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Soybean allergy: Effect of genetic modification (GM), heat and enzymatic treatment on overall allergenicity

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SOYBEAN ALLERGY: EFFECT OF GENETIC MODIFICATION (GM), HEAT AND ENZYMATIC TREATMENT ON OVERALL ALLERGENICITY

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SOYBEAN ALLERGY: EFFECT OF GENETIC MODIFICATION (GM), HEAT AND ENZYMATIC TREATMENT ON OVERALL ALLERGENICITY

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Soybean allergy affects approximately 0.4% of children worldwide. At least 16 proteins in soybean bind IgE from some soybean allergic subjects. Although the relative allergenic importance and abundance of individual proteins in soybean varieties is not understood, the allergenicity assessment guideline for the safety of genetically modified (GM) food crops (Codex, 2003) includes assessing potential increases in expression of endogenous allergens in an allergenic crop like soybeans that might be due to insertion of the new DNA. The studies described in this dissertation included comparison of binding of IgE from individual soybean allergic subjects to proteins in three transgenic soybean lines, their respective near-isogenic and other commercial lines. The results indicated no evidence that the transgenic soybean lines present an increased risk for soybean allergic subjects especially since those with soybean allergy should avoid all soybeans. Furthermore, based on the observed variation among commercial lines, it is not clear that similar tests are useful to evaluate food safety for typical GM varieties.

Soybean products are widely used in food because of their functionality, nutritional properties and low cost. Some soybean ingredients are processed either by heat treatment or enzymatic hydrolysis to attain desirable functional properties or in some cases to reduce the allergenicity. However, few studies have investigated the effect various processing conditions have on allergenicity of soybean products and their efficacy in

reducing allergenicity of soybean. Additional studies described in this dissertation evaluated potential changes in IgE binding to soybean proteins that are heat-treated under conditions that mimic some commercial processing or undergo enzyme hydrolysis. Results indicated that majority of thermal treatment conditions utilized in making soybean products will not affect their allergenicity and hydrolysis of soybean proteins by different enzymes does not make them less allergenic compared to the untreated proteins and may increase their allergenicity.

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CHAPTER 1: LITERATURE REVIEW

SOYBEAN

Soybean (Glycine max), a crop native to China and Southeast Asia, has a high nutritional value due to the high concentration of oil (18-25%) and protein (38-50%) and is a popular food all over the world (Muller et al., 1998). Production and consumption of soy products in western countries have increased dramatically in the last decade. In Asian countries soybean is processed into various fermented and non-fermented food stuffs such as soy sauce, miso, natto, yogurts, kinako, protein crisp, desserts, baby food, and soy milk which is further processed into tofu, aburage and yuba. The use of soybean-based food in western countries has gained popularity recently because of the health benefits of soybean. In western countries relatively unprocessed soybeans are mainly used in soy milk, tofu, soy sprouts and edamame, while soy protein products that are textured are used in soy and tofu burgers, soy sausages, chicken nuggets, soy ice cream, yogurt and various other products (Hammond and Jez, 2011). In the US, the area planted with soybeans is second only to corn. Globally 38% of the total soybean crop is grown in US, followed by Brazil (25%), Argentina (19%), China (7%), India (3%), Canada (2%), and Paraguay (2%) (Singh et al., 2008). In a recent survey approximately 33% of Americans recalled consuming soybean products at least once a month (Michelfelder, 2009). Soy protein formulas are generally introduced into the diet early in life particularly for infants with an intolerance or allergy to cow's milk. Soybean based products are also used as the primary protein source for those with several other disorders such as lactose intolerance and severe gastroenteritis in infants (Businco et al., 1992).

Mature soybean seed contains approximately 35% protein, 31% carbohydrates, 17% fat, 5% mineral and 12% moisture (L'Hocine and Boye, 2007). Soybean protein contains adequate amounts of essential amino acids; histidine, isoleucine, leucine, lysine, phenylalanine, tyrosine, threonine, tryptophan and valine. However, the combined amount of lysine and methionine (sulfur containing amino acid) falls below the recommended daily intake for a balanced diet. Soybean is particularly high in lysine, which is a limiting amino acid in rice, wheat or corn (Erdman and Fordyce, 1989). Several health benefits have been reported to be associated with consumption of soybean. For example, consumption of soybean has been reported to lower plasma cholesterol (Anthony et al., 1996), prevent cancer (Kennedy, 1998), improve bone mineral density (Kreijkamp-Kaspers et al., 2004) and provide protection against bowel and kidney disease (Friedman and Brandon, 2001). These health benefits are attributed to the presence of isoflavones, saponins, proteins, and peptides in soybean (Friedman and Brandon, 2001; Michelfelder, 2009; Xiao, 2008). The Food and Drug Administration (FDA) has authorized the use on food labeling of health claims on the association between soy protein and a reduced risk of coronary heart disease (http://www.fda.gov/food/labelingnutrition/labelclaims/healthclaimsmeetingsignificantscientificagreementssa/ucm074740.htm

Although soybean is well utilized by human, it has a low nutritional value compared to milk, which is attributed to the presence of anti-nutritional factors in soybean including inhibitors of digestive enzymes and lectins and its low content of the essential amino acid L-methionine. The low methionine content of soy protein is particularly important since it is chemically modified during food processing and storage,

further reducing nutritional quality (Friedman and Brandon, 2001). Also protein-bound methionine in some plant foods is poorly utilized. To overcome this problem, several soybean lines are being investigated as options to increase the methionine-rich proteins (Friedman and Brandon, 2001). Protease inhibitors present in raw soybean can bind to proteolytic digestive enzyme thereby reducing the intestinal digestive process. Two major types of protease inhibitors are found in soybean including Kunitz inhibitor and Bowman-Birk inhibitor. Kunitz trypsin inhibitor is heat labile and has a molecular weight of approximately 20 kDa. It has a strong inhibitory effect against trypsin and weak action against chymotrypsin. Bowman-Birk inhibitor is a heat stable inhibitor with a molecular weight of approximately 8 kDa and can inhibit both trypsin and chymotrypsin strongly (DiPietro and Liener, 1989). Although heat treatment can inactivate these inhibitors, commercially available soybean products have been shown to contain approximately 5-20% of trypsin inhibitor activity found in raw soy (Erdman and Fordyce, 1989). Despite their anti-nutritional effects, soybean protease inhibitor particularly Bowman-Birk inhibitor has been reported to have anti-carcinogenic and anti-inflammatory properties (Kennedy, 1998). Other important anti-nutritional factors found in soybean are lectins or plant agglutinins. These are carbohydrate-binding proteins that have hemagglutinating properties. They are present in several legume species including soybean and can exert anti-nutritional effects and cause growth depression in agriculturally important species (Etzler, 1985).

SOYBEAN ALLERGY

Soybean is one of the eight allergenic foods or groups of foods (peanuts, soybeans, tree nuts, milk, egg, fish, crustaceans, and wheat) that are thought to cause nearly 90% of food-allergic reactions in the US (Goodman et al., 2005). Soybean allergy affects approximately 0.4% of children worldwide (Savage et al., 2010). The prevalence and incidence of soy allergy in the general population is unknown and is likely to be dependent on local dietary habit and exposure (Sicherer et al., 2000). The prevalence is considered to be higher in Asian countries compared to Europe and US and it has been reported to be the fifth most common food allergen causing anaphylaxis in Japan (Ito et al., 2011). Several reports suggest wide discrepancies in prevalence of soybean allergy. A study including a large cohort of atopic children, where soybean was fed early in life for up to several months, showed that soy allergy is not common in children with atopic disorders and also rare in children fed soy early in life (Bruno et al., 1997). In another study designed to determine the prevalence of soy allergy in 704 atopic children in Italy, out of 131 soy skin prick positive children, only 6% showed positive oral challenge with soybean, representing 1.1% of the atopic children (Magnolfi et al., 1996). Soybean allergy has also been reported to occur in a minority of young children with cow's milk allergy (CMA). Zeiger et al. (1999) found that 14% of IgE mediated cow's milk allergic children also have soy allergy based on double blind placebo controlled food challenge (DBPCFC), open challenge, and history of anaphylactic reaction to soy. Most children with soy allergy tend to outgrow their allergy later in life. It has been shown that the median age at which tolerance develops is around 10 years (Savage et al., 2010). The rate of soy allergy resolution depends on soy specific IgE levels and children with higher soy

specific IgE levels tend to have persistent soy allergy (Savage et al., 2010). Although soy allergy is considered an early onset disease, the disease can occur later in life, which may be due to cross-reactivity with peanut or birch pollen (Savage et al., 2010). In a study by Mittag et al. (2004) soybean allergy has been shown to be prevalent in adult patients allergic to birch pollen, and the allergy is due to cross-reactivity or shared IgE binding of the soybean allergen Gly m 4 with the birch pollen allergen Bet v 1. Unlike mild oro-pharyngeal symptoms in patients with pollen related food allergy, patients with marked allergies to birch pollen showed systemic reactions in DBPCFC to soybean (Mittag et al., 2004).

Clinical manifestations of soy allergy range from severe enterocolitis to atopic eczema and immediate IgE-mediated systemic multi-system reactions (Sicherer et al., 2000). While still a common allergen, life threatening reactions to soy are quite rare and much lower in prevalence than severe reactions to peanut (Cantani and Lucenti, 1997). A recent study was conducted by Rolinck-Werninghaus et al. (2012), where oral food challenge was performed in 869 children using cow's milk, egg, wheat and soy. In contrast to 4 and 14% of milk- and egg-allergic children respectively who showed severe reaction at the first dose (3 mg), most of the severe reactions to soy in soy-sensitized children occurred at larger doses (>1.1 gram) (Rolinck-Werninghaus et al., 2012). Nevertheless, a few cases of anaphylactic reactions including food dependent exercise induced anaphylaxis have been reported due to consumption of soybean (Adachi et al., 2009; David, 1984; Foucard and Malmheden Yman, 1999; Magnolfi et al., 1996; Moroz and Yang, 1980; Senna et al., 1998; Taramarcaz et al., 2001).

SOYBEAN ALLERGENS

At least 16 soybean proteins with molecular weights ranging from 14 kDa to 70 kDa have been shown to bind IgE from sera of patients with atopic dermatitis (Ogawa et al., 1991). Soybean Gly m Bd 30K was identified as a major allergenic component of soybean seed protein with 65% of soy-sensitive patients with atopic dermatitis showing specific IgE binding (Ogawa et al., 1993; Helm et al., 1998). Gly m Bd 30K is a 34-kDa oil body associated cysteine protease from soybean, also known as soybean vacuolar protein P34 (Ogawa et al., 1993; Kalinski et al., 1992). It is a monomeric insoluble glycoprotein of 257 amino acids and is often found attached by a disulfide bond to the 7S globulin proteins of soybeans (Wilson et al., 2005). This protein accounts for 5% of total seed cotyledon protein of soybean. It accumulates during seed maturation and is processed to a 32 kDa protein by removal of its amino terminus on the fourth through sixth days of seedling growth (Herman et al., 1990; Ogawa et al., 1993). B-cell epitope mapping with overlapping peptides of P34 identified five immuno-dominant IgE binding linear epitopes; while conformational epitopes have not been identified (Helm et al., 1998). Soybean allergen Gly m Bd 28K is a minor soybean glycoprotein component shown to be recognized by 25% of soybean allergic subject. It has a molecular mass of 26 kDa and an iso-electric point of 6.1 (Ogawa et al., 2000; Tsuji et al., 1997; Tsuji et al., 2001). The glycan moiety of this glycoprotein is similar to that of other glycoprotein allergens including bromelain, horseradish peroxidase, ascorbate oxidase and Gly m Bd 30K (Tsuji et al., 1997). The glycan at Asn 20 was demonstrated to bind IgE (Hiemori et al., 2000). A C-terminal peptide fragment of Gly m Bd 28 K, a 23 kDa glycoprotein, has also been

shown to bind IgE from soybean sensitive patients, primarily due to the glycan moiety (Hiemori et al., 2004).

Soybean glycinin (Gly m 6) and β-conglycinin (Gly m 5), the major seed storage proteins of soybean, have been implicated as the major allergens in soybean (Holzhauser et al., 2009; Ito et al., 2011). Soybean glycinin is a hexameric protein of approximately 350 kDa and composed of six non-identical subunits (Adachi et al., 2003). The subunit composition of soybean glycinin varies according to cultivar (Mori et al., 1981). Five major kinds of subunits have been identified in glycinin; A1aB1b, A2B1a, A1bB2, A3B4, and A5A4B3 (Adachi et al., 2003). Each of these subunits is composed of an acidic chain of 37-42 kDa, pI=4.2-4.8 (A1a, A1b, A2, A3, A4, A5) and a basic chain of 17-20 kDa, pI=8-8.5 (B1a, B1b, B2, B3, B4) linked by a single disulfide bond (Petruccelli and Anon, 1995a; Sathe et al., 1987; Maruyama et al., 2003). An early study by Pedersen and Djurtoft (1989) indicated that the acidic glycinin chain is mainly responsible for IgE binding with sera from soy allergic patients. However, IgE binding to the basic chain of glycinin has also been reported (Helm et al., 2000a). In a recent study, 36% of study subjects with positive DBPCFC and with history of anaphylaxis to soy had specific IgE to glycinin (Holzhauser et al., 2009). A recent Japanese study found that 58% of children with severe allergic reactions to soybean (n=33) exhibited IgE binding to Gly m 6 (Ito et al., 2011). Eleven linear epitopes have been identified in glycinin by B-cell epitope mapping among which, four are immuno-dominant (Helm et al., 2000b). The IgE binding epitopes of the acidic chain of glycinin were shown to be identical to that of major peanut allergen Ara h 3 in other studies (Beardslee et al., 2000; Xiang et al., 2002).

Soybean β-conglycinin (Gly m 5) is a trimeric protein of approximately 150-200 kDa, composed of different combinations of three subunits; α (67 kDa), α'(71 kDa) and β (50 kDa). All of the monomers include a single asparagine-linked glycosylation site and these are usually glycosylated by addition of a polymannose or complex glycan (Maruyama et al., 2003). In a study by Ogawa et al. (1995), the α subunit of β-conlgycinin (Gly m Bd 60k) was shown to be recognized by IgE from 25% of sera from soybean sensitive patients with atopic dermatitis. Although both the α' and β subunits are homologous to the α subunit, no IgE binding was seen to either of these subunits (Ogawa et al., 1995). However, another study used IgE immunoblot analysis with soybean-allergic patient sera and showed that the α' and β subunits of β-conglycinin can also be recognized by IgE and are therefore likely allergens (Krishnan et al., 2009). Another recent study by Holzhauser et al. (2009) also showed all three subunits of β-conglycinin may be bound by IgE from subjects who were allergic to soybean as demonstrated by DBPCFC. In a recent Japanease study, 67% of children with severe allergic reactions to soybean (n=33) showed IgE reactivity against β-conglycinin (Ito et al., 2011). β-conglycinin has also been identified as the soybean allergen responsible for food dependent exercise induced anaphylaxis induced during consumption of tofu (Adachi et al., 2009).

Soybean profilin (Gly m 3) is a 14 kDa protein from soybean. This protein has been isolated and identified by PCR based c-DNA cloning and has been shown to bind IgE from 69% of soybean sensitive patient sera (Rihs et al., 1999). Only the full length profilin and not profilin fragments showed IgE binding indicating the importance of conformational epitopes in IgE binding to this protein. Cross-reactivity of soybean

recombinant Gly m 3 (rGly m 3) with the birch pollen profilin allergen Bet v 2, has also been shown (Mittag et al., 2004; Rihs et al., 1999). However, soybean profilin has not been demonstrated to elicit clinical food allergy. The Bet v 1 homologous PR-10 protein of soybean, Gly m 4 is another important soybean allergen. This protein was first described by Crowell et al. (1992) as a stress induced protein in soybean also known as SAM 22 (starvation associated message 22). It is a 16 kDa protein, which shows a 53% sequence identity with the major birch pollen aeroallergen, Bet v 1; 58% with the major hazelnut food allergen Cor a 1.0401; 53% with the major apple food allergen Mal d 1; and 54% with the major cherry food allergen Pru a v (Crowell et al., 1992; Kleine-Tebbe et al., 2002). The study by Kleine-Tebbe et al. (2002), using sera from patients with severe oropharyngeal and anaphylactic reactions to a soy-containing nutritional supplement drink showed high IgE levels to rSAM 22 indicating that this soybean protein was responsible for the severe adverse reaction to this soybean product. Both rSAM22 and rBet v 1 fully inhibited IgE binding to soy protein isolate in Enzyme Allergosorbent tests (EAST) and immunoblotting inhibition assays. Additionally rSAM22 induced mediator release from basophils of patients with birch pollen allergy indicating cross-reactivity between soybean SAM 22 (or Gly m 4) and the birch pollen allergen Bet v1 (Kleine-Tebbe et al., 2002). Another study by Mittag et al. (2004) found that 71% of patients allergic to birch pollen with high titers of Bet v 1–specific IgE were sensitized to Gly m 4, further confirming cross-reactivity between Gly m 4 and Bet v 1 (Mittag et al., 2004). A recent study reported that soybean-dependent pollen-food cross-reaction in children due to ingestion of soy milk was likely due to the presence of high concentrations of Gly m 4 in moderately processed soy milk (Kosma et al., 2011). The 2S

albumin from peanut, Ara h 2, is one of the major food allergens in one of the most allergenic food crops. Some investigators speculate that 2S albumins are likely to be major allergens in other legumes. However, a study using 23 soybean allergic patients sera demonstrated that the two isolated 2S albumins of soybean (AL 1 and AL 3) are minor allergens at best, based on serum IgE binding (Lin et al., 2006)

At least five IgE binding proteins have been identified in soybean lecithin (P7, P12, P57, P39 and STI), which is widely used as emulsifier in processed food (Xiang et al., 2008). Out of the five IgE binding proteins, P39 soybean allergen has been well characterized. It is a hydrophobic protein associated with the matrix of the protein bodies and possesses four conserved cysteine residues (Gu et al., 2001; Xiang et al., 2008). Several aeroallergens have also been identified in soybean that can cause sensitization by inhalation. This includes Kunitz soybean trypsin inhibitor (KSTI) from soybean flour. The KSTI is a 20 kDa protein with a pI of 4.5 and has been shown to be recognized by sera from 68% of bakers suffering from workplace-related respiratory symptoms and sensitized to soybean (Baur et al., 1996; Quirce et al., 2002). The KSTI has also been reported to be a minor soybean allergen responsible for causing allergic reaction after ingestion of soybean-containing products (Moroz and Yang, 1980; Burks et al., 1994). Several soybean hull allergens such as Gly m 1.0101 (Gly m 1A), Gly m 1.0102 (Gly m 1B), and Gly m 2 have also been identified as aeroallergens in soybean. These were reportedly responsible for very common airway allergies around the shipping port in Barcelona, Spain (Codina et al., 1997, 1999).

GENETICALLY MODIFIED SOYBEANS AND THEIR ALLERGENICITY ASSESSMENT

Recombinant DNA technology allows for the transfer of gene from one species into another, bypassing biological barriers for recombination and genetic exchange (Cockburn, 2002). Different methods are used for the introduction of DNA into plants including electroporation, particle bombardment and infection with modified recombinant vectors such as *Agrobacterium tumefaciens* (Goodman et al., 2005). The introduced gene produces an additional protein that confers the trait of interest in resistance to virus, fungi and to improve nutritional quality (Cockburn, 2002). The potential of GM crops is manifold. Transgenic technology has not only reduced environmental degradation by decreasing the use of pesticides, but also has the potential to increase crop yield thereby fight global hunger (Tester and Langridge, 2010). Plants have also been developed by introduction of genes encoding industrial and pharmaceutical proteins thereby reducing production costs compared to microbial fermentation or purification from the natural sources (Goodman et al., 2005). The first commercial GM crop introduced in US was the FLAVR SAVR tomato in 1994 that had delayed ripening characteristics (Kok and Kuiper, 2003). Several other GM crops that underwent regulatory evaluation during 1994-1996 include herbicide-tolerant soybean, insect-resistant and herbicide-tolerant cotton, insect-resistant and herbicide-tolerant maize, insect-resistant potato, virus-resistant papaya and virus-resistant squash (Goodman et al., 2005). In 2007, GM crop production covered 143 million hectors of land in 23 countries. GM soybean occupied 50% of global biotech crop area in 2007 followed by maize (31%), cotton (13%), and canola (5%) (Holst-Jensen, 2009; Magaña-Gómez and

de la Barca, 2008). United States (50%) holds the major share of GM crop area followed by Argentina (17%), Brazil (13%), India (6%), Canada (6%), and China (3%) (Qaim, 2009). The production of GM crops in European countries is much lower than those listed above. Spain is the only country that grows GM crops on a significant scale. Several GM crops that have been developed in the past few years have never been commercialized. Many are awaiting regulatory approval or have been withdrawn from the market because of consumer acceptance or marketing problems. Examples include insect resistant Bt eggplant, Bt cauliflower and Bt cabbage in India and golden rice (rice containing pro-vitamin A) in Asian countries (Qaim, 2009).

Approximately 90% of soybeans grown in the US are now GM cultivars (http://www.gmocompass.org/eng/agri_biotechnology/gmo_planting/506.usa_cultivation_gm_plants_2009.html). Most genetic modification in soybean is targeted to increase the yield such as crop varieties that resist pest and disease and tolerant to herbicides rather than improving nutritional or quality parameters (Sten et al., 2004). Herbicide tolerant soybean is the dominant GM soybean crop and accounts for 70% of global soybean production. It is currently grown in United States, Argentina, Brazil, and other South American countries (Qaim, 2009). One of the most widely grown herbicide tolerant GM soybean varieties is the Roundup Ready soybean that is resistant to glyphosate, the active ingredient in Roundup agricultural herbicides. This GM soybean is produced by introduction of the glyphosate-tolerant CP4 EPSPS coding sequence, derived from the common soil bacterium *Agrobacterium sp*. strain CP4, into the soybean genome (Gizzarelli et al., 2006).

Although GM technology provides several benefits, there is opposition to the development and use of GM crops particularly in European and some Asian countries. Several issues are being debated regarding GM crops. Potential issues include economic issues, environmental impact, ethical and social considerations and public confidence in regulatory procedures. However, the major concerns are food safety and environmental risk. The potential human health risks due to the use of GM food crops include toxicity, allergenicity, instability of the inserted gene, and negative effects on nutrition (Qaim, 2009; Magaña-Gómez and Calderon de la Barca, 2008). Therefore GM crops are subjected to rigorous safety evaluation before they could be approved by regulators for market release. The purpose of this assessment is to avoid transferring a gene encoding a major allergenic protein or toxin into a food crop to ensure the safety of the food for human consumption (Goodman et al., 2005). Since 1990, several national and international organizations [International Food Biotechnology Council (IFBC) and the International Life Sciences Institute (ILSI), the Organization for Economic Cooperation and Development (OECD), the Food and Agricultural Organization (FAO), the World Health Organization (WHO)] and several biotechnology industries have been working to put forward strategies for safety assessment of GM food crops (Martens, 2000). In the US, three principal agencies regulate GM crops; the Food and Drug Administration (FDA), the Animal and Plant Health Inspection Service (APHIS) of the US Department of Agriculture (USDA), and the Environmental Protection Agency (EPA). In Europe, an independent agency, the European Food Safety Authority (EFSA), funded by the European community is responsible for assessing the safety of GM crops and to communicate their findings within the European community (Goodman and Hefle, 2005).

The guidelines for safety assessment of GM food crops were first outlined in the 1992 FDA Federal register. Soon after, the iFBC-ILSI drafted recommendations in 1996 and the FAO/WHO followed in 2001 with some modification. These earlier recommendations were based on a stepwise decision tree approach. The current recommended guidelines for safety evaluation of GM proteins are outlined in the Codex Alimentarius Commission Guidelines, Alinorm 3/34 (2003), which recommends a weight of evidence approach rather than a specific decision tree approach for safety assessment of GM crops (Goodman et al., 2005; Goodman et al., 2008). Guidelines from the US, Japan and the European Union (through EFSA), have adopted requirements that are generally consistent with Codex (Goodman et al., 2008). In spite of all efforts made to ensure the safety of GM foods before their release into the market for human consumption, there have been isolated cases in which novel foods that were not approved for a specified use ended up in the market. For example, the case of Starlink® corn, a GM corn variety engineered to express the Cry 9 C gene from *Bacillus thuringiensis* providing resistance from the European corn borer. This GM crop was approved for use in animal feed and non-food industrial uses, but not for human consumption in the US. The US EPA did not approve the Cry 9 C protein for human consumption because of an assumed higher potential ability of the protein to cause allergic reactions due to stability of the protein in pepsin at acidic pH. However, despite this restriction, Starlink corn ended up in the processed food chain and resulted in significant food chain disruptions and added costs to remove the grain and seeds from the agricultural and food system (Lin et al., 2003).

Food allergy is an important public health issue that affects approximately 6% of children and 3-4% of adults in US (Riascos et al., 2010). Approximately 90,000 individual cases of food-induced anaphylaxis reactions require emergency room treatment per year in the US, and there may be 150–200 food allergy related deaths per year (Clark et al., 2011; Goodman et al., 2005). The assessment of potential allergenicity of GM crops is important since protecting individuals with food allergy from accidental exposure to an allergen is very important from a food safety point of view (Goodman et al., 2008). The focus should be to ensure that the allergenicity of the GM variety is not greater than that of the traditionally produced crop (Goodman et al., 2005). There are four possible ways in which a novel protein may increase the risk of allergenicity; by being an allergen itself, by cross-reacting with a known allergen, by acting as a de-novo allergen not exposed previously to the human or by increasing the endogenous allergenicity of the target allergic plant population. However, the primary risk is introduction of a known allergen or a cross-reacting allergen (Deraman and Kimber, 2009). For example, early in the development of a nutritionally enhanced GM soybean by introduction of Brazil nut 2S albumin, testing discovered that the 2S albumin is the major allergen in the Brazil nut (Nordlee et al., 1996). Continued development and commercial release of that product would have introduced a major risk for individuals allergic to Brazil nuts as soybeans are used in many processed foods. However, due to the results of the safety study by Nordlee et al., (1996), Pioneer Hi-Bred, the developer, stopped all development of this transgenic line.

The Codex Alimentarius Guidelines, Alinorm 3/34 (2003) outline the current recommended procedures for allergenicity assessment of a new protein(s) produced in the

GM event. The guidelines involve evaluation of the source of the gene for allergenicity, protein sequence comparison to known allergens, evaluation of stability of the protein to digestion, and when indicated based on the allergenicity of the source of the gene or high identity to a known allergen, serum IgE testing would be required. In addition, there would be additional concerns and probably a demand for further undefined testing if the protein was stable to digestion by pepsin or if it was abundant in the food grade materials (Goodman et al. 2008). The Codex guidelines also recommend evaluating the plant for potential changes in the overall allergenicity as measured by various antigen-specific serum IgE tests of the food materials from the GM plant; compared to the near isogenic event, or to other varieties that are genetically similar; if the GM plant species is one that is known to be a common source of food allergy (Goodman et al., 2008). The purpose is to evaluate any unintended effect the genetic modification may have on the plant by modulating directly or indirectly the level of accumulation of endogenous allergens thereby increasing the allergic potential of the GM crop compared to the non-GM counterpart (Rouquie et al., 2010). Several studies have been done to evaluate endogenous allergenicity of various GM soybean varieties. A study conducted by Burks and Fuchs (1995) compared the endogenous allergenicity of glyphosate tolerant and commercial soybean varieties by IgE immunoblotting using a pool of sera from five soybean allergic patients. No differences in IgE binding were observed between the GM soybean and the commercial or parental soybean lines (Burks and Fuchs, 1995). Another study by Gizzarelli et al. (2006) compared the same GM soybean variety to a wild type variety by IgE immunoblotting and enzyme linked immunosorbent assay (ELISA) using a pool of sera from 10 soybean allergic subjects. No quantitative or qualitative

differences were found in the IgE recognition of the GM soybean compared to the wild type soybean. The same study also used a Balb/c mouse model to identify any differences in the GM and wild type soybean. The levels of IgE and IgG1 antibodies produced by GM soybean sensitized mice were found to be comparable to those obtained from the wild type soybean sensitized mice (Gizzarelli et al., 2006). Another recent study was conducted using 2-dimensional gel electrophoresis (2D PAGE) to separate proteins based on isoelectric point and molecular mass followed by quantitative measurement of spot densities between the GM and non-GM soybean lines. Comparisons were made only of spots identified as the endogenous allergenic proteins with spot identities confirmed by MALDI-TOF to compare the endogenous allergenicity of an herbicide tolerant GM soybean with a non-GM near-isogenic counterpart and three commercial soybean lines. No biologically significant differences in the level of endogenous allergens in the GM soybean were found (Rouquie et al., 2010).

An important consideration for studies attempting to evaluate potential differences in the endogenous allergenicity of varieties of a crop is the clear fact that no limits of acceptable variance in allergen content are established. Differences in expression of proteins in plants could be due to genetic variations (e.g. gene mutations) or variation produced by epigenetic mechanisms and the latter is influenced by the environment where the plants are grown (Jaenisch and Bird, 2003; Ruebelt et al., 2006). Variation in the post translational modification of proteins has also been shown to occur among plant varieties (Campbell et al., 2011). A study by Conde Hernández et al., (2002) investigating the allergenicity of 16 cultivars of olive trees using olive pollen sensitized subjects found a difference in allergenicity among cultivars by skin prick test and specific

IgE determination, which can be attributed to factors such as eco-environment and crop management (Conde Hernandez et al., 2002). In the case of soybean, Yaklich et al. (1999) indicated that P34 is highly conserved in soybean varieties. Sera from soybean sensitized human and a monoclonal antibody against P34 were used in immunoassays that found no difference in the expression and level of P34 in several soybean cultivars representing the public cultivars released between 1947 and 1988 in North America (Yaklich et al., 1999). Consistent with this, another study reported finding no differences in P34 allergen distribution among 16 soybean genotypes (Xu et al., 2007). However, several other studies have found differences in the expression or concentration of proteins including allergenic proteins among soybean varieties. Quantitative variation in soybean glycinin and β-conglycinin, two major allergens and storage proteins of soybean, has been observed among high protein soybean lines indicating that genomic differences are responsible for the synthesis of different quantities of subunits and polypeptides of these two proteins in different soybean lines (Yaklich, 2001). In a study by Codina et al. (2003), 10 soybean varieties were investigated for differences in the content of hull allergens responsible for causing respiratory allergy from dust near loading docks using 21 subjects sensitized to soybean hull. It was found that the allergen content (Gly m 1 and Gly m 2) of some varieties were lower as determined by in vitro tests. However, this lower allergen content would not reflect clinical sensitivity since all 10 varieties showed similar wheal and flare skin test reactions in soybean sensitized subjects (Codina et al., 2003). In another recent study, the distribution of three major soybean allergens (Gly m Bd 60K, Gly m Bd 30K and Gly m Bd 28K) were compared among 16 soybean genotypes (including wild and cultivated genotypes) by separating allergens by 2D

PAGE and subsequently identifying by LC-MS/MS. Considerable heterogeneity in the distribution of the α subunit of β-conglycinin was found among these 16 soybean genotypes (Xu et al., 2007).

Based on published differences in allergen and protein content among varieties of a single crop such as soybeans, it is clear further data are necessary to establish the natural variability of allergen levels from non-GM food varieties that are currently commercially available and consumed by non-allergic individuals. That data can then be used to establish a range of acceptability for later evaluation of the allergen content and differences when considering a specific GM and non-GM comparator. Studies on the natural variation in allergen levels among crop varieties grown in different environments are essential to conclude that significant differences occur in the endogenous allergen content produced by genetic modification as well as providing information on the level of allergens currently consumed (Goodman et al., 2008; Rouquie et al., 2010)**.**

SOYBEAN PROCESSING

Good nutritional qualities and physiochemical properties have led to the application of soy proteins in a diversity of processed food products. Approximately 60% of processed foods are estimated to contain ingredients derived from soybean (Hou and Chang, 2004). Soybean proteins are used in food in various forms including relatively unprocessed soy flour, soy protein isolates (SPI), soy protein concentrates (SPC) and various texturized products (Friedman and Brandon, 2001). In the mid-1990s world production of soy flour was estimated to be 2,300,000 metric tons, SPC was 150,000 metric tons and SPI was 200,000 metric tons (Lusas and Riaz, 1995). Soy flour contains approximately 40-50%

protein and is prepared from soybeans after dehulling and milling, either as active full fat soy flour, enzyme inactive full fat flour, or defatted soy flour. Defatted soy flour is produced through hexane extraction of the oils. Soy flour is the least refined form of soy protein (Singh et al., 1995; Hammond and Jez; Lusas and Riaz, 1995). Soy protein concentrates, which contains approximately 70% protein, is prepared from defatted soybean flakes or flour after either extraction with aqueous ethyl alcohol (60-80%), extraction with water at the isolelectric point of soy protein (pH 4.5) or denaturing the protein with moist heat before extraction with water. The SPC has a reduced flavor level compared to soy flour since the processes used to prepare the concentrate remove some of flavor constituents (Singh et al., 2008). Soy protein isolate is the most refined form of soy protein containing approximately 90% protein and is prepared by precipitating protein from an alkaline extract (NaOH, pH 7-8.5) of defatted soybean flakes or flour with hydrochloric acid or phosphoric acid at pH 4.5 (Lusas and Riaz, 1995; Wolf, 1970). The 11S globulin (60%) and 7S globulin (30%) proteins together are the main components of SPI (Lusas and Riaz, 1995; Renkema et al., 2000). Texturized soy protein is produced by thermoplastic extrusion of flours, grits, and protein concentrates under heat and pressure to form chips, chunks, flakes, and a variety of other shapes used as meat substitutes or analogs. The texturization process can denature soy protein, inactivate trypsin inhibitors present in soybean as well as help in controlling bitter flavor (Singh et al., 2008; Lusas, 1996).

The functionality of soybean proteins is an important attribute that makes them a popular choice to be used in food applications. For example, the gel forming property of soy proteins is one of the most important functional properties, particularly for food such

as Tofu (Hou and Chang, 2004). Soybean proteins are used in various baked products to provide specific functional properties such as improved texture, moisture, fat retention and emulsification. When heated, proteins in SPI denature and form a matrix that can bind substantial quantities of water, so they may be used as an egg or milk replacer in baked products. Similarly the hydrophilic nature of soy protein is responsible for enhancing the viscosity and moistness in baked products. Soy flour and isolates can improve the dough strength and elasticity of yeast-leavened products (Klein et al., 1995). Defatted soy flour is the most widely used ingredient in bakery products as a partial replacement for dry milk. The flour has high fat and water absorption capacity, and when incorporated at 2-4% can improve the water holding capacity and sheeting properties of sweet dough. Up to 0.5% (flour-weight basis) of enzyme active soy flour and 3% of defatted soy flour are permitted in standardized bakery foods. Soy flour may be incorporated at up to 12% with wheat flour to improve the protein quantity and quality of bread. Enzyme active soy flour with lipoxidase activity can bleach the carotenoid pigments in dough thereby producing whiter breadcrumbs. Soy flour can also be used at a level of 5-20% in cookies to improve their nutritional values and extend the self-life without changing their sensory qualities (Riaz, 1999; Singh et al., 2008).

Soy proteins in processed meat products can act as an emulsifier. While SPI is the most effective soy protein product used for this purpose, soybean flour and SPC can provide the desired texture in coarsely chopped meats including meat patties, sausages, meatballs, chili, Salisbury steaks, pizza topping, and meat sauces. About 3.5% of soy flour and SPC and 2% SPI can be used in cooked or fresh sausage. Up to 8% of these soy protein ingredients can be used in chili con carne and about 12% in spaghetti with meat

balls or Salisbury steak. Various SPI are used in dairy products because of their fine particle size and dispensability. Soybean proteins are also used in soups, gravies and sauces, confections, imitation nut meats, coffee creamer, ice cream, low fat spreads, chocolate products and yogurts (Lusas and Riaz, 1995; Singh et al., 2008).

Food and food ingredients are subjected to a wide variety of processing treatments in order to improve sensory quality, to remove or inactivate toxins, microbes and anti-nutritional factors and to modify their properties to suit the end use (improve functionality and digestibility). Several thermal and non-thermal processing methods are applied during food manufacture. Thermal processing includes application of moist or dry heat and non-thermal processing methods include fermentation, germination, enzyme hydrolysis and ultra-filtration to name just a few of the various unit operations commonly used in food production (Thomas et al., 2007). Heat treatment of soybean is commonly used in the manufacture of products such as soy milk, tofu, texturized soy proteins and fermented soy products such as Tempeh. Soy milk is traditionally heated to 93-100^{o}C for 30 min to destroy anti-nutritional factors and to improve flavor (Kwok et al., 2002; Golbitz, 1995; Kowk and Niranjan, 1995). It has been shown that heating soy milk at a temperature higher than 90^{o}C increases the dispersion stability of soy milk protein and soy milk emulsions. The increased stability is due to the denaturation of glycinin and β-conglycinin present in soymilk and subsequent formation of soluble aggregates due to disulfide bonding (Shimoyamada et al., 2008). Heat processing may also improve the functionality of soybean proteins for use as food ingredients (Sorgentini et al. 1995). Enzymatic hydrolysis of food proteins is a biochemical procedure that is used to impart certain functionalities to food as well as in preparing hypoallergenic food (Lusas and

Riaz, 1995; L'Hocine and Boye, 2007). The reduced solubility of soybean proteins at acidic pH (close to the iso-electric point (4.5) of major seed storage proteins glycinin and β-conglycinin) limits their use as functional ingredients in moderately acidic foods such as citric beverages and salad dressings. The hydrolysis of soybean proteins with proteases can increase the protein solubility thereby providing functional properties that depends on protein solubility such as foaming and emulsifying properties (Molina Ortiz and Wagner, 2002). Several investigators have used various protease enzymes to treat soybean proteins to improve their solubility and to achieve the desirable functionality. Calderon de la Barca, et al. (2000) treated defatted soybean flour with the enzyme, chymotrypsin, to improve protein solubility, emulsifying and foaming properties. In another study, SPI was treated with enzymes such as Alcalase, α-chymotrypsin, trypsin, liquozyme and rennet to improve its solubility, emulsifying capacity and its ability to undergo thermal aggregation (Kim et al., 1990). SPI has also been treated with enzymes such as papain and bromelain to improve its solubility and foaming properties (Molina Ortiz et al., 2000). A combination of heat and enzymatic treatment has also been used to improve the functional properties of soybean proteins. Molina Ortiz and Wagner (2002) heat-treated SPI at 90^{o}C for 30 min followed by treatment with the enzyme bromelain. An improvement in solubility and foaming property was observed under this combination treatment (Molina Ortiz and Wagner, 2002). In another study, selective hydrolysis of soybean β-conglycinin and glycinin was achieved by treatment with a combination of heat and enzymes such as papain and pepsin. It was observed that selective hydrolysis altered functional properties such as the viscosity, emulsifying activity, whippability, and gel forming ability of soybean protein. A study by Tsumura et al. (2005) indicates that

SPI foaming properties are improved by selective hydrolysis of glycinin and β-conglycinin. High pressure treatment has recently been used to improve the emulsifying and gelation properties of soybean proteins (Molina et al., 2001; Wang et al., 2008). A combination of heat and high pressure has been shown to alter the textural property of gels formed by SPI, 7S and 11S globulin (Molina and Ledward, 2003).

EFFECT OF FOOD PROCESSING ON ALLERGENICITY

Various processing steps applied during food manufacture can alter the allergenicity of food. Changes could be due to inactivation or destruction of epitope structures, formation of new epitopes, or improved access of previously hidden epitopes (Paschke and Besler, 2002). However, processing methods may not affect the allergenicity of all allergens in a similar manner (Paschke and Besler, 2002). Understanding the impact of food processing and food structure on allergenic potential is crucial in managing allergen risks in the food chain. Our current knowledge of the impact of food processing on allergen structure indicates that it is hard to predict how different allergens respond to various food processing treatments. Several studies have indicated a wide variation in the allergenicity of processed food products. In a study to investigate the allergenicity of 20 peanut products, 15 processed peanut products were found to be allergenic by the RAST inhibition assay using peanut allergic patient sera indicating that the various processing treatments (shelling, blanching, dry roasting, oil roasting, toasting, grinding, defatting) used in preparation of those products have negligible effects on allergenicity (Nordlee et al., 1981). A market-based study with 8 different soybean products involving various processing treatments (soy flour, roasted soybean, SPI, soy grit, soy milk, defatted soy

flour, SPC) and IgE binding using seven soybean allergic patient sera showed highly variable results. None of the processes resulted in the abolition of IgE binding and roasted soybean showed enhanced IgE binding to a 20 kDa band with sera from two patients (Herian et al., 1990). In another study, commercially available soy flour, soy milk, two infant formula milk products (hydrolyzed and unhydrolyzed) and texturized soy protein products were tested for serum IgE binding using sera from nine soy allergic individuals. The infant formula soy milk did not show any IgE binding; however, IgE binding was observed for the other soy milk with all nine soy allergic sera. Texturized soy protein (produced by heating, mechanical pressure and acid treatment (pH 4.5) of soy protein) showed IgE binding with seven out of nine sera tested (Franck et al., 2002). Based on the findings of these studies it can be concluded that since commercially available food products undergo complex industrial processing steps involving a combination of heat, enzymatic treatment and texturization, it is difficult to interpret the effect the individual processing steps can have on the allergenicity of proteins/allergens by these kinds of studies. Certain allergens are frequently labile to common food processing technologies while others are not. For example, IgE binding to cross-reactive Bet v1 related food allergens in general are lost by processing as the epitopes of these allergens are primarily conformational and the allergens are easily denatured. The prolamine super family proteins including 2S albumins and non-specific lipid transfer proteins are quite stable to denaturation due to conserved cysteine residues, which form intra-chain disulfide bonds (Mills et al., 2009).

Various thermal processes applied during food manufacture may lead to alteration of protein conformation and reduction of IgE binding to conformational epitopes.

Additionally some processes can lead to the creation of new epitopes. For example, the formation of glycation adducts by the Maillard reaction (covalent cross-linking between lysine residues of protein with reducing sugars present in food) can lead to the formation of new immunologically reactive structures (Paschke, 2009; Paschke and Besler, 2002). The heat induced denaturation and alteration of conformational epitopes can explain the loss of 90% of the immuno-reactivity of the heat labile birch-pollen-related allergens of hazelnuts such as Cor a1.04 and Cor a 2 (Hansen et al., 2003), whereas the absence of conformational epitopes can explain IgE binding to the lipid transfer protein of maize after thermal treatment at 100^{o}C for 160 min (Pastorello et al., 2003). In the case of peanut allergy, it has been observed that the cooking method can influence the allergenicity of peanut proteins. A study conducted by Beyer et al., (2001) indicated that frying (5 min for Valencia peanuts and 10 min for Florunner) and boiling (100^{o}C for 20 min) peanut resulted in the reduction in IgE reactivity to the major peanut allergens Ara h 1, Ara h 2 and Ara h 3, whereas roasting (170^{o}C, 20 min) resulted in increased reactivity to the same allergens. This could explain the apparent lower prevalence of peanut allergy in China where fried or boiled peanuts are primarily consumed compared to the US where peanut is usually consumed after roasting (Beyer et al., 2001). A study by Maleki et al. (2000) showed that proteins from roasted peanuts bound IgE from peanut allergic subjects at an approximately 90-fold higher level compared to raw peanut, which they attributed to protein modification by the Maillard reaction. In another study, boiling peanuts in water (100^{o}C, 30 min) reduced the median IgE binding by 1.5- to 2-fold compared to raw or roasted peanuts. This decrease was attributed to the loss of soluble proteins in the water used for boiling the peanuts (Mondoulet et al., 2005). For other

legume allergens such as lentil, chickpea and lupine, the reduction of IgE binding to proteins has been observed by prolonged autoclaving, whereas little modification in IgE binding has been observed by boiling (Alvarez-Alvarez et al., 2005; Cuadrado et al., 2009). A few studies have investigated the effect of heat processing on the allergenicity of soy flour or on individual soybean proteins. In a study by Shibasaki et al. (1980) soybean glycinin, β-conglycinin and 2S globulin were heat treated at 80^{o}C, 100^{o}C and autoclaved at 120^{o}C for 30 min prior to testing IgE binding by RAST and RAST inhibition using five soy allergic sera. A reduction in IgE binding to glycinin and β-conglycinin and a slight increase in IgE binding to 2S globulin was observed under these heat treatment conditions (Shibasaki et al., 1980). In another study where soy flour extract, purified 11S globulin and 7S globulin fractions of soybean were heat treated at 37^{o}C for 1 hour, 56^{o}C for 1 hour and 100^{o}C for 5 min, 20 min and 60 min, no differences in IgE or IgG binding compared to control samples were observed using a pool of soybean allergic patient sera (Burks et al., 1992). In another study by Muller et al. (1998), three of six patients used in the study showed specific IgE against cooked soybean extract (100^{o}C for 2 hours) by the enzyme allergosorbent test (EAST) while the other three sera failed to bind. The protein extract of raw soybean had a reduced inhibitory capacity of approximately 40% for inhibition of IgE binding to the heated soybean proteins by EAST inhibition assay using a pool of soybean allergic sera (Muller et al., 1998). IgE from the same serum pool recognized six protein bands from heat-treated soybean with strongest binding to a 39 kDa band (Muller et al., 1998).

Enzymatic hydrolysis can lead to the alteration of allergenicity in two different ways. The enzyme used for hydrolysis could itself be a potent allergen. Further enzymatic hydrolysis of food proteins can lead to loss of epitope structure thereby reducing allergenicity (Paschke and Besler, 2002). The prerequisites for removal of allergenicity are sufficient contact between the allergenic epitope and the enzyme and sufficient control of undesirable side effects that may affect sensory quality or functionality (De Angelis et al., 2010). However, the enzymatic hydrolysis of protein does not always lead to a reduction in allergenicity. The initial breakdown of proteins can expose new antigenic epitopes, which can result in increased allergenicity. With the progress of hydrolysis however, proteins are broken down to a greater degree resulting in lessening of their allergenic properties (Nagodawithana, et al., 2010). Porcine trypsin and chymotrypsin are frequently used to prepare hypoallergenic formulas, as well as other enzymes of bacterial and fungal origin. These enzymes cleave proteins at different sites due to favored binding in the catalytic sites and depending on the exposure of those sites. Thus digestion often leads to residues of peptides of different lengths with more or less residual IgE binding capacity (Fritsche, 2003). Sequential hydrolysis with the enzymes Alcalase and flavourzyme were shown to reduce IgE binding to lentil and chickpea protein hydrolysates (Cabanillas et al., 2010; Clemente et al., 1999). Alcalase, pepsin and trypsin have also been used to hydrolyze and reduce the immunoreactivity of pea protein extract (Szymkiewicz and Jedrychowski, 2005). Protease, elastase and trypsin have been shown to eliminate IgE binding to hazelnut proteins (Wigotzki et al., 2000). A few studies have focused on the use of enzymatic hydrolysis on reduction of the allergenicity of soybean proteins. Tsumura et al. (1999) hydrolyzed SPI using a commercially

available *Bacillus sp*. protease. Degradation of the soybean allergens Gly m Bd 30K and Gly m Bd 28K was observed using an alkaline protease, porleather FG-F (Tsumura et al., 1999). A reduction in IgE binding with serum from soybean sensitive subjects was also observed using the same protease (Tsumura et al., 1999). In another study, soybean 11S globulins were hydrolyzed by sequential treatment with the enzymes, pepsin and chymotrypsin. Although a reduction of IgE binding was observed after this treatment, it was not completely eliminated (Lee et al., 2007). In a study by Van Boxtel et al. (2008), the effects of heating and pepsin digestion on the IgE binding to Ara h 3 of peanut and glycinins of soybean were investigated. Both proteins were found to be relatively stable to denaturation, having denaturation temperatures ranging from 70 to 92^{o}C. However, the IgE binding capacity of both allergens was shown to be markedly degraded within 10 min of peptic digestion as no IgE binding was observed with any samples (van Boxtel et al., 2008).

Evaluation of the allergenicity of food proteins, rather than simply IgE binding to soluble proteins, must be done following processing to understand food safety. The conditions used to extract proteins from both raw and processed foods is an important determinant for appropriate interpretation of effects of the processing on the allergenicity of a given food material. The inefficient extraction of proteins from processed food products can happen due to matrix effects including impaired solubility of denatured proteins from other constituents, cross-linking through glycation and binding of hydrophobic proteins to the food matrix. Some of the studies reporting reduced allergenicity of various foods do not seem to control for possible poor extraction of allergenic proteins and most use water-soluble extraction methods followed by antibody

detection. A study conducted by Poms et al. (2004) utilized different temperature and time combinations for dry and oil roasting of peanuts. They found that elevated roasting temperatures resulted in a greater influence on the solubility of peanut proteins with a reduction in protein yield of 50-75% from dry roasted peanuts and 75-80% from oil roasted peanuts. Their study demonstrated that the extraction efficiency of the allergenic proteins varied by using a variety of extraction buffers (Poms et al., 2004). Quite often it is necessary to optimize sample extraction in order to ensure that an analytical result represents the true impact of a process on allergenic activity. Normally the allergic subject would consume a whole food material and not an aqueous extract, so the tests to measure the impact of processing on allergens must reflect likely true exposure to the proteins that cause allergy (Poms et al., 2004).

Physio-chemical methods such as SDS-PAGE and peptide profiling can be used to assess the degree of hydrolysis after heat and enzymatic processing of allergenic proteins. However, immunological methods are more suitable to determine the allergenicity of the resulting peptides or proteins obtained (Fritsche, 2003). In vitro IgE binding tests such as radioallergosorbent test (RAST), RAST inhibition test, immunoelectrophoresis methods and ELISA are commonly used to evaluate any reduction in allergenicity obtained after heat or enzymatic hydrolysis (Host and Halken, 2004). In vitro tests are often quick, inexpensive and without a threat to human or animal subjects. However, certain limitations of in vitro immunological methods include alteration of three dimensional structures, destruction of epitopes by the adsorption of allergens to solid matrix, susceptibility of the results to interference by the presence of IgG of similar specificity as IgE and a lack of correlation between positive IgE binding

results with expression of clinical symptoms due to several factors such as presence of cross-reactive carbohydrate determinants, low affinity binding, one IgE binding epitope and poor spatial orientation of epitopes (Kaul et al., 2007; Ladics et al., 2008). Functional assays such as basophil histamine release, skin prick tests, patch tests and challenge tests would evaluate the biological consequence of any IgE binding detected by in vitro immunological methods and thereby help in providing a true picture of any reduction in allergenicity achieved by various processing treatments (Host and Halken, 2004; L' Hocine and Boye, 2007). Several in vitro functional assays can be used to measure the biological activity of allergens. They are based on in vitro activation of basophils sensitized with IgE and measurement of release of histamine and/or sulphidoleukotriene or expression of basophil surface activation markers CD63 or CD 203c when exposed to the allergen or allergen-containing material. Specific mediators can be measured by ELISA based methods or by flow cytometry to investigate the up regulation of activation markers on the basophil surface (Crockard and Ennis, 2001; Ebo, 2009; Poulsen, 2001). The CD63 protein is a member of the transmembrane-4 super family that is expressed on basophils, mast cells, macrophages, and platelets. In resting basophils, it is attached to the intracytoplamic granules. Activation of basophils with IgE and allergen leads to expression of the CD63 on the surface of basophils. The CD203c protein is expressed only on IgE-bearing basophils, mast cells, and their progenitors. Similar to CD63, they are up-regulated on the surface of mast cells and basophils by activation with allergen or anti-IgE (Hamilton and Franklin Adkinson, 2004). Mediator release assays with basophils can be performed either by incubating heparinized whole blood from an allergic individual with allergen or after stripping endogenous IgE from basophils of non-allergic

donors prior to incubating with serum IgE from appropriately allergic donors, followed by stimulating with allergen (Hamilton and Franklin Adkinson, 2004; Kleine Budde et al., 2001). The primary advantage of using the stripped basophil assays rather than fresh basophils isolated from specifically allergic donors is that serum IgE from donors around the world or serum collected 10 years before may be used with IgE-stripped basophils, whereas basophils from allergic donors must be used within 24 hrs of drawing blood samples (Kleine Budde et al., 2001; Poulsen, 2001). The disadvantage is that additional controls are needed to demonstrate that the stripped basophils are not activated by non-allergen related signaling. Another option for evaluating the biological activity of allergens is by measuring the release of β-hexosaminidase (present within granules in basophils and released along with histamine during an allergic reaction) from humanized RBL (rat basophilic leukemia) cells that have been sensitized by addition of appropriately allergic sera. Three versions of hRBL cells have been generated by transforming a rat basophil line with human genes expressing α, β and γ chain (RBL SX-38) or only the α-chain (RBL-30/25 and hEI_a-2B12) of human Fc epsilon RI receptors (Dibbern et al., 2003; Vogel et al., 2005; Ladics et al., 2008). These cells have been shown to be useful in exploring IgE- allergen interactions using sera from peanut specific subjects and peanut extract or peanut allergens Ara h 2 and Ara h 1 (Dibbern et al., 2003). Some of the limitations of using a humanized RBL assay are lack of consistency, reduced IgE binding capacity over time and a tendency of being effective only with sera containing high concentrations of allergen specific IgE (Ladics et al., 2008; Palmer et al., 2005).

The best way to determine the biological activity of an allergen is by challenging food allergic patients with their specific food allergen in a double-blind, placebo-

controlled food challenge (DBPCFC). DBPCFC is considered the gold standard for diagnosis of allergy. However, the possibility of a severe anaphylactic reaction occurring during a food challenge limits the use of this method as a routine way for allergenicity assessment. Other limitations of using this method are the involvement of ethical issues in using human subjects, the challenges are time consuming, labor intensive, costly and a uniform challenge protocol (minimum dose, maximum dose, assessment of symptoms and challenge vehicle) is still lacking. In addition, the low prevalence of allergy to any one allergen makes a coordinated and timely test of reactivity for any but the most common allergenic foods impossible to perform. Also these tests need to be conducted in well-equipped hospitals or other healthcare settings to take appropriate measures in case severe anaphylactic reactions occur (Asero et al., 2007; Bindslev-Jensen, 2001; Sicherer and Sampson, 2006; Taylor et al., 2004).

The use of animal models, such as a rodent model, may be an alternative way of evaluating the potential allergenicity of processed food products or novel food proteins. Some advantages of using a rodent model are that they have a well characterized immune system, tests would not risk the health or life of human subjects, both exposure and challenges may be carefully controlled. The question is whether the rodent model would accurately predict the response of the allergic human subject. Rodents can be sensitized by oral, intra-peritoneal, dermal or sub-cutaneous routes, with or without the use of adjuvants (Ladics et al., 2008). However, tests of rodent models for allergenic risk assessment have not been demonstrated to accurately predict the sensitizing potential or active allergenicity of dietary proteins when purified proteins are used (Ladics et al., 2008). Tests with whole, highly allergenic foods have demonstrated similar responses to

those of allergic humans in the type and severity of the immunological responses, but the responses seem to be variable and strain-dependent. There are additional limitations for some allergenic foods because of the bulkiness and nutritional content of the whole food limits the amount of protein of interest that is administered during the challenge when they are fed to rodents (Constable et al., 2007; Ladics et al., 2008).

The major portion of this dissertation focuses on the evaluation of potential changes in the endogenous allergenicity of three newly developed transgenic soybean lines using serum samples from soybean sensitized patients by in vitro IgE immunoblot and inhibition ELISA to evaluate potential risks to soybean allergic individuals from the GM products. Further the effect of heat processing and enzymatic hydrolysis on the allergenicity of endogenous soybean proteins was evaluated by in vitro IgE binding tests as well as a mediator release assay using hRBL cell lines with soybean sensitive patient sera to find out whether any of these processes have the potential to increase or decrease the allergenicity of soybean proteins.

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CHAPTER 2: THE EFFECT OF GENETIC MODIFICATION ON SOYBEAN ALLERGENICITY

INTRODUCTION

Soybean is one of the eight allergenic foods or groups of foods (peanuts, soybeans, tree nuts, milk, egg, fish, crustaceans, and wheat) that are thought to cause nearly 90% of food-allergic reactions in the US (Goodman et al., 2005). Soybean allergy affects approximately 0.4% of children worldwide (Savage et al., 2010). The prevalence and incidence of soy allergy is likely to be dependent on local dietary habits and exposure (Sicherer et al., 2000). At least 16 IgE binding proteins of molecular weights ranging from 14 kDa to 70 kDa has been identified in soybean and the major seed storage proteins are likely to be the major allergens (Ballmer-Weber et al., 2007; Holzhauser et al., 2009; Ogawa et al., 1991; Ogawa et al., 1993).

Approximately 90% of soybeans grown in the USA are now genetically modified (GM)(http://www.gmocompass.org/eng/agri_biotechnology/gmo_planting/506.usa_cultivation_gm_plants_2009.html). Most of the genetic modifications in the current collection of transgenic soybeans are targeted to herbicide resistance or insect protection and to improve agricultural efficiencies (Sten et al., 2004). All newly developed transgenic crop lines need to undergo a rigorous safety evaluation before regulatory approval. The safety evaluation of transgenic food crops described in the Codex Alimentarius Guidelines (Codex, 2003) includes assessment of the potential allergenicity to reduce potential risks of transferring a gene that codes for an allergenic protein. The risk of allergic reactions to a specific food produced from a transgenic organism that included an allergen from

another source (e.g. peanut or wheat) into a different food crop (e.g. rice) would be increased; but more importantly those with allergies to the transferred protein would be at great risk because they would not avoid foods that normally do not cause their reactions (Goodman et al., 2005; Goodman et al., 2008). Not only is the safety of the protein expressed by the introduction of the new gene inserted into transgenic crop is evaluated, but potential unintended changes in the transgenic crop due to gene insertion are also evaluated. Those include possible disruption of endogenous genes as well as altered expression of endogenous genes. Further, if the inserted gene encodes an enzyme, the possibility that it will increase or decrease key metabolites or produce a novel metabolite with potential health consequences is evaluated. Possible changes in the endogenous levels of allergens expressed in the food fractions of the transgenic plant are measured if that species is a known source of food allergy, e.g. soybean (Barros et al.; Cellini et al., 2004). Therefore the Codex Alimentarius Guidelines, Alinorm 3/34 (2003), and many countries including the U.S., Japan, the European Union (through EFSA) that have adopted requirements that are generally consistent with the Codex recommend evaluating a transgenic plant for potential changes in the overall allergenicity by comparing them to a near-isoline or other genetically similar lines (Goodman et al., 2008). Although explicit procedures for such an evaluation are not provided, the comparison is generally performed by measuring and comparing antigen-specific serum IgE binding to proteins in the food material from the transgenic plant; compared to a near-isoline and/or to other lines that are genetically similar by immunoblot and ELISA procedure using sera from donors allergic to soybeans (Goodman and Leach, 2004; Goodman et al., 2008; Hoff et al., 2007). Proteomic techniques such as two dimensional poly-acrylamide gel

electrophoresis (2D-PAGE) have also been used to compare the protein/allergen expression profile of transgenic and non-transgenic soybean seeds (Brandao et al., 2010; Rouquie et al., 2010). However, in vitro IgE binding studies with allergic sera could give a more complete answer to the question of whether there are differences in the expression of allergenic proteins as a combination of tests with native, denatured and reduced extracts, and tested with sera from a number of specifically allergic subjects. It is highly likely that different allergic subjects can exhibit markedly different IgE recognition patterns and some allergic subjects may exhibit IgE binding to a minor protein(s) that may be differentially expressed in some lines. In addition, their IgE may recognize some isoforms of allergens and not others due to one or a few amino acid differences between isoforms. Yet it is also important to remember that not all proteins identified by IgE binding are capable of eliciting an allergic response. Some may express only one epitope, others may have very low affinity binding and still others have specific carbohydrate determinants (CCD) that are not likely to cause an allergic reaction (Goodman et al., 2008; Hoff et al., 2007). If the protein is expressed in a crop that is always cooked, it is also important to understand whether the epitopes are conformational structures that might be destroyed by heating.

In studies described in this chapter, potential changes in the endogenous allergenicity of three transgenic soybean lines were evaluated by comparing them to their near-isolines and other commercial soybean lines by using sera from soybean sensitive patients. One study (study 1) was sponsored by BASF (Research Triangle Park, NC, USA) and two studies (study 2 and 3) were sponsored by Bayer CropScience AG

(Monheim am Rhein, Germany). Rather than focusing on a few proteins as probable major allergens, these studies were designed to test for any antigen-specific IgE binding using direct binding from individual soybean allergic subjects using 1D-immunoblots for qualitative assessment of possible differences between soybean extracts. Gels were run under both reducing and non-reducing conditions to differentiate IgE recognition of conformational and linear epitopes. Further inhibition ELISA was conducted for evaluating any quantitative differences in IgE binding potential between the soybean lines, which allows for the evaluation of aggregate IgE binding differences. In addition, blots with proteins separated by 2D-PAGE were carried out, which allows for separation of proteins both according to their molecular weight and isoelectic point (pI) and therefore increases the possibility of finding differences in IgE binding patterns (Lilley et al., 2002). Minor changes in post-translational processing including differential C-terminal proteolysis, modified carbohydrate structures, differential phosphorylation will result in slight differences in migration.

It is also important to consider that consumption of soybean food products does not pose a risk of allergy except to those who are already sensitized (Goodman et al., 2008). Non-allergic consumers can consume soybeans ad libitum, without risk. Further, those with allergy to soybeans are at risk if they consume soybeans and they therefore should avoid eating any soybean to avoid the risk of allergic reactions. The results of these studies provide some data regarding the natural variation of soybean allergen content. If there are even modest differences in the allergen content of various soybean

varieties, there is no practical risk consequence as those with soybean allergy should be avoiding consumption of all soybean foods.

MATERIALS AND METHODS

Human sera

Soybean allergic and control sera from historical clinical samples collected for research purposes were used in the studies described in this chapter. Additional samples procured from PlasmaLab International, a U.S. FDA approved facility, were also used in these studies. The University of Nebraska Institutional Review Board has approved the use of these samples in these studies (reviewed and approved for the Goodman laboratory). The allergic patients utilized in Study 1 had soybean specific IgE levels ranging from 0.8 to 47 kU/L (Appendix A), those utilized in Study 2 had a soybean specific IgE level ranging from 3-71.6 kU/L (Appendix C) and those used in Study 3 had a soybean specific IgE level ranging from 1.12-71.6 kU/L (Appendix E) as measured by either the ImmunoCAP® (Phadia, Uppsala, Sweden, recently purchased by Thermo Scientific) or the IMMULITE® system (Siemens AG, Erlangen, Germany). Most of the soybean allergic subjects also had history of allergic reactions to peanut and significant peanut specific IgE levels ranging from 1.94 to 100 kU/L as assayed by ImmunoCAP® or IMMULITE®. Three of the six control subjects utilized in these studies had reported allergies to other legumes such as lupine and pea, one had no allergic symptoms and two subjects had asthma. However, none of the control subjects reported soybean specific symptoms (Appendices B, D and F).

Test proteins and extracts

Nearly identical methods were used for all the three serum studies. For brevity, the assay descriptions that are identical are described together, although the studies were performed at different times. Transgenic lines were the only GM line used in this study. All other soybean lines (near-isolines and commercial lines) were non-transgenic. The transgenic line used in Study 1 was BPS-CV127-9 /3411-T (referred to as transgenic line I in this chapter). It is an herbicide-tolerant soybean tolerant to the imidazolinone class of agricultural herbicides. The near-isoline used in Study 1 was 3410-I and the three commercial soybean lines used in this study were 3415-M/MON8001, 3416-C/CD217 and Conquista (referred to as commercial lines 1, 2 and 3 respectively in this chapter). For Study 2, the transgenic line A5547-127 soybean, also known as LibertyLink® soybean (referred to as transgenic line II in this chapter) was used, which is tolerant to the glufosinate ammonium class of agricultural herbicide and in Study 3 transgenic line FG72 (referred to as transgenic line III in this chapter), another herbicide tolerant transgenic line was used. The near-isoline used was A5547 for Study 2 and Jack for study 3. The three commercial lines utilized in Studies 2 and 3 were Stine 2686-6, Stine 2788 and Stine 3000-0 (referred to as commercial lines 4, 5 and 6 respectively in this chapter).

For one dimensional poly-acrylamide gel electrophoresis (1D-PAGE) and immunoblots, full fat flour of all three transgenic soybean lines (transgenic line I, II and III), their near-isolines (near isogenic lines) and similar use commercial soybean lines were extracted at room temperature for 2 hours at a 1:10 w/v ratio with 1X PBS (11.9mM phosphate, 137mM sodium chloride, 2.7mM potassium chloride, pH 7.4) containing

protease inhibitor (ThermoScientific, Rockford, IL, USA, Cat # 78415). The extracts were clarified by centrifugation using an Eppendorf centrifuge (model 5810R) at 18514 g for 30 min, and then filtered by gravity through Whatman 2V filter papers. The soluble protein content of the extracts were determined by the Lowry method using a DC protein assay kit (BioRad, Hercules, CA, USA, Cat # 500-0113, reagent A, Cat # 500-0114, reagent B). Bovine serum albumin (BioRad, Hercules, CA, USA, Cat # L9704881) was used as a standard, with the absorbance read at 750 nm. Three control samples (navy bean, peanut and corn) utilized in the immunoblot along with the soybean samples were also extracted and tested for protein content as indicated above. For 2D-PAGE immunoblots, soybean samples were extracted by a trichloroacetic acid (TCA)/acetone precipitation method modified from Natarajan et al. (2005). A powdered sample of 0.1 g of full-fat soybean powder was mixed with 1 mL of cold acetone (Fisher Scientific Rockford, IL, USA, Cat # FL-08-0704, stored at -20^{o}C for at least 1 hour and used cold) containing 10% TCA solution (Sigma Aldrich, St. Louis, MO, USA, Cat # T0699-100ML) and 2% 2-mercaptoethanol (BioRad, Hercules, CA, USA, Cat #161-0710), and stored at -20^{o}C overnight. The mixture was centrifuged at 10,000 x g for 30 min at 4^{o}C. The supernatant was discarded and the pellet was washed with cold acetone by vortexing and then centrifuged at 10,000 g for 10 min at 4^{o}C, which was repeated twice. The protein pellet was dried in a chemical fume hood for 30 min to fully eliminate the acetone. The dried pellet was then dissolved in 1.5 ml of 8 M urea (Invitrogen, Carlsbad, CA, USA, Cat #15505-035) and 2% CHAPS (Invitrogen, Carlsbad, CA, USA, Cat # ZC10003) by vortexing a few times and then mixing on a rotary shaker at room temperature for 1 hour. The solution was then centrifuged at 10,000 x g for 30 min to remove any undissolved

particles. The soluble protein content of the samples was determined by the Bradford assay (BioRad, Hercules, CA, USA, Cat # 500-0205) using bovine serum albumin as a standard, with the absorbance read at 595 nm.

SDS-PAGE and Immunoblotting

For 1D-PAGE, the soybean and control extracts were diluted in Laemmli SDS-sample buffer (Boston BioProducts, Ashland, MA, Cat # BP-111NR) to a protein content of 10 µg per well except the peanut extract was loaded at 2 µg per well. Samples were separated under both reducing (2-mercaptoethanol and heating at ~ 95°C for 5 minutes) and non-reducing conditions using Novex 10-20% tris-glycine gels (Invitrogen, Carlsbad, CA, USA, Cat # EC61355). A 4µl sample of pre-stained Precision Plus molecular weight marker proteins (BioRad, Hercules, CA, USA, Cat # 161-0374) was run in one lane on each gel. Electrophoresis was performed with a constant 125 Vdc for 105 min. Following separation, proteins in the gels were fixed with 7% acetic acid, 40% methanol for one hour, then stained for a minimum of 6 hours in Colloidal Brilliant blue G250 (Sigma, St Louis, MO, USA, Cat # B2025). Background staining was reduced by submerging gels for one minute in 10% acetic acid, 25% methanol and followed by multiple changes of 25% aqueous methanol. Images were captured using white light in a Kodak Gel Logic 440 Image Station.

For 2D-PAGE, a Bio Rad PROTEAN IEF Cell (BioRad Hercules, CA, USA, Cat # 165-4001) was used for the first dimensional separation of the proteins based to their isoelectric points. Samples of two of the three transgenic soybeans, near-isoline controls and two sets of commercial lines were prepared as describe above. The samples

representing 25 µg of TCA/Acetone precipitated protein were diluted to a final volume of 125 µl with IEF sample buffer [8M urea, 2% CHAPS, 50 mM DTT (Fisher Bioreagents, Pittsburg, PA, USA, Cat # BP172-5) and 0.5% ampholyte (BioRad, Hercules, CA, USA, Cat # 163-1112)] and then applied to individual troughs of the IEF focusing tray (BioRad, Hercules, CA, USA, Cat # 165-4030). Individual 3-10 non-linear IEF strips (BioRad, Hercules, CA, USA, Cat # 163-2002) were placed into the trough of each sample well gel side down and the strips were then covered with 1 ml of mineral oil (BioRad, Hercules, CA, USA, Cat # 163-2129). Active rehydration was performed at 50 Vdc for 12 hours to equilibrate the strips and initiate protein migration followed by 250 Vdc run for 15 min, 4000 vdc ramping for 2 hours and finally a 4000 Vdc limit step was used until 30,000 integrated volt-hours was reached. The protein focusing pattern was retained by holding at 500 Vdc. Following IEF, the strips were equilibrated for 15 min with 1 ml of dithiothreitol (DTT) equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 130 mM DTT) and then 15 min in 1 ml of iodoacetamide equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 135 mM iodoacetamide) for reduction and acetylation. Separation in the second dimension was accomplished by placing each strip in the 7 cm well of NuPAGE® Novex 4-12% Bis-Tris ZOOM® Gels (Invitrogen, Carlsbad, CA, USA, Cat # NP0330BOX), sealing the well with 0.5% agarose (Invitrogen, Carlsbad, CA, USA, Cat # 15510-019) as well as loading 4 µl of pre-stained Precision Plus molecular weight marker proteins onto the small well and then separating proteins with constant 150 Vdc for 60 min. Representative gels were stained with Coomassie Blue as described earlier.

For immunoblotting, the separated proteins from the second-dimension gels were electro-transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA, Cat. # LC3675) at 25 Vdc for 90 min. Protein transfer was confirmed by staining with Ponceau S. The membranes were then rinsed in deionized water followed by blocking with 5% non-fat dry milk (NFDM) in PBS containing 0.05% Tween 20 (PBST) in sealed plastic bags for at least one hour. Individual human sera were diluted appropriately (1:10 or 1:20 v:v), in 2.5% NFDM in PBST and allowed to block for 1 hour in a polypropylene tube at room temperature before adding to the blocked membrane in the plastic bag for overnight incubation at room temperature. Unbound antibody was removed from the membranes by washing four times for 5 min each with fresh PBST. Bound IgE was detected using mouse monoclonal horse radish peroxidase (HRP) conjugated anti-human IgE (SouthernBiotech, Birmingham, AL: clone B3102E8 Cat # 9160-05), diluted 1:1000 with 2.5% NFDM in PBST. The unbound secondary antibodies were removed by washing the membranes four times with fresh PBST. Detection was achieved using Supersignal West Dura Extended Duration chemiluminescent substrate (Pierce, Rockford, IL, USA, Cat # 34076) and emitted light was captured using the Kodak Gel Logic 440 image station with multiple exposures. A nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA, Cat # 645239) spotted with diluted purified IgE, then blocked with 5% NFDM in PBST and incubated with the secondary antibody and substrate similarly to the immunoblots was exposed along with the immunoblots to help evaluate signal strength across samples.

IgE ELISA

IgE ELISA was performed in Study 1 and 3. For each study, an equal protein pool of the transgenic, near-isoline and three commercial lines were used to coat ELISA plates as a solid phase antigen. For Study 1, equal volumes of six sera (714, 716, 719, 721, RG LEG 118 and 297) and one-half volumes of two sera (715 and RE LEG 103) were pooled and a pool of six non-soybean allergic human samples (Appendix B) was used as the negative control. For Study 3, samples of sera CC 10, 9735-RE and 20197-BH were added in equal volumes and serum of subject 19392-CS was added at one-quarter volume. A pool of 4 non-soybean allergic human samples was used as the negative control. Sera of subjects chosen for the pool were previously characterized and selected for diverse qualitative protein IgE binding patterns by 1D-immunoblotting and were adjusted in volume based on direct IgE binding to soybean in the direct ELISA to provide a relative balanced pool where no individual dominated soybean-specific IgE binding. For the inhibition ELISA, a standard inhibition curve was generated using the same soybean pool as the inhibitor that was used to coat the plates, but with six different concentrations of protein used in replicate sample wells with a fixed volume of pooled soy allergic sera. Similar inhibition curves were produced for each individual soybean line extract using equivalent concentrations of protein from individual soybean samples to generate line-specific inhibition curves. For inhibition, the wells of a microtiter plate were coated with 100 µl of the soybean pool diluted with pH 10 carbonate-bicarbonate buffer (10 µg protein/ml) and incubated overnight at 4^{o}C. The plate was washed four times with 300 µl of PBST and then blocked with 100 µl of 1% BSA in PBST for 1 hour at 37^{o}C. For direct binding ELISA, individual soybean allergic sera and control sera were diluted 1:20 (v:v)

with 1% BSA in PBS and allowed to incubate in tubes with the blocking buffer at room temperature for 1 hour to absorb any BSA-specific IgE. After one hour, 100 µl of the diluted sera was added to triplicate wells and incubated at 37^{o}C for 2 hours prior to washing and detection. For inhibition ELISA, serially diluted soybean extracts were added to and mixed with diluted serum pool (1:5 v:v) in micro-centrifuge tubes to achieve final concentrations of inhibitor at: 0, 0.04, 0.2, 1, 5, 25 or 125 µg soybean protein per 100 µl solution (per well). These were held at room temperature for two hours to allow IgE binding to soluble soybean inhibitor proteins before adding the mixture to pre-determined wells of the ELISA plate. Duplicate dilution series were prepared for each soybean sample (standard pool and each soybean line). After rinsing the blocked wells once with PBST, 100 µl of pooled soy allergic sera and sera-inhibitor mixture samples from duplicate dilutions were added to the wells. Control wells that did not receive diluted soybean serum included wells without serum and positive (e.g. peanut coated wells with peanut serum) and negative control serum samples (soybean coated wells, received sera from those without soybean allergy) added to three replicate wells, respectively. The plates were then incubated at 37^{o}C for 2 hours to allow IgE binding. For both the direct and inhibition ELISA, the wells after sera incubation were washed four times with 300 µl of PBST and incubated with 100 µl of 1:5000 dilution of HRP conjugated mouse monoclonal antihuman IgE in blocking solution for 1 hour at 37^{o}C. After another wash step, 100 µl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma-Aldrich, St. Louis, MO, USA, Cat # T0440) was added to each well and incubated for 20 min in the dark. The reactions were stopped after 20 min by adding 100 µl of 1 N sulfuric acid to the wells. The absorbance values were measured at 450 nm using a BioTek PowerWave XS2 microplate reader

(Winooski, VT, USA). The inhibition ELISA experiments were repeated two more times on different days for a total of 3 replicates per study.

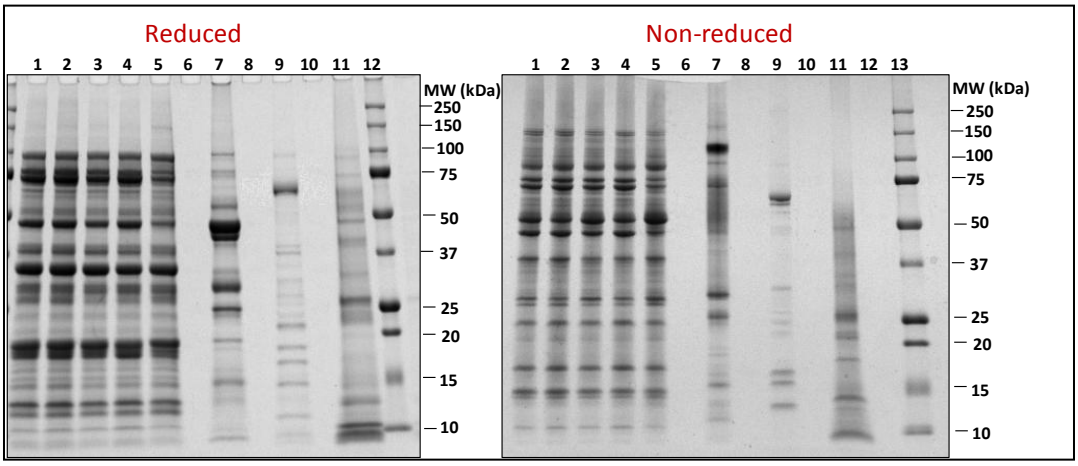
Statistical analysis

For each study the data of dose of inhibitor and absorbance reading of each well were compiled and calculations completed using Microsoft Office Excel 2007 (Microsoft, Seattle, WA, USA). Individual inhibition lines were constructed for each standard and sample set. The soybean concentration inhibitor dose that resulted in 50% inhibition of maximum binding values (EC50) were calculated for comparison of relative IgE binding values by using a logistic response model to fit the inhibition values for each replicate of each soybean sample (standard pool and five lines) using regression analysis. In determining the relative concentration of IgE binding proteins in the extracts, the mean EC50 values and standard deviations were calculated from the three replicate values for each soybean sample (standard pool and each line). In order to compare samples from individual soybean lines with respect to EC50 values, an unbalanced one-way ANOVA was carried out on the means for individual lines (omitting the standard pool). Three types of lines (transgenic, near-isoline and commercial) were compared in the ANOVA, and the Error Mean Square was calculated as the variation among the commercial lines. This Error variance can be interpreted as an estimate of variation in a reference population of conventional lines. The transgenic lines were compared to the near-isolines and also to the mean of the commercial lines, using this measure of variability as error. The GLM Procedure of SAS (version 9.1) was used for the analysis, and a 95% level ($p<0.05$) was chosen for significance.

RESULTS

Samples

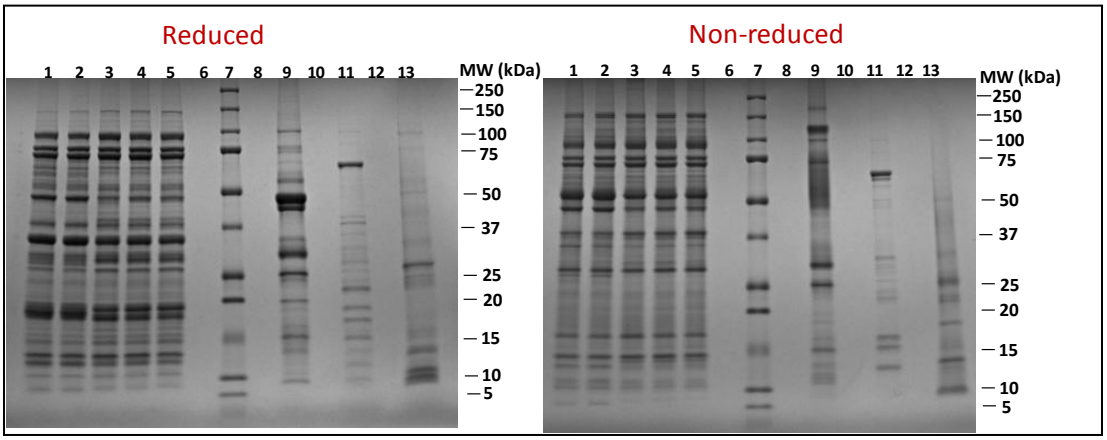
All powdered soybean flour samples were extracted as described in the Materials and Methods. The average protein content of the sample extracts ranged from 7.4 mg/ml to 17.8 mg/ml for PBS extracted samples and from 8.1 mg/ml to 11.6 mg/ml for TCA/acetone precipitated samples. With 1D-PAGE, no obvious qualitative differences in protein expression among the transgenic, near-isoline and the commercial lines were found in Coomassie stained gels for proteins separated under either reducing or non-reducing conditions for any of the three transgenic soybean lines (Figures 1A, 1B and 1C). However, some minor intensity differences were observed in specific band between individual soybean lines. Similarly with the 2D-PAGE, only minor qualitative differences in protein expression were observed among the transgenic, near-isoline and the commercial lines as demonstrated by Coomassie staining of protein (Figures 2A, 2B).



Lanes

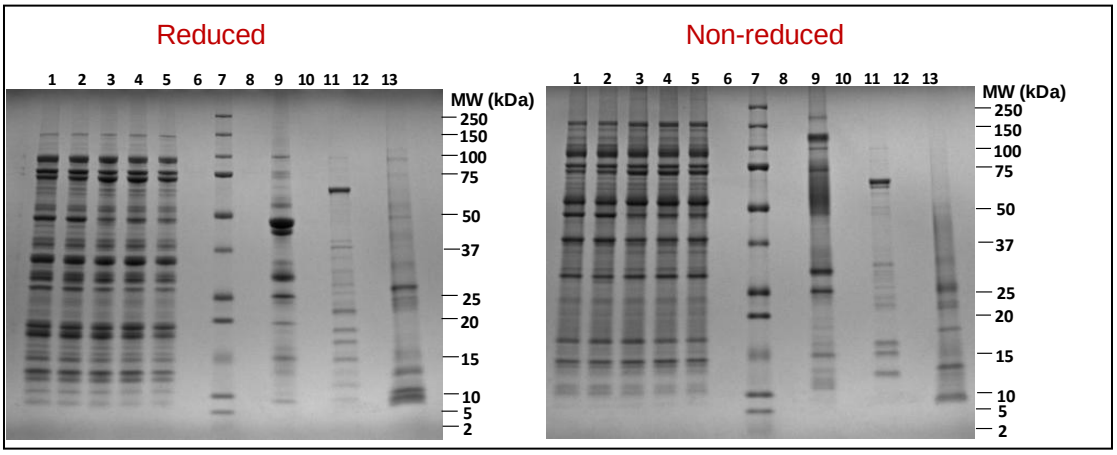
1- Near-isoline (10 µg)
2- Transgenic line I (10 µg)
3- Commercial line 1 (10 µg)
4- Commercial line 2 (10 µg)
5- Commercial line 3 (10 µg)
6- Empty
7-Navy bean (10 µg)
8- Empty
9- Peanut (2 µg)
10-Empty
11-Corn (10 µg)
12- Molecular weight Marker(reduced), empty (non-reduced)
13- Molecular weight marker (non-reduced)

Figure 1A. 1D-PAGE gel of soybean and control extracts under reducing and non-reducing conditions for transgenic line I. Proteins separated were stained with Brilliant Blue G-colloidal stain following electrophoresis.



Lane 1- Near-isoline(10µg)
Lane 2- Transgenic line II (10µg)
Lane 3- Commercial line 4 (10µg)
Lane 4- Commercial line 5 (10µg)
Lane 5- Commercial line 6 (10µg)
Lane 6- Empty
Lane 7- Molecular weight marker
Lane 8- Empty
Lane 9- Navy bean (10 µg)
Lane 10- Empty
Lane 11- Peanut (2 µg)
Lane 12- Empty
Lane 13- Corn (10 µg)

Figure 1B. 1D PAGE gel of soybean and control extracts under reducing and non-reducing conditions for transgenic soybean line II. Proteins separated were stained with Brilliant Blue G-colloidal stain following electrophoresis.



Lane 1- Transgenic line III (10µg)
Lane 2- Near-isoline (10µg)
Lane 3- Commercial line 4 (10µg)
Lane 4- Commercial line 5 (10µg)
Lane 5- Commercial line 6 (10µg)
Lane 6- Empty
Lane 7- Molecular weight marker
Lane 8- Empty
Lane 9- Navy bean (10 µg)
Lane 10- Empty
Lane 11- Peanut (2 µg)
Lane 12- Empty
Lane 13- Corn (10 µg)

Figure 1C. 1D PAGE gel of soybean and control extracts under reducing and non-reducing conditions for transgenic soybean line III. Proteins separated were stained with Brilliant Blue G-colloidal stain following electrophoresis.

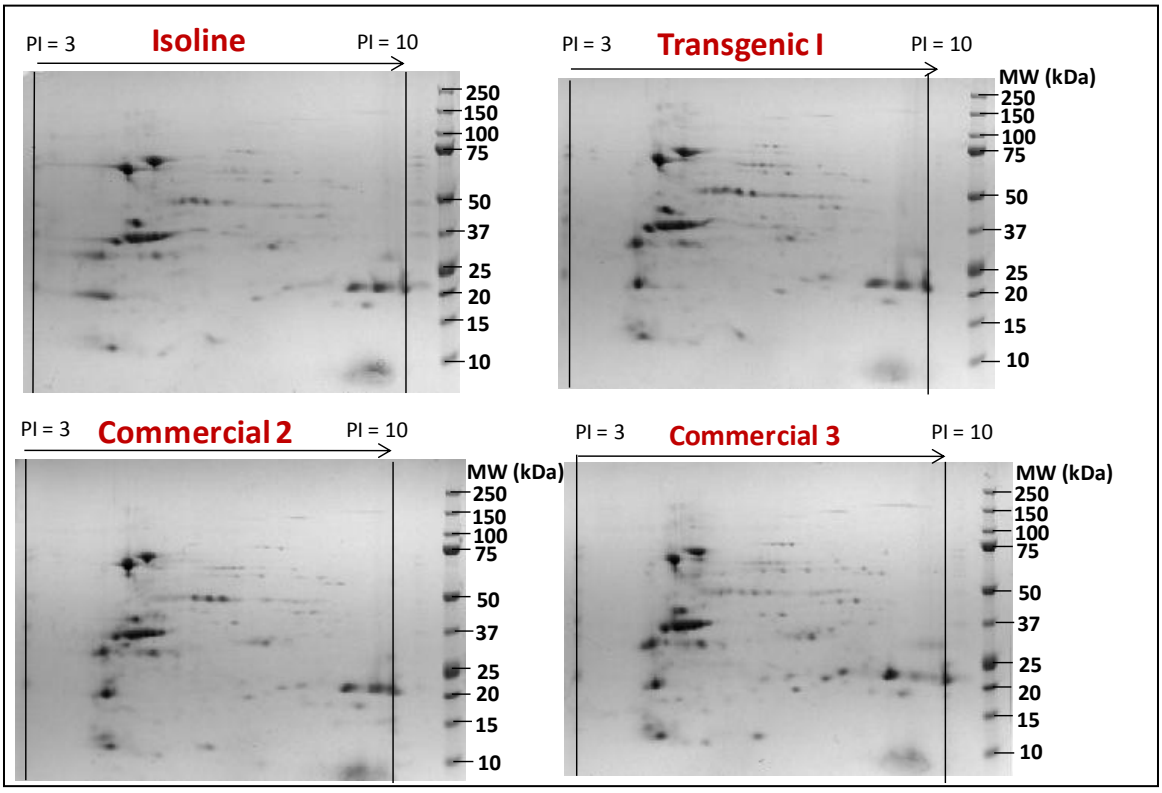


Figure 2A. 2D-PAGE gel of transgenic soybean line I, near-isoline and two commercial lines. 25 µg of protein were separated first according to their isoelectric points and then according to their size. Proteins separated were stained with Brilliant Blue G-colloidal stain following electrophoresis.

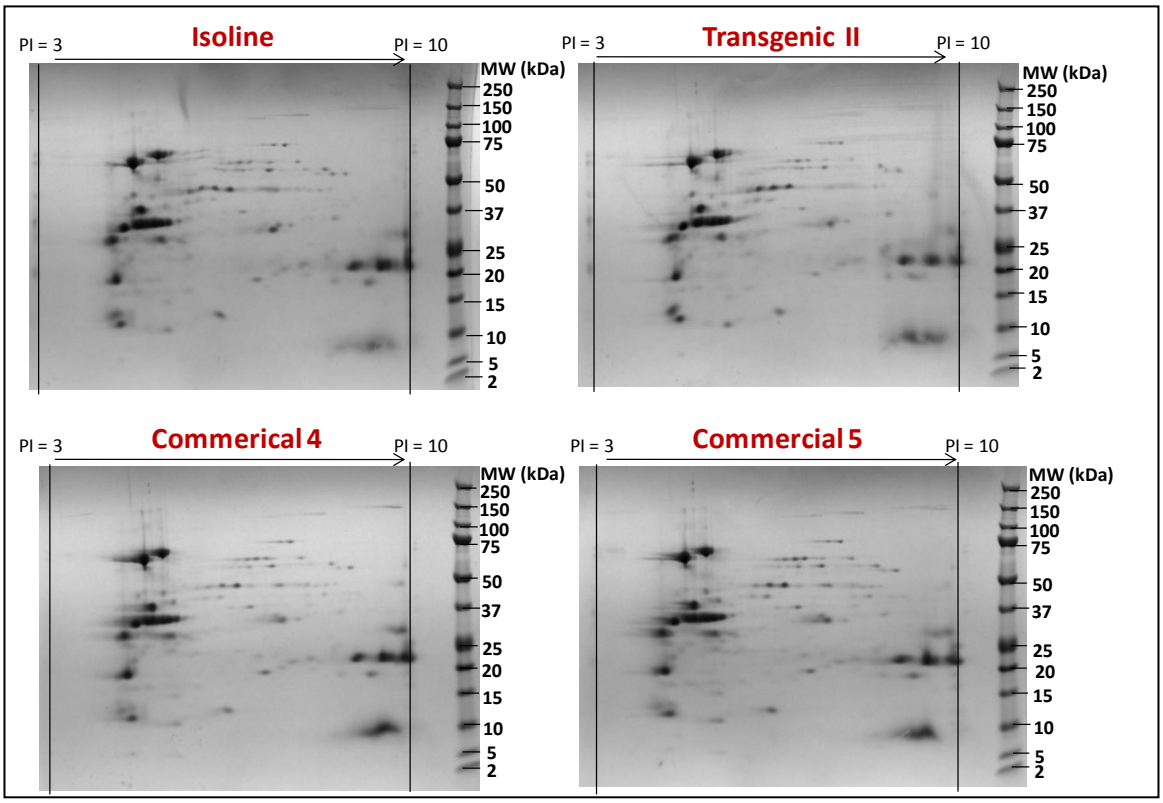


Figure 2B. 2D PAGE gel of transgenic soybean line II, its near isoline and two commercial lines. 25 µg of protein were separated first according to their isoelectric points and then according to their size. Proteins separated were stained with Brilliant Blue G-colloidal stain following electrophoresis.

Qualitative comparison of IgE binding by 1D-immunoblot

Protein samples of extracts of the three transgenic soybean lines, their near-isolines and the commercial lines supplied by study sponsors were separated by 1D-PAGE and subsequently transferred to PVDF membranes for immunoblotting. Control IgE dot blots were incubated with the same diluted anti-IgE and exposed along with the immunoblots to provide an indication of the relative exposure time and sensitivity. Figure 3 shows the IgE binding pattern of nine individual serum samples with transgenic soybean line I, near-isoline and other commercial lines. As described here, the IgE binding patterns differ between the reducing and non-reducing conditions for some of the subjects. Immunoblotting with serum 715 provides a good illustration of the difference in binding patterns under reducing and non-reducing conditions (Figure 3). Importantly for every subject there was from one to a few dominant IgE binding bands; however, typically there were also a few minor, less intense bands. No clear differences were noted in IgE binding to proteins of the transgenic soybean line I (lane 2) compared to the near-isoline (lane 1) using sera from nine soybean allergic subjects. Qualitatively, IgE binding to commercial soybean lines 1 (lane 3) and 2 (lane 4) did show a few differences, although primarily in relative band intensities (e.g. serum 714, reducing condition). In four out of nine sera, a minor IgE binding band was only visible in one commercial line (commercial line 2) under reducing condition (the low MW band in lane 4 with sera 719, 721, 716, 714). Further a clear IgE binding band was visible at approximately 60 kDa in the transgenic (lane 2), near-isoline (lane 1) and commercial line 3 (lane 5) but not in commercial line 1 and 2 (lane 3 and 4) under both reducing and non-reducing condition for sera RG LEG 118.

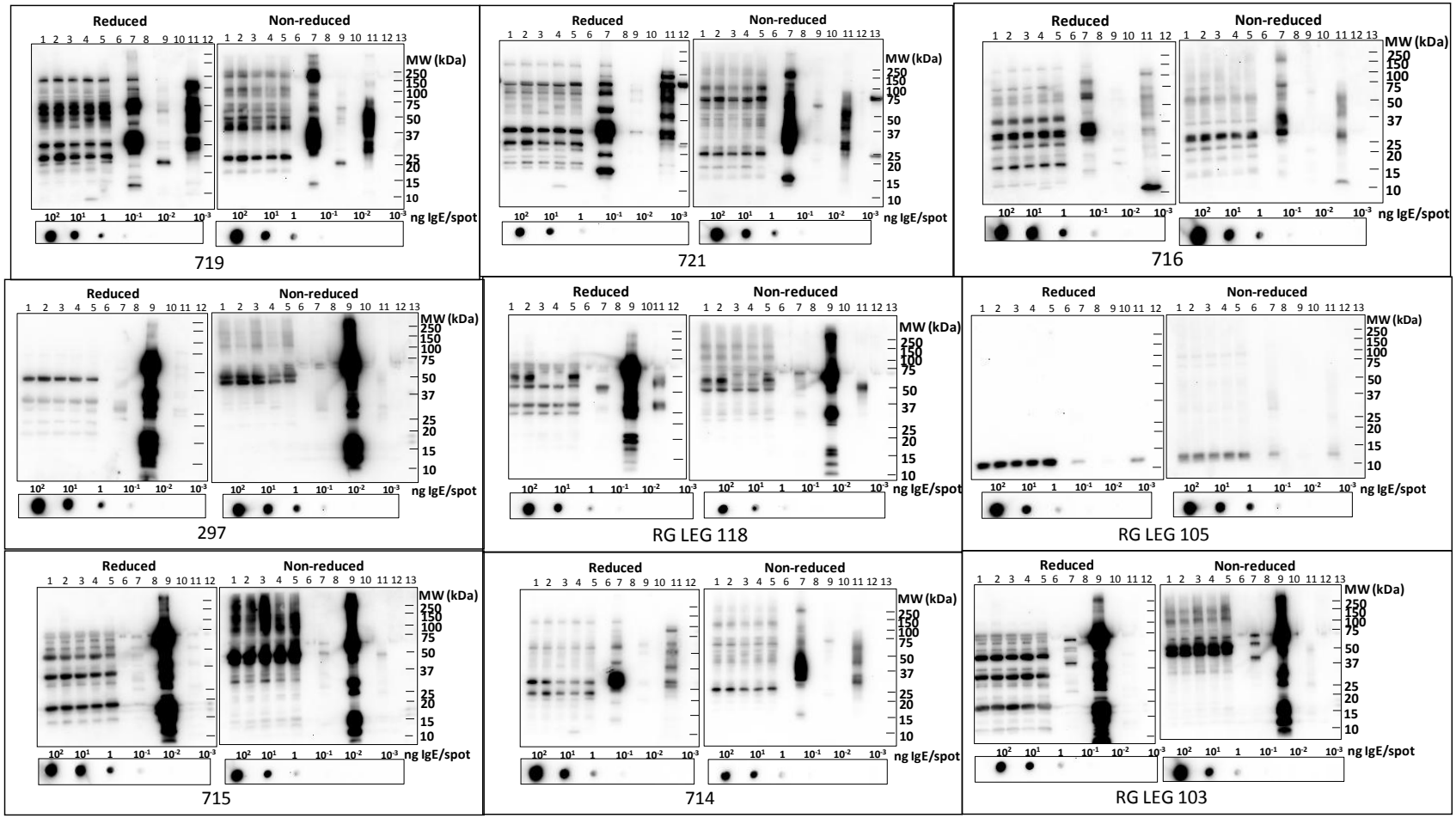


Figure 3. IgE immunoblots of transgenic soybean line I, near-isoline and commercial line 1, 2 and 3 separated by reducing and non-reducing SDS PAGE with nine soybean allergic sera. Lane 1, near-isoline; Lane 2, transgenic line I; Lane 3, commercial line 1; Lane 4, commercial line 2; Lane 5, commercial line 3; Lane 6, empty; Lane 7, navy bean; Lane 8, empty; Lane 9, peanut; Lane 10, empty; Lane 11, corn; Lane 12, molecular weight marker (reduced) and empty (non-reduced); Lane 13; molecular weight marker (non-reduced).

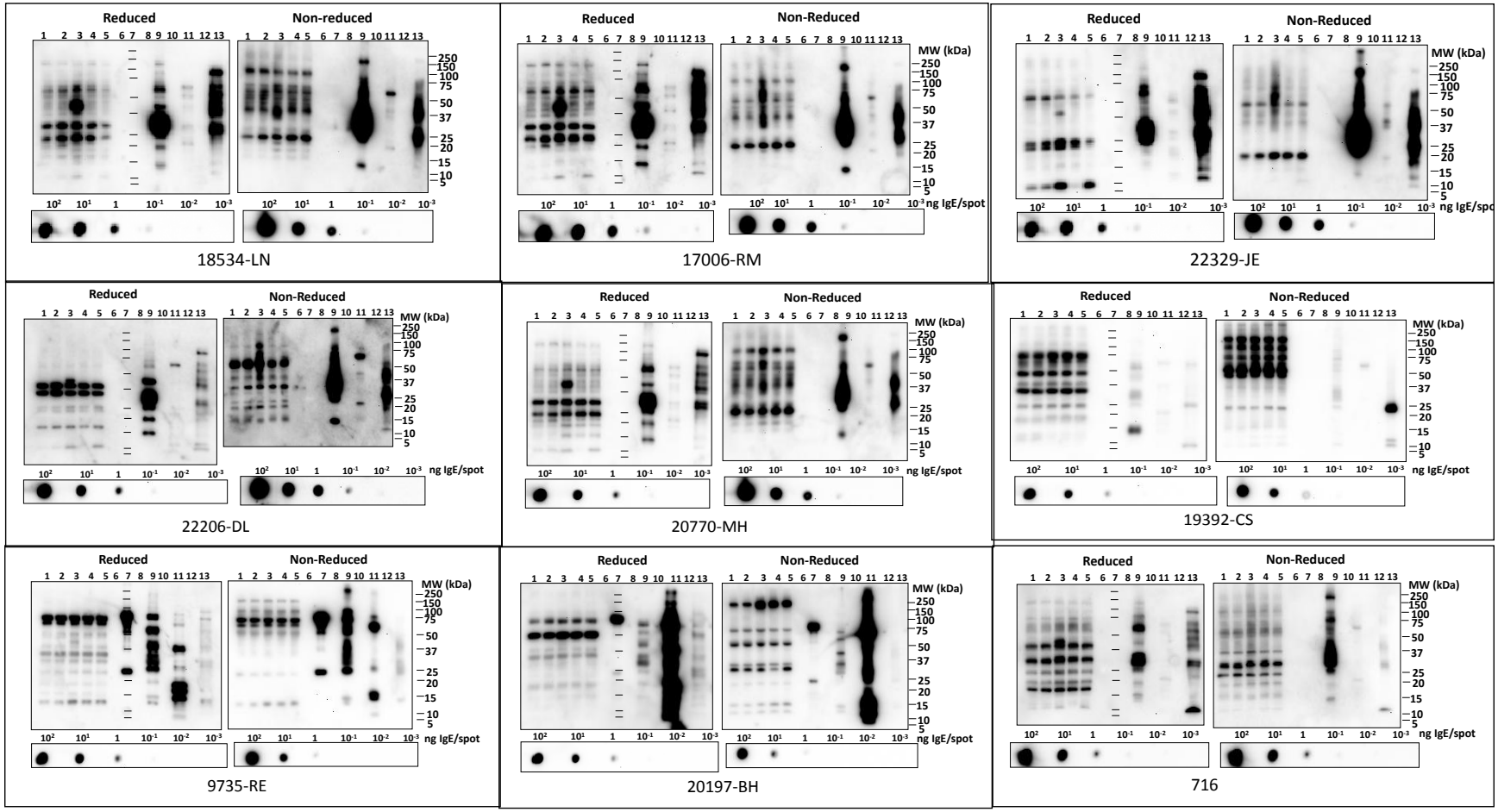


Figure 4. IgE immunoblot of transgenic soybean line II, near-isoline and commercial lines 4, 5 and 6 separated by reducing and non-reducing SDS PAGE with 9 soybean allergic sera. Lane 1, near-isoline; Lane 2, transgenic line II; Lane 3, commercial line 4; Lane 4, commercial line 5; Lane 5, commercial line 6; Lane 6, empty; Lane 7, molecular weight marker; Lane 8, empty; Lane 9, navy bean; Lane 10, empty; Lane 11, peanut; Lane 12, empty; Lane 13, corn.

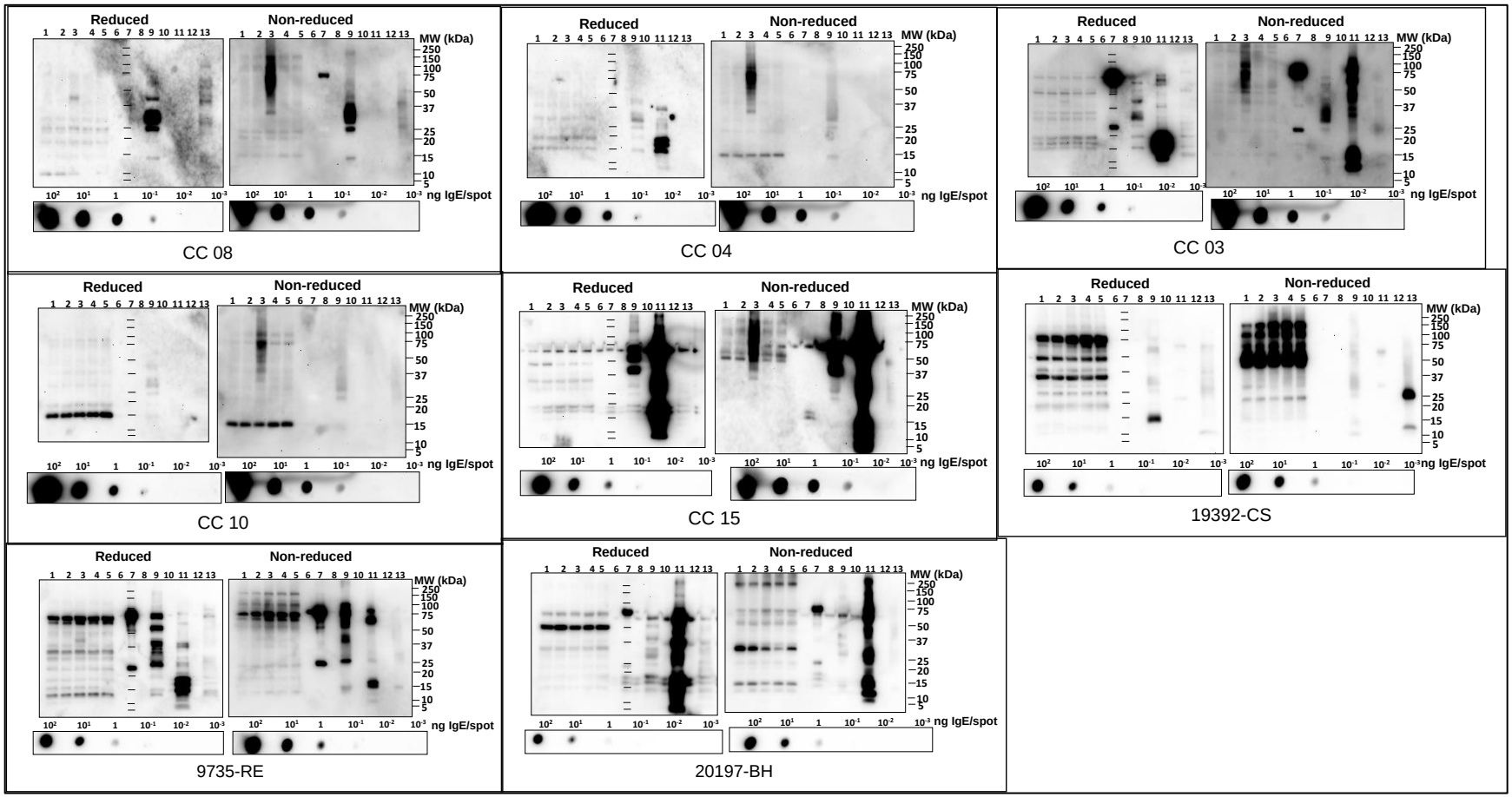


Figure 5. IgE immunoblot of transgenic soybean line III, near-isoline and commercial lines 4, 5 and 6 separated by reducing and non-reducing SDS PAGE with 8 soybean allergic sera. Lane 1, transgenic line III; Lane 2, near-isoline; Lane 3, commercial line 4; Lane 4, commercial line 5; Lane 5, commercial line 6; Lane 6, empty; Lane 7, molecular weight marker; Lane 8, empty; Lane 9, navy bean; Lane 10, empty; Lane 11, peanut; Lane 12, empty; Lane 13, corn.

Figure 4 shows the immunoblots of transgenic soybean line II, its near-isoline and three commercial lines (4, 5 and 6) with nine individual soybean allergic serum samples. Similar to transgenic line I, the IgE binding patterns differed in the apparent molecular masses between reducing and non-reducing gels (e.g. serum 22206-DL, reducing gels showed two major bands at 35 and 40 compared to four or five bands from 16 to 120 kDa in non-reducing gels). This would be expected for some bound proteins contain intra- or inter-peptide disulfide bonds, or comprise of two or more subunits that were joined by a disulfide bridge. For example, glycinin includes a basic and an acid subunit joined by a single disulfide bond. Running the gels under reducing condition results in separation of those subunits and some subjects would be expected to bind IgE to one or both subunits migrating at lower molecular weight (MW) than in non-reducing gels. The patterns of IgE binding did not differ noticeably between the transgenic (lane 2), near-isoline (lane 1) and non-transgenic commercial lines (lane 3, 4 and 5), but for subjects that also had notable IgE binding to bands at approximately 30-35 kDa in navy bean (lane 9), an additional high intensity band was evident 40 kDa in reducing and 37 kDa and 70 kDa in non-reducing gels (likely cross-reactive carbohydrate (CCD) epitope) for the commercial line 4 (Figure 4, lane 3, serum samples 18534-LN, 17006-RM, 22329-JE, 22206-DL, 20770-MH, 9735-RE).

With the transgenic line III, the IgE binding pattern differed remarkably between some subjects (Figure 5). The IgE from subject CC10 showed a single dominant band at about 15 kDa, which is likely to represent Gly m 4, the Bet v 1 homologue in soybeans. In addition, in the non-reducing blot, there is a significant signal at 75 kDa only in

commercial line 4 (lane 3). That pattern is markedly different than the pattern for subject 19392-CS, which appears to have the highest level of binding and the most diverse band pattern. No clear differences were noted in IgE binding to proteins of the transgenic soybean line III (lane 1) compared to its non-transgenic counterpart (lane 2) using sera from eight soybean allergic subjects tested here (Figure 5). However, commercial line 4 did show differences in IgE binding mostly under the non-reducing condition, which was observed in five out of eight sera tested (Figure 5, lane 3, serum CC 08, CC 04, CC 03, CC 10, CC 15). All of these sera that showed intense binding to the high molecular weight protein in commercial soybean line 4 also showed IgE binding to navy bean extract (lane 9) at a position that likely represents the lectin phytohemagluttinin (PHA) and may involve CCD binding.

Qualitative comparison of IgE binding by 2D-immunoblot

Protein samples of extracts of transgenic soybean line I and II, their near-isolines and two commercial lines (2 and 3 for transgenic line I, 4 and 5 for transgenic line II) were separated by 2D-PAGE and subsequently transferred to PVDF membrane for immunoblotting with individual soybean allergic sera (Figures 6, 7, 8, 9). The individual serum samples are indicated in the Figures and the spots showing IgE binding have been circled and numbered. IgE binding was observed to a number of spots for all of the four soybean samples with all individual soybean allergic sera. For the transgenic soybean line I (Figures 6, 7), the IgE binding patterns were similar for the transgenic and near-isoline for all the subjects. Commercial line 3 also showed similar IgE binding patterns as the transgenic and near-isoline for all the subjects. However, commercial line 2 showed some

additional IgE binding spots or absence of some common spots compared to the other three soybean lines with four out of eight soybean allergic sera (Spot # 18 and 19 in Figure 6A, Spot # 22 and 23 in Figure 6B, Spot # 21 and absence of spot 20 in Figure 6C and spot # 17 and 19 and absence of spot 4 in Figure 6D). For the transgenic soybean line II (Figures 8, 9), a number of differences were observed in the 2D IgE binding pattern among the four lines with some serum samples. For example, with serum 18534-LN (Figure 8B) an extra IgE binding spot (spot #7) was observed only with the near-isoline. Similarly with six out of ten serum samples including 20770-MH (Figure 8A, spot # 6), 18534-LN (Figure 8B, spot # 6), 22329-JE (Figure 8D, spot # 5), 17006-RM (Figure 9F, spot # 5), 22206-DL (Figure 9G, spot # 4), 9735-RE (Figure 9H, spot # 4), IgE binding to commercial line 4 included strong binding to an acidic spot at approximately 45 kDa that was not present in the other three extracts. The same sera showed IgE binding to bands at approximately 30-35 kDa with navy bean (Figure 4, lane 9) in 1D-immunoblots, which is likely due to CCD binding. In addition, with serum 20197-BH (Figure 8C), two associated light spots were present in the transgenic soybean while not visible in the near-isoline (spot #11). However, these spots were present in both of the commercial lines, which also showed several differences with the same serum compared to the transgenic and the near-isoline. For example, the absence of spot # 1 and presence of two low-intensity additional spots (Spot # 14 and 15) in commercial line 4 and 5 (Figure 8C). With serum from subject 22329-JE (Figure 8D), an apparent IgE binding spot was only visible in the transgenic line (spot # 2) while not visible in the three non-transgenic soybean lines. The serum from the same subject also demonstrated intense IgE binding to the likely CCD epitope in commercial line 4 (Spot # 5).

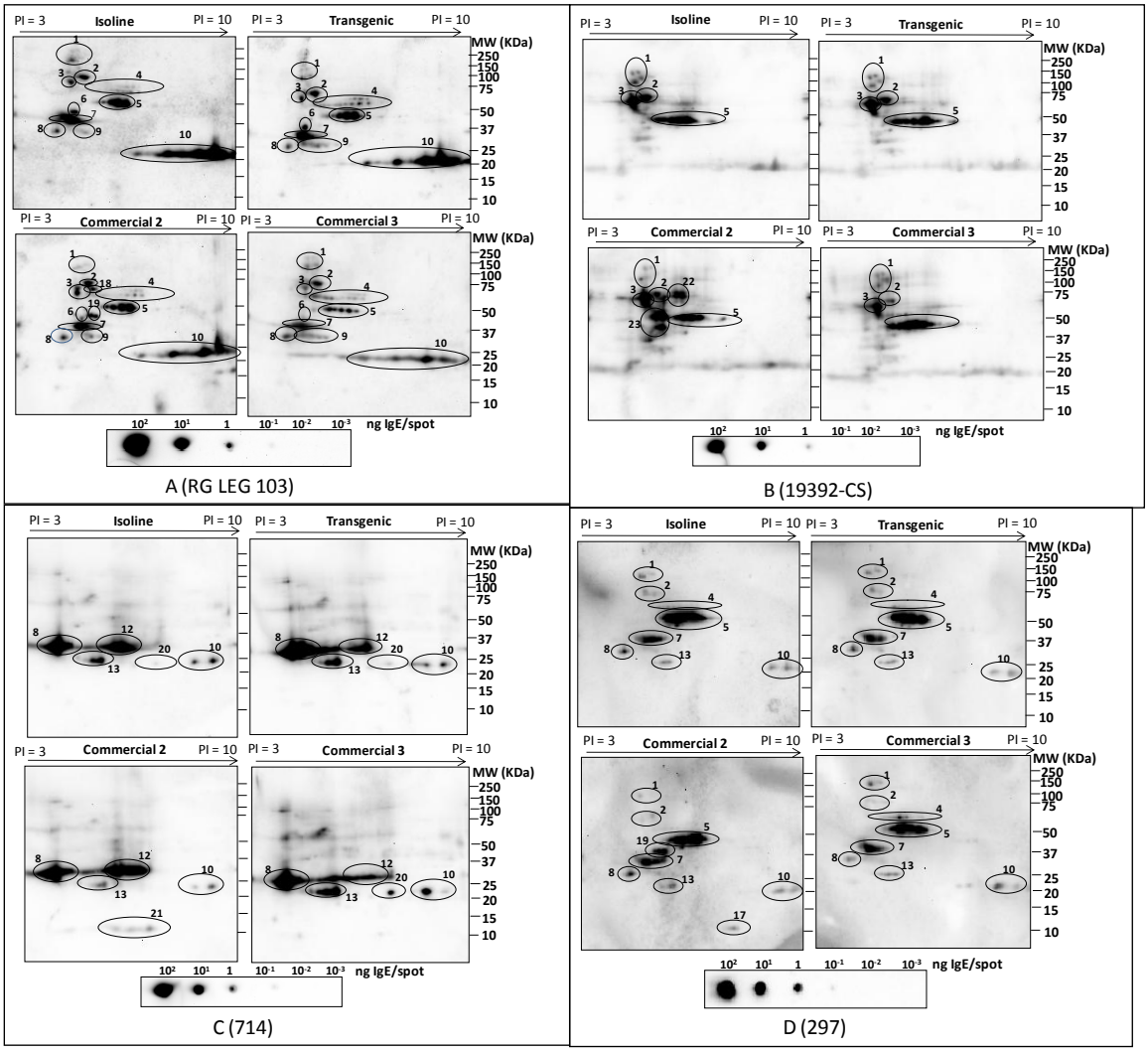


Figure 6. 2D-PAGE immunoblots of transgenic soybean line I, near-isoline and commercial lines 2 and 3 using individual soy allergic sera RG LEG 103 (figure 6A), 19392-CS (figure 6B), 714 (Figure 6C) and 297 (Figure 6D). Figure demonstrates additional spots that were only visible in commercial line 2 (Spot # 18 and 19 in Figure 6A, Spot # 22 and 23 in Figure 6B, Spot # 21 and absence of spot 20 in Figure 6C and spot # 17 and 19 and absence of spot 4 in Figure 6D).

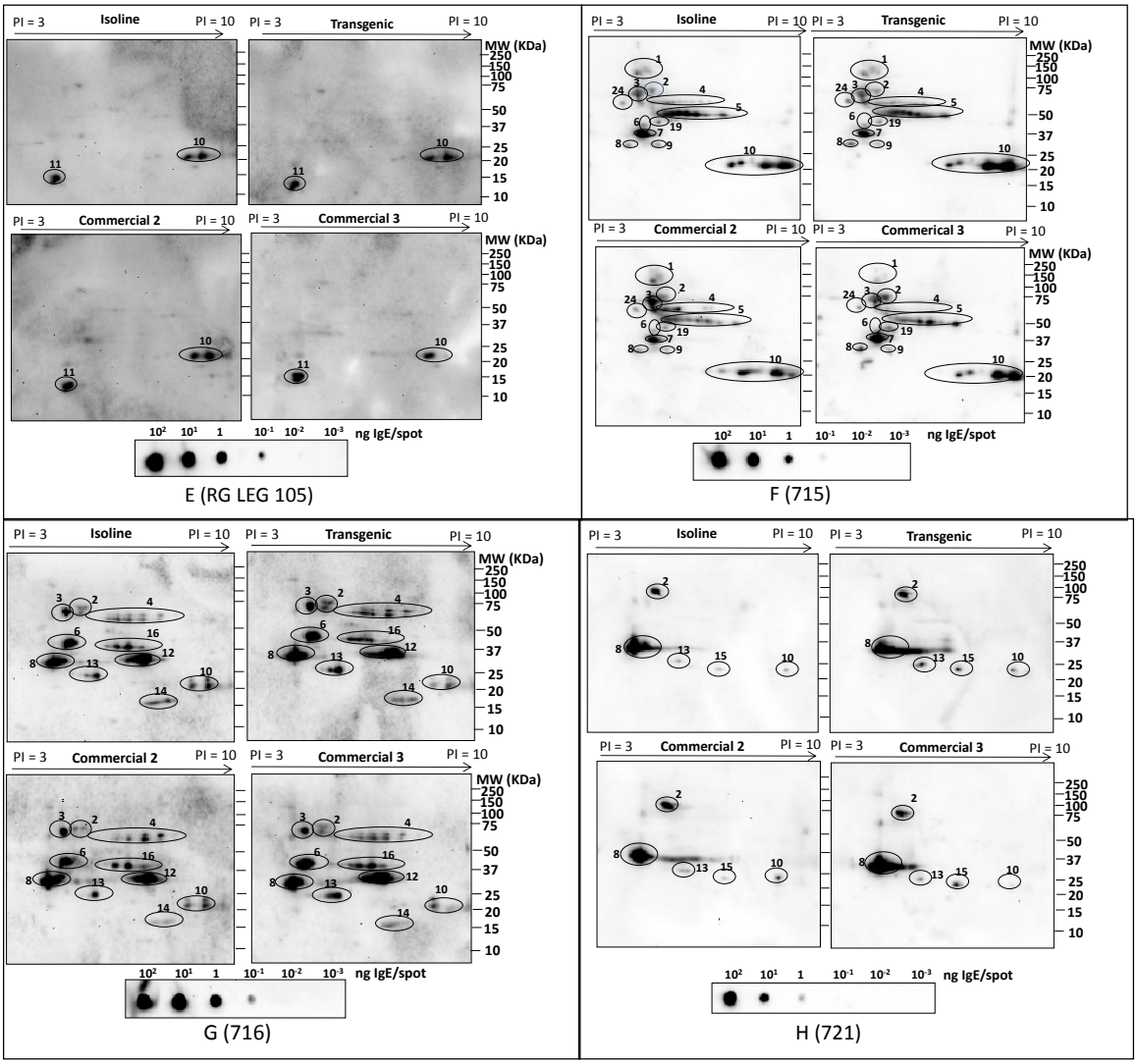


Figure 7. 2D-PAGE immunoblots of transgenic soybean line I, near-isoline and commercial lines 2 and 3 using individual soy allergic sera RG LEG 105 (figure 7E), 715 (figure 7F), 716 (Figure 7G) and 721 (Figure 7H).

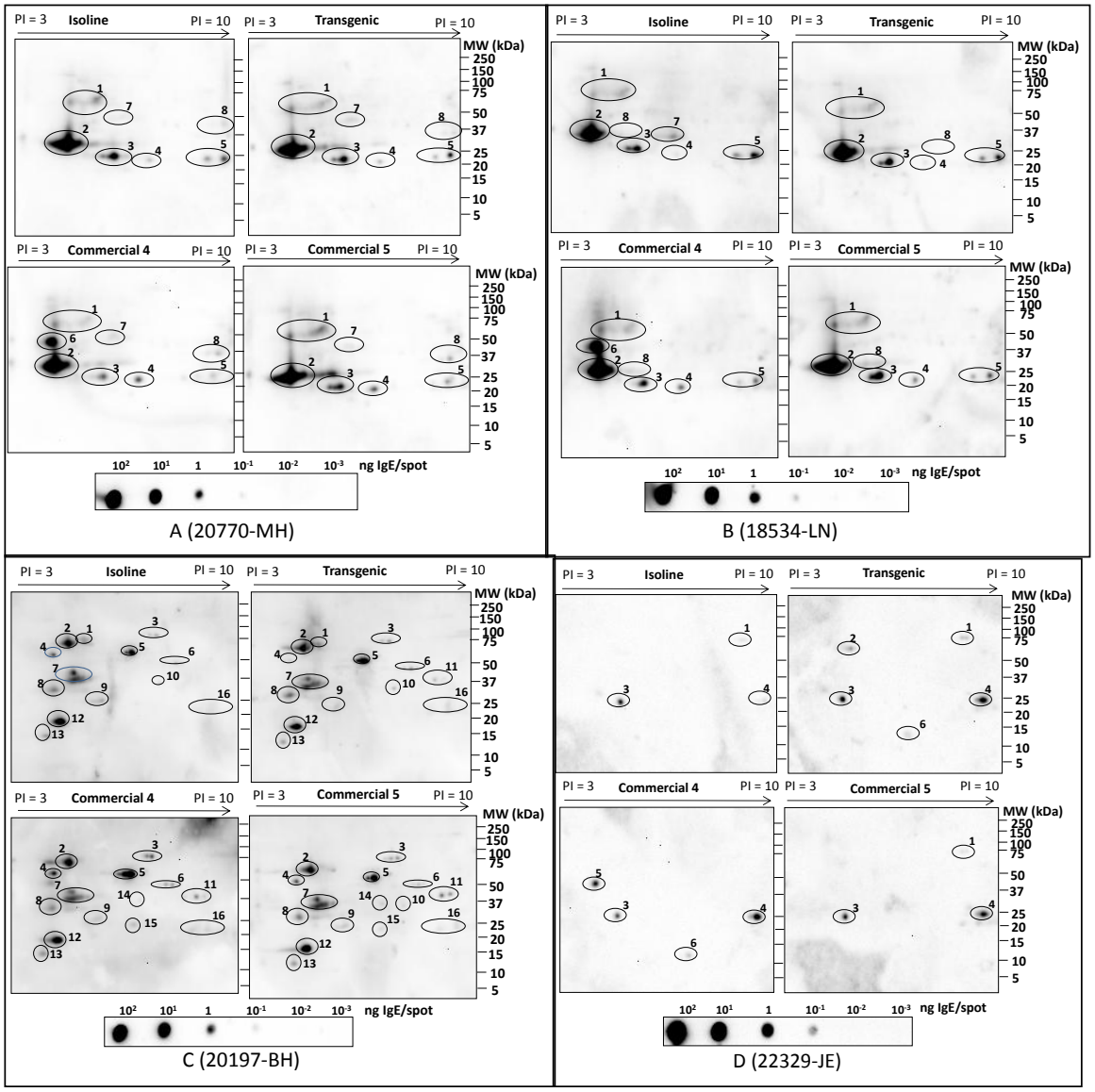


Figure 8. 2D-PAGE immunoblots of transgenic soybean line II, near-isoline and commercial lines 4 and 5 using individual soy allergic sera 20770-MH (Figure 8A), 18534-LN (Figure 8B), 20197-BH (Figure 8C) and 22329-JE (Figure 8D). Figure demonstrates additional IgE binding spot at approximately 45 kDa for commercial line 4 (Figure 8A, spot # 6; Figure 8B, spot # 6; Figure 8D, spot # 5).

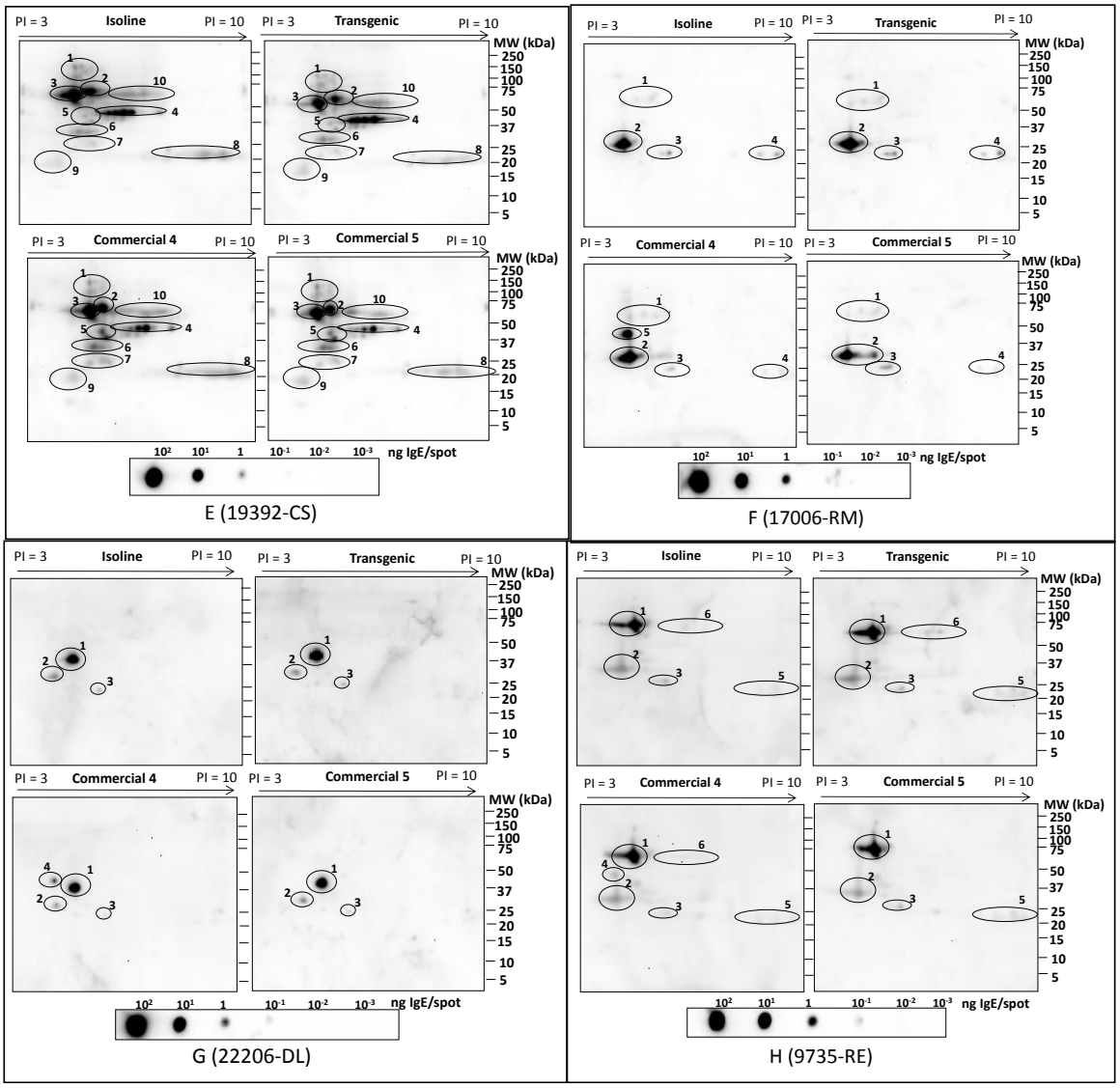


Figure 9. 2D-PAGE immunoblots of transgenic soybean line II, near-isoline and commercial lines 4 and 5 using individual soy allergic sera 19392-CS (Figure 9E), 117006-RM (Figure 9F), 22206-DL (Figure 8G) and 9735-RE (Figure 9H). Figure demonstrates additional IgE binding spot at approximately 45 kDa for commercial line 4 (Figure 9F, spot # 5; Figure 9G, spot # 4; Figure 9H, spot # 4).

IgE inhibition ELISA assay

In order to select soybean allergic sera for IgE inhibition, each individual serum sample was tested for IgE binding to non-denatured soybean proteins using direct binding to pooled soybean extract (pool of the transgenic, isoline and commercial lines) by ELISA (results not shown). Based on the direct ELISA results a serum pool was made, which was utilized in the inhibition ELISA to provide standard inhibition curves. The means across replicates for inhibition are plotted in Figure 10 for transgenic soybean line I and Figure 11 for transgenic soybean line III. The study sponsor did not want an ELISA inhibition assay for transgenic soybean line II. The fit of the linear regression lines was accurate for each replicate of each sample with $r^2 \geq 0.96$ for each regression (data not shown). For the study with transgenic soybean line I, commercial line 3 had slightly more inhibiting capacity at all concentrations than all other soybean lines (Figure 10). For the transgenic soybean line III, the most noticeable difference between samples in the graph is the decrease in inhibition at high inhibitor concentrations for commercial line 4 and to a lesser extent for the standard pool (Figure 11). The inhibition values between soybean lines are so close that it is nearly impossible to distinguish the other individual points and lines.

The mean EC50 and standard deviation for transgenic soybean line I, near-isoline and commercial lines 1, 2 and 3 are shown in Table 1. From Table 1 data there is less than a two-fold difference in the EC50 values across the soybean lines, with observed potency being greatest for commercial line 3 (EC50 = 3.11 µg/well) and least for commercial line 1 (EC50 = 5.39 µg/well). The EC50 values of the transgenic line I and

near-isoline were similar (4.31 vs. 4.68 µg/well, respectively; p = 0.846) and fall within the range for commercial lines. For the transgenic soybean line III (Table 2), differences in the mean EC50 values among the 5 soybean samples were not statistically significant when compared to intra-assay error (F = 2.07; P = 0.177). Similarly, genetic variance among EC50s for the commercial lines was estimated to be 0 when compared to intra-assay variance (F = 0.21; P = 0.814). There was less than a two-fold difference in the EC50 values across the soybean lines, with observed potency tending to be higher for the commercial lines 4, 5 and 6 (EC50 = 0.67, 0.69, 0.75 µg/well respectively) and least for the transgenic line III and its non-transgenic counterpart (EC50 = 0.96, 0.88 µg/well respectively). The EC50 values of transgenic soybean line III and its near-isoline were similar (0.96 vs. 0.88 µg/well, respectively, t = 0.73; P = 0.489). It should be noted that the similarity of EC50 values does not reflect the differences seen at higher inhibitor concentrations where the results for the commercial line 4 are different from the other four samples.

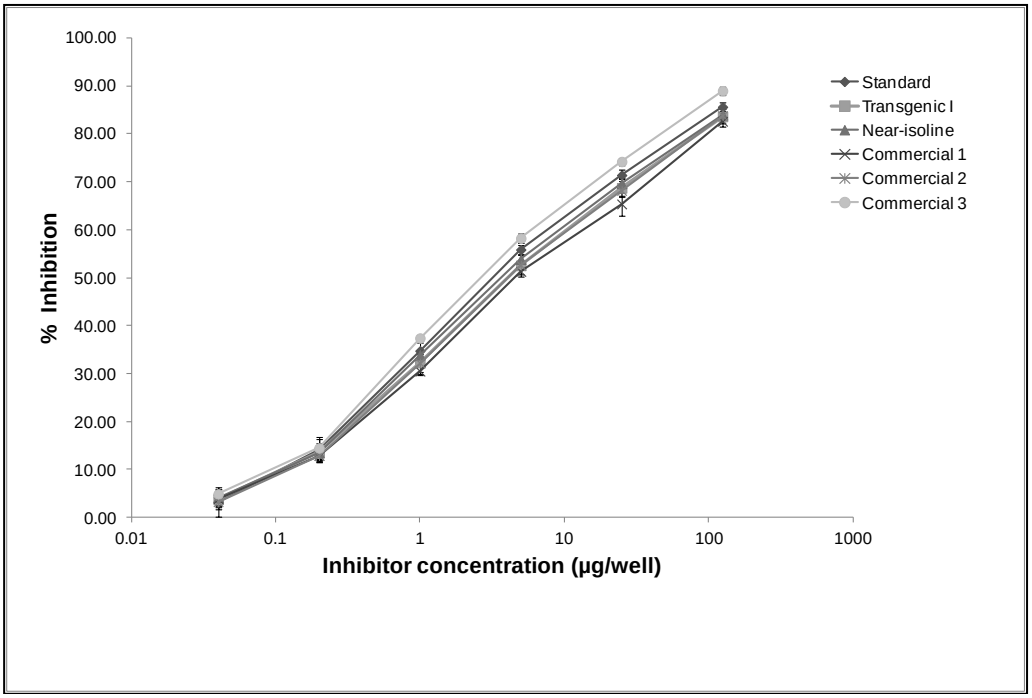


Figure 10. Inhibition ELISA IgE binding curves with transgenic soybean I, near-isoline and commercial lines 1, 2 and 3 compared to a pooled standard soybean extract.

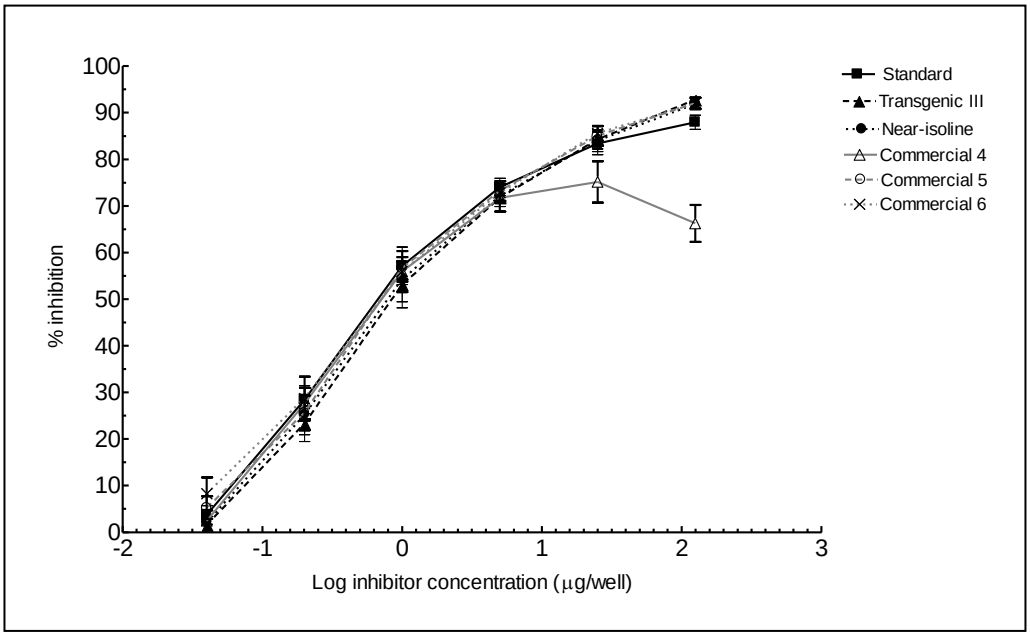


Figure 11. Inhibition ELISA IgE binding curves with transgenic soybean III, near-isoline and commercial lines 4, 5 and 6 compared to a pooled standard soybean extract.

Table 1. IgE Inhibition ELISA EC50 values of transgenic soybean line I, near-isoline and commercial lines 1, 2 and 3: Average of 3 Assays.

Sample	EC50 (µg/well)	Std Dev
Pooled Soy Standard	3.86	0.45
Transgenic I	4.68	0.7
Near-isoline	4.31	0.11
Commercial 1	5.39 *	0.27
Commercial 2	4.76	0.35
Commercial 3	3.11 @	0.19

*Significantly different from the Standard Pool, Dunnett, $p<0.05$

@ Significantly different than all other soybean lines, Tukey, $p<0.05$

Table 2. IgE Inhibition ELISA EC50 values of transgenic soybean line III, near-isoline and commercial lines 4, 5 and 6: Average of 3 Assays.

Sample	EC50 (µg/well)	Std Dev
Pooled Soy Standard	0.693	0.197
Near-isoline	0.877	0.320
Transgenic III	0.969	0.414
Commercial 4	0.665	0.232
Commercial 5	0.746	0.140
Commercial 6	0.693	0.064

DISCUSSION

The *Codex Alimentarius* Commission guideline for safety assessment of foods derived from recombinant-DNA plants (CAC/GL 46-2003, as described in Foods Derived from Modern Biotechnology, 2009), states that the composition of the GM plant including key toxicants and (endogenous) allergens, whose toxic potency and level may be significant to health, should be compared to the composition of the conventional counterpart, harvested under similar conditions. If clear differences are found between the transgenic and comparator lines, further evaluation of the biological significance of the differences would likely be required. It is well known that soybean can cause adverse reactions in humans who are allergic to soybeans, and that soybean allergy is much more common in infants and young children compared to adults (Savage et al., 2010; Sicherer et al., 2000). While common, severe reactions are relatively rare (Sicherer, 2011; Imamura et al., 2008; Sicherer and Sampson, 2010; Rolinck-Werninghaus et al., 2012). Several allergenic proteins have been recognized in soybean primarily based on their reactivity to IgE antibodies from soybean sensitive patients, however, the major seed storage proteins (conglycinins and glycinins) seem to be the most important food allergens (Herian et al., 1990; Holzhauser et al., 2009; Ogawa et al., 1991; Ito et al., 2011). In the current studies, potential changes in endogenous allergenicity (IgE binding) of three unrelated transgenic soybean lines were evaluated by comparing them to their non-transgenic, near isogenic counterpart (near-isoline) as well as other commercial lines of soybeans. Qualitative comparison was performed by separating soybean proteins by 1D- and 2D-immunoblots and quantitative comparison was done by inhibition ELISA. For 2D-immunoblots, no attempt was made to measure the spots quantitatively as such a comparison would require

multiple 2D-PAGE blots for each sample to control for technical variance, which was not possible due to limited volumes of appropriately sensitized serum donors and the great difficulty in recruiting new donors. From the results of the qualitative and quantitative analysis of all three transgenic soybean lines, no significant differences in IgE binding, which is used as a measure of potential allergenicity, were observed between the transgenic soybean lines and their near-isoline comparators. The only clear difference found by the qualitative analysis was a low intensity spot in 2D-immunoblot of transgenic soybean line II using serum sample 22329-JE (Figure 8D spot # 2). This spot was not present in other soybean lines tested with the same serum. This unique spot was a minor IgE-binding spot with a low intensity compared to other IgE binding spots found in 2D-immunoblot with the same sera. This minor difference should not be considered biologically significant regarding the allergenicity of transgenic soybean line II, for the following reasons: The intensity of this IgE-binding spot is close to the limit of detection, compared to the standard IgE dilution; the IgE spot is minor compared to other IgE binding spots observed with this serum; finally this difference is observed with only one of ten sera tested by immunoblotting. There were no significant differences in IgE binding, as measured by ELISA-inhibition, between any of the transgenic soybean lines and their corresponding near-isogenic lines.

The most important finding from these studies was the variation that was observed among the non-transgenic commercial lines (both qualitative and quantitative) that are already present in market. In the first study with transgenic soybean line I and commercial lines 1, 2 and 3, commercial line 2 showed a minor IgE binding band in ID-

immunoblots with four out of nine sera used (Figure 3). The same commercial line also showed differences in IgE binding spot patterns in 2D-immunoblot with four out of eight sera used (Figure 6). Furthermore, commercial lines 1 and 2 showed absence of a 50 kDa IgE binding band in the 1D-PAGE immunoblot with one soybean allergic serum, which was present in the other three soybean lines (Figure 3, serum RG LEG 118). Similarly in the study comparing the transgenic soybean line II and the commercial lines 4, 5 and 6, it was the commercial line 4 that showed an extra, strong IgE binding band under both reducing and non-reducing 1D-immunoblots as well as a corresponding size spot in 2D-immunoblots from using six out of ten sera (Figure 4, 8, 9). IgE binding to this extra band and spot may be due to the presence of a CCD epitope on a protein in commercial line 4 compared to the other soybeans. However, this hypothesis is merely implied by the correlation between the appearance of the extra soybean band and the presence of binding to the phytohemagglutinin (PHA) sized bands in the navy bean extract. The CCD presence was not verified by mass spectrometry. Further, commercial line 4 also showed a downward inhibition trend when used at higher concentrations in the ELISA inhibition (Figure 11). This trend might be due to low affinity binding of IgE to high molecular weight proteins in commercial line 4, as the IgE bound to soluble inhibitor would be expected to release over time at 37°C during incubation with the plate. If the released IgE bound to a solid phase antigen, the binding would go up during the longer exposure. Taking into account the variation in IgE binding to the soybean commercial lines used in these studies it is logical to first establish how much natural variability exists in allergen levels from a broad selection of non-transgenic soybean lines consumed by non-soybean-allergic individuals, before measuring and comparing allergen levels in transgenic lines to

their near-isogenic comparator. Without this data, the measurement of allergen levels in transgenic lines will not provide an accurate comparison for evaluating crop safety. It is important to consider that the amount of allergen required for sensitization is not known. It is likely to differ markedly between individuals and between routes of sensitization. Other factors are also thought to contribute to sensitization. Thus the consideration should be whether the level of allergen should be measured to judge possible differences in risk for elicitation of an allergic response. However, it is clear from studies that there are significant differences in natural endogenous allergen content of many crops. It is also clear that individuals allergic to a given food crop must avoid food derived from that crop and that raw material selection does not hinge on the concentration of endogenous allergens.

Differences in expression of proteins in plants could be due to genetic variations (e.g. gene mutations), variation produced by epigenetic mechanisms or by environmental factors that modulate gene expression, such as exposure to drought or pathogens (Jaenisch and Bird, 2003; Ruebelt et al., 2006). Several studies have substantiated the effect that the environment and genetics can have on the proteomic profile of plant seeds and how it can affect the GM safety assessment data (Batista and Oliveira; Ruebelt et al., 2006; Zolla et al., 2008). Variation in post translational modification of proteins has also been shown to occur among plant varieties (Campbell et al., 2011). Therefore information on the natural variability in allergenic protein expression and also the effects of processing on the conformation and IgE binding to the proteins are critical in designing and correctly interpreting potential biologically relevant changes in

endogenous allergens between transgenic and non-transgenic plant varieties (Ruebelt et al., 2006).

In conclusion, considering the natural variation in allergen content observed and based on the qualitative and quantitative results in the studies described in this chapter, the serum IgE binding pattern to proteins of three transgenic soybean lines evaluated here were not substantially different from the IgE binding to proteins in other non-transgenic soybean lines. Therefore, these transgenic soybean lines should be considered as safe as the non-transgenic soybean lines. The natural variation of allergens has not been systematically studied in diverse commercial soybean lines and a limit of acceptable variation has not been established. Studies on natural variation in allergens among soybean lines grown in different environments are essential to be able to set acceptability limits for transgenic crop lines. Furthermore, the importance of these kinds of studies should be re-evaluated considering the fact that soybean food products do not pose a risk of allergy except for those who are already sensitized to soybean. There are no practical risk consequences to large increases in soybean allergen expression, including 3 or more fold increases, as those with soybean allergy should be avoiding consumption of all soybean foods.

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APPENDIX A: SOYBEAN ALLERGIC HUMAN SERA USED FOR EVALUATING TRANSGENIC SOYBEAN LINE I

Serum #	Reported food allergies and symptoms of past reactions	Total IgE kU/L	Soy-specific IgE (ImmunoCAP* or IMMULITE#) kU/L
297	soybean and peanut: anaphylaxis	nd	soy: 0.8#; peanut: 70#
714	soybean: symptoms not specified	nd	soy: 15.9#; peanut: 22#
715	soybean: oral, dermal, respiratory; peanut: anaphylaxis	644	soybean: 17.8*; peanut: 100*
716	soybean: symptoms not specified; peanut, no information	nd	soybean: 7.2#; peanut: 18#
719	soybean and wheat: asthma	1406	soybean: 22*; peanut: 23*
721	soybean: no information; peanut: reported but symptoms not specified	14725	soybean: 47*; peanut 44*
RG-LEG-103	soybean: no information; peanut: hives, throat swelling	1032	soybean: 12.2*, 1.7#; peanut: 100#
RG-LEG-105	soybean: oral itch, facial edema, breathing difficulty	1023	soybean: 2.3*; peanut: 5*
RG-LEG-118	soybean and peanut: hives and edema of face, throat and tongue	915	soybean: 6.6*; peanut: 100*
19392-CS	Angioedema, vomit, EOS G; milk, egg, meat, fruit, peaches, pears, (?profilin?)	nd	soy: 68#; peanut: 15#

* = Phadia ImmunoCAP® Total and specific IgE as kU/L

= Siemens IMMULITE® specific IgE as kU/L

** = Total IgE = 644 kU/L (ImmunoCAP)

nd= not detected

APPENDIX B: NON-SOYBEAN ALLERGIC CONTROL SERA USED FOR EVALUATING TRANSGENIC SOYBEAN LINE I

Serum #	Reported food allergies and symptoms of past reactions	Total IgE kU/L	Soy-specific IgE (ImmunoCAP or IMMULITE) kU/L
RG-71	allergic to lupin, but no symptoms to soybean or peanut	nd	soybean: 1.5*; peanut: 1.5*
RG-73	allergic to pea (no claim of allergy, but weak skin test positive to peanut and soy)	nd	Soybean: 0.7*; peanut: 15*
RG-74	allergic to lupin, oral symptoms to peanut, no symptoms to soybean	nd	soybean: nd; peanut <0.35*
SNP	no known allergies	nd	Nd
RS-ID-1	Asthma	nd	soybean: nd; German cockroach 31*
RS-ID-3	Asthma	nd	soybean: nd; German cockroach 42*

nd = not determined

* = Phadia ImmunoCAP® Total and specific IgE as kU/L

APPENDIX C: SOYBEAN ALLERGIC HUMAN SERA USED FOR EVALUATING TRANSGENIC SOYBEAN LINE II

Serum number	Gender; Age (years)	Reported food allergies and symptoms of past reactions	Soybean-specific IgE (ImmunoCAP* or IMMULITE#) kU/L	Peanut-specific IgE (ImmunoCAP* or IMMULITE#) kU/L
17006-RM	Male; 48	Soybean: no information; peanut: no information	7.8*	Nd
22206-DL	Female; 29	Nuts, beans and seeds	7.8*	Nd
18534-LN	Female; 52	Nuts, beans and seeds	17.3*	
715	Male; 19	Soybean: oral, dermal, respiratory; peanut: anaphylaxis**	17.8*	100*
716	Male; 29	Soybean: symptoms not specified; peanut, no information	7.2#	18#
9735-RE	Male: 58	Anaphylaxis to peanut, soybean, causes sore throat, itchy mouth, queasy stomach	5*	58*
20197-BH	Male; 39	Itchy throat with nuts and raw veggies	3*	95*
19392-CS	Male; 40	Nuts, beans and seeds	71.6*	Nd
22329-JE	Male; 25	No recorded history	8.6*	Nd
20770-MH	Male; 26	Nuts, beans and seeds; soybean; peanut: causes throat closes	16.4*	Nd

* = Phadia ImmunoCAP® Total and specific IgE as kU/L

= Siemens IMMULITE® specific IgE as kU/L

** = Total IgE = 644 kU/L (ImmunoCAP)

nd= not detected

APPENDIX D: NON-SOYBEAN ALLERGIC CONTROL SERA USED FOR EVALUATING TRANSGENIC SOYBEAN LINE II

Serum number	Reported food allergies and symptoms of past reactions	Soybean-specific IgE (ImmunoCAP*) kU/L	Peanut-specific IgE (ImmunoCAP*) kU/L
RG-71	Allergic to lupin, but no symptoms to soybean or peanut	1.5*	1.5*
RG-73	Allergic to pea (no claim of allergy, but weak skin test positive to peanut and soy)	0.7*	15*
RG-74	Allergic to lupin, oral symptoms to peanut, no symptoms to soybean	nd	<0.35*
SNP	No known allergies	nd	Nd

nd = not determined

* = Phadia ImmunoCAP® Total and specific IgE as kU/L

APPENDIX E: SOYBEAN ALLERGIC HUMAN SERA USED FOR EVALUATING TRANSGENIC SOYBEAN LINE III

Serum number	Gender; Age (years)	Reported food allergies and symptoms	Soybean-specific IgE Immuno-CAP™ kU/L	Peanut-specific IgE Immuno-CAP™ kU/L
9735-RE	Male: 58	Anaphylaxis to peanut, soybean, causes sore throat, itchy mouth, queasy stomach	5	58
20197-BH	Male; 39	Itchy throat with nuts and raw veggies	3	95
19392-CS	Male; 40	Nuts, beans and seeds	71.6	Nd
CC 10	Female; 44	Peanut (a, b), Soybean (a,b,d), Milk (a,b,c), Almond (a), Walnut (a), Hazelnut (a,b,d), Celery root (a), Anaphylaxis with hazelnut. Food challenge positive to soybean hazelnut and celery. Positive skin prick test to soybean.	6.7	18.9
CC 03	Male; 45	Peanut (a,d), Soybean (a,d), Fish (a,d,b,c,e), Walnut (a,d), Hazelnut (a), Pepper(a,b), Anaphylaxis to fish. Food challenge positive to soybean and peanut. Skin prick test positive to soybean, birch pollen, grass and hazelnut.	1.2	21.5
CC 04	Female; 44	Peanut (a,b,c,d,e), Soybean (b,d,e), Almond (a,b,c,d), Walnut (a,b,c,d), Hazelnut (a,b,c,d,e), Celery root (a,b,c,d). Food challenge positive to soybean. Skin prick test	0.6	1.9

		positive to soybean, birch, housedust mite, grass.		
CC 08	Female; 32	Peanut (d), Soybean (a,c), almond, walnut, hazelnut (a,c), celery root (a,c). Food challenge positive to soybean, negative to hazelnut and celery. Skin prick test positive to soybean, peanut, hazelnut and celery.	1.1	2.1
CC 15	Male; 42	Lentil (a), Peanut (a,c), Soybean (a), Almond (a). Food challenge positive to soybean and peanut. Skin prick test positive to soybean and peanut.	1.1	84.6

TM=Trademark of Phadia, Uppsala, Sweden

nd = not done

Symptoms: a: Mouth (lip swelling, itching, swollen tongue, swelling of throat); b: Skin (itching, hives, eczema); c: Gastrointestinal tract (abdominal cramping, vomiting, diarrhea, flatulence); d: Lower respiratory tract (coughing, wheezing, severe chest tightening/difficulty breathing; e: Systemic anaphylaxis, requiring epinephrine or emergency room care.

APPENDIX F: NON-SOYBEAN ALLERGIC CONTROL SERA USED FOR EVALUATING TRANSGENIC SOYBEAN LINE III

Serum number	Gender; Age (years)	Reported food allergies and symptoms of past reactions	Soybean-specific IgE (Immuno CAP) kU/L	Peanut-specific IgE (Immuno CAP) kU/L
CC 11	Female; 27	Green pea (a), carrot (a), almond(a), walnut (a), Hazelnut (a,b), peach, cherry, kiwi (a). Food challenge positive to hazelnut, celery and carrot. Skin prick test positive to hazelnut, carrot and grass pollen.	0.1	0.4
CC 12	Female; 58	Peanut (a,b), carrot (a,d,b), almond (a,d,b), walnut and hazelnut (a,d,b), celery(a) , anaphylaxis (strawberry). Food challenge positive to hazelnut and carrot. Skin prick test positive to hazelnut, carrot, celery and birch pollen.	0.1	0.6
CC 16	Female; 23	Carrot (a,b), hazelnut (a,b), apple (a). Food challenge positive to hazelnut. Skin prick test positive to hazelnut, carrot and birch pollen.	0.04	0.1
SNP		No known allergies	Nd	

Symptoms: a: Mouth (lip swelling, itching, swollen tongue, swelling of throat), b: Skin (itching, hives, eczema), c: Gastrointestinal tract (abdominal cramping, vomiting, diarrhea, flatulence), d: Lower respiratory tract (coughing, wheezing, severe chest tightening/difficulty breathing, e: Systemic anaphylaxis, requiring epinephrine or emergency room care.

CHAPTER 3: THE EFFECT OF HEAT PROCESSING ON SOYBEAN ALLERGENICITY

INTRODUCTION

Processed fractions of soybean protein including soy flour, soy protein concentrate (SPC) and soy protein isolates (SPI) are widely used in processed foods because of their nutritional value and functionality. Processed soybean ingredients are used in various baked products, meat products and dairy products to provide specific functional properties such as improved texture, moisture and fat retention, foaming properties and emulsification (Klein et al., 1995; Lusas and Riaz, 1995; Singh et al., 2008). Thermal processing of soy based food ingredients is commonly used to inactivate anti-nutritional components including trypsin inhibitors, improve protein digestibility and impart characteristic flavors to products (Amigo-Benavent et al., 2008). Heat processing has also been used to improve or gain certain functional properties as well as to reduce the allergenic activity of soy-based food products (Anderson and Wolf, 1995; Lusas and Riaz, 1995; Sorgentini et al. 1995; L'Hocine and Boye, 2007). However, very few studies have systematically investigated the effect of heat processing on the allergenicity of soy flour or individual soy proteins (Burks et al., 1992; Shibasaki et al., 1980; Muller et al., 1998). In these studies, the allergenicity of soybean proteins has been interpreted based on in vitro IgE binding using soy allergic sera. IgE binding against allergenic structures does not correlate well with the expression of clinical symptoms which may be due to the presence of cross-reactive carbohydrate determinants, which may be bound by IgE with low affinity binding, the presence of only one IgE binding epitope, or ineffective spatial

orientation of the IgE epitopes. Positive IgE binding detected by in vitro IgE binding tests should be evaluated further for clinical relevance (Ladics et al., 2008). The study conducted by Burks et al., (1992) has used a pool of sera to investigate changes in IgE binding after soybean processing. Using pooled sera may not be a proper method to investigate reduction in allergenicity as there is a chance that one highly sensitive allergic serum in the pool may dominate the others and the result seen may be representation of binding by that one dominant individual. Further a low levels of IgE against some epitopes found in one serum sample may get diluted by other sera in the pool. Heat treatment can cause unfolding of proteins, exposing hydrophobic and sulfhydryl groups located in the interior of the molecule, which can result in irreversible protein aggregation, thus leading to a decrease in solubility (Renkema et al., 2000). However, the extractability of soybean proteins following heat treatment has not been performed in studies claiming to evaluate potential reduction in allergenicity. The proteins of heat treated materials in most studies have only been measured by extraction under very mild conditions (e.g. phosphate buffered saline [PBS], pH 7.4, low salt). It is possible that most of the reduction of IgE binding previously reported following heat treatment of soybean ingredients is simply due to protein aggregation and insolubility. If so, soybean products that are heat treated may not have easily extractable and detectable allergens, but would still contain the insoluble allergens that could cause allergic reactions if the solid food is ingested.

In this study, soybean samples heat treated under different conditions were evaluated for IgE binding by extracting the proteins using a variety of extraction buffers

for testing by immunoblotting to maximize protein solubilization for a more complete evaluation of potential allergenicity compared to extraction in simple PBS. The allergenicity was evaluated by IgE immunoblot using individual soybean allergic serum samples. In addition, a basophil mediator release assay was performed to further evaluate the potential biological relevance of any IgE binding detected by immunoblotting. The results of this study provide additional information on potential changes in the allergenicity of various heat treatments of soybean ingredients performed to alter their potential risk for soybean allergic consumers.

MATERIALS AND METHODS

Human sera

Historical serum samples collected from consenting soybean allergic and non-soybean allergic human subjects collected under Institutional Review Board oversight at various clinical institutions were used in this study. Additional serum samples procured from PlasmaLab International, a U.S. FDA approved facility, were used in this study. The University of Nebraska Institutional Review Board approved the use of these samples in these and similar studies (reviewed and approved for Goodman laboratory). Samples from eight soybean allergic subjects and one control subject without soybean allergy were used in this study (Table 1). The allergic patients utilized in this study had soybean specific IgE level ranging from 3-68 kU/L as measured by ImmunoCAP® or IMMULITE® systems. Most of the soybean allergic subjects also had peanut specific allergic reactions and significant peanut specific IgE levels ranging from 15 to100 kU/L. The control subject used in this study has no reported food allergies.

Table 1. Soybean allergic and control sera used to evaluate heat treated soybean products

Serum	**Reported food allergies**	**Soybean-specific IgE (ImmunoCAP**	**Peanut-specific IgE (ImmunoCAP**
18534-LN	Nuts, beans and seeds	17.30	nd
9735-RE	Anaphylaxis to peanut, soybean, causes sore throat, itchy mouth, queasy stomach	5	58
20197-BH	Itchy throat with nuts and raw veggies	3	95
19392-CS	Angioedema, vomit, EOS G; milk, egg, meat, fruit, peaches, pears, (?profilin?)	68	15
20770-MH	Throat swelling with peanut	38	43
24033/20431	Peas, peanut, soy, lentil, sulfur drugs, garbanzo beans; anaphylactic shock from peanut, eczema, hives	Nd	nd
23736-AM/20300	all trees, grass, peanuts, cats, rabbits	15.3	>100
20247-LA/20160	buckwheat, rice, rye, celery, lettuce, orange, crab, parsley, tomato, almond, coconut, peanut, pecan, sesame, corn, pea, whitebean, carrot, potato, wheat, oat, soybean	14.9	15.6
Control serum (RP)	No known allergies	Nd	nd

nd- not detected

Heat processing of soybean samples

Three different heat treatment conditions were used in this study. Soybean flour dispersed in PBS was treated under various temperature and time combination; soybean seeds were dry roasted or oil roasted prior to extraction. Raw (unprocessed) Vinton 81 soybean seeds were used in the study. For heat processing of soybean flour, soybean seeds were ground to a fine powder in a SPEX CertiPrep 6850 freezer mill under liquid nitrogen, to make full fat flour. The soybean flour samples were dispersed in 0.01M PBS, pH 7.4 at concentrations of 5% and 50% w/v. The dispersed samples were incubated at 80^{o}C and 100^{o}C in a water bath for 15, 30, 60 or 120 min. The samples were then cooled immediately and centrifuged using an Eppendorf centrifuge 5810R at 10,000 x g for 30 min at 4^{o}C to separate insoluble and aggregated material from soluble material. After centrifugation any supernatant (cooking water) obtained was stored at ¬20^{o}C until further analysis. The pellets were thawed at room temperature and extracted (1:10 or 1:5 w/v) using different extraction buffers. Dry and oil roasting of soybean seeds were performed according to the method described by Boge et al. (2009). Raw Vinton 81 soybean seeds were soaked in distilled water (1:3 w/v) for 20 hours at 4^{o}C. The water left from soaking of the soybeans (soaking water) was stored at -20^{o}C for further analysis to measure leached proteins. The soaked soybean samples were then dry roasted in a conventional oven at 171^{o}C (340^{o}F) for 30, 60 or 90 min or they were roasted in canola oil at 171^{o}C (340^{o}F) for 2, 5 or 8 min. After dry or oil roasting, the soybean seed samples were ground in a freezer mill under liquid nitrogen and were then extracted with specific extraction buffers detailed below.

Eight different extraction buffers were used to extract soybean proteins after processing. The extraction buffers used were, 1) 0.01M PBS, pH 7.4, 2) 0.01M PBS with 0.5M NaCl, pH 7.4, 3) non-reducing Laemmli buffer, 4) reducing Laemmli buffer [2X buffer composition: 100mM Tris HCl, pH 6.8, 200mM DTT (not included under non-reducing condition), 4% SDS and 10% glycerol] 5) 100 mM Tris HCl buffer, pH 6.8, 6) 0.01M PBS with 0.2% Tween 20, pH 7.4, 7) 0.01M PBS with 2% CHAPS, pH 7.4, and 8) borate buffer (0.1M H_3BO_3, 0.025N Na_2 B_4 O_7, 0.075M NaCl, pH 8.45). Extraction was carried out at room temperature for 2 hours. After extraction the samples were centrifuged at 10,000 x g for 30 min at 4^{o}C using an Eppendorf centrifuge 5810R. The soluble protein content of the extracts was estimated by the Lowry method (BioRad, Hercules, CA, USA, Cat # 500-0113, reagent A, Cat # 500-0114, reagent B) for all the extraction buffers except for the CHAPS containing buffer for which Bradford protein assay (BioRad, Hercules, CA, USA, Cat # 500-0205) was used. BSA (BioRad, Hercules, CA, USA, Cat # L9704881) was used as a standard in both the assays. The untreated soybean flour sample was also extracted with each of the extraction buffers.

SDS-PAGE and IgE immunoblotting

The protein profiles of the heat treated and control soybean samples were examined after separating the proteins by SDS-PAGE using XCell *SureLock*™ Mini-Cell (Invitrogen, Carlsbad, CA, USA). Samples were run under reducing conditions by diluting the high concentration extracts to a protein content of 10 μg per 10 □L in 6x Laemmli SDS-sample buffer (Boston Bio-products, Ashland, MA, Cat # BP-111NR), containing the reducing agent 2-mercaptoethanol (Bio-Rad, Hercules, CA, USA, Cat # 161-0710).

Samples were heated at 100°C for 5 min. Samples were also run under non-reducing conditions by diluting to 10 µg per 10 □L in 6x Laemmli SDS-sample buffer (Boston Bio-products, Ashland, MA, Cat # BP-111NR), without mercaptoethanol and without heating prior to loading in the gel. Samples were loaded in wells of a 10-20% tris glycine gel (Invitrogen, Carlsbad, CA, USA, Cat # EC61355BOX) so that non-reducing samples were spaced at least one lane away from samples that included reducing agent. Protein sizes were estimated based on migration of known proteins from a 4 µl sample of Precision Plus protein standards (BioRad, Hercules, CA, USA, Cat # 161-0374) that were loaded in the same gel. Electrophoresis was carried out at a constant 125 V for 1.5 hours. The proteins separated in the gels were then fixed in a solution of 7% acetic acid and 40% methanol in water and then stained with Brilliant Blue G-colloidal (Sigma, St Louis, MO, USA, Cat # B2025) for at least 2 hours. After staining, the gels were destained for one min in 10% acetic acid and 25% methanol in water and and then the gels were washed with multiple changes of 25% methanol until the background was clear of blue dye.

For immunoblotting, the separated proteins from unfixed, unstained gels were electro-transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA, Cat. # LC3675) at 25 V for 90 minutes. The membranes were blocked with 5% non-fat dry milk (NFDM) in PBS containing 0.05% Tween 20 (PBST) for at least one hour. Individual human sera (eight individual soybean allergic and one control sera) were diluted 1:10, in 2.5% NFDM in PBST and allowed to block for 1 hour before incubating with the blocked membrane for overnight at room temperature. Unbound antibody was removed from the membranes by washing four times, 5 min each with PBST. Bound IgE

was detected using monoclonal horseradish peroxidase (HRP) conjugated anti-human IgE (SouthernBiotech, Birmingham, AL: clone B3102E8 Cat # 9160-05), diluted 1:1000 with 2.5% NFDM in PBST. Unbound secondary antibodies were removed by washing the membranes four times with PBST. Detection was achieved using Supersignal West Dura Extended Duration substrate (Pierce, Rockford, IL, USA, Cat # 34076) and capturing emitted light by Kodak Gel Logic 440 image station with multiple exposures. A nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA, Cat # 645239) was spotted with serially diluted purified IgE, air-dried, then blocked with 5% NFDM in PBST and incubated with the secondary antibody and substrate used to detect immunoblotsand exposed along with the immunoblots to evaluate signal strength between experimental blots.

Mediator release assay

Humanized rat basophilic leukemia (hRBL) cells (RBL-703/21) developed by transfecting an immortalized RBL with the alpha-chain of the human FcεRI gene to present the high-affinity receptor for human IgE (Vogel et al., 2005) were used for mediator release assays. The hRBL cells were maintained in 50 ml culture flasks in a 5% CO_2 incubator at 37°C with minimal essential media (MEM) from Invitrogen (Carlsbad, CA, USA, Cat #10370), supplemented with 5% (v/v) fetal calf serum (Invitrogen, Carlsbad, CA, USA, Cat # 10100), and 1.0% L-glutamine-Penicillin-Streptomycin (Sigma, St Louis, MO, USA, Cat # G6784). A 20X Tyrode's buffer (provides physiological condition in tissue culture) was prepared by adding 80.0 g of NaCl (JT Baker Cat #4058-05), 2.0 g KCl (Sigma, St Louis, MO, USA, Cat # P5405), 0.65 g of

NaH_2PO_4.2 H_2O (Sigma, St Louis, MO, USA, Cat # 71505), 1.0 g $MgCl_2$. $6H_2O$ (Sigma, St Louis, MO, USA, Cat # M2393), 4.0 g $CaCl_2$. 2 H_2O (Sigma, St Louis, MO, USA, Cat # C7902) and 24.0 g HEPES (Sigma, St Louis, MO, USA, Cat # H3375) to water with a final volume of 500 ml. Tyrode's wash buffer was prepared by adding 0.50 g D -(+)-Glucose (Sigma, St Louis, MO, USA, Cat # G7528) to 25 ml of the 20X Tyrode buffer and approximately 450 ml dH_2O and the pH was adjusted to pH 7.15 using 3 M NaOH, followed by addition of 0.5 g of bovine serum albumin (BSA, Sigma, St Louis, MO, USA, Cat # A9647) before adjusting to a final volume of 500 ml. Antigen challenge buffer (ACB) was prepared by adding 0.50 g of glucose to 25 ml of 20X Tyrode's buffer and 250 ml of deuterium oxide (Thermo Fisher scientific, Rockford, IL, USA, Cat# 16630-1000), adjusting the pH to 7.45 with 3 M NaOH prior to adding 0.5 g BSA and adjusting to a final volume of 500 ml with dH_2O. Lysis buffer was prepared fresh with 1% by volume,Triton X-100 (Fisher Scientific, Rockford, IL, USA, Cat # 161-0407) in 1X PBS. Substrate solution was prepared from 8.9 g of Na_2HPO_4. 2 H_2O (Sigma, St Louis, MO, USA, Cat # 30412), 0.65 g of P-nitrophenyl N–acetyl-β-D-glucosaminide (Sigma, St Louis, MO, USA, Cat # N9376) in water and the pH adjusted to 4.5 with citric acid buffer before adjusting the final volume to 500 ml with dH_2O. Stop solution (0.5M glycine, pH 10.7) was prepared with fresh glycine (Sigma, St Louis, MO, USA, Cat # G7126).

Mediator (β-hexosaminidase) release assays were carried out according to the method of Kaul et al. (2007). Adherent cells in stationary phase were dislodged by application of 0.01 M EDTA in MEM for 30-45 minutes. Dislodged cells were washed

twice with fresh media and diluted to a cell density of 2.0 X 10^6 cells/ml. Cells (50 µl) were seeded into wells of a 96 well micro titer plate (Fisher scientific, Rockford, IL, USA, Cat #167008) followed by the addition of 50 µl of serum or plasma (diluted 1:10 with the MEM) to allow binding of IgE to the FcεRI during 12 hours of incubation at 5% CO_2 and 37°C. Cells were then washed twice with Tyrode's wash buffer before challenging replicate wells with one of five concentrations of soluble antigen (heat treated and control samples) diluted in allergen challenge buffer (10 µg/ml, 1 µg/ml, 0.1 µg/ml, 0.01 µg/ml or 0.001 µg/ml) of soluble antigen. The plates were then incubated in a water bath (37°C) for 1 hour before transferring 30 µl of cell supernatant from each well to corresponding wells of an untreated polystyrene 96-well micro plate (Thermo Scientific, Rockford, IL, USA, Cat # 269620) which contained 50 µl per well of substrate. Substrate conversion was allowed to proceed at 37°C in a water bath for one hour. Reactions were stopped by the addition of 100 µl of stop solution before reading absorbance values at 405 nm. Absorbance values of samples were adjusted to a baseline reference by subtracting readings from IgE sensitized cells that were not exposed to antigen (serum negative controls). Cells sensitized by the addition of purified human IgE (Fisher, scientific, Rockford, IL, USA, Cat #ab65866) and cross-linked by addition of anti-human IgE (Sigma, St Louis, MO, USA, Cat # I6284) were used as positive control. Additional wells with cells sensitized with the test serum and challenged by anti-IgE instead of the antigen extract were used as an individual subject control for total serum IgE release. Test sample readings were expressed as a percentage of total (complete) release as well as total serum IgE release.

RESULTS

Heat treatment of 5% (w/v) and 50% (w/v) soybean flour samples dispersed in PBS

Soluble protein concentrations of extracts were determined for all soybean sample treatments. As demonstrated from the soluble protein content of the supernatant of the 5% soy flour heat treated and control samples (Figure 1), it is clear that the pellets retained proteins that were extractable by the more robust extraction buffers. Non-reducing Laemmli buffer resulted in greater protein extraction from the heat treated sample pellets among all the extraction buffers followed by the Tween 20 containing buffer. The NaCl containing buffer resulted in the least amount of protein being extracted from the pellets. For the 50% soy flour samples (Figure 2), non-reducing Laemmli buffer resulted in more efficient protein extraction from the heat treated samples compared to the other two extraction buffers. However, the 0.5M NaCl buffer was better for extracting proteins from the control (unheated) sample. In order to investigate whether there was differential solubility, heat treated and control samples were separated by SDS-PAGE and compared. Figure 3 (A-D) shows the protein profile of the 5% w/v heat treated soy flour supernatant as well as the pellets extracted with the three extraction buffers. Figure 4 shows the protein profiles of 50% w/v heat treated soy flour samples extracted with the same extraction buffers. Based on the stained gel patterns from SDS-PAGE, the 5% soy flour supernatants (Figure 3A) under reducing conditions demonstrated that the protein profiles of the heat treated samples (lane B-E and lane I-L) were similar to those of the control sample (lane A) except for few protein bands (bands 1, 2, 5, 6), whose intensity declined as the heat treatment was prolonged. These bands became almost invisible when the samples were heat treated at 100^{o}C for 120 min (lane L). Under non-reducing

conditions a similar trend was observed. The intensity of protein bands 1, 3, 4, 5, 6, 9, 10 and 13 declined as the heat treatment duration was increased. Additionally some new protein bands not present in the control sample become visible in the heat treated samples (bands 2, 7, 8, 11, 12 and 14). When pellets were extracted with non-reducing Laemmli buffer (Figure 3B), some new protein bands appeared in both the heat treated and control samples that were not observed in the supernatant (band 2 in the control sample (lane A) and band 16 in the heat treated samples (lane C-M) under non-reducing conditions. For pellets extracted with the higher salt buffer (Figure 3C), an additional band, 15 was observed under non-reducing conditions in the control sample (lane A). This band was not present in the supernatant nor in samples that were extracted with non-reducing Laemmli buffer. Other bands (3, 4, 7) different from supernatant were also observed under reducing conditions. Some differences were also observed when the pellets were extracted with buffer containing Tween 20 (Figure 3D; under reducing conditions, bands 3, 4 not observed in the supernatant and band 8 not observed with any other extraction buffers). For the 50% soy flour samples (Figure 4), the intensity of most of the protein bands decreased as the heat treatment conditions increased. A similar protein profile was observed for samples extracted with Tween 20 containing buffer and salt containing buffer. With the non-reducing Laemmli buffer, some proteins in the heat treated samples were extracted more efficiently compared to the other two extraction buffers (e.g. bands 1 and 2 under reducing conditions and bands 1 and 3 under non-reducing conditions). Similar to the 5% soy flour sample, some new protein bands also appeared in the 50% soy flour sample following heat treatment (band 6 under reducing conditions and bands 6, 7 and 8 under non-reducing conditions).

The soluble protein content as well as the SDS-PAGE profile of the pellets extracted with Laemmli buffer with reducing agent was comparable to that of Laemmli buffer without reducing agent. Additionally, the pattern from pellets extracted with Tris HCl, Borate buffer, 1X PBS and PBS with 2% CHAPS were similar to that produced from the pellet extracted from PBS with Tween 20 (not shown). Based on obvious protein extraction differences in the SDS-PAGE profile of proteins extracted with Laemmli buffer without reducing agent, PBS with Tween 20 and the PBS with 0.5M NaCl, these three extraction buffers were used for subsequent immunoblot analysis as well as in RBL assay to find out differences in the allergenicity of the heat treated samples compared to the control sample.

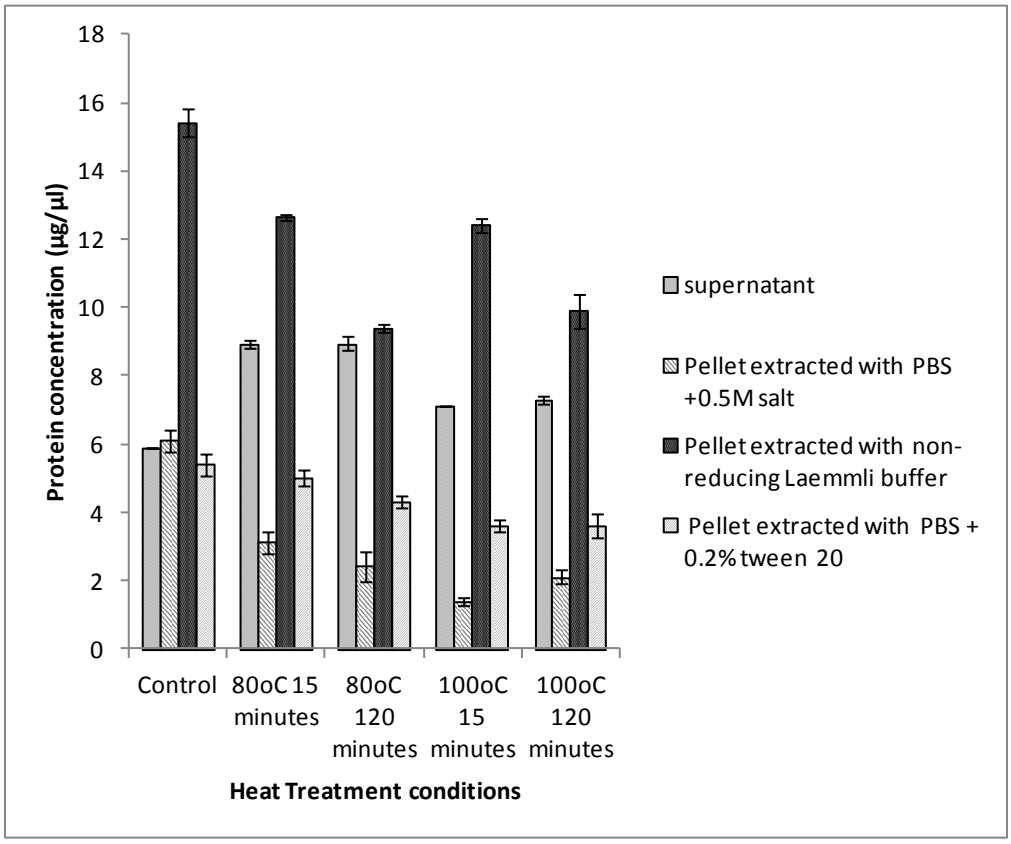

Figure 1. Soluble protein content (µg/µl) of the supernatants and pellets extracted with PBS containing salt, non-reducing Laemmli buffer and PBS containing Tween 20 of 5% (w/v) soy flour dispersion control sample and samples heat treated at 80^{o}C and 100^{o}C for 15 and 120 min. Soluble protein content was determined using the Lowry method.

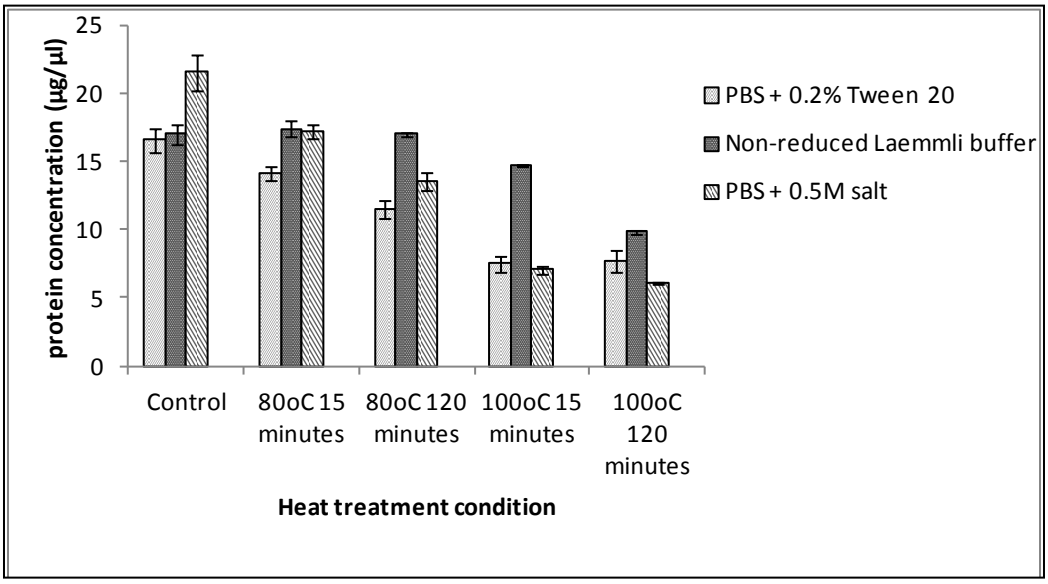

Figure 2. Soluble protein content (µg/µl) of the 50% (w/v) soy flour dispersion control sample and samples heat treated at 80°C and 100°C for 15 and 120 min extracted with PBS containing salt, non-reducing Laemmli buffer and PBS containing Tween 20. Soluble protein content was determined using the Lowry method.

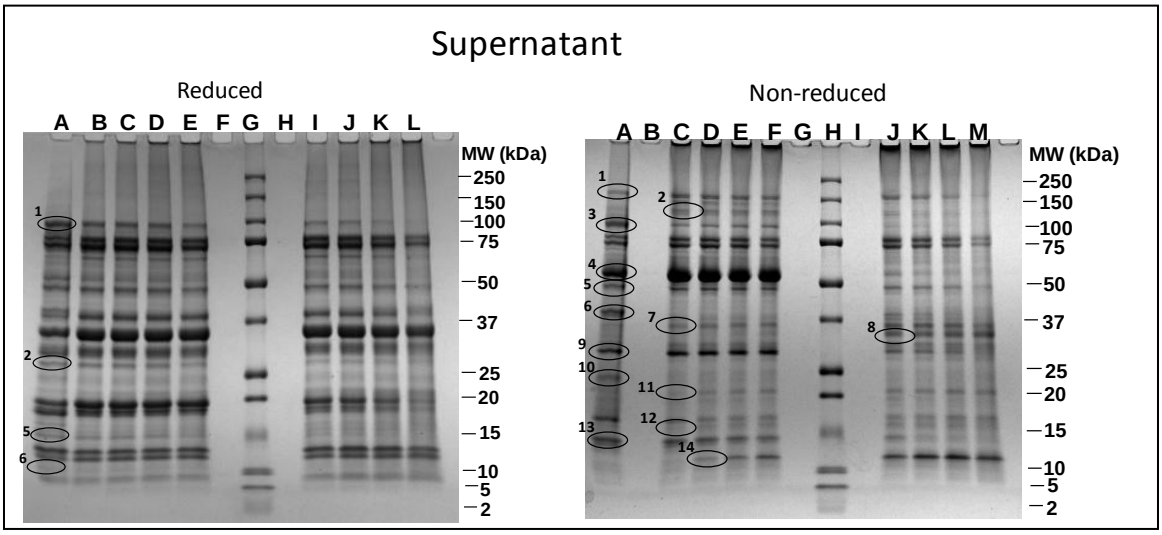

Figure 3A. SDS-PAGE stained gel of the supernatants of the 5% w/v soy flour samples dispersed in PBS and heat treated under various temperature and time combinations compared to the control samples. Samples (10 μg protein/lane) were run under both reducing and non-reducing conditions, then gels were fixed and stained with Brilliant Blue G-colloidal stain. Reduced gel lanes: A, unheated control; B- E, heat treatment at 80^{o}C for 15, 30, 60 and 120 min respectively; F, empty; G, molecular weight marker; H, empty. I-L, heat treatments at 100^{o}C for 15, 30, 60 or 120 min respectively. Non-reduced gel lanes: A, Unheated control; B, empty; C-F, heat treatments at 80C for 15, 30, 60 or 120 min respectively; G, Empty; H, molecular weight marker; I, empty; J-M, heat treatment at 100^{o}C for 15, 30, 60 or 120 min respectively.

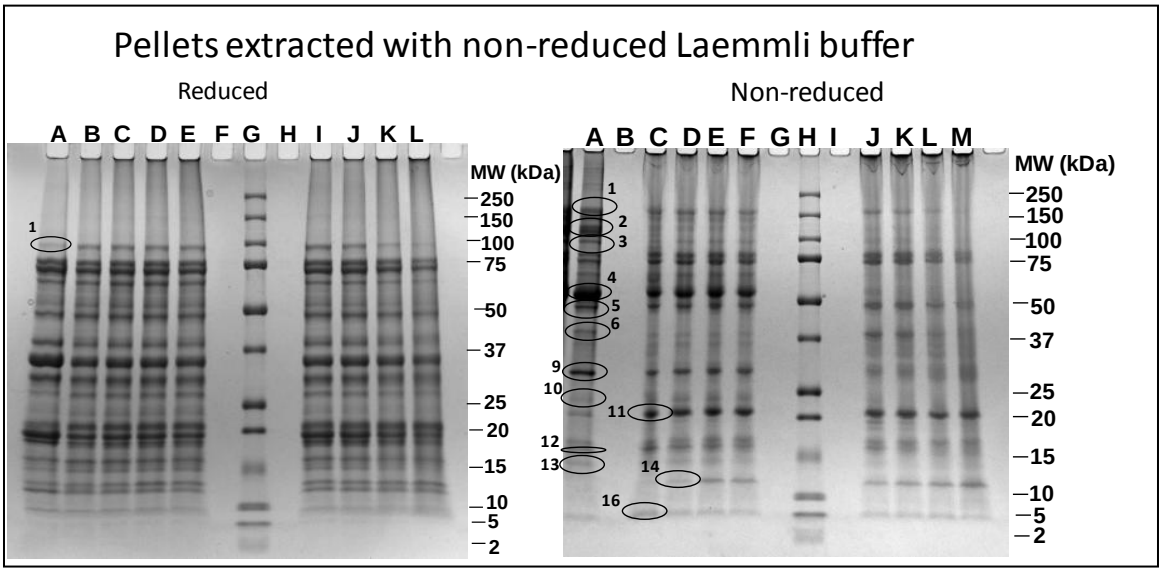

Figure 3B. SDS-PAGE stained gels with extracts of pellets from 5% w/v soy flour samples dispersed in PBS and heat treated under various temperature and time combinations compared to the control samples extracted with non-reducing Laemmli buffer. Lane designations are same as in Figure 3A for both reducing and non-reducing conditions.

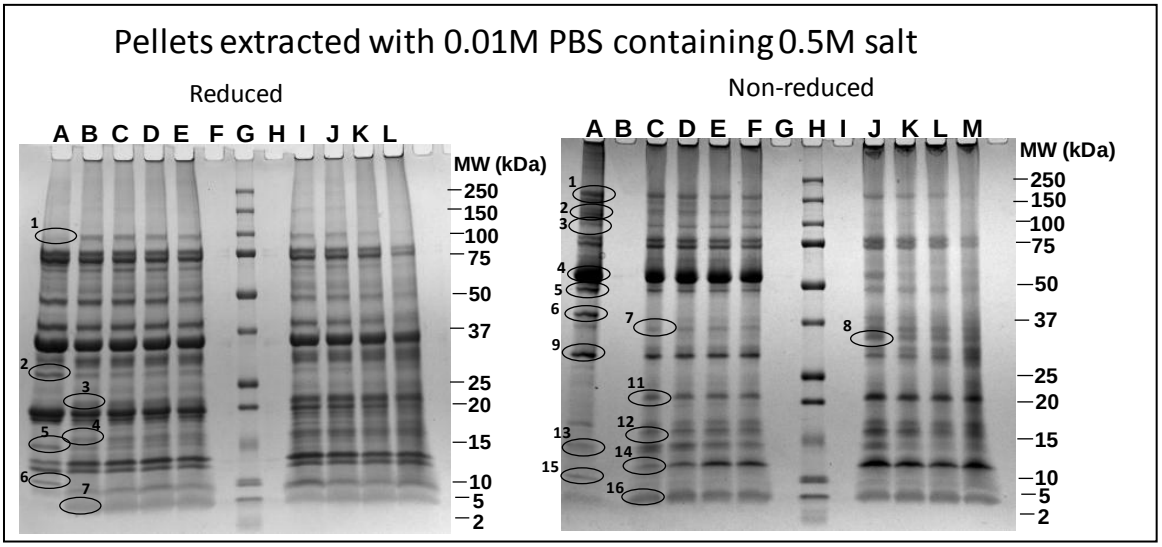

Figure 3C. SDS-PAGE stained gels with extracts of pellets from 5% w/v soy flour samples dispersed in PBS and heat treated under various temperature and time combinations compared to the control samples extracted with NaCl containing buffer. Lane designations are same as in Figure 3A for both reducing and non-reducing conditions.

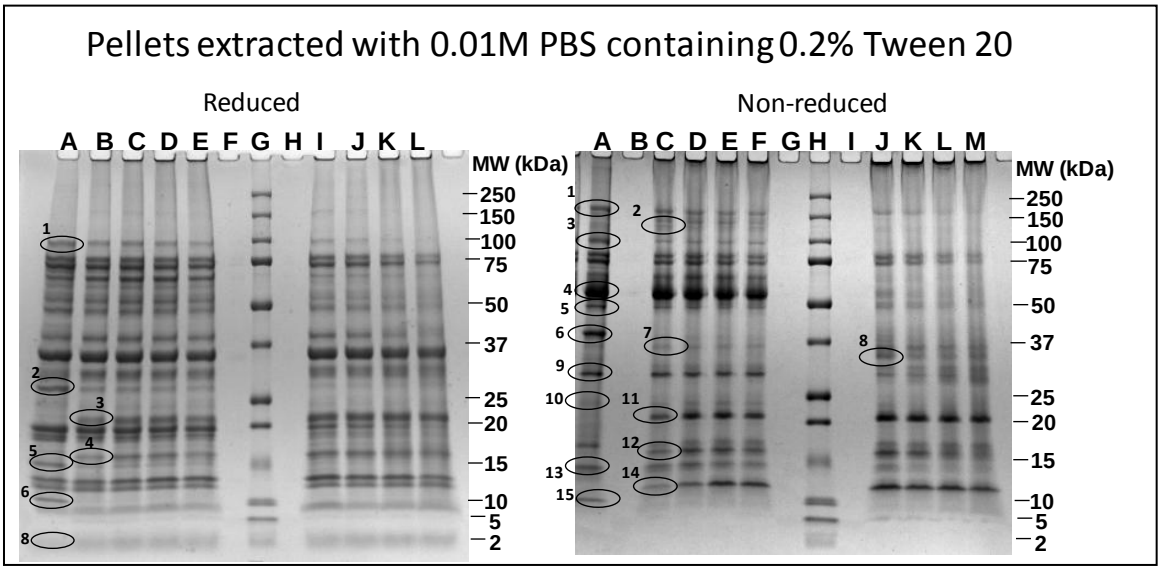

Figure 3D. SDS-PAGE stained gels with extracts of pellets from 5% w/v soy flour samples dispersed in PBS and heat treated under various temperature and time combinations compared to the control samples extracted with Tween 20 containing buffer. Lane designations are same as in Figure 3A for both reducing and non-reducing conditions.

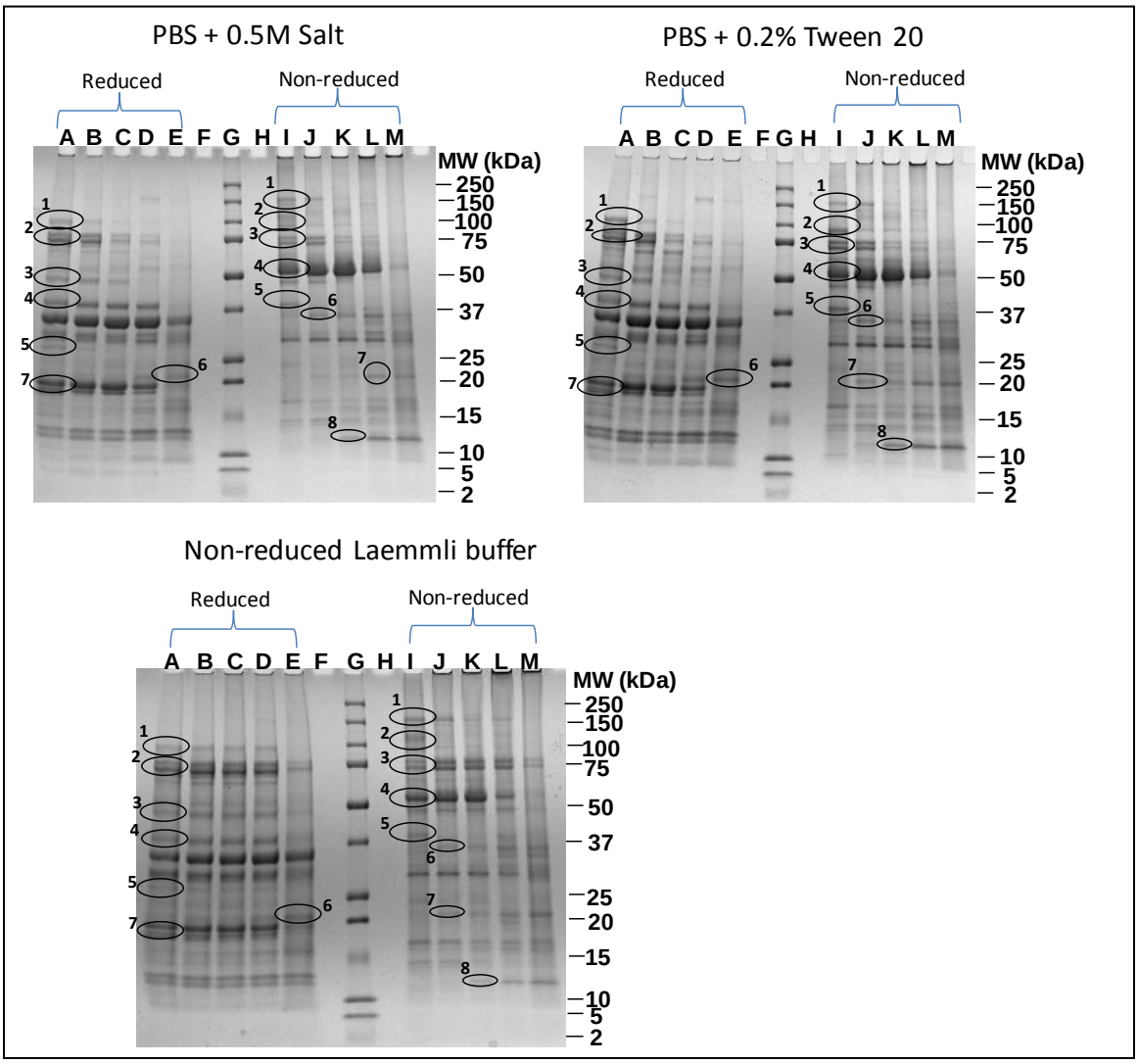

Figure 4. SDS-PAGE stained gels of the 50% w/v soy flour samples dispersed in PBS and heat treated under various temperature and time combinations extracted with 0.01M PBS containing 0.5M salt, 0.01M PBS containing 0.2% Tween 20 and non-reducing Laemmli buffer. Samples (10 μg protein/lane) was run under both reducing and non-reducing conditions, then gels were fixed and stained with Brilliant Blue G-colloidal stain. Lanes: A, unheated control; B, heat treated at 80^{o}C for 15 min; C, heat treated at 80^{o}C for 120 min; D, heat treated at 100^{o}C for 15 min; E, heat treated at 100^{o}C for 120 min; F, empty; G, Molecular weight marker; H, empty; I, unheated control; J, heat treated at 80^{o}C for 15 min; K, heat treated at 80^{o}C for 120 min; L, heat treated at 100^{o}C for 15 min; M, heat treated at 100^{o}C for 120 min.

The same sample extracts and conditions used for the SDS-PAGE stained gels were used for IgE immunoblotting experiments with serum samples from eight soybean allergic patients to evaluate potential differences in IgE binding patterns and possibly allergy to differentially processed soybean ingredients for a number of allergic subjects. Figure 5A shows the immunoblot of the 5% soy flour heat treated and control samples blotted with serum 19392-CS. For the supernatant under reducing conditions, strong IgE binding was observed to bands of 75 kDa, 50 kDa and 35 kDa (bands 1, 2 and 3) in the control sample (lane A) as well as the samples heat treated at 80^{o}C for 15 or 120 min (B, C) or 100^{o}C for 15 min (D). The sample heat treated at 100^{o}C for 120 min (E) showed very faint IgE binding to the 50 kDa band. However, when the pellets were extracted with non-reducing Laemmli buffer, strong IgE binding was also observed to the 50 kDa protein band in the same samples. With all other extraction buffers, very faint binding was seen to the three bands (1, 2 and 3) for the 100^{o}C, 120 min heat treated sample. This indicates that non-reducing Laemmli buffer is efficient in extracting proteins from the pellets that were insoluble and not present in the supernatant compared to the buffers containing 0.5 M NaCl or Tween 20. Under non-reducing conditions, strong IgE binding was observed to higher molecular weight protein bands ranging from 50 to 150 kDa for both the control and all of the heat treated samples in the supernatants, indicating that no apparent changes occurred in IgE binding epitopes of these proteins due to heat processing. Only a slight reduction in IgE binding was seen for sample heat treated at 100^{o}C for 120 min. However, IgE binding was observed to a 25 kDa protein band (band 4) only in the control (unheated) supernatant indicating that this protein epitope is labile under all of the heat treatment conditions used in this study. Interestingly, a faint IgE binding band was

observed for supernatant samples at approximately 20 kDa (band 5) for all of the heat treatments, but not the control sample, indicating a new epitope may have been created under heat treatment, or alternatively that the protein was dissociated from other components in the soybean matrix. The second possibility seems plausible since extraction of the pellets with the salt containing buffer produced a faint IgE binding band at 20 kDa, which also became visible in the control sample indicating differential solubility of the protein rather than creation of a new epitope by heat treatment.

Immunoblots applying the same serum (19392-CS) to the extracts of the 50% soy flour samples (Figure 6A) showed high intensity IgE binding to the 35, 50 and75 kDa bands under reducing conditions for both the control (A) as well as the samples heat treated at 80^{o}C for 15 (B) and 120 min (C). Although IgE binding to the 75 kDa and the 50 kDa bands was reduced for the sample heated at 100^{o}C for 15 min (D) and extracted with the NaCl and Tween 20 containing buffers, intense IgE binding was observed to both the bands when the sample was extracted with non-reducing Laemmli buffer. This observation again indicated better extraction efficiency for the non-reducing Laemmli buffer. For the sample heat treated at 100^{o}C for 120 min (E), a reduction in IgE binding was observed to all of the three bands. Under the non-reducing condition, strong IgE binding to the larger protein bands (50 to 150 kDa) in the control samples were also drastically reduced when the sample was heat treated at 100^{o}C for 120 min.

The immunoblotting results of serum 20247-LA with the 5% soy flour samples (Figure 5B) showed strong IgE binding to a 35 kDa protein band (band 1) under reducing conditions for both the control and heat treated samples in the supernatant as well as in all

of the three extraction buffer extracted pellets. The intensity of IgE binding to a protein band of 25 kDa (band 3) was reduced as the heat treatment times were increased. In the supernatants, strong IgE binding was observed to two protein bands of approximately 30 and 22 kDa (band 2 and 4 respectively), which were visible only in the heat treated and not in the control samples. Pellets extracted with Tween 20 and salt containing buffers also showed intense IgE binding to the 30 kDa band (band 2) only in the heat treated sample. However, when the pellets were extracted with non-reducing Laemmli buffer IgE binding was seen to both band 2 and 4 in the control sample as well. Strong IgE binding was observed to band 4 in the control sample when pellets were extracted with salt containing buffer. These results again indicate that differential solubility of proteins in different extraction buffers is responsible for the apparent differences in IgE binding. Under non-reducing conditions, IgE binding to most protein bands observed in the control sample were reduced in samples with increased heat treatments. However, strong IgE binding to the 30 kDa band (band 5) was observed only in the sample heat treated at 80^{o}C for 15 mins (lane B). This may indicate creation of a potential new IgE binding epitope in that particular protein due to heat treatment. IgE binding to this band was observed in four out of eight sera.

The IgE binding pattern of serum 20247-LA with the 50% soy flour samples differed from that observed for the 5% heat treated samples (Figure 6B). Under reducing condition, intense IgE binding was observed to four bands of approximately150, 70, 30 and 20 kDa (bands 1, 2, 3, 4 respectively) with both the control (lane A) and the 80^{o}C 15 min (B) heat treated sample for all of the three extraction buffers. For sample heat treated

at 80^{o}C for 120 min (C) and for both the 100^{o}C heat treated samples (D and E) extracted with the Tween 20 and NaCl containing buffer, IgE binding to band 1 and 2 was reduced drastically and IgE binding intensity to band 3 and 4 was lower compared to the control sample. However, when the samples were extracted with non-reducing Laemmli buffer, IgE binding to all of the four bands in the heat treated samples was comparable to the control sample except for the 100^{o}C 120 min heat treated sample, which showed binding only to the 30 kDa band (band 3). Under non-reducing condition, intense IgE binding was seen to four protein bands of approximately 100, 70, 30 and 22 kDa (bands 5, 6, 7, 8 respectively) for both control (A) and the 80^{o}C 15 min (B) heat treated samples. Although IgE binding to these bands were reduced for all other heat treated samples extracted with salt and Tween 20 containing buffer, non-reducing Laemmli buffer extracted samples showed high intensity IgE binding to these bands for all samples except for the 100^{o}C 120 min heat treated sample (E).

Figure 5C shows the IgE immunoblot of serum 20431 with the 5% soy flour samples. With this serum strong IgE binding was observed to bands of molecular weights ranging from 50 to 100 kDa for both the control and heat treated supernatant as well as the pellets extracted with the three extraction buffers indicating the stability of these IgE binding proteins to the heat treatment conditions used. Additionally faint IgE binding bands (band circled in red) were observed only in the heat treated sample indicating creation of some new IgE binding epitopes in some proteins undergoing heat processing. For the 50% soy flour samples, immunoblot results with the same sera (Figure 6C) under reducing condition showed strong IgE binding to three protein bands of approximately 75, 50 and

25 kDa (bands 1, 2, 3) for the control sample (A) and comparable IgE binding to all of the three bands was observed for the 80^{o}C heat treated samples (B and C). For the samples extracted with the Tween 20 and salt containing extraction buffers, IgE binding to these bands were reduced for the 100^{o}C 15 min heat treated sample (D) and was completely abolished for the 100^{o}C 120 min heat treated sample (E). However, with the non-reducing Laemmli buffer extracted samples, IgE binding to all three bands for all of the heat treated samples were comparable to the control sample. Under non-reducing conditions, the control sample extracted with NaCl and Tween 20 containing buffer showed strong IgE binding to three protein bands of approximately of 150, 100 and 45 kDa (bands 4, 5, 6 respectively), which was reduced as the heat treatment temperature and time were increased. For the non-reducing Laemmli buffer extracted samples, although IgE binding to the 45 kDa band (band 6) was observed for the control as well as for all of the heat treated samples, strong IgE binding to the 150 and 100 kDa (bands 4, 5) bands was observed only for the 80^{o}C heat treated samples (B and C). These differences in IgE binding patterns among the non-reducing Laemmli buffer and NaCl containing buffer again indicated the role of differential solubility of some proteins in salt containing buffer (better at extracting proteins from control samples) and non-reducing Laemmli buffer (better at extracting proteins from heat treated samples).

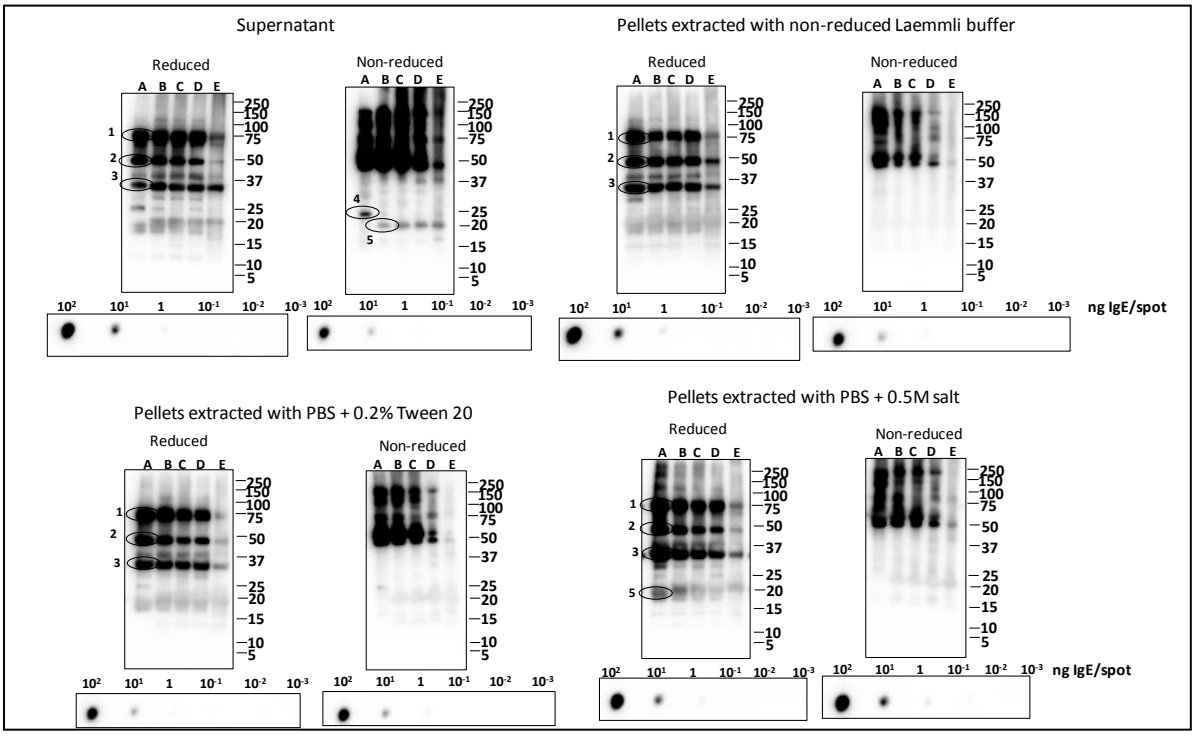

Figure 5A. IgE immunoblot of 5% w/v soy flour dispersed in PBS and heat treated at various temperature and time combinations compared to the unheated control soy flour sample using serum 19392-CS. Unstained proteins from both non-reducing and reducing SDS-PAGE gels loaded with 10 µg of each protein extract were transferred onto PVDF membranes. The membranes were blocked with 5% NFDM in PBST followed by incubation with 1:10 dilution of plasma and 1:1000 dilution of monoclonal anti-human IgE. Images were captured using a Kodak imaging system after adding chemiluminescent substrate to the membranes. Lanes: A, control soy flour sample dispersed in PBS; B, soy flour dispersion heat treated at 80^{o}C for 15 min; C, soy flour dispersion heat treated at 80^{o}C for 120 min; D, soy flour dispersion heat treated at 100^{o}C for 15 min; E, soy flour dispersion heat treated at 100^{o}C for 120 min.

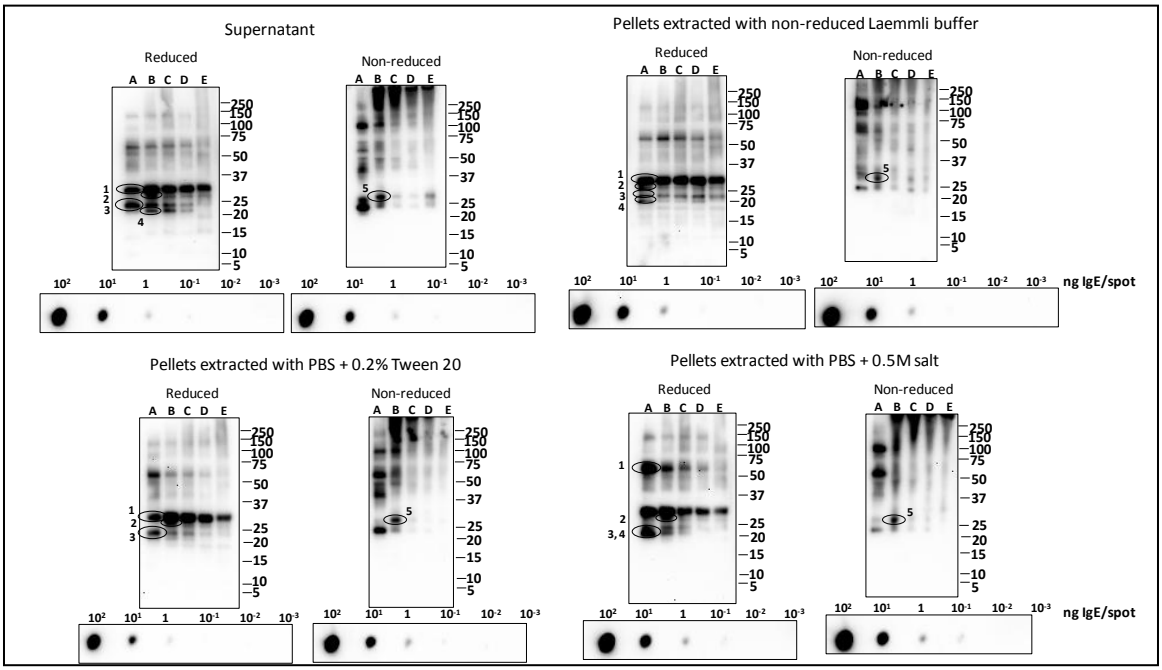

Figure 5B. IgE immunoblot of 5% w/v soy flour dispersed in PBS and heat treated at various temperature and time combinations compared to the unheated control soy flour sample using serum 20247-LA. Unstained proteins from both non-reducing and reducing SDS-PAGE gel loaded with 10 µg of each protein extract were transferred onto PVDF membranes. The membranes were blocked with 5% NFDM in PBST followed by incubation with 1:10 dilution of plasma and 1:1000 dilution of monoclonal anti-human IgE. Images were captured using a Kodak imaging system after adding chemiluminescent substrate to the membranes. Lanes: A, control soy flour sample dispersed in PBS; B, soy flour dispersion heat treated at 80^{o}C for 15 min; C, soy flour dispersion heat treated at 80^{o}C for 120 min; D, soy flour dispersion heat treated at 100^{o}C for 15 min; E, soy flour dispersion heat treated at 100^{o}C for 120 min.

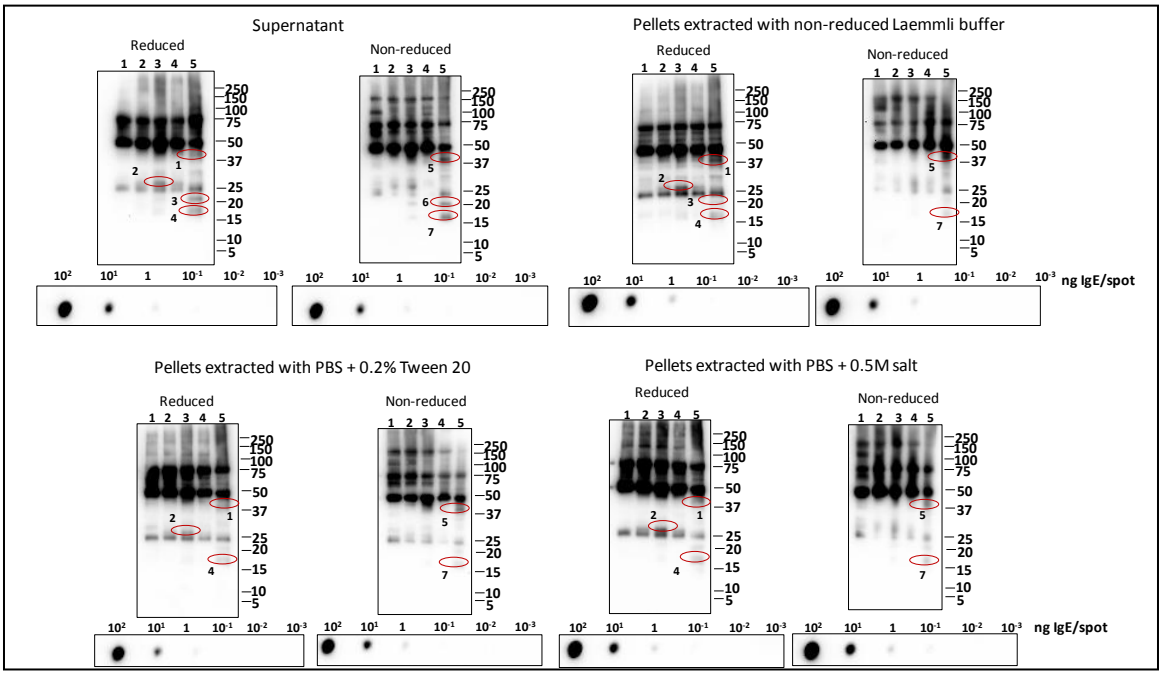

Figure 5C. IgE immunoblot of 5% w/v soy flour dispersed in PBS and heat treated at various temperature and time combinations compared to the unheated control soy flour sample using serum 20431. Unstained proteins from both non-reducing and reducing SDS-PAGE gel loaded with 10 µg of each protein extract were transferred onto PVDF membranes. The membranes were blocked with 5% NFDM in PBST followed by incubation with 1:10 dilution of plasma and 1:1000 dilution of monoclonal anti-human IgE. Image was captured using Kodak imaging system after adding chemiluminescent substrate to the membranes. Lanes: A, control soy flour sample dispersed in PBS; B, soy flour dispersion heat treated at 80^{o}C for 15 min; C, soy flour dispersion heat treated at 80^{o}C for 120 min; D, soy flour dispersion heat treated at 100^{o}C for 15 min; E, soy flour dispersion heat treated at 100^{o}C for 120 min.

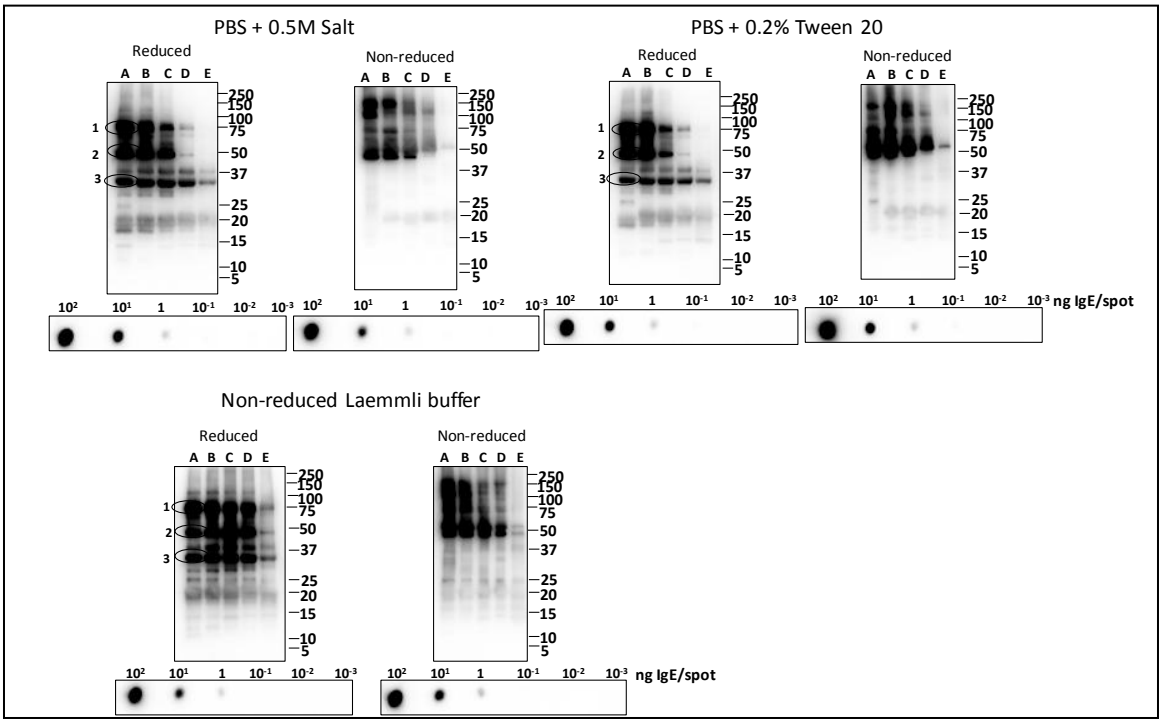

Figure 6A. IgE immunoblot of 50% w/v soy flour dispersed in PBS and heat treated at various temperature and time combinations compared to the unheated control soy flour sample using serum 19392-CS. Unstained proteins from both non-reducing and reducing SDS-PAGE gel loaded with 10 µg of each protein extract were transferred onto PVDF membranes. The membranes were blocked with 5% NFDM in PBST followed by incubation with 1:10 dilution of plasma and 1:1000 dilution of monoclonal anti-human IgE. Image was captured using Kodak imaging system after adding chemiluminescent substrate to the membranes. Lanes: A, control soy flour sample dispersed in PBS; B, soy flour dispersion heat treated at 80^{o}C for 15 min; C, soy flour dispersion heat treated at 80^{o}C for 120 min; Lane D, soy flour dispersion heat treated at 100^{o}C for 15 min; Lane E, soy flour dispersion heat treated at 100^{o}C for 120 min.

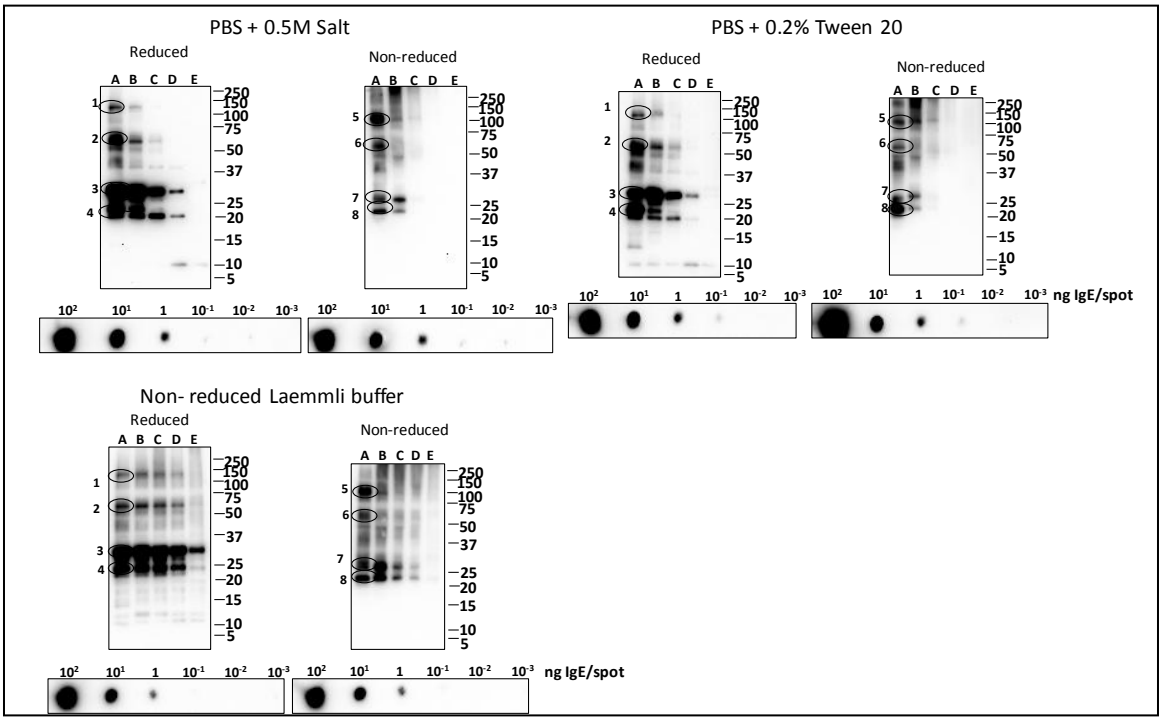

Figure 6B. IgE immunoblot of 50% w/v soy flour dispersed in PBS and heat treated at various temperature and time combinations compared to the unheated control soy flour sample using serum 20247-LA. Unstained proteins from both non-reducing and reducing SDS-PAGE gel loaded with 10 µg of each protein extract were transferred onto PVDF membranes. The membranes were blocked with 5% NFDM in PBST followed by incubation with 1:10 dilution of plasma and 1:1000 dilution of monoclonal anti-human IgE. Images were captured using Kodak imaging system after adding chemiluminescent substrate to the membranes. Lanes: A, control soy flour sample dispersed in PBS; B, soy flour dispersion heat treated at 80^{o}C for 15 min; C, soy flour dispersion heat treated at 80^{o}C for 120 min; Lane D, soy flour dispersion heat treated at 100^{o}C for 15 min; Lane E, soy flour dispersion heat treated at 100^{o}C for 120 min.

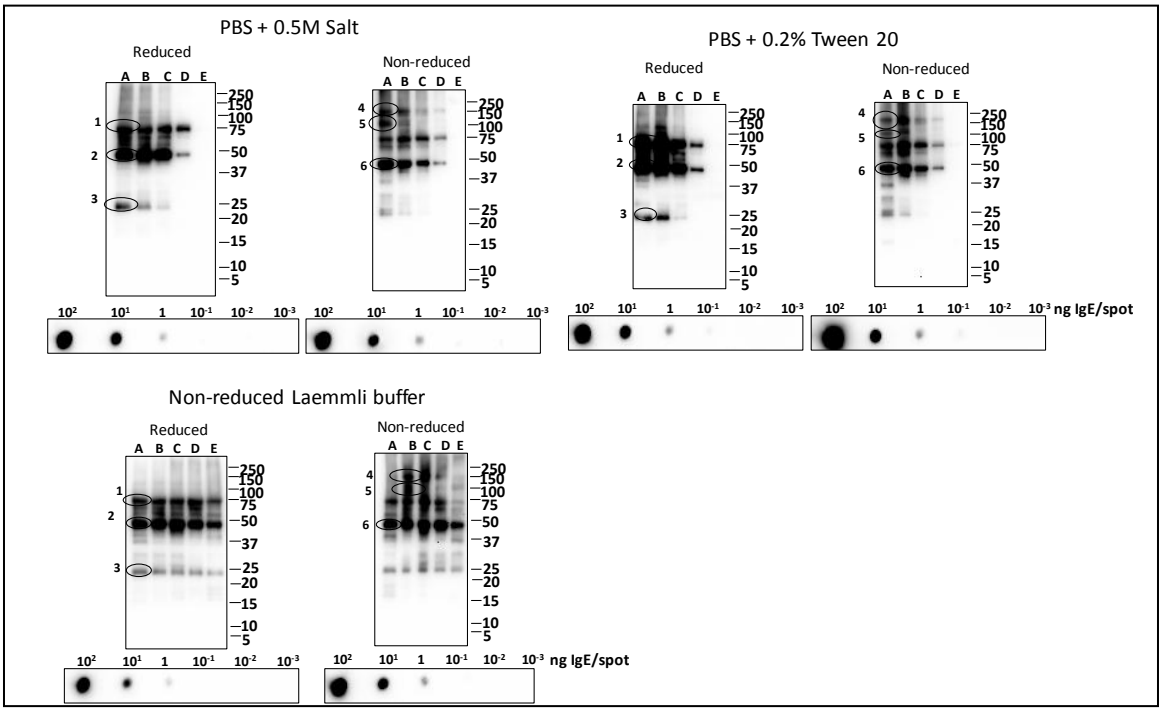

Figure 6C. IgE immunoblot of 50% w/v soy flour dispersed in PBS and heat treated at various temperature and time combinations compared to the unheated control soy flour sample using serum 20431. Unstained proteins from both non-reducing and reducing SDS-PAGE gel loaded with 10 µg of each protein extract were transferred onto PVDF membranes. The membranes were blocked with 5% NFDM in PBST followed by incubation with 1:10 dilution of plasma and 1:1000 dilution of monoclonal anti-human IgE. Images were captured using Kodak imaging system after adding chemiluminescent substrate to the membranes. Lanes: A, control soy flour sample dispersed in PBS; B, soy flour dispersion heat treated at 80^{o}C for 15 min; C, soy flour dispersion heat treated at 80^{o}C for 120 mins; Lane D, soy flour dispersion heat treated at 100^{o}C for 15 min; Lane E, soy flour dispersion heat treated at 100^{o}C for 120 min.

To evaluate the biological significance of the IgE binding differences visible in immunoblots among the heat treated and control soybean flour samples, a mediator release assay was performed with serum 19392-CS, the highest IgE binding human sample. The hRBL cells were sensitized with 1:10 dilution of serum 19392-CS followed by challenges with different concentrations of the antigen extracts diluted in the antigen challenge buffer. Figure 7 and 8 shows the β-hexosaminidase release as a percentage of total serum IgE release (obtained by sensitizing the cells with serum and challenging with anti-IgE) from the hRBL cells with the 5% and 50% heat treated and control soy flour samples respectively extracted with non-reducing Laemmli buffer and NaCl containing extraction buffer. For the 5% soy flour samples, comparing the mediator release at 0.1 µg/ml of antigen concentration, it was observed that the control supernatant as well as the supernatant of samples heat treated at 80^{o}C for 15 and 120 min showed a mediator release of approximately 60%. Both these heat treated sample pellets extracted with non-reducing Laemmli buffer also showed a similar mediator release as the control pellet (approximately 60%) However, a reduced mediator release was observed for the 80^{o}C heat treated sample pellets compared to the control sample pellets extracted with the NaCl containing buffer (approximately 25% vs 45%). For both the supernatant and the pellets extracted with NaCl containing buffer, the 100^{o}C, 15 min heat treated sample gave a very low mediator release (≤ 20%); whereas the same sample extracted with non-reducing Laemmli buffer resulted in a release of approximately 35%. These observations indicate that the samples heat treated at 80^{o}C for 15 min and 120 min and the sample heat treated at 100^{o}C for 15 min still retain IgE binding and apparent allergenicity. In accordance with the immunoblot results (Figure 5A), the 100^{o}C 120 min heat treated

samples (both the supernatant and sample pellets extracted with NaCl containing buffer and non-reducing Laemmli buffer) resulted in a lower mediator release (< 10%) compared to the control sample. With the 50% soy flour, the control as well as all the heat treated samples except for the 100^{o}C 120 min heat treated sample gave a mediator release ranging from 25-60% for both the NaCl containing buffer and non-reducing Laemmli buffer extracts. Although the 100^{o}C 120 min heat treated sample showed a release of less than 10% when extracted with the NaCl containing buffer, release from the same sample extracted with non-reducing Laemmli buffer was comparable to the other samples (approximately 40%). These results indicate that the 100^{o}C 120 min heat treated sample still retains allergenicity even though a lower IgE binding was observed with the same sample in immunoblot (Figure 6A).

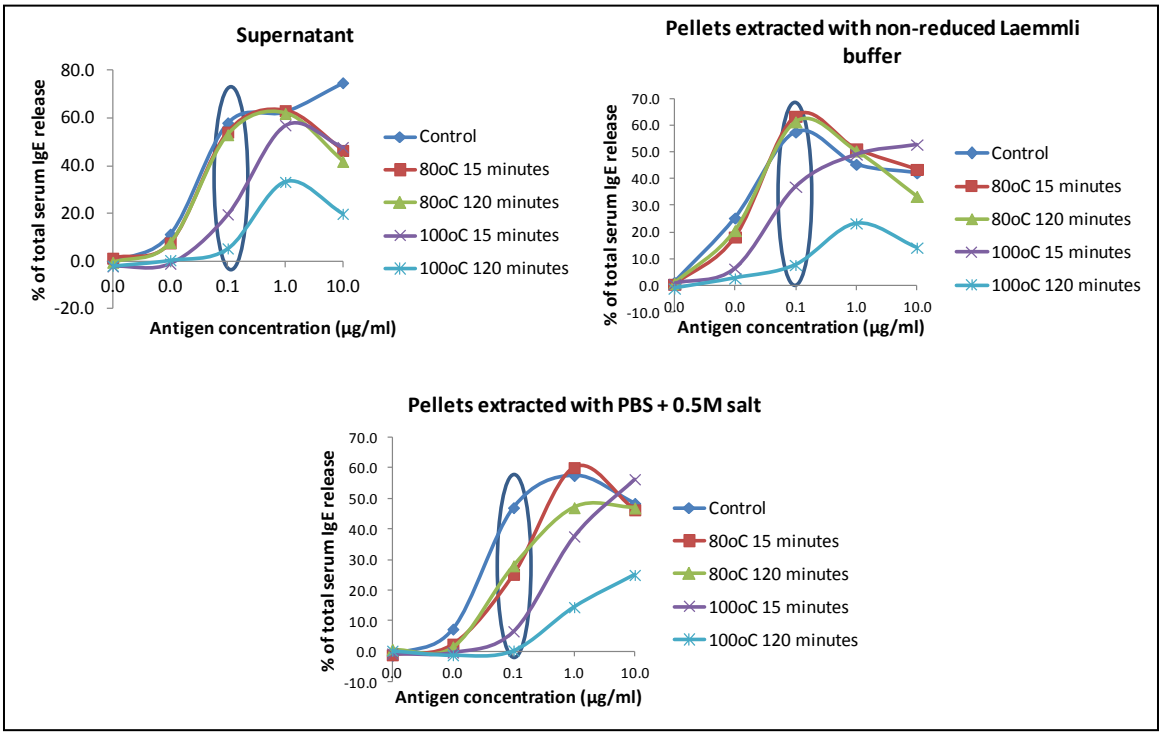

Figure 7. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with 5% w/v soy flour suspension control and heat treated samples. Humanized RBL-clone 703/21 were sensitized with IgE from serum 19392-CS (diluted 1: 10) and were challenged with 100 µl of 5% w/v soy flour suspension control samples as well as the sample heat treated at 80^{o}C and 100^{o}C for 15 and 120 min. 0.001 µg/ml to 10 µg/ml of antigen was used to challenge the cells as shown in the graph. Absrobance values were measure at 405 nm and β-hexosaminidase release was expressed as percentage of total serum IgE release.

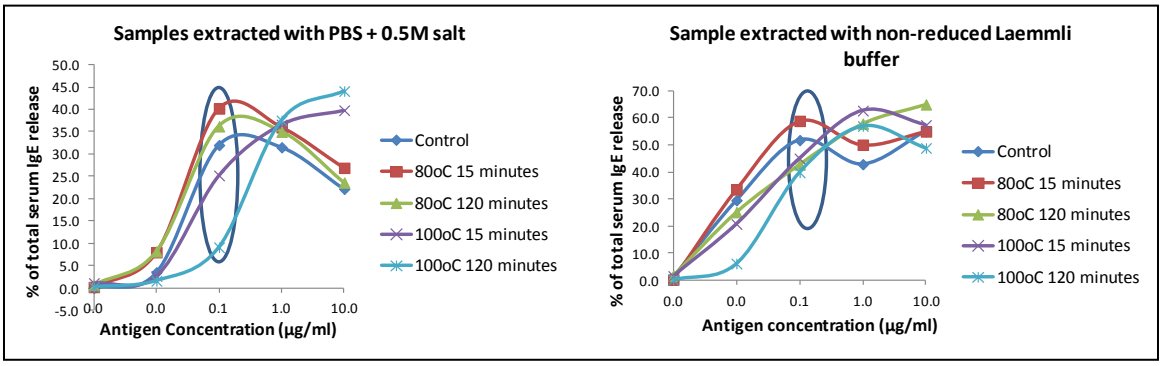

Figure 8. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with 50% w/v soy flour suspension control and heat treated samples. Humanized RBL-clone 703/21 were sensitized with IgE from serum 19392-CS (diluted 1: 10) and were challenged with 100 µl of 50% w/v soy flour suspension control samples as well as the sample heat treated at 80^{o}C and 100^{o}C for 15 and 120 min. 0.001 µg/ml to 10 µg/ml of antigen was used to challenge the cells as shown in the graph. Absrobance values were measure at 405 nm and β-hexosaminidase release was expressed as percentage of total serum IgE release.

Dry and oil roasting of soybean seeds

Three different extraction buffers (PBS + 0.5M salt, non-reducing Laemmli buffer and PBS + 0.2% Tween 20) were used to extract proteins from the dry and oil roasted soybean seed samples. For both samples, as the heat treatment was prolonged the soluble protein content of the samples was reduced. Out of all of the three extraction buffers, the non-reducing Laemmli buffer resulted in a higher recovery of soluble proteins from the heat treated samples (Figure 9 and 10). The total protein content of the dry and oil roasted samples was determined by the Dumas method using a LECO instrument at University of Nebraska (Table 2 and 3). After adjusting for moisture loss during the baking process, the total protein content of all of the roasted soybean seed samples was found to be similar to the unheated control soybean sample. This observation indicates that the lower protein content observed in the Lowry assay for the roasted soybean samples is because of aggregation and denaturation of soybean proteins making them insoluble and thereby un-extractable by the extraction buffers used.

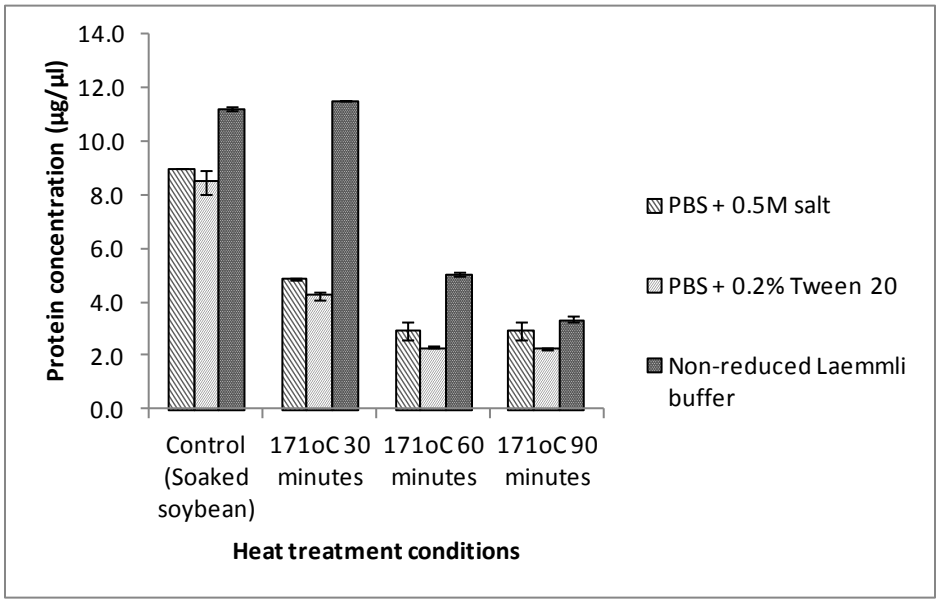

Figure 9. Soluble protein content by Lowry (µg/µl) of dry roasted soybean seeds. Soaked soybean seeds were roasted in a conventional oven at 171°C for 30, 60 and 90 min and were extracted with salt containing buffer, Tween 20 containing buffer and non-reducing Laemmli buffer.

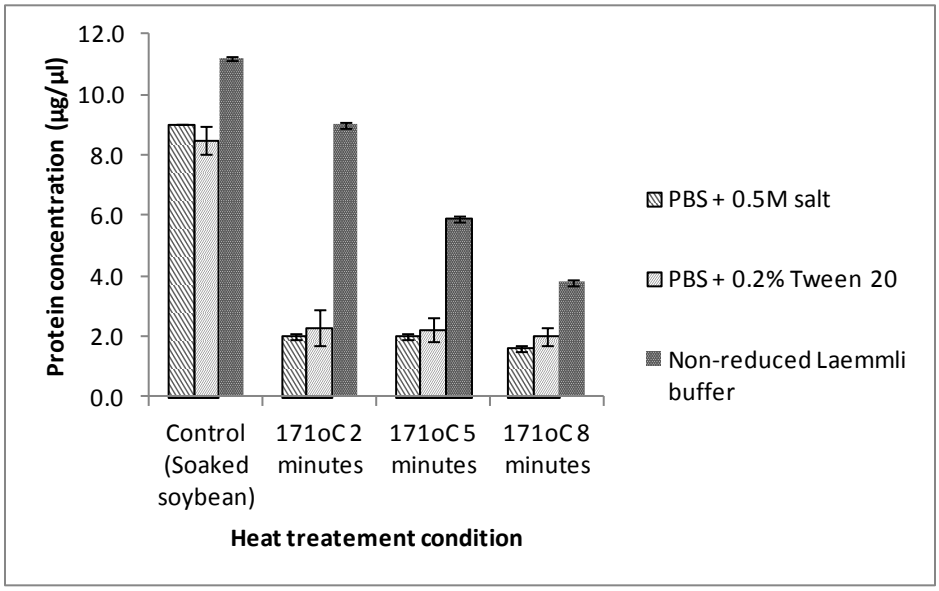

Figure 10. Soluble protein content by Lowry (µg/µl) of oil roasted soybean seeds. Soaked soybean seeds were roasted in canola oil at 171°C for 2, 5 and 8 min and were extracted with salt containing buffer, Tween 20 containing buffer and non-reducing Laemmli buffer.

Table 2. Total protein content of dry roasted soybean samples by Dumas (LECO) method

Sample	Moisture loss (%)	Protein content by LECO (%)	Adjusted protein content (%)
Soaked soybean (control)		15.7	
Dry roasted for 30 minutes	38.6	24.4	14.9
Dry roasted for 60 minutes	61.5	38.7	14.9
Dry roasted for 90 minutes	69.1	40.4	12.5

Table 3. Total protein content of oil roasted soybean samples by Dumas (LECO) method

Sample	Moisture loss (%)	Protein content by LECO (%)	Adjusted protein content (%)
Soaked soybean (control)		15.7	
Oil roasted for 2 minutes	28.7	24.1	17.2
Oil roasted for 5 minutes	55.5	29.7	13.2
Oil roasted for 8 minutes	60.7	31.3	12.3

The SDS-PAGE profile of the dry and oil roasted soybean samples showed a number of differences compared to the control sample (Figure 11 and 12). The collected soaking water collected was also separated by SDS-PAGE to find out whether there is any protein loss due to the soaking process. 10 µg of each protein was loaded unto gels under both reducing and non-reducing condition. From Figure 11 it can be observed that the soybean seeds that were dry roasted for 30 min (lanes D and M) showed a similar protein profile as the control (soaked soybean) sample (C and L) except for disappearance of a few high molecular weight protein bands. Although the seeds dry roasted for 60 min (E and N) showed considerable reduction in protein band intensity compared to the control samples when extracted with Tween 20 or NaCl containing buffer, non-reducing Laemmli buffer resulted in better protein extraction from the same sample. For the 90 min dry roasted sample (F and O), no protein bands were observed in SDS-PAGE with any of the three extraction buffers. With all three extraction buffers, some new protein bands (bands 5, 2, 3) appeared in the heat treated sample. Although the overall protein extraction was better with the non-reducing Laemmli buffer, some protein bands were better extracted by the NaCl containing buffer compared to the non-reducing Laemmli buffer (e.g. band 5 and 3 under non-reducing condition). The SDS-PAGE protein profile of the samples extracted with the Tween 20 containing buffer was found to be comparable to that of the NaCl containing buffer. A protein band of approximately 10 kDa (band 1) was observed in the soaking water (B) under reducing condition. A similar molecular weight band (band 6) was also observed in the 30 min dry roasted sample (D) when the gel was run under reducing condition. Under non-reducing condition a 12 kDa band (band 4) was observed only in the soaking water (K).

For the oil roasted soybean samples (Figure 12), again the non-reducing Laemmli buffer was found to be better at extracting proteins from the 2 min (lane C and K) and 5 min (D and L) oil roasted samples compared to the other two extraction buffers. However, a reduction in band intensity was observed for the 8 min oil roasted sample (E and M). Similar to dry roasted soybean samples, new protein bands (bands 1, 2, 4, 5) also appeared in the oil roasted samples.

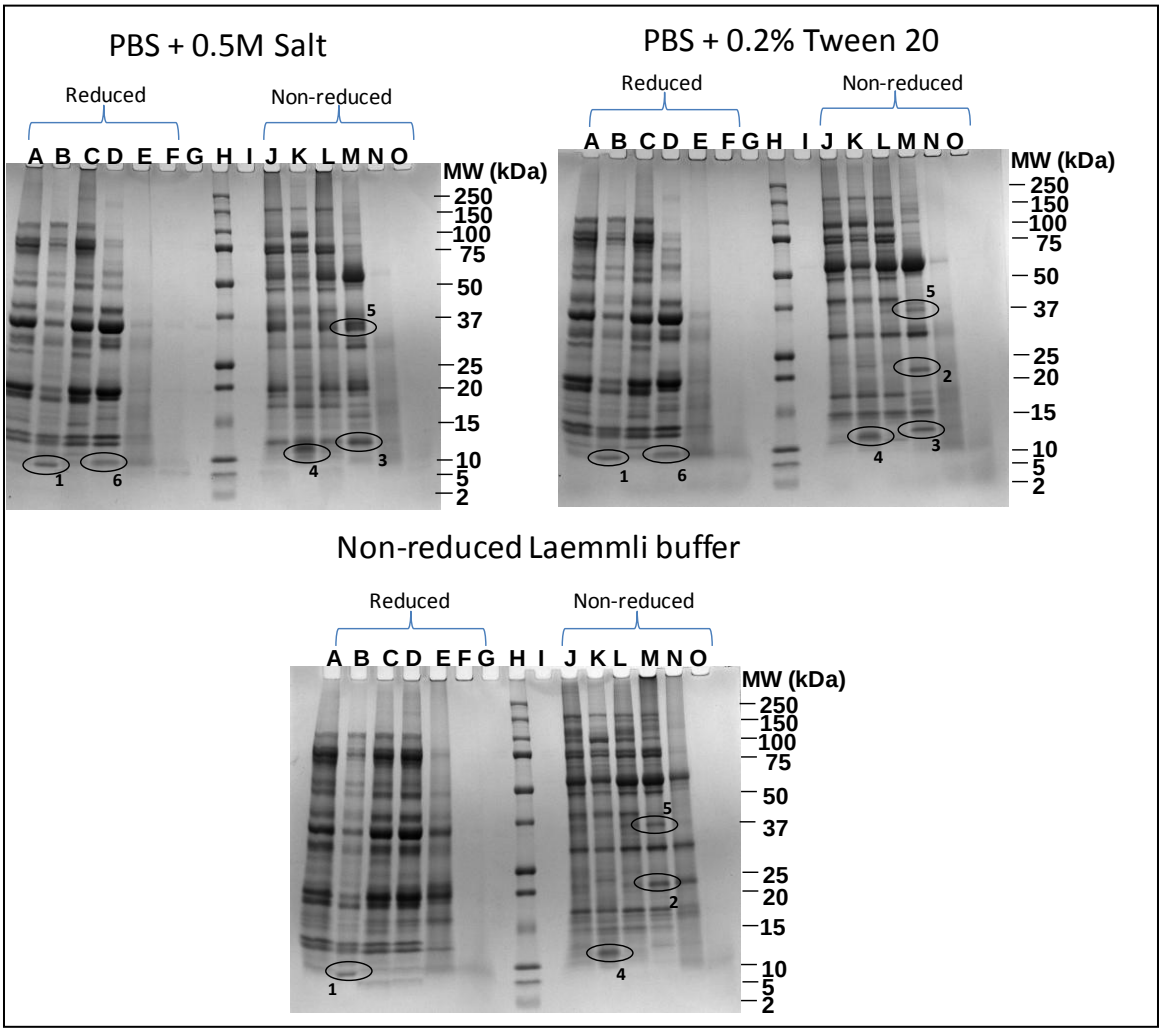

Figure 11. SDS-PAGE stained gels of dry roasted soybean seed samples compared to the control samples extracted with 0.01M PBS containing 0.5M salt, 0.01M PBS containing 0.2% Tween 20 and non-reducing Laemmli buffer. Samples (10 μg protein/lane) were run under both reducing and non-reducing conditions, then gels were fixed and stained with Brilliant Blue G-colloidal stain. .Lanes: A, whole soybean flour extract; B, soaking water; C, soaked soybean (control); D, dry roasted at 171^{o}C for 30 min; E, dry roasted at 171^{o}C for 60 min; F, dry roasted at 171^{o}C for 90 min; G, empty; H, molecular weight marker; I, empty; J, whole soybean flour extract; K, soaking water; L, soaked soybean (control); M, dry roasted at 171^{o}C for 30 min; N, dry roasted at 171^{o}C for 60 min; O, dry roasted at 171^{o}C for 90 min.

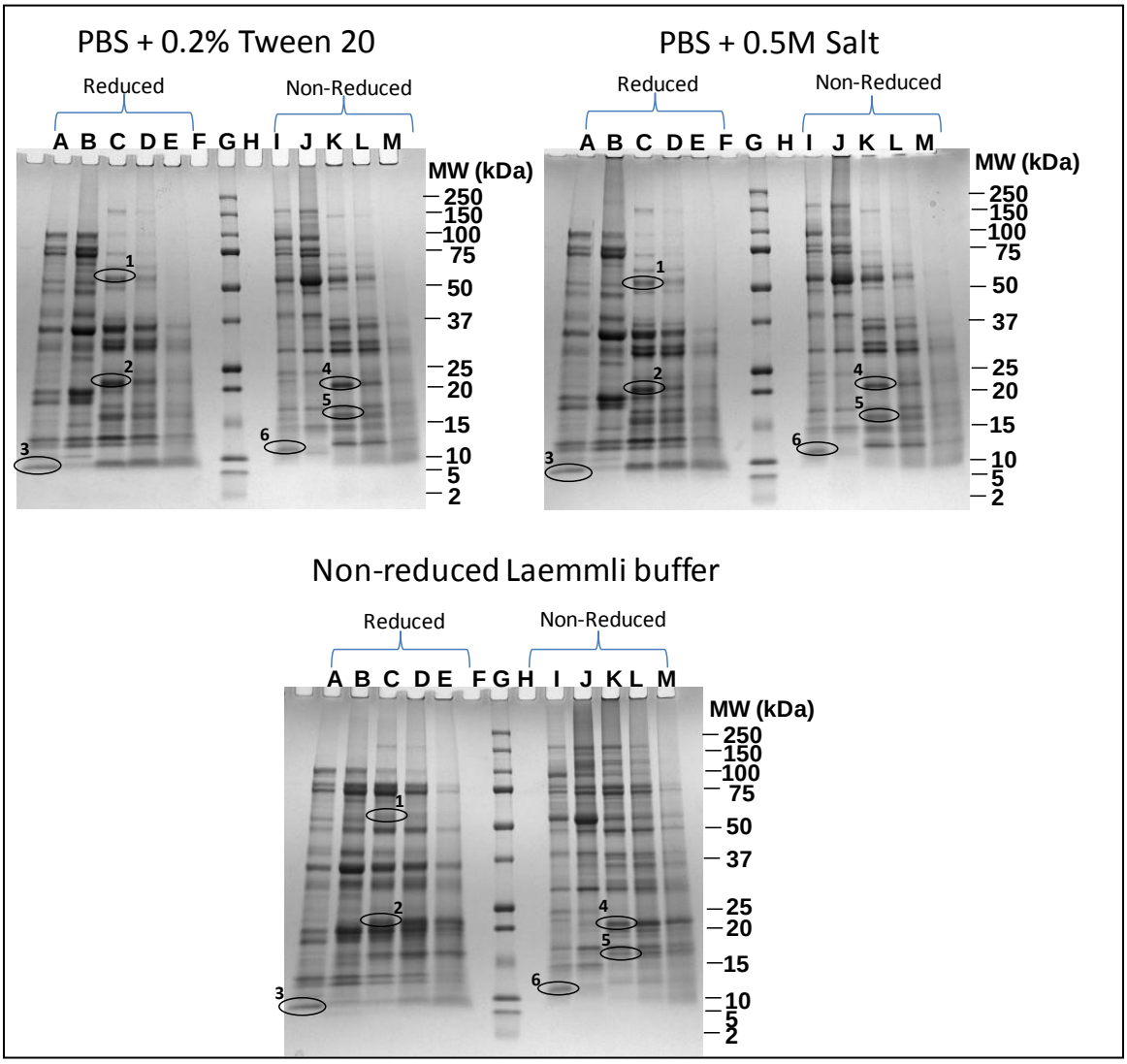

Figure 12. SDS-PAGE stained gels of oil roasted soybean seed samples compared to the control samples extracted with 0.01M PBS containing 0.5M salt, 0.01M PBS containing 0.2% Tween 20 and non-reducing Laemmli buffer. Samples (10 µg protein/lane) were run under both reducing and non-reducing conditions, then gels were fixed and stained with Brilliant Blue G-colloidal stain. Lanes: A, soaking water; B, soaked soybean (control); C, oil roasted at 171^{o}C for 2 min; D, oil roasted at 171^{o}C for 5 min; E, oil roasted at 171^{o}C for 8 min; F, empty; G, molecular weight marker; H, empty; I, soaking water; J, soaked soybean; K, oil roasted at 171^{o}C for 2 min; L, oil roasted at 171^{o}C for 5 min; M, oil roasted at 171^{o}C for 8 min.

Since both the NaCl containing as well as Tween 20 containing buffer resulted in a similar protein profile in SDS-PAGE for both the dry and oil roasted soybeans seeds, immunoblot was performed only with the non-reducing Laemmli buffer and the NaCl containing extraction buffer to find out any differences in IgE binding due to the roasting process. Figure 13 shows the immunoblots of dry roasted soybean seeds with serum 19392-CS (panel I) and serum 18534-LN (panel II). With serum 19392-CS, the 30 min dry roasted soybean seed (lane C) showed strong IgE binding comparable to the control sample (B) when the samples were extracted with either the NaCl containing or the non-reducing Laemmli buffer. The 60 min dry roasted sample (D) also showed intense IgE binding to some protein bands when the sample was extracted with non-reducing Laemmli buffer. However, the 90 min dry roasted sample (E) did not show any IgE binding with both extraction buffers. This may indicate the destruction of IgE binding epitopes or an inability of either extraction buffer to extract proteins due to insoluble aggregate formation as a result of the prolonged roasting process. With serum 18534-LN although the 30 min dry roasted sample (C) extracted with non-reducing Laemmli buffer showed strong IgE binding comparable to the control sample (B), both the 60 min (D) and 90 min (E) roasted sample showed a significant reduction in IgE binding for either extraction buffers used.

The immunoblot of the 2 min (C) and 5 min (D) oil roasted samples extracted with non-reducing Laemmli buffer showed strong IgE binding comparable to the control sample (B) with serum 19392-CS (Figure 14, panel I). The 8 min oil roasted soybean seeds (E) extracted with the same buffer showed an overall reduction in IgE binding

compared to the control sample (B); however, strong IgE binding was still observed to a band of approximately 50 kDa under non-reducing condition and two bands of 50 and 35 kDa under reducing condition. With serum 20770-MH (Figure 14, panel II), the 2 min (C) and 5 min (D) oil roasted samples showed a drastic reduction in IgE binding when extracted with NaCl containing buffer whereas the non reducing Laemmli buffer extracted samples still retained IgE binding to a 24 kDa band under non-reducing condition and two bands of molecular weight of 30 and 25 kDa under reducing condition. IgE binding to the 8 min oil roasted sample (E) was reduced drastically compared to the control sample with both extraction buffers.

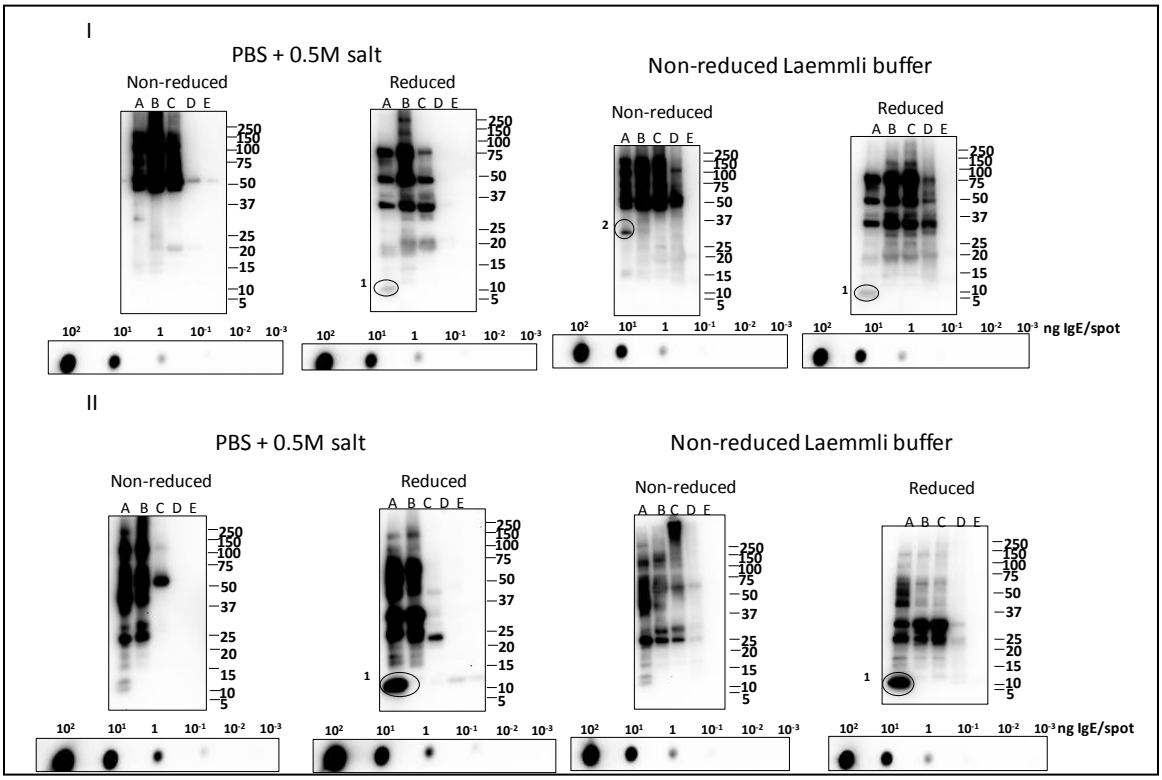

Figure 13. IgE immunoblot of dry roasted soybean seeds with serum 19392-CS (Panel I) and 18534-LN (Panel II). Unstained proteins from both non-reducing and reducing SDS-PAGE gel loaded with 10 µg of each protein extract were transferred onto PVDF membranes. The membranes were blocked with 5% NFDM in PBST followed by incubation with 1:10 dilution of plasma and 1:1000 dilution of monoclonal anti-human IgE. Image was captured using Kodak imaging system after adding chemiluminescent substrate to the membranes. Lanes: A, soaking water; B, soaked soybean seeds (control); C, soybean seeds dry roasted at 171^{o}C for 30 min; D, soybean seeds dry roasted at 171^{o}C for 60 min; E, soybean seeds dry roasted at 171^{o}C for 90 min.

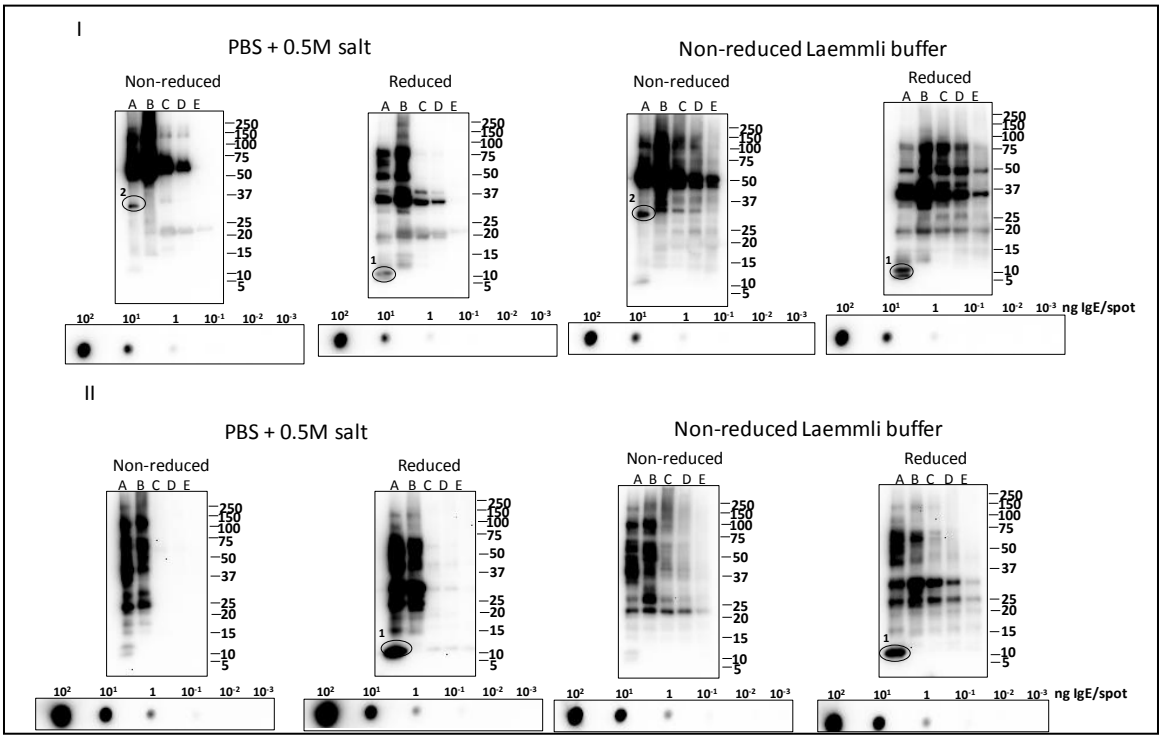

Figure 14. IgE immunoblot of oil roasted soybean seeds with serum 19392-CS (Panel I) and 20770-MH (Panel II). Unstained proteins from both non-reducing and reducing SDS-PAGE gel loaded with 10 µg of each protein extract were transferred onto PVDF membranes. The membranes were blocked with 5% NFDM in PBST followed by incubation with 1:10 dilution of plasma and 1:1000 dilution of monoclonal anti-human IgE. Image was captured using Kodak imaging system after adding chemiluminescent substrate to the membranes. Lanes: A, soaking water; B, soaked soybean seeds (control); C, soybean seeds oil roasted at 171^{o}C for 2 min; D, soybean seeds oil roasted at 171^{o}C for 5 min; E, soybean seeds oil roasted at 171^{o}C for 8 min.

With six out of eight sera used in the immunoblots, intense IgE binding at approximately 10 kDa was observed only for the soaking water (Figure 13 and 14, lane A, band 1). The identity of this IgE binding band in the soaking water was verified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) by the analytical core facility, at the University of Nebraska. The Mascot (Matrix Sciences) output search for mass identity matches indicated a match to soybean hull allergen Gly m 1, GI: 123506, from the non-redundant NCBI database (sequence with identified peptide coverage (red) shown below). This indicates that soybean hull allergen Gly m 1 is leached out of soybean into the water that was used to soak the soybean seeds before they were dry or oil roasted.

Band 1 (Soybean Gly m 1) GI: 123506

ALITRPSCPD LSICLNILGG SLGTVDDCCA LIGGLGDIEA IVCLCIQLRA LGILNLNRNL QLILNSCGRS YPSNATCPRT

The biological significance of the IgE binding observed by immunoblot analysis of the dry and oil roasted soybean seeds was evaluated by hRBL cell assay. Figure 15 shows the β-hexosaminidase release compared to the total serum IgE release from the hRBL cell for the dry roasted soybean samples using serum 19392-CS. Considering the mediator release at 1µg/ml antigen concentration, the control samples (soaked soybean) extracted with both the non-reducing Laemmli buffer and the NaCl containing buffer showed a release of approximately 50%. The soybean samples that were dry roasted for 30 min resulted in a β-hexosaminidase release comparable to or higher than the control

sample (approximately 50% when extracted with NaCl containing buffer and 70% with non-reducing Laemmli buffer). The β-hexosaminidase release from the 60 min dry roasted sample was lower (approximately 20%) than the control sample when extracted with the NaCl containing buffer. However, when extracted with non-reducing Laemmli buffer the same sample showed a release comparable to the control sample (approximately 45%). The 90 min dry roasted sample resulted in a very low β-hexosaminidase release (< 20%) with both extraction buffers. This result correlated with the IgE immunoblot finding where reduced IgE binding compared to the control sample was observed for the 90 min dry roasted sample with the same sera (Figure 13, Panel I). Figure 16 shows the β-hexosaminidase release compared to the total serum IgE release from the hRBL cell for the oil roasted soybean samples using serum 19392-CS. A high β-hexosaminidase release (approximately 35-50%) was observed from the 2 min and 5 min oil roasted samples extracted with either the NaCl containing buffer or non-reducing Laemmli buffer, although the release was lower compared to the control sample (approximately 60%). For the 8 min oil roasted sample, while the NaCl containing extraction buffer showed a release of approximately 20%, the non-reducing Laemmli buffer showed a release of approximately 30%. This reduction in mediator release by the 8 min oil roasted sample compared to other samples again correlated with the IgE immunoblot result with the same sera where IgE binding was observed only to one single protein band under the non-reducing condition and two bands under the reducing condition (Figure 14, panel I).

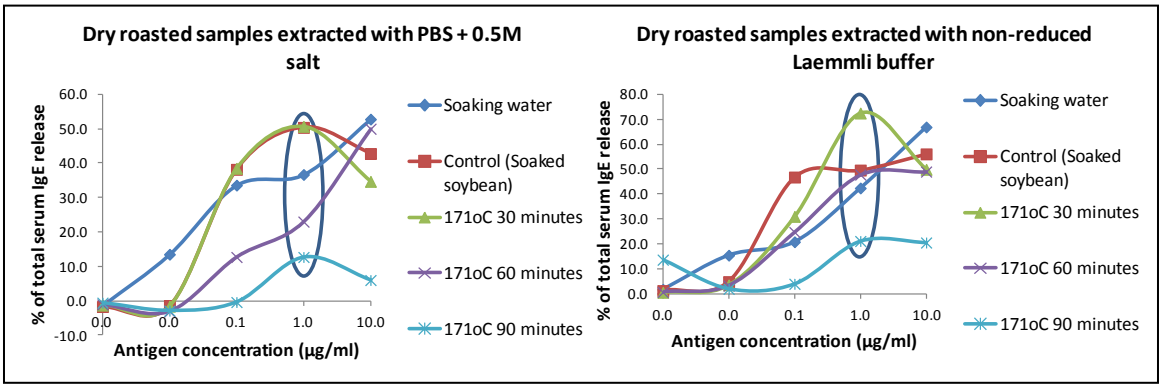

Figure 15. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with dry roasted soybean seeds and control samples. Humanized RBL-clone 703/21 were sensitized with IgE from serum 19392-CS (diluted 1: 10) and were challenged with 100 µl of individual samples. 0.001 µg/ml to 10 µg/ml of antigen was used to challenge the cells as shown in the graph. Absorbance values were measure at 405 nm and β-hexosaminidase release was expressed as percentage of total serum IgE release.

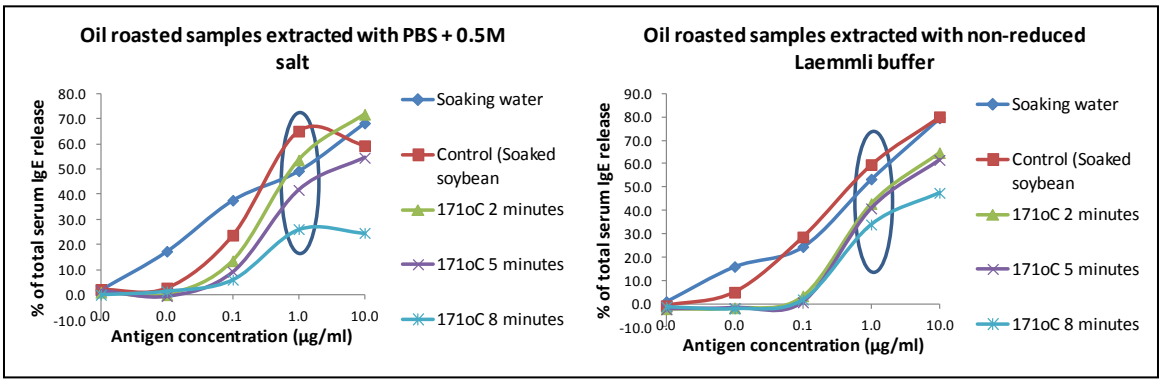

Figure 16. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with oil roasted soybean seeds and control samples. Humanized RBL-clone 703/21 were sensitized with IgE from serum 19392-CS (diluted 1: 10) and were challenged with 100 µl of individual samples. 0.001 µg/ml to 10 µg/ml of antigen was used to challenge the cells as shown in the graph. Absorbance values were measure at 405 nm and β-hexosaminidase release was expressed as percentage of total serum IgE release.

DISCUSSION

Soybean is an important source of dietary oil and protein that is gaining popularity in Asian as well as western countries. Soybean protein fractions in various processed forms are widely used in food because of their functional, nutritional properties and low cost. Some soybean ingredients are processed either by heat treatment or enzymatic hydrolysis to attain desirable functional properties or in some cases with the intent of reducing the allergenicity. Heat treatment by far is the most important process utilized during preparation of soybean food products. Heat treatment helps in removing anti-nutritional factors presents in soybean, imparts characteristic flavors to the product and improves a number of functional properties (gel forming, foaming and emulsification) of soybean proteins (Anderson and Wolf, 1995). Heat treatment is a common process involved in the preparation of soy products such as soy milk, tofu and texturized soybean products. Soybean protein products used as soy flour, soy protein isolates and concentrates are used in various baked products, yeast-leavened products, processed meat products and dairy products, have undergone various degrees of heat processing (Riaz, 1999; Singh et al., 2008; Klein et al., 1995). Keeping in mind the wide range of heat processing conditions that are encountered during the manufacturing of soybean products, it is essential to have an understanding of the impact of various heat treatment conditions on the allergenicity of soybean proteins. This could help in managing the risk of allergy to sensitive individuals in the population. Although several studies have been done in the past to investigate the effect that heat processing could have on allergenicity of soybean products (Burks et al., 1992; Shibasaki et al., 1980; Muller et al., 1998), there are several

shortcomings in the methods used in evaluating the allergenicity, such as the use of pooled sera for in vitro IgE binding studies and lack of investigation of the extractability of proteins after heat processing. The most important shortcoming is that the interpretation of allergenicity is based only on in vitro IgE binding tests such as ELISA or immunoblotting. Since results of in vitro IgE binding assays often do not correlate with food allergic reactions and can either over or under-predict allergic activity, assays confirming the biological activity of IgE such as skin prick tests or basophil histamine release should be performed to answer very specific questions about the ability of proteins to cross-link IgE on basophils and mast cells and induce release of vasoactive mediators. In our study, the allergenicity of heat processed soybean products were evaluated after extracting proteins with eight different extraction buffers and the potential allergenicity was interpreted based on the results of in vitro IgE immunoblots using individual soybean allergic patient sera as well as by a functional assay (mediator release assay using hRBL cell lines).

Most of the in vitro methods utilized to assess the presence of food specific IgE rely on soluble or extractable forms of food proteins. Heat treatment can result in denaturation and unfolding of protein leading to surface exposure of hydrophobic groups and formation of covalent complexes. This leads to decreased protein solubility/extractability. Thermal induced unfolding and aggregation of β-conglycinin has been shown to occur at a temperature above 75^{o}C and that of glycinin at a temperature above 60^{o}C (Mills et al., 2001; Mills et al., 2003). Inter and intra molecular covalent cross linking among proteins can occur during heat processing due to chemical

modification of proteins by the Maillard reaction (involving reaction of reducing sugar with free amino groups of proteins) resulting in further reduction in solubility (Schmitt et al., 2010). The use of detergents and reducing agents has been shown to maximize protein extraction from insoluble pellets (Schmitt et al., 2010). In our study, reducing and non-reducing formulations of Laemmli buffer (with or without beta-mercaptoethanol) were used for protein extraction. Our results showed a large difference in protein extraction between Laemmli buffer (non-reducing) and all other extraction buffers. Laemmli buffer was able to extract the maximum amount of protein from heat treated soybean products compared to all other extraction buffers (figure 1, 2, 9, 10). Laemmli buffer extracted samples showed higher IgE binding by immunoblot compared to the other extraction buffers and were also able to provide a higher mediator release from the heat treated samples in hRBL cell assay. Although Laemmli buffer resulted in higher overall protein solubilization, it was also observed that some proteins were extracted more efficiently by NaCl containing buffer especially from the unheated soybean samples (Figures 3B, 3C, 11, 12). This difference led to different interpretations with regard to allergenicity of heat treated products. For example, considering the immunoblot of 50% soy flour PBS dispersion, heat treated at various temperature and time combinations and incubated with serum 20431 (figure 6C), the Laemmli buffer extract showed high intensity IgE binding to two high molecular weight protein bands (bands 4, 5) only for the 80^{o}C heat treated samples. Considering this result alone, one could conclude that the heat treatment condition has augmented the allergenicity of the soybean proteins by creating new epitopes in those proteins. However, when the same samples were extracted with NaCl containing extraction buffers, IgE binding to those two high molecular weight

protein bands were also observed in the control sample. This indicates that the differential solubility of soybean proteins in the different extraction buffers is the cause of the differences in the IgE binding pattern observed in immunoblot (Figure 6C). Therefore it is essential to evaluate proteins extracted with different extraction buffers and not just a single buffer while making any interpretation on allergenicity since consumers will include both soluble and insoluble proteins. Evaluation of the more complex extracts is necessary to understand the protein mixture that the consumer is exposed to.

In general, in our study heat treatment of 5% and 50% soy flour dispersions at 80^{o}C showed IgE binding comparable to the control sample by immunoblot with eight soybean allergic sera (Figures 5, 6). The same samples also showed comparable mediator release in the hRBL assay as the control sample (Figures 7, 8). For the 50% soy flour sample heat treated at 100^{o}C for 2 hours, although a reduction in IgE binding was observed in the immunoblot, a comparable mediator release was observed as the other samples indicating that the sample still retained essentially full allergenicity (Figure 8). With the dry and oil roasted samples, except for the soybean samples that were dry roasted for 90 min, all other samples retained allergenicity as shown by a strong IgE binding in immunoblots and by high mediator release (> 20% at 1 µg/ml antigen concentration) in the hRBL assay (Figures 13, 14, 15, 16). The low IgE binding and mediator release shown by the 90 min dry roasted sample could either be due to the destruction in allergenic epitopes under high temperature or possibly that some soybean proteins after heat treatment at such a high temperature were un-extractable even with the harsh Laemmli buffer. Since both the immunoblots and hRBL assay utilized in this study

rely on soluble antigens, whether the insoluble complexes formed after heat processing will retain residual allergenicity is still a question. Double blind placebo controlled food challenge (DBPCFC) with soybean sensitive patients using whole heat processed products could give a complete answer to this question. However, the availability of willing soybean allergic patients to undergo a challenge test and lack of proper medical facility and expertise to conduct a challenge test limited the utilization of this method in this study. Nevertheless, the relevance of soluble antigen in elicitation of an allergic reaction has been shown by several studies (Roth-Walter et al., 2008; Martos et al., 2011). Although aggregated antigens are essential for allergic sensitization by their ability to enter into Peyer's patches through M cells, they are poor elicitors since from Peyer's patches they are transported into draining mesenteric lymph nodes and thereby bypass the lamina propria or fail to reach the systemic circulation, which limits their ability to contact tissue mast cells or blood basophils. It is the soluble antigens that are required for allergic provocation by their ability to transcytose across the epithelium more easily and come in contact with the sensitized lamina propria mast cells (Roth-Walter et al., 2008). Heat-induced aggregation of milk allergens has been shown to prevent their absorption through enterocytes and subsequent onset of anaphylactic reactions in mice (Roth-Walter et al., 2008). Heating of an egg allergen, ovalbumin, has also been shown to completely abrogate the transcytosis of immunologically intact forms of allergen across the epithelial monolayer due to aggregate formation (Martos et al., 2011). Therefore it is possible that dry roasting of soybean seeds for 90 min may reduce the elicitation capacity of the soybean proteins (due to an inability of the soluble portion to degranulate mast cells as shown by reduced mediator release in the hRBL assay in our study and the

probable inability of the insoluble complexes that should be formed due to heat treatment to transcytose through enterocytes to come in contact with sensitized lamina propria mast cells).

In conclusion, the results from this study showed that most of the heat processing methods that soybean proteins encounter will not affect their allergenicity. Soaking soybean seeds before processing removes soybean hull allergen Gly m 1 from soybean. Dry roasting of soybean proteins for a longer period of time (90 min in our study) may reduce their elicitation capacity as shown by a lower IgE binding by immunoblot as well as a lower mediator release by hRBL assay in this study. However, clinical challenge tests with soybean allergic individual could provide a more confirmative result. Another important finding from our study is that choice of extraction buffer while making a soluble extract could make a difference in interpretation of results from in vitro assays that utilize soluble antigens.

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CHAPTER 4: THE EFFECT OF ENZYME HYDROLYSIS ON SOYBEAN ALLERGENICITY

INTRODUCTION

Soybean proteins are modified by processing that may include heating, solvent extraction, pH adjustment, enzymatic hydrolysis or a combination of processes to produce ingredients such as soy flour and soy protein isolates (SPI) to improve their functionality as food ingredients (Singh et al., 2008; Lusas and Riaz, 1995). The concentration of allergenic proteins present in soybean and the ability to induce allergic reactions in sensitized individuals is likely to depend on the degree of processing (Kosma et al., 2010). Enzymatic hydrolysis of soybean proteins is a common process used by industry to improve functional properties and has been used to reduce allergenicity in making hypoallergenic soybean products (Host and Halken, 2004; Lusas and Riaz, 1995; L'Hocine and Boye, 2007). The reduced solubility of soybean proteins at acidic pH (close to the isoelectric point (4.5) of major seed storage protein glycinins and β-conglycinins) limits their use as functional ingredients in moderately acidic foods such as citric beverages and salad dressings. Hydrolysis of soybean proteins with proteases can increase protein solubility thereby providing functional properties that depends on protein solubility such as foaming and emulsifying properties (Molina Ortiz and Wagner, 2002; Adler-Nissen, 1976). Several enzymes have been used by investigators to improve the functionality of SPI and soy flour. Calderon de la Barca, et al. (2000) treated defatted soybean flour with chymotrypsin to improve protein solubility, emulsifying and foaming properties. In another study, SPI was treated with enzymes such as Alcalase, α-

chymotrypsin, trypsin, liquozyme and rennet to improve solubility, emulsifying capacity and the ability to undergo thermal aggregation (Kim et al., 1990). SPI has also been treated with enzymes such as papain, bromelain, and cucurbita to improve solubility and foaming properties (Molina Ortiz et al., 2000). Since enzyme hydrolysis of protein does not always lead to reduction in allergenicity and can result in an increase in allergenicity because of exposure of new antigenic epitopes due to protein breakdown, it is essential to evaluate the allergenicity of various hydrolyzed soybean products. However, there are only a few studies, where the allergenicity of a few soybean proteins (Gly m Bd 30K, Gly m Bd 28K, 11s globulin) have been investigated after enzymatic hydrolysis by in vitro IgE binding tests (Tsumura et al., 1999; Lee et al., 2007). Furthermore, the effect of hydrolysis with several other enzymes including Alcalase, papain and bromelain on the overall allergenicity of SPI or soybean flour that are commonly used to make functional soy protein products, has not been investigated.

In this study, the overall allergenicity of soybean flour and SPI treated with several enzymes commonly used to improve functionality or to make hypoallergenic products were evaluated by both IgE binding studies as well as by the basophil mediator release assay using soybean allergic patient sera. The results of this study may provide information on the efficacy of any of the enzymes in making hypoallergenic soy protein hydrolysate products.

MATERIALS AND METHODS

Sample preparation

Defatted soybean flour extract and solubilized SPI were used as starting material for enzyme hydrolysis. The soybean seeds were ground in a SPEX CertiPrep 6850 freezer mill under liquid nitrogen to make flour. The flour was then defatted using a hexane extraction method. A 1:20 (w/v) ratio of flour to hexane was placed in a shaking water bath at 50^{o}C for 30 min and the process was repeated three more times to remove lipids. Following air-drying in a chemical fume hood, the defatted soy flour was extracted with 0.01M PBS (1:10 w/v) at room temperature for 2 hours followed by clarification by centrifuging at 10,000 g for 30 min using a table top centrifuge.

Preparation of SPI from defatted soybean flour was carried out according to the procedure by Molina Ortiz and Wagner, (2002) and Sorgentini et al. (1995). Defatted soybean flour was extracted with alkaline water (1:10 w/v) at room temperature for 2 hours after adjusting the pH of water to 8.0 with 2N NaOH. After extraction the solution was centrifuged at 10,000 x g for 30 min at 4^{o}C using a table top centrifuge. The pellet was discarded and the supernatant was adjusted to a pH of 4.5 using 2N HCl. In this condition an isoelectric precipitate was formed, which was separated by centrifuging at 5000 x g for 10 min at 4^{o}C. The precipitate was resuspended in 0.01M PBS (5% w/v) and the pH was adjusted to pH 8.0 using 2N NaOH. The precipitate was dissolved by vortexing and subsequently shaking at room temperature for 1 hour, and stored at -20^{o}C. The protein content of the defatted soy flour extract and that of the SPI was determined by the Lowry method (BioRad, Hercules, CA, USA, Cat # 500-0113, reagent A, Cat #

500-0114, reagent B) using BSA (BioRad, Hercules, CA, USA, Cat # 500-0205) as a standard.

Human sera

Soybean allergic and non-soybean allergic control serum samples collected by PlasmaLab International, an FDA licensed blood collection company, were used in this study (Table 1). Use of these serum samples has been approved by the UNL Institutional Review Board. The allergic patients utilized in this study have soybean specific IgE level ranging from 3-68 kU/L as measured by ImmunoCAP® (Phadia, now Thermo Scientific, Uppsala, Sweden) or IMMULITE® (Siemens Healthcare, Erlangen, Germany), allergen-specific IgE test systems. Most of the soybean allergic subjects also reported peanut specific allergic reactions and have significant peanut specific IgE levels ranging from 15 to100 kU/L. The control subject used in this study did not report any food allergies.

Table 1. Soybean allergic and control serum used to evaluate enzyme hydrolyzed soybean proteins

Serum	Reported food allergies	Soybean-specific IgE (ImmunoCAP	Peanut-specific IgE (ImmunoCAP
18534-LN	Nuts, beans and seeds	17.30	Nd
9735-RE	Anaphylaxis to peanut, soybean, causes sore throat, itchy mouth, queasy stomach	5	58
20197-BH	Itchy throat with nuts and raw veggies	3	95
19392-CS	Angioedema, vomit, EOS G; milk, egg, meat, fruit, peaches, pears, (?profilin?)	68	15
20770-MH	Throat swelling with peanut	38	43
24033/20431	Peas, peanut, soy, lentil, sulfur drugs, garbanzo beans; anaphylactic shock from peanut, eczema, hives	Nd	Nd
23736-AM/20300	all trees, grass, peanuts, cats, rabbits	15.3	>100
20247-LA/20160	buckwheat, rice, rye, celery, lettuce, orange, crab, parsley, tomato, almond, coconut, peanut, pecan, sesame, corn, pea, whitebean, carrot, potato, wheat, oat, soybean	14.9	15.6
Control serum (RP)	No known allergies	Nd	Nd

nd- not detected

Enzyme hydrolysis of soybean samples

Five different enzymes including Alcalase® (Novozymes, Bagsvaerd, Denmark), papain, bromelain, trypsin and chymotrypsin were purchased from Sigma (Sigma-Aldrich, Saint Louis, MO) and were used to hydrolyze the SPI and defatted soy flour extract samples. The concentrations and hydrolysis conditions (pH and temperature) used for hydrolysis of SPI and soy flour extract with different enzymes are summarized in Table 2. These hydrolysis conditions were chosen based on previously published studies (Kim et al., 1990; Oritz, 2000; Cabanillas et al., 2010). Hydrolysis was carried out for 5, 15, 30 or 60 min with each enzyme and after hydrolysis the enzymes were inactivated by rapidly heating the samples at 95^{o}C for 5 min. The samples after hydrolysis were aliquoted and stored at -20^{o}C until further analysis. Two different types of control samples were used along with the hydrolyzed samples for analysis. Heat-controlled samples were prepared for SPI and soy flour extracts and incubated at the respective incubation temperatures for each enzyme along with the test samples, and then they were also heated at 95^{o}C for 5 min. In addition, untreated extracts of SPI and soy flour were analyzed along with treated samples, as unheated controls. An enzyme only control was also analyzed along with the treated and control extracts.

Table 2. Hydrolysis conditions of SPI and defatted soy flour extracts with different enzymes

Enzyme	Concentration	Hydrolysis condition
Alcalase® (Protease from *Bacillus licheniformis*, Sigma # P4860), Activity = 2.4U/g	0.2U of enzyme/gm of protein	50^{o}C and pH 8.00
Papain from papaya latex (Sigma # P4762, Activity ≥ 10 units/mg solid)	4 parts of protein mixed with 1 part of 0.168 mg/ml of enzyme solution (for SPI), 4 parts of protein mixed with 1 part of 0.197 mg/ml of enzyme solution (for soy flour)	40^{o}C and pH 8.00
Bromelain from pine apple stem (Sigma # B4882), Activity= 3-7 units/mg protein	4 parts of protein mixed with 1 part of 0.168 mg/ml of enzyme solution (for SPI), 4 parts of protein mixed with 1 part of 0.197 mg/ml of enzyme solution (for soy flour)	40^{o}C and pH 8.00
Trypsin (Sigma, Trypsin from bovine pancreas # T8003)	2% of SPI or defatted soy flour extract (w/w)	37^{o}C and pH 8.00
Chymotrypsin (Sigma, α-chymotrypsin from bovine pancreas # C4129)	2% of SPI or defatted soy flour extract (w/w)	37^{o}C and pH 8.00

1D- and 2D-PAGE and IgE immunoblotting

The hydrolyzed SPI and defatted soy flour extracts and control samples were diluted using Laemmli SDS-sample buffer (Boston BioProducts, Ashland, MA) to a allow loading 10 µg of soybean protein (10 µl /well) in SDS-PAGE gel. Samples were run under both reducing (2-mercaptoethanol and heating at ~ 95°C for 5 minutes) and non-reducing conditions using a Novex 10-20% tris-glycine gels (Invitrogen, Carlsbad, CA). A pre-stained Precision Plus molecular weight marker protein sample (BioRad, Hercules, CA, USA) was run in a separate lane to estimate protein size. Electrophoresis was accomplished at a constant 125 V for 105 min. The proteins separated in the gels were then fixed in a solution of 7% acetic acid, 40% methanol in water and stained with Brilliant Blue G-colloidal (Sigma, St Louis, MO, USA, Cat # B2025) for at least 2 hours. After staining, the gels were destained for one min in 10% acetic acid, 25% methanol in water, and then multiple changes of 25% methanol until the background was clear of blue dye.

For two dimensional (2D) PAGE, a BioRad PROTEAN IEF Cell (BioRad Hercules, CA, USA, Cat # 165-4001) was used for the first dimensional separation of the proteins based on their iso-electric points. Sample consisting of 25 µg of heated control SPI or SPI digested with Alcalase, trypsin or chymotrypsin for 60 min were diluted to 125 µl with IEF sample buffer [8M urea, 2% CHAPS, 50 mM DTT (Fisher Bioreagents, Pittsburg, PA, USA, Cat # BP172-5) and 0.5% ampholyte (BioRad, Hercules, CA, USA, Cat # 163-1112)] and then applied to individual troughs of the IEF focusing tray (BioRad, Hercules, CA, USA, Cat # 165-4030). Individual pI 3-10 linear IEF strips (BioRad, Hercules, CA, USA, Cat # 163-2000) were placed into the trough of each sample well and

focusing was carried out after covering the strip in each well with 4 ml of mineral oil (BioRad, Hercules, CA, USA, Cat # 163-2129). An active rehydration was performed at 50 V for 12 hours followed by 250 V run for 15 min, 4000 V ramping for 2 hours and finally a 4000 V limit step was used until 34,000 integrated Vhr was reached. Proteins were then maintained in position with a constant application of 500 V until morning. The strips were then equilibrated for 15 min in 2.5 ml of DTT equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 130 mM DTT) and then 15 min 2.5 ml iodoacetamide equilibration buffer (6 M urea, 2% SDS, 0.375 M Tris-HCl, pH 8.8, 20% glycerol, 135 mM iodoacetamide) for reduction and acetylation. Separation in the second dimension was carried out by placing the focused strips into the 7cm wide well and 4 µl of pre-stained Precision Plus molecular weight marker proteins into the small well of NuPAGE® Novex 4-12% Bis-Tris ZOOM® Gels (Invitrogen, Carlsbad, CA, USA, Cat # NP0330BOX). The wells were sealed with molten 0.5% agarose (Invitrogen, Carlsbad, CA, USA, Cat # 15510-019). Electrophoresis was accomplished at a constant 150 V for 60 min. Staining of proteins in the gels after electrophoresis was performed using EZBlue™ gel stain (Sigma, St Louis, MO, USA, Cat # G1041).

For immunoblots, the separated proteins from unstained gels were electro-transferred to polyvinylidene difluoride (PVDF) membranes (Invitrogen, Carlsbad, CA, USA, Cat. # LC3675) at 25 V for 90 min using Novex® transfer buffer (Invitrogen, Carlsbad, CA, USA, Cat # LC 3675). The protein transfer was verified by staining the membranes with Ponceau S stain (Sigma, St Louis, MO, USA, Cat# P7170). The membranes were then blocked with 5% non-fat dry milk (NFDM) in PBS containing

0.05% Tween 20 (PBST) for at least one hour. Individual human sera (eight individual soybean allergic and one control sera) were diluted 1:10, in 2.5% NFDM in PBST and allowed to bind to the NFDM for 1 hour before incubating with the blocked membrane for overnight at room temperature. Unbound antibody was removed from the membranes by washing four times 5 min each with PBST. Bound IgE was detected using monoclonal horse radish peroxidase (HRP) conjugated anti-human IgE (SouthernBiotech, Birmingham, AL: clone B3102E8 Cat # 9160-05), diluted 1:1000 with 2.5% NFDM in PBST. The unbound secondary antibodies were removed by washing the membranes four times with PBST. Detection was achieved using Supersignal West Dura Extended Duration substrate (Pierce, Rockford, IL, USA, Cat # 34076) and capturing emitted light with a Kodak Gel Logic 440 image station with multiple exposures. A nitrocellulose membrane (Invitrogen, Carlsbad, CA, USA, Cat # 645239) spotted with diluted purified IgE (Human IgE, monoclonal with kappa light chain, ABCAM, Inc., Cambridge, MA, Cat # AB65866-100) then blocked with 5% NFDM in PBST and incubated with the secondary antibody and substrate as immunoblots was exposed along with the immunoblots to help evaluate signal strength.

Mediator release assay using a humanized rat basophilic leukemia (hRBL) cell line

Humanized rat basophilic leukemia (hRBL) cells (RBL-703/21) were used for the mediator release assay. The procedure for maintenance of the cells and preparation of buffers for use in the assay were similar to as described in Chapter 3. For the assay, adhered cells in stationary phase were dislodged by application of 0.01 M EDTA in MEM for 30-45 min. The cells were washed twice with fresh media and diluted to a cell

density of 2.0 X 10^6 cells/ml and 50 µl (1 X 10^5 cells) was seeded into each well of a 96 well micro titer plate followed by sensitization with 50 µl of individual human plasma (diluted 1:10 with the MEM). Sensitization was carried out by incubating the plates at 5% CO_2 at 37°C for approximately 12 hours. After 12 hours incubation cells were washed with Tyrode's wash buffer twice and challenged with five different concentrations (10 µg/ml, 1 µg/ml, 0.1 µg/ml, 0.01 µg/ml and 0.001 µg/ml) of the hydrolyzed SPI and defatted soy flour extracts as well as the control extracts diluted in allergen challenge buffer. The plates were then incubated in a water bath (37°C) for 1 hour. After one hour incubation 30 µl of cell supernatant was added to 50 µl of substrate in untreated polystyrene 96-well micro plates (Thermo Scientific Nunc # 12-565-226) and the plates were incubated at 37°C in a water bath for one hour. Reactions were stopped by adding 100 µl stopping solution and the absorbance of samples was measured at 405 nm. Absorbance values of samples challenged with antigen were adjusted to a baseline reference by subtracting readings from IgE sensitized cells that were not exposed to antigen (serum negative controls). Cells sensitized by human IgE (#ab65866 from Abcam Inc., Cambridge, MA) and cross-linked by anti-human IgE (Sigma # I6284) were used as positive control. Further cells sensitized with the test serum and cross-linked by anti-IgE instead of the antigen extract were used as a positive control for total serum IgE release. Test sample readings were expressed as a percentage of total (complete) release as well as serum IgE release.

RESULTS

Hydrolysis of SPI and soy flour extract with Alcalase

Alcalase is a serine endopeptidase from *Bacillus licheniformis*. The enzyme used in this study had a specific activity of 2.4 Anson units (AU) per gram. Extracts of SPI and defatted soy flour were digested with this enzyme at a concentration of 0.2 AU/ gram of protein. After hydrolysis the samples were run under both reducing and non-reducing SDS-PAGE. Figure 1A shows the SDS-PAGE pattern of the hydrolyzed and control SPI and soy flour extracts. Both the unheated and heated control samples under reducing conditions showed multiple bands between molecular weight of 10 to 100 kDa. After hydrolysis most of the higher molecular weight bands disappeared and at the same time an increase in low molecular weight protein bands ranging from 6-10 kDa appeared in the stained gel. Intense staining to some protein bands (approximately 20 kDa and two bands at 12-13 kDa) remained in the hydrolyzed samples. The staining pattern for the proteins bands appearing in SDS-PAGE was similar for all the three time points of hydrolysis (5, 30 and 60 min) except for a slight reduction in band intensity with increase in hydrolysis time. Under non-reducing conditions, the heated control sample showed some differences in SDS-PAGE band pattern compared to the unheated control (e.g. disappearance of a band of approximately 50 kDa). All hydrolyzed samples showed a reduction in band intensity compared to the control samples. Additionally, new protein bands of approximately 6-12 kDa appeared in all the hydrolyzed samples as was evident in samples analyzed under reducing conditions.

In order to find out whether there are differences in the IgE binding patterns of protease treated samples as might be expected from stained gel patterns, immunoblotting

was performed using eight individual soybean allergic patient sera and one non-soybean allergic control sera. Figure 1B shows the IgE binding pattern of Alcalase hydrolyzed and control SPI and defatted soy flour samples with serum 19392-CS. Both the heated and unheated control samples of 5% SPI and defatted soy flour extract showed a complex pattern of IgE binding ranging from 30 kDa to 75 kDa under reducing conditions. With the SPI sample IgE binding to most of the protein bands was reduced after treatment with Alcalase. However, IgE binding was still similar for two bands of approximately 50 and 35 kDa in the sample hydrolyzed for 5 min. Furthermore, strong IgE binding to a band of approximately 20 kDa appeared following digestion. The defatted soy flour extract control samples also showed a complex pattern of IgE binding to protein bands ranging from approximately 30 to 75 kDa under reducing conditions and IgE binding to the hydrolyzed samples was reduced compared to the control samples. Similar to the 5% SPI samples, strong IgE binding also appeared to a protein at approximately 20 kDa in all hydrolyzed samples. However, unlike SPI, IgE binding to the 50 kDa band remained in all hydrolyzed samples and binding to the 35 kDa band remained in the 5 min and 30 min hydrolyzed samples. Under non-reducing conditions, for both the 5% SPI and defatted soy flour samples the intense IgE binding observed to the higher molecular weight protein bands ranging from 35 to 250 kDa in both the unheated and heated control samples was reduced when samples were treated with Alcalase, although faint IgE binding to a 50 kDa band still retained in the hydrolyzed soy flour extract (Figure 1B).

With serum 20431 (Figure 1C), under both reducing and non-reducing conditions, both the unheated and heated control SPI showed strong IgE binding to protein bands ranging

from 50- 250 kDa and to a lower molecular weight protein band of approximately 25 kDa. After treatment with Alcalase most of the IgE binding to the higher molecular weight protein bands was markedly reduced. However, IgE binding to the 25 kDa band remained in all hydrolyzed samples. For the defatted soy flour sample most of the IgE binding seen to the control samples was reduced with Alcalase treatment. IgE binding to two protein bands of 50 and 25 kDa still remained in all hydrolyzed samples and IgE binding to a band of 35 kDa appeared only in the sample hydrolyzed for 5 min, but not longer (Figure 1C). With all other sera used in the immunoblot analysis, IgE binding to both the 5% SPI and soy flour samples treated with Alcalase was markedly reduced compared to the control samples (not shown).

To evaluate whether these reductions in IgE binding as well as appearance of new IgE binding bands in the hydrolyzed samples as observed in the immunoblots have any biological significance, mediator release assays were performed using the hRBL cell line. Figure 1D shows the β-hexosaminidase release results expressed as a percent of total serum IgE (anti-IgE induced) release of the Alcalase hydrolyzed and control 5% SPI and defatted soy flour extract samples using serum 19392-CS. Results of the β-hexosaminidase release using 1µg/ml antigen concentration, it can be observed that the unheated control sample showed a release of approximately 20% for the SPI sample and 30% for the defatted soy flour extract sample. The β-hexosaminidase release was markedly reduced for the heated control (8% release for SPI and 12% for defatted soy flour extract) even though both samples showed similar IgE binding in immunoblots with the same sera (Figure 1B). SPI samples treated with Alcalase resulted in a similar mediator

release as the heated control sample except for the 60 min hydrolyzed sample, which showed a slightly lower release compared to shorter digestion samples. Similar to the hydrolyzed SPI samples all hydrolyzed defatted soy flour samples also showed a mediator release (approximately 12%) similar to the heated control indicating no effect of hydrolysis on the release.

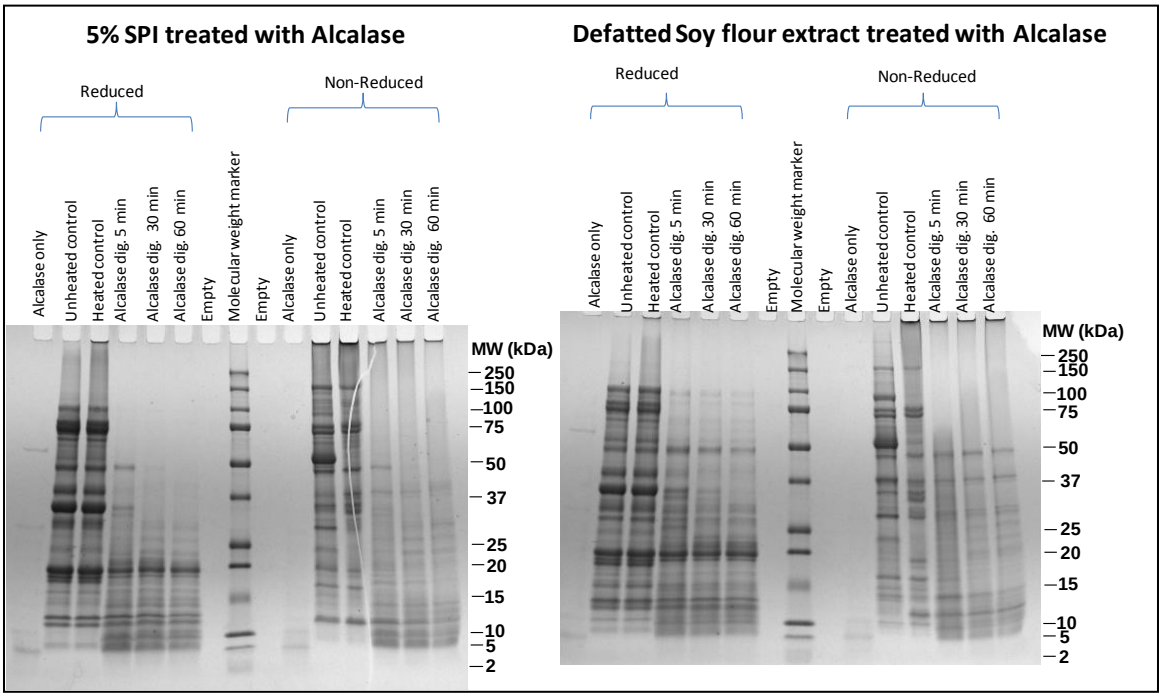

Figure 1A. SDS-PAGE stained gel of 5% SPI and defatted soy flour extracts hydrolyzed for 5, 30 or 60 min with Alcalase compared to the unheated and heated control samples. Samples (10 μg/lane) were run under both reducing and non-reducing conditions. Proteins in gels were then fixed and stained with Brilliant Blue G-colloidal stain.

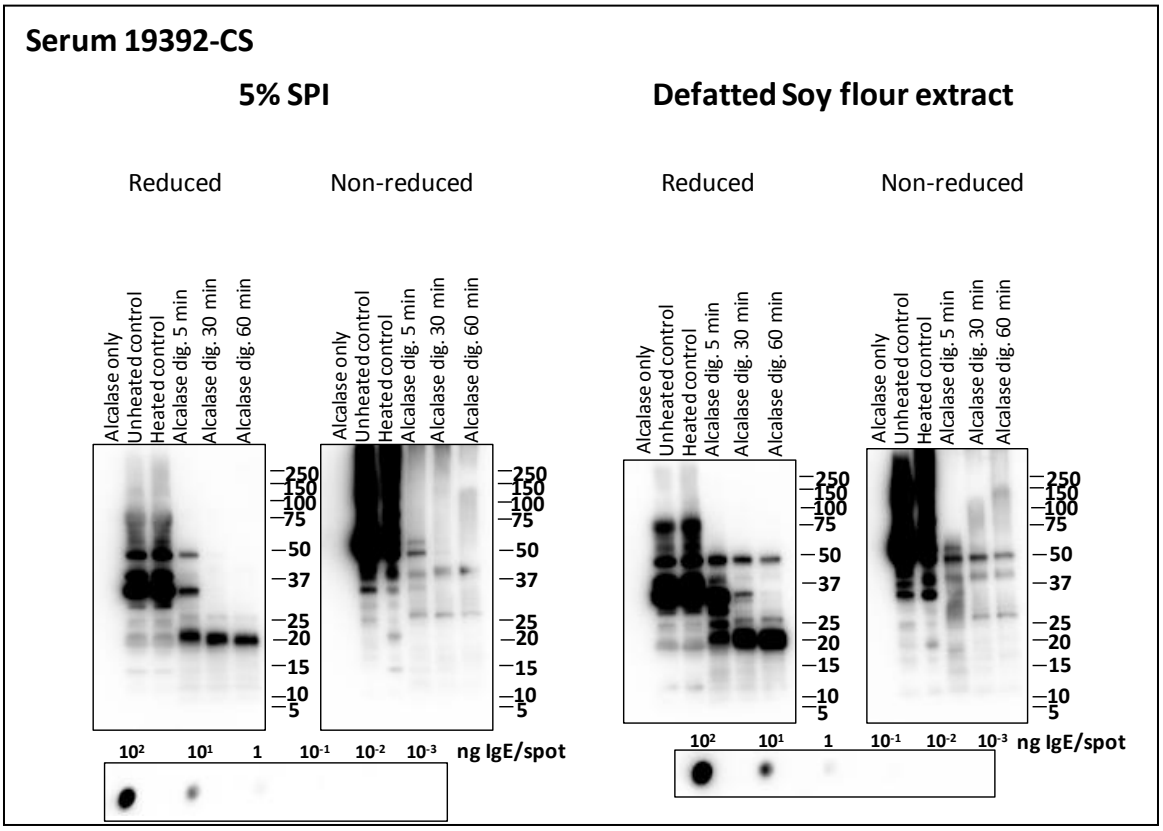

Figure 1B. IgE immunoblots of 5% SPI and defatted soy flour extract treated for 5, 30 or 60 min with Alcalase compared to the control samples, using serum 19392-CS. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

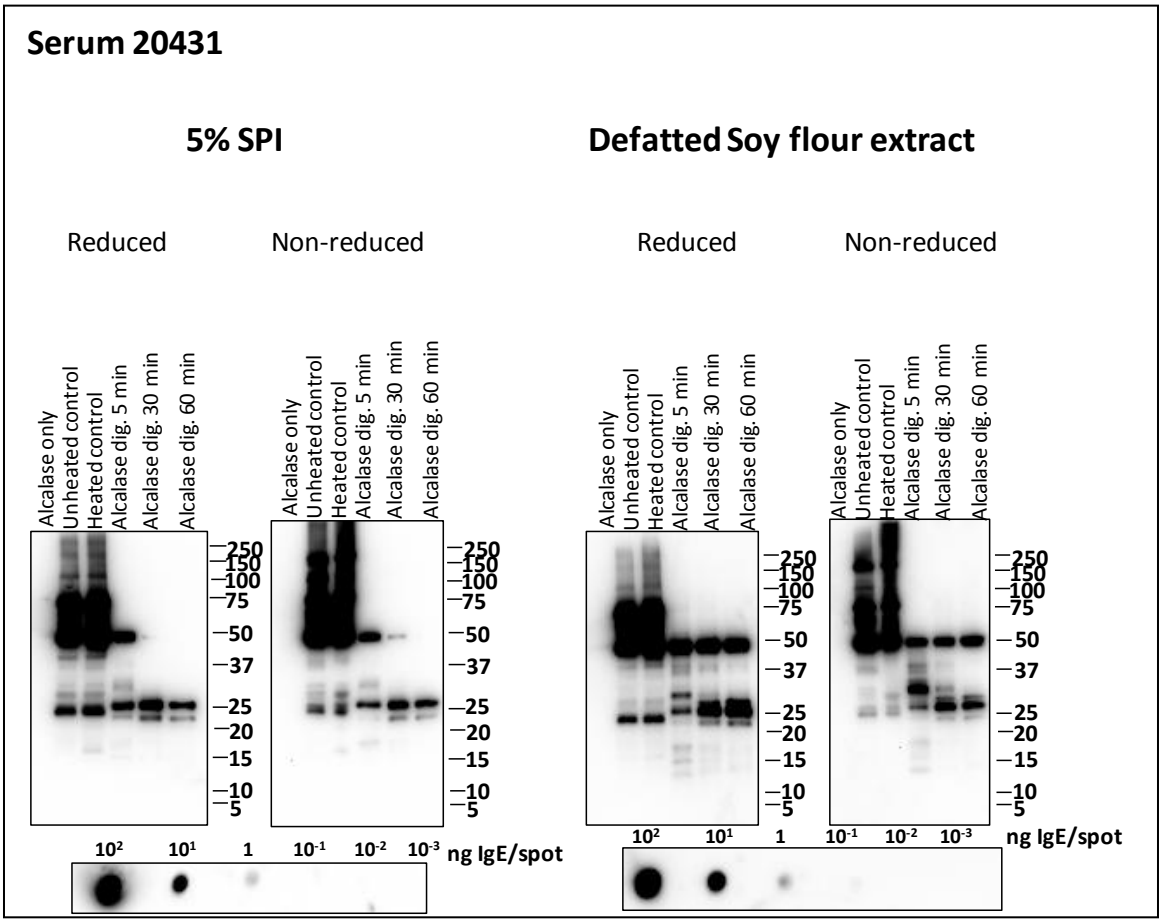

Figure 1C. IgE immunoblots of 5% SPI and defatted soy flour extract treated for 5, 30 or 60 min with Alcalase compared to the control samples, using serum 20431. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

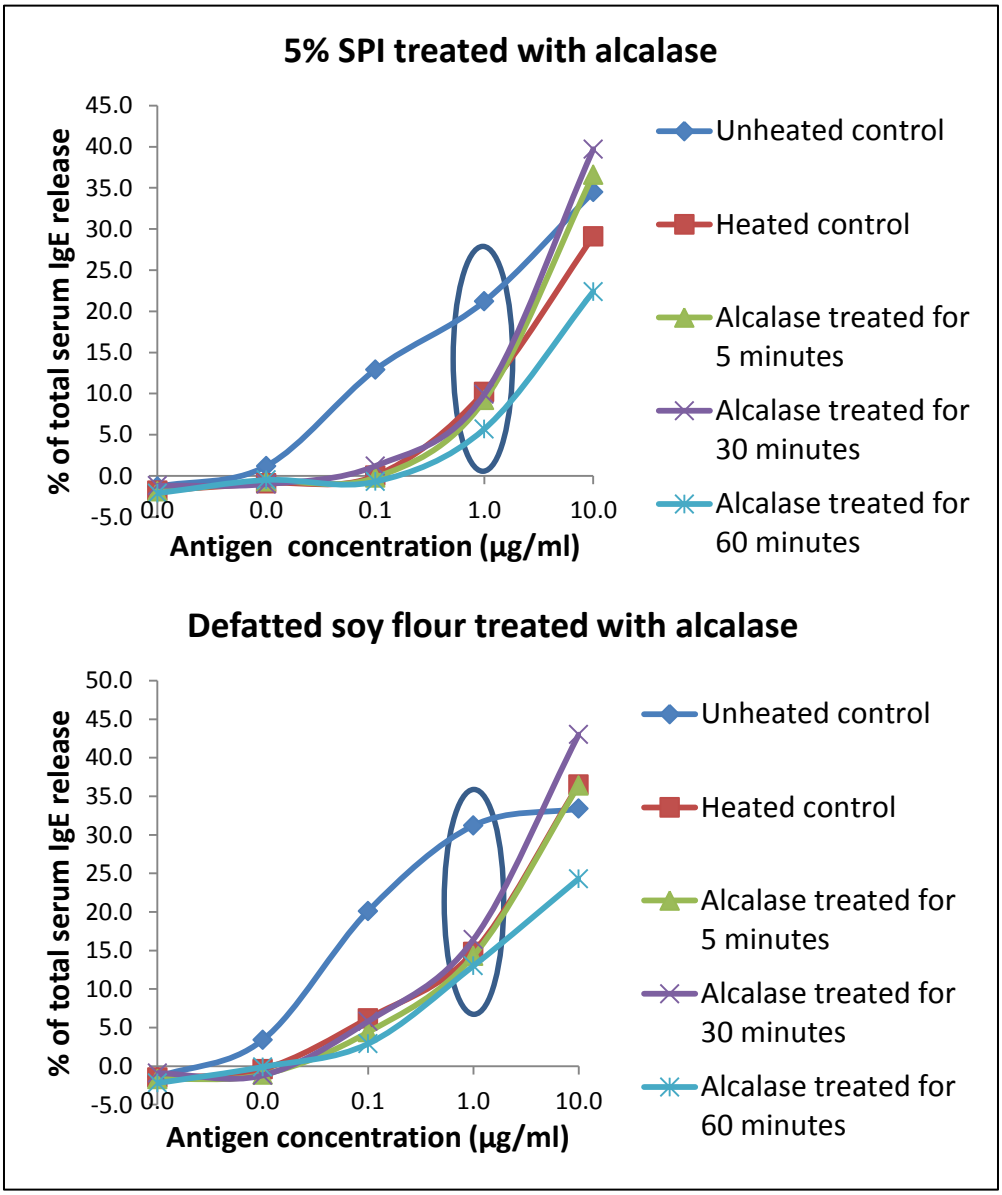

Figure 1D. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with Alcalase hydrolyzed and control SPI and soy flour extract samples. Humanized RBL-clone 703/21 cells were sensitized with IgE from serum 19392-CS (diluted 1: 10) and were challenged with 100 µl of alcalse hydrolyzed and control SPI and defatted soy flour extract samples. The challenge doses corresponding to 0.001 µg/ml to 10 µg/ml of undigested soybean antigen was used to challenge the cells as shown in the graph. Absorbance values were measure at 405 nm and β-hexosaminidase release is expressed as a percentage of total serum IgE release (cells sensitized with serum and challenged with anti-IgE)

Hydrolysis of SPI and soy flour extract with papain

Papain, a cysteine protease from papaya latex, was used to hydrolyze SPI and defatted soy flour extracts and the effect of hydrolysis on soybean protein allergenicity was evaluated. Figures 2A and 2B show the SDS-PAGE profiles of the papain digested SPI and soy flour extract samples respectively in addition to the unheated and heated control samples. Under both reducing and non-reducing conditions the unheated and heated SPI and soy flour extract control samples showed multiple protein bands ranging from 12 to 100 KDa, which were reduced in intensity and number of bands in the digested samples. Some protein bands (e.g. approximately 25 kDa and two bands at approximately 14 kDa under reducing condition) still remained visible in the digested samples and some new lower molecular weight protein bands of approximately 10 kDa appeared in all the digested samples. A slight difference in band intensity was observed among the samples digested for longer times. All samples were analyzed by IgE immunoblots using eight soybean allergic sera to evaluate differences in IgE binding patterns. Figure 2C shows the immunoblot patterns of the papain digested and control SPI and defatted soy flour extract samples with serum 19392-CS. With this serum the strong IgE binding observed to both the unheated and heated control SPI and soy flour extract samples was markedly reduced when the samples were digested with papain. Very faint binding to a 35 kDa band under reducing conditions and a 50 kDa band under non-reducing conditions remained in all hydrolyzed SPI samples. With the hydrolyzed soy flour extract, strong IgE binding was observed to the 50 kDa band under non-reducing conditions for samples that were digested with papain for 30 and 60 min and under reducing condition IgE binding to the 35 kDa band remained in all hydrolyzed samples. Figure 2D shows the IgE immunoblots of the

papain digested SPI and soy flour extract samples with serum 20431. All papain digested SPI samples showed a complete absence of IgE binding with this serum both under reducing and non-reducing conditions. However, IgE binding to a 50 kDa band remained in the digested defatted soy flour extracts. IgE immunoblots with all other sera showed a complete absence of IgE binding to the papain digested samples (not shown). A mediator release assay was used to evaluate the biological significance of IgE binding observed in blotting experiments with serum 19392-CS (Figure 2E). The β-hexosaminidase release assay with hRBL cells challenged with a dose of 1 µg/ml antigen concentration produced a release of approximately 45% using unheated control SPI and soy flour extract samples while the heated control resulted in a comparatively lower release of approximately 20%. All papain digested SPI and defatted soy flour extract samples showed β-hexosaminidase release comparable to the heated control samples in spite of showing a strong reduction in IgE binding by immunoblots (Figure 2C).

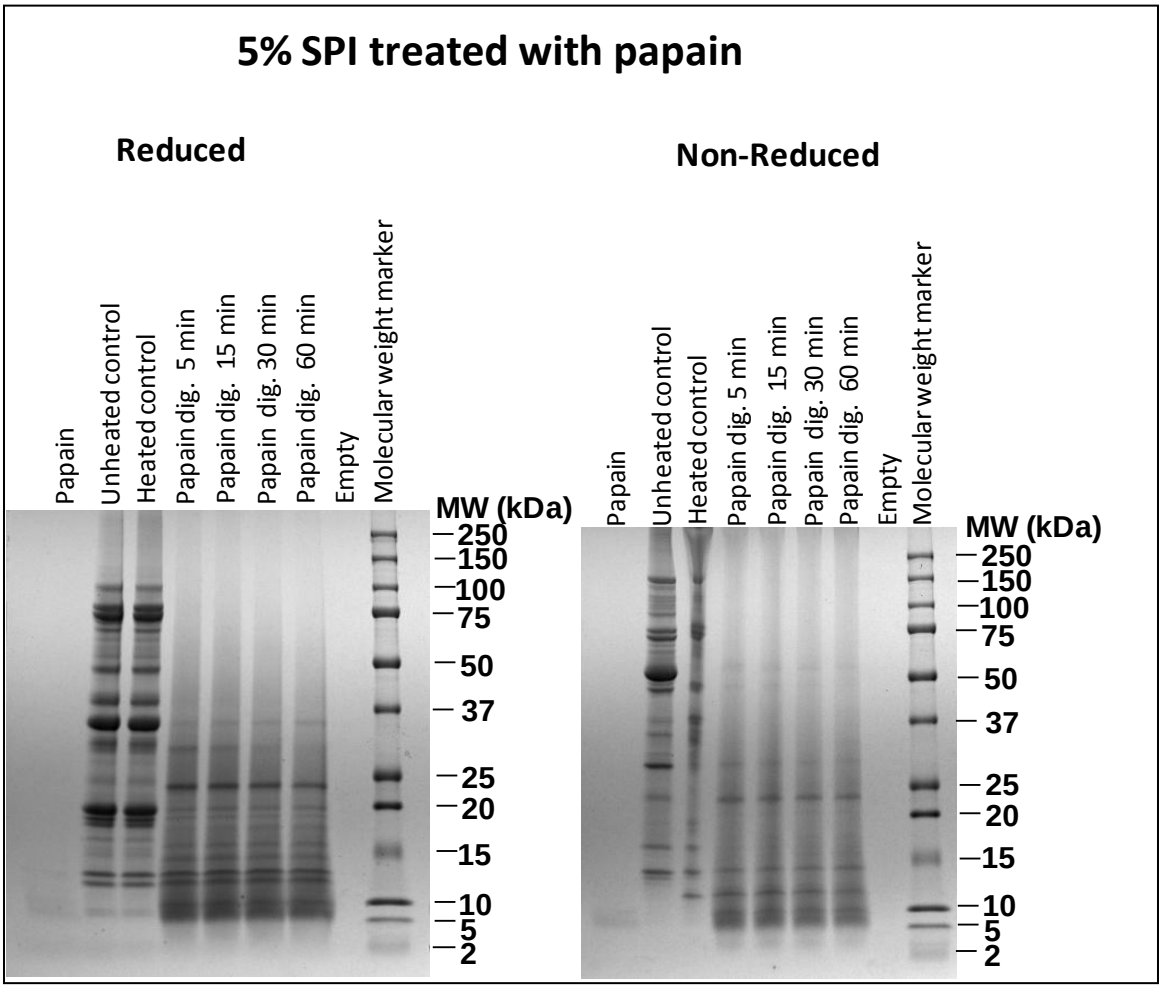

Figure 2A. SDS-PAGE stained gel of 5% SPI hydrolyzed for 5, 15, 30 or 60 min with papain compared to the unheated and heated control samples. Samples (10 μg protein/lane) were run under both reducing and non-reducing conditions, then the gels were fixed and stained with Brilliant Blue G-colloidal stain.

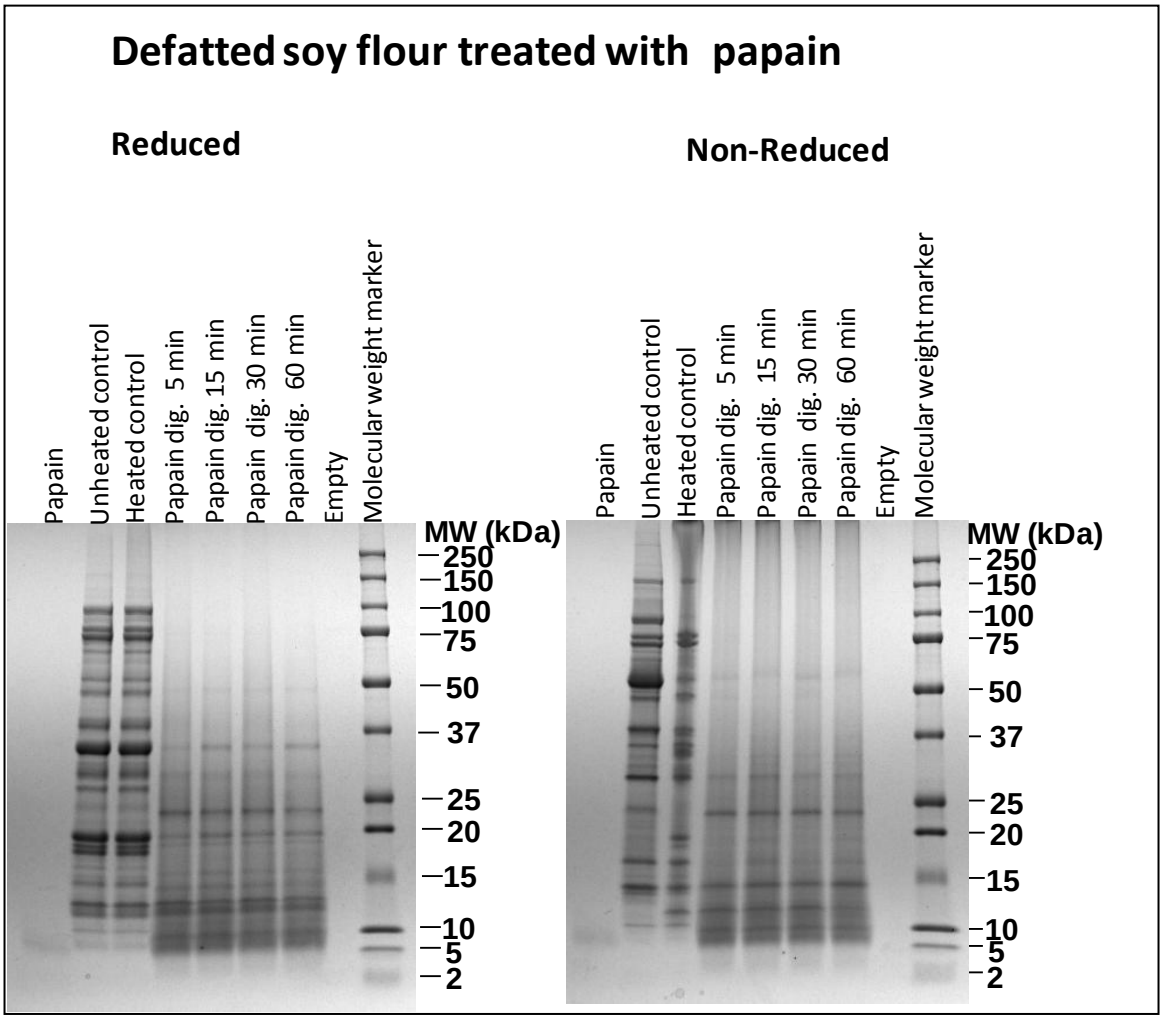

Figure 2B. SDS-PAGE stained gel of defatted soy flour extract hydrolyzed for 5, 15, 30 or 60 min with papain compared to the unheated and heated control samples. Samples (10 μg protein/lane) were run under both reducing and non-reducing conditions, then gels were fixed and stained with Brilliant Blue G-colloidal stain.

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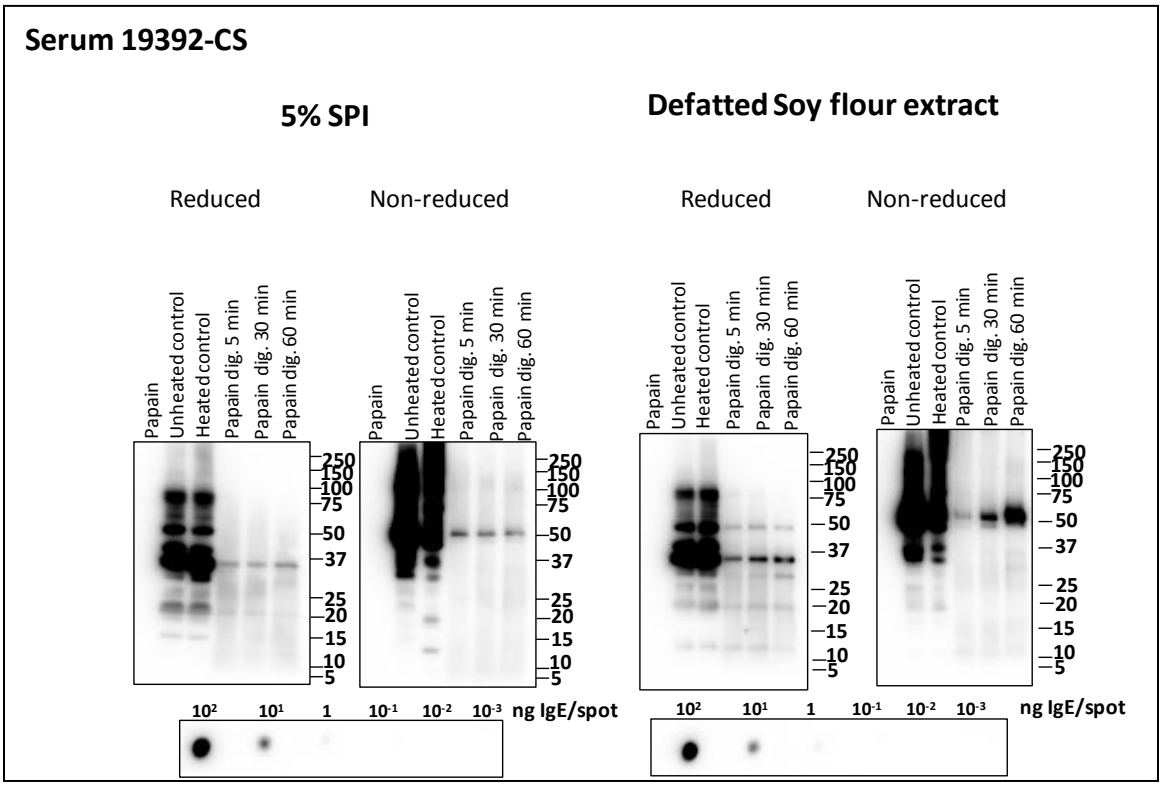

Figure 2C. IgE immunoblots of 5% SPI and defatted soy flour extract treated for 5, 30 or 60 min with papain compared to the control samples, using serum 19392-CS. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

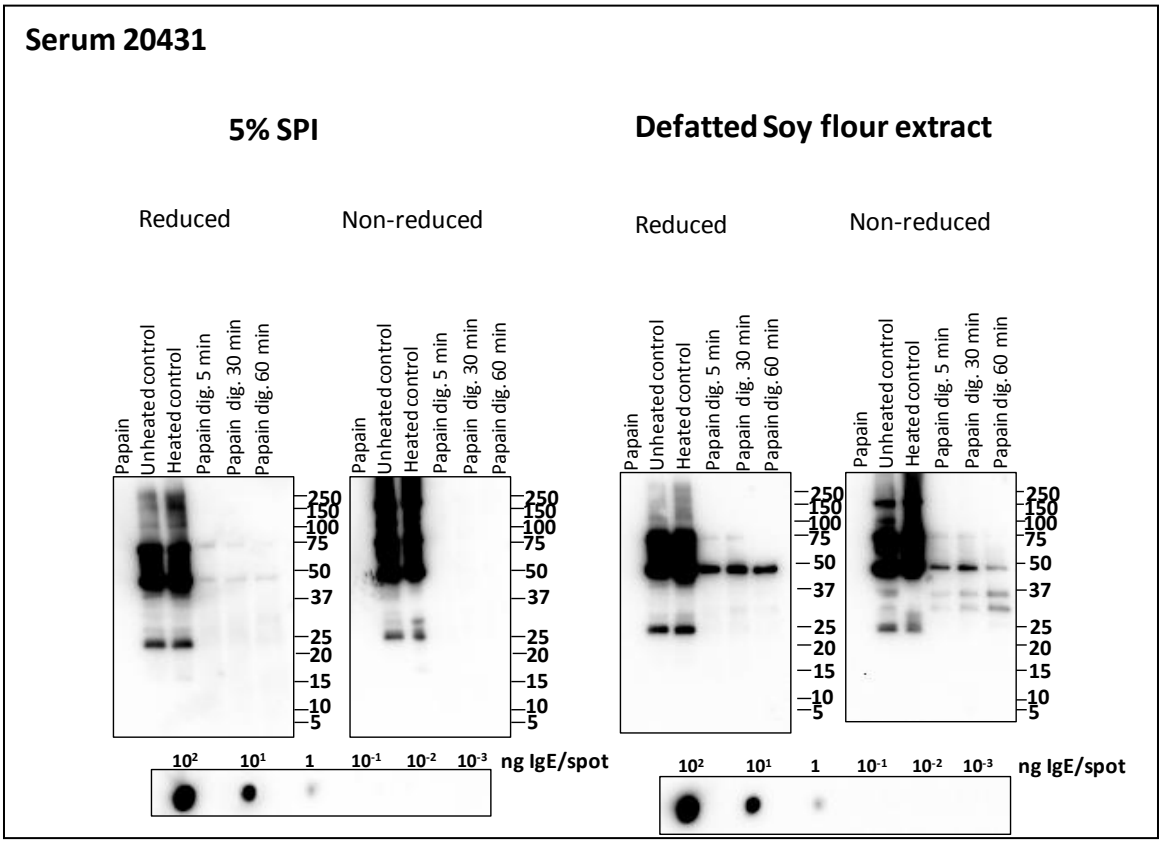

Figure 2D. IgE immunoblots of 5% SPI and defatted soy flour extract treated for 5, 30 or 60 min with papain compared to the control samples, using serum 20431. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

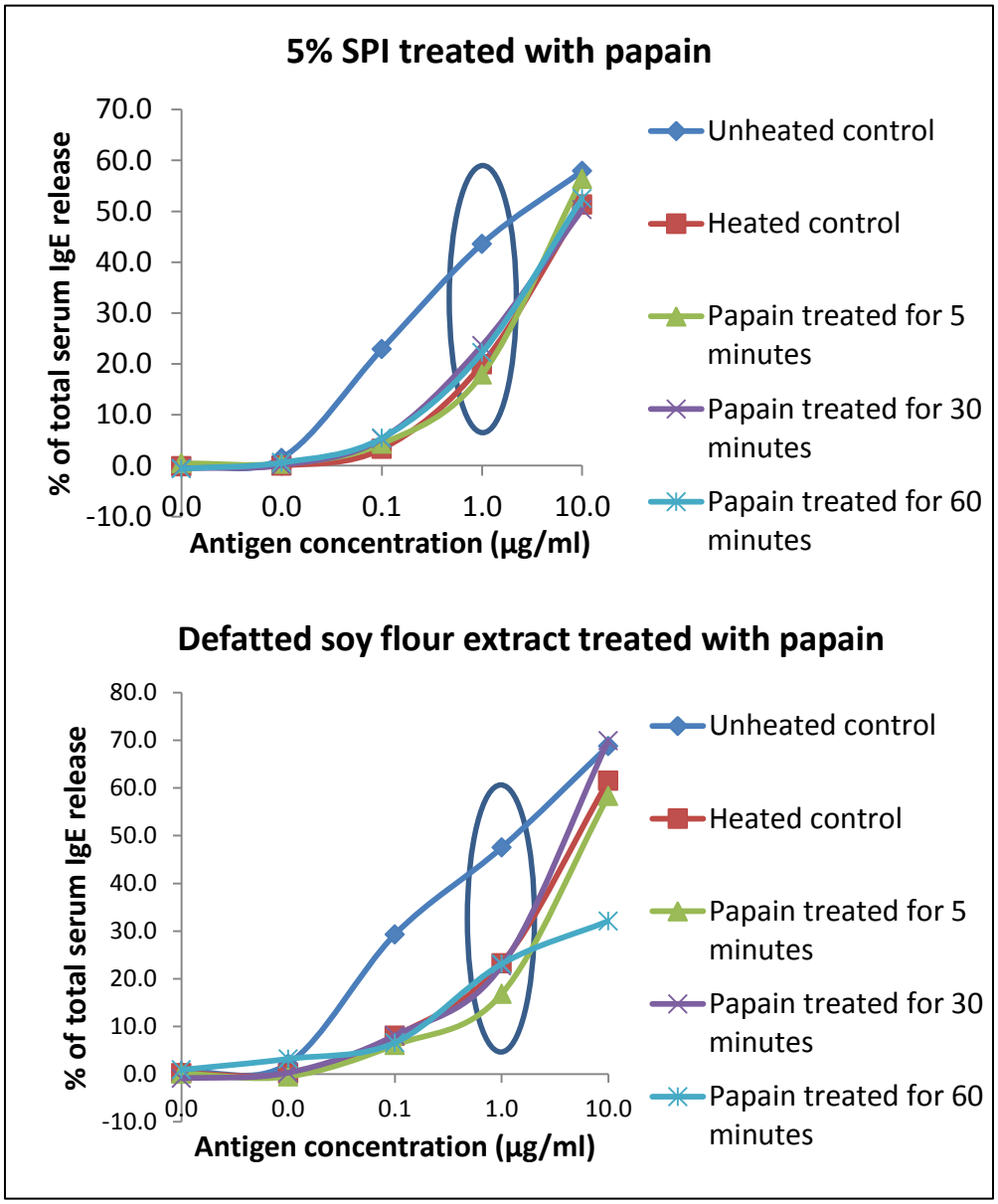

Figure 2E. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with papain hydrolyzed and control SPI and soy flour extract samples. Cells of humanized RBL-clone 703/21 were sensitized with IgE from serum 19392-CS (diluted 1: 10) and were challenged with 100 µl of papain hydrolyzed and control SPI and defatted soy flour extract samples Representing original protein concentrations of 0.001 µg/ml to 10 µg/ml of antigen. Absrobance values were measure at 405 nm and β-hexosaminidase release was expressed as percentage of total serum IgE release (cells sensitized with serum and challenged with anti-IgE)

Hydrolysis of SPI and soy flour extract with bromelain

Bromelain from pineapple stem was used to hydrolyze the SPI and defatted soy flour extract samples. The protein profile of hydrolyzed samples was evaluated by separating them by both reducing and non-reducing SDS-PAGE (Figure 3A and 3B). Additionally, IgE immunoblotting was performed using eight soybean allergic and one control patient sera following transfer of the proteins from unstained gels onto PVDF membranes as described previously. Digestion of both SPI and defatted soy flour extracts with bromelain resulted in the elimination of some higher molecular weight protein bands that are visible in both the unheated and heated control samples. However, some lower molecular weight protein bands are still visible in digested samples (approximately 12, 14, 20 and 35 kDa under reducing condition and 12, 14, 37 and 45 kDa under non-reducing condition). Additionally some new lower molecular weight protein bands of approximately 5-13 kDa, appeared in the stained gels of the digested samples under both reducing and non-reducing conditions. Figure 3C shows the immunoblot analysis of the bromelain hydrolyzed SPI and defatted soy flour samples with serum 19392-CS. Most of the IgE binding seen to the control samples with this serum was retained in the hydrolyzed samples under both reducing and non-reducing conditions. Additionally a complex pattern of new IgE binding bands appeared in the hydrolyzed samples mostly under reducing conditions including bands of approximately 30, 45 and 55 kDa in hydrolyzed SPI samples and 22. 30, 45 and 55 kDa in hydrolyzed defatted soy flour extract samples. With serum 20431 (Figure 3D), IgE binding to a band of 75 kDa observed under reducing conditions and three bands of 75, 100 and 150 kDa under non-reducing condition observed in the control samples were eliminated when both SPI and defatted soy flour extract samples were hydrolyzed with

bromelain. However, IgE binding to a protein band of 50 kDa remained in all hydrolyzed samples. Additionally two new IgE binding protein bands of 25 and 30 kDa appeared in all hydrolyzed samples. Out of six other soy allergic sera that were used in IgE immunoblot tests, only serum 9735-RE showed a complete absence of IgE binding to the bromelain hydrolyzed SPI and defatted soy flour extract (Figure 3E) whereas all other sera still retained most of the IgE binding. Furthermore, four out of eight sera showed strong IgE binding to a 12 kDa band that appeared only in the hydrolyzed SPI samples under reducing conditions (Figure 3F).

In accordance with the immunoblot results, mediator release from hRBL cells sensitized with serum 19392-CS and stimulated with samples that correspond to 1 μg/ml antigen concentration revealed no reduction in β-hexosaminidase release for hydrolyzed samples of SPI relative to the heated control sample whereas a slight reduction in release was obtained for hydrolyzed defatted soy flour samples compared to the heated control (Figure 3G). Interestingly both the SPI and defatted soy flour samples that were treated with bromelain for 30 min showed an increase in β-hexosaminidase release compared to the heated control (Approximately 50% vs 40% for defatted soy flour sample and 30% vs 15% for the SPI sample).

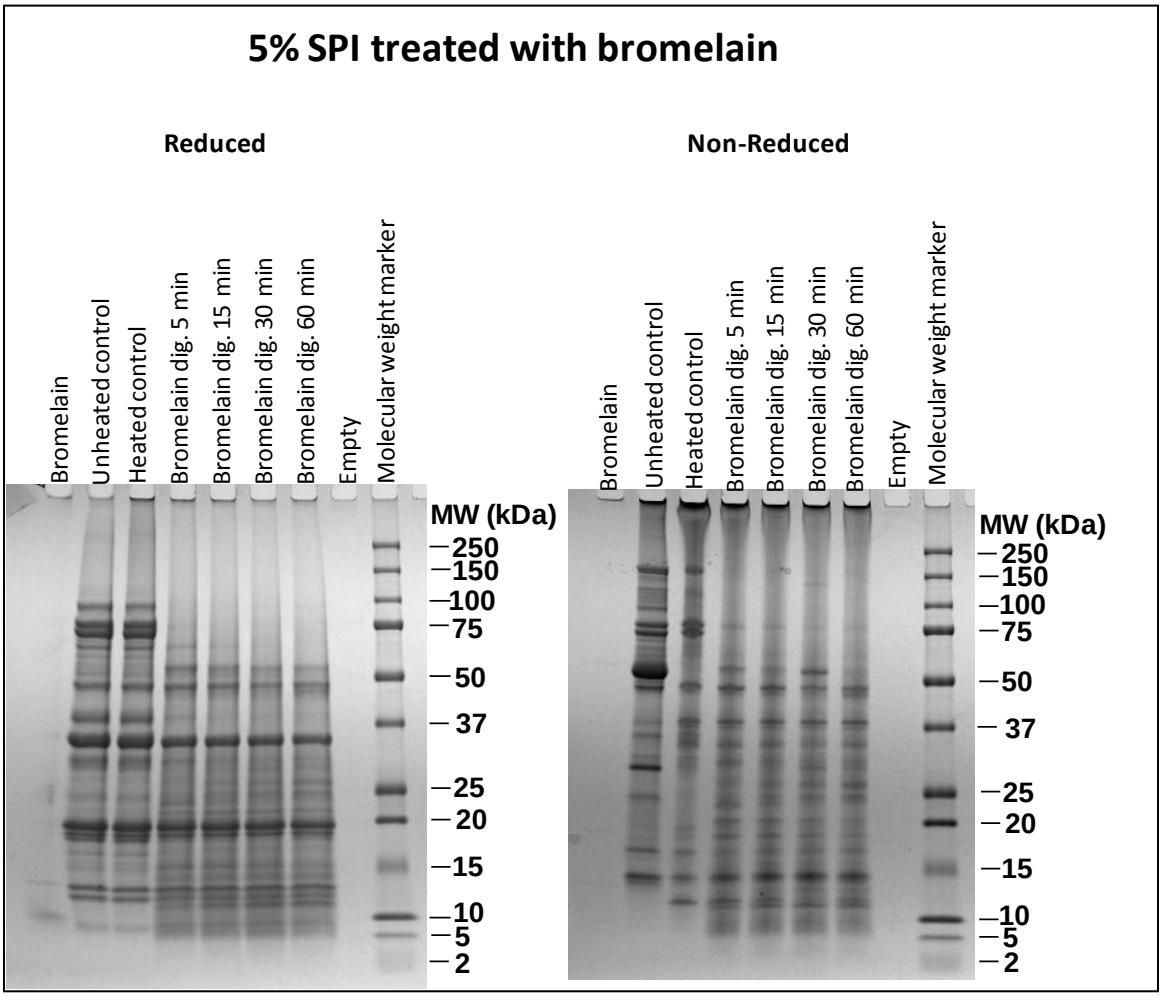

Figure 3A. SDS-PAGE stained gel of 5% SPI hydrolyzed for 5, 15, 30 or 60 min with bromelain compared to the unheated and heated control samples. Identical samples (10 μg protein/lane) were run under both reducing and non-reducing conditions, and then gels were fixed and stained with Brilliant Blue G-colloidal stain.

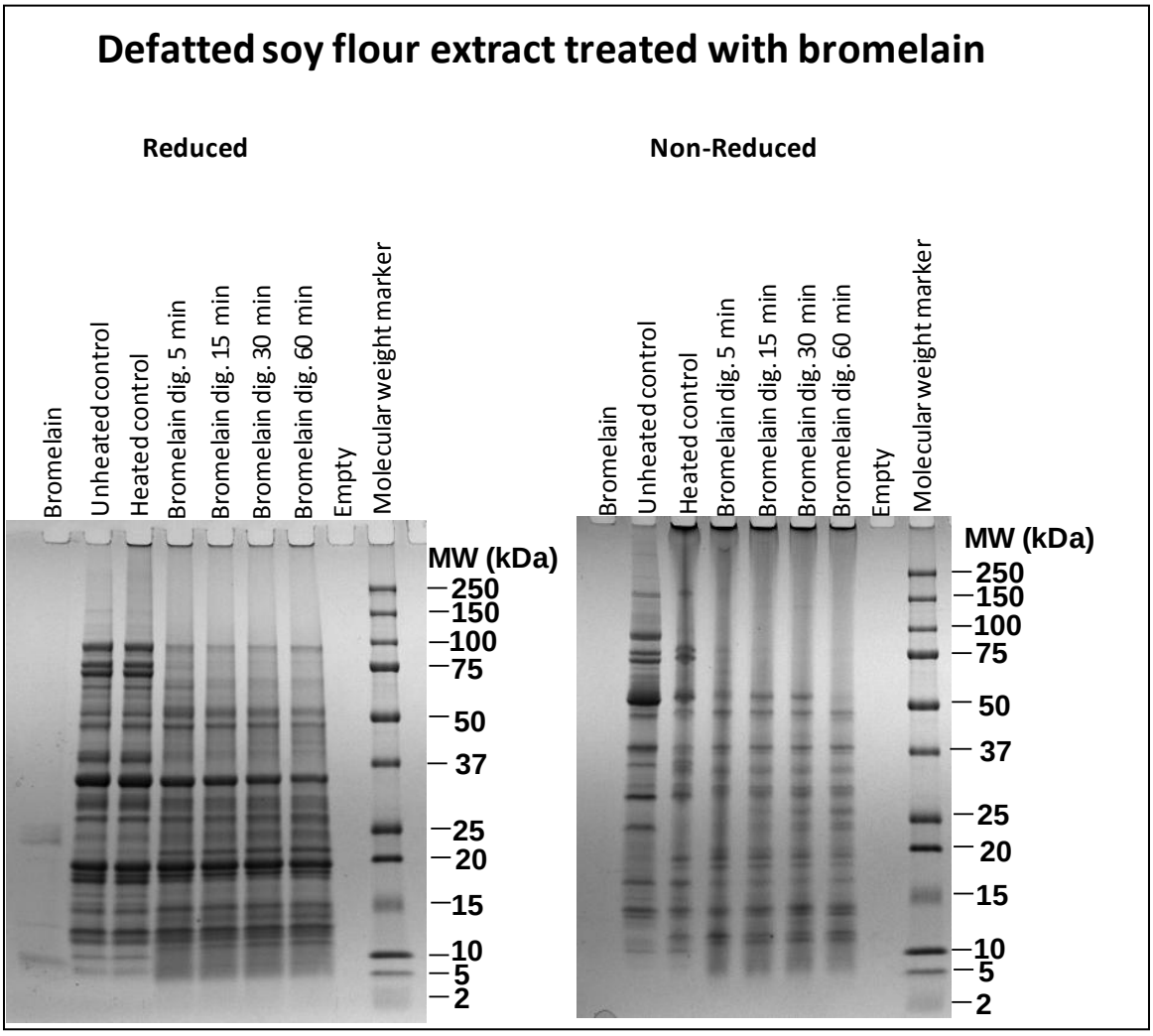

Figure 3B. SDS-PAGE stained gel of defatted soy flour extract hydrolyzed for 5, 15, 30 or 60 min with bromelain compared to the unheated and heated control samples. Identical samples (10 μg protein/lane) were run under both reducing and non-reducing conditions, and then gels were fixed and stained with Brilliant Blue G-colloidal stain.

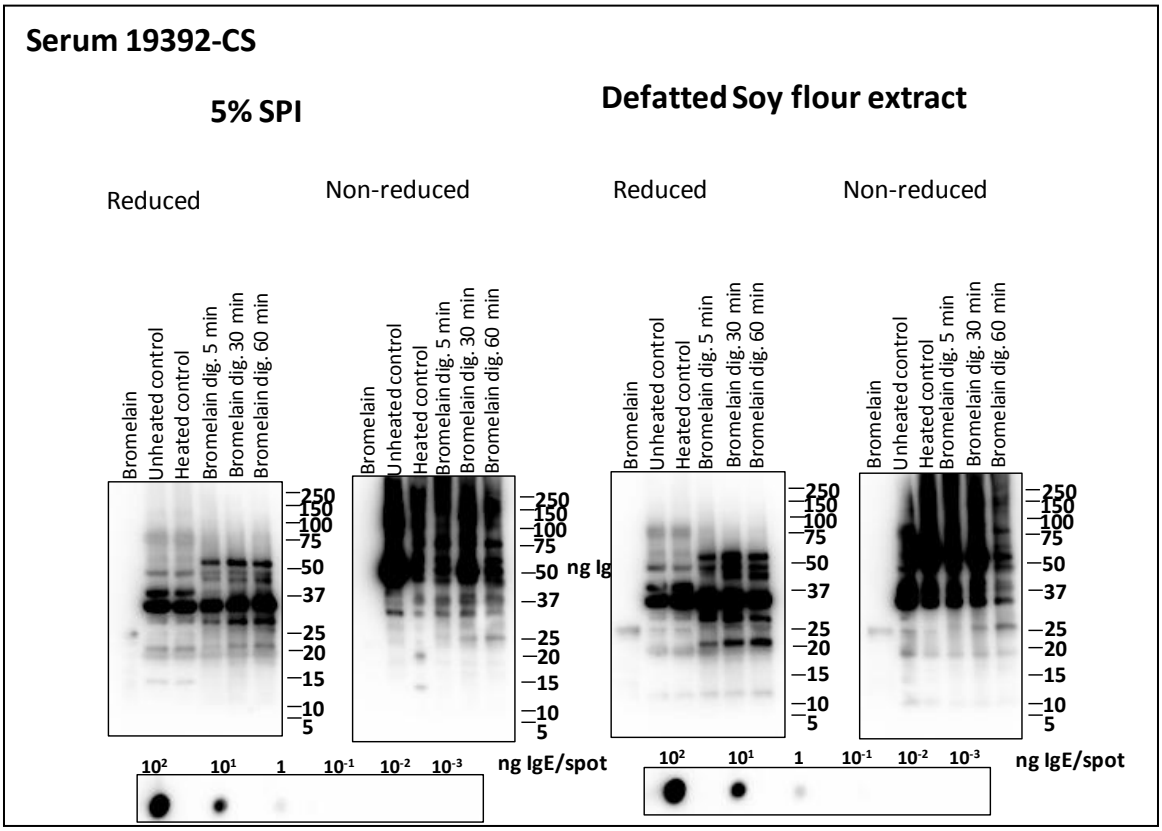

Figure 3C. IgE immunoblots of 5% SPI and defatted soy flour extract treated for 5, 30 or 60 min with bromelain compared to the control samples, using serum 19392-CS. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

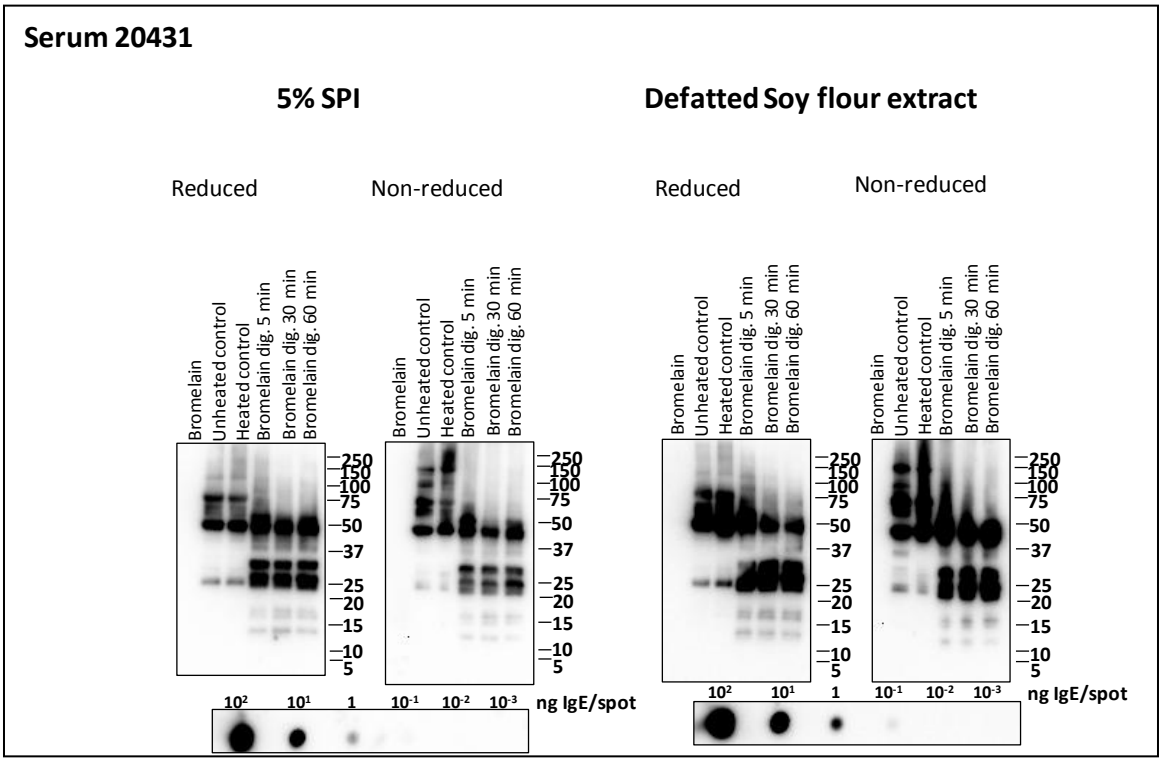

Figure 3D. IgE immunoblots of 5% SPI and defatted soy flour extract treated for 5, 30 or 60 min with bromelain compared to the control samples, using serum 20431. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

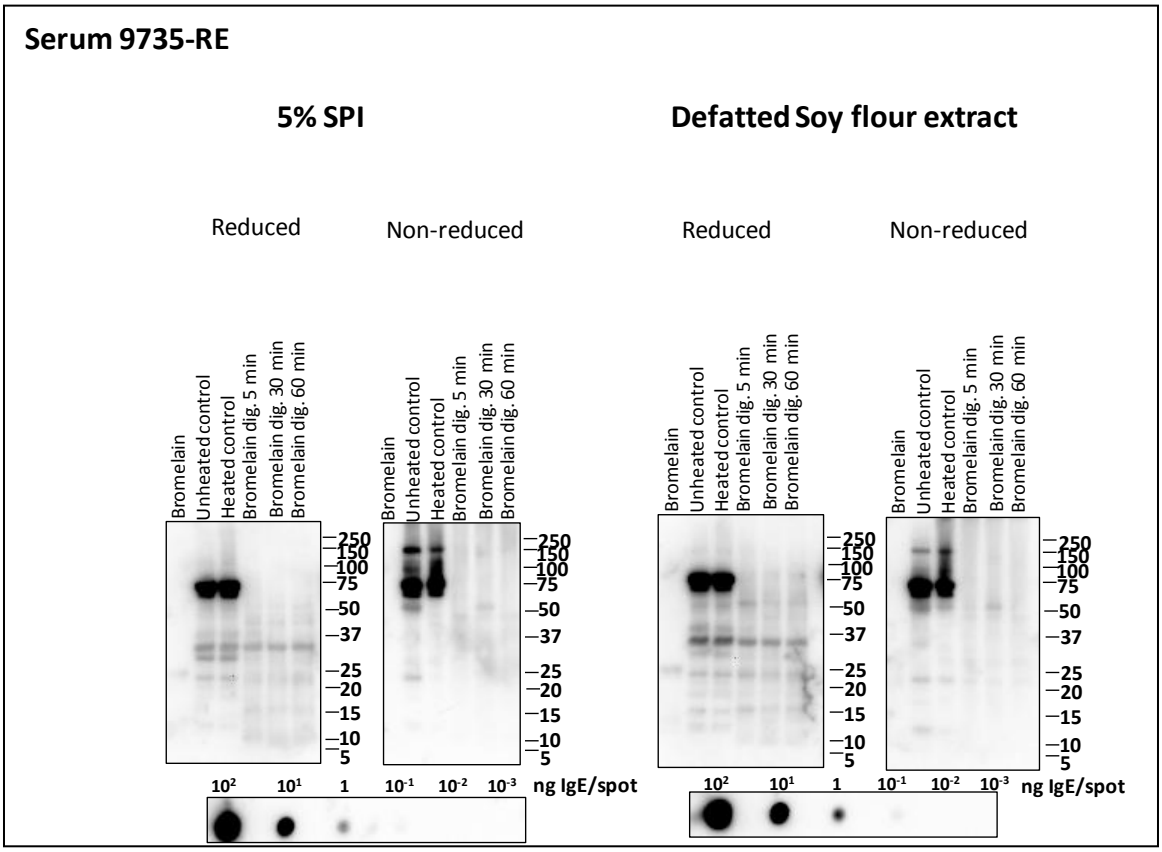

Figure 3E. IgE immunoblots of 5% SPI and defatted soy flour extract treated for 5, 30 or 60 min with bromelain compared to the control samples, using serum 9735-RE. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

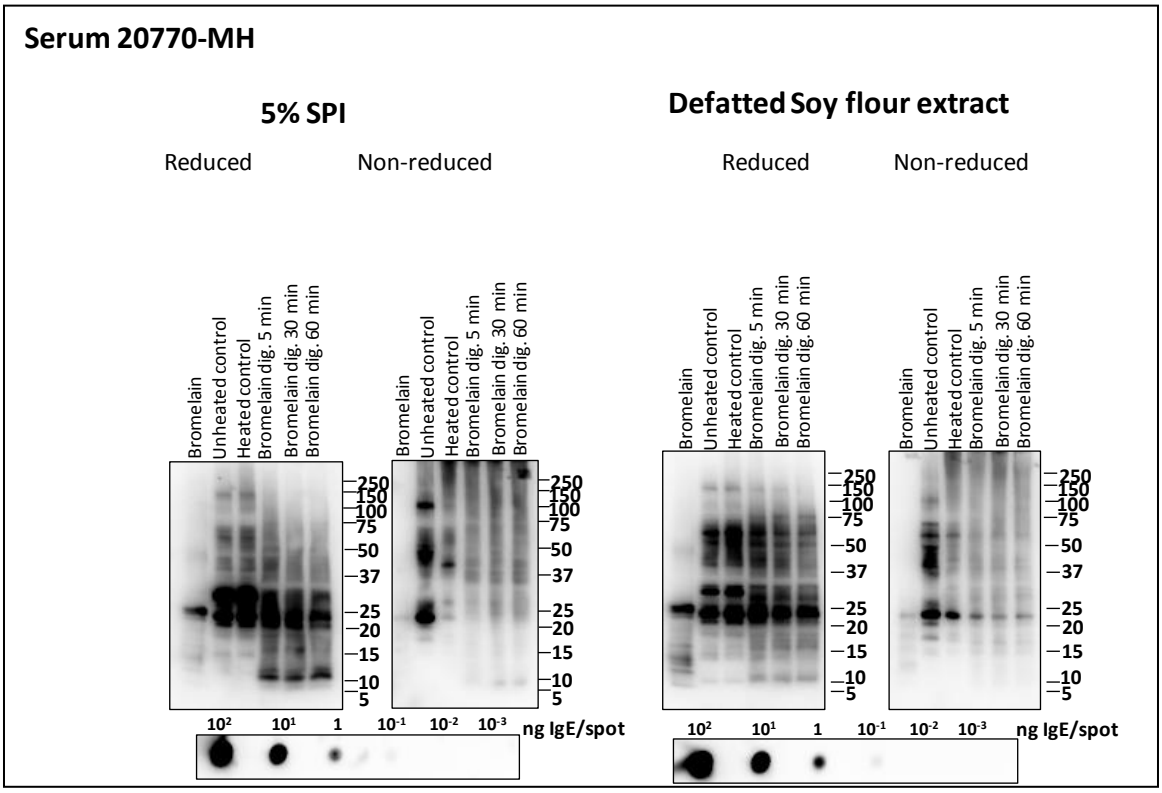

Figure 3F. IgE immunoblots of 5% SPI and defatted soy flour extract treated for 5, 30 or 60 min with bromelain compared to the control samples, using serum 20770-MH. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

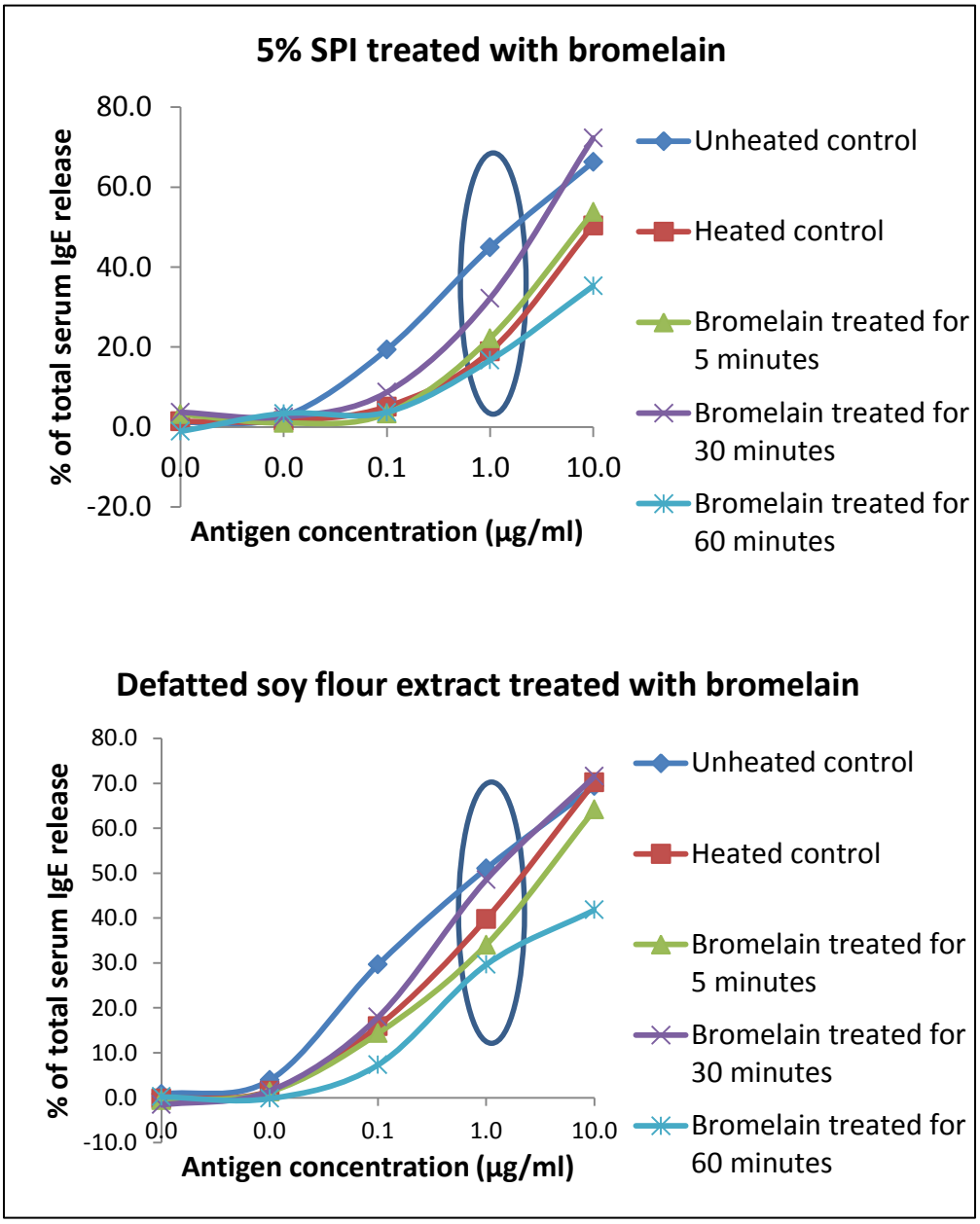

Figure 3G. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with bromelain hydrolyzed and control SPI and soy flour extract samples. Cells of humanized RBL-clone 703/21 were sensitized with IgE from serum 19392-CS (diluted 1: 10) and challenged with 100 µl of bromelain hydrolyzed and control SPI and defatted soy flour extract samples. Doses of 0.001 µg/ml to 10 µg/ml of antigen were used to challenge the cells as shown in the graph. Absrobance values were measure at 405 nm and β-hexosaminidase release is expressed as a percentage of total serum IgE release (cells sensitized with serum and challenged with anti-IgE).

Hydrolysis of SPI and soy flour extracts with trypsin and chymotrypsin

Trypsin and chymotrypsin from bovine pancreas were used to hydrolyze the SPI and defatted soy flour extract samples in separate digestion samples. Any changes in the apparent allergenicity of the hydrolyzed proteins were evaluated by IgE immunoblot as well as mediator release assay. Figures 4A and 4B show the SDS-PAGE protein profiles of the trypsin or chymotrypsin digested SPI and defatted soy flour extract samples respectively. Trypsin hydrolysis of SPI and defatted soy flour extracts resulted in the disappearance or reduction of most of the high molecular weight protein bands compared to the control samples. However, a number of protein bands remained constant throughout the hydrolysis by trypsin under reducing and non-reducing conditions (e.g. 18-20 kDa under reducing conditions and a 30 kDa band under non-reducing conditions) and were present in undigested controls. Additionally some new protein bands appeared at 5 min of hydrolysis and remained constant in both SPI and soy flour samples (e.g. 24 kDa band under reducing conditions and three adjacent bands ranging from 24-26 kDa under non-reducing conditions). A similar result was obtained when both SPI and defatted soy flour samples were hydrolyzed with chymotrypsin. For example, a 20 kDa and a 50 kDa band remained resistant to hydrolysis and a new protein band of 25 kDa appeared in the hydrolyzed samples under reducing conditions.

The IgE immunoblots with serum 19392-CS for 5% samples of SPI and defatted soy flour extract samples digested with trypsin and chymotrypsin are shown in Figures 4C and 4D. A marked reduction in IgE binding to the trypsin digested SPI samples was observed compared to the control samples under both reducing and non-

reducing condition although strong IgE binding to a 20 kDa band remained under reducing conditions (Figure 4C). The mediator release assay using the same sera however, did not result in any differences in β-hexosaminidase release for all the trypsin hydrolyzed SPI samples compared to the heated control sample (Figure 4E). Some differences in IgE immunoblotting patterns were observed for trypsin digested defatted soy flour extract compared to the trypsin digested SPI with serum 19392-CS (Figure 4D). Under reducing conditions, most of the IgE binding to protein bands ranging from 35 to 75 kDa still remained after 5 min and 15 min of trypsin digestion followed by marked reduction at 30 min and 60 min for soy flour extract. Under non-reducing conditions IgE binding to a 50 kDa band remained in the 5 min and 15 min trypsin hydrolyzed extract, however binding was completely abolished when the extract was digested with trypsin at 30 and 60 min. Additionally two new IgE binding bands (24 and 30 kDa) appeared in the trypsin digested soy flour extract when the gel was run under reducing conditions, and a minor band appeared with a similar profile under non-reducing conditions (Figure 4D). The mediator release assay of the trypsin hydrolyzed defatted soy flour sample with the same sera showed a slight reduction in β-hexosaminidase release compared to the control samples when stimulated with either 1 or 10 microgram equivalents of digested sample (Figure 4E).

Chymotrypsin digested SPI (Figure 4C) and defatted soy flour extract (Figure 4D) retained most of the IgE binding that was observed in case of the control samples with serum 19392-CS. An interesting observation was that IgE binding to a 20 kDa band was obviously stronger for chymotrypsin digested SPI compared to the control samples (Figure

4C). This increase was not observed in case of chymotrypsin digested defatted soy flour extract (Figure 4D). The mediator release assay with the serum 19392-CS also reflected this difference between the chymotrypsin digested SPI and defatted soy flour extract (Figure 4F). While all the chymotrypsin digested SPI samples showed a higher β-hexosaminidase release compared to the heated control sample at 1 µg/ml antigen concentration, the release with the chymotrypsin hydrolyzed defatted soy flour samples was lower compared to the heated control (Figure 4F).

With serum 20431 (Figure 4G), IgE binding to the higher molecular weight protein bands ranging from 50-150 kDa that was observed for the control SPI samples was eliminated or markedly reduced when they were treated with trypsin. Relatively faint IgE binding to a 50 kDa band remained in the hydrolyzed samples (mostly under reducing conditions) and faint IgE binding to a 25 kDa band that was observed in the control sample was strongly augmented in all the trypsin hydrolyzed SPI samples. Additionally a new IgE binding protein band (15 kDa) was observed for all the trypsin hydrolyzed SPI samples (Figure 4G). In contrast to the SPI samples most of the IgE binding to high molecular weight protein bands still remained in all the trypsin hydrolyzed defatted soy flour extract samples except for the sample that was hydrolyzed for 60 min (Figure 4H). However, similar to the SPI samples IgE binding to a 25 kDa band was increased considerably compared to the control samples. Chymotrypsin digestion of SPI (Figure 4G) and defatted soy flour extract (Figure 4H), although results in a reduction in IgE binding to higher molecular weight protein bands compared to control samples, all hydrolyzed samples still retained IgE binding to a 50 kDa protein band. Similar to the trypsin digested

results, SPI and defatted soy four extract samples digested with chymotrypsin also showed stronger IgE binding to a 25 kDa band compared to the control samples. Additionally a new IgE binding band of 35 kDa appeared in all chymotrypsin digested SPI and defatted soy flour samples, which was stronger under non-reducing conditions. Immunoblots with other soybean allergic sera resulted in similar IgE binding to all the trypsin and chymotrypsin digested SPI and defatted soy flour samples as the control samples (not shown).

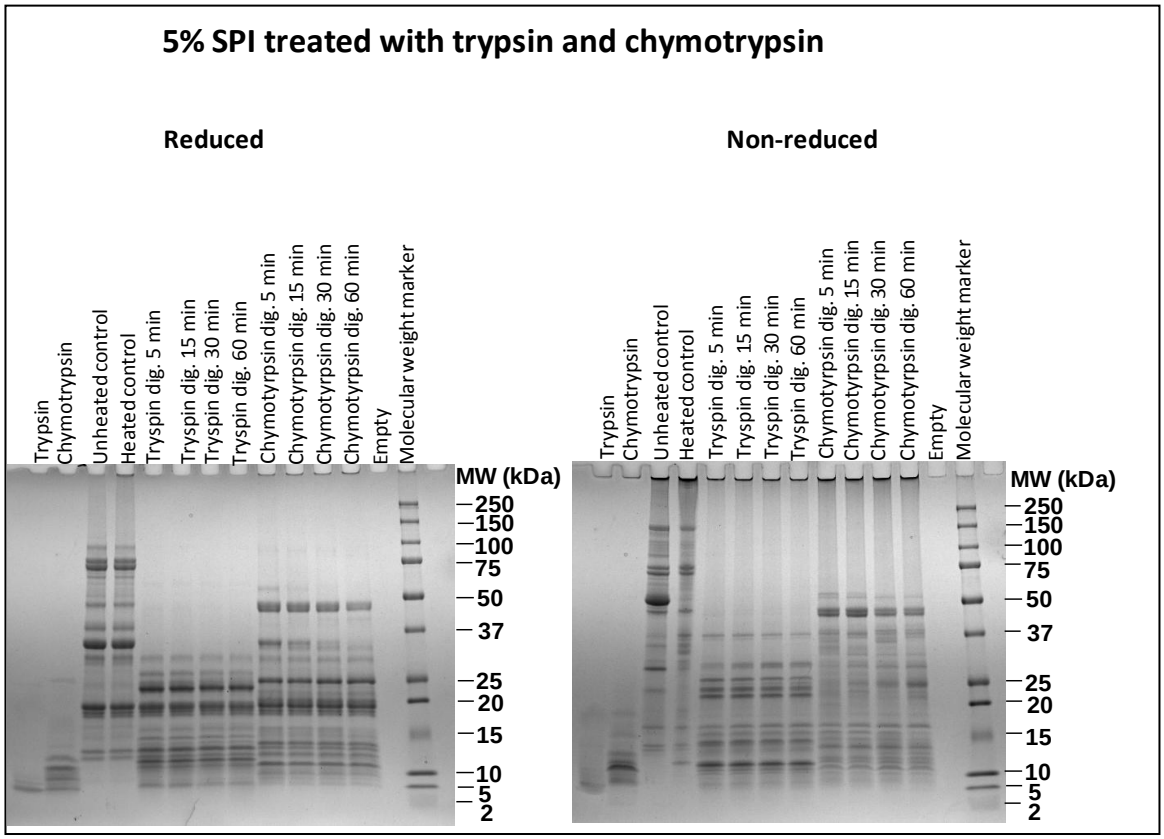

Figure 4A. SDS-PAGE stained gel of 5% SPI hydrolyzed for 5, 15, 30 or 60 min with trypsin or chymotrypsin compared to the unheated and heated control samples. Samples (10 μg protein/lane) were run under both reducing and non-reducing conditions, then gels were fixed and stained with Brilliant Blue G-colloidal stain.

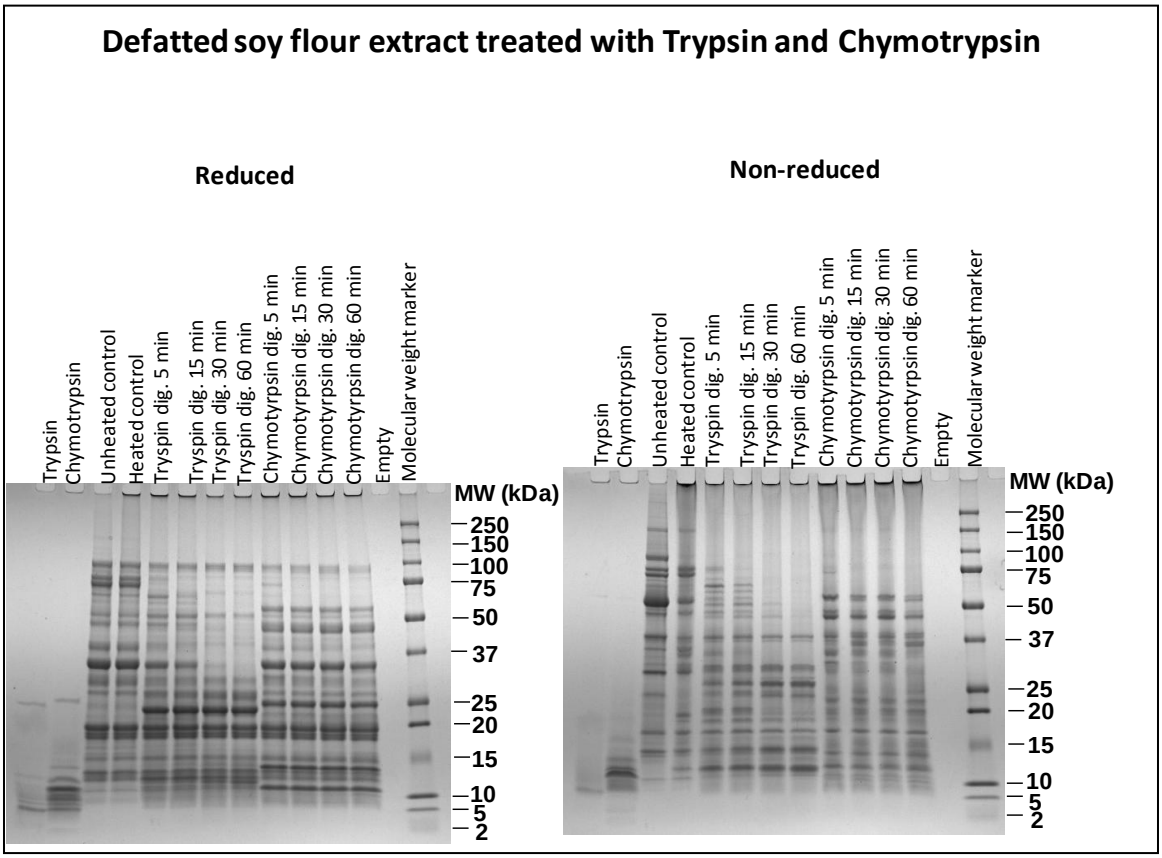

Figure 4B. SDS-PAGE stained gel of defatted soy flour extract hydrolyzed for 5, 15, 30 or 60 min with trypsin or chymotrypsin compared to the unheated and heated control samples. Samples (10 μg protein/lane) were run under both reducing and non-reducing conditions, then gels were fixed and stained with Brilliant Blue G-colloidal stain.

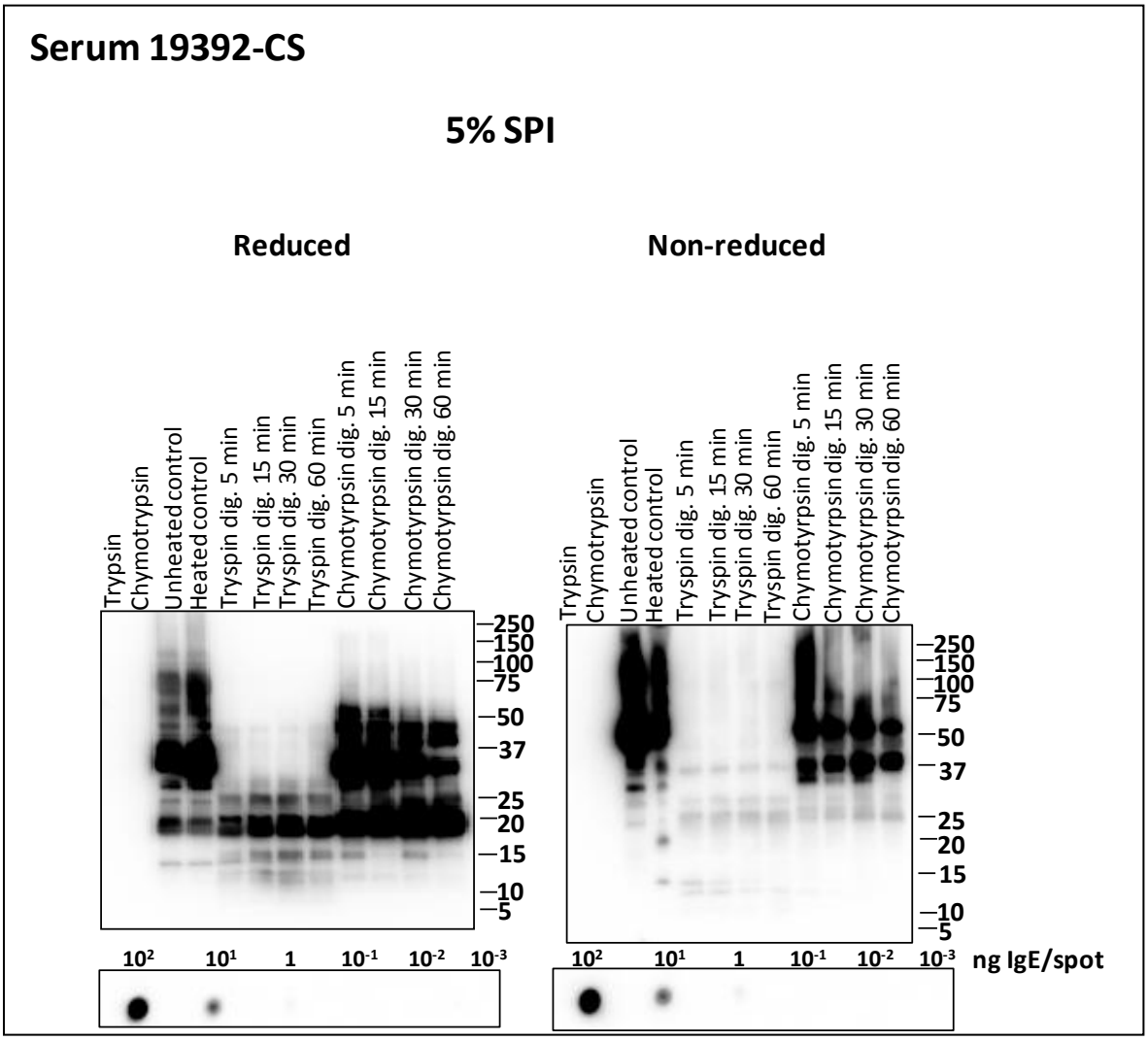

Figure 4C. IgE immunoblot of 5% SPI treated for 5, 15, 30 or 60 min with trypsin or chymotrypsin compared to the control samples, using serum 19392-CS. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

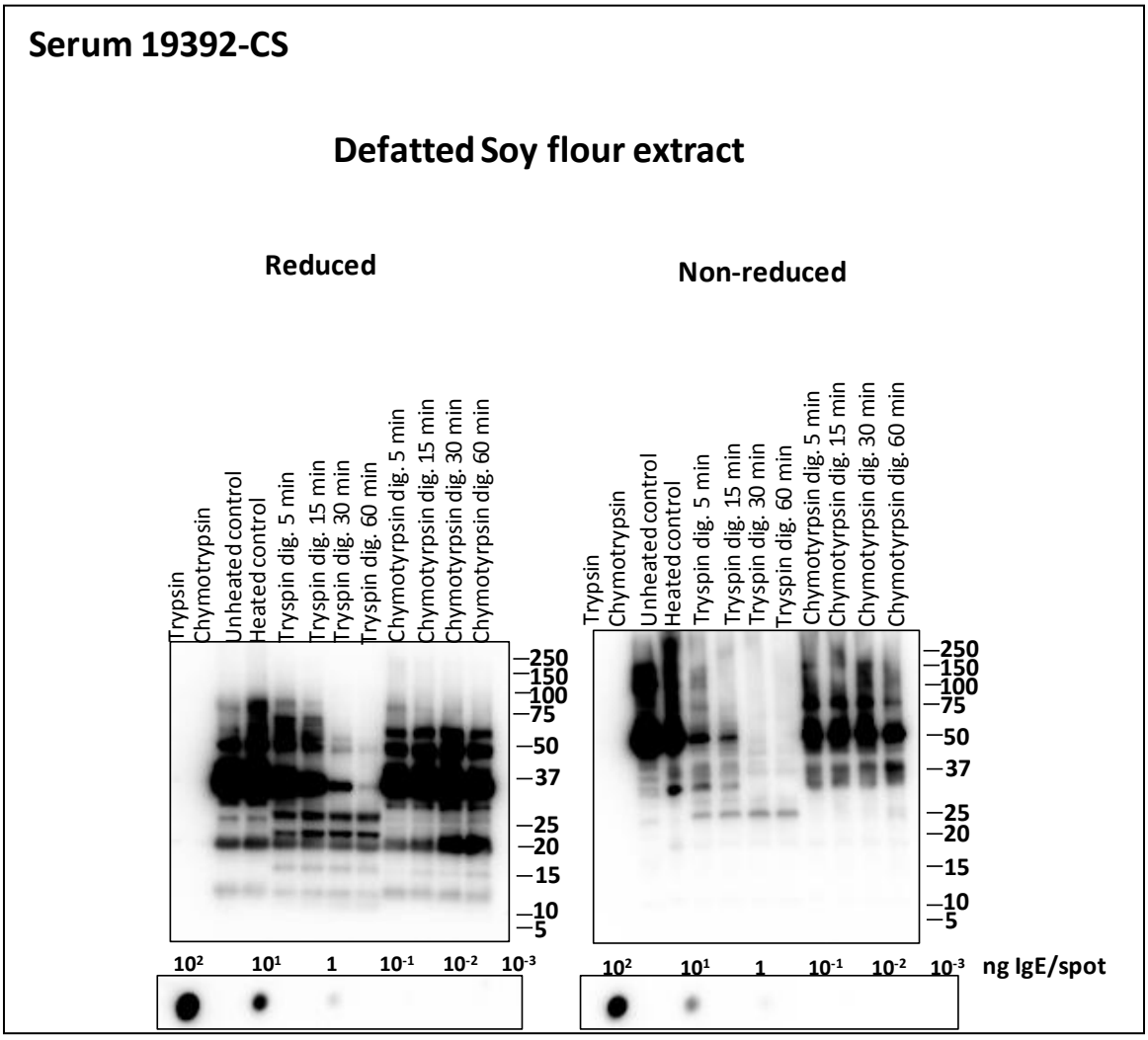

Figure 4D. IgE immunoblot of defatted soy flour extract treated for 5, 15, 30 or 60 min with trypsin or chymotrypsin compared to the control samples, using serum 19392-CS. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

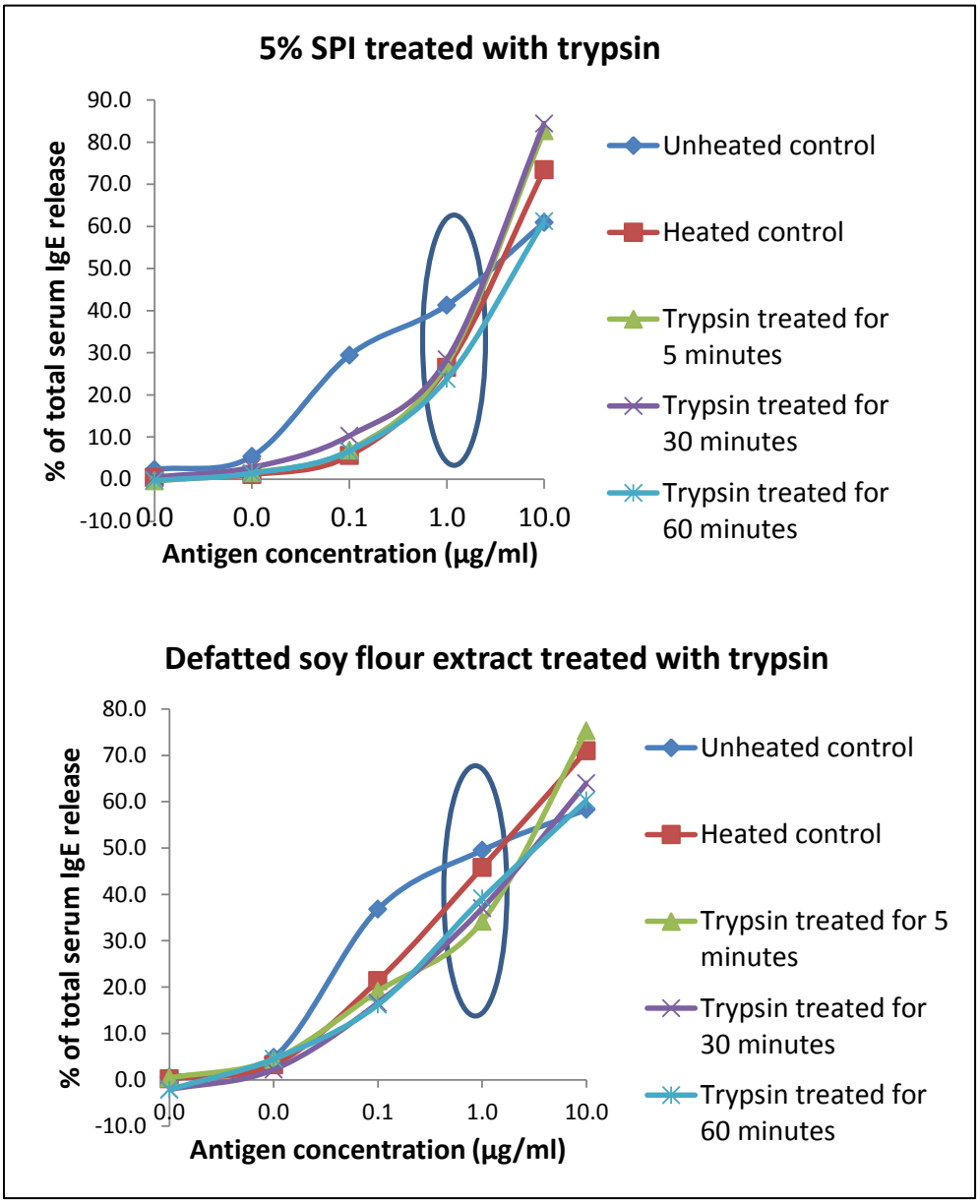

Figure 4E. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with trypsin hydrolyzed and control SPI and soy flour extract samples. Humanized RBL-clone 703/21 were sensitized with IgE from serum 19392-CS (diluted 1: 10) and were challenged with 100 µl of trypsin hydrolyzed and control SPI and defatted soy flour extract samples. 0.001 µg/ml to 10 µg/ml of antigen was used to challenge the cells as shown in the graph. Absrobance values were measure at 405 nm and β-hexosaminidase release was expressed as percentage of total serum IgE release (cells sensitized with serum and challenged with anti-IgE).

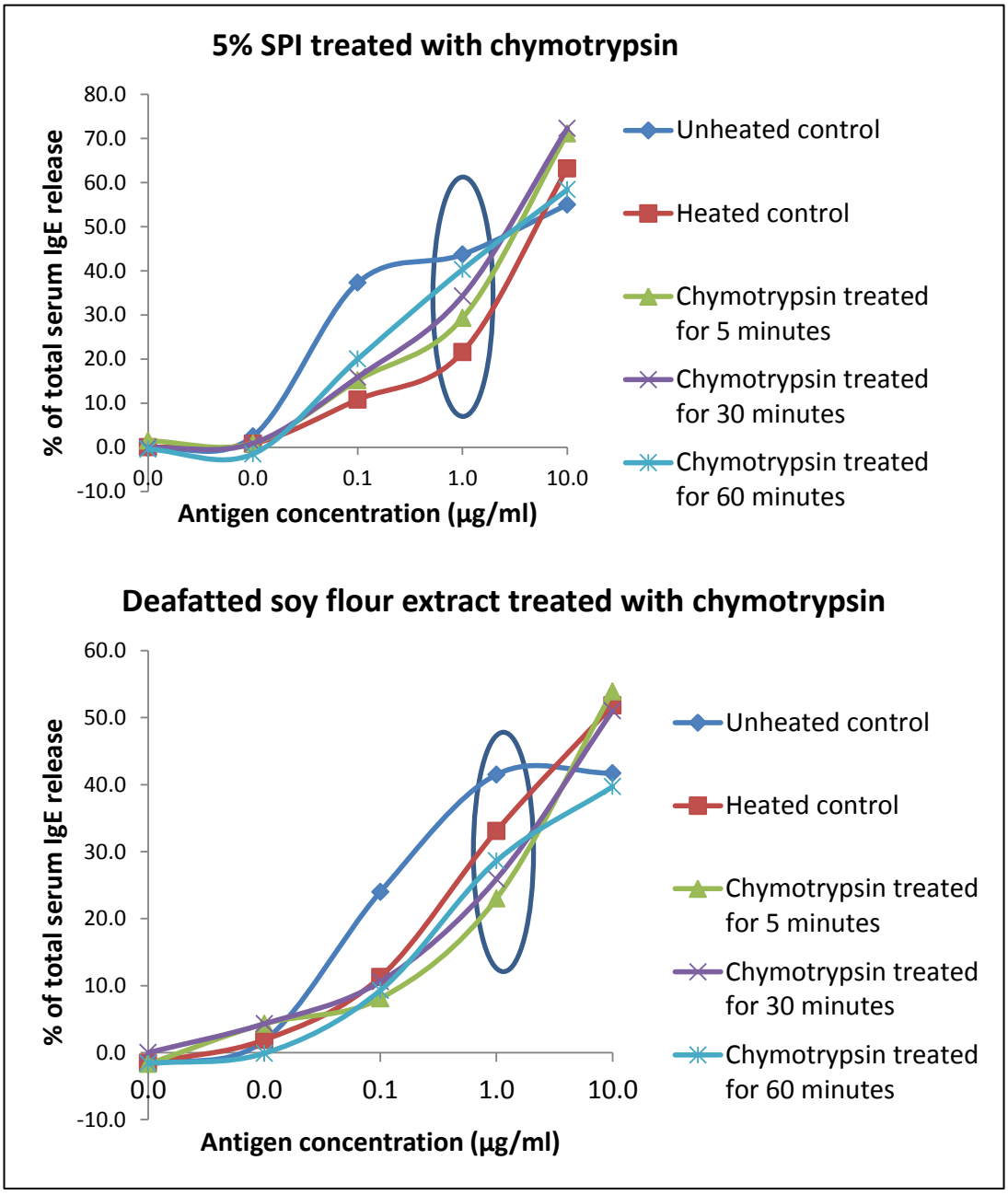

Figure 4F. β-hexosaminidase release from humanized RBL-703/21 cells, sensitized with IgE from serum 19392-CS and challenged with chymotrypsin hydrolyzed and control SPI and soy flour extract samples. Humanized RBL-clone 703/21 were sensitized with IgE from serum 19392-CS (diluted 1: 10) and were challenged with 100 µl of chymotrypsin hydrolyzed and control SPI and defatted soy flour extract samples. 0.001 µg/ml to 10 µg/ml of antigen was used to challenge the cells as shown in the graph. Absrobance values were measure at 405 nm and β-hexosaminidase release was expressed as percentage of total serum IgE release (cells sensitized with serum and challenged with anti-IgE).

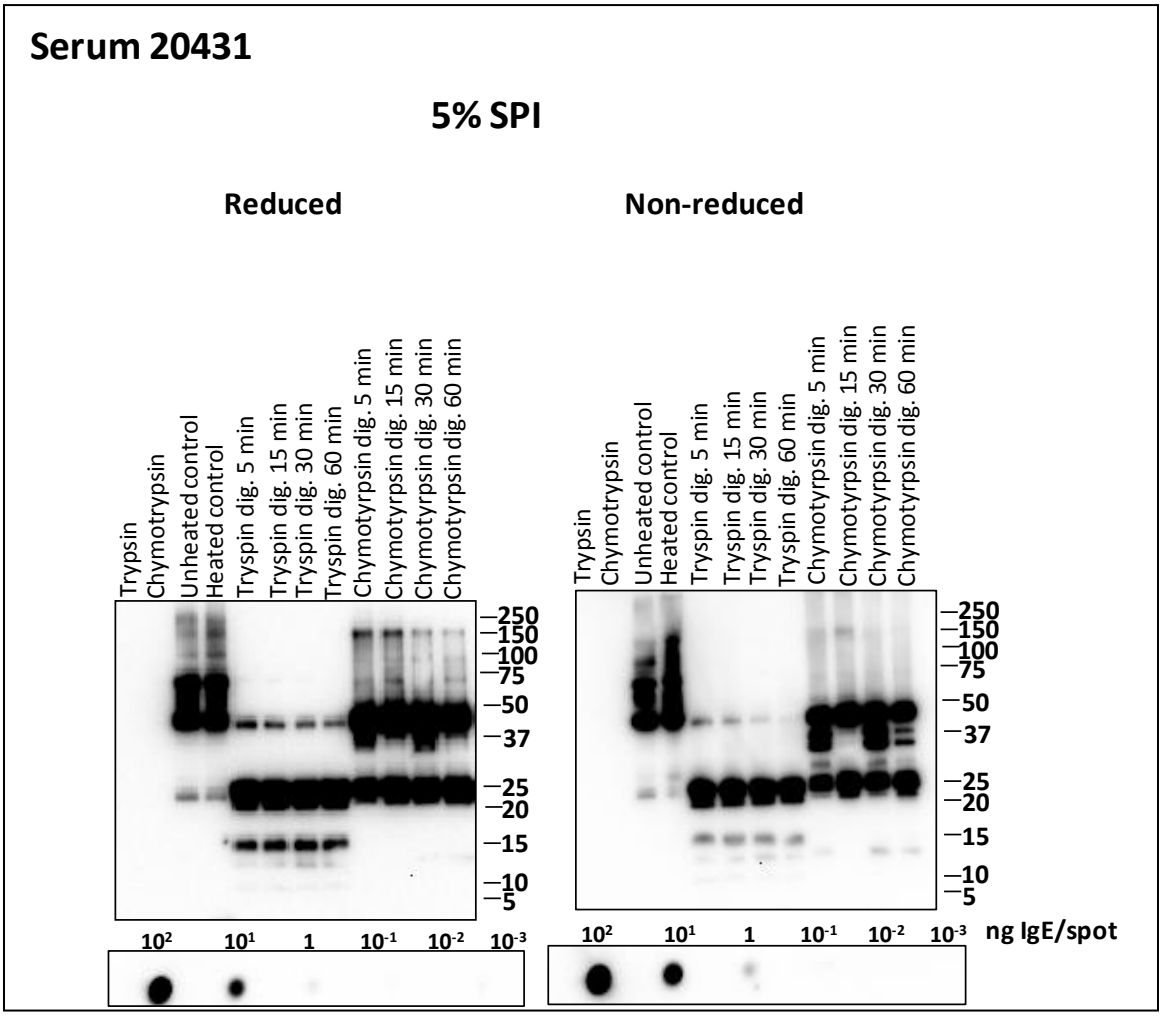

Figure 4G. IgE immunoblot of 5% SPI treated for 5, 15, 30 or 60 min with trypsin or chymotrypsin compared to the control samples, using serum 20431. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

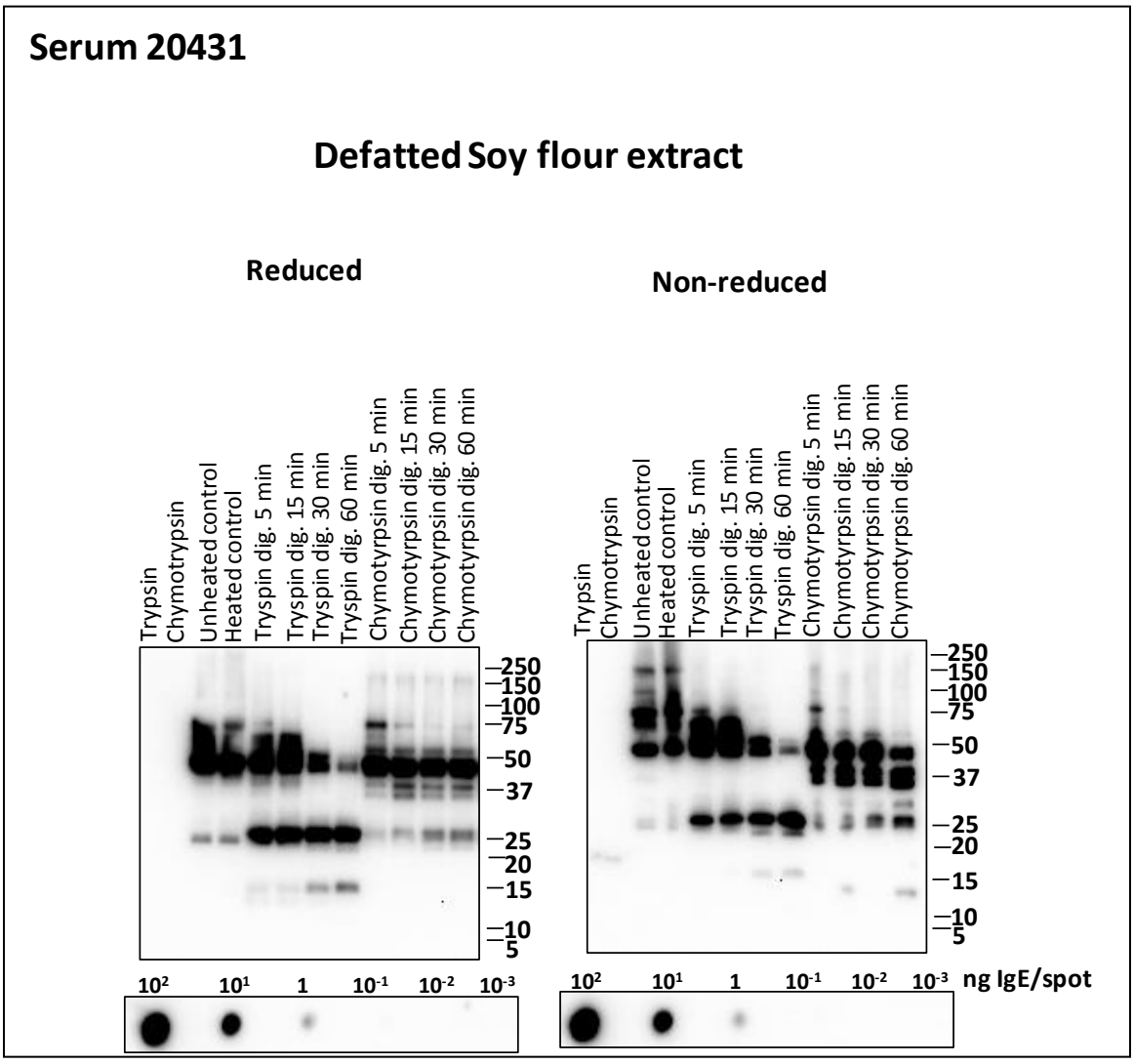

Figure 4H. IgE immunoblot of defatted soy flour extract treated for 5, 15, 30 or 60 min with trypsin or chymotrypsin compared to the control samples, using serum 20431. Samples (10 µg pre-treatment protein) were treated as indicated for each lane, then separated by SDS-PAGE in both non-reducing and reducing gels prior to transfer onto PVDF membranes. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

Additional investigations were undertaken in an attempt to identify the proteins from bands that appeared after initial hydrolysis. The strong IgE binding observed at approximately 20-25 kDa in the Alcalase, trypsin or chymotrypsin hydrolyzed 5% SPI samples with serum 19392-CS (Figure 1B, 4C) and for the trypsin or chymotrypsin hydrolyzed samples with serum 20431 (Figure 4G) may either represent creation of a fragment from the higher molecular weight proteins or may represent uncovered epitopes that were masked in undigested samples. To answer the question 2D gel electrophoresis of samples was performed to more effectively isolate proteins, followed by immunoblotting with serum 19392-CS for the Alcalse, trypsin or chymotrypsin digested SPI and serum 20431 for the trypsin or chymotrypsin digested SPI. Samples included both the heated control SPI and the SPI samples hydrolyzed for 60 min. Figure 5A shows the stained gel after separating the proteins by 2D gel electrophoresis. From the stained gel it can be observed that most of the higher molecular weight protein spots from approximately 37 to 75 kDa (upper left pane) that are observed in the control SPI sample (upper left panel) were no longer visible when the samples were treated with the three enzymes (other three panels of Figure 5A). With the Alcalase treated SPI very faint protein spots were observed around 25 kDa and 12 kDa. A number of lower molecular weight protein spots ranging from 10-20 kDa were observed when the samples were treated with trypsin or chymotrypsin. A protein spot of approximately 25 kDa at a pI=4 (Spot #1) and two spots of similar molecular weight and pI values (Spots # 2, 3) appeared in the trypsin and chymotrypsin digested samples respectively (right two panels, Figure 5A). These spots do not appear to be present in the control SPI sample. Further spots of approximately 50 kDa appeared in both the control and the chymotrypsin hydrolyzed SPI

(# 10) indicating the resistance of these spots to digestion by chymotrypsin. Immunoblots with serum 19392-CS (Figure 5B) showed strong IgE binding to protein spots ranging from 37-150 kDa in the control SPI sample (upper left panel). These dominant IgE binding spots were completely absent when the samples were treated with Alcalase and trypsin (upper right and lower left panels). However, there was a faintly visible spot at approximately 50 kDa (# 10) in the chymotrypsin hydrolyzed sample (lower right panel). With both trypsin and chymotrypsin hydrolyzed samples, relatively strong IgE binding was observed to the 25 kDa, pI= 4 spots (# 1, 2, 3) that seem to correspond to the similarly marked spots visible in the stained gels (Figure 5A). The Alcalase hydrolyzed sample also showed strong IgE binding to a spot of similar molecular weight (25 kDa) and pI=4 that is marked as spot # 9, which was not visible in the stained gel. Furthermore, the immunoblot of the trypsin hydrolyzed sample showed modest IgE binding to three spots of approximately 23 kDa, with pI values between 6-7 (spots # 4, 5, 6). IgE binding to those spots was very faint in the control SPI sample. With serum 20431 (Figure 5C) again strong IgE binding was observed to the spots at 25 kDa, pI=4 (#1, 2, 3) only in the trypsin and chymotrypsin hydrolyzed samples. Further similar to serum 19392-CS, IgE binding was observed at 50 kDa (Spot # 10) for chymotrypsin digested as well as the control samples indicating the resistance of these IgE binding spots to digestion by chymotrypsin. These results were consistent with the results obtained from 1D immunoblots with both the sera (Figures 1B, 4C, 4G).

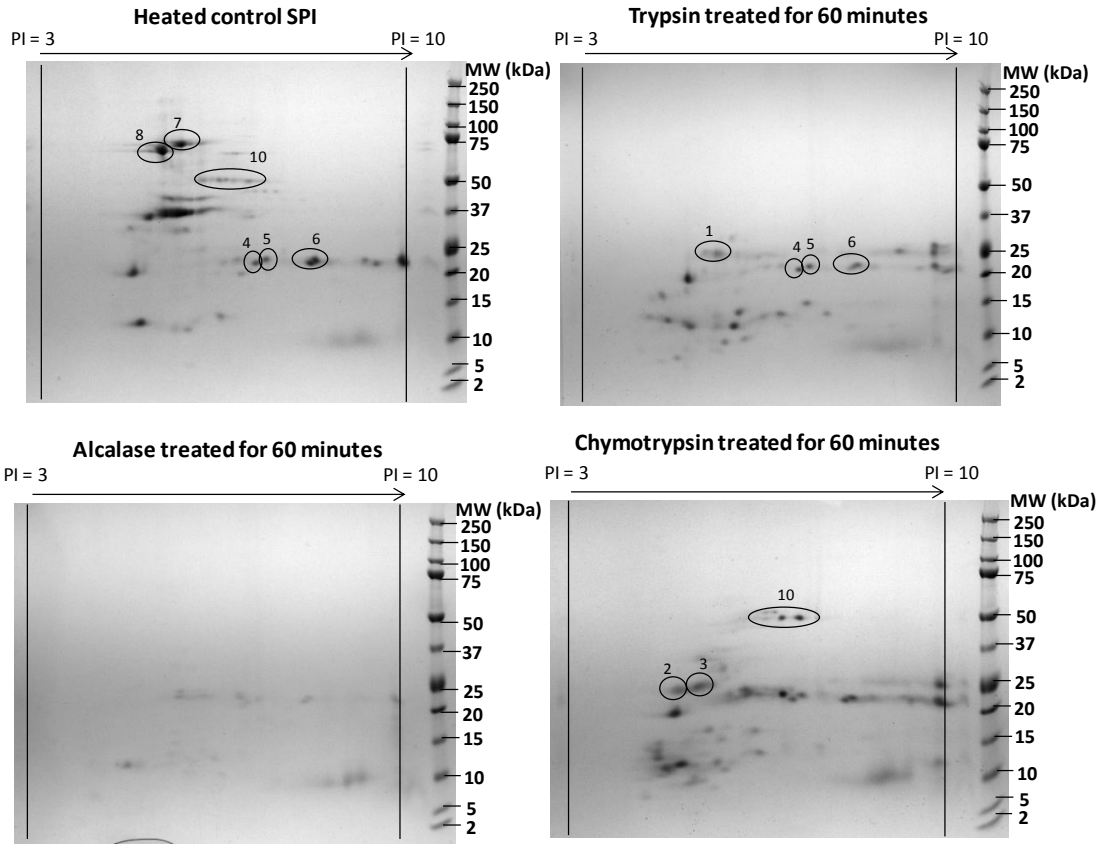

Figure 5A. Two dimensional gel electrophoresis of heated control SPI and SPI treated with Alcalase, trypsin or chymotrypsin for 60 min. Samples representing 25 µg of protein from the original undigested extracts were separated according to their isoelectric point using linear 3-10 IPG strips after dilution with rehydration buffer (8M urea, 2% CHAPS, 50mM DTT and 0.5% ampholyte). Separation in the second dimension was performed using SDS-PAGE. After protein separation the gels were fixed and then stained with Brilliant blue G colloidal and images were captured using Kodak gel logic 440 imaging system with white light illumination.

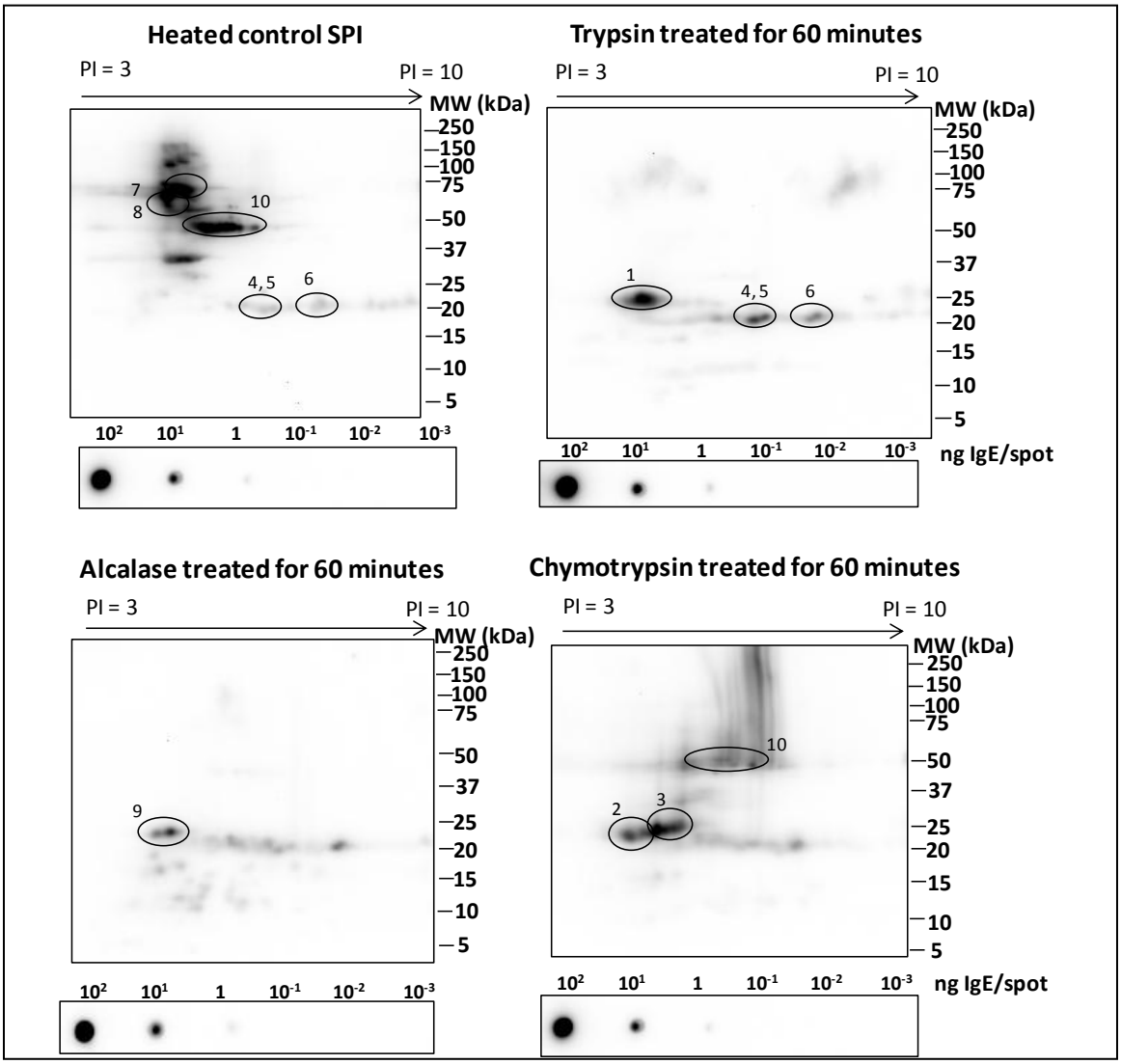

Figure 5B. Immunoblot of heated control SPI, Alcalase, trypsin or chymotrypsin treated SPI separated by two dimensional gel electrophoresis using serum 19392-CS. Samples representing 25 µg of protein from the original undigested extracts were separated according to their isoelectric point using linear 3-10 IPG strips after dilution with rehydration buffer (8M urea, 2% CHAPS, 50mM DTT and 0.5% ampholyte). Separation in the second dimension was performed using SDS-PAGE. The proteins from the gel were transferred onto a PVDF membrane. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

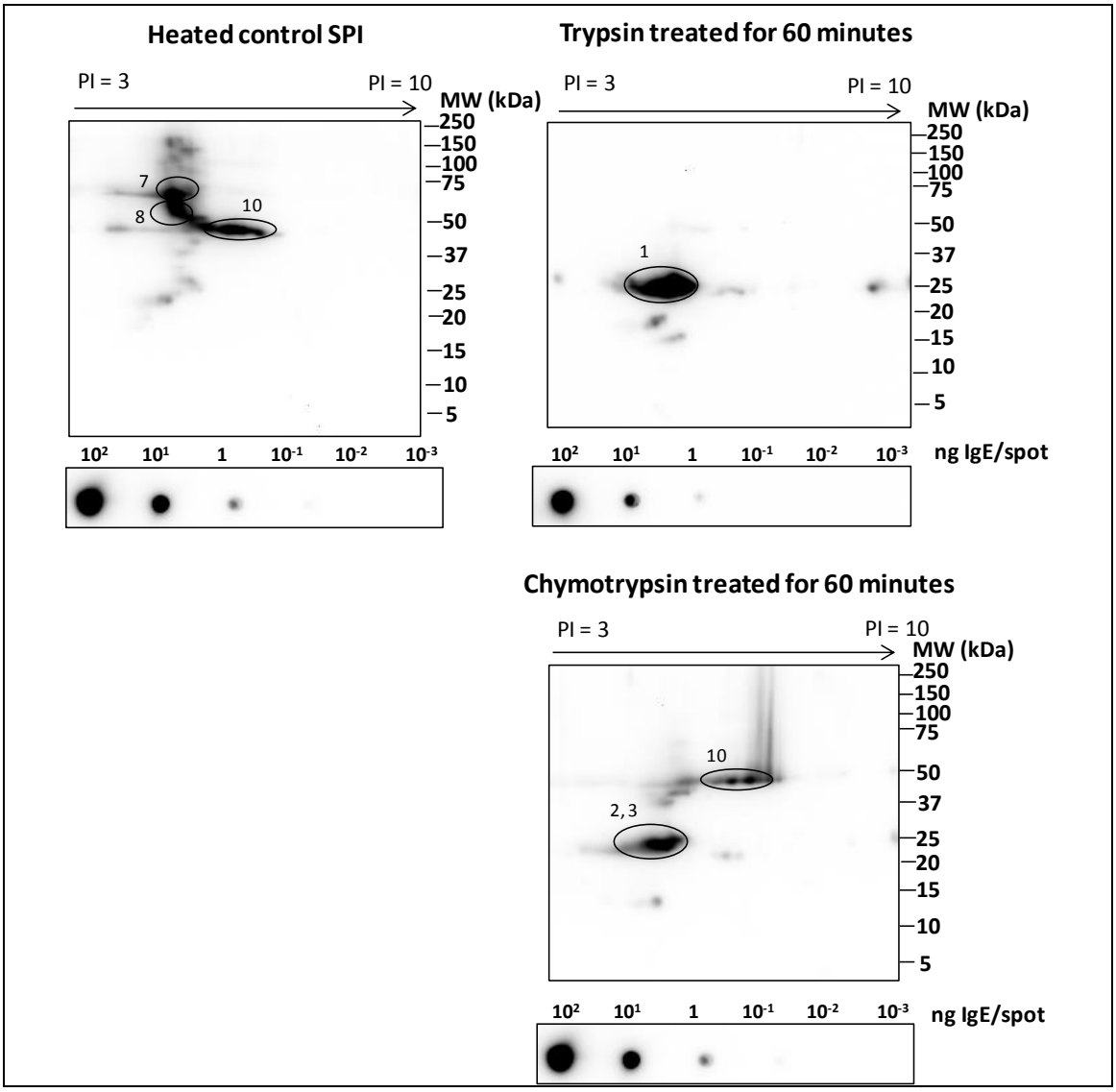

Figure 5C. Immunoblot of heated control SPI, trypsin or chymotrypsin treated SPI separated by two dimensional gel electrophoresis using serum 20431. Samples representing 25 µg of protein from the original undigested extracts were separated according to their isoelectric point using linear 3-10 IPG strips after dilution with rehydration buffer (8M urea, 2% CHAPS, 50mM DTT and 0.5% ampholyte). Separation in the second dimension was performed using SDS-PAGE. The proteins from the gel were transferred onto a PVDF membrane. A 1:10 diluted sample of serum was incubated with each membrane and following washing, bound IgE was detected using monoclonal anti-human IgE-HRP conjugate with detection by ECL. Images of the light emissions were captured using a Kodak imaging system as described in the text.

The 25 kDa, pI=4 spots marked on the immunoblots of Figure 5C (spot #1, 2, 3) in the trypsin and chymotrypsin hydrolyzed samples were not visible in the control sample. The strong IgE binding to these spots with both sera used in immunoblotting indicates the creation of a protein fragment of high IgE binding capacity due to hydrolysis of some high molecular weight proteins in SPI. Furthermore, the three spots at 23 kDa, pI=6-7 (Figure 5, spot # 4, 5, 6) showed stronger IgE binding for the trypsin hydrolyzed sample compared to the control sample with serum 19392-CS although the spots are visible in both the control and trypsin hydrolyzed sample stained gels. This indicates these proteins were likely modified to some extent by trypsin digestion, exposing hidden epitopes that resulted in stronger IgE binding. These strong IgE binding protein spots in Figure 5 (# 1, 2, 3, 4, 5, 6) were excised from a Coomassie stained gel and identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) by the analytical core facility at the University of Nebraska (Dr. Nandakumar). The high molecular weight protein spots (# 7, 8) showing strong IgE binding in the control sample and the 50 kDa spot (# 10) showing IgE binding both in the control and chymotrypsin digested sample with both serum 19392-CS and 20431 were also analyzed by LC- MS/MS. The highest scoring matches are shown here. The identified spots with peptide coverage (red) are indicated in the sequences below. The Mascot (Matrix Sciences) program output search for mass identity matches indicated matches to Glycinin G1 protein for spot # 4, 5, 6 from the non-redundant NCBI database showing coverage strictly in the basic chain of the protein (starting point shown by arrow). Spots # 1 and 3 showed a match with the alpha subunit of β-conglycinin whereas spot # 2 showed a match with the alpha’ subunit of β-conglycinin indicating that these spots in the trypsin and chymotrypsin hydrolyzed samples are fragments of the alpha’ and alpha

subunits of β-conglycinin (Spot # 7 and 8 respectively) that were visible in the control SPI stained gel and showed intense IgE binding with both the serum used in immunoblot. Spot # 10, the 50 kDa spot that remained resistant to digestion by chymotrypsin was identified as beta subunit of β-conglycinin (Figure 5).

Spot 4 (Glycinin G1 protein) GI:255221

MAKLVFSLCF LLFSGCCFAF SSREQPQQNE CQIQKLNALK PDNRIESEGG LIETWNPNNK
PFQCAGVALS RCTLNRNALR RPSYTNGPQE IYIQQGKGIF GMIYPGCPST FEEPQQPQQR
GQSSRPQDRH QKIYNFREGD LIAVPTGVAW WMYNNEDTPV VAVSIIDTNS LENQLDQMPR
RFYLAGNQEQ EFLKYQQEQG GHQSQKGKHQ QEEENEGGSI LSGFTLEFLE HAFSVDKQIA
KNLQGENEGE DKGAIVTVKG GLSVIKPPTD EQQQRPQEEE EEEEDEKPQC KGKDKHCQRP
RGSQSKSRRN GIDETICTMR LRHNIGQTSS PDIYNPQAGS VTTATSLDFP ALSWLRLSAE
FGSLRKNAMF VPHYNLNANS IIYALNGRAL IQVVNCNGER VFDGELQEGR VLIVPQNFVV
AARSQSDNFE YVSFKTNDTP MIGTLAGANS LLNALPEEVI QHTFNLKSQQ ARQIKNNNPF
KFLVPPQESQ KRAVA

Spot 5 (Glycinin G1 protein) GI:255221

MAKLVFSLCF LLFSGCCFAF SSREQPQQNE CQIQKLNALK PDNRIESEGG LIETWNPNNK
PFQCAGVALS RCTLNRNALR RPSYTNGPQE IYIQQGKGIF GMIYPGCPST FEEPQQPQQR
GQSSRPQDRH QKIYNFREGD LIAVPTGVAW WMYNNEDTPV VAVSIIDTNS LENQLDQMPR
RFYLAGNQEQ EFLKYQQEQG GHQSQKGKHQ QEEENEGGSI LSGFTLEFLE HAFSVDKQIA
KNLQGENEGE DKGAIVTVKG GLSVIKPPTD EQQQRPQEEE EEEEDEKPQC KGKDKHCQRP
RGSQSKSRRN GIDETICTMR LRHNIGQTSS PDIYNPQAGS VTTATSLDFP ALSWLRLSAE
FGSLRKNAMF VPHYNLNANS IIYALNGRAL IQVVNCNGER VFDGELQEGR VLIVPQNFVV
AARSQSDNFE YVSFKTNDTP MIGTLAGANS LLNALPEEVI QHTFNLKSQQ ARQIKNNNPF
KFLVPPQESQ KRAVA

Spot 6 (Glycinin G1 protein) GI:255221

MAKLVFSLCF LLFSGCCFAF SSREQPQQNE CQIQKLNALK PDNRIESEGG LIETWNPNNK
PFQCAGVALS RCTLNRNALR RPSYTNGPQE IYIQQGKGIF GMIYPGCPST FEEPQQPQQR
GQSSRPQDRH QKIYNFREGD LIAVPTGVAW WMYNNEDTPV VAVSIIDTNS LENQLDQMPR
RFYLAGNQEQ EFLKYQQEQG GHQSQKGKHQ QEEENEGGSI LSGFTLEFLE HAFSVDKQIA
KNLQGENEGE DKGAIVTVKG GLSVIKPPTD EQQQRPQEEE EEEEDEKPQC KGKDKHCQRP
RGSQSKSRRN GIDETICTMR LRHNIGQTSS PDIYNPQAGS VTTATSLDFP ALSWLRLSAE
FGSLRKNAMF VPHYNLNANS IIYALNGRAL IQVVNCNGER VFDGELQEGR VLIVPQNFVV
AARSQSDNFE YVSFKTNDTP MIGTLAGANS LLNALPEEVI QHTFNLKSQQ ARQIKNNNPF
KFLVPPQESQ KRAVA

Spot 1 (β-conglycinin alpha subunit) GI:15425633

MRARFPLLLL GVVFLASVSV SFGIAYWEKQ NPKHNKCLQS CNSERDSYRN QACHARCNLL
KVEKEEECEE GEIPRPRPRP QHPEREPQQP GEKEEDEDEQ PRPIPFPRPR QPRQEEEHEQ
REEQEWPRKE EKRGEKGSEE EQDGREHPRP HQPHDEDEEQ DERQFPFPRP PHQKESEERK
QEEDEDEEQQ RESEESESSE SQRELRRHKN KNPFHFGSNR FETLFKNQYG RIRVLQRFNQ
RSPQLQNLRD YRILEFNSKP NTLLLPNHAD ADYLIAILNG TAILSLVNND DRDSYRLQSG
DALRVPSGTT YYVVNPDNNE NLRLITLAIP VNKPGRFESF FLSSTEAQQS YLQGFSRNIL
EASYDTKFEE INKVLFSREE GQQQGEQRLQ ESVIVEISKE QIRALSKRAK SSSRKTISSE
DKPFNLRSRD PIYSNKLGKF FEITPEKNPQ LRDLDIFLSI VDMNEGALLL PHFNSKAIVI
LVINEGDANI ELVGLKEQQQ EEQQEEQPLE VRKYRAELSE QDIFVIPAGY PVVVNATSNL
NFFAIGINAE NNQRNFLAGS QDNVISQIPS QVQELAFLGS AQAVEKLLKN QRESYFVDAQ
PKKKEEGNKG RKGPLSSILR AFY

Spot 2 (β-conglycinin alpha'-subunit) GI:341603991

MMRARFPLLL LGVVFLASVS VSFGIAYWEK QNPSHNKCLR SCNSEKDSYR NQACHARCNL
LKVEEEEECE EGQIPRPRPQ HPERERQQHG EKEEDEGEQP RPFPFPRPRQ PHQEEEHEQK
EEHEWHRKEE KHGGKGSEEE QDEREHPRPH QPHQKEEEKH EWQHKQEKHQ GKESEEEEED
QDEDEEQDKE SQESEGSESQ REPRRHKNKN PFHFNSKRFQ TLFKNQYGHV RVLQRFNKRS
QQLQNLRDYR ILEFNSKPNT LLLPHHADAD YLIVILNGTA ILTLVNNDDR DSYNLQSGDA
LRVPAGTTYY VVNPDNDENL RMITLAIPVN KPGRFESFFL SSTQAQQSYL QGFSKNILEA
SYDTKFEEIN KVLFGREEGQ QQGEERLQES VIVEISKKQI RELSKHAKSS SRKTISSEDK
PFNLRSRDPI YSNKLGKLFE ITPEKNPQLR DLDVFLSVVD MNEGALFLPH FNSKAIVVLV
INEGEANIEL VGIKEQQQRQ QQEEQPLEVR KYRAELSEQD IFVIPAGYPV VVNATSDLNF
FAFGINAENN QRNFLAGSKD NVISQIPSQV QELAFPGSAK DIENLIKSQS ESYFVDAQPQ
QKEEGNKGRK GPLSSILRAF Y

Spot 3 (β-conglycinin alpha subunit) GI:15425633

MRARFPLLLL GVVFLASVSV SFGIAYWEKQ NPKHNKCLQS CNSERDSYRN QACHARCNLL
KVEKEEECEE GEIPRPRPRP QHPEREPQQP GEKEEDEDEQ PRPIPFPRPR QPRQEEEHEQ
REEQEWPRKE EKRGEKGSEE EQDGREHPRP HQPHDEDEEQ DERQFPFPRP PHQKESEERK
QEEDEDEEQQ RESEESESSE SQRELRRHKN KNPFHFGSNR FETLFKNQYG RIRVLQRFNQ
RSPQLQNLRD YRILEFNSKP NTLLLPNHAD ADYLIAILNG TAILSLVNND DRDSYRLQSG
DALRVPSGTT YYVVNPDNNE NLRLITLAIP VNKPGRFESF FLSSTEAQQS YLQGFSRNIL
EASYDTKFEE INKVLFSREE GQQQGEQRLQ ESVIVEISKE QIRALSKRAK SSSRKTISSE
DKPFNLRSRD PIYSNKLGKF FEITPEKNPQ LRDLDIFLSI VDMNEGALLL PHFNSKAIVI
LVINEGDANI ELVGLK**EQQQ EEQQEEQPLE VR**KYRAELSE QDIFVIPAGY PVVVNATSNL
NFFAIGINAE NNQRNFLAGS QDNVISQIPS QVQELAFLGS AQAVEKLLKN QRESYFVDAQ
PKKKEEGNKG RKGPLSSILR AFY

Spot 7 (β-conglycinin alpha'-subunit) GI:341603991

MMRARFPLLL LGVVFLASVS VSFGIAYWEK QNPSHNKCLR SCNSEKDSYR NQACHARCNL
LKVEEEEECE EGQIPRPRPQ HPERERQQHG EKEEDEGEQP RPFPFPRPRQ PHQEEEHEQK
EEHEWHRKEE KHGGKGSEEE QDEREHPRPH QPHQKEEEKH EWQHKQEKHQ GKESEEEEED
QDEDEEQDKE SQESEGSESQ REPRRHKNKN PFHFNSKRFQ TLFKNQYGHV RVLQRFNKRS
QQLQNLRDYR ILEFNSKPNT LLLPHHADAD YLIVILNGTA ILTLVNNDDR DSYNLQSGDA
LRVPAGTTYY VVNPDNDENL RMITLAIPVN KPGRFESFFL SSTQAQQSYL QGFSKNILEA
SYDTKFEEIN KVLFGREEGQ QQGEERLQES VIVEISKKQI RELSKHAKSS SRKTISSEDK
PFNLRSRDPI YSNKLGKLFE ITPEKNPQLR DLDVFLSVVD MNEGALFLPH FNSKAIVVLV
INEGEANIEL VGIKEQQQRQ QQEEQPLEVR KYRAELSEQD IFVIPAGYPV VVNATSDLNF
FAFGINAENN QRNFLAGSKD NVISQIPSQV QELAFPGSAK DIENLIKSQS ESYFVDAQPQ
QKEEGNKGRK GPLSSILRAF Y

Spot 8 (β-conglycinin alpha subunit) GI:15425633

MRARFPLLLL GVVFLASVSV SFGIAYWEKQ NPKHNKCLQS CNSERDSYRN QACHARCNLL
KVEKEEECEE GEIPRPRPRP QHPEREPQQP GEKEEDEDEQ PRPIPFPRPR QPRQEEEHEQ
REEQEWPRKE EKRGEKGSEE EQDGREHPRP HQPHDEDEEQ DERQFPFPRP PHQKESEERK
QEEDEDEEQQ RESEESESSE SQRELRRHKN KNPFHFGSNR FETLFKNQYG RIRVLQRFNQ
RSPQLQNLRD YRILEFNSKP NTLLLPNHAD ADYLIAILNG TAILSLVNND DRDSYRLQSG
DALRVPSGTT YYVVNPDNNE NLRLITLAIP VNKPGRFESF FLSSTEAQQS YLQGFSRNIL
EASYDTKFEE INKVLFSREE GQQQGEQRLQ ESVIVEISKE QIRALSKRAK SSSRKTISSE
DKPFNLRSRD PIYSNKLGKF FEITPEKNPQ LRDLDIFLSI VDMNEGALLL PHFNSKAIVI
LVINEGDANI ELVGLKEQQQ EEQQEEQPLE VRKYRAELSE QDIFVIPAGY PVVVNATSNL
NFFAIGINAE NNQRNFLAGS QDNVISQIPS QVQELAFLGS AQAVEKLLKN QRESYFVDAQ
PKKKEEGNKG RKGPLSSILR AFY

Spot 10 (β-conglycinin beta subunit) GI: 341603993

MMRVRFPLLV LLGTVFLASV CVSLKVREDE NNPFYFRSSN SFQTLFENQN GRIRLLQRFN
KRSPQLENLR DYRIVQFQSK PNTILLPHHA DADFLLFVLS GRAILTLVNN DDRDSYNLHP
GDAQRIPAGT TYYLVNPHDH QNLKIIKLAI PVNKPGRYDD FFLSSTQAQQ SYLQGFSHNI
LETSFHEINR VLFGEEEEQR QQEGVIVELS KEQIRQLSRR AKSSSRKTIS SEDEPFNLRS
RNPIYSNNFG KFFEITPEKN PQLRDLDIFL SSVDINEGAL LLPHFNSKAI VILVINEGDA
NIELVGIKEQ QQKQKQEEEP LEVQRYRAEL SEDDVFVIPA AYPFVVNATS NLNFLAFGIN
AENNQRNFLA GEKDNVVRQI ERQVQELAFP GSAQDVERLL KKQRESYFVD AQPQQKEEGS
KGRKGPFPSI LGALY

DISCUSSION

Soybean is a popular and widely used food protein source that is often processed by hydrolysates prior to addition to foods since hydrolysis has been shown to increase nutritional quality, flavor and functionality of soybean proteins (Sun, 2011). Among the two commonly used methods to obtain protein hydrolysates (acid and enzymatic), enzymatic hydrolysis is mostly preferred to hydrolyze soybean proteins since it prevents formation of undesirable side products. Further functionality of the final product can be controlled by selection of specific enzymes and reaction conditions (Sun, 2011). Several enzymes derived from plant, bacteria or fungal sources have been utilized to hydrolyzed soybean proteins in order to improve their functionality (Calderon de la barca, et al. 2000; Kim et al., 1990; Molina Ortiz et al. 2000). Protein hydrolysate formulas have also been developed with the aim to produce hypoallergenic foods that have reduced sensitizing or elicitating capacity. Enzymatic hydrolysis of food proteins can lead to alteration of epitope structure thereby reducing allergenicity (Paschke and Besler, 2002). Sequential hydrolysis of chickpea protein isolate and lentil protein extract with enzymes Alcalase and flavourzyme has been shown to reduce IgE binding to lentil and chickpea protein hydrolysates (Cabanillas et al., 2010; Clemente et al., 1999). Enzymes such as Alcalase, pepsin and trypsin has been used to hydrolyze and reduce immunoreactivity of pea protein extract (Szymkiewicz and Jedrychowski, 2005). Furthermore, enzymes such as protease, elastase and trypsin have been shown to eliminate IgE binding to hazelnut proteins (Wigotzki et al., 2000). In case of soybeans several enzymes such as *Bacillus sp*. Protease porleather FG-F, pepsin and chymotrypsin has been shown to reduce IgE binding to hydrolyzed soybean proteins using soybean sensitive patient sera. However,

one of the major drawbacks in these studies has been the interpretation that allergenicity is only based on in vitro IgE binding as measured by ELISA or immunoblotting procedures. Yet, IgE binding does not always correlate with expression of clinical symptoms as IgE binding may be to poorly reactive cross-reactive carbohydrate determinants, low affinity binding, the occurrence of individual isolated IgE binding epitopes or ineffective binding due to very close or distant location of epitopes (Ladics et al., 2008). Therefore the final confirmation of reduced allergenicity should be from the results of functional assays such as basophil histamine release assay, skin prick test or double blind placebo controlled food challenge (DBPCFC).

In this study, soybean proteins in the form of defatted soy flour and SPI were hydrolyzed by five different enzymes (Alcalase, papain, bromelain, trypsin and chymotrypsin) that are commonly used to improve the functionality of soybean proteins as well as in making hypoallergenic protein formulas. The allergenicity of soybean proteins after hydrolysis was evaluated by in vitro IgE binding by immunoblot as well as a functional assay, which measured the release of β-hexosaminidase from hRBL cell lines. From the IgE immunoblot results with eight soybean allergic patient sera, an overall reduction in IgE binding to protein bands was observed for the soybean samples hydrolyzed with Alcalase, papain and trypsin. However, IgE binding was still retained for a few soybean proteins in the hydrolyzed samples. These proteins are probably more resistant to digestion than other immunoreactive proteins. With bromelain and chymotrypsin, most of the IgE binding seen with the control sample was retained in the hydrolyzed samples (observed with six out of eight soybean allergic sera used in immunoblot) indicating that digestion with these enzymes has little impact on the

immunoreactivity of soybean proteins. Furthermore, with two of the sera (19392-CS and 20431) new IgE binding bands appeared in the samples hydrolyzed with Alcalase, bromelain, trypsin and chymotrypsin. This increase in IgE binding observed compared to control sample could represent an increase in immunoreactivity and possibly allergenicity, either due to creation of some new protein epitopes due to protein hydrolysis or exposure of some already present hidden epitopes making them more accessible to IgE antibodies. From the 2D immunoblot results of this study it was concluded that both of these processes resulted in increased IgE binding. With the samples used in this study, three spots at approximately 25 kDa, pI=4 (# 1, 2, 3) were identified as fragments of alpha' and alpha subunits of β-conglycinin by LC-MS/MS. These spots were only in the trypsin and chymotrypsin digested samples in 2D gels (Figure 5A). These spots showed strong IgE binding in immunoblot with two sera (Figure 5B and 5C) indicating creation of new protein epitopes by enzyme hydrolysis of the β-conglycinin protein. Further with trypsin hydrolyzed sample, with one serum, increased IgE binding compared to the control sample was observed with three spots at approximately 23 kDa, pI 6-7, which was already present in the control sample (Spot # 4, 5, 6, Figure 5A, 5B, identified as glycinin basic chain in LC-MS/MS) indicating exposure of some hidden epitopes in the glycinin protein by treatment with trypsin.

Despite the overall reduction in IgE binding to protein bands observed in case of soybean samples hydrolyzed with papain, Alcalase and trypsin, all of them showed a similar or slightly reduced mediator release as the control sample in hRBL assay. This indicates that the protein fragments remaining after hydrolysis with these enzymes can still cross-link IgE antibodies on basophils or mast cells and lead to elicitation of an

allergic reaction even if they were not detected by IgE immunoblot. Furthermore, bromelain hydrolysis of defatted soy flour and SPI and chymotrypsin hydrolysis of SPI lead to an increase in mediator release compared to the control sample indicating that digestion of soybean proteins with these enzymes can increase elicitation capacity of soybean proteins.

Another important finding from this study was that the form of soy protein product subjected to hydrolysis condition is important in determining the effect of hydrolysis on protein allergenicity. For example, with serum 19392-CS, chymotrypsin hydrolysis of SPI resulted in a strong increase in IgE binding at approximately 20-25 kDa compared to the control sample, whereas the IgE binding at the same molecular weight when defatted soy flour extract was digested with chymotrypsin was moderately increase (Figure 4C, 4D). These new strong IgE binding bands in the chymotrypsin digested SPI was identified as fragments of the alpha and alpha' subunits of β-conglycinin by 2D gel electrophoresis followed by LC-MS/MS analysis. This observation was also reflected in the mediator release assay results where chymotrypsin digested SPI resulted in a higher mediator release compared to the control sample whereas a reduction in mediator release was observed with chymotrypsin digested defatted soy flour extract (Figure 4F). Since SPI is composed mostly of glycinin and β-conglycinin proteins (~90% of the total protein), which are the two major allergenic proteins of soybeans (Holzhauser et al., 2009), it is possible that preparation of SPI resulted in concentration of these two allergic proteins in the SPI preparation and its subsequent digestion with chymotrypsin resulted in creation of fragments with strong IgE binding as well as higher mediator release capacity compared to undigested SPI.

In conclusion, the finding from this study indicated that none of the enzymes that are used in making functional soybean protein products or hypoallergenic soybean protein hydrolysate are effective in reducing the allergenicity of soybean proteins. In fact some of the hydrolysates (bromelain and chymotrypsin hydrolyzed soybean proteins) have a potential to increase the allergenicity of soybean proteins. Hydrolysis of different forms of protein preparations may have different effect on allergenicity as demonstrated by an increase in allergenicity of chymotrypsin digested SPI and not defatted soy flour in our study. Most importantly our study demonstrated that in vitro IgE binding tests alone cannot be confirmatory methods to conclude on allergenicity of protein hydrolysates and function assays such as mediator release assay (used in this study) are essential for confirmation of allergenicity.

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