allergy ranges from 1.6% to 3.2% in infants and young children (Hill, Hosking, & Heine, 1999; Osterballe et al., 2005; Pereira et al., 2005). Egg

allergy typically occurs mostly in the first two years of life and two thirds of the allergic infants will become tolerant after seven years of age (Heine, Laske, & Hill, 2006). The four major egg white allergens are: ovomucoid (Gal d1; 11%), ovalbumin (Gal d2, 55%), ovotransferrin (Gal d3, 12%), and lysozyme (Gal d4, 3%) (Perelmutter, 2001). Ovomucoid is a glycoprotein consisting of 186 amino acids with a molecular weight (MW) of 28 kDa (Lin & Feeney, 1972; Kato et al., 1987b; Urisu et al., 1997). Ovalbumin, with a MW of 45 kDa, is composed of 386 amino acids containing a single carbohydrate moiety and accounts for 54% of the total egg white proteins (Nakai, 1989). Ovotransferrin, with a MW of 77 kDa, contains 12 disulfide bonds and 2.6% carbohydrate moieties (Mine & Rupa, 2004b). Lysozyme, with a MW of 14.3 kDa, contains 129 amino acids and four disulfide bonds (Mine & Rupa, 2004a). It has been well established that ovomucoid is the dominant egg allergen (Bernhiselbroadbent et al., 1994; Urisu et al., 1999; Kovacs-Nolan et al., 2000).

Effects of food processing, such as moist heating, dry heating, ultrafiltration, irradiation, high hydrostatic pressure, enzymatic hydrolysis, and fermentation, on food IgE binding ability has been extensively explored (Sathe & Sharma, 2009; Poms & Anklam, 2004). Protease digestion is the most popular strategy for reducing the IgE binding ability of food allergen. However, extensive digestion of food allergens will result in the loss of protein functional properties, which will limit their applications in the food industry; therefore, preparing hypoallergenic egg products while maintaining their excellent functionality still remains to be a challenge. Recent studies showed that IgE binding ability of milk

proteins could be reduced by over 70% by fermentation of *L. acidophilus* and *S. thermophilus subsp. salivarius* (Kleber, Weyrich, & Hinrichs, 2006). Fermentation of soybean proteins by *L. plantarum*, commonly used in many fermented food products, was reported to reduce soybean IgE binding ability up to 96% (Frias et al., 2008). The IgE binding ability of wheat flour was reduced up to 80% by the fermentation of *L. brevis* MS-99 (Leszczynska et al., 2009). Lactic acid bacteria fermentation is widely used in food preparation and/or preservation. However, fermentation of egg white proteins has not been extensively studied. The high pH environment and the presence of several antimicrobial proteins lysozyme, avidin, and ovotransferrin, inhibit growth of bacteria in egg white (Yadav & Vadehra, 1977). The objective of the study was to determine if fermentation of lactobacilli could reduce the IgE binding ability of egg white with the aim to develop a hypoallergenic egg product. The effect of fermentation on the egg white proteins were evaluated using the Ellman method, Ninhydrin method, SDS-PAGE, enzyme-linked immunosorbent assay (ELISA), fluorescence FITC labeling, and MALDI-TOF-MS analysis.

2.2. MATERIALS AND METHODS

2.2.1. Materials

Hen eggs (lucerne^{TM} premiere large) were purchased from a local supermarket (Edmonton, AB, Canada). Plasma from egg allergy patients was obtained from PlasmaLab International (Everett, WA, USA). Ovomucoid was obtained from Neova (Abbotsford, BC, Canada). Agar, beef extract, malt extract,

peptone, tryptone, and yeast extract were purchased from Bacto™ (Sparks, MD, USA). Coomassie Brilliant Blue R-250, glycine, Laemmli sample buffer, Precision Plus Protein standards, and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad (Hercules, CA, USA). Acetic acid, D-glucose, K_2HPO_4, KH_2PO_4, methanol, $NaH_2PO_4.H_2O$, $Na_2HPO_4.7H_2O$, NaOH, and tris base were bought from Fisher (Ottawa, Canada). Ninhydrin, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB), β-mercaptoethanol, acetone, bovine serum albumin (BSA), cystein HCl, D-maltose, fluorescein isothiocyanate (FITC), fructose, glycine, goat-anti-human IgE conjugated with alkaline phosphate, KH_2PO_4, KIO_3, L- glutathione, $MgCl_2$, $MgSO_4$, NH_4Cl, p-nitrophenyl phosphate (pNPP), sodium carbonate, sodium carbonate-bicarbonate, and Tween-80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this research were obtained from Sigma-Aldrich and were of analytical grade.

2.2.2. Strains preparation

Lactobacillus sakei LS8 (Institute of Food Technology, University of Hohenheim, Stuttgart, Germany), *Lactobacillus sanfranciscensis* DSM20451, *Lactobacillus delbrueckii* subsp. *delbrueckii* (CHR Hansen), *Streptococcus thermophilus* (CHR Hansen), *Lactobacillus bulgaricus* subsp. *delbrueckii* (American Type Culture Collection (ATCC) 11842), *Streptococcus thermophilus* (SKYR2), and *Streptococcus thermophilus* (Danish yoghurt) were used in this research. All of the strains were initially grown in the autoclaved modified de Man, Rogosa, Sharpe medium (mMRS) broth, which contained 10 g/L peptone, 5 g/L beef extract, 5 g/L yeast extract, 10 g/L maltose, 5 g/L fructose, 5 g/L

glucose, 4 g/L K_2HPO_4, 2.6 g/L KH_2PO_4, 3 g/L NH_4Cl, 1 g/L Tween-80, 0.5 g/L cystein HCl, 10 g/L malt extract, 0.2 g/L $MgSO_4$, and 0.05 g/L $MnSO_4$ in distilled water. The solid mMRS substrate, used for cell counts, also contained 15 g agar per litre of broth. In order to inoculate lactobacilli into egg white solution at a consistent concentration, 100 µL stationary phase cultures (grown for 24 h or 48 h) were added into 10 mL fresh mMRS broth and then grown for 16 h to reach bacteria concentration of (2±1) $x10^8$ Colony Forming Unit (CFU)/mL. Cells of lactobacilli were washed with sterilized 0.9% saline before inoculating to egg white as follows: 10 mL mMRS lactobacilli solution were centrifuged, and the pellet was collected; 10 mL of sterilized 0.9% saline was added to, mixed, and centrifuged; and the pellet was again suspended in 10 mL of sterilized 0.9% saline (Loponen et al., 2008). The strains in mMRS broth, in egg white samples, and on mMRS agars were grown either at 30 ^oC or at 37 ^oC at atmosphere.

2.2.3. Preparation of egg white solutions for fermentation

Eggs were whiped with 70% ethanol and a flame was applied to sterilize before breaking egg shells and egg white was separated with egg yolk manually in a sterilized cabinets (Biosafety hood) (Canadian cabinets, Ottawa, Canada). Egg white was mixed either with 100 g/L D-maltose or 100g/L tryptone at the ratio of 9:1 (v/v). The pH of these samples was adjusted to 6.0 by slowly adding 3 M HCl while stirring the egg white rapidly. Bacteria in saline was mixed with the egg white samples at the ratio of 1:9 (v/v) and the mixtures were incubated in either 30 ^oC (*L. sakei* and *L. sanfranciscensis*) or 37 ^oC (*L. delbrueckii* subsp.

delbrueckii) at atmosphere for up to 96 h of fermentation. All of the processes were implemented in sterile conditions and all samples were prepared in triplicate.

2.2.4. Ascertainment of bacteria growth in egg white solutions

Cell counts and the pH of an egg white solution are two indicators of lactobacilli growth. The samples for these two measurements were prepared from the egg white solutions that were withdrawn at 0, 24, 48, 72, and 96 h of inoculation. For measuring the cell count of lactobacilli in the egg white solutions, withdrawn samples were sequentially diluted with sterilized 0.9% saline and 0.1 mL of each dilution was applied and sprayed onto mMRS agar plates. Those plates were incubated at 30 ^{o}C or 37 ^{o}C for 48 h before the cells were counted. The pH of egg white was accomplished by immediately measuring the pH of the withdrawn samples by accumet- excel XL15 pH/mV/Temperature Meter (Fisher Scientific, Ottawa, Canada).

2.2.5. Measurement of total free thiol groups

The total free thiol content of egg white proteins was determined according to the Ellman method (Ellman, 1959). The Ellman's reagent was prepared by applying 40 mg of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) in 10 mL of 500 mM sodium phosphate buffer (pH 7.0). Egg white samples were diluted 20 times by 100 mM sodium phosphate buffer (pH 8.0, containing 1.5% SDS); after mixing at room temperature for 1 h, the samples were centrifuged at 10,000 g for 10 min by Eppendorf Centrifuge 5417C (Eppendorf AG, Hamburg, Germany). Then 200 µL of the supernatant was mixed with 400 µL of 50 mM sodium phosphate buffer (pH 8.0), and 30 µL Ellman's reagent was added. The

mixtures were incubated in dark for 30 min. The absorbance of the samples was read at 412 nm with a spectrophotometer (Molecular Devices, Sunnyvale, California). Reduced L- glutathione was used as the standard for the free thiol group calculation.

2.2.6. Determination of amino nitrogen content

The amino nitrogen content of egg white sample was determined by the Ninhydrin method (ASBC, 1992). Egg white was diluted 200 times by 100 mM sodium phosphate buffer (pH 8.0). After centrifugation (10,000 g, 10 min, Eppendorf Centrifuge 5417C, Eppendorf AG, Hamburg, Germany), 200 µL of supernatant was mixed with 100 µL of ninhydrin color reagent (prepared by dissolving 5 g of Na_2HPO_4, 6 g of KH_2PO_4, 0.5 g of ninhydrin, and 0.3 g of fructose in 100 mL of distilled water at a pH of 6.7). After incubating in boiling water bath for 16 min, the mixtures were cooled at room temperature for 20 min, and then 500 µL of the dilution solution (0.2% KIO_3 in 40% ethanol) was added to each mixture. The absorbance was determined with a spectrophotometer (Molecular Devices, Sunnyvale, California) at 570 nm and glycine was used as the standard for amino nitrogen content calculation.

2.2.7. Enzyme-linked immunosorbent assay (ELISA)

The IgE binding ability of fermented egg whites was analyzed by ELISA using plasma from 4 egg allergy patients (PlasmaLab international, Everett, WA, USA) (Frias et al., 2008). Egg white samples were diluted to a protein concentration of 5 mg/mL in 50 mM sodium carbonate buffer (pH 9.6, containing 0.6 M NaCl). Then 100 µL of diluted egg white sample was applied into C96

Maxisorp plate (NUNC-IMMUNO, Denmark), the plate was kept at 4 ^{o}C overnight, and washed four times with 200 μL 0.05 M Tris-HCl buffer (pH 7.2, containing 0.1% Tween-20) by SkanWasher 400 (Molecular Devices, Sunnyvale, U.S.A). For blocking the unoccupied space of wells, 200 μL of 1 M PBS containing 2% BSA was added and kept in room temperature for 1 h. After washing, 100 μL of diluted human plasma (1:100, v/v, in 1 M PBS containing 1% BSA) was applied onto each well and the plate was incubated with gentle shaking at 37 ^{o}C for 2 h, followed by four times washing. And then 100 μL of the diluted goat-anti-human IgE conjugated with alkaline phosphate (diluted 1,000 times in 0.05 M Tris-HCl buffer, pH 8, containing 1% BSA, 0.1% Tween-20, and 1 mM $MgCl_2$) was added to each well, and the plate was incubated at room temperature for 2 h with gentle shaking. After that, the plate was washed for 5 times to remove all the remaining unbound antibodies, and the bound antibodies were detected by adding 100 μL of p-nitrophenyl phosphate (pNPP) solution, a color reagent. After 30 min of incubation, 100 μL of 3 M NaOH was added to stop the color reaction, and the plate was read with a spectrophotometer (Molecular Devices, Sunnyvale, California). In order to obtain most accurate results, each sample was applied to eight wells for analysis and the highest and lowest absorbance values for each sample were discarded.

2.2.8. SDS-PAGE analysis of egg white proteins

Egg white proteins were analyzed by SDS-PAGE (Laemmli, 1970). Egg white samples were diluted with 100 mM Tri-HCl buffer (pH 8, containing 1% (w/v) SDS) to the concentration of 2 mg/mL. After adding 45 μL of Laemmli

sample buffer and 5 µL of β-mercaptoethanol to 50 µL diluted egg white, the mixture was heated at 95 ^{o}C for 15 min with gentle shaking by Eppendorf Thermomixer R, (Hauppauge, NY, U.S.A), and centrifuged at 14,000 g for 5 min with Eppendorf Centrifuge 5417C (Eppendorf AG, Hamburg, Germany). A 25 µL of supernatant was loaded to 12% SDS-PAGE ready gel (Bio-rad Laboratories, Hercules, CA). Precision Plus Protein standards, a broad range molecular weight proteins of 10, 15, 20, 25, 37, 50 75, 100, 150, 250 kDa, was loaded as well. The gel was run at 200 V, for approximately 45 min, until the front dye reached the bottom. The gel was stained in methanol/ acetic acid /water (5:1:4) with 1/500 (w/v) Coomassie Brilliant Blue R-250 for 2 h, destained with methanol/acetic acid/water (5:1:4) with 5 washes each at 15 min, and then washed with de-ionized water. The gels were scanned using an Alpha Innotech gel scanner (Alpha Innotech Corp., San Leandro, U.S.A) with FluorChem SP software.

2.2.9. SDS-PAGE analysis of egg white containing FITC labelled ovomucoid

Ovomucoid was labelled and purified according to the manufacture's manual of Fluoro Tag^{TM} FITC Conjugation Kit (Sigma-Aldrich, St. Louis, USA). Ovomucoid (5 mg/mL) was mixed with FITC (1 mg/mL), all prepared in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.0), at a ratio of 4/1 (v/v). The mixture was covered with aluminum foil to protect the sample from light and was incubated at room temperature for 2 h with gentle shaking. And then the labelled ovomucoid was purified by Sephadex G-25M column (bed volume: 9.1 mL, bed height: 5 cm, and maximal sample volume: 1.5 mL) and eluted by 10 mM PBS

(pH 7.4). Absorbance at 280 nm and 495 nm of each eluted fractions were measured by spectrophotometer NanoDrop 2000C (Thermoscientific, Wilmington, U.S.A) to select the fractions that contain the conjugation of ovomucoid with FITC.

Fermentation of egg whites supplemented with labeled ovomucoid solution (5%, v/v) was performed as above using *L. delbrueckii* subsp. *delbrueckii*. After 48 h incubation covered with aluminum foil, samples were taken for SDS-PAGE analysis as described above and the fluorescence of FITC was read with Typhoon Variable Mode Imager (Sunnyvale, CA, USA).

2.2.10. Matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS) analysis of fermented egg white proteins

MALDI-TOF-MS was used to determine the molecular weights of egg white proteins before and after fermentation. Samples were prepared according to the procedure of two-layer sample preparation (Dai & Whittal, 1999). The first thin matrix layer was formed by loading 0.7 µL of 10 mg/mL 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 80% acetone / 20% methanol (HPLC grade) (v/v) onto a clean MALDI target. The matrix layer was spread and dried immediately after loading. Then 2 µL diluted egg white sample was mixed with 2 µL of saturated sinapinic acid in 50% acetonitrile/ 50% water (v/v) and 1 µL of this mixture was applied onto the first layer of the matrix. After drying, the sample was desalted by adding 5 µL of water onto top of the dry spot and the liquid was blown off by an air pulse after 10 seconds; this was repeated for five

times. Each sample was applied onto six spots, and at least three spots of each sample were analyzed. MALDI analysis was carried out on Applied BioSystems Voyager Elite MALDI (Foster City, CA, USA) time of flight mass spectrometer in a positive linear ion mode.

2.2.11. Statistical analysis

All data was analyzed based on one-way analysis of variance (ANOVA) by Dunnett test by GraphPad Prism (GraphPad Sofeware, Inc. California). The significant differences were determined using Dunnett test at $p < 0.05$ (Dunnett, 1955).

2.3. RESULTS

2.3.1. Growth of lactobacilli in egg whites

The maximum pH for the growth of *L. sanfranciscensisis* was previously reported to be 6.6 (Ganzle, Ehmann, & Hammes, 1998). However, the pH of egg white is approximately 9; therefore, the pH of the egg white must be lowered to approximately 6 to promote the growth of lactic acid bacteria. Our preliminary experiment showed that the addition of tryptone was necessary to keep lactic acid bacteria grow in egg white. Effect of fermentation time on the growth of lactobacilli and the pH change of egg white solution was shown in Figure 2.1. The cell count of *L. sanfranciscensis* and *L. sakei* maintained nearly a constant level of $1x10^8$ CFU/mL up to 96 h of incubation. The cell count of *L. delbrueckii* subsp. *delbrueckii*, however, dropped approximately 1000 times within the first 24 h;

increased gradually to their inoculated level after 48 h of incubation ($1X10^6$ CFU/mL); and then leveled off up to 96 h. The pHs of the egg white solutions fermented by *L. sanfranciscensis* and *L. sakei* were lowered from 6 to 5 within a 48 h period and then maintained at 5 up to 96 h of incubation. The pH of egg white fermented by *L. delbrueckii* subsp. *delbrueckii* did not significantly change within the first 24 h incubation, while started to drop gradually to pH 5 after 48 h incubation.

2.3.2. Effect of fermentation on the contents of free thiol and amino nitrogen

Many egg white proteins contain disulfide bonds and it is known that *L. sanfranciscensis* produces a glutathione-reductase that reduces the disulfide bounds in gluten (Vermeulen et al., 2006). Although *L. sanfranciscensis, L. sakei*, and *L. delbrueckii* subsp. *delbrueckii* can grow in tryptone supplemented egg white, the total free thoil content of egg white solution was not affected by fermentation (Figure 2.2A).

We measured the content of primary amines of fermented egg white in order to reveal whether there was protein degradation during fermentation. The content of amino nitrogen was increased to approximately 30%, compared to the control, in *L. delbrueckii* subsp. *delbrueckii* fermented egg white, after 48 h of incubation, and then leveled off for up to 96 h; but there were no changes in *L. sanfranciscensis* and *L. sakei* fermented egg white (Figure 2.2B).

2.3.3. Effect of fermentation on the IgE binding ability of egg white proteins

The effect of fermentation time on the IgE binding ability of egg white proteins is shown in Figure 2.3. Fermenting egg white with *L. sanfranciscensis or L. sakei* did not affect the IgE binding ability of the egg white. However, the IgE binding ability of egg white fermented by *L. delbrueckii* subsp. *delbrueckii* was significantly reduced after 48 h of incubation while further incubation up to 96 h did not further reduce it.

2.3.4. SDS-PAGE analysis of egg white proteins

SDS-PAGE was used to determine if protein degradation occurred during fermentation. There was no evidence of change in all of the fermented egg white proteins (Figure 2.4). The intensity of the FITC labeled ovomucoid in *L. delbrueckii* subsp. *delbrueckii* fermented egg white was reduced compared to the egg white without fermentation (Figure 2.5). However, no new fluorescence band was generated during fermentation.

2.3.5. MALDI-TOF-MS analysis of fermented egg white proteins

The effect of fermentation on the molecular weight of egg white proteins analyzed by MALDI-TOF-MS is shown in Figure 2.6. According to the molecular weight of egg white proteins, labeled peak 1 represents lysozyme, peak 2 represents ovomucoid, peak 3 represents ovotransferrin after being broken down into two equivalent domains during ionization, peak 4 represents ovalbumin, and peak 5 represents ovotransferrin. MALDI-TOF-MS analysis of fermented egg

white showed that no significant degradation occurred to the egg white proteins during fermentation. However, the molecular weight of the highest ovomucoid peak decreased from 29,250 Da to 29,012 Da, and the molecular weight range of the main peak domains reduced from 28,415—29,750 Da to 28,070—29,690 Da.

2.4. DISCUSSION

2.4.1. Growth of lactobacilli in egg white

By nature, the bio-function of egg white is to protect the embryo from infection of microorganism and to provide necessary nutrients for the embryo to develop (Kovacs-Nolan, Phillips, & Mine, 2005). Egg white contains approximately 88% water, 9.7~10.6% proteins, 0.4~0.9% of carbohydrate, 0.5~0.6% of ash, and 0.03% of lipids (Mine, 2002). Although the pH of egg white in freshly laid egg is 7.6~7.9, it will rise up to 9.7 due to the diffusion of solubilized CO_2 during storage (Belitz, Grosch, & Schieberle, 2008). The high pH environment and the presence of several antimicrobial proteins such as lysozyme and avidin in egg white indicate that egg white is not an ideal substrate for the growth of lactobacilli (Nath & Baker, 1973). To overcome this, the pH of egg white was decreased to 6, and maltose was provided as carbohydrate source; however, all seven tested strains did not grow. Further supplementation of tryptone made three of them grow in egg white, which indicated that egg white proteins could not be used as nitrogen source for lactobacilli although egg white is rich in protein.

The growth of lactobacilli in egg white was reflected as a reduction in pH and cell counts (Figure 2.1). Cell counts of *L. sanfranciscensis* and *L. sakei* remained constantly during fermentation up to 96 h while that of *L. delbrueckii subsp. delbrueckii* reduced rapidly within the first 24 h. The rapid reduction of *L. delbrueckii subsp. delbrueckii* within the first 24 h might be due to autolysis. Endogenous peptidoglycan hydrolases (PGHs), autolysins, can degrade the cell wall peptidoglycan causing bacterial autolysis (Smith, Blackman, & Foster, 2000). One single strain may be able to produce several PGHs with diverse specificities such as N-acetylmuramidases (lysozymes), N-acetylglucosaminidases, N-acetylmuramyl-L-alanine amidases, and endopeptidases (Lortal et al., 1997; Smith, Blackman, & Foster, 2000); which form a complex enzymatic PGHs system. The autolysis always occurs during nutriment starvation or other unfavorable environmental conditions. *L .delbrueckii* has been proved to be able to generate at least two autolysins itself (Kang, Laberge, & Simard, 2003). The presence of lysozyme in egg whites may be the major cause of the autolysis of *L. delbrueckii* subsp. *delbrueckii* within 24 h.

Even with the supplementation of tryptone, only three strains of out of total seven tested could grow in acidified egg white. The presence of lysozyme, the major antimicrobial protein in egg protein, may be the major reason for the difficulty in growing lactobacilli. Lysozyme is effective mainly against Gram-positive bacteria and lactobacilli are Gram-positive bacteria. It was previously reported that lysozyme at a concentration greater than 50 μg/mL could inhibit the growth of *L. helviticus* in milk and its effective antimicrobial concentrations range

from 100 to 400 μg/mL, while the concentration of lysozyme is 34 mg/mL in egg white (Bottazzi et al., 1978; Kovacs-Nolan et al., 2005); therefore, the content of lysozyme in egg white is high enough to inhibit the growth of lactobacilli. Acidification of egg white may not eliminate the inhibitory activity of lysozyme, since it was previously reported that lysozyme's inhibitory activity against Gram-positive bacteria was slightly affected by changing pH from 7 to 5 (Ibrahim et al., 1996). To explore the potential of lactobacilli for reducing egg white IgE binding ability, further study is needed to ferment egg white using lysozyme-deleted egg white.

2.4.2. Effect of fermentation on the contents of free thiols and amino nitrogen

The presence of disulfide bonds plays an important role in stabilizing the structure of proteins (Breiteneder & Mills, 2005). Proteins are more likely to be denatured and their digestibility improved if the disulfide bonds are cleaved, resulting in a decrease in the proteins' allergenicity. In sourdough fermentation, *L. sanfranciscensis* produces a glutathione-reductase that reduces the extracellular low molecular weight glutathione disulfide (GSSG) to glutathione (GSH). The accumulation of GSH further reduces the disulfide bonds in gluten to free thiols (Vermeulen et al., 2006). Egg white proteins contain high content of disulfide bonds. Our previous study showed that ovotransferrin was reduced by *L. sanfranciscensis* when egg white was fermented in sourdough (Loponen et al., 2008). We expected that *L. sanfranciscensis*, rather than *L. sakei* or *L. delbrueckii* subsp. *delbrueckii*, should be able to alter the free thiol levels of egg white

proteins. However, our results showed that the content of total free thiols was not affected in fermented egg whites (Figure 2.2A). It should be pointed out that the presence of the GSH and related SH compounds in wheat flour functions as essential intermediate substances that enable the glutathione-reductase to reduce the disulfide bonds in gluten (Li, Bollecker, & Schofield, 2004); however, since no low molecular weight sulphydryl compounds exist in egg white to serve as intermediates, the glutathione-reductase from *L. sanfranciscensis* could not reduce the egg white proteins through thiol exchange.

L. sanfranciscensis, *L. sakei*, and *L. delbrueckii* subsp. *delbrueckii* can only grow in tryptone, but not maltose, supplemented egg whites, which implies that nitrogen in egg white proteins could not be used as the nitrogen source for lactobacilli. Therefore, supplementation of an external nitrogen source, such as tryptone, is necessary for the growth of bacteria. Our results showed that the content of amino acid nitrogen was significantly increased in *L. delbrueckii* subsp. *delbrueckii* fermented egg white within 48 h, but not in *L. sanfranciscensis* and *L. sakei* fermented egg whites. The increase in amino nitrogen in *L. delbrueckii* subsp. *delbrueckii* fermented egg white within 48 h may indicate that the strain developed certain types of mechanisms to cope with the environment and therefore started to grow after 24 h. However, the increase in amino acid nitrogen content in *L. delbrueckii* subsp. *delbrueckii* fermented egg white might not be due to protein degradation as revealed by SDS-PAGE and MALDI-TOF-MS (Figures 2.4 &2.6). It is very likely that the cleavage of tryptone peptides were responsible for most of the increased amino nitrogen content. One speculation is that the

inoculated *L. delbrueckii* subsp. *delbrueckii* may secrete some extra enzymes to produce more available short peptides and/or free amino acids by degrading tryptone for surviving although only a small portion of them were still viable after 24 h. This strain repopulated back to initial inoculation concentration after 48 h, which shows that the essential peptides and/or amino acids for cells growth may be sufficient in the medium. As a result, those enzymes production may stop because of the feedback repression. Feedback repression is a widespread phenomenon in microorganism regulating the synthesis of amino acids that are used for building macromolecules such as enzymes; it prevents the overproduction of some unessential primary metabolites (Wang et al., 1949). Therefore, the amino nitrogen content of egg white solution stayed in the same level from 48 to 96 h as no more enzymes were generated during that period

2.4.3. Effect of fermentation on the IgE bind ability of egg white proteins

Of the three lactobacilli tested, only *L. delbrueckii* subsp. *delbrueckii* could significantly reduce the IgE binding ability of egg white proteins after 48 h of incubation, to 50% of its original binding capacities based on ELISA results using plasma from four egg allergy patients. Since there was a rapid decline in cell counts within the first 24 h of fermentation, no pronounced reduction in the IgE binding ability of the egg white occurred. Certain mechanisms for *L. delbrueckii* subsp. *delbrueckii* to cope with the environment in egg white, such as enzymes generation, probably contributed to the reduction of egg white IgE binding ability within 48 h of fermentation. In addition to increasing amino

nitrogen content, the enzymes from *L. delbrueckii* subsp. *delbrueckii* may also cleave some linear epitopes in egg white allergens.

Ovomucoid is the predominant allergen in egg white. Our study showed that ovomucoid was the only protein that displayed obvious molecular weight changes during fermentation with *L. delbrueckii* subsp. *delbrueckii* by MALDI-TOF-MS analysis (Figure 2.6). Ovomucoid is composed of three tandem domains that all are homologous to the pancreatic secretary trypsin inhibitor (Kato et al., 1987). Each domain is cross-linked by three intra-domain disulfide bonds, although ovomucoid contains no inter-domain disulfide bonds. As a glycoprotein, glycan moiety accounts for 25-30% of its total molecular weight (Beeley, 1971). The main saccharines, N-acetylglucosamine (GlcNAc), mannose, galactose, and N-acetylneuramic acid (NeuNAc), represent 14~16, 6.5~8.5, 0.5~4, and 0.04~2.2% of the total molecular weight of ovomucoid, respectively (Beeley, 1971; Kato et al., 1987). Compared to the natural ovomucoid, the chemically deglycosylated form is very sensitive to heating but retains its trypsin inhibitory activity; this phenomenon indicates that the carbohydrate moiety also contributes to the stabilization of ovomucoid against heat (Yamamoto et al., 1998). Even after peptic digestion, the fragments of ovomucoid still preserve the allergenic properties (Kovacs-Nolan et al., 2000). Therefore, ovomucoid is very resistant to heat denaturation and digestive enzymes, which make this protein a stable allergen (Matsuda, Watanabe, & Nakamura, 1982; Kato et al., 1987). For the first time, our study showed that fermentation of lactobacilli could modify ovomocoid, leading to the reduction in IgE binding ability of egg white.

The precise molecular weight of ovomucoid could not be determined by MALDI-TOF-MS due to the presence of glycan moieties and isomers, while it seems that there was a decrease in molecular weight of ~ 238 Da after fermentation (Figure 2.6). The reduction in the fluorescence intensity of the ovomucoid band in the fermented egg white indicated that it is very likely that the moieties adjacent to the FITC might be removed by enzymes produced by the *L. delbrueckii* subsp. *delbrueckii* (Figure 2.5). The fluorescence FITC labeling could react with the amine ends, N-terminals of the proteins and some amino acids residues with primary amines in their side chains; therefore the exact molecule modification of ovomucoid during fermentation needs to be characterized further.

Exopeptidases from lactic acid bacteria, with molecular weight of 80~100 kDa, were reported to be able to cleave off N-terminal amino acids residues of broad substrates with various peptide chain lengths (Raksakulthai & Haard, 2003; Tan, Poolman, & Konings, 1993; Wohlrab & Bockelmann, 1994; Pritchard & Coolbear, 1993). Exopeptidases from *L. delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* have been characterized (Eggimann & Bachmann, 1980; Bockelmann, Schulz, & Teuber, 1992). It is likely that *L. delbrueckii* subsp. *delbrueckii* can also produce exopeptidases that may cleave the peptide at the N-terminal of ovomucoid.

Many linear epitopes have been characterized from ovomucoid (Mine & Zhang 2002), but this protein was only slightly modified during fermentation of *L. delbrueckii* subsp. *delbrueckii* based on the result from MALDI-TOF-MS (Figure 2.6). *L. delbrueckii* subsp. *delbrueckii* was reported to be able to produce some

sorts of carbohydrate enzymes; for example, *L. delbrueckii* subsp. *delbrueckii* KCTC 1047 can produce β-glucosidase and possesses glucoside-hydrolyzing activity, as it can hydrolyze isoflavone glucosides, genistin, and daidzin. After fermentation, all of the glycosidic bonds in isoflavones were cleaved by β-glucosidase from *L. delbrueckii* subsp. *delbrueckii* (Choi, Kim, & Rhee, 2002). The reduction of egg white IgE binding ability after 48 h of incubation may be also due to cleavage of glycan moiety from ovomucoid since: 1) SDS-PAGE analysis did not show obvious protein degradation, and 2) fermentation of labeled ovomucoid did not show new fluorescence band despite there was a reduction in intensity after fermentation. Although a previous study reported that chemical deglycosylation of ovomucoid did not affect its IgE binding ability (Besler, Steinhart, & Paschke, 1997). Gu et al (1989) discovered that the immunological properties of deglycosylated ovomucoid can be affected by heat treatment more easily than native ovomucoid. Another recent research also confirmed that the deglycosylated ovomucoid is very sensitive to heating compared to the natural one (Yamamoto et al., 1998). This phenomenon indicates that the carbohydrate moiety contributes to the stabilization of ovomucoid. Essentially, the binding sites of food allergens towards major histocompatibility complex (MHC) molecule in the antigen presenting cells are amino acids rather than glycan (Hannigan, Moore, & Quinn, 2009). Nevertheless, the removal of glycan moieties by carbohydrate enzymes from *L. delbrueckii* subsp. *delbrueckii* benefits the proteases from the same strain to access to the linear epitopes in ovomucoid. Further study is needed

to characterize the structural changes of ovomucoid to understand the mechanism of change.

2.5. CONCLUSION

Egg white is not an appropriate substrate for the growth of lactobacilli. Acidification of egg white to pH of ~ 6.0 and supplementation of typtone are necessary to grow the bacteria. Of three strains tested, only *L. delbrueckii* subsp. *delbrueckii* could significantly reduce the egg white IgE binding ability after 48 h incubation. Cell counts of this strain decreased significantly at the first 24 h of fermentation, but started to regain their numbers to the initial level at 48 h. The reduction of egg white IgE binding ability was related to ovomucoid, the dominant egg allergen, as evident in the change of molecular weight distribution analyzed by MALDI-TOF-MS and reduction of intensity of FITC labeled ovomucoid after fermentation. As a very stable egg allergen, for the first time our study showed the fermentation could modify the structure of ovomucoid. Our next step in research is to determine whether the reduction of egg white IgE binding ability is caused by the cleavage of N-terminal peptide, the carbohydrate moieties, or some other structural changes of ovomucoid. Further study also includes determining the functionalities of fermented egg whites. Our research provides industry a new applicable approach to develop hypoallergenic fermented egg product.

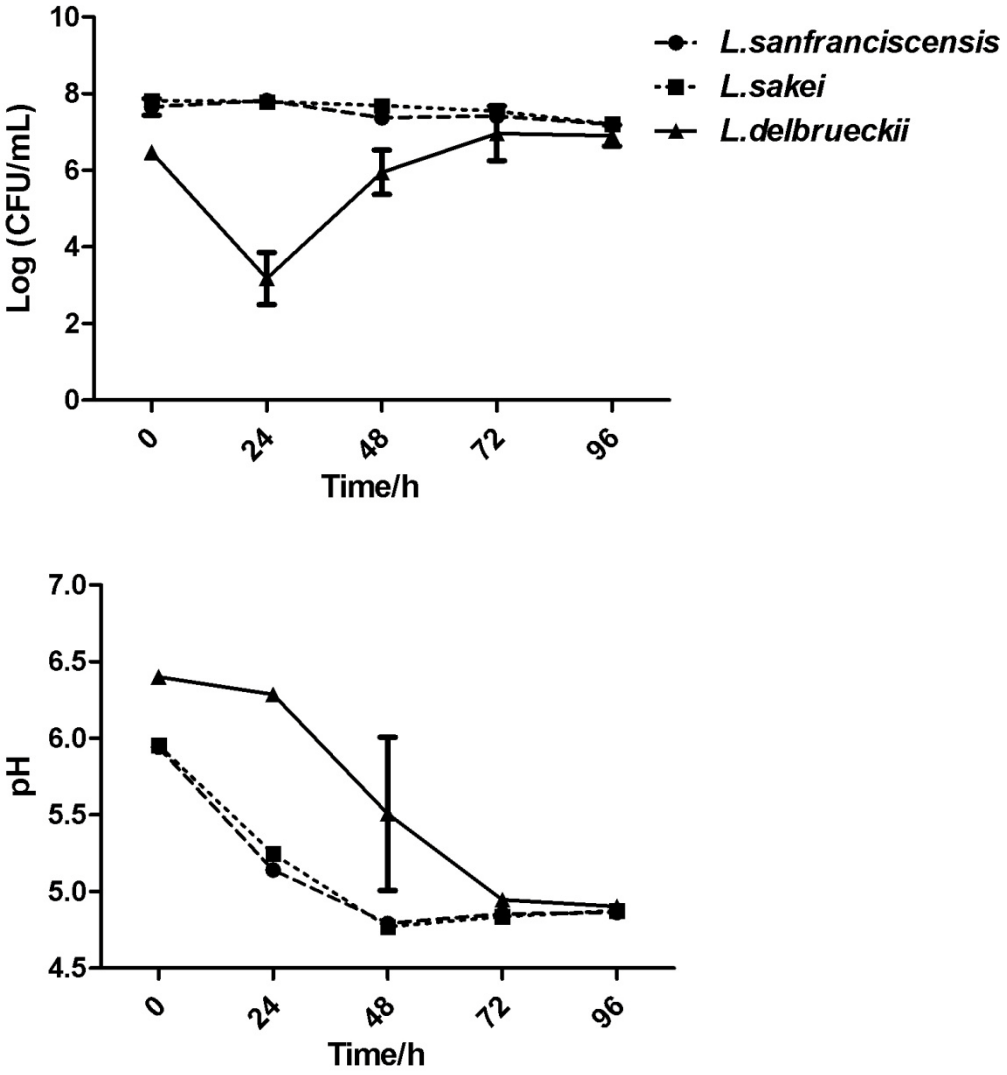

Figure 2. 1 Effect of fermentation time on cell counts and pHs of egg white fermented by lactic acid bacteria in the presence of tryptone. Results are means ± standard deviation of three independent fermentations. The statistical analysis by Dunnett's test of one way ANOVA indicated that the amounts of *L. delbrueckii* showed significant difference at 24 h of incubation. The pH of egg white fermented with *L. sanfranciscensis* and *L. sakei* was significantly different at 24, 48, 72, and 96 h and that of *L. delbrueckii* showed significant difference at 72 and 96 h.

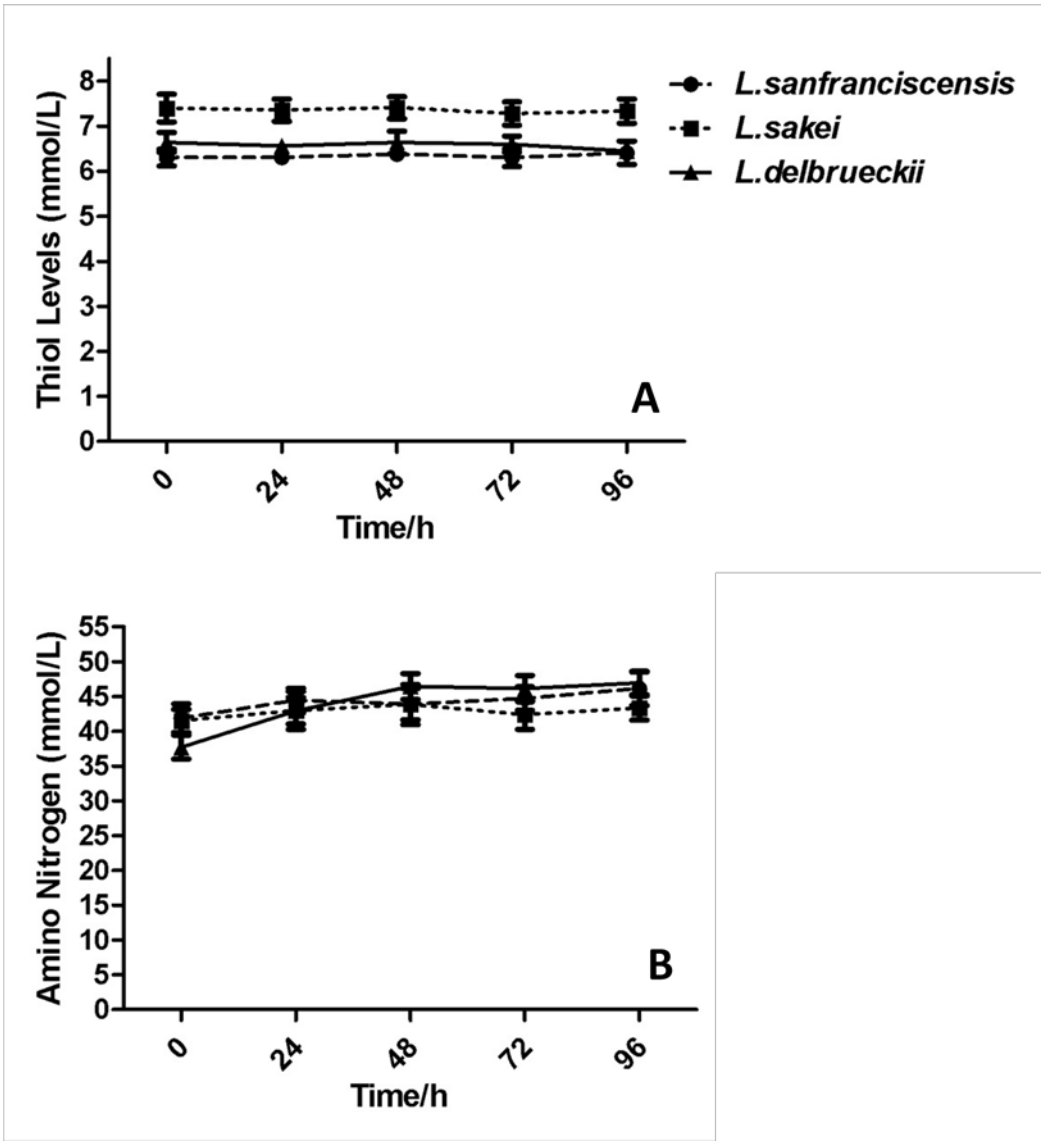

Figure 2. 2 Effect of incubation time on the contents of total free thiol (A) and amino nitrogen (B) of fermented egg whites. Results are means ± standard deviation of triplicate analyses each of three independent fermentations. The statistical analysis by Dunnett's test of one way ANOVA indicated that the amino nitrogen content of *L. delbrueckii* fermented egg white showed significant difference at 24, 48, 72, and 96 h of incubation.

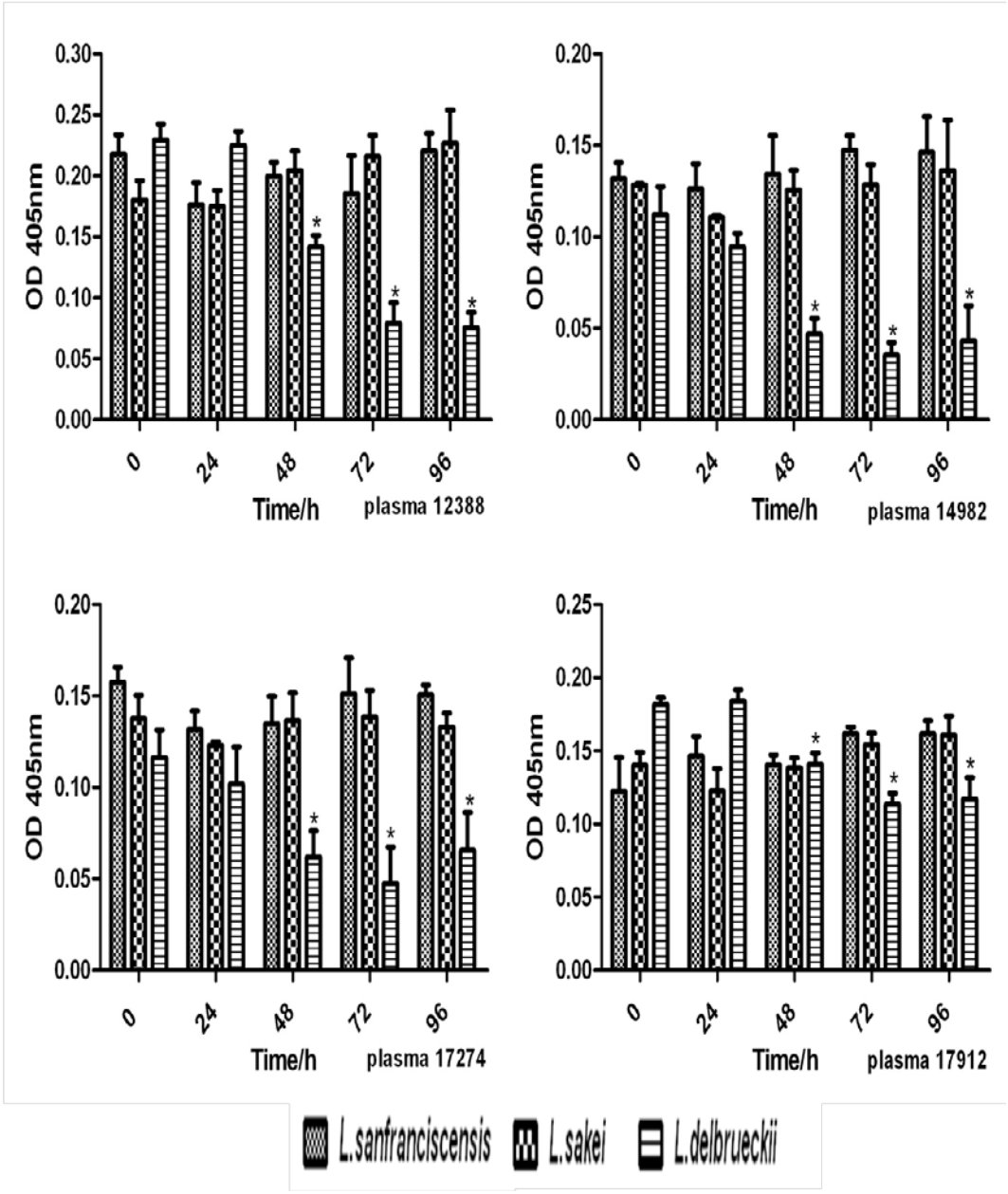

Figure 2. 3 Effect of fermentation time on the IgE binding ability of egg white proteins. Results are means ± standard deviation of replicate analyses each of three independent fermentations. The statistical analysis by Dunnett's test of one way ANOVA indicated that the IgE binding ability of *L. delbrueckii* fermented egg white showed significant difference (p<0.001) at 48, 72, and 96 h of incubation towards all four plasmas from egg allergy patients (labelled with star).

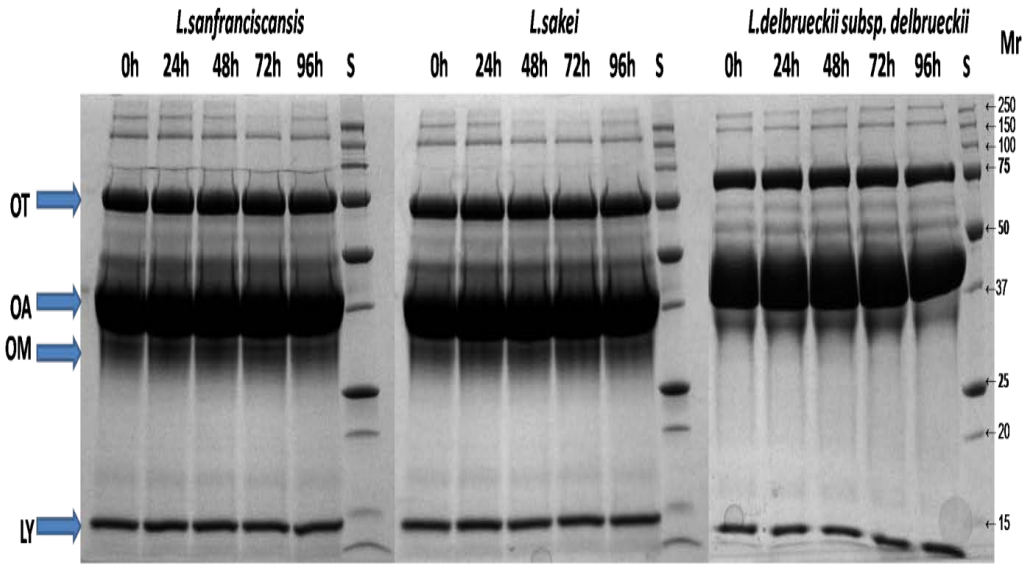

Figure 2. 4 SDS-PAGE analysis of effect of fermentation time on egg white solution with tryptone. OT-Ovotransferrin; OA-Ovalbumin; OM-Ovomucoid; LY-Lysozyme; S-Molecular weight marker proteins (Mr indicated on the right in kDa)

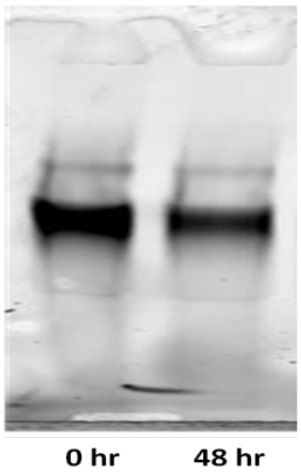

Figure 2. 5 SDS-PAGE analysis of effect of *L. delbrueckii* subsp. *delbrueckii* fermentation on the modification of FITC labelled ovomucoid in acidic egg white solution containing tryptone.

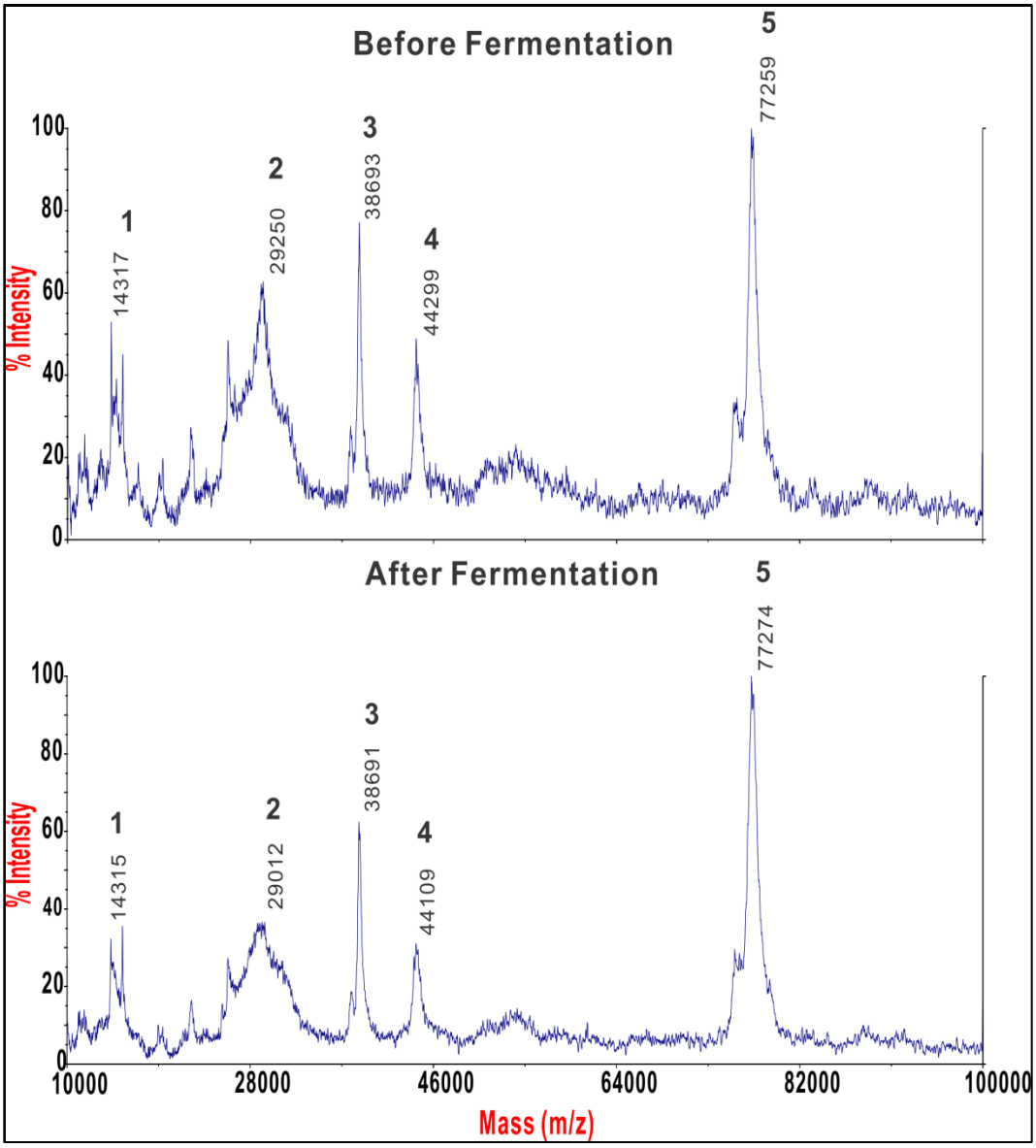

Figure 2. 6 MADLI-TOF-MS analysis of effect of *L. delbrueckii* subsp. *delbrueckii* fermentation on the modifications of egg white proteins. Peak 1 represents lysozyme, peak 2 represents ovomucoid, peak 3 represents ovotransferrin after being broken down into two equivalent domains during ionization, peak 4 represents ovalbumin, and peak 5 represents ovotransferrin.

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CHAPTER 3 EFFECT OF *ASPERGILLUS ORYZAE* FERMENTATION ON IGE BINDING ABILITY OF EGG WHITE PROTEINS

3.1. INTRODUCTION

Egg white contains four major egg allergens that have been characterized as Gal d 1-4: ovomucoid (Gal d 1, 11%), ovalbumin (Gal d 2, 54%), ovotransferrin (Gal d 3, 12%), and lysozyme (Gal d 4, 3.4%) (Perelmutter, 2001). Using 34 sera from adults who had egg allergy history; Aabin et al (1996) reported that ovotransferrin and ovomucoid had higher IgE binding ability than ovalbumin and lysozyme. The greater IgE binding ability of ovotransferrin and ovomucoid was also recently confirmed (Jacobsen et al., 2008). It was estimated that about 35% of 52 egg allergy patients are sensitive to lysozyme (Fremont et al., 1997). Ovomucoid is now recognized as the dominant egg allergen. Many egg allergic patients are sensitive to heated egg white (~40% of egg allergic patients are able to tolerant egg in baked goods), but 94.1% of 74 subjects who are allergic to egg white show no reaction to the challenge of heated ovomucoid-depleted egg white (Urisu et al., 1997). Compared with other egg white proteins, ovomucoid is insensitive to heat and digestive enzymes; heating at 80 ^{o}C diminished the IgE binding ability of ovalbumin (Kim et al 2002) whereas ovomucoid showed IgE binding ability even after heating at 100 ^{o}C (Deutsch & Morton, 1956). Ovomucoid contains three tandem domains; each domain is homologous to pancreatic secretary trypsin inhibitor and possesses three intra-domain disulfide

bonds. Those characteristics make this protein becomes resistant to heat denaturation and digestive enzymes (Matsuda, Watanabe, & Nakamura, 1982; Kato et al., 1987). Ovomucoid also comprises carbohydrate moieties up to 25 ~ 30% of its total molecular weight (Beeley, 1971; Kato et al., 1987), and they contribute to stabilization of ovomucoid against heat (Yamamoto et al., 1998).

The sites on allergen proteins that can bind to IgE are called epitopes. There are two types of epitopes: conformational and linear. Conformational epitopes are made up of several separated peptide chains, which may be quite distant in primary sequences but are linked loosely by three-dimensional structures (Van Regenmortel, 1992). The secondary or tertiary structures of the conformational epitopes are critical for their binding ability to IgE (Bannon, 2004), so conformational epitopes may be destroyed by protein denaturation during the thermal process, irradiation, and/or high pressure treatment (Sathe & Sharma, 2009). The binding ability of liner epitopes is based on the integrity of their primary sequences, so they are to some extent stable against heat treatment and partial proteolysis (Van Regenmortel, 1992; Dominguez et al., 1990). Linear epitopes are prevalent in food allergens since they can be presented to immune system after the heating and/or digestion (Bannon, 2004). The dominant egg allergen, ovomucoid, contains at least eight linear epitopes for IgE/T-cell (Mine & Zhang, 2002); damaging all of them is not an easy task. It is also very difficult to destroy the conformational epitopes in ovomucoid, because the compact structure makes this protein very resistant to heat denaturation.

Destroying both linear and conformational epitopes are essential in reducing the protein IgE binding ability. Many types of food processing such as heating, high pressure treatments, irradiation, enzymes hydrolysis, and fermentation can reduce IgE binding ability of proteins (Yamamoto et al., 2010; Kume & Matsuda, 1995; Sathe & Sharma, 2009; Hannigan, Moore, & Quinn, 2009). Fermentation has been used widely in the food products. The IgE binding ability of β-lactoglobulin in yogurt has been substantially reduced compared to the one in unfermented milk (Ehn et al., 2004). *Aspergillus oryzae* has been employed for thousands of years for preparing many Asian traditional foods: soy sauce, *sake* (a rice wine), *miso* (fermented soy seasoning), and more; and they are all produced by *koji*. *Aspergillus oryzae* is used to prepare *koji*, in which contains various carbohydrate enzymes (such as glucoamylase, α-amylase, and α-glucosidase) and proteases (Hesseltine & Wang, 1978; Ishida et al., 2000; Mudgett & Annunziato, 1986). The generation of numerous types of proteases from *Aspergillus oryzae* can lead to hydrolyzing of food proteins and changing of their IgE binding ability. It was reported that *miso* is less allergenic than raw soybeans (Herian, Taylor, & Bush, 1993). The IgE binding ability of soybean proteins against the sera of soy sensitive patients has been substantially diminished after treating with the protease from *Aspergillus oryzae* (Yamanishi et al., 1996). Recent study also showed that fermentation of soybean by *Aspergillus oryzae* could significantly reduce their IgE binding ability (Frias et al., 2008). We want to ascertain whether the enzymes from *Aspergillus oryzae* can cleave the linear epitopes and diminish the IgE binding ability of egg white proteins,

especially ovomucoid that is quite resistant to heat treatment and trypsin hydrolysis. The structure of proteins will be changed when they are cleaved by enzymes, and their conformational epitopes are also very likely to be damaged then.

The objective of this study was to determine if fermentation of *Aspergillus oryzae* could reduce the IgE binding ability of egg white with the aim to develop a hypoallergenic egg product. The effect of fermentation on the egg white proteins were evaluated using the Ninhydrin method, SDS-PAGE, enzyme-linked immunosorbent assay (ELISA), fluorescence FITC labeling, and MALDI-TOF-MS analysis.

3.2. MATERIALS AND METHODS

3.2.1. Materials

Hen eggs (lucerne^{TM} premiere large) were purchased from a local supermarket (Edmonton, Canada). *Aspergillus oryzae* ATCC 1011 and *Aspergillus oryzae* ATCC 16868 were bought from Microfungus Collection and Herbarium, University of Alberta (Edmonton, Canada). Plasmas from egg allergy patients were obtained from PlasmaLab international (Everett, WA, USA). Ovomucoid was obtained from Neova (Abbotsford, BC, Canada). Agar, malt extract, and peptone were purchased from Bacto^{TM} (Sparks, MD, USA). Coomassie Brilliant Blue R-250, glycine, Laemmli sample buffer, Precision Plus Protein standards, and sodium dodecyl sulfate (SDS) were obtained from Bio-rad (Hercules, CA, USA). Acetic acid, D-glucose, K_2HPO_4, KH_2PO_4, methanol,

$NaH_2PO_4.H_2O$, $Na_2HPO_4.7H_2O$, NaOH, and tris base were bought from Fisher Scientific (Ottawa, Ontario, Canada). Ninhydrin, β-mercaptoethanol, acetone, bovine serum albumin (BSA), cystein, fluorescein isothiocyanate (FITC), goat-anti-human IgE conjugated with alkaline phosphate, p-nitrophenyl phosphate (pNPP), and Tween-80 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this research were all obtained from sigma and of analytical grade.

3.2.2. Preparation of egg white solutions for fermentation

Hen eggs (lucerne^{TM} premiere large) were purchased from a local supermarket (Edmonton, Canada). Eggs were whiped with 70% ethanol and flame was applied to sterilize before breaking egg shells, and egg white was separated with egg yolk manually in a sterilized cabinets (Biosafety hood) (Canadian cabinets, Ottawa, Canada). The pH of egg white was adjusted to 6.0 by slowly adding 3 M HCl while stirring rapidly.

3.2.3. Strains preparation

Aspergillus oryzae ATCC 1011 and *Aspergillus oryzae* ATCC 16868 (Microfungus Collection and Herbarium, University of Alberta) were first inoculated from stock cultures into mould growing broth (pH 5.0~5.5) containing 20 g/L malt extract, 1 g/L peptone, and 20 g/L glucose; and they were incubated at 25 ^{o}C with gentle shaking for 5 days. After that, some mycelium balls (mycelium aggregates that were formed naturally during incubation) were harvested and washed with sterilized 0.85% saline for 3 times and were directly

inoculated into acidified egg white (one mycelium aggregate into 20 mL egg white solution). At the same time, some mycelium balls were inoculated onto moulds growing agar (20 g agar per litre broth) in flasks that were then covered with two layers of aluminum foil and grow at 25 ^{o}C for 5 days. After mycelium grown well on the agar, 10 mL sterilized 0.85% saline was poured onto the agar. The flasks were shaken intensively and mycelium was pressed with sprayer to make moulds conidia spores soluble and spread in the saline. The saline with conidia spores was filtered in sterile condition and then inoculated into acidified egg white (2 mL saline with conidia spores into 18 mL egg white solution). The remaining saline with conidia spores was kept at -20 ^{o}C for future use. After inoculation of mycelium or conidia spores, egg white was incubated in a shaker (New Brunswick Scientific, Edison, New Jersey) at 25 ^{o}C with gentle shaking. Egg white samples were withdrawn for analysis at 0, 24, 48, 72, 96, and 120 h intervals of fermentation. No visible mycelium was observed in egg white incubated with conidia spores for up to 120 h fermentation. All samples were prepared in duplicate.

3.2.4. Determination of amino nitrogen

The amino nitrogen content of egg white sample was determined by the Ninhydrin method (ASBC, 1992). Egg white was diluted 200 times by 100 mM sodium phosphate buffer (pH 8.0). After centrifugation (10,000 g, 10 min, Eppendorf Centrifuge 5417C, Eppendorf AG, Hamburg, Germany), 200 µL of supernatant was mixed with 100 µL of ninhydrin color reagent (prepared by dissolving 5 g of Na_2HPO_4, 6 g of KH_2PO_4, 0.5 g of ninhydrin , and 0.3 g of

fructose in 100 mL of distilled water, pH 6.7). After incubating in boiling water bath for 16 min, the mixtures were cooled in room temperature for 20 min, and then 500 μL of the dilution solution (0.2% KIO_3 in 40% ethanol) was added to each mixture. The absorbance was determined with a spectrophotometer (Molecular Devices, Sunnyvale, California) at 570 nm, and glycine is used as the standard for amino nitrogen content calculation.

3.2.5. Enzyme-linked immunosorbent assay (ELISA)

The IgE binding ability of fermented egg white was analyzed by ELISA (Frias et al., 2008) using plasma from 4 egg allergy patients (PlasmaLab international, Everett, WA, USA). Egg white samples were diluted to a protein concentration of 5 mg/mL in 50mM sodium carbonate buffer (pH 9.6, containing 0.6 M NaCl). Then 100 μL of diluted egg white sample was applied into C96 Maxisorp plate (NUNC-IMMUNO, Denmark), the plate was kept at 4 ^{o}C overnight, and washed four times with 200 μL 0.05 M Tris-HCl buffer (pH 7.2, containing 0.1% Tween-20) by SkanWasher 400 (Molecular Devices, Sunnyvale, U.S.A). For blocking the unoccupied space of wells, 200 μL of 1 M PBS containing 2% BSA was added and kept in room temperature for 1 h. After washing, 100 μL diluted human plasma (1:100, v/v, in 1M PBS containing 1% BSA) was applied onto each well and the plate was incubated with shaking at 37 ^{o}C for 2 h, followed by four times washing. And then 100 μL of diluted goat-anti-human IgE conjugated with alkaline phosphate (diluted 1,000 times in 0.05 M Tris-HCl buffer, pH 8, containing 1% BSA, 0.1% Tween-20, and 1 mM $MgCl_2$) was added to each well, and the plate was incubated at room temperature for 2 h

with shaking. After that, the plate was washed for 5 times to remove all the remaining unbound antibodies, and the bound antibodies were detected by adding 100 μL of p-nitrophenyl phosphate (pNPP) solution, a color reagent. After 30 min of incubation, 100 μL of 3 M NaOH was added to stop the color reaction, and the plate was read with a spectrophotometer (Molecular Devices, Sunnyvale, California). In order to obtain most accurate results, each sample was applied to eight wells for analysis and the highest and lowest absorbance values for each sample were discarded.

3.2.6. SDS-PAGE analysis of egg white proteins

Egg white proteins were analyzed by SDS-PAGE (Laemmli, 1970). Egg white samples were diluted with 100 mM Tri-HCl, pH 8, with 1% (w/v) SDS to the concentration of 2 mg/mL. After adding 45 μL of Laemmli sample buffer and 5 μL of β-mercaptoethanol to 50 μL diluted egg white, the mixture was heated at 95 ^{o}C for 15 min with gentle shaking by Eppendorf Thermomixer R (Hauppauge, NY, U.S.A). After centrifugation at 14,000 g for 5min by Eppendorf Centrifuge 5417C (Eppendorf AG, Hamburg, Germany), 25 μL sample supernatant was loaded to 12% SDS-PAGE ready gel (Bio-rad Laboratories, Hercules, CA). Precision Plus Protein standards, a broad range molecular weight proteins of 10, 15, 20, 25, 37, 50 75, 100, 150, 250 kDa, was loaded as well. The gel was run at 200 V until the dye front reached the bottom (around 45 min). The gel was stained in methanol/ acetic acid /water (5:1:4) (v/v/v) with 1/500 (w/v) Coomassie Brilliant Blue R-250 for 2 hour, destained with methanol/acetic acid/water (5:1:4) (v/v/v) for 5 washes each of 15 minutes, and washed with de-ionized water at the

end. The gels were scanned using an Alpha Innotech gel scanner (Alpha Innotech Corp., San Leandro, U.S.A) with FluorChem SP software.

3.2.7. SDS-PAGE analysis of egg white containing FITC labelled ovomucoid

Ovomucoid was labeled and purified according to the manufacture's manual of Fluoro Tag^{TM} FITC Conjugation Kit (Sigma-Aldrich, St. Louis, USA). Ovomucoid (5 mg/mL) was mixed with FITC (1 mg/mL), all prepared in 0.1 M sodium carbonate-bicarbonate buffer (pH 9.0), at a ratio of 4/1 (v/v). The mixture was covered with aluminum foil to protect the sample from light and was incubated at room temperature for 2 h with gentle shaking. And then the labelled ovomucoid was purified by Sephadex G-25M column (bed volume: 9.1 mL, bed height: 5 cm, and maximal sample volume: 1.5 mL) and eluted by 10 mM PBS (pH 7.4). Absorbance at 280 nm and 495 nm of each eluted fractions were measured by spectrophotometer NanoDrop 2000C (Thermoscientific, Wilmington, U.S.A) to select the fractions that contain the conjugation of ovomucoid with FITC.

Growth of mycelium of *Aspergillus oryzae* in acid egg white supplemented with labeled ovomucoid was performed as previous statement but in small scale. Egg white solution, pH 6, was mixed with purified FITC labeled ovomucoid solution at the ratio of 9:1 (v/v). The washed mycelium of *Aspergillus oryzae* was then inoculated into the mixtures. After 48h incubation covered with aluminum foil with gentle shaking, samples were taken for SDS-PAGE analysis

as described above and the fluorescence of FITC was read with Typhoon Variable Mode Imager (Sunnyvale, CA, USA).

3.2.8. Matrix-assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS) analysis of fermented egg white proteins

MALDI-TOF-MS was used to determine the molecular weights of egg white proteins before and after fermentation. Samples were prepared followed by the procedure of two-layer sample preparation (Dai & Whittal, 1999). The first thin matrix layer was formed by loading 0.7 µL of 10 mg/mL 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) in 80% acetone / 20% methanol (HPLC grade) (v/v) onto a clean MALDI target. The matrix layer was spread and dried immediately after loading. Then 2 µL diluted egg white sample was mixed with 2 µL of saturated sinapinic acid in 50% acetonitrile/ 50% water (v/v) and 1µL of this mixture was applied onto the first layer of the matrix. After drying, the sample was desalted by adding 5 µL of water onto top of the dry spot and the liquid was blown off by an air pulse after 10 seconds; this was repeated for five times. Each sample was applied onto six spots, and at least three spots of each sample were analyzed. MALDI analysis was carried out on Applied BioSystems Voyager Elite MALDI (Foster City, CA, USA) time of flight mass spectrometer in a positive linear ion mode.

3.2.9. Statistical analysis

All data was analyzed based on one-way analysis of variance (ANOVA) by Dunnett test by GraphPad Prism (GraphPad Sofeware, Inc. California). The significant differences were determined using Dunnett test at $p < 0.05$ (Dunnett, 1955).

3.3. RESULTS

3.3.1. Amino nitrogen content of fermented egg white

Effect of fermentation time on the amino nitrogen content of egg white solution was shown in Figure 3.1. When acid egg white solution was inoculated with conidia spores or mycelium of *Aspergillus oryzae* ATCC 1011 or ATCC 16868, the total free amino nitrogen content did not change for up to 120 h of fermentation, which indicated that there was no significant protein degradation occurred during fermentation.

3.3.2. IgE binding ability of fermented egg white

Effect of fermentation time on the IgE binding ability of egg white proteins by *Aspergillus oryzae* was shown in Figure 3.2. The higher the absorbance in ELISA test, the higher the IgE binding ability of egg white samples. The IgE binding ability of egg white proteins was significantly reduced within the first 24 h after incubation of mycelium of *Aspergillus oryzae*, using plasmas from four egg allergy patients. The mycelium of *Aspergillus oryzae* ATCC 16868 almost eliminated the IgE binding ability of egg white proteins after 24 h of

fermentation. While inoculated with conidia spores, the IgE binding ability of egg white proteins was decreased gradually but not completely eradicated till 120 h of fermentation; and IgE from some plasma can still bind to egg white inoculated with conidia spores after 120 h fermentation. Our results showed that the fermentation of egg white by mycellium of *Aspergillus oryzae* is more efficient and effective than their conidia spores in reducing IgE binding ability of egg whites.

3.3.3. Analysis of egg white proteins by SDS-PAGE

Effect of fermentation time on egg white proteins was shown in Figure 3.3. Four egg white allergens were not significantly altered during fermentation for up to 120 h by either mycelium or conidia spores of *Aspergillus oryzae* ATCC 1011 and ATCC 16868. As the IgE binding ability of egg white proteins was almost eliminated after mycelium fermentation, FITC labeled ovomucoid was fermented with egg white to determine if this dominant egg allergen was degraded. The fluorescence intensity of ovomocoid band was not affected during fermentation, compared to the one in acidified egg white without fermentation, and there was no new fluorescence band generated during fermentation (Figure 3.4).

3.3.4. MALDI-TOF-MS analysis of Aspergillus oryzae fermented egg white proteins

Effect of *Aspergillus oryzae* fermentation on the molecular weight of egg white proteins analyzed by MALDI-TOF-MS was shown in Figure. 3.5.

According to the molecular weight of egg white proteins, peak 1 represents lysozyme, peak 2 represents ovomucoid, peak 3 represents ovotransferrin after being broken down into two equivalent domains during ionization, peak 4 represents ovalbumin, and peak 5 represents ovotransferrin. Similar to our observation from SDS-PAGE, significant protein degradation was not observed in mycelium of *Aspergillus oryzae* fermented egg whites with the exception of the peak 2 where the highest peak switched from 28,973 Da to 27,271 Da and 27,582 Da respectively, and the main peaks domain shifted from 25,490—29,970 Da to 25,130—29,770 Da and 25,150—29,725 Da, respectively.

3.4. DISCUSSION

Aspergillus oryzae is known to be able to secret various types of proteases and carbohydrate enzymes, which are responsible for the formation of the textural and flavor features of many fermented food products (Machida, Yamada, & Gomi, 2008; Smith, 1978). However, the content of amino nitrogen content was not increased during fermentation of egg white by either mycelium or spores of *Aspergillus oryzae* ATCC 1011 and ATCC 16868; which indicated that egg white proteins were not degraded during fermentation by *Aspergillus oryzae* (Figure 3.1).

Although fermentation of egg whites by both *Aspergillus oryzae* ATCC 1011 and ATCC 16868 nearly eliminated the IgE binding ability of egg white proteins within 24 h (Figure 3.2), the cause of this reduction was not evident. The change of amino nitrogen content was used for the assessment of protein degradation, but the content was not affected during fermentation (Figure 3.1).

Furthermore, SDS-PAGE analysis also supported that there was no obvious protein degradation occurred during fermentation (Figure 3.3). The fluorescence intensity of FITC labeled ovomucoid was not affected during fermentation (Figure 3.4), which implied that there may not be cleavage of FITC adjacent moieties in ovomucoid as we observed in *L. delbrueckii* fermented egg whites. No obvious migration of fluorescence bands was observed after fermentation, which indicated that the labeled ovomucoid has not been substantially degraded by fermentation. This was also in agreement with the results of MALDI-TOF-MS showing there was no obvious protein degradation in fermented egg whites (Figure 3.5). However, change of the shape of ovomucoid peak was obvious between *Aspergillus oryzae* fermented egg whites and acidified egg whites; it is not known if this change is caused by the carbohydrate moiety cleavage that might be responsible for the reduction of IgE binding ability. *Aspergillus oryzae* is able to produce numerous carbohydrate active enzymes, such as α-amylase, α – galactosidase, and β-xylosidases (Annunziato, Mahoney, & Mudgett, 1986; Suganuma, Fujita, & Kitahara, 2007; Hashimoto et al., 1999); which may remove various carbohydrate moieties in egg white proteins. Many egg proteins are glycoproteins and the dominant egg allergen, ovomucoid, also contains 25~30% carbohydrate moiety. It is possible that some of carbohydrate moieties in ovomucoid were removed by fermentation resulting in the reduction of IgE binding ability. Although chemical deglycosylated ovomucoid with molecular weight between 20.7 and 21.5 kDa was reported to possess the same strong IgE binding ability as the natural one (Besler, Steinhart, & Paschke, 1997), two

studies confirmed that the immunological properties of deglycosylated ovomucoid can be affected by heat treatment more easily than native ovomucoid (Gu et al., 1989; Yamamoto et al., 1998). The removal of glycan moieties by carbohydrate enzymes from *Aspergillus oryzae* may benefit the proteases from the same strain to access to the linear epitopes in ovomucoid.

It's interesting to note that only the dominant egg allergen, ovomucoid, rather than the other egg white proteins, was modified during fermentation. It seems that slight structural modification of ovomucoid led to significant IgE binding ability reduction of whole egg white. Compared to other major egg white proteins, ovomucoid is more resistant to hydrolysis because of its primary sequence and conformational structure. Ovomucoid contains three tandem domains and each of them is homologous to pancreatic secretary trypsin inhibitor (Kato et al., 1987). Although ovomucoid contains no inter-domain disulfide bonds, each domain is cross-linked by three intra-domain disulfide bonds, rendering ovomucoid resistant to heat denaturation and digestive enzymes (Matsuda, Watanabe, & Nakamura, 1982; Kato et al., 1987). Losing several carbohydrate moieties and/or peptide sequences of ovomucoid alone cannot explain the tremendous reduction of its IgE binding ability as there are eight linear epitopes along the primary sequence of ovomucoid (Mine & Zhang, 2002). It is not known how the slight structural modifications and/or cleavages significantly affect the IgE binding ability of ovomucoid.

In our preliminary experiment, we also inoculated the mycelium of *Aspergillus oryzae* into nature egg white of pH 9; however, no reduction of egg

white IgE binding ability was observed in those trials. The pH in egg white might have a huge effect on either the generation of enzymes or the activity of the enzymes from the mycelium of *Aspergillus oryzae*. It is evident that the mild acid pH condition in egg white is favorable for the reduction of egg white IgE binding ability, and this phenomenon is in accordance with the claims from Ferea & Botstein (1999) who reported that more protease genes of *Aspergillus oryzae* function in acidic pH. Further study is needed to characterize the structural modification of ovomucoid and enzymes produced in *Aspergillus oryzae* fermentation that are responsible for the observed change of IgE binding ability.

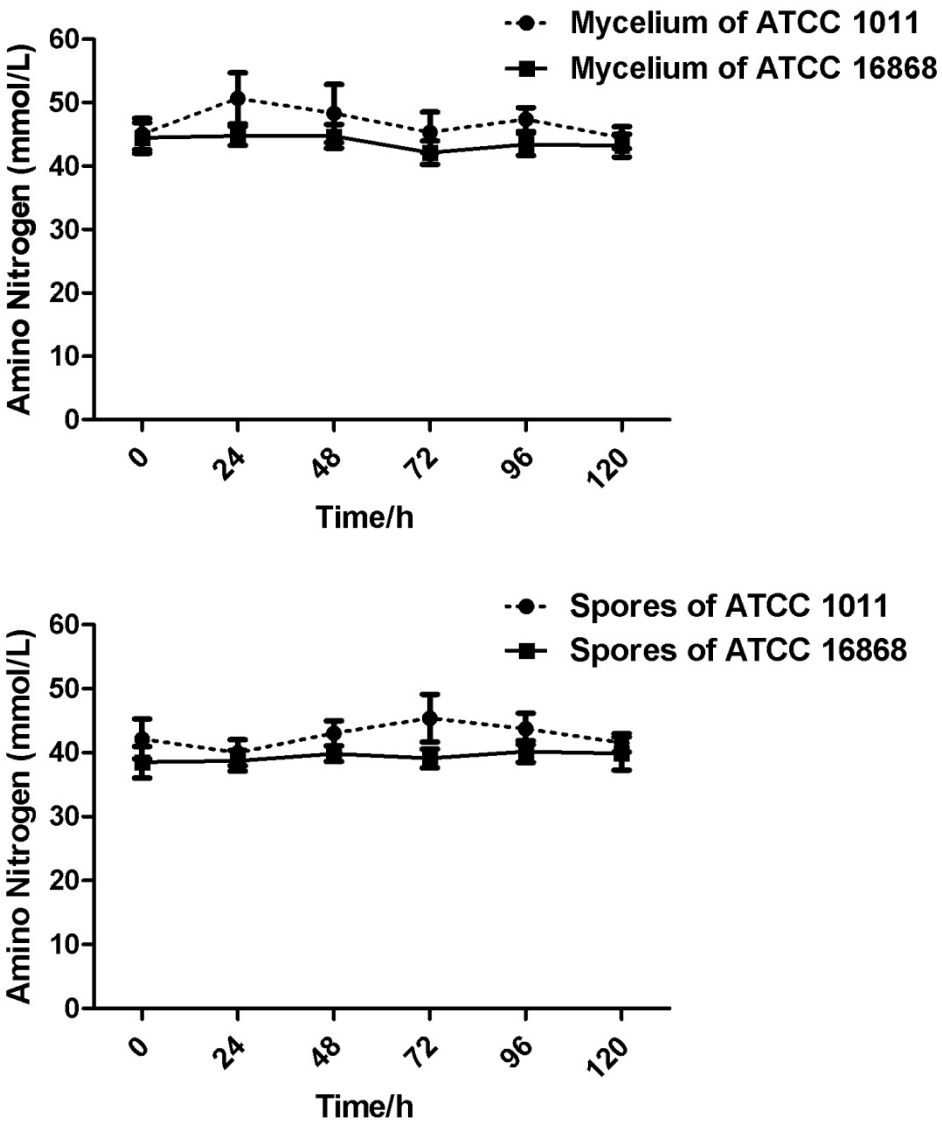

Figure 3. 1 Effect of fermentation time on the amino nitrogen content of *Aspergillus oryzae* fermented egg white. Results are means ± standard deviation of triplicate analyses each of two independent fermentations. The statistical analysis by Dunnett's test of one way ANOVA showed no significant difference of amino nitrogen content of egg white during 120 h fermentation.

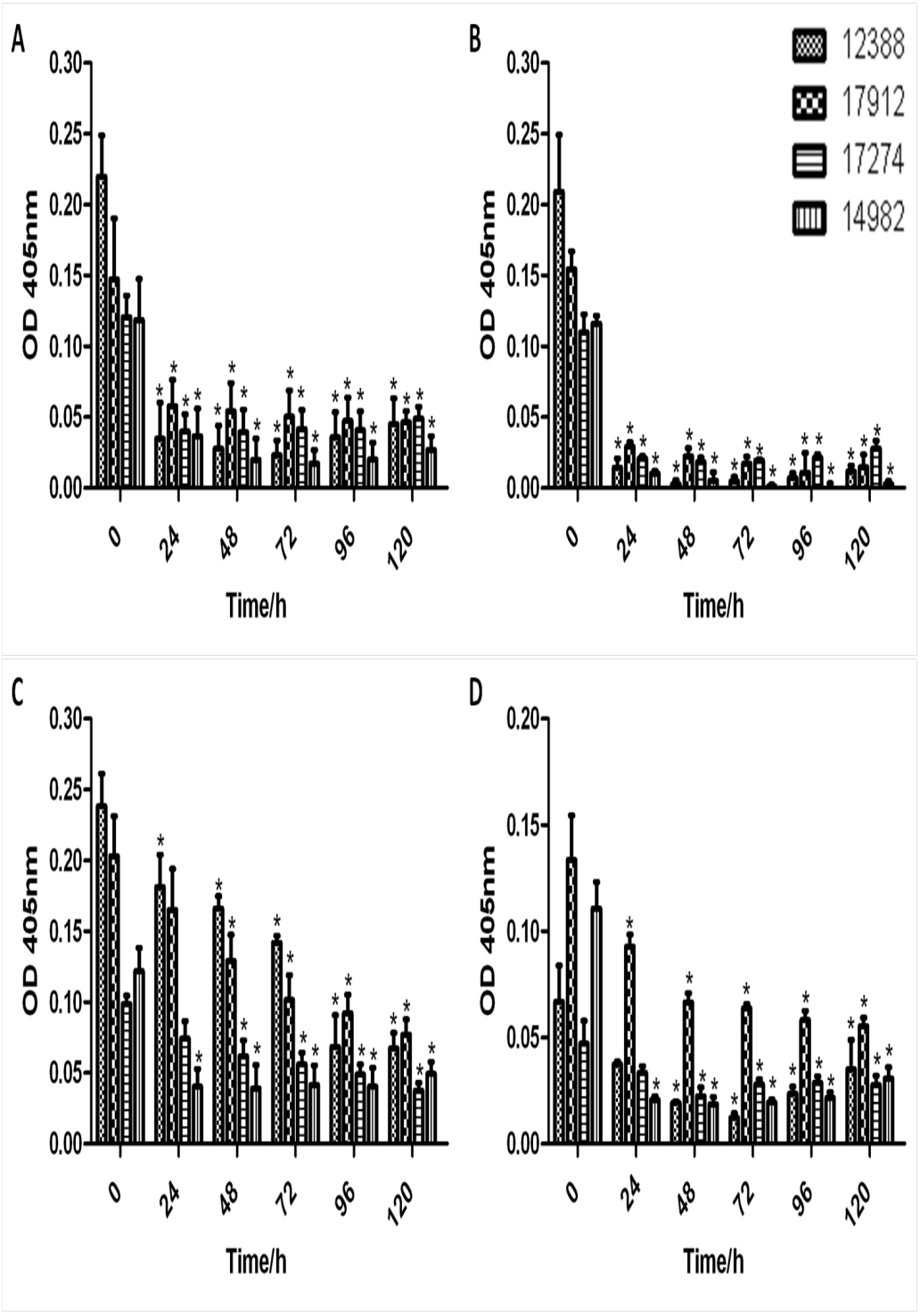

Figure 3. 2 Effect of fermentation time on the IgE binding ability of egg white proteins by *Aspergillus oryzae* fermentation. Results are means ± standard

deviation of triplicate analyses each of two independent fermentations using 4 egg allergy patients' plasma (#12388, 17912, 17274 and 14982). A. Egg white inoculated with mycelium of ATCC 1011. B. Egg white inoculated with mycelium of ATCC 16868. C. Egg white inoculated with conidia spores of ATCC 1011. D. Egg white inoculated with conidia spores of ATCC 16868. The statistical analysis by Dunnett's test of one way ANOVA indicated that the IgE binding ability of egg white fermented with mycelium of ATCC 1011 and ATCC 16868 showed significant difference ($p<0.001$) at 24, 48, 72, and 96 h of incubation towards all four plasmas from egg allergy patients (labelled with star).

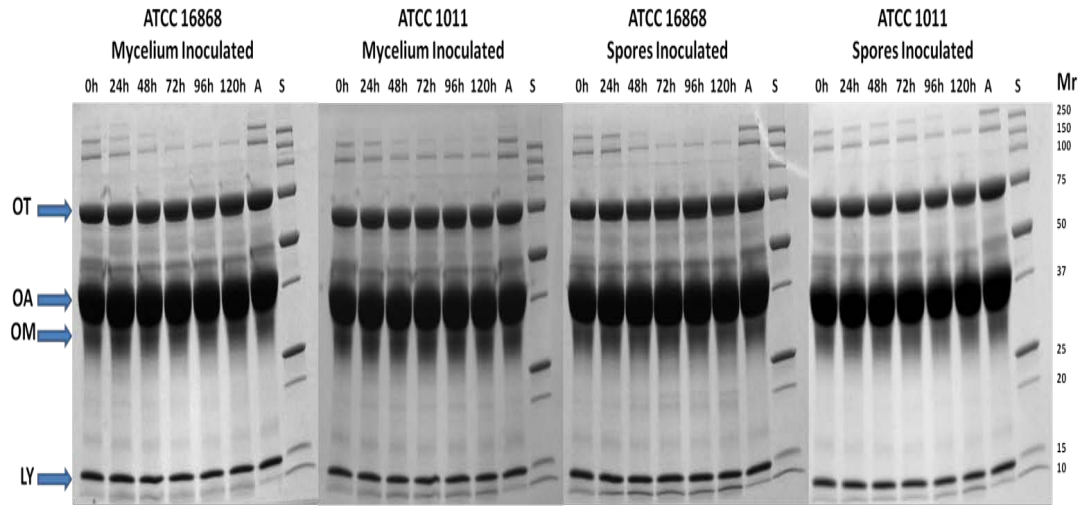

Figure 3. 3 SDS-PAGE analysis of effect of *Aspergillus oryzae* fermentation time on acid egg white. OT-Ovotransferrin; OA-Ovalbumin; OM-Ovomucoid; LY-Lysozyme. S-Molecular weight marker proteins (Molecular weights are indicated on the right in kDa). A- Acidic egg white with pH 6 before fermentation.

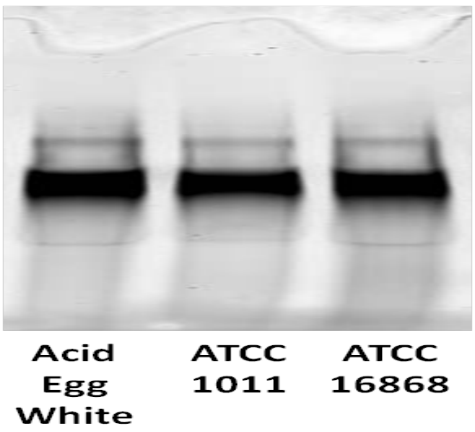

Figure 3. 4 SDS-PAGE analysis of FITC labeled ovomucoid during fermentation with mycelium of *Aspergillus oryzae* ATCC 1011 or ATCC 16868.

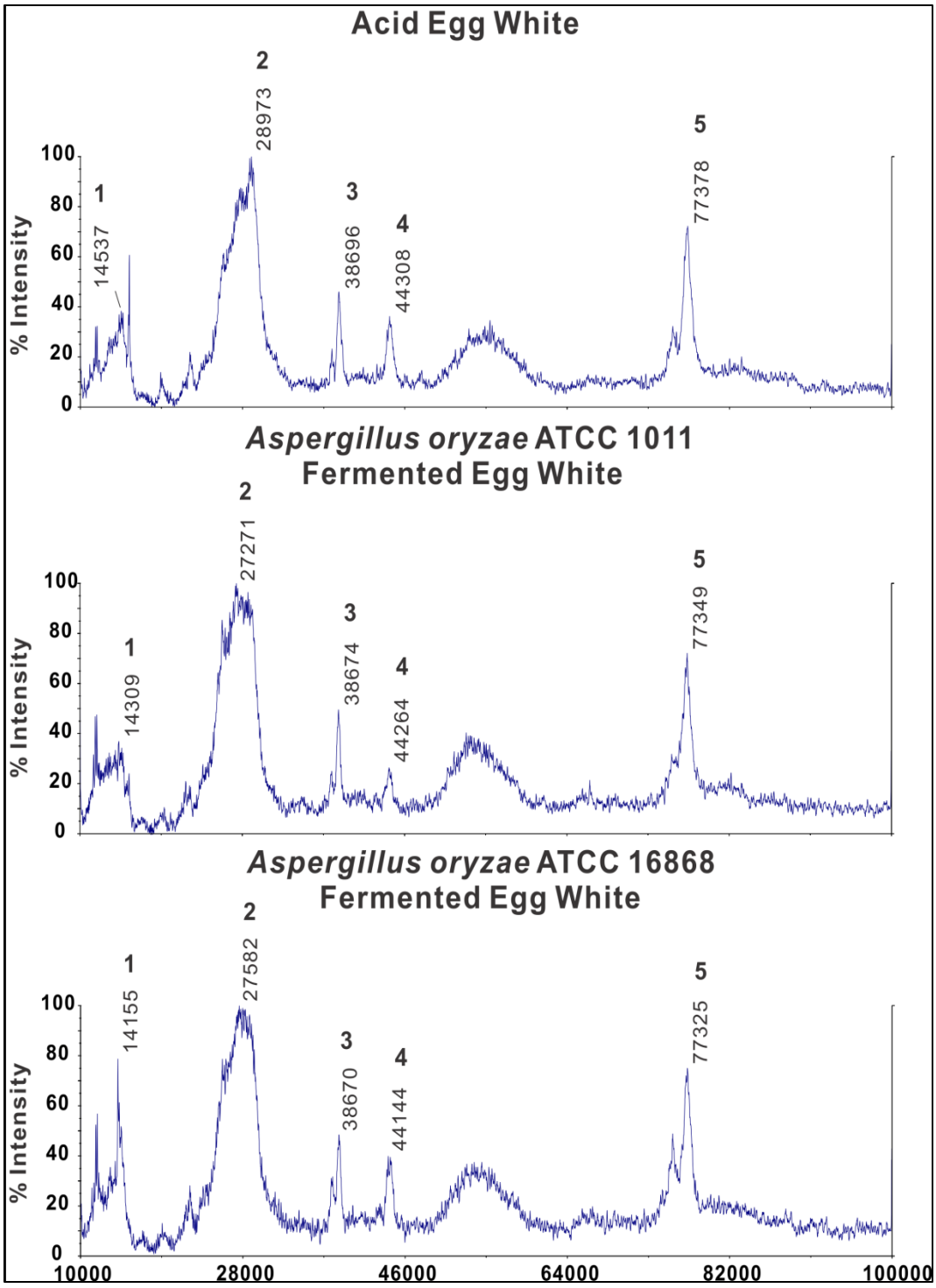

Figure 3. 5 MADLI-TOF-MS analysis of egg white proteins after fermentation with *Aspergillus oryzae* ATCC 1011 or ATCC 16868. Acid egg

white without fermentation and acid egg white fermented with the mycelium of ATCC 1011 or ATCC 16868 were analyzed. Peak 1 represents lysozyme, peak 2 represents ovomucoid, peak 3 represents ovotransferrin after being broken down into two equivalent domains during ionization, peak 4 represents ovalbumin, and peak 5 represents ovotransferrin.

3.5. REFERENCES

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CHAPTER 4 FINAL REMARKS

4.1. ONE MAJOR FOOD ALLERGEN: EGG WHITES

4.1.1. Egg whites

Egg white is composed of 88% water and 9.7~10.6% (w/w) proteins. The major proteins in egg white include: ovalbumin (54%, w/w of dry matter), ovotransferrin (12%), ovomucoid (11%), ovomucin (3.5%), lysozyme (3.4%), G2 globulin (4%), G3 globulin (4%), ovoinhibitor (1.5%), ovoglycoprotein (1%), ovoflavoprotein (0.8%), ovomacroglobulin (0.5%), cystatin (0.05%), and avidin (0.05%) (Mine, 2007). Carbohydrate, lipids, and minerals account for 0.4~0.9%, 0.03%, and 0.5~0.6% of the dry matter of egg white, respectively.

By nature, the bio-function of egg white is to protect the embryo from infection of microorganism and to provide necessary nutrients for the embryo to develop (Kovacs-Nolan, Phillips, & Mine, 2005). Although the pH of egg white in freshly laid egg is 7.6~7.9, it will rise up to 9.7 due to the diffusion of solubilized CO_2 during storage (Belitz, Grosch, & Schieberle, 2008). Lysozyme can inhibit Gram-positive bacteria by cleaving the β-1, 4-glycosidic linkage between N-acetylmuraminic acid and N-acetylglucosamine of the polysaccharide components in their cell wall (Mine, 2008). The high pH environment and the presence of antimicrobial proteins such as lysozyme in fresh egg white indicate that egg white is not an ideal substrate for the growth of lactobacilli (Nath & Baker, 1973).

4.1.2. Significance of producing hypoallergenic egg white products

Egg whites are widely used in many food products, because of their excellent foaming, gelling, and emulsion properties. Egg white will become milk white and form a thermo-irreversible gel coagulum after being heated. The denaturation and coagulation of egg white give angel cakes and puddings their characteristic textural qualities. Egg white is also an excellent food foaming agent as it possesses supreme foaming capacity and stability that are caused by the interactions among various egg white proteins. The combined effects of thermal coagulation and foaming enable the products with egg white to maintain stable textural properties (Mine, 1995). Egg white is everywhere, in bakery products, sauces, ice cream, cookies, and so on. Regulation requires manufactures to label any food product containing egg ingredients, however, cross-contamination of egg ingredients during handling, storage, transportation, machinery operation or misconduct in manufacturing may lead to inclusion of egg ingredient without appropriate labeling. Currently, there is no treatment for egg allergy and the most common method to eliminate the food IgE binding ability is to use protease to hydrolyze the food allergens. Extensive enzymatic digestion will cause the food materials to lose their functionalities, which may limit their applications in food industry. Therefore, it remains a challenge to prepare hypoallergenic egg white products while maintaining its premium functionalities. However, fermentation may provide a possibility.

4.2. A SUMMARY OF PRESENT RESEARCH RESULTS

4.2.1. Reduction of egg white IgE binding ability after fermentation

L. sanfranciscensis, *L. sakei*, and *L. delbrueckii* subsp. *delbrueckii* managed to sustain in acidic egg white supplemented with tryptone up to 96 h; and *L. delbrueckii* subsp. *delbrueckii* could remarkably reduce the IgE binding ability of egg white proteins after 48 h of incubation, to 50% of its original binding ability based on ELISA results using plasma from four egg allergy patients.

The IgE binding ability of egg white proteins was significantly reduced within the first 24 h after incubation of mycelium of *Aspergillus oryzae*, using plasma from four egg allergy patients. The mycelium of *Aspergillus oryzae* ATCC 16868 almost eliminated the IgE binding ability of egg white proteins after 24 h of fermentation.

4.2.2. The modifications of ovomucoid during fermentation

Compared to other major egg white proteins, ovomucoid is more resistant to hydrolysis because of its primary sequence and conformational structure. Ovomucoid contains three tandem domains and each of them is homologous to pancreatic secretary trypsin inhibitor (Kato et al., 1987). Although ovomucoid contains no inter-domain disulfide bonds, each domain is cross-linked by three intra-domain disulfide bonds, rendering ovomucoid resistant to heat denaturation

and digestive enzymes (Matsuda, Watanabe, & Nakamura, 1982; Kato et al., 1987).

Our study showed that ovomucoid was the only protein that displayed obvious molecular weight changes but not extensive degradation during fermentation of *L. delbrueckii* subsp. *delbrueckii* by MALDI-TOF-MS analysis. Furthermore, the intensity of the FITC labeled ovomucoid in *L. delbrueckii* subsp. *delbrueckii* fermented egg white was reduced compared to the one in egg white without fermentation, which indicated that the moieties adjacent to the FITC might be removed by enzymes from *L. delbrueckii* subsp. *delbrueckii*. Because the FITC may react with the amine ends of proteins, N-terminals of ovomucoid were possibly cleaved. On the other hand, ovomucoid was not extensively degraded and the three tandem domains were probably still connected during fermentation based on the MADLI-TOF-MS analysis.

For egg white fermented with *Aspergillus oryzae*, again only the dominant egg allergen, ovomucoid, rather than the other egg white proteins, was modified tremendously. However, the band intensity of FITC labeled ovomucoid was not affected during fermentation, compared to the one in acidified egg white without fermentation. As a result, the FITC adjacent moieties in ovomucoid may not be removed by the enzymes from *Aspergillus oryzae*. According to MALDI-TOF-MS, the molecular weight declines of ovomucoid during *Aspergillus oryzae* fermentations were ~1.5 kDa and the other parts of ovomucoid seemed not being cleaved.

The removal of FITC adjacent moieties of ovomucoid by the enzymes from *L. delbrueckii* subsp. *delbrueckii* resulted in up to 50% egg white IgE binding ability reduction; the slight cleavages of ovomucoid, removing moieties of ~1.5 kDa, almost led to the IgE binding ability elimination of whole egg white when the mycelium of *Aspergillus oryzae* was inoculated. Ovomucoid was reported to contain eight linear IgE/B-cell epitopes (Mine & Zhang, 2002) and it is not known how the slight structural modifications and/or cleavages affected the IgE binding ability of this protein substantially.

4.3. RECOMMENDATIONS FOR FURTHER RESEARCH

Ascertaining the exact moieties that have been removed from ovomucoid during the fermentation will help us to better understand the mechanism of IgE bind ability reduction of egg white. In order to achieve this, ovomucoid needs to be purified from unfermented and fermented egg white, hydrolyzed by digestive enzymes, and analyzed by MALDI-TOF-MS.

Functionalities of fermented egg white such as emulsion capacity and stability and forming capacity and stability need to be determined.

4.4. REFERENCES

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