REDUCTION OF WHEAT ALLERGEN POTENCY BY PULSED ULTRAVIOLET LIGHT, HIGH HYDROSTACTIC PRESSURE AND NON-THERMAL PLASMA

By

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To my parents, Jaikrishna and Vidya Nooji, and my husband, Pradeep Rao

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LIST OF ABBREVIATIONS

HHP	High hydrostatic pressure
HMW	High molecular weight
lgE	Immunoglobulin E
LMW	Low molecular weight
NTP	Non-thermal plasma
PUV	Pulsed ultraviolet light

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Wheat allergy is known to elicit adverse immune reaction in children and adults. Ingestion and inhalation of wheat proteins can cause Baker's asthma, urticaria, atopic dermatitis, gastrointestinal symptoms and wheat dependent exercise induced anaphylaxis (WDEIA) in hypersensitive individuals. Allergenic proteins are present in the entire wheat protein fractions which are mainly characterized into; water/salt soluble albumin and globulin fractions and insoluble gluten fraction. Total avoidance has been utilized in an attempt to reduce allergic reactions but, is often impractical or ineffective; thus research focusing on using processing technologies to alter food allergens is gaining more and more attention. Various thermal and non-thermal processing techniques have been utilized to alter the structure of the allergenic proteins. One of the major disadvantage of conventional thermal processing over non-thermal processing is it causes undesirable effects on the nutritive and sensory qualities. Pulsed ultraviolet light (PUV), high hydrostatic pressure (HHP) and non-thermal plasma (NTP) are non-thermal processing methods which can potentially alter wheat protein conformation and hence, reduce the immunereactivity. The main objective of this study was to assess the effect of PUV alone or in combination with heat, HHP and NTP on the IgE binding of

wheat protein extract. Wheat protein extract was subjected to PUV at 3pulses for 30 s, 60 s, 90 s, 120 s and 120 s followed by boiling. In addition, HHP (21°C and 70°C for 5 and 15 min) and NTP (1, 3 and 5 min) were also applied to wheat proteins extracts. The control (untreated), PUV, HHP and NTP treated samples were analyzed by SDS-PAGE, western blots, dot blots and indirect ELISA. Allergen potency indicated by IgE binding was determined in blots and ELISA. The PUV, HHP and NTP treatments indicated noticeable difference in the proteins profile demonstrated by SDS-PAGE. A significant reduction in IgE binding was observed in PUV (90 s), HHP (21°C and 70°C for 5 min) and NTP (5 min) treated samples as demonstrated by indirect ELSIA. The maximum reduction in IgE binding was achieved by PUV (46%) and HHP (42%) treatments. These findings indicate that non-thermal processing methods can be implemented to reduce wheat allergen potency.

CHAPTER 1
INTRODUCTION

Food allergies are abnormal immunological responses to normally harmless foods or food components, which are almost always protein based. Reactions that occur within minutes to an hour are considered immediate hypersensitivity reactions and are mediated by immunoglobulin E (IgE) antibodies. Delayed hypersensitivity reactions take place between 6-24 hours or more after ingestion and are cell-mediated. Various studies suggest that the prevalence of food allergy in United States is approximately 1.0% - 4.0% (Sampson 1999; Sicherer and others 2003). Food allergy is a constant problem in the population which includes children, adolescents and adults who are allergic to certain foods. According to the Food Allergen Labeling and Consumer Protection Act (FALPCA) (FDA 2009), an estimated 2% of adults and 5% of young children and infants suffer from allergy in Unites States. The prevalence of food allergy amongst children under 18 years has increased by 18% from 1997 to 2007 (Branum, CDC 2008). The FDA (2009) has listed eight major foods that are responsible for approximately 90% of overall food allergic reaction which includes - milk, eggs, fish, crustacean shellfish, tree nuts, peanuts, wheat and soybeans.

Wheat is an essential part of the diet in United States and several other countries throughout the world, including China, Japan, Germany, and India (Pomeranz 1988). According to FAO (2008), China is the leading wheat producing country followed by India, United States, and Russia, with average wheat production at 112, 78, 68, and 63 million metric tons, respectively. Wheat is among the major cereals cultivated throughout the world along with rice, maize, barley, rye, sorghum, oats and millet. Wheat is mostly milled into a flour form and, is consumed in the form of bread, biscuits,

pasta products, breakfast cereals, couscous, chapathi (Indian bread), tortilla and many other wheat based products.

Wheat is now recognized as a source of allergens and is responsible for various allergenic reactions (Mittag and others 2004; Sicherer 2002; Scibilia and others 2006; Sampson 1999). Allergic reactions to wheat manifest in children as well as in adults (Battais and others 2005; Matsuo and others 2004; Scibilia and others 2006). Although many researchers (Sicherer 2002) (Takizawa and others 2001) have put forward the theory that children outgrow wheat allergy with age, adults were also found to suffer severely from wheat allergens (Scibilia and others 2006; Sampson 1999). Some of the adverse reactions demonstrated by individuals allergic to wheat are urticaria, atopic dermatitis, baker's asthma, gastrointestinal symptoms and anaphylaxis (Takizawa and others 2001; Pastorello and others 2007; Luis and others 1990; Varjonen and others 1994; Simonato and others 2001). Currently there is no medical prevention or cure for food allergies. Therefore, the best way in food allergy management is complete elimination of allergenic foods.

Food proteins are main food components that are required for both functional properties of foods and nutritional importance in the human diet. The functional properties of proteins are numerous, as they are used for emulsifying agents, viscoelastic properties, textural properties, solubility, gelation, thickness swelling, and many more (Sikorski 2001). In terms of their nutritional importance, food proteins contain amino acids essential for human growth, maintenance, repair and metabolism (Sikorski 2001). However, as previously mentioned they are responsible for adverse

reaction in individuals allergic to them. Therefore, for certain individuals choosing food proteins can be restrictive.

According to the FDA's food label regulation the eight foods should be declared on the food label; nonetheless, some of the allergens may be accidently transported in the food product, or may be present in unspecified manner as a derivative of food allergens (FDA 2005). There are certain cases due to cross-contamination where the individual reacts to a minor amount of allergens present in the food. In these instances, most often food products are manufactured on equipment which is also used to produce other allergen containing foods; this is called cross-contamination, and allergenic proteins are introduced into the food inadvertently. Other times, consumers may unknowingly purchase foods which contain allergenic proteins like, children, and adults who have less knowledge regarding allergens (Vierk and others 2007). As described, food proteins, including allergenic proteins, can be used for their functional and organoleptic properties which can be present in the form (hydrolyzed wheat proteins as meaty flavor enhancer, whey and casein powders, soy lecithin and so on) that are not obvious as a food ingredient and or too difficult/technical to assimilate (Vierk and others 2007).

There have been numerous attempts to reduce or eliminate food allergens through processing and technology. Some of these methods have proven to be effective and include deamidation of amino acids, enzymatic hydrolysis, step-wise polishing, high pressure, irradiation methods, and thermal treatment (boiling, extrusion, cooking) (Sicherer 2002; Zhenxing and others 2007b; Mondoulet and others 2005; Ehn and others 2004; Handoyo and others 2008; Davis and Williams 1998). However, the demand for new technologies which can produce less-allergenic food products is

becoming more vital. Thermal treatment, although sometimes effective in reducing food allergens, can make the food products lose their quality by affecting the taste, texture and other organoleptic properties. Non-thermal technologies can be advantageous because they often have minor effect on the overall product's quality (Chung and others 2008; Yang and others 2010; Messens and others 1997). The main objective of this study was to analyze the effect of pulsed ultraviolet light (PUV), high hydrostatic pressure (HPP), and non-thermal plasma (NTP) technology on major wheat allergen reactivity. The wheat protein extracts were treated with PUV, HHP and NTP and protein profiles were analyzed by gel electrophoresis. The IgE reactivity was investigated by Western blot, dot blot and indirect ELISA (enzyme linked immunosorbent assay) with pooled human plasma from patients with clinical history of wheat allergy.

CHAPTER 2
LITERATURE REVIEW

Food Allergy and Allergens

Consumption of foods with allergenic components can cause various disorders in hypersensitive individuals. Food allergy can be caused by inhalation or ingestion of an allergen and, is immune-mediated. Non-immune mediated reactions are known as “food intolerances” (Sicherer 2002; Sampson 2004).

The immune mediated food allergy can be further categorized into IgE mediated and non-IgE mediated allergy (Asero and others 2007). The IgE or immunoglobulin E antibody is involved in inducing immediate reactions following the intake of food allergens (Tanabe 2007). During the initial exposure of the food, the allergen binds to T-cells which subsequently activate B-cells. The B-cells produce and release IgE which is cross-linked to mast cells or basophils (Tanabe 2007). This process is called sensitization. Upon re-exposure to the allergen, IgE cross links the allergen which causes degranulation of the mast cells. Chemical mediators such as histamine and prostaglandin are released that ultimately cause allergic reactions in the individual. This process results in the adverse reactions seen in hypersensitive individuals, like anaphylaxis, urticaria, gastrointestinal symptoms and other various adverse reactions (Tanabe 2007; Sampson 1999, 2004). Some of the most common food allergen and have been mentioned in Table 2-1.

Extensive studies have been conducted over the past few decades in order to identify the actual cause of IgE mediated food allergies. According to these studies and the current insight of food allergy, it is now understood that the IgE binds to the specific site on the allergen or the surface of allergen protein known as an epitope (Tanabe

2007). The epitope is categorized into linear and conformational epitopes depending on the epitope structure (Bannon 2004; Davis and Williams 1998). The cross reactivity between the epitope and the allergen is determined by the structure of the allergen protein and the sequence of the amino acids (Bannon 2004; Davis and Williams 1998).

In order to understand allergen epitopes, a short review on protein conformation is required. The structure of the protein is categorized as primary (amino acid sequence), secondary, tertiary and quaternary structure. The primary structure refers to the linear sequence of amino acids connected to each other via peptide bonds. It forms the back bone for other higher structure of proteins. The secondary structure refers to the three dimensional organizations of segments of polypeptide chain. It includes (a) α-helix (b) β-sheet and (c) random coil. The α-helix is stabilized by hydrogen bonding and is an organized structure where the hydrogen bonding occurs within single protein chain. The β-sheet is in the form of zigzag and each β-sheet strand is connected via hydrogen bonds (Damodaran and others 1996). The stretched proteins combine to form β-pleated sheets. The random coil arrangement has no ordered pattern along the polypeptide chain. This kind of structure is formed when the amino acid side chains prevent formation of α-helix or β-sheet. The tertiary structure is a three dimensional organization where the linear protein chain with secondary structure segments folds into three dimensional arrangement. The quaternary structure contains more than one polypeptide chain. More than one polypeptide chains are linked with each other via non-covalent interaction (Damodaran and others 1996).

A linear epitope is composed of a short sequence of amino. A conformational epitope is composed of various amino acids on the protein that are brought together by

folding or determined by secondary, tertiary or quaternary structure of the protein. Therefore, for the IgE to crosslink the conformational epitope, secondary and tertiary structure of protein is necessary; whereas IgE binding to linear epitopes can entail the primary structure of the protein as well (Bannon 2004). During heating, hydrolysis and other physical/chemical treatments there can be a great effect on protein structure; some of the proteins' secondary and tertiary structures are disrupted, exposing smaller amino acid sequence to the exterior of the protein (Sathe and Sharma 2009). These once-buried structures are hydrophobic sites on proteins that are hidden from surfacing water or other solvent (Tanabe 2007; Sampson 1999) and can contain linear epitopes that become exposed upon denaturation. Conversely, this conformational change can destroy conformational epitopes.

According to a study involving 20 children having a history of cow's milk allergy, patients exposed to milk allergens had significantly higher IgE reactivity to linearized proteins (linear epitopes) compared to proteins in their native state (conformational epitope) (Vila and others 2001). The linear epitopes were revealed by treating the native milk proteins (α-casein, β-casein, α-lactoglobulin and β-lactoglobulin) with DTT (dithiothreitol), which denatured the secondary and tertiary structure. Similarly in another study by (Järvinen and others 2007), IgE binding to linear and conformational epitopes was observed in 37 patients, in the 16 who had already outgrown the allergy, as well as those with persistent allergy to egg. High IgE reactivity was observed to both linear and sequential epitopes in patients with persistent allergy compared to patients with tolerance. The author also speculated that linear-epitope-binding must be considered in order to identify the actual allergenic nature of food proteins. Since the

allergen protein undergoes digestion that includes enzymatic hydrolysis the linear epitope is exposed in the gut due to disruption of secondary and tertiary structure (Pasini and others 2001; De Zorzi and others 2007).

The Application Support Center provides a group of general purpose styles to help you format your document and give your thesis or dissertation a continuity of appearance. Styles cannot do everything but they can be used for general formatting purposes. Each style created by the ASC is listed in Table 2-1.

History of Wheat

The origin of wheat is believed to be from the hybridization of an Eurasion emmer - type wheat and wild species of grass. Durum wheat or *Triticum turgidum* is closely related to *Triticum dicoccoides* (hexaploid wheat), and is native to wild emmer wheat. Modern cultivated wheat belongs to two species: (1) hexaploid bread wheat, *T. aestivum*, and (2) tetraploid, hard or durum-type wheat, *T. turgidum*. Wheat tends to grow well in temperate climate and is grown throughout the year in some part of the World (Pomeranz 1988; Carver 2009). From January to March it is cultivated in Australia, Argentina and India; between April and June it is grown in Mexico, Japan, China, and some parts of United States and from July to September it is grown in France, Germany, England, Canada and Norway; finally from October to December it is cultivated in Finland, South Africa, Burma, and Argentina. Many countries in the world grow wheat as their main source of food supply (Pomeranz 1988).

Wheat and related cereals like rice, barley and rye are an important part of the diet in Europe, Asia, United States and other parts of the world (Pomeranz 1988). In addition to its nutritional importance, wheat is widely utilized in the baking industry for its excellent viscoelastic properties (Ahmedna and others 1999). Hexaploid wheat; *Triticum*

aestivum, is used for the production of bread flour, and tetraploid; *T.turgidum*, hard or durum type wheat, which is mainly used for pasta products Table 2-2 illustrates the nutritive value of different cereals, which indicates wheat is comparatively high in proteins versus other cereals. Some of the most common wheat based products include bread, biscuits, pasta, couscous, pita and chapathi/naan (Indian bread). Wheat is most commonly divided into winter or spring wheat which refers to the season during which the crop is grown. The other most commonly used commercial classification is hard or soft wheat that refers to the kernel strength and hardness. Durum wheat is the best example of hard wheat and is used in the pasta and noodle products. Soft wheat is preferred for products like bread, biscuits and pastries.

Wheat Kernel Structure

A wheat kernel is approximately 5 to 8 mm in length and 2.5 to 4.5 in width. In the technological perspective wheat kernel or seed mainly consists of bran, endosperm, and the germ (Figure 2-1). The wheat bran consists of a pericarp, which is the outermost hard layer, and closely adheres to the seed coat. Beneath a layer of nuclear tissue is the aleurone layer (Figure 2-2 and 2-3). The starchy endosperm is beneath the aleurone layer. During milling, the germ, bran and the endosperm can be separated and used for varieties of products. The whole wheat flour consists of nutrients from all the layers (Pomeranz 1988). The white flour is produced by separating bran and germ from the endosperm, and solely using the endosperm portion. Table 2-3 illustrates, the chemical composition of endosperm, bran and germ and clearly shows wheat endosperm contains high amount of starch compared to other parts. Therefore, the endosperm is widely utilized for the production of wheat flour for baking. The germ is present on the dorsal side of the wheat kernel and accounts for 2-4% of the kernel

weight. The germ or embryo transports nutrients during sprouting and germination of the seed and it will grow into new plant, absorbing nutrients from the endosperm during its development (Eliasson and Larsson 1993).

Wheat Proteins

Wheat and other related cereals proteins are mainly divided based on their solubility. According to the Osborne classification that is most widely adopted, wheat, barley, rye and other cereals are classified into albumin, globulin, prolamin and glutelin protein fraction depending on their solubility in different solvents (Shewry and others 1986; Osborne 1907). The wheat proteins are also classified into two major groups, the storage proteins which consist of gluten proteins and cytoplasmic or metabolically active proteins i.e. albumin and globulin fraction.

Albumins and Globulins

Albumin and globulin fraction are classified as water and salt soluble proteins and together comprises to about 10 to 15% of total wheat proteins. The albumin proteins present in wheat are soluble in water and account for 10% of total wheat proteins whereas salt soluble globulin accounts for only 5%. Albumins can be easily extracted by dissolving the whole wheat flour in water and the supernatant derived is considered as albumin fraction. Lockehart and Bean (1995) performed a sequential extraction of albumin in deionized water in the ratio 1:5 (w/v). The resulting supernatant was considered as albumin fraction. The globulin fraction is extracted in dilute salt solution (NaCl). The albumin and globulin proteins are also known as cytoplasmic proteins and contain high amount of amino acids, especially lysine, compared to other wheat proteins. These proteins contain metabolic enzymes, enzymes that hydrolyze proteins, enzyme inhibitors; α amylase/trypsin inhibitors and phytohemaglutenins.

Storage Proteins

The water/salt-insoluble gluten protein fraction of wheat is the main storage protein of the wheat kernel and accounts for 50 to 80% of total wheat proteins, also known as prolamins. Gluten can be prepared by washing the whole wheat in dilute salt solution to remove any water/salt soluble albumin and globulin fraction. The resulting sticky mass is known as gluten that can be subdivided into two major protein groups: (1) gliadins and (2) glutenins (Field and others 1982; Tatham and others 2000; Shewry and others 1986; Osborne 1907)

The monomeric gliadins containing single polypeptide chains are soluble in 70% (v/v) ethanol and comprises of α-, β-, γ-, and ω- gliadins based on their mobility in gel electrophoresis. The α-, β-, γ- gliadins have less proline, glutamine and phenylalanine but the sulfur containing amino acids - cysteine and methionine - are involved in intra/inter molecular disulfide linkage (Tatham and others 2000). The ω- gliadin has little or no sulfur containing amino acids but, has high amount of glutamine, proline and phenylalanine. During the treatment with reducing agents like DTT (Di thiotriol) or β-mercaptoethanol the disulfide bonds are cleaved resulting in more linear structure of amino acid sequence. The remaining proteins can be extracted in dilute acetic acid or dilute alkali and are known as glutenin fraction. Glutenin is a high molecular weight protein consisting of subunits stabilized by disulfide bonding. Reducing glutenin yields low molecular (LMW) and high molecular weight (HMW) subunits. Both subunits differ in molecular weight and amino acids. The molecular weight of HMW glutenin subunits can be larger than 70 kDa whereas, LMW subunits are smaller than 50 kDa. The polymeric glutenin fractions are high molecular weight proteins containing polypeptide chains bonded by intermolecular disulfide bonds. During the reduction of the disulfide bonds

the glutenin fraction results in low molecular weight (LMW) and high molecular weight (HWM) glutenin subunits (Shewry and others 1986; Pomeranz 1988).

Another way of distinguishing wheat gluten proteins are sulfur-rich (S-rich), S-poor and high molecular weight prolamins (Figure 2-4). The S-rich group contains α-, β-, γ-gliadins corresponding to 32 to 42 kDa and LMW glutenin subunits range from 36 to 44 kDa. The S-poor group contains ω- gliadin corresponding to 44 to 72 kDa. The third group is HWM prolamins comprising of HMW glutenin subunits (64 to 136 kDa) (Shewry and others 1986; Tatham and others 2000).

Gluten is usually extracted in acid, bases and alcohol. Chemical detergents such as sodium dodecly sulfate (SDS), urea, dithiothreitol (DTT) are often added to assist in extraction; however, these chemicals will disrupt the disulfide bonds resulting in non-native proteins. For these reasons extracting native wheat gluten can be problematic if proper solvent is not used. Furthermore, extraction allow apparition of the wheat gluten into gliadin and glutenin subunits by extracting these fractions separately. Glutenin subunits (including low and high molecular subunits) can be extracted in dilute acid (e.g. acetic acid, HCl) and gliadins can be efficiently extracted in 70% alcohol solvent.

Wheat Allergy

IgE-mediated allergy to wheat occurs after the ingestion or inhalation of wheat and related cereals like barley, rye, oats etc. Depending on the route of exposure, wheat may demonstrate as a classic IgE-mediated food allergy affecting skin, respiratory tract, gut, wheat dependent exercise induced anaphylaxis(WDEIA), occupational asthma also known as baker’s asthma, contact urticaria or atopic dermatitis. In children wheat is the causative factor for several immunological reactions like atopic dermatitis, nausea, abdominal pain and other gastrointestinal symptoms and anaphylaxis (Inomata 2009).

Studies indicate that most children outgrow the hypersensitivity to wheat by the age of 3 to 5 years unlike peanut, milk and eggs which have been shown to persist throughout the life time (Sicherer 2002). But the prevalence of wheat allergy in adults can be severe and fatal and therefore when considering wheat as a food allergen both children and adults should be given equal importance in diagnosis and treatment (Mittag and others 2004; Matsuo and others 2004; Scibilia and others 2006).

Baker's Asthma

Baker's asthma is an occupational allergy caused in bakery workers due to the inhalational of wheat flour. It is an IgE mediated allergy where the individual has specific IgE to wheat flour and the inhalation of wheat flour results in adverse reaction. The major allergens responsible for adverse reaction in individuals having baker's asthma are proteins from water-and-salt soluble fraction (α-amylase inhibitor family) (James and others 1997; Luis and others 1990). An immunoblotting study by Weiss and others (1993) showed extensive IgE binding to albumin and globulin polypeptides whereas lower IgE binding to gluten protein fractions. Another study by Mittag and others (2004) also showed similar results that illustrated that the water/salt soluble albumin and globulin fraction had high IgE reactivity in individuals having the symptoms of baker's asthma; however, no major allergen was identified. This shows that the individuals suffering from baker's asthma may have adverse reactions to various subfractions of albumin and globulin proteins.

Wheat-Dependent Exercise-Induced Anaphylaxis

Anaphylaxis is a severe form of food allergy, which can be life-threatening. Anaphylaxis reactions occurs when intake of foods like wheat, shellfish, peanuts, treenuts, egg, and milk (Sampson 1998). WDEIA reaction occurs after the intake of

wheat followed by exercise within 2 to 3 hrs. Scientists demonstrated that IgE reactivity of ω-5 gliadin (65 kDa) was major protein involved in WDEIA using skin prick tests, ELISA and immunoblotting (Mittag and others 2004; Palosuo and others 2003; Matsuo and others 2004).

Wheat Allergen Proteins

According to published literature, the allergen responsible for inducing the adverse reaction is glycoprotein range between 10-60 kDa (Watanabe and others 2001; Sanchez and others 1992). Glycoproteins are low molecular weight water-soluble proteins and are often stable to heating, acid treatment and proteases. Therefore these allergens are still active in the body even after undergoing extensive heat, enzymatic hydrolysis or other chemical treatment. Upon reaching the gut the allergenic protein can elicit the adverse reaction. Watanabe and others (2001) performed a study on the IgE reactivity of wheat glycoproteins. The IgE reactive protein was found to be a 60-kDa glycoprotein which was present in wheat α-amylase inhibitor (water/salt soluble protein). Hypoallergenic wheat flour was produced by the enzymatic treatment of the 60-kDa glycoprotein with actinase and cellulase. However, a 16-kDa glycosylated subunit of α-amylase inhibitor has shown to have substantial IgE reactivity in patients with Baker's asthma (Sanchez and others 1992; Garcia and others 1996).

Several researchers have demonstrated IgE binding to various fractions of wheat. From these different studies it should be noted that entire wheat protein fractions have been showed to elicit IgE reactivity in wheat allergic individuals, depending on the route and extent of exposure. A study done by Simonato and others (2001) showed IgE binding to soluble and insoluble protein fractions. This study examined 20 patients (atopic and non-atopic) with irritable bowel syndrome and other symptoms after the

ingestion of wheat. The immunoblotting results of this study recognized several IgE binding protein bands in water/salt soluble fraction. The authors also mentioned that a 16-kDa protein was the most frequently observed band in more than 50% of the sera; whereas, in the salt-insoluble proteins the IgE reacted to 42 kDa protein band.

Wheat α-Amylase/Trypsin Inhibitor and ω-5 Gliadin

Wheat α-amylase/trypsin inhibitor has been frequently reported in its involvement in wheat allergic patients and is confirmed as the major allergen in Baker's asthma. Pastorello and others (2007) investigated the IgE reactivity in three wheat protein fraction namely albumin/globulin, gliadin and glutenin. The strongest IgE binding was noted in α-amylase inhibitor along with IgE binding to lipid transfer protein (LTP) and LMW glutenin subunits. Similarly Simonato and others (2001) confirmed that cereal α-amylase/trypsin inhibitor corresponding to 16-kDa was the major allergen involved in atopic patients with positive results in skin prick test. Individuals with atopic dermatitis showed reactivity to low molecular weight proteins, particularly a 26-kDa protein as demonstrated by immunoblotting. Other IgE bands which ranged from 7 kDa-84 kDa were classified as minor allergens (Varjonen and others 1994). Five children with wheat allergy reacted to 15-kDa wheat protein as analyzed by Western blotting (James and others 1997). The results confirm that the low molecular weight protein is responsible for eliciting adverse reaction after the ingestion and inhalation of wheat in wheat allergic patients and in individuals with baker's asthma. In another study an intense IgE activity was observed to 26-, 38- and 69-kDa in children suffering from atopic dermatitis (AD). The results were confirmed by performing skin prick tests and radioallergsorbent test (RAST) (Varjonen and others 1995). A higher immuno reactivity was also found with gliadins, LMW glutenin subunits and various bands in albumin/globulin fraction; yet, the

sera had significantly lower IgE reactivity in HMW glutenin subunits (Battais and others 2003). The immunoblots of proteins from the albumin/globulin fraction ranged between 15-70 kDa, and includes α-amylase/trypsin inhibitor. The allergen reactivity appears to be varied depending on the age of the individual and symptoms. The major allergen associated with WDEIA patients is ω-5 gliadin, which has reported several times in individuals suffering from anaphylaxis followed by exercise (Tatham and Shewry 2008; Matsuo and others 2004). According to the study by Battais and others (2005) IgE reactivity to ω-5 gliadin was higher in adult patients with WDEIA and more than 50% (adults and children) reacted to the same allergen with urticaria. In the case of children there were no specificity in IgE reactivity to single allergen; however, reactivity to α-,β-, and γ-gliadin and extensive reactivity to the albumin/globulin fraction in children with atopic dermatitis was noted.

Lipid Transfer Protein (LTP)

Upon encounter with an allergen,the IgE binds to specific sequence of amino acid on the allergen which is known as epitope. The major IgE binding epitope has been recognized by Maruyama and others (1998) that consists of Gln-Gln-Gln-Pro-Pro (Gln = glutamine; pro = proline) which is confirmed as the major IgE binding site in wheat allergic individuals and is present in LMW glutenin subunits.

Research in Developing Reduced Allergenic Products

To date an extensive amount of research has been conducted in the following fields: identifying the IgE-mediated allergen associated with wheat allergy, managing the wheat allergy, treatment and various processing and methods that could potentially be used to produce non-allergenic and/or wheat products with reduced allergen potency. Processing methods used to reduce allergen potency are particularly of

interest because they could potentially help a large population of wheat allergy sufferers. Some of processing methods have shown to be effective; however; there are no hypoallergenic or reduced allergen wheat products currently in the market. This shows a huge demand for producing safer and healthier wheat based products that can be consumed by adults and children suffering from wheat allergy.

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Various efforts have been made to reduce the allergen reactivity of certain foods. During the processing conditions the allergen reactivity may be altered due to a change in protein structure. These modifications may ultimately result in the alteration of epitopes to which IgE would normally bind. Thermal treatment on food allergens is extensively researched due to its wide applicability in food processing. Heat treatment alters the native structure of proteins that result in denaturation. Denaturation of proteins modifies secondary and tertiary structure and results in formation and breakage of covalent and non-covalent bonds. Conformational epitopes are associated with IgE

binding to the secondary and tertiary structure; therefore modified structure may reduce an adverse immune response.

Heating food proteins result in unfolding which can be reversible or irreversible depending on the type of protein, temperature and extent of heating. The native structure of proteins has hydrophobic bonds buried inside whereas the hydrophilic sites are on the surface. Heating can lead to new inter/intramolecular interaction in proteins that includes, hydrogen bonding, electrostatic interaction, disulfide bonding. These interactions can be irreversible leading in unfolding and random coil conformation. However, this may not apply for all the proteins due to the treatment conditions, and therefore, unfolded proteins may refold resulting in new covalent/non-covalent interactions and regaining of allergenic activity. Some food allergens (proteins) are stable to heating, and their allergenic properties may not be affected.

Maillard reaction is a chemical reaction between the free amino group of peptide chain and reducing sugar during heating at sufficiently high temperature. The stages involved in Maillard reaction are complex are not completely understood. However, some of the distinctive stages are sugar and amine condensation and Amadori rearrangement. The second stage involves sugar dehydration and fragmentation, and the final stage includes the formation of heterocyclic nitrogen compounds. Some of the Maillard reaction intermediates are Schiff base, hydroxyl methyl furfural, aldehydes and ketones. The final products which impart the brown color that is often desired in cooked products are called melanoidins. Wheat undergoes the Maillard reaction during baking, boiling, fermentation, etc where browning is desired for the flavor components and appeal. Furthermore, the reaction can involve covalent/non-covalent modification in

proteins that contribute to allergenicity. De Zorzi and others (2007) studied the effect of pasta drying temperature (60 - 110°C) on the digestibility and allergenicity of wheat allergens. They found a change in protein solubility due to the formation disulfide and hydrophobic interaction, yet the interactions were reversible when they were subjected to digestive conditions. In contrast, heating at ultra-high temperature (110°C) resulted in decreased in proteins solubility that was irreversible. The immunological results indicated a reduction in the IgE reactivity when pasta was heated up to 80°C;pasta heated above this temperature showed increase in IgE reactivity. Heating proteins at high temperature in the presence of reducing sugar may result in the formation of new immunologically reactive structures in the protein. These new structures that have IgE and/or allergen activity are referred as neo antigens (Davis and others 2001).

The temperature at which the proteins are treated can have major effect on its structure and functionality. Bread is one of the examples of heat treated wheat product. During baking the bread crumb and crust reach different temperature. The temperature of the crust reaches almost to 200°C; whereas the crumb's temperature reachs approximately 100°C. Therefore the effect on proteins can be varied depending on the temperature and treatment time (Wal 2003). It was found that, compared to crumb, the crust contained proteins which showed lowered solubility following baking. Since the crust reached very high temperatures, there could be new irreversible covalent interaction involving aggregation, cross-linking and Maillard-type reaction. In contrast the crumb was more soluble due to significantly lower temperature. The interaction involved in crumb could be disulfide bonding and hydrophobic interaction due to

denaturation (Pasini and others 2001) that can be cleaved with a reducing agent (Davis and Williams 1998).

Beta-lacto globulin is one is of the major allergens present in milk and was shown to have reduced allergenicity when heated at 70°C (Ehn and others 2004). The researchers speculated that the IgE reactivity was decreased with increases in temperature (90°C); however, the heat treated milk still retained allergen reactivity to a minor extent.

A traditional roasting of peanuts involves heating at 140°C for 40 min whereas boiling of peanuts takes place at 100°C (Mondoulet and others 2005) for a shorter period of time. The roasted peanuts were confirmed to have significantly higher IgE reactivity to Ara h 1 and Ara h 2 (two major allergens present in peanuts) compared to boiled peanuts. Thus, it should be noted that heating proteins at certain temperature (80-100°C) causes denaturation of proteins resulting in reduced IgE binding. On the other hand, there is considerable difference in the IgE reactivity when proteins are heated at very high (>100°C) which may contribute to the formation of peptide fragments and ultimately amino acids that may or may not have adverse immune response (Korhonen and others 1998; Davis and others 2001; Davis and Williams 1998). These studies indicate that temperature is a large component involved in changing the allergen reactivity of proteins.

Wheat is consumed as whole wheat and white flour. Another processing method which separates the wheat into different components is called step-wise polishing. The endosperm of wheat, which contains high starch content, is separated by removing the outer layer and the, -bran (pericap and aleurone). The resulting high starch wheat flour

is extensively utilized in baked products, pasta, pastry etc. Whole wheat kernels that underwent stepwise polishing to remove the outer layer that contained high concentrations of the water/salt-soluble albumin and globulin fraction was ultimately separated (Handoyo and others 2008). Immunoblotting results demonstrated a reduction in IgE activity of the inner most fraction due to the lower concentration of albumin/globulin.

Gamma irradiation is a newer technique that has been researched in recent year for its ability in altering the proteins involved in allergenic response. The effect of gamma radiation was speculated on allergen in milk (β-lactoglobulin), chicken egg (albumin) and shrimp (tropomyosin) (Byun and others 2002). The irradiation dose ranged between 3 to 10 kGy, and the IgE binding activity was demonstrated by imunoblotting and ELISA. The IgE binding epitope was structurally altered and a reduced IgE binding was observed in all of the samples. (Zhenxing and others 2007b) found that irradiated shrimp (7-10 kGy) myosin showed a variation in the protein structure. A new band at 45 kDa was generated in irradiaded samples at 7 and 10 kGy demonstrated by SDS-PAGE. Pen a1, one of the major allergen in shrimp (36 kDa) existed in all the irradiated samples observed in SDS-PAGE results. The IgE reacted to the newly generated band (45 kDa) at 7 and 10 kGy, conversely the band at 15 kGy confirmed by Immunoblotting. ELISA and Immunoblotting indicate a reduction in IgE binding to Pen a 1 with increase in the dose level. Gamma irradiated (1-15 kGy) shrimp in conjunction with heat (100°C) has shown to have reduce IgE binding to shrimp allergens(5 – 30 fold) but, the radiation treatment alone was not effective in reducing the allergenicity (Zhenxing and others 2007a). On the other hand, gamma irradiated (1-

25 kGy) followed by thermal treatment (autoclaving, roasting, blanching, microwaving) almond, cashew and walnut showed no difference in the allergenicity (Su and others 2004). This shows the stability of some proteins that is stable to both radiation and thermal treatment. Wheat gliadin fraction, especially ω-5 gliadin, elicits an immune response in sensitive individuals – especially in WDEIA individuals (Maruyama and others 1998; Matsuo and others 2004; Simonato and others 2001). Irradiation treatment with Cobalt 60 (2.2-12.8 kGy) has shown to increase the allergenic response of the wheat gliadin fraction as confirmed by immunoblotting and ELISA. Specifically, IgE reactivity increased with dose levels of 12.2 kGy with higher IgE binding to whole wheat compared to the gliadin fraction alone, indicating the interaction of proteins in the presence of other proteins.

Similar results were obtained by microwave heating (30–150 kJ) of wheat gliadin fraction (Leszczynska and others 2003). A high immunological activity was confirmed at 40 kJ but, the response to allergen proteins decreased at 90 and 150 kJ detected by Immunoblotting and ELISA.

Deamidation of wheat proteins is performed in order to improve the functionality of the product - namely the emulsion stability, foaming capacity, solubility, and water-holding capacity compared to native proteins (Mimouni and others 1994). Deamidation is achieved either enzymatically or chemically. Degree of hydrolysis is the term used to define the number of peptide bonds cleaved during hydrolysis. Increase in the percentage is proportional to increase in the amount of fragmented proteins and better solubility. Acid deamidation of wheat gluten is found to have reduced IgE binding (Maruyama and others 1990). The gluten was deamidated with 30%, 50% and 90%-

demidation. The results indicated a significant reduction in IgE binding confirmed by dot blot.

Ultraviolet and Pulsed Ultraviolet Light Technology

Ultraviolet (UV) radiation has been widely utilized in the food industries as a pasteurization technology (Krishnamurthy and others 2008). Currently there are two types of UV mode: continuous and pulsed. Continuous UV light consists of wavelength region between 200 and 400 nm. The UV spectrum is further divided into short wave (UVC) from 200 to 280 nm, medium wave (UVB) from 280 to 320 and long wave (UVA) from 320 to 400 nm. The PUV radiation encompasses a wide range spectrum, from vacuum UV to far infrared radiation with the wavelength range between 100-1100 nm. Although both methods have been utilized in microbial inactivation, the effectiveness of PUV is two to four times that of continuous UV technology. The inactivation mechanism for PUV includes from UV, infrared and visible range; whereas, the inactivation mechanism for continuous is comparatively limited (Krishnamurthy and others 2008). Another major drawback of continuous UV is the source of UV generation. Typically mercury is used to generate UV radiation and might result in some residual mercury deposition in the food. On the other hand, PUV equipment consists of a discharge lamp filled with xenon or krypton gas. Electrical energy is stored in a capacitor and is released into the lamp in short bursts that consists of xenon or krypton. The intense energy causes the Ionization of gas within the lamp and results in the release of high energy radiation ranging in wavelength between 100 and 1100 nm.

PUV light pasteurization

PUV has been effectively utilized in inactivation of the microorganisms. Krishnamurthy and others (2007) investigated the effect of PUV on inactivating

Staphylococcus aureus in milk and reported 0.55- to 7.26 $\log_{10}$ reduction. The results indicate no significant rise in temperature when the sample was placed at a distance of 5 cms from the quartz window. In another study by McDonald and others (2000) on inactivation of *Bacillus subtilis* spores PUV was three times more effective compared to continuous UV light.

Effect of PUV on food allergens

Due to PUV's high energy level that encompasses energy from UV, infrared and visible region, therefore the synergistic effect of the three regions might be effective in altering many chemical bonds (Krishanmurthy and others 2008). Table 2-4 shows various chemical bonds that exist in biomolecules. From this Table (2-4) it is clear that PUV produces sufficiently high energy that can effectively cleave covalent bonds. Therefore, PUV might alter the conformational structure of allergen proteins resulting in reduced immunoreactivity (Chung and others 2008; Yang and others 2010). On the other hand, continuous UV consists of energy from UV region only (Krishnamurthy and others 2008). Therefore, the maximum wavelength in continuous UV-irradiation is 400 nm from UV-A region. Cho and Koji (2010) reported that UV-irradiation on beta-lactoglobulin showed increase in the IgE-binding when treated for 4, 8, and 12 hr. However, beta-lactoglobulin irradiated for 32 hr indicated decrease in IgE binding. Few researchers have employed PUV to reduce the allergen potency. Chung and others (2008) have illustrated that the PUV treatment of peanut extracts and liquid peanut butter caused a six-to seven-fold reduction in IgE binding compared to the control. In a recent study by Yang and others (2010), PUV was applied on soybean extracts and the results indicated a remarkable decrease in IgE binding . The researchers noted that the optimal treatment time was 4 min at 13.2 cm from the PUV lamp.

High Hydrostatic Pressure Technology

High hydrostatic pressure (HHP) is a non-thermal technology which utilizes water or a dilute aqueous solution (~ 100 to 1000 MPa) as a pressure transmitting medium. The liquid surrounding the food inside the pressure vessel is compressed; and; the resulting high hydrostatic pressure is applied uniformly to the food product. This is a great advantage of this technology, considering that the product is treated evenly. There might be small rise in the temperature due to adiabatic compression; however, the temperature rise is in significant in most cases unless an external heat treatment is applied. Foods subjected to HHP have been shown to have improved functional properties. For example, pressure treated ovalbumin gels had better texture and elasticity compared to heat-induced gels (Yaldagard and others 2008). Similarly, pressure treated soymilk at 500 MPa for 10 min showed better emulsifying activity and stability compared to heat-treated soymilk at 100°C for 19 min (Messens and others 1997). In comparison to thermal processing, HHP can preserve the taste, flavor, color and nutritional quality of the treated food. Furthermore, food proteins can be structurally modified upon pressurization causing changes in food allergen reactivity. The proteins under high pressure can undergo reversible and/or irreversible alteration in the structure resulting in denaturation, aggregation or gel formation. Non-covalent bonds are highly susceptible to pressure treatments whereas; covalent bonds may or may not be affected high pressure.

Effect of HHP on food allergens

High pressure treatment on rice grains resulted in the loss of some of the major allergens and resulted in hypoallergenic rice grains (Kato and others 2000). The results indicated that high pressure in combination with protease treatment was able to

completely solubilize major rice allergens (Glb33, α-Glb, and Alb 16) and effectively remove them from the rice, resulting in less-allergenic rice. Soybean sprouts obtained from HHP-treated seeds showed reduced antigenicity. Conversely, pressurized soybean seeds and tofu showed increase in the antigenecity compared to untreated ones.

Non-Thermal Plasma Technology

In the recent years, NTP has been utilized in food processing as an innovative method for microbial inactivation. Plasma is defined as highly energized matter in a gaseous state. The NTP equipment mainly consists of two electrodes with dielectric discharge material, a power outlet and a treatment chamber. A gas, such as air, argon, or nitrogen, is supplied between the electric fields. During plasma discharge, the energized matter reacts with gas or food molecules to generate charged particles in the form of positive ions, negative ions, free radicals, electrons and quanta of electromagnetic radiation (photons). High-energy electrons cause ionization of the energized matter as well as the excitation of the particles present. Therefore, there might physical damage taking place when the biological material which is ultimately ruptured leading to the inactivation of microorganisms. The electrons cause ionization and dissociation of molecules leading in alteration in the orientation of ions and molecules. Therefore, it has been speculated that the main mechanism behind the microbial inactivation is destruction of DNA by UV irradiation, volatilization of components by UV photons and erosion of spore by free radicals.

Reactive oxygen species (ROS) are generated by NTP and readily diffuse into the cell surface. It has been speculated that ROS has profound damaging effect on the microbial cell. The oxidative stress caused by NTP can rupture the cell membrane and

hence cell death. NTP was employed in inactivating *E.coli* on almonds and the results reported indicate approximately 5-log reduction after 30 sec treatment at 30 kV and 2000 Hz (Deng and others 2007). The *E.coli* O 157:H7 is one of common food borne pathogen and was effectively inactivated by pulsed NTP up to 7 log units (Montenegro and others 2002). The power required for NTP is very low compared to other inactivation technology and capital cost is minimal compared to other sterilization technology. Therefore, non-thermal plasma treatment is considered effective and economical in inactivation microorganisms.

Table 2-1. Major food allergen isolated and characterized (Sampson 1999)

Cow's milk caseins	α_s-Caseins,	
	β–Casein,	
	κ- Casein	
Whey	β-Lactoglobulin,	
	α-Lactoglobulin	
Chicken egg white	Ovomucoid,	(Gal d 1)
	ovalbumin,	(Gal d 2)
	ovatransferrin	(Gal d 3)
Peanut	Vicilin,	(Ara h 1)
	conglutin,	(Ara h 2)
	glycinin	(Ara h 3)
Soybean	Vicilin,	
	conglycinin	(Gly m 1)
Fish	Parvalbumin	(Gad c 1 [cod]; Sal s 1 [Salmon])
Shrimp	Tropomyosin	(Pen a 1; Pen i 1; Met e 1)
Brazil nut	2S albumin	(Ber m 1)
Walnut	2S albumin	(Jug r 1)
Rice	α-Amylase inhibitor	(Ory s 1)
Wheat	α-Amylase inhibitor	
Barley	α-Amylase inhibitor	(Hor v 1)
Buckwheat	11 S globulin	(Fag e 1)
Mustard	2S albumin	(Sin a 1 [yellow]; Bra j 1 [oriental])
Celery	Pathogenesis-related	(Api g 1)
	protein	(Api g 2)
	Profilin	
Potato	Patatin	(Sol t 1)
Carrot	Pathogenesis-related protein	(Dau c 1)
Apple	Pathogenesis-related	(Mal d 1)
	protein,	(Mal d 2)
	Profilin	

Table 2-2. Nutritional value of few selected cereals (Pomeranz 1988)

Cereal	Energy (kJ)	Protein (g)	Fat (g)	Carbohydrates +Fiber (g)	Calcium (mg)	Iron (mg)	Thiamin (mg)	Riboflavin (mg)	Niacin (mg)
Wheat-Hard	1,390	13.8	2.0	70	37	4.1	0.45	0.13	5.4
Wheat-Soft	1,390	10.5	1.9	74	35	3.9	0.38	0.08	4.3
Rice	1,495	7.5	1.8	77	15	1.4	0.33	.05	4.6
Maize	1,490	9.5	4.3	73	10	2.3	0.45	0.11	2.0
Barley	1,390	11.0	1.8	73	33	3.6	0.46	0.12	5.5
Rye	1,335	11.0	1.9	73	38	3.7	0.41	0.16	1.3
Oats	1,625	11.2	7.5	70	60	5.0	0.50	0.15	1.0
Millets	1,485	9.7	3.4	73	32	4.5	0.50	0.12	3.5

Table 2-3. Chemical composition of endosperm, bran, and germ (Eliasson and Larsson 1993)

Whole Wheat	Endosperm	Germ	Aleurone	Bran (pericarp)
8.2-12.1	5.8-16.2	24.3-31.1	18.4-24.3	2.85-7.60
1.8	0.5-0.8	3.65-9.47	11.1-17.2	1.7-5.1
9.0	1.4	8.6	43.0	17.1-73.3
1.8	1.6-2.2	5.05-18.8	6.0-9.89	0.0-1.03
59.2	63.4-72.6	0.0	0.0	0.0

Table 2-4. Strength of common bonds in Biomolecules(Krishnamurthy and others 2008)

Chemical Bond Type	Wavelength	Bond Dissociation Energy (kJ/mole)
N≡N	129	930
C≡C	147	816
C=O	168	712
C=N	195	615
C=C	196	611
P=O	238	502
O–H	259	461
H–H	275	435
P–O	286	419
C–H	289	414
N–H	308	389
C–O	340	352
C–C	344	348
S–H	353	339
C–N	408	293
C–S	460	260
N–O	539	222
S–S	559	214

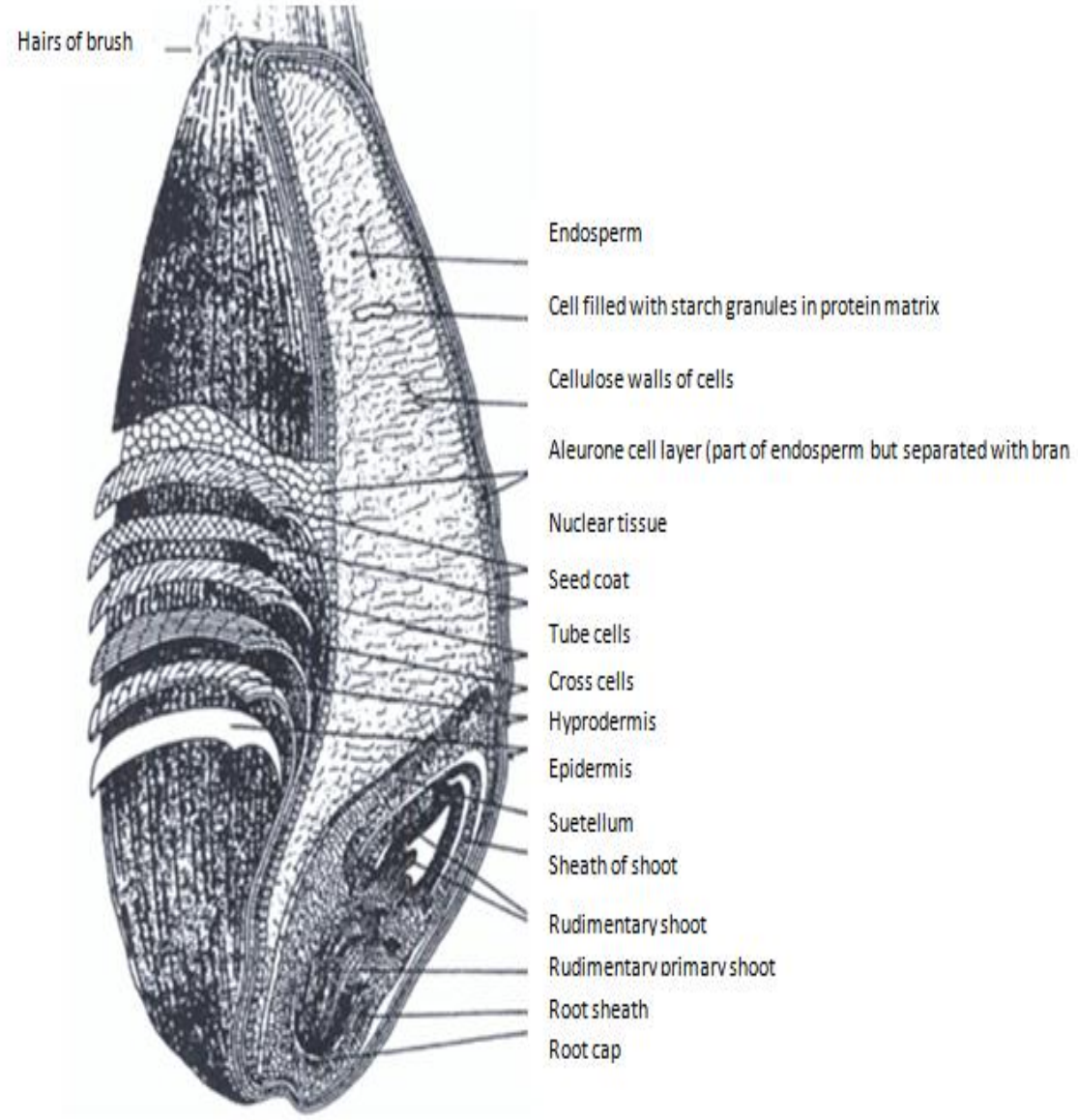


Figure 2-1. Longitudinal view of the wheat kernel (Cornell and Hoveling 1998)

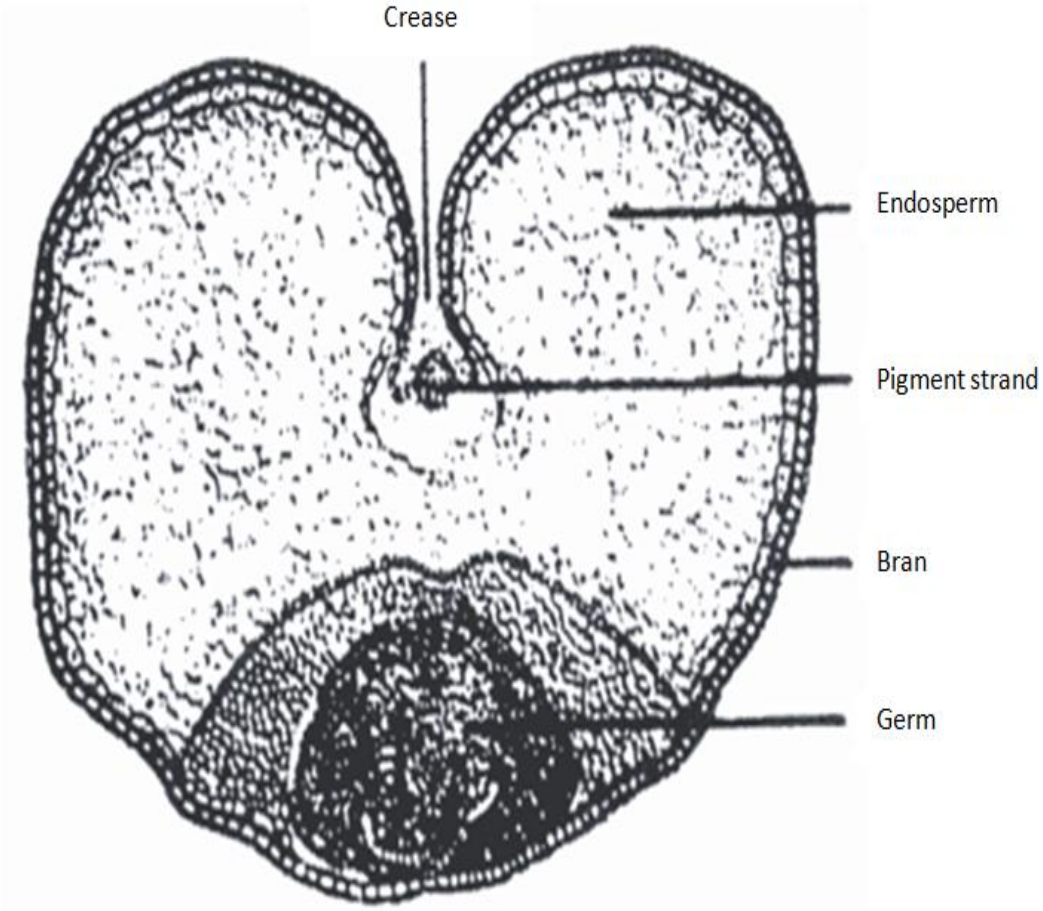


Figure 2-2. Cross sectional view of wheat kernel (Cornell and Hoveling 1998)

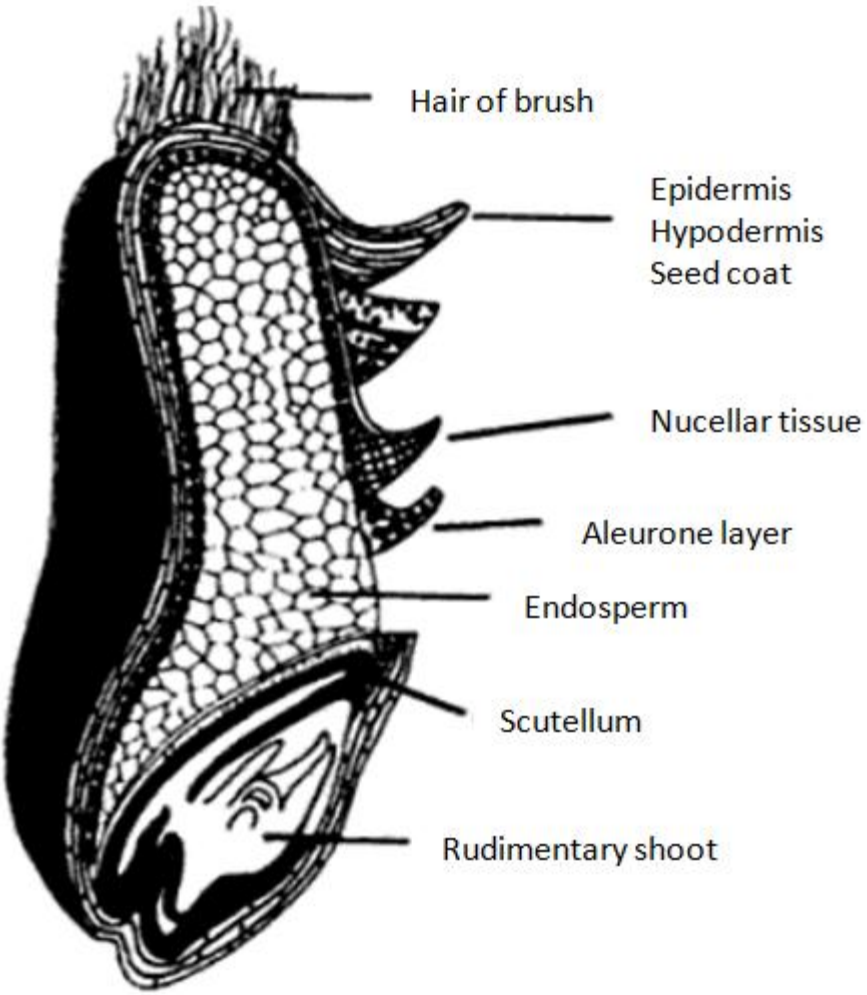


Figure 2-3. Cross sectional view of wheat kernel (Eliasson. and Larsson. 1993)

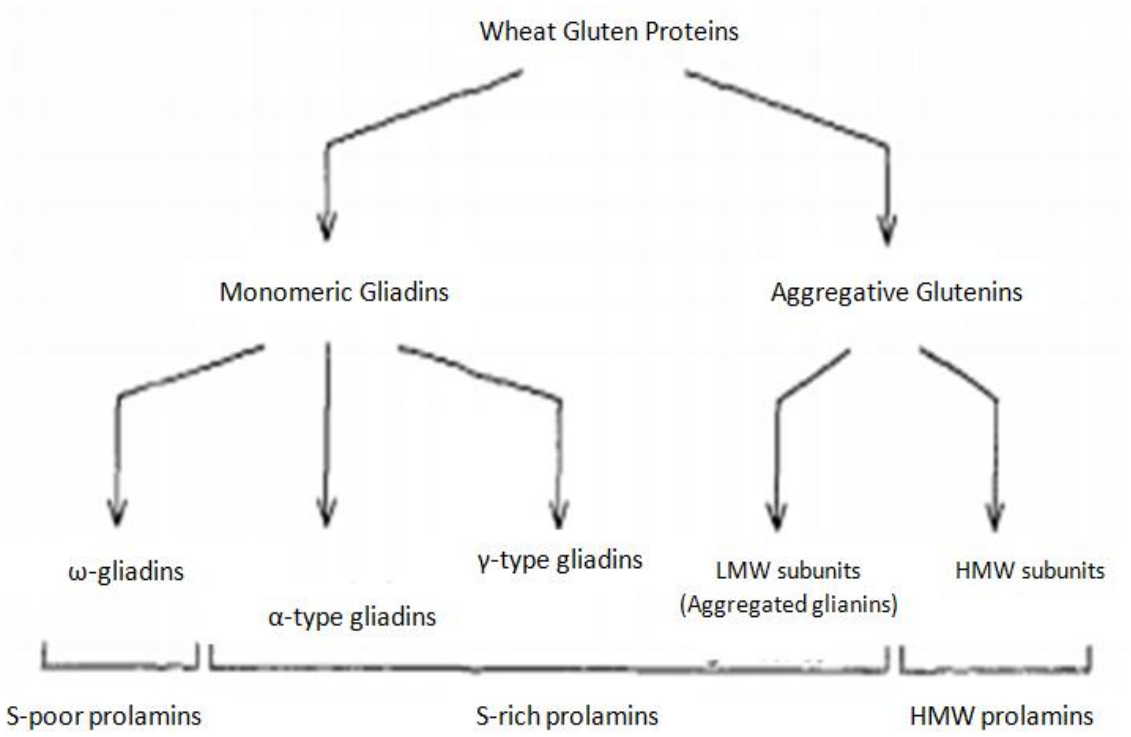


Figure 2-4. Classification of wheat gluten (Tatham Arthur S and others 2000)

CHAPTER 3
MATERIALS AND METHODS

Chemical Reagents

The standard reagents phosphate buffered saline (PBS) 10 X solution, sodium acetate tri-hydrate, sodium chloride and acetic acid were purchased from Fisher Scientific (Fair Lawn, NJ).

Reagents for SDS-PAGE

Tris/Glycine/SDS running buffer containing 24 mM Tris, 192 mM glycine and 0.1% (w/v) SDS, pH 8.3 was obtained from Bio-Rad Laboratories (Hercules, CA). Leammli sample buffer, 2-Mercaptoethanol Electrophoresis Purity Reagent, Precision Plus Protein All Blue Standards were purchased from Bio-Rad Laboratories. Gel Code Blue Safe Protein Satin was obtained from ThermoScientific (Rockford, IL).

Reagents for Western Blot and Dot Blot

Tris/Glycine Buffer containing 25 mM Tris, 792 mM glycine, pH 8.3 was purchased from Bio-Rad Laboratories. Starting Block ™ T20 (TBS) Blocking Buffer, 1-Step ™ Chloronapthol, Super Signal ® West Pico Chemiluminescent Substrate (an enhanced chemiluminescent substrate for detection of HRP) was purchased from ThermoScientific. Methanol HPLC Grade, Tris Buffered Saline (TBS) containing Tris 25mM, NaCl 0.13 and KCl 0.0027 M pH 7.4 and Tween ®20 was obtained from Fisher Scientific.

Reagents for Indirect Enzyme Linked Immunosorbent Assay (ELISA)

Stable Peroxide Substrate Buffer and O-phenylenediamine dihydrochloride (OPD) was purchased from Thermo Scientific.

Wheat Protein Extraction

Albumin and Globulin Fraction

The albumin/globulin fraction was extracted from commercial whole wheat flour (Pillsbury® whole wheat flour) purchased from the local grocery store. The extraction procedure was followed according to Simonato and others (2001) with few variations. A weight of 5g whole wheat flour was mixed with 0.5 M NaCl buffer. The mixture was stirred with magnetic stirrer for 1 hr at 4°C followed by centrifugation at 12000g for 20 min at 4°C. The resulting supernatant was saved as water/salt soluble, albumin and globulin fraction at -20°C for the experiment.

Glutenin Fraction

Wheat gluten powder was purchased from Sigma-Aldrich- (St.Louis, MO). The glutenin fraction was extracted in acetate buffer according to Takeda (2001) with few variations. The gluten powder was dissolved in acetate buffer at pH 4 in a ratio 1:4 (w/v). The mixture was stirred with magnetic stirrer on vortex at room temperature for 5 min. Two variations of samples (supernatant and homogenate) were used to analyze SDS-PAGE and Western blot. For the homogenate, the sample was homogenized using a hand held BioHomogenizer (Biospec, Bartlesville, OK) for 4 min at high speed. For the supernatant, the samples were centrifuged at 8000 x g for 10 min and only the supernatant was collected for further analysis.

Total Soluble Wheat Protein

Commercial whole wheat flour (Pillsbury® Whole Wheat Flour) was purchased from local grocery store to extract the total soluble wheat proteins. The extraction procedure was followed according to (James and others 1997) with few variations. Five grams of whole wheat flour was dissolved in 100 ml phosphate buffered saline (PBS),

pH 7.4 for 24 hours (4°C). The mixture was centrifuged at 11,000xg for 20 min at 4°C. The resulting supernatant was dialyzed against distilled water for 24 to 36 hours. The water was replaced with fresh deionized water every 10 hrs. The dialysate was collected in plastic tubes and stored at -20°C for subsequent use in the experiments.

Pooled Human Plasma Samples

Wheat Allergic Plasma

Pooled human plasma from three individuals containing specific IgE antibodies to wheat was obtained from Plasma Lab International, Everett, WA. Wheat IgE specific plasma was used to detect the IgE binding demonstrated by Western blot, dot blot, and Indirect ELISA.

Control Plasma

Control plasma of individuals with no known history of any allergy to any food was obtained from Plasma Lab International. Control plasma was utilized in indirect ELISA analysis to ensure there is no non-specific binding. The absorbance value of control plasma was negated from the samples to prevent error from non-specific binding.

Equipments

Pulsed Ultraviolet Light (PUV) Source

Xenon Steripulse® - XL 3000 (Xenon Corp, Wilmington, Ma) was utilized to treat the samples (Figure 3-1 A and 3-1 B). The PUV unit produced polychromatic light in the wavelength range between 100 and 1100 nm. The energy from the PUV lamp included 54% from UV region, 26% from visible region and 20% from infrared region. The PUV equipment mainly consisted of a 16 inch linear clear fused quartz PUV lamp, treatment chamber, control module and cooling blower.

High Hydrostatic Pressure

A laboratory-scale high-pressure unit (Figure 3-2 A) Avure PT-1 (Avure Technologies, Kent, WA) monitored with DASYLab ® 7.0 software (Figure 3-2 B) (DASYTECH, Bedford, NH) was utilized in the study.

Non-Thermal Plasma

The NTP system included two major components, NTP reactor chamber and high voltage power supply. Additionally, it included electrical parameter measurement and control devices (Figure 3-3). The NTP was generated by dielectric discharge between the two electrodes. The NTP reactor was the planar configuration of the dielectric barrier discharge reactor with two dielectric layers. The electrodes in the NTP chamber were covered with dielectric plates (epoxy resin board). The thickness of the dielectric layer was 0.062 inches and the radius of the discharge chamber was 1 inch with height of 0.25 inches.

Centrifuge and Spectrophotometer

Sorvall® RC-5B Refrigerated Superspeed Centrifuge, Du Pont instruments (Wilmington, DE) was utilized to centrifuge samples. Spectramax 340[384] spectrophotometer (Molecular Devices, Inc. Sunnyvale, CA) was utilized to read the absorbance of the samples.

PUV-Treatment

The settings for the PUV treatment were 3 pulses/sec with the pulse width of 360 µs. The distance between the samples and PUV lamp was adjusted manually to 10cm. The energy released by PUV lamp according to the manufacturer's claim is 1.27 Joules/cm^2 at 1.9 cm from the quartz window. A volume of 5 ml of sample was placed in round aluminium dish with diameter of 6.5 cm. The samples were placed directly under

the PUV lamp. To make the sample tray stationary a custom designed holder was used to push the samples such that it gets the maximum energy from the PUV source. A cooling blower was switched on during the entire treatment to prevent the overheating of the PUV lamp and to reduce the ozone accumulation in the pilot plant. This method was followed for all the samples to obtain the same energy each time. The samples were exposed to PUV with different treatment time. The initial and final temperatures of the samples were recorded using hand held thermometer.

PUV-Treatment on Wheat Albumin and Globulin

A volume of 5 ml of albumin/globulin proteins were treated with PUV for different time. The initial and the final surface temperatures were measured using a hand-held infrared thermometer. The samples were weighed before and after treatment to account for moisture loss caused by heating of the sample. The treatment conditions included control (untreated), boiled (5 min at 100°C), PUV 30 s, PUV 60 s, PUV 90 s and PUV (90s) followed by boiling for 5 min at 100°C. The samples were subjected to SDS-PAGE and Western blotting.

PUV-Treatment on Wheat Gluten

A volume of 5 ml of wheat gluten (homogenized and supernatant) was treated under the PUV at a distance of 20.5 cm from the UV lamp. The initial and the final surface temperatures were measured using a hand-held infrared thermometer. The samples were weighed before and after treatment to account for moisture loss during treatment. The treatment conditions for homogenized samples included control (untreated), boiled (5 min at 100°C), PUV 45 s and PUV 45 s followed by boiling at 100°C for 5 min. The treatment conditions for supernatant gluten samples were control

(untreated), boiled at 100°C for 5 min and PUV 45 s. The samples were subjected to SDS-PAGE and Western blotting.

PUV-Treatment on Total Soluble Wheat Proteins

Total soluble wheat proteins (5 ml) were treated with the continuous PUV unit. A customized ruler designed by the fellow student was used to insert the samples under the UV lamp. The samples were placed exactly under the PUV lamp every time so that high energy PUV radiation can be absorbed by the samples and also for the accuracy of the PUV-treatment. The distance between the PUV lamp and samples was adjusted to 20.4 cm with 3 pulses/sec. The treatment conditions were control (untreated), boiled at 100°C for 5 min, PUV 30 s, PUV 60 s, PUV 90 s, PUV 120 s and PUV 120 s followed by boiling at 100°C for 5 min. The initial and final temperatures of the samples were measured. The initial and final weight of the sample was measured and the moisture loss was calculated.

HHP-Treatment

The samples were shipped to University of Delaware at controlled temperature (4°C) for the HHP treatment. The samples (1.5 ml) were transferred to a sterile polypropylene pouch (Fisher Scientific) heat sealed and placed in another pouch to prevent it from leaking. HHP treatments were conducted at 600 MPa and the treatment time included 5 and 15 min at 21°C and 70°C respectively. Pressure increase rate was approximately 22 MPa/s, and pressure release was immediate. After the treatment the samples were sealed in plastic pouches and shipped to University of Florida at 4°C. The samples were transferred into plastic tubes and stored at -20°C until it was used for the experiment.

NTP-Treatment

The NTP-treatment on wheat proteins was conducted in University of Minnesota. The NTP unit was designed by Dr. Ruan and his colleagues. The system included 30 kV power supply with frequency of 60 Hz (Figure 3-3). A volume of 12.87 ml was treated with NTP. The samples were subjected to NTP by placing it between two electrodes and the treatment time included 1, 3 and 5 minutes. The samples were transferred into glass vial and shipped to University of Florida. Upon receiving the package at 4°C the samples were transferred into vials and stored at -20°C until further experiments.

Protein Assay

Protein concentration of treated and control samples were measured according to Bradford Assay with the Coomassie Plus Protein Assay Reagent Kit (Pierce Boitechnology, Rockford, IL). The protein concentration of unknown samples was estimated by reference to absorbances (595 nm) obtained for a series of standard protein Bovine Serum Albumin (BSA) dilutions (0.2 to 0.8 mg/ml), which were assayed alongside the unknown samples. A standard curve was plotted for each BSA standard vs. its concentration in mg/ml. The standard curve was used to determine the concentration of treated and control wheat protein samples.

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

The control and treated wheat samples were separated by a 4-20% Tris-HCl precast gels (30 and 50 µl) (Bio-Rad Laboratories) according to the method of Laemmli (1970). The protein samples were diluted in loading buffer (2% SDS, 25% glycerol 0.01% Bromophenol Blue, and 62.5 mM Tris-HCl, pH 6.8, 10% 2-mercaptoethanol) in ratio 1:1 and boiled for ≈5 min at 95°C and cooled to room temperature. The precast gels were assembled on to a Mini-PROTEAN ®TetraSystem connected to PowerPac

Basic (Bio-Rad). The electrophoresis tank was filled with SDS-PAGE running buffer Tris/Glycine/ SDS Buffer containing 25mM Tris, 192 mM glycine and 0.1% (w/v) SDS pH 8.3. The standard protein marker (10 µl) was loaded in the first well and the subsequent wells were loaded with control and treated samples(~20-25 µg) using gel loading tips. The proteins were electrophoresed at ~190 Volts for about 45 min or until the dye front reached the bottom of the well. The bromophenol blue dye present in the sample buffer helped to monitor the electrophoresis process. The gel was either stained with Gel Code Blue Safe Protein Stain (5-10 hrs) for analyzing the protein expression or transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Immobilon-P Transfer Membrane, Millipore Corporation, Billerica, MA).

Western Blot

After performing gel electrophoresis, the resolved proteins were blotted on to a PVDF membrane. The gel was placed in the deionized water and equilibrated in ice cold transfer buffer (pH 8.3), consisting of 25 mm Tris, 192 mm glycine and 20% aqueous methanol. The membrane dimension of 7 x 8 inch was utilized for blotting. The membranes were wetted in methanol to hydrate and rinsed with deionized water. The membrane was incubated in ice cold transfer buffer for 5-10 min. Filter paper of approximately the same dimension as membrane was used to sandwich the gel and the membrane. The filter paper was submerged in transfer buffer and placed in 4°C until ready for transferring. The Tras-Blot ®SD Semi-Dry Transfer Cell and Power Pac HC (Bio-Rad) was utilized for transferring the proteins. The filter paper was placed on the transfer cell. The well hydrated membrane was placed on the filter place followed by placing the gel on the membrane. To have proper contact between the gel and membrane and for better transfer of proteins the air pockets between the gel and the

membrane was removed by firmly pressing the membrane-gel sandwich with a glass rod. Another filter paper was placed on the gel to complete the sandwiching. The transfer cell was closed to begin transferring. The Transblot was run at 15 volts for 30 min at the room temperature. After transferring the proteins to the PVDF membrane the membrane was blocked in the blocking buffer for 1 hr, on the shaker, at room temperature. Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies to the membrane. The membrane was rinsed 2 times for 10 min with TBS containing 0.5% Tween-20 on a shaker. Primary antibody with a history of wheat allergy is diluted in blocking buffer in a ratio 1:4. (1 ml of antibody in 3 ml of blocking buffer). The membrane is incubated in the diluted primary antibody overnight on a shaker at 4°C. Care is taken to completely submerge the membrane in the antibody solution to enable adequate homogenous covering of the membrane and shaking is required to prevent uneven binding. The membrane is washed 2 times for 5 – 10 min with TBS Tween-20 to remove residual primary antibody that could cause high background and poor detection. The horse radish peroxidase conjugated secondary antibody contains enzyme (peroxidase) is used to detect the secondary antibody was diluted (1:1000) in blocking buffer. The membrane was incubated in secondary antibody solution, the amount (3-5 ml) used depending on the size of the membrane to attain complete coverage of the membrane. The incubation time is 1-2 hrs at room temperature, with agitation. The secondary antibody was poured off and the membrane was rinsed 3 times for 10 min with TBS Tween-20. To visualize the protein bound to the membrane, the membrane was incubated in 1-Step^{TM} Chloronapthol (ThermoScientific,) or in Electrochemiluminescence (ECL) reagent (SuperSignal ® West Pico

Chemiluminescent substrat, ThermoScientific). For visualizing the bound protein with chloronapthol, the membrane was placed on plain counter. Using the transfer pipets (graduated 0.3 ml Small Bulb, Samco Scientific, Mexico) the membrane was covered with chloronapthol. Once the entire membrane is covered with reagent, it is incubated for 20-30 min at room temperature. After 30 min of incubation the IgE bound samples can be visualized. The membrane was scanned immediately to analyze the results.

Chemiluminescent detection uses an enzyme to catalyze a reaction that results in the production of visible light. The procedure was followed according to Millipore Immunodetection Technique. An equal volume of Supersignal® West Pico Luminol/ Enhancer Solution (Thermoscientific) and Supersignal® West Pico Stable Peroxide Solution (Thermoscientific) was mixed in a test tube. The membrane was incubated in the ECL reagent mixture for 5 min. After pouring off the excess solution the membrane was placed the cassette and the air bubbles were gently smoothed out and taken to the dark room. In the dark room the autoradiography film (Classic Autoradiography Film BX, Molecular Technologies, St.Louis, MO) was placed on top of the membrane and the cassette was closed. The exposure time was between 30 sec- 4min. Membrane was fed into the developer. The X-ray film was scanned immediately for analyzing the results.

Dot Blot

Dot blot is a similar technique as Western blot for detecting, analyzing and identifying the IgE bound proteins. However, in this technique the protein samples are not separated electrophoretically but, are spotted through circular templates directly onto the nitrocellulose membranes (0.45 µm, 8 cm x 8 cm, Thermoscientific). A grid was drawn with a pencil to indicate the region the blot was done. Protein was spotted at the center of the grid at amounts 2.4 and 1.2 µg determined using Bradford assay. The

samples were applied slowly to minimize the area the solution penetrates (3-4 mm diameter). The membrane was left for drying. The membrane blocked in blocking buffer (3-4 ml) at room temperature, on rocker for 30 min. The blocked membrane was thoroughly washed with TBST two times for 10 min. Incubation time in primary antibody (1:80) is overnight (10-14 hrs) at 4°C on a rocker to ensure even binding. Excess antibody was poured in water containing bleach (30%) followed by washing the membrane in TBST two times for 10 min to remove residual antibody. The next step was to incubate the membrane in secondary antibody 1:1000 or 1:3000 for 1-2 hrs at room temperature, with agitation. The membrane was washed with TBST three times for 10 min with agitation to remove excess antibody. To visualize the IgE bound samples the membrane was incubated in ECL reagent for 5 min. After pouring off the excess solution the membrane was placed in the cassette and the air bubbles was gently smoothed out and taken to the dark room. In the dark room the autoradiography film was placed on top of the membrane and the cassette was closed. The exposure time was between 30 sec- 4min. Membrane was fed into the developer. The X-ray film was scanned immediately for analyzing the results with densitometry software.

Indirect ELISA

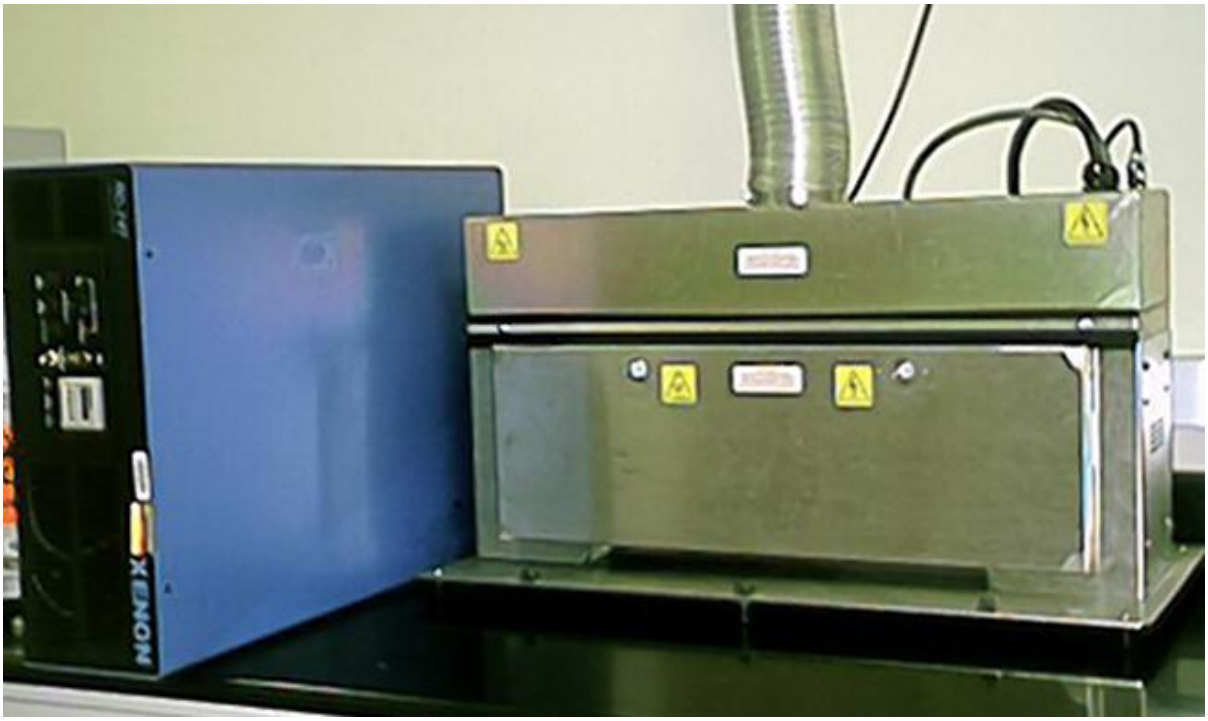
The control, PUV-, HPP- and NTP-treated samples were diluted to a final concentration of 20 µg/ml in PBS (pH 7.4). A volume of 100 µl of each sample was coated onto a 96 well microtiter plate (Costar, EIA/RIA, No Lid, 96 Well Easy Wash™ Certified High Binding Non-Sterile, Polystyrene, Corning Incorporated, Corning, NY) with a manual repeating pipette (Original Model 4780 Repeating Pipette, Eppendorf, Gremany) . The plate was covered with an adhesive plastic (Whatman™ Microplate Devices Uniseal™, Adhesive Clear Ployster Seal Film, 0.05 mm, GE Healthcare UK

Limited, Buckinghamshire, UK) and incubated for 2 hrs at 37°C. The samples coated onto the plate were removed and the plate was washed twice with TBST by filling approximately 200 µl in each well and subsequently removing the TBST by flicking the plate over the sink. The remaining drops were removed by patting the plate on a paper towel few times. A volume of 200 µl of blocking buffer was added to each well that contained the samples to ensure that all remaining available binding surfaces of the plastic wells are covered. The plate was covered with plastic adhesive and incubated at 37°C for at least 2 hrs. Following blocking, plate was washed twice with TBST. A 100 µl of primary antibody diluted in PBS at 1:5 (1ml of primary antibody in 4 ml of PBS, pH 7.4) was added to each well using a repeating pipette. The plate was covered with adhesive plastic and incubated for 2 hrs at 37°C. After removing the antibody, the plate was cleaned for any residual antibody by washing with TBST at least 2-3 times similar to the first wash. Care was taken to prevent any contamination between the wells. A volume of 100 µl of mouse anti-human IgE-HRP antibody diluted in PBS (1:3000) was added to each well, with multichannel pipette and incubated at 37°C for 1 hr. After removing the secondary antibody the same wash procedure was followed. Just before detection a 100 µl of freshly prepared substrate solution containing 10% of Stable Peroxide Substrate Buffer and OPD (0.5 mg/ml) was added to each well and incubated for 10-30 min. The reaction was stopped with 2.5 N sulfuric acid (50-100 µl) and the absorbance was read on microplate reader at 490 nm. For unstopped reaction the absorbance was read at 450 nm.

Statistical Analysis

Absorbance data were entered into Excel and sorted by treatment time and condition. The experiments were conducted in triplicates with three repetitions. The data

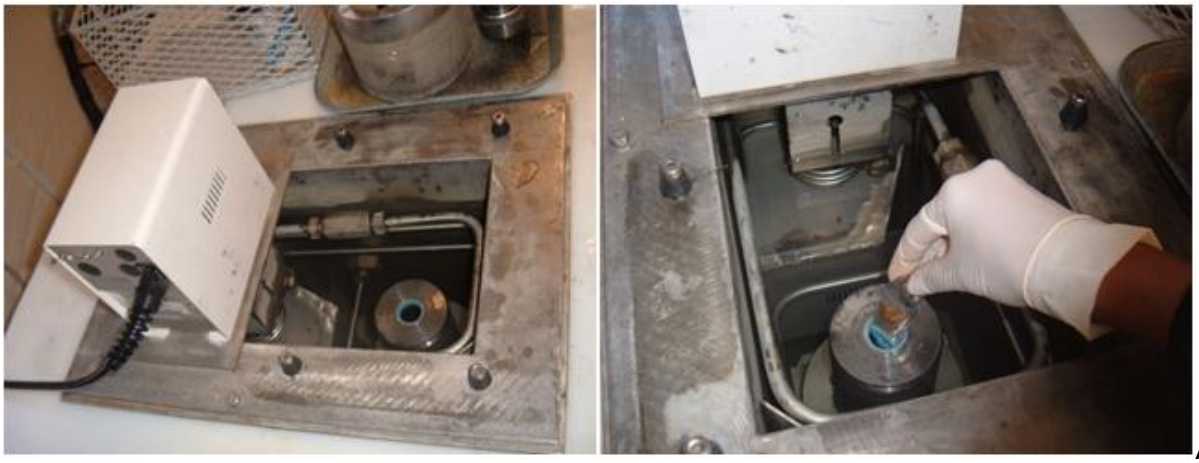
were then analyzed by SAS 9.2 software (Cary, NC). Data were sorted by treatment time and condition. Analysis of variance (ANOVA) of two ways, Duncan's test, Least significant difference (LSD) at $\alpha = 0.05$ using SAS software. Additionally Tukey's Tests at $\alpha = 0.05$ was performed for the accuracy of the results and to determine significant difference. All the samples (PUV, HHP and NTP) were compared with the control (untreated).

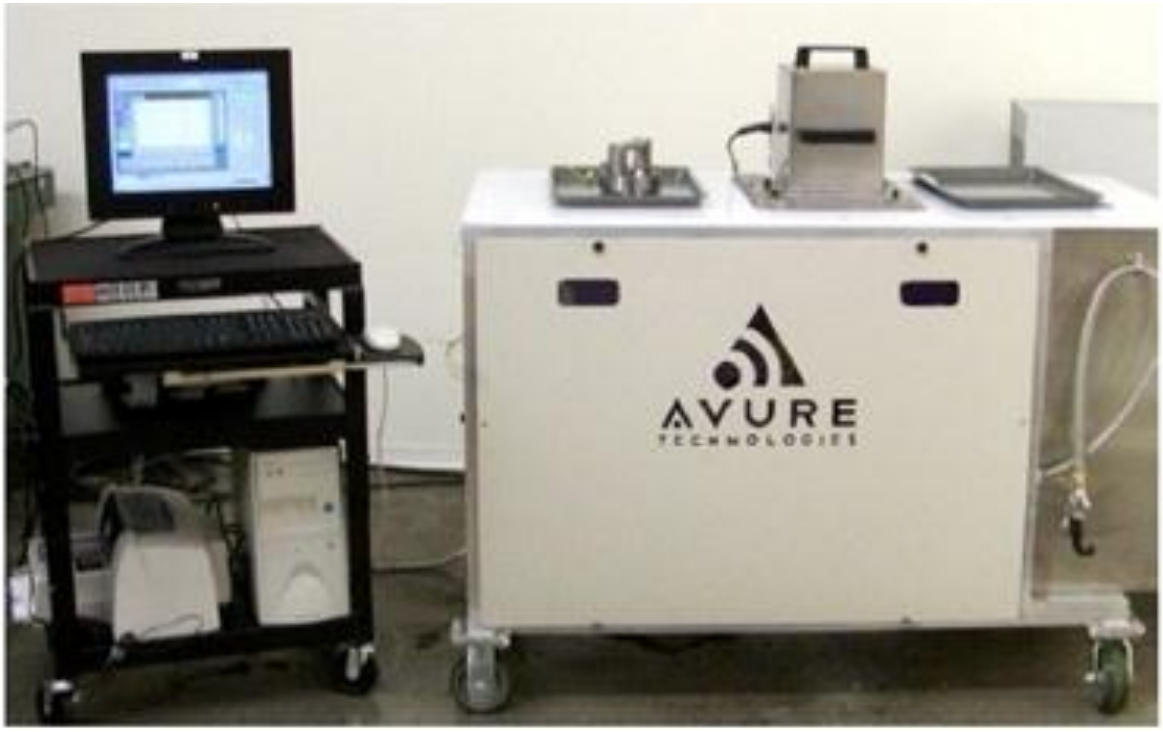


A

B

Figure 3-1. PUV Equipments A) Xenon® Steripulse XL-3000 batch system, B) Xenon® Steripulse XL-3000 continuous system (Photo courtesy of Dr. Yang, University of Florida)


A



B

Figure 3-2. HHP equipments A) Lab scale HHP unit, B) DASYLab ® 7.0 software used for HHP-treatment (Photo courtesy of Dr. Chen, University of Delaware)

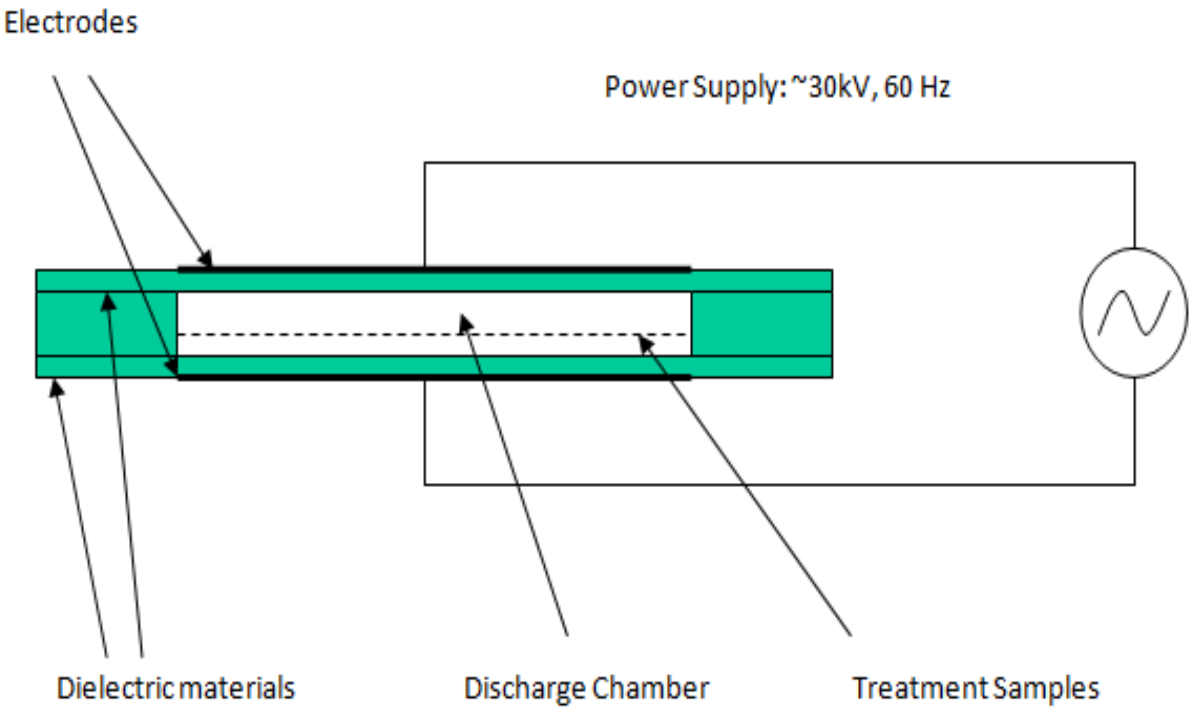


Figure 3-3. NTP Treatment set up (Photo courtesy of Dr. Ruan, University of Minnesota)

CHAPTER 4
RESULTS AND DISCUSSION

Effect PUV-Irradiation on Albumin and Globulin

Temperature Measurement During PUV-treatment and the Effect on Sample Volume

The sample temperature increased gradually with increase in treatment time. The temperature data is presented in Table 4-1. The final temperature of the samples were 52.4 °C, 64.8 °C, 73.0 °C, and 74.7 °C when treated for 30, 60, 90, and 120 s, respectively, when a sample volume of 5 ml was kept at a distance of 20.5 cm from the PUV lamp. As the treatment time increased the sample volume decreased approximately to about 20% when treated for 120 s (Table 4-1).

SDS-PAGE of PUV-Treated Albumin and Globulin

Electrophoreis was performed under reducing conditions in the presence of 2-mercaptoethanol to determine the nature of proteins in their non-native state. The SDS-PAGE (Figure 4-1) results illustrate the protein profile of control, boiled, PUV-treated (30, 60 and 90 s) and PUV (90 s) followed by boiling samples. For the control samples (lane 1) the bands ranged between 6 kDa- 75 kDa. Albumin and globulin samples seem to be affected by PUV-treatment; the variations in the protein profile were detected in comparison with untreated sample. Such variations might be due to PUV-treatment, since the instantaneous temperature rise could be much higher than temperature measured. Krishnamurthy and others (2008) have reported that there was significant rise in the temperature when the milk sample was treated with PUV at a distance of 8 cm for 180 s. Visually, PUV-treatment for 30 s (lane 3) resulted in lesser amount of protein likely due to protein fragmentation. The fragment proteins could be smaller than the radius of the SDS-PAGE gel hence, were excluded from during electrophoresis.

These results are in agreement with those obtained for beta-lactoglobulin treated by UV-irradiation (Cho and Koji 2010). However, beta-lactoglobulin was treated with continuous UV that has energy from UV regions and comparatively low energy level than PUV. Therefore, proteins fragmentation was observed after 4 to 8 hr of UV-exposure. Whereas, in this study merely 30 s of PUV-exposure time caused protein fragmentation. As PUV-treatment time was increased, aggregated protein bands were observed on top of the SDS-PAGE gel. As it has been described earlier that electrophoresis was performed in the presence of 2-mercaptoetahnol therefore, these aggregated proteins give further indication that it is PUV induced cross-linking that does involve S-S bonds (Cho and Koji 2010; Cooper and Davidson1965). The PUV-treated (90 s) sample followed by boiling did not show any apparent difference in protein profile compared to rest of the PUV-treated (> 30 s) samples.

Western Blotting of Wheat Albumin and Globulin

Immunoblotting of wheat albumin/globulin (Figure 4-2) with pooled human plasma from individuals allergic to wheat recognized 5 bands in the control (untreated) and boiled samples, which ranged between 15 kDa – 60 kDa. The lower molecular-weight protein (15 kDa) has been confirmed as α-amylase/trypsin inhibitor in previous studies (Mittag and others 2004; Pastorello and others 2007; Simonato and others 2001). Other bands might be LTP present in the water/salt soluble fraction and other minor allergens (Pastorello and others 2007). Lower IgE-binding to PUV-treated sample for 30 s (lane 3) was observed. In regards to longer PUV treatment time, a decrease in immunoreactivity was apparent (Yang and others 2010). There was negligible IgE reactivity in PUV (60 s and 90 s) treated samples in bands corresponding to 12 – 25 kDa, and there was minimal IgE binging to the 35-kDa band. Additionally, PUV

treatment followed by boiling showed no IgE reactivity to any proteins except some minimal reactivity to the 35-kDa band. The susceptibility of wheat albumin/globulin antibody binding epitopes to PUV treatment was apparent with increase in treatment time.

Effect PUV-Irradiation on Gluten

SDS-PAGE of PUV-Treated Gluten

The PUV-treated gluten extracts after treating with PUV light were analyzed by SDS-PAGE under denaturing conditions (Figure 4-3). The gluten homogenate samples were treated with PUV for 45 s and PUV (45 s) treatment followed by boiling 100°C for 5 min. For gluten supernatant samples only PUV (45 s) was used for analysis.

The SDS-PAGE pattern of the gluten homogenate sample for control (untreated) bands ranged between 14 kDa – 110 kDa. On the contrary, the PUV treatment for 45 s was noticeably different compared to control samples. Rapid disappearance of the bands was noted in this sample. Similarly, the PUV 45s followed by boiling also showed identical SDS-PAGE results. These results show that PUV treatment is likely effective in reducing the protein solubility and as shown by SDS-PAGE. The formation in irreversible aggregation might result in high molecular weight proteins that cannot be resolved in SDS-PAGE (Chung and others 2008). Control (untreated) gluten supernatant samples showed disappearance of the major proteins corresponding to HMW and LMW gluten subunits whereas, the boiled and PUV 45 showed all the major proteins ranged between 14 kDa-110kDa. Glutenin is a polymeric protein invlolved in S-S interchange, upon reduction it is separated into LMW and HMW-glutenin subunits. However, the present study did not indicate distinct band on SDS-PAGE. This might due to an error while pipetting or other experimental error. A more accurate

characterization of glutenin samples was observed in boiled samples. Therefore, the PUV-treated sample was compared with boiled sample. The PUV-treated (45 s) showed decrease in band definition. These results show a likelihood of PUV-induced fragmentation which did not result in SDS-PAGE gel (Chung and others 2008; Yang and others 2010; Krishnamurthy and others 2008).

Western Blot of PUV-Treated Gluten

The gluten sample (homogenate and supernatant) were analyzed for the IgE reactivity using the pooled human plasma of individuals with a history of wheat allergy for the control (untreated), boiled and PUV-treated samples (Figure 4-4). The antibody against wheat recognized a 15 kDa band in control (untreated), boiled and PUV (45 s) homogenate sample. These protein might be an α-amylase/trypsin inhibitor protein residue present in the gluten fraction (Pastorello and others 2007). However, the IgE no longer recognized the band in the PUV (45 s) treated sample followed by boiling. These results suggest that gluten fraction are stable to PUV treatment but are susceptible when it is combined with heating at 100°C.

The gluten supernatant sample showed IgE reactivity to control and boiled samples at 14 kDa, which is possibly α-amylase/trypsin inhibitor yet there was no IgE binding after PUV treatment for 45 s. From these results we can notice that homogenate samples is more stable to PUV due to the presence of other proteins whereas, the supernatant samples contain only glutenin subunits that are highly susceptible to PUV-treatment.

PUV-Treated Total Soluble Wheat Protein

SDS-PAGE of PUV-Treated Wheat Proteins

In order to understand the nature of the proteins after PUV-treatment, the total soluble wheat proteins were analyzed by SDS-PAGE (Figure 4-5). The treatment conditions included control (untreated), boiled (100°C for 5 min), and PUV treatment for 30 s, 60 s, 90 s and 120 s. An additional treatment condition was PUV (120 s) followed by boiling.

Analysis of SDS-PAGE results of PUV-treated sample indicated apparent distinction between the control and PUV-treated sample. Control (untreated) sample showed bands ranging from 10 to 70 kDa. At 30 s after PUV exposure, a slight reduction in protein band was observed compared to the control. These modifications are mainly due to UV-induced protein fragmentation (Cho and Koji 2010; Cooper and Davidson1965; Krishnamurthy and others 2008). With increase of irradiation period, PUV-irradiation caused irreversible protein aggregation demonstrated by SDS-PAGE. PUV-treatment followed by boiling (lane 7) indicated further increase in aggregation. A smear was observed in PUV-treated (> 30 s) samples suggesting PUV-induced high molecular weight aggregates (Gennadios and others 1998; Cooper and Davidson 1965; Krishnamurthy and others 2008). In the present study, initial exposure to PUV-irradiation resulted in fragmentation whereas, prolonged exposures to PUV (>30 s) resulted in irreversible high molecular weight aggregates that are covalently cross-linked.

Dot Blot of PUV-Treated Wheat Proteins

Dot blot was conducted by loading the proteins on nitrocellulose membrane to analyze and compare the IgE reactivity of control with PUV-treated proteins with

different treatment times (Figure 4-6). Incubation of the spotted membrane with pooled human plasma of wheat allergic patients showed IgE binding in control (1 and 2) and PUV (30 s) (3). The control (boiled) sample showed minimal decrease IgE reactivity compared to PUV (30 s) and control. On the contrary, there was no IgE binding to any of the PUV-treated samples with the exposure time above 30 s. Similarly, there was no IgE reactivity to PUV (120 s) followed by boiling. This experiment was repeated 3 times to ensure the consistency of the results obtained. There was also strong non-specific binding to the membrane, which did not contain the proteins. The IgE bound proteins showed dark spots, which could be clearly distinguished from the non-specific binding. The proteins that did not react to IgE in this case PUV-treated (> 30 s) showed blank spot (4, 5, 6 and 7) which was noticeably different from the non-specific bound membrane sites. The dot blot results illustrates that the PUV-treatment for 30 s did not alter the wheat allergen reactivity. The immune reactivity of PUV-treated (30 s) allergens was similar to control (untreated) whereas, the boiled samples showed remarkable decrease in IgE binding. Therefore, it can be concluded that wheat proteins susceptibility increases with increased PUV exposure time. These effects of PUV have been attributed to alteration of tertiary and secondary structure primarily caused by UV-region (Cho and Koji 2010; Davidson and Cooper1967; Cooper and Davidson1965). The effects of PUV has been credited to irreversible aggregation of proteins (Chung and others 2008; Yang and others 2010) and thus, alteration of conformational epitope binding sites.

Indirect ELISA of PUV-Treated Wheat Proteins

To further elucidate the contribution of PUV on conformational IgE binding epitope Indirect ELISA was performed (Figure 4-7). The treatment conditions were boiling

(100°C for 5 min), PUV treatment (30 s, 60 s, 90 s and 120 s) and PUV (120 s) followed by boiling.

The IgE-binding capacity was measured as absorbance at 450 nm. Higher absorbance value indicates higher IgE binding and vice versa. According to the indirect ELISA results, there was no significant ($P \leq 0.05$) difference between the control, boiled and PUV treatment for 30 s. On the contrary, the IgE binding to PUV 60, 90, and 120 s was significantly different. The additional treatment group of PUV (120 s) followed by boiling also showed no significant difference ($P \leq 0.05$).There was approximately 40-50% reduction in IgE binding in PUV-treatment for 60, 90 and 120 s compared to control (untreated) sample. These results indicate that PUV is effective in reducing IgE binding capacity when the exposure time is above 30 s. However, when the PUV (120 s) is followed by boiling the IgE binding capacity increases considerably.

HHP-Treated Total Soluble Wheat Proteins

SDS-PAGE of HHP-Treated Wheat Proteins

Total soluble wheat protein was treated with HPP at 600 MPa. The treatment was conducted in laboratory scale HHP unit. The treatment conditions were control (untreated), HHP (21°C for 5 min), (21°C for 15 min), (21°C for 30 min), (70°C for 5 min) and (70°C for 15 min). Gel electrophoresis was performed in the presence of 2-mercaptoethanol in order to analyze the nature of the proteins following HHP-treatment (Figure 4-8). For control (lane 6) sample the band ranged between 10 kDa-150 kDa. The boiled sample showed mild smearing in the upper part of the gel but, was very similar to the protein profile of the control sample. The HHP-treated samples showed a remarkable difference in SDS-PAGE. The wheat proteins seem to be sensitive to high pressure treatment. High hydrostatic pressure has been speculated to cause

denaturation and/or aggregation. The denatured proteins are involved in extensive hydrophobic interaction that can include strong covalent interactions or weak di- sulfide bonding resulting in aggregation. The SDS-PAGE showed similarity in the proteins profile of HHP-treated samples. The 14 kDa α-amylase/trypsin inhibitor had a distinctive band in all the HHP treatment groups. From the HHP treatment on wheat proteins that there is extensive inter molecular hydrophobic interaction, which led to high molecular weight protein aggregation.

Western Blot of HHP-Treated Wheat Proteins

In immunoblotting experiments all the proteins samples treated with HHP were shown to react with IgE. In particular the IgE reacted to two bands a 10 kDa and 14 kDa (Figure 4-9). The former one is probably LTP proteins whereas, the later one is α-amylase/trypsin inhibitor which are mainly considered as major protein responsible for IgE reactivity in wheat allergic patients. There was minimal difference in IgE binding to HHP 21°C for 5 min and 21°C for 15 min compared to other HHP-treated samples. These data indicated a slight reduction in the IgE reactivity. There was also mild IgE reactivity to 2 other bands corresponding to 35 kDa and 45 kDa which are likely LTP present in LMW glutenin subunits. From the Western blotting results, we found that HHP does not alter IgE binding epitopes and change allergen reactivity.

Dot Blot of HHP-Treated Wheat Proteins

The dot blot results (Figure 4-10) indicate IgE reactivity to all the HHP treated samples. However, there was minimal reduction in the IgE binding in HHP treated at 70°C for 15 min compared to control (untreated) sample.

Indirect ELISA of HHP-Treated Wheat Proteins

The indirect ELISA results indicate a significant reduction in IgE binding in HHP treatment for 21°C for 5 min and 70°C for 5 min (Figure 4-11). However there was no significant difference between the control and other HHP treated samples. The results do not display any trend in the reduction in IgE binding either with time or temperature. Nonetheless, the results indicate the susceptibility of wheat proteins to HHP at lower or room temperature for short duration. The lower temperature and shorter treatment time has massive effect on wheat allergen protein structure compared to longer duration at higher temperature. There was approximately 30-40% reduction in the IgE binding capacity in HHP treatment at 21°C for 5 min and 70°C for 5 min.

NTP-Treated Total Soluble Wheat Proteins

SDS-PAGE of NTP-Treated Wheat Proteins

SDS-PAGE was conducted on NTP treated samples in reducing condition. Analysis of SDS-PAGE results of NTP treated samples in reducing conditions showed that NTP treatment for 1 min was similar to the control (untreated) samples (Figure 4-12). After they were exposed for 3 min and 5 min showed marked reduction in the protein profile on the SDS-PAGE. There was a noticeable reduction in 14 kDa band corresponding to α-amylase/trypsin inhibitor in NTP treatment for 3 min 5 min. During the NTP treatment the free radicals generated can have massive effect on proteins conformation resulting in fragmentation. The fragmented proteins are comparatively smaller in size than the pores of the gel and can easily escape from the gel during the initial electrophoresis.

Western Blot of NTP-Treated Wheat Proteins

As expected the membrane incubated in the pooled human plasma of individual allergic to wheat showed higher IgE reactivity in control and NTP treatment for 1 min but, reduced IgE reactivity in NTP treatment for 3 min and 5 min (Figure 4-13). The proteins profile showed reduction in the protein recovered during SDS-PAGE and therefore there must be less amount of proteins transferred to membrane hence reduced IgE binding. The western blot exhibited IgE reactivity to several bands ranging form10 kDa to 60 kDa. The low molecular weight (around 4 k Da) is present in all the NTP treated samples and do not indicate reduction in IgE reactivity. On the contrary, other low and high molecular weight bands (10 kDa, 35 kDa and 60 kDa) exhibited reduction in IgE binding with increase in treatment time.

Dot Blot Results of NTP-Treated Wheat Proteins

The dot blot results (Figure 4-14) give better understanding of NTP treated proteins in non-reducing conditions. The treatment condition included control (untreated), NTP treatment for 1 min, 3 min and 5 min. The results illustrate the IgE binding to control (untreated) and NTP treatment for 1 min. In contrast, the NTP treatment for 3 min and 5 min indicated negligible IgE reactivity.

Indirect ELISA Results of NTP-Treated Wheat Proteins

To further validate the results indirect ELISA was conducted on NTP treated samples (Figure 4-15). Both NTP treated for 1 min and 3 min did not show significant difference in the IgE reactivity but, the IgE binding was lower in both groups compared to control (untreated). Data showed that NTP treatment for 1 min and 3 min has approximately 25% reduction in IgE binding expressed as absorbance value. Furthermore, the NTP treatment for 5 min indicated significant reduction in the IgE

binding approximately 37% reduction in the absorbance value. These results explain that higher exposure to NTP has immense effect on the IgE binding proteins. The free radicals generated during NTP exposure might mask the conformational site and/or disrupt the conformational binding eptiope. Therefore, the allergen is no longer present or no longer recognized by the IgE to elicit adverse immune response.

Discussion

Effect of PUV-Treatment on Wheat Proteins

In the previous study it has been shown that UV and PUV have notable effects on conformational and aggregation properties of proteins. Taking these facts into account, the present study evaluated the IgE-binding activity of PUV-treated samples. Some of the authors (Davidson and Cooper 1967; Cooper and Davidson1965) have reported that UV-irradiation disrupt collagen molecules resulting in low-molecular-weight fragments. The authors also mentions the formation of insoluble precipitates resulting from excessive UV-treatment. In the present study initial exposure to PUV-irradiation (30 s) resulted in fragmentation. On the other hand, increase in the PUV-treatment time resulted in the insoluble high-molecular-weight aggregates demonstrated by SDS-PAGE gel. The present data are in good agreement with previously reported studies (Cho and Koji 2010; Chung and others 2008; Yang and others 2010; Gennadios and others 1998). Cho and others (2010) have also mentioned that UV-irradiation cleaves covalent bonds leading to formation of fragments when treatment time was 4 and 8 hr. However, the author observed the irreversible aggregation when after 32 hr irradiation. There are several reports on the effect of gamma irradiation on wheat proteins (Srinivas and others 1972; Leszczynska and others 2003). According to Srinivas and others (1972) gamma-irradiation on wheat protein resulted low molecular weight entities

indicating the susceptibility of wheat protein to irradiation-treatment. Another study (Leszczynska and others 2003) on the immunoreactivity of gamma-irradiated wheat gliadin indicated increase in the immunoreactivity with increase in dose level.

To elucidate the effect on IgE-binding activity Western blot, dot blot and indirect ELISA was performed. The IgE-binding activity of PUV-treated sample decreased with increase in treatment time. The maximum reduction in immunoreactivity was observed in 60 s whereas; further increase in PUV-irradiation time did not result in reduced IgE-binding activity (Yang and others 2010). The present study illustrated that IgE-binding activity of PUV-treated sample was lower compared to control sample. Since UV generates free radicals (Cooper and Davidson1965; Krishnamurthy and others 2008) particularly hydroxyl radicals, super oxide radicals, and peroxides, which may interact with water molecule present in the samples and modify the proteins structure resulting in aggregation and/or fragmentation. Nonetheless, which is the main mechanism involved in this study that leads to the reduction in allergen potency is unclear.

From the results it is postulated that PUV-treatment caused conformational changes of proteins resulting in fragmentation, denaturation, aggregation, (Chung and others 2008; Yang and others 2010; Krishnamurthy and others 2008) and ultimately lost their immunogenicity. However, PUV alter the linear protein structure is still needs to be evaluated.

Effect of HHP-Treatment on Wheat Proteins

High hydrostatic pressure treatment on total soluble wheat proteins at 600 MPa showed reduction in IgE binding to HHP 21°C 5 min and 70°C 5 min. There was approximately 30 to 40% reduction in IgE binding expressed as absorbance value at 450 nm. However, HHP treatment for longer duration or higher temperature resulted in

no significant difference. The ultrahigh pressure (200 to 600 MPa) treatment on β-lactoglobulin a major milk allergen resulted in increase in antigenicity with increase in the pressure and holding time (Kleber and others 2007). Furthermore, pressure treatment denatured the milk proteins and the proteins unfolding might expose the linear epitope resulting in enhanced IgE binding in the ultrahigh pressure treated proteins. On the contrary, pressurization treatment on rice grains in the solution released allergenic proteins to its surrounding fluid (Kato and others 2000). The pressurized rice grains were better in quality and resulted in reduced allergen reactivity. Based on the present results it is postulated that HHP effect on food proteins varies from the type, pressure, temperature and duration of treatment. The present study however did not indicate major difference in the IgE reactivity on western blot and dot blot. But, the indirect ELISA indicates significant reduction in two HHP treated samples at 21°C and 70°C for 5 min. Therefore, it is difficult to denote which condition is better suited in reducing the wheat allergen potency.

Effect of NTP-Treatment on Wheat Proteins

NTP treated wheat proteins illustrate a remarkable decrease in allergen reactivity in dot blot. The results indicate a significant reduction in the IgE binding in NTP treatment for 5 min. The dot blot and ELISA results show similarity in the results whereas, the wetern blot results show IgE binding in NTP samples with minimal reduction at 3 and 5 min. This apparent discrepancy is due to the presence of reducing agent in SDS-PAGE. The reducing agent denatures the proteins that might reveal the linear IgE binding epitope on SDS-PAGE results. But, in ELSA and dot blot the IgE binds to the conformational and/or linear IgE binding epitope. However, it is unclear as to what is main mechanism involved in the decreasing the IgE binding.

Table 4-1. Temperature and time measured during PUV-treatment

Time	Initial Temperature (°C)	Final Temperature (°C)	Initial Volume (5 mL)	Final Volume (5 mL)
30	21.1	52.4	5	4.8
60	22.0	64.8	5	4.65
90	21.1	73.0	5	4.4
120	21.1	74.7	5	4.1

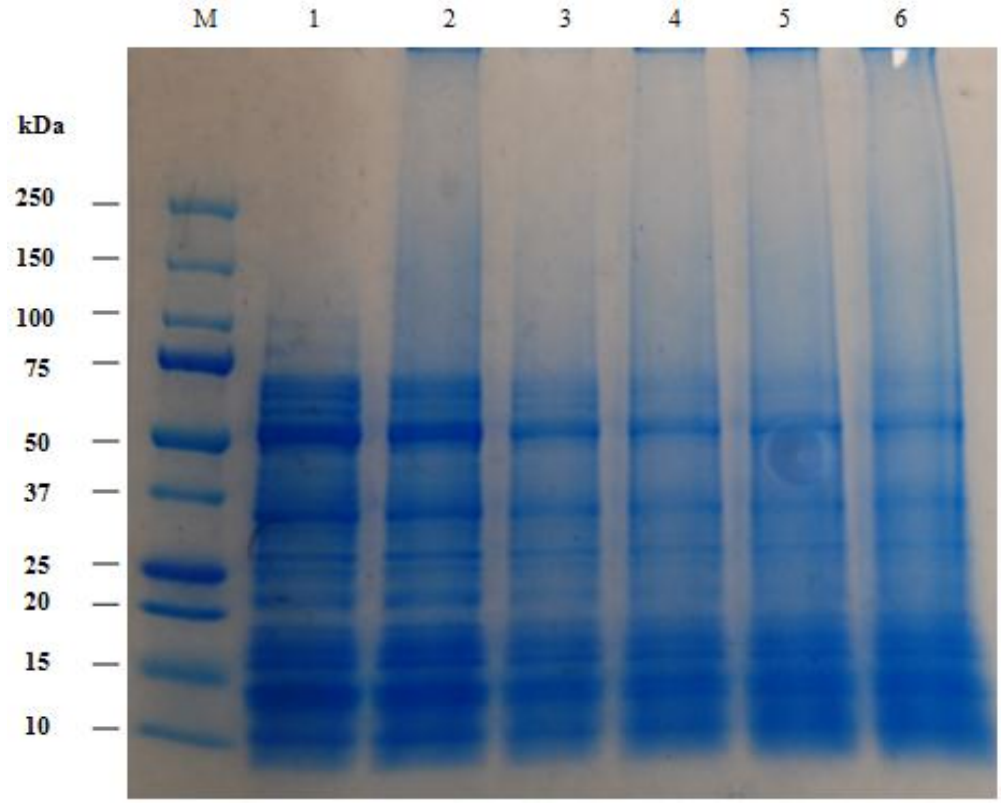


Figure 4-1. SDS-PAGE analysis of PUV treated wheat albumin and globulin; Lanes: (1) Control (2) Boiled (3) PUV 30 s (4) PUV 60 s (5) PUV 90 s (6) PUV+Boiling (Photo taken by author)

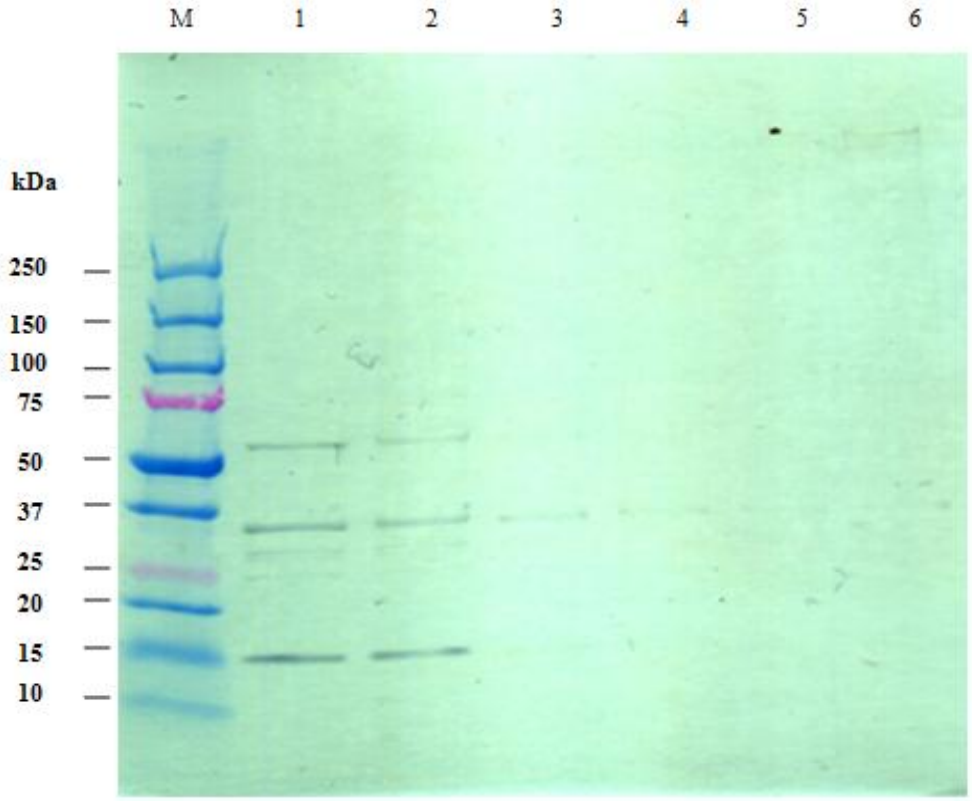


Figure 4-2. Western blot analysis of PUV treated wheat albumin and globulin; Lanes: (1) Control (2) Boiled (3) PUV 30 s (4) PUV 60 s (5) PUV 90 s (6) PUV+Boiling (Photo taken by author)

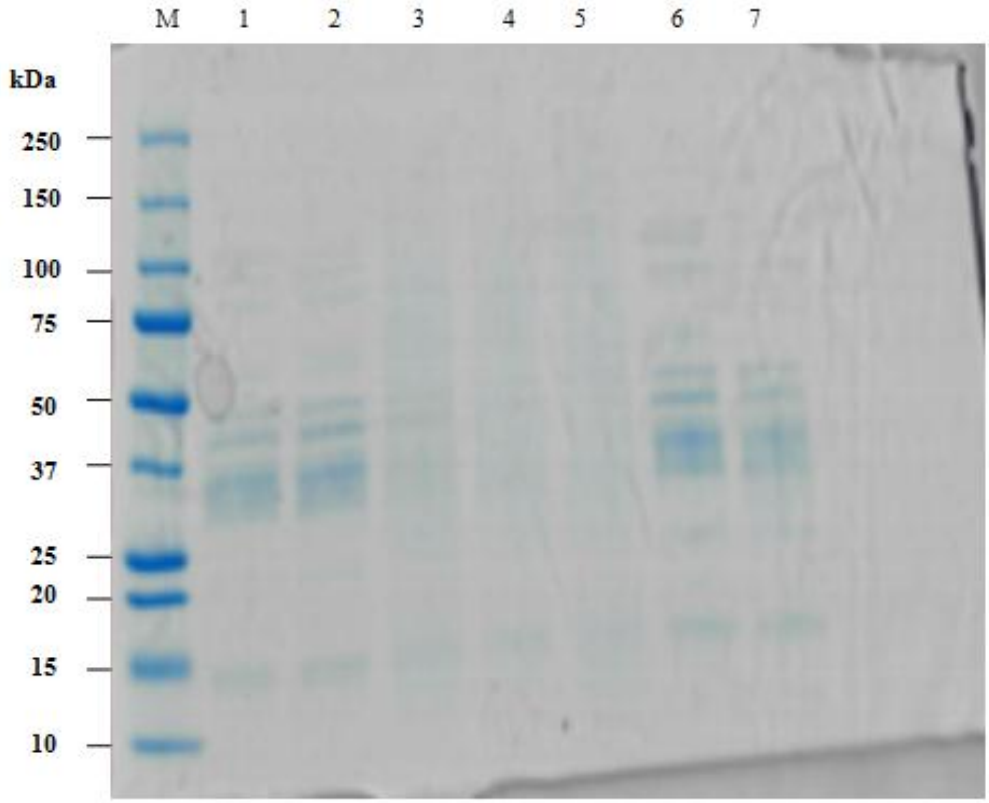


Figure 4-3. SDS-PAGE analysis of PUV treated wheat gluten;
Lanes: (1)-(4) Gluten homogenised (1) Control (2) Boiled (3) PUV (45 s) (4) PUV+Boiling. Lane (5)-(7) Gluten supernatant (5) Control (6) Boiled (7) PUV (45 s) (Photo taken by author)

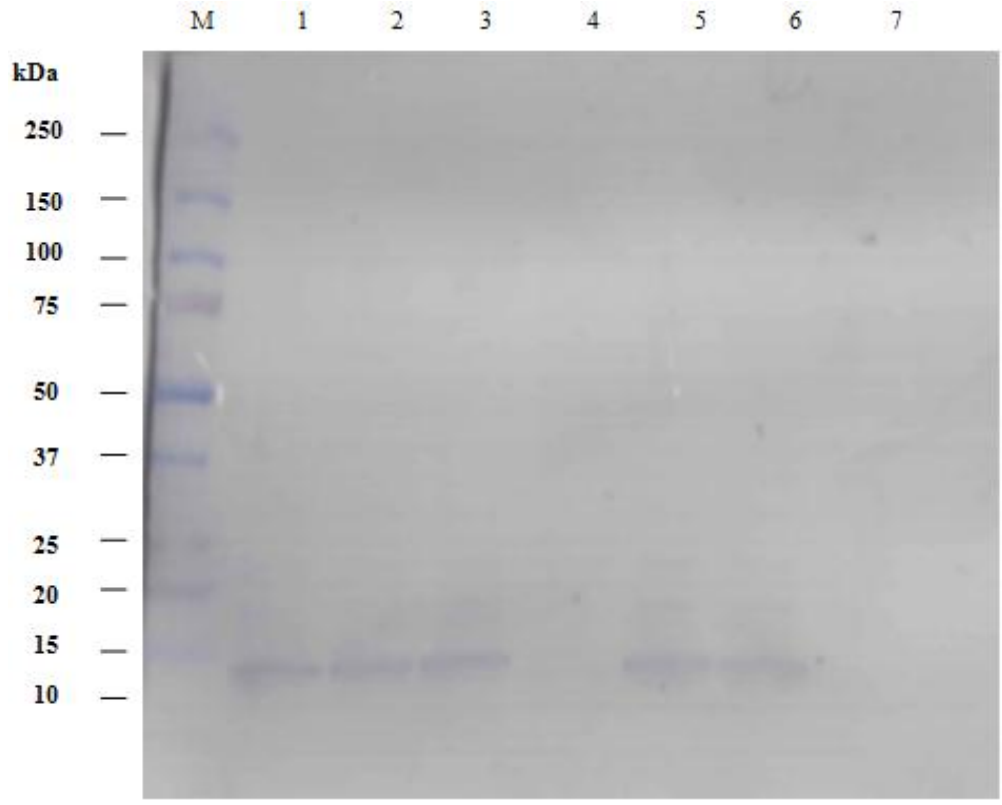


Figure 4-4. Western blot analysis of PUV treated wheat gluten;
Lanes: (1) – (4) Gluten homogenised (1) Control (2) Boiled (3) PUV (45 s) (4) PUV+Boiling. Lanes (5)-(7) Gluten supernatant (5) Control (6) Boiled (7) PUV (45 s) (Photo taken by author)

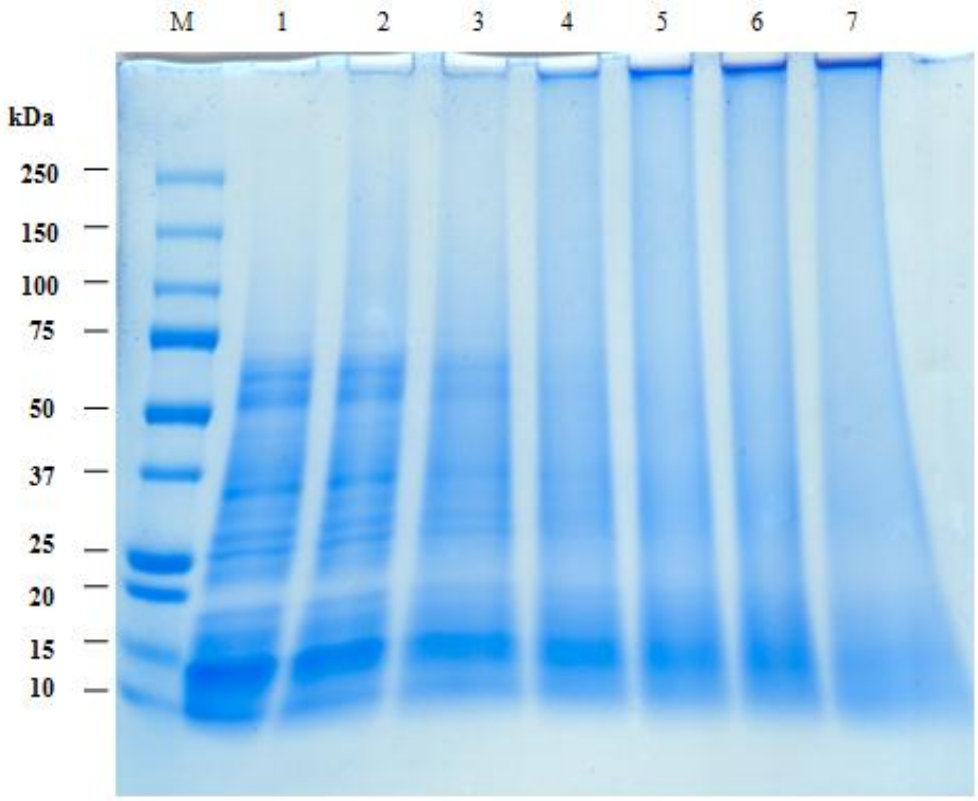


Figure 4-5. SDS-PAGE analysis of PUV treated total soluble wheat proteins; Lanes: M= Marker (1) Control (2) Boiled (3) PUV 30 s (4) PUV 60 s (5) PUV 90 s (6) PUV 120 s (7) PUV 120 s+Boiling (Photo taken by author)

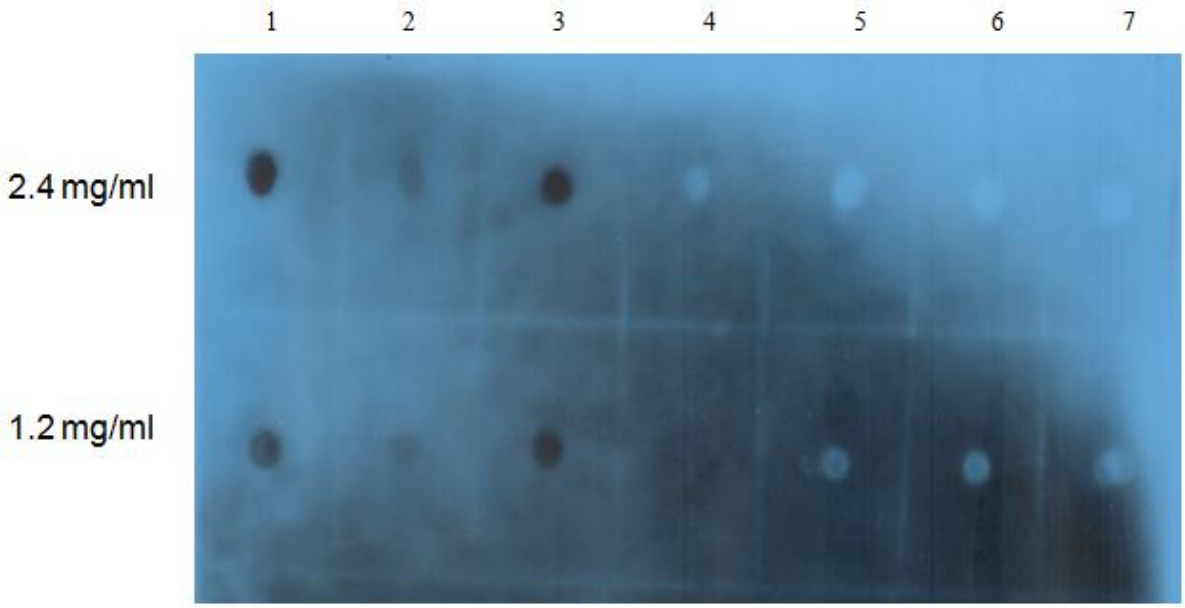


Figure 4-6. Dot blot results of PUV treated total soluble wheat proteins;
(1) Control (2) Boiled (3) PUV 30 s (4) PUV 60 s (5) PUV 90 s (6) PUV 120 s
(7) PUV 120 s + boiling (Photo taken by author)

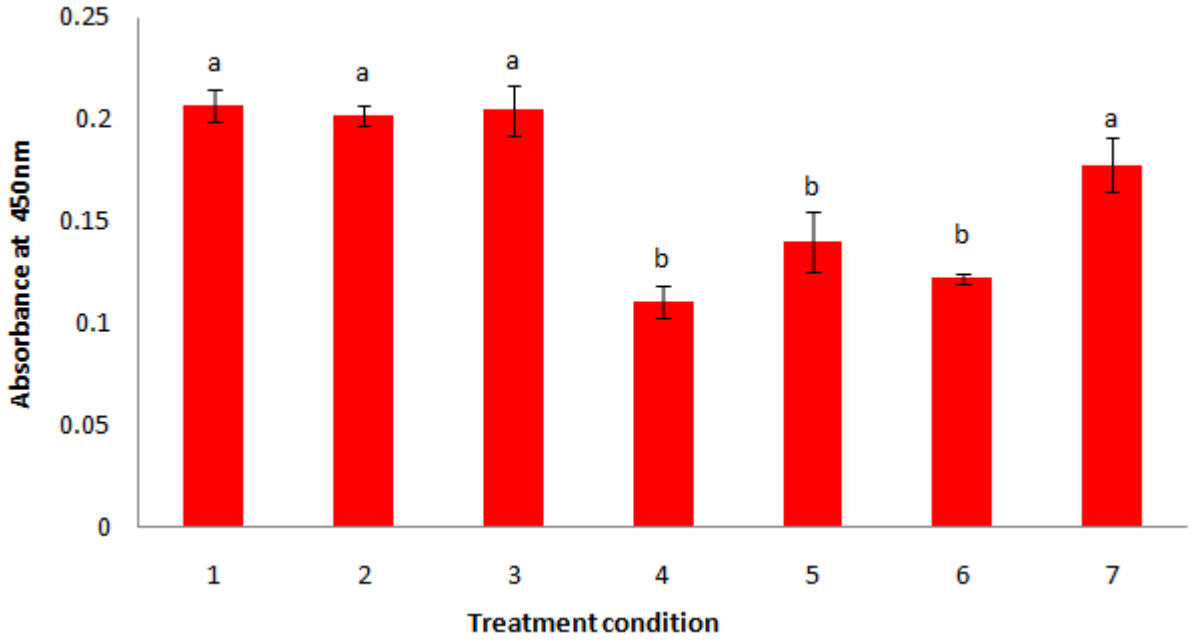


Figure 4-7. Immunoreactivity of PUV treated total soluble wheat proteins determined by indirect ELISA; (1) Control (untreated) (2) Boiled (3) PUV 30 s (4) PUV 60 s (5) PUV 90 s (6) PUV 120 s (7) PUV (120 s)+Boiling. Data represents mean of 4 measurements (n=4) and standard error mean (SEM) are represented (bars). Data with same letters are not statistically different from each other ($p < 0.05$)

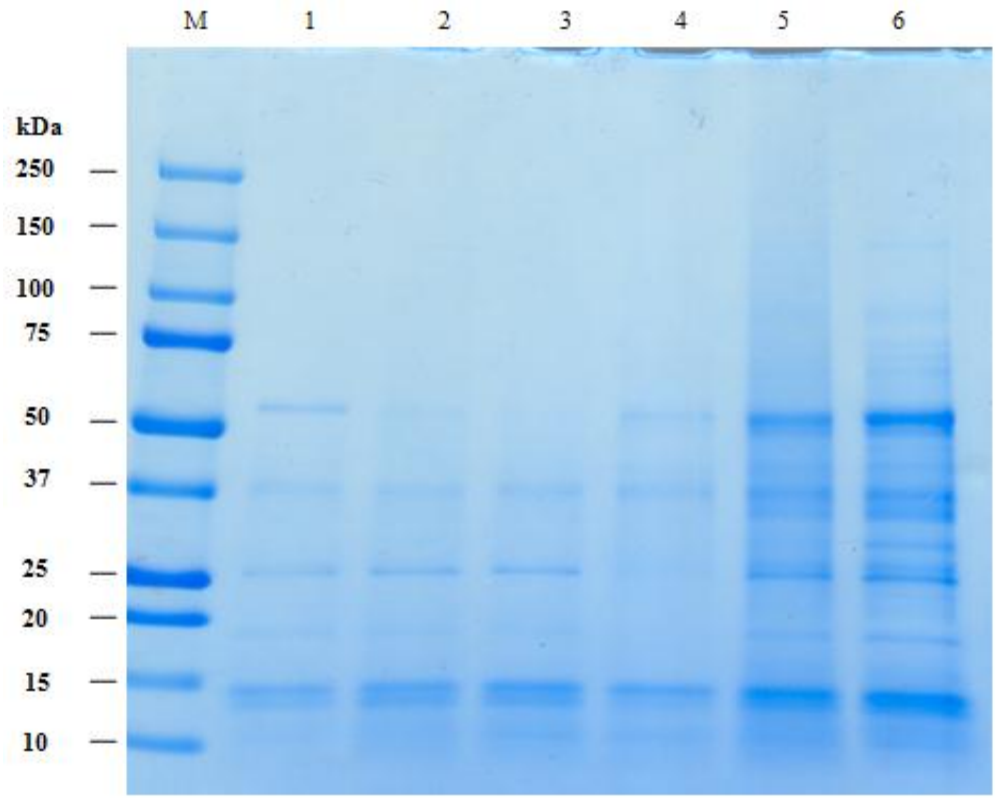


Figure 4-8. SDS-PAGE analysis of HHP treated total soluble wheat protein; Lanes: M= Marker (1) HPP 21°C, 5 min (2) HPP 21°C, 15 min (3) HPP 21°C, 30 min (4) HPP 70°C, 5 min (5) HPP 70°C, 15 min (6) control (Photo taken by author)

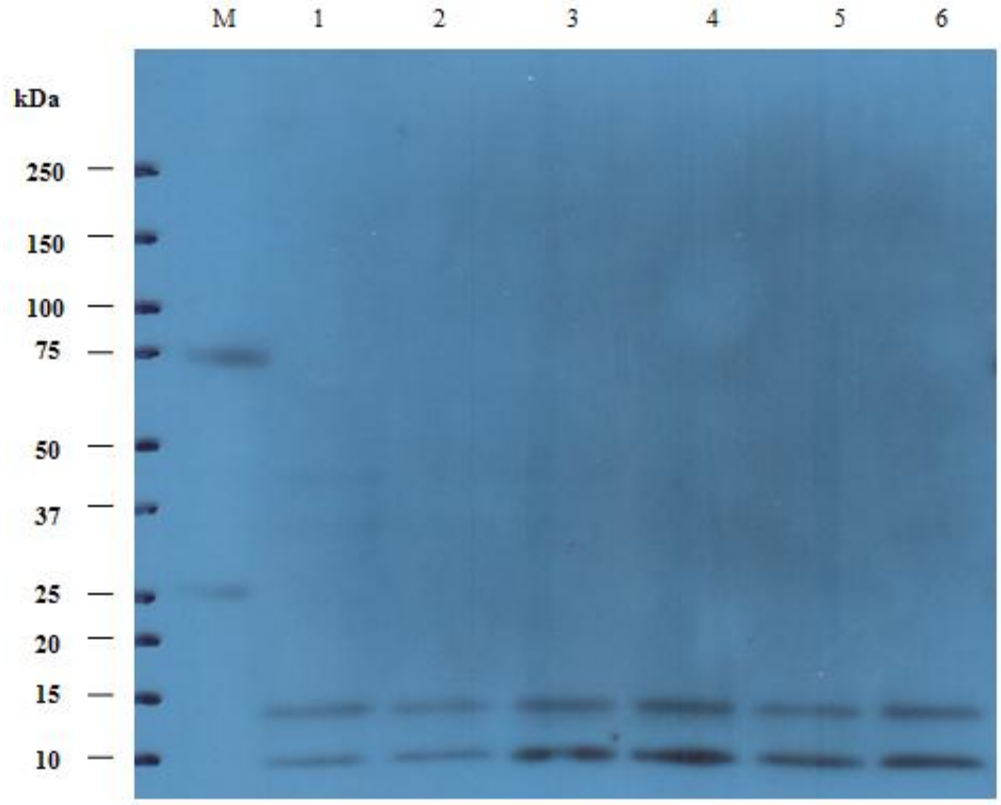


Figure 4-9. Western blot analysis of HHP treated total soluble wheat protein; Lanes: M= Marker (1) HPP 21°C, 5 min (2) HPP 21°C, 15 min (3) HPP 21°C, 30 min (4) HPP 70°C, 5 min (5) HPP 70°C, 15 min (6) control (Photo taken by author)

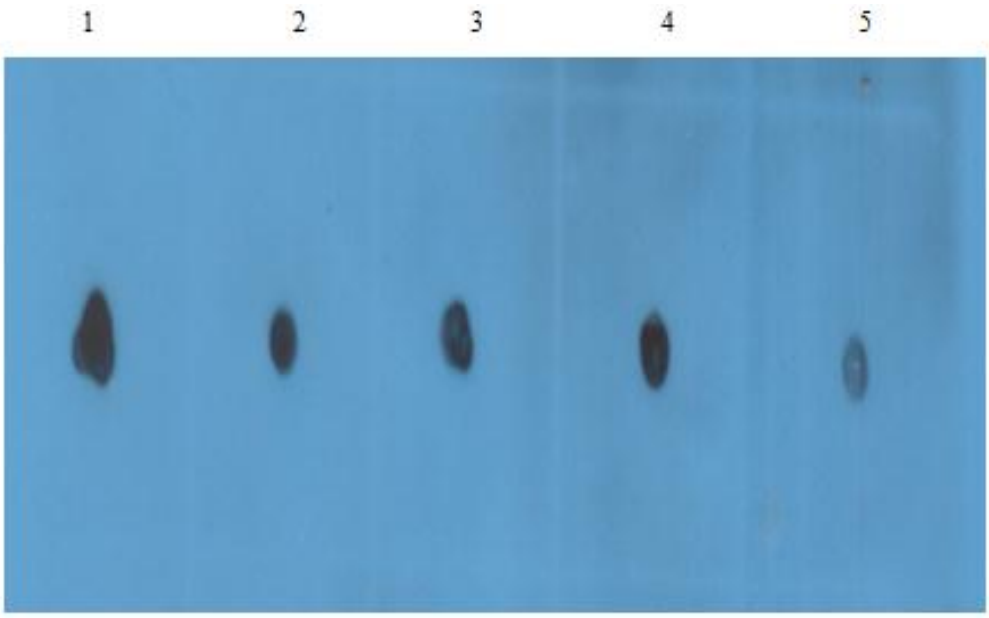


Figure 4-10. Dot blot results of HHP treated total soluble wheat protein;
(1) Control (2) HPP 21°C, 5 min (3) HPP 70°C, 5 min (4) HPP 21°C, 15 min
(5) HPP 70°C, 15 min (Photo taken by author)

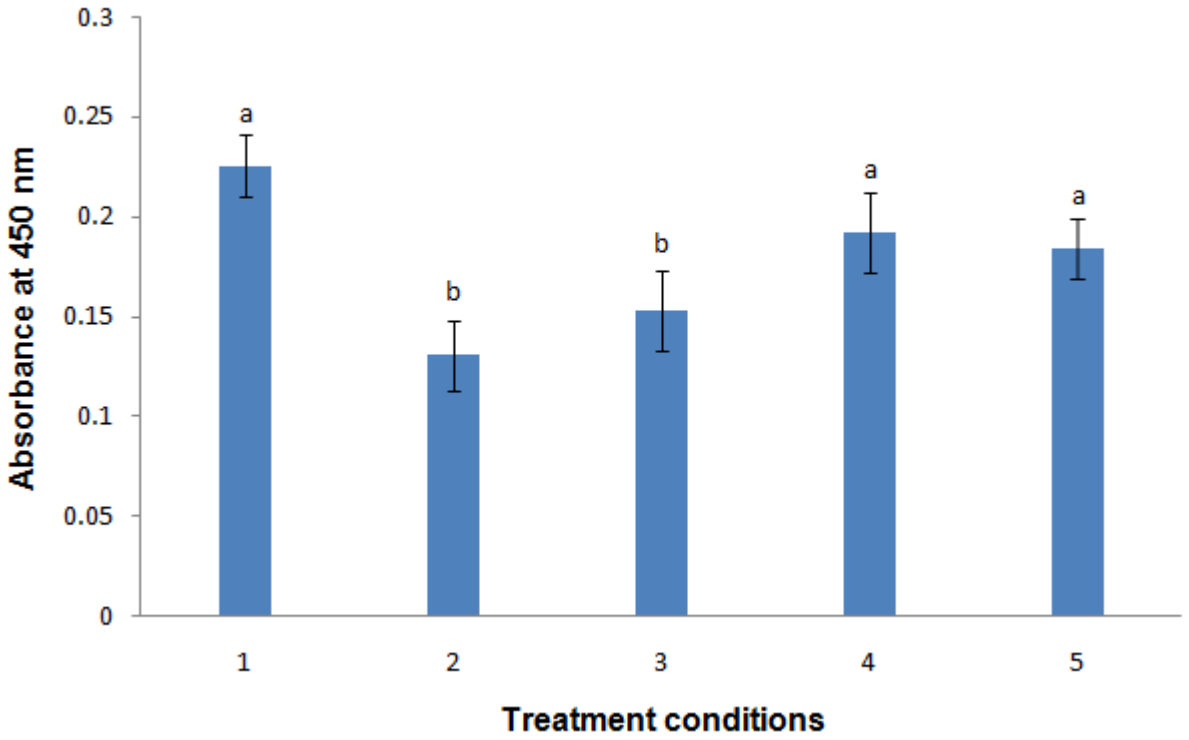


Figure 4-11. Immunoreactivity of HHP treated total soluble wheat proteins determined by indirect ELISA; (1) Control (untreated) (2) HHP 21°C, 5 min (3) HHP 70°C, 5 min (4) HHP 21°C, 15 min (5) HHP 70°C, 15 min. Data represents mean of 12 measurements (n=12) and standard error mean (SEM) are represented (bars). Data with same letters are not statistically different from each other ($p < 0.05$)

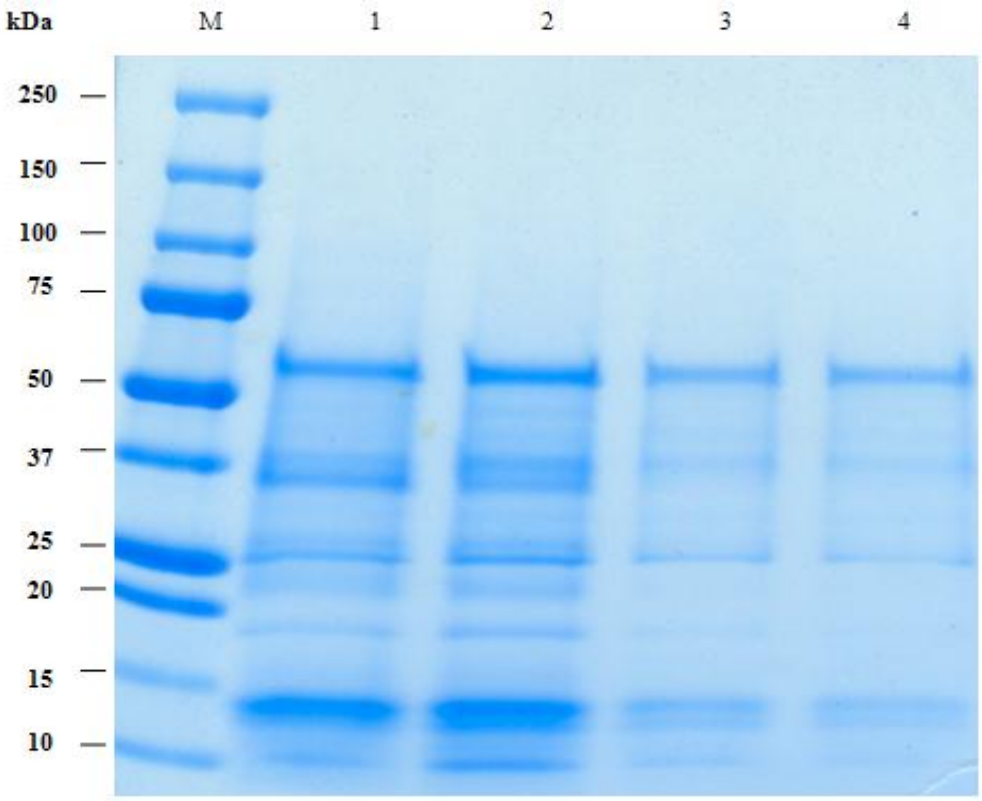


Figure 4-12. SDS-PAGE analysis of NTP treated total soluble wheat protein; Lanes: M= Marker (1) control (2) NTP 1 min (3) NTP 3 min (4) NTP 5 min (Photo taken by author)

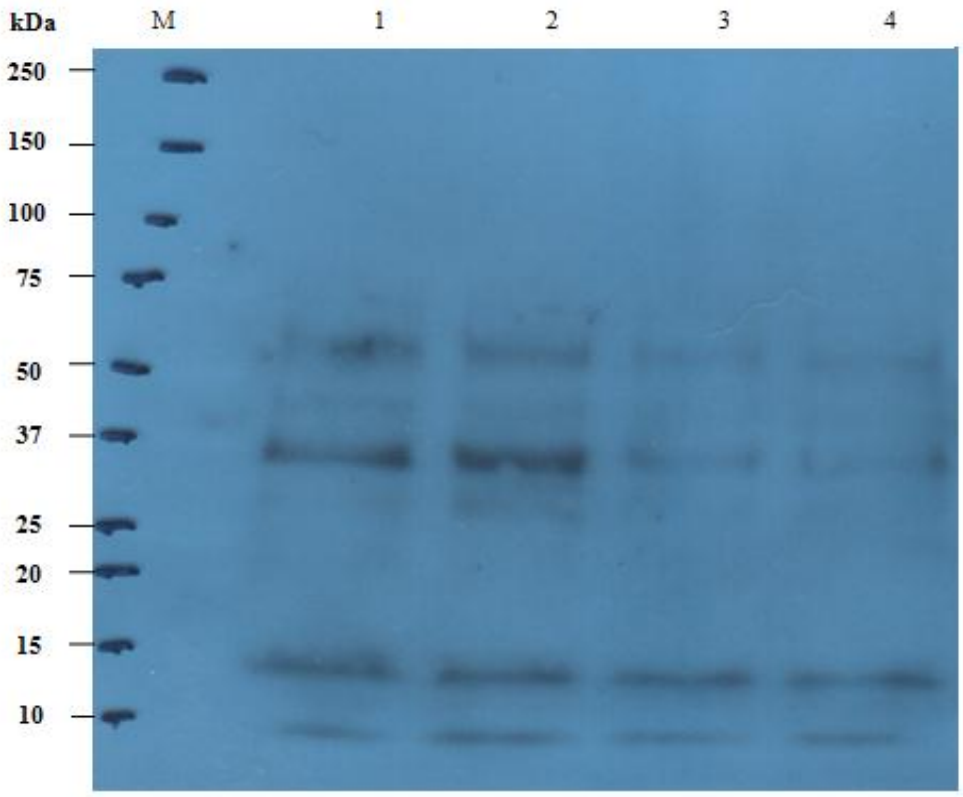


Figure 4-13. Western blot analysis of NTP treated total soluble wheat protein; Lane: M= Marker (1) Control (2) NTP 1 min (3) NTP 3 min (4) NTP 5 min (Photo taken by author)

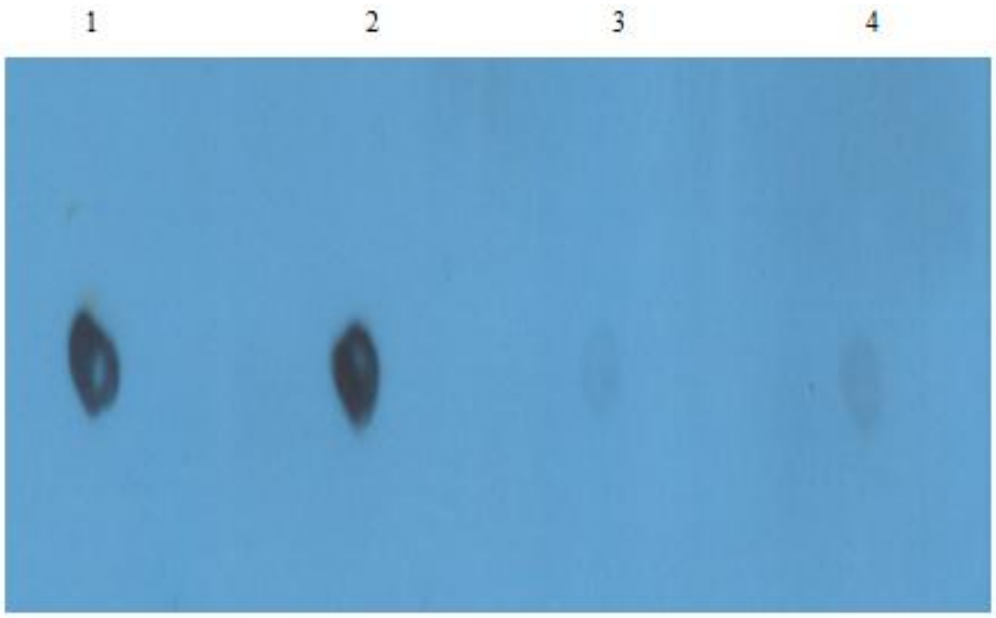


Figure 4-14. Dot blot results of total soluble wheat proteins treated with HPP; (1) Control (2) NTP 1 min (3) NTP 3 min (4) NTP 5 min (Photo taken by author)

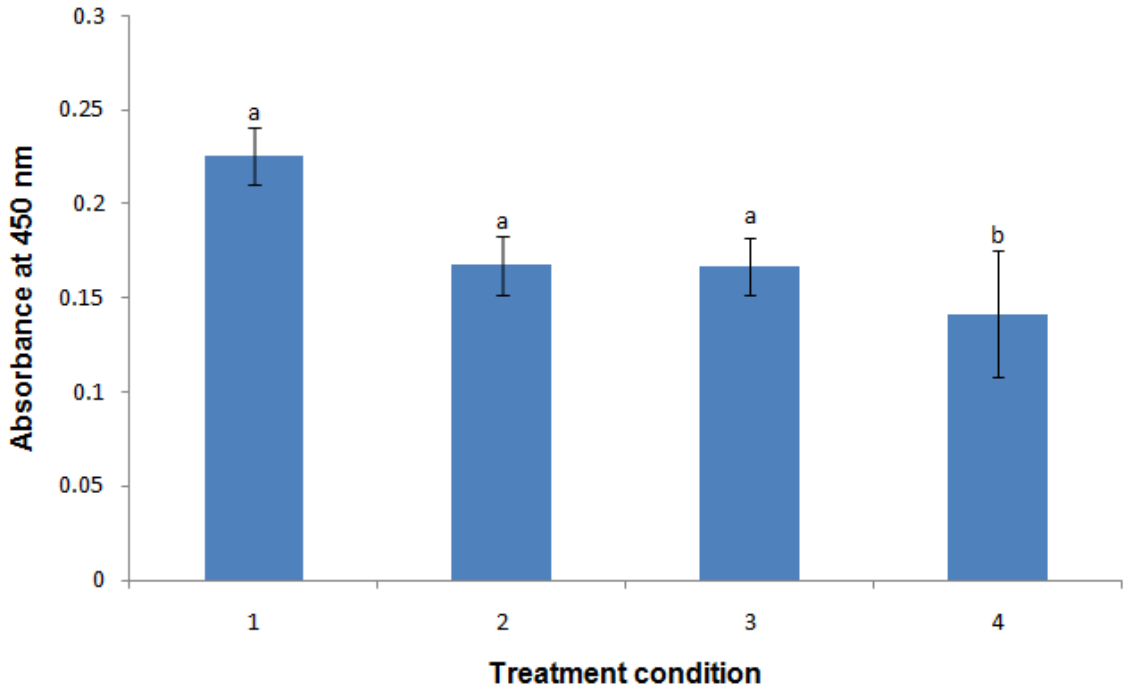


Figure 4-15. Immunoreactivity of NTP treated total soluble wheat proteins determined by indirect ELISA; (1) Control (2) NTP 1 min (2) NTP 3 min (4) NTP 5 min. Data represents mean of 12 measurements (n=12) and standard error mean (SEM) are represented (bars). Data with same letters are not statistically different from each other ($p < 0.05$)

CHAPTER 5
SUMMARY AND CONCLUSION

PUV, HHP and NTP technology were applied to wheat proteins. The effect of PUV indicated a significant reduction in the IgE reactivity at 60 and 90 s. Increasing the exposure time did not result in further reduction decrease. The optimal treatment condition were 60 and 90 s at 20.5 cm from the PUV lamp at 3 pulses/sec. From HHP results it can be conclude that HHP-treated wheat proteins demonstrated a reduction in IgE at 21 °C and 70 °C with treamtment time being 5 min for both the temperature. NTP-treatment on wheat proteins resulted in remarkable decreace in IgE binding demonstrated by dot blot. The highest percentage reduction in IgE binding was obtained by PUV- approximately 46% followed by HHP- and NTP-treatment approximately 42% and 37%. Non-thermal-treatment may produce conformational and chemical changes in major wheat allergen structure that have influence on their immunoreactivity.

The results obtained show that non-thermal technology may be employed for the reduction of major wheat allergen potency. However, future experiments such as *invivo* studies are essential in order to examine the actual nature of non-thermally treated products in the human digestive tract. The future work should also include sensory and microbial analysis.

APPENDIX
THE ANOVA PROCEDURE

The ANOVA Procedure

Class Level Information

Class	Levels	Values
PUV treated sample	7	Boil Control PUV sixty PUV thirty PUV ninety PUV one twenty PUV+Boil

Number of observations 28

The ANOVA Procedure

Dependent Variable: Result Result

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	6	0.04105236	0.00684206	17.08	<.0001
Error	21	0.00841250	0.00040060		
Corrected Total	27	0.04946486			

R-Square	Coeff Var	Root MSE	Result Mean
0.829930	12.02611	0.020015	0.166429

Source	DF	Anova SS	Mean Square	F Value	Pr > F
PUV_treated_sample	6	0.04105236	0.00684206	17.08	<.0001

The ANOVA Procedure

Duncan's Multiple Range Test for Result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	21
Error Mean Square	0.000401

Number of Means	2	3	4	5	6	7
Critical Range	.02943	.03090	.03183	.03249	.03297	.03335

Means with the same letter are not significantly different.

Duncan Grouping	Mean	N	PUV_treated_sample
A	0.20700	4	Control
A	0.20475	4	PUV thirty
A	0.20225	4	Boil

A	0.17775	4	PUV+Boil
B	0.14025	4	PUV ninety
B	0.12225	4	PUV one twenty
B	0.11075	4	PUV sixty

Means and Descriptive Statistics 1

PUV treated sample	Mean of RESULT	Std. Dev. of RESULT	Std. Error of RESULT	Variance of RESULT
Boil	0.20225	0.009811	0.004905	.000096250
Control	0.20700	0.015748	0.007874	.000248000
PUV sixty	0.11075	0.016070	0.008035	.000258250
PUV thirty	0.20475	0.024391	0.012195	.000594917
PUV ninety	0.14025	0.029960	0.014980	.000897583
PUV one twenty	0.12225	0.005123	0.002562	.000026250
PUV+Boil	0.17775	0.026133	0.013066	.000682917

The ANOVA Procedure

Class Level Information

Class	Levels	Values
HHP treated sample	5	Control HP21°15m HP21°C5m HP70°C15m HP70°C5m

Number of observations 60

The ANOVA Procedure

Dependent Variable: Result Result

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	0.06403710	0.01600928	4.27	0.0044
Error	55	0.20628343	0.00375061		
Corrected Total	59	0.27032053			

R-Square	Coeff Var	Root MSE	Result Mean
0.236893	34.54807	0.061242	0.177267

Source	DF	Anova SS	Mean Square	F Value	Pr > F
HHP_treated_sample	4	0.06403710	0.01600928	4.27	0.0044

The ANOVA Procedure

Duncan's Multiple Range Test for Result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	55
Error Mean Square	0.003751

Number of Means	2	3	4	5
Critical Range	.05011	.05270	.05442	.05566

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	HHP_treated_ sample
	A	0.22557	12	Control
B	A	0.19207	12	HP21°15m
B	A	0.18457	12	HP70°C15m
B	C	0.15332	12	HP70°C5m
	C	0.13082	12	HP21°C5m

HHP treated sample	Mean of RESULT	Std. Dev. of RESULT	Std. Error of RESULT	Variance of RESULT
Control	0.22557	0.053751	0.015517	.002889155
HP21°15m	0.19207	0.068034	0.019640	.004628628
HP21°C5m	0.13082	0.060701	0.017523	.003684651
HP70°C15m	0.18457	0.052116	0.015045	.002716101
HP70°C5m	0.15332	0.069531	0.020072	.004834505

1

The ANOVA Procedure

Class Level Information

Class	Levels	Values
NTP treated samples	4	Control NTP 1 m NTP 3 m NTP 5 m

Number of observations 48

The ANOVA Procedure

Dependent Variable: Result Result

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	3	0.04570773	0.01523591	2.71	0.0565
Error	44	0.24739561	0.00562263		
Corrected Total	47	0.29310334			

R-Square	Coeff Var	Root MSE	Result Mean
0.155944	42.78596	0.074984	0.175254

Source	DF	Anova SS	Mean Square	F Value	Pr > F

NTP_treated_samples	3	0.04570773	0.01523591	2.71	0.0565

The ANOVA Procedure

Duncan's Multiple Range Test for Result

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	44
Error Mean Square	0.005623

Number of Means	2	3	4
Critical Range	.06170	.06488	.06697

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	NTP_treated_samples
A		0.22557	12	Control
B	A	0.16740	12	NTP 1 m
B	A	0.16657	12	NTP 3 m
B		0.14148	12	NTP 5 m

Means and Descriptive Statistics 1

NTP treated samples	Mean of RESULT	Std. Dev. of RESULT	Std. Error of RESULT	Variance of RESULT
Control	0.22557	0.05375	0.015517	0.002889
NTP 1 m	0.16740	0.05495	0.015864	0.003020
NTP 3 m	0.16657	0.05338	0.015411	0.002850
NTP 5 m	0.14148	0.11718	0.033827	0.013731

The ANOVA Procedure

Class Level Information

Class	Levels	Values
PUV_treated_samples	4	Control PUV ninety PUV sixty PUV thirty

Number of observations 48

The ANOVA Procedure

Dependent Variable: Results Results

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F

Model	3	0.06330673	0.02110224	3.50	0.0230
Error	44	0.26507021	0.00602432		
Corrected Total	47	0.32837694			

R-Square	Coeff Var	Root MSE	Results Mean
0.192787	38.24965	0.077617	0.202921

Source	DF	Anova SS	Mean Square	F Value	Pr > F
PUV_treated_samples	3	0.06330673	0.02110224	3.50	0.0230

The ANOVA Procedure

Duncan's Multiple Range Test for Results

NOTE: This test controls the Type I comparisonwise error rate, not the experimentwise error rate.

Alpha	0.05
Error Degrees of Freedom	44
Error Mean Square	0.006024

Number of Means	2	3	4
Critical Range	.06386	.06716	.06932

Means with the same letter are not significantly different.

Duncan Grouping		Mean	N	PUV_treated_samples
A		0.23773	12	Control
A		0.23732	12	PUV thirty
B	A	0.18390	12	PUV sixty
B		0.15273	12	PUV ninety

Means and Descriptive Statistics 1

PUV treated samples	Mean of RESULTS	Std. Dev. of RESULTS	Std. Error of RESULTS	Variance of RESULTS
Control	0.23773	0.04192	0.012102	0.001758
PUV ninety	0.15273	0.04641	0.013399	0.002154
PUV sixty	0.18390	0.11013	0.031791	0.012128
PUV thirty	0.23732	0.08976	0.025912	0.008057

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BIOGRAPHICAL SKETCH

Jyotsna K Nooji was born and raised in Karnataka, India. She is the second of two children born to Jaikrishna and Vidya Nooji. She graduated from University of Mysore, India and received her master's in Food Science and Human Nutrition in June 2006. After that in spring 2009, Jyotsna enrolled as a master's student in the Food Science and Human Nutrition Department at University of Florida.

Jyotsna is an active member of Institute of Food Technologists (IFT), and has presented her thesis IFT, Chicago, 2010. She has also presented an oral presentation at IFT. In her free time, Jyotsna enjoys painting, sketching, cooking, and travelling. She has a strong interest in baking, wine industry, and food product development, and intends to pursue her career in that discipline.