



Limited hydrolysis combined with controlled Maillard-induced glycation does not reduce immunoreactivity of soy protein for all sera tested



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ABSTRACT

Combining proteolysis and Maillard-induced glycation was investigated to reduce the immunoreactivity of soy protein. Soy protein was hydrolyzed by Alcalase following response surface methodology utilizing three variables, temperature, time, and enzyme:substrate ratio, with the degree of hydrolysis (DH) and percent reduction in immunoreactivity as response variables. Western blots and ELISA were used to evaluate immunoreactivity using human sera. Data were fitted to appropriate models and prediction equations were generated to determine optimal hydrolysis conditions. The hydrolysate produced under optimized conditions was subjected to glycation with dextran. Hydrolysate produced under optimal conditions had 7.8% DH and a percent reduction in immunoreactivity ranging from 20% to 52%, depending on the sera used. Upon glycation, immunoreactivity was further reduced only when using serum that had the highest soy-specific IgE. This work revealed limitations and provided premises for future studies intended to prove the potency of the combined modification approach to produce a hypoallergenic protein ingredient.

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1. Introduction

In 1999, the Food and Drug Administration (FDA) approved a health claim that “25 g of soy protein a day, as part of a diet low in saturated fat and cholesterol, may reduce the risk of heart disease”. As a consequence, soy protein-based foods, including processed meat, energy bars, infant formulas, and dairy product replacements, are among the fastest growing categories in the food industry. However, one of the major drawbacks for the expanded use of soy protein is its allergenicity. Soybean is recognized by the FDA as one of “the big eight” food allergens, with about 0.5% of the U.S. population having an allergy to soy (Sicherer & Sampson, 2006). While allergic reactions to soy mostly include hives, rhinitis, and inflammation, incidences of anaphylactic shock have also occurred resulting in death (Foucard & Malmheden Yman, 1999). Currently, with the No Observed Adverse Effect Level (NOAEL) of soy being as low as 0.0013 mg of soy flour (Becker, Brasseur, Bresson, Flynn, & Jackson, 2004), avoidance of soy is the only recommendation given to allergic individuals. But with over 6000 different food products containing soybean and/or peanuts (Helm, Burks, & Herman, 2002), avoidance of soy containing food

can be challenging. Therefore, despite the health benefits of soy protein, the utilization of soy protein ingredients may gradually decrease over time with the continued increase in the incidences of soy protein allergy. In order to overcome this problem, it is crucial to develop techniques that can eliminate soy protein allergenicity through investigating various protein modification approaches.

While it is hard to eliminate soybean allergy as a crop (Riascos, Arthur, Sandra, & Burks, 2010), it is possible to reduce the allergenicity of soy protein ingredients by modifying the protein structure. Several hypoallergenization techniques, including enzymatic hydrolysis and Maillard-induced glycation, have been exploited to reduce the allergenicity of soy protein ingredients. In order to reduce allergenicity, extensive hydrolysis ($\geq 25\%$) has been employed (Calderon De La Barca, Wall, & Lopez-Diaz, 2005; Tsumura, Kugimiya, Bando, Hiemori, & Ogawa, 1999). Extensively hydrolyzed protein ingredient is mainly used in infant formula and has had limited if any applications in other processed foods. Apart from the bitter taste (Muraro, 2001), functionality of an extensively hydrolyzed soy protein is adversely affected (Tsumura et al., 1999). Additionally, hydrolyzed products vary in their residual allergenicity, which is a function of the extent of hydrolysis and the selective destruction of the epitopes. While hydrolysis may destroy certain epitopes, it may result in the exposure of other epitopes buried within the three-dimensional

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structure of the protein (L'Hocine & Boye, 2007), and may result in increased allergenicity (Panda, Tetteh, Pramod, & Goodman, 2015).

One of the major problems to address protein allergenicity in the food industry is the flavor and functionality of a protein ingredient that has been extensively hydrolyzed to make it hypoallergenic. Hence, the need to investigate other ways to modify the protein, such as Maillard-induced glycation (Arita, Babiker, Azakami, & Kato, 2001; Babiker, Hiroyaki, Matsudomi, & Iwata, 1998; Usui, Tamura, Nakamura, Ogawa, & Muroshita, 2004). While allergenicity of a major soy protein allergen, Gly m Bd 30 K (also known as P34) was reduced upon glycation (Babiker et al., 1998; Usui et al., 2004), the extent of alteration of potential epitopes of other soy proteins is not known. Additionally, the glycation conditions used were rather extensive. While protein limited glycation may have functional benefits, extensive glycation may have detrimental effects on quality, including development of advanced glycation end products, lysine blockage, and reduced digestibility.

Limited degree of hydrolysis (DH < 8%), which may maintain flavor and functionality, may reduce allergenicity of soy protein, however not to an acceptable level; and may result in the exposure of originally buried epitopes. Similarly, limited Maillard-induced glycation, may improve functionality, and reduce, yet not eliminate allergenicity. Combining the two mechanisms, proteolysis and Maillard glycation, under controlled and mild conditions may have a synergistic effect on reducing allergenicity, while maintaining quality.

The objectives of this work were: 1) Determine optimal hydrolysis conditions for maximum reduction of soy protein immunoreactivity using response surface methodology; 2) Determine the effect of limited and controlled Maillard-induced glycation on the allergenicity of soy protein hydrolysate produced under optimized conditions.

2. Materials and methods

2.1. Materials

Minimally heat-treated and defatted soy flour and Alcalase 2.4L (2.4 AU-A/g) were kindly provided by Archer Daniels Midland (Decatur, IL, USA) and Novozymes (Denmark), respectively. Biotinylated Mouse anti-Human IgE was purchased from Abcam (Cambridge, England). Polyvinylidene fluoride (PVDF) was purchased from Millipore (Billerica, MA, USA). Enhanced chemiluminescent substrate was obtained from Pierce Scientific (Rockford, IL, USA). Microplates were purchased from Corning (Tewksbury, MA, USA). Goat anti-human IgE was purchased from Kirkland and Perry Laboratories (Gaithersburg, MD, USA). Streptavidin alkaline phosphatase, p-nitrophenol phosphate, streptavidin peroxidase, and dextran were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular weight standards, Laemmli buffer, Tris/Glycine/SDS buffer, and TGX Precast Gels were purchased from BioRad (Hercules, CA, USA). Other reagents were purchased from Fisher Scientific (Waltham, MA, USA).

2.2. Patient recruitment and sera collection

Clinical laboratory services were provided by Clinical Translational Science Institute at the University of Minnesota following Institutional Review Board approval. Self-reported soybean-allergic subjects were screened by reviewing their skin prick and/or radioallergoabsorbent test results. Once a history of soy allergy was confirmed, blood samples were collected. Serum samples were sent to Quest Diagnostics (Woodale, IL, USA) to analyze for the soy specific IgE, IgE total, and IgG total. A total of 12 subjects were screened and 5 with soy specific IgE levels of

3.5 kU/L or greater were chosen for participation (Table S1). A soy specific IgE level of ≥ 3.5 kU/L is considered high according to the manufacturer of the ImmunoCap assay used by Quest to determine soy specific IgE levels. Up to 100 mL of blood was drawn from the 5 participants at once or on multiple visits depending on body weight. Serum was separated from blood by centrifugation, aliquoted, and stored at -80°C for later use.

2.3. Soy protein isolate (SPI) production from defatted soy flour

Defatted soy flour was used to prepare SPI following the method outlined by Margatan, Ruud, Wang, Markowski, and Ismail (2013). The protein content of the lyophilized sample was 91.8% as determined following the Dumas method (AOAC 990.03) using a nitrogen analyzer (LECO, St. Joseph, MI, USA).

2.4. Response surface methodology (RSM) design

Soy protein isolate (SPI) was subjected to enzymatic hydrolysis following RSM utilizing a central composite design (CCD) consisting of 3 variables, temperature (x_1), time (x_2), and enzyme to substrate ratio (E:S) (x_3). Alcalase was used as it has potential to reduce soy protein allergenicity (Panda et al., 2015). The pH was held constant at 7.5, which is within the optimum range for Alcalase. The CCD design consisted of an embedded factorial design and axial points. The distance from the center point to a factorial point is ± 1 , while the distance from the center to an axial point is $\pm$ the absolute value of alpha ($\alpha = 1.63$). The design was generated using R 3.01.2 "Good Sport" and consisted of 20 different experimental runs with 6 repeated center points, 8 factorial points, and 6 axial points (Table 1). Preliminary experiments were conducted to determine central points that would lead to a DH between the targeted range of 2–8%. The temperature levels selected did not exceed the optimum for the Alcalase. The relationship between the independent variables and the responses, DH and %reduction in immunoreactivity, was determined using Design Expert 8.0 software (Stat Ease, Minneapolis, MN). Response surface plots were generated using R. Prediction equations were generated for all responses with Eqs. (1)–(3) as examples of first order response, first order with interaction response, and second order response. The conditions at which the responses were optimal were then determined to validate the model. To validate the model, experimental data were obtained using the predicted optimum levels for each experimental factor.

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \varepsilon \quad (1)$$

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (2)$$

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_1 x_1^2 + \beta_2 x_2^2 + \beta_3 x_3^2 + \varepsilon \quad (3)$$

y is the dependent variable (%Reduction in immunoreactivity or % DH), x_i is the level of the independent variable, β_0 is the linear coefficient predicted by the model, β_i is the linear term, $\beta_{ixi} x_i$ represents the interaction term, while β_i^2 represents the quadratic term predicted by the model, ε is the error associated with the model.

2.5. Hydrolysis of SPI using pH stat method

Soy protein isolate was subjected to enzymatic hydrolysis by Alcalase 2.4L following the pH-stat methodology (Adler-Nissens, 1986) using a DL22 titrator (Mettler Toledo, Columbus, OH, USA). Dispersions of SPI in DDW (5% protein w/v) were prepared in triplicate and the pH adjusted to 7.5. The dispersions were preheated for 15 min prior to the addition of Alcalase. Alcalase was added

Table 1

Central composite design for the hydrolysis conditions using Alcalase 2.4 L, showing experimental and predicted DH values for the 15 SPH samples produced.

Coded Level			Natural Levels ^a			SPH code ^b	DH ± SD ^c	Predicted ^d DH
x1	x2	x3	x1	x2	x3			
0	0	0	55	45	0.2	9	6.06 ± 0.0021	6.23
0	0	0	55	45	0.2	9	6.49 ± 0.0033	6.23
0	0	0	55	45	0.2	9	6.19 ± 0.0019	6.23
0	0	0	55	45	0.2	9	6.21 ± 0.0016	6.23
0	0	0	55	45	0.2	9	6.10 ± 0.0025	6.23
0	0	0	55	45	0.2	9	6.39 ± 0.0046	6.23
−1	−1	−1	45	25	0.1	1	1.17 ± 0.0004	1.19
1	−1	−1	65	25	0.1	6	3.64 ± 0.0018	3.28
−1	1	−1	45	65	0.1	5	3.12 ± 0.0011	3.01
1	1	−1	65	65	0.1	10	6.77 ± 0.0010	6.24
−1	−1	1	45	25	0.4	7	3.79 ± 0.0006	4.15
1	−1	1	65	25	0.4	13	9.61 ± 0.0014	9.56
−1	1	1	45	65	0.4	8	6.06 ± 0.0008	7.04
1	1	1	65	65	0.4	15	14.6 ± 0.0029	14.3
−1.63	0	0	38.67	45	0.2	4	2.49 ± 0.0014	1.64
1.63	0	0	71.33	45	0.2	11	7.58 ± 0.0053	8.67
0	−1.63	0	55	12.38	0.2	2	2.03 ± 0.0008	2.45
0	1.63	0	55	77.66	0.2	12	8.03 ± 0.0041	7.85
0	0	−1.63	55	45	0.06	3	2.28 ± 0.0034	2.50
0	0	1.63	55	45	0.62	14	13.1 ± 0.0034	12.31
Optimized Conditions			65	65	0.12	–	7.82 ± 0.026	7.50

^a x1 represents temperature (°C); x2 represents time (min); x3 represents enzyme to substrate ratio (E:S; mL of enzyme: 100 g protein).^b Soy protein hydrolysate sample codes based on degree of hydrolysis (DH), with 1 being given for the sample with the lowest DH and 15 being given to the sample with the highest DH.^c SD: standard deviation, n = 3.^d Predicted values obtained using the best fitted RSM model.

to obtain a specified E:S ratio (Table 1). The pH was maintained at 7.5 throughout the incubation period. The hydrolysates were then boiled for 5 min in order to inactivate the enzyme, and were lyophilized. Lyophilized samples were analyzed for protein content using a nitrogen analyzer following the Dumas method, and kept at −20 °C. The protein content of the hydrolysates ranged from 86% to 94%. The DH was calculated following the formula reported by Adler-Nissens (1986). Following RSM analysis, a soy protein hydrolysate (SPH) (DH = 7.8%) was produced under the optimized enzymatic conditions determined.

2.6. Glycation of SPI and SPH

Both SPI and SPH produced under optimized hydrolysis conditions were subjected to Maillard-induced glycation. Glycation of SPI was carried out to determine the effect of glycation on the allergenicity of intact protein. This information was used to assess whether or not controlled Maillard-induced glycation and limited hydrolysis have a synergistic effect on reducing the allergenicity of soy protein. Both SPI and SPH were mixed with dextran (narrow molecular weight range with an average of 10 kDa; 2.3 dextrose equivalent) in 1:4 ratio (w/w), dissolved in a phosphate buffer (0.1 M, pH 7), and lyophilized. The lyophilized samples were incubated, in triplicate, in a desiccator at 60 °C and water activity (a_w) of 0.43 for 24, 48, 72, 96, and 120 h. Control SPI and SPH samples were incubated without dextran under the same conditions. Dextran (10 kDa) was chosen because it allowed better control of the rate of Maillard reaction, and resulted in minimal glycation, improved functionality of the protein, and preserved digestibility and nutritional quality (Wang & Ismail, 2012). The a_w was set at 0.43 below the optimum for the Maillard reaction (0.60–0.85) in order to control the reaction and avoid the propagation to advanced stages. A protein to carbohydrate ratio of 1:4 was selected based on preliminary work. The incubation temperature of 60 °C was selected to avoid protein denaturation. Samples were pulled out at different time intervals, analyzed for protein content, and stored at −20 °C for later analysis.

2.7. Amadori compounds, browning, fluorescence and loss of free amino groups

Monitoring of Amadori compounds and browning in partially glycosylated soy protein (PGSP) and partially glycosylated SPH (PGSPH) samples was carried out as outlined by Wang and Ismail (2012). Fluorescence index was determined as outlined by Rao, Rocca-Smith, and Labuza (2013). Percent of free amino groups was determined based on the o-phthalaldehyde (OPA) method outlined by Goodno, Swaisgood, and Catignani (1981) using lysine as a standard (5–200 µg/mL).

2.8. Sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein/peptide distribution was monitored using SDS-PAGE as outlined by Laemmli (1970). Each sample was dissolved in DDW (~10.60 mg protein/mL), and an aliquot was mixed 1:1 (v/v) with Laemmli buffer under reducing (0.75 M β-mercaptoethanol) conditions and boiled for 5 min. Aliquots (5 µL; containing ~26.5 µg protein) and pre-stained molecular weight standards (10 µL) were loaded onto wells of a pre-cast 4–20% gradient gels. Gels were electrophoresed at 150 V for approximately 1 h and then were stained using Coomassie Brilliant Blue solution. Molecular Imager Gel Doc XR system (Biorad, Hercules, CA, USA) was used to scan the gels. To determine the band intensities, the image acquired was uploaded into Image J according to the method outlined by Ge, Bahaie, Kang, Hosseinkhani, and Ha (2010). Percent reductions in protein band intensities, after background subtraction, were determined in triplicate (i.e. average measurement from three gels).

2.9. Western blot

Western blots were performed based on the method outlined by Eigenmann, Burks, Bannon, and Sampson (1996). Samples were prepared for electrophoresis as outlined above. Transfer onto a PVDF membrane was carried out using transfer buffer (48 mM Tris,

39 mM Glycine, 20% Methanol w/v, 0.0375% w/v SDS) at room temperature and at 300 A for 2 h. In order to confirm a complete transfer, gels after transfer were stained with Coomassie blue and scanned as outlined above. Membranes were blocked by incubating with 0.5% bovine gelatin in PBS (10 mM phosphate, 0.138 M NaCl, 2.7 mM M KCl, pH 7.4)-Tween 20 (0.1% w/v) overnight at 4 °C, followed by washing with PBS-Tween 20 (0.1%). The membranes were then incubated with serum (1:8–1:50 dilution) for 2 h at room temperature. Membranes were washed with PBS-Tween 20 (0.1% w/v) for 25 min and incubated at room temperature with secondary biotinylated mouse anti-human IgE (1:3000) for 1 h, followed by washing with PBS-Tween 20. Membranes were then incubated with streptavidin-peroxidase (diluted 1:2000 using 0.1% BSA/PBS w/v) for 30 min at room temperature, and washed with PBS-Tween 20. All blots were developed by enhanced chemiluminescent substrate and scanned with a film processor. Determination of band intensities was carried out as described above.

2.10. Enzyme linked immunosorbant assay (ELISA)

Samples were prepared in triplicates (5 µg protein/mL PBS). Wells of a Costar 3370 non-treated, sterile polystyrene microtiter plate were coated each with 100 µL of each prepared sample and left overnight at 4 °C. Plates were washed 4 times in between each step using an automatic plate washer (Stat Fax, Orlando, FL, USA) with PBST (PBS with 0.05% Tween 20 w/v). Plates were blocked using 200 µL/well of 3% w/v bovine serum albumin (BSA) + 0.05% w/v Tween 20 was carried out at room temperature for 1 h. Diluted (1:10–1:50 using PBST) serum (100 µL) was added to each well and the plate was incubated at room temperature for 1 h. An aliquot (100 µL) of diluted secondary antibody (1:250 using tris buffered saline (TBS), 50 mM Tris, 0.138 M NaCl, 2.7 mM KCl, pH 8.0, and 0.01% Tween 20, w/v) was added to each well and the plate was incubated at room temperature for 1 h. An aliquot (100 µL) of diluted streptavidin alkaline phosphatase (1:500 in TBS and 0.01% Tween 20, w/v) was added to each well and the plate was incubated at room temperature for 30 min. Lastly, p-nitrophenol phosphate (100 µL) was added to each well, and the plate was incubated at room temperature for 30 min. Absorbance was read at 405 nm in a microplate reader (Biotek, Winooski, VT, USA). Negative sera with all assay components, secondary antibody with no sera but with all remaining assay components (to correct for non-specific binding), and blank (all reagents with no antigen) were run for each type of protein/antigen used to make the necessary corrections. Reduction in immunoreactivity of the protein samples was determined using Eq. (4).

$$\% \text{Reduction in immunoreactivity} = 100 \times (\text{Corrected ABS}_{\text{SPI}} - \text{Corrected ABS}_{\text{SPH or PGSP or PGSPH}}) / \text{Corrected ABS}_{\text{SPI}} \quad (4)$$

ABS is the absorbance; Corrected ABS is equivalent to $\text{ABS}_{\text{positive sera}} - \text{ABS}_{\text{2nd antibody}}$.

2.11. Statistical analysis

Response surface analysis was done using Design Expert 8.0 taking into account first order, first order with interactions, and second order model terms. Response surfaces with appropriate model fit were used to assess the influence of three independent variables (time, temperature, and E:S) on the %DH and %reduction in immunoreactivity. Model selection was first based on an insignificant lack of fit p-value ($P \geq 0.05$) for first order, first order with interactions, and second order. When a model was found significant the next highest order model was chosen. When an insignificant

lack of fit p-value was not found for any models, the model sum sequential p-value ($P \leq 0.05$) was taken into consideration, which provides information about the number of terms included in the model that are found to be significant ($P \leq 0.05$). The highest order model that included the greatest number of significant model terms was selected, as a greater number of significant model terms are desirable to assess which factors have the greatest effect on the response variable. The highest order model with a significant ($P \leq 0.05$) model sum of squares p-value was chosen. Lastly, adjusted R^2 was used to signify the power of the model. The models generated were used to determine the optimized hydrolysis conditions that would lead to the greatest reduction in immunoreactivity while the DH would not exceed 8%. Three-dimensional response surface plots for the different models were generated using R version 3.01.2 “Good Sport” by holding one independent variable constant and setting the other two independent variables to levels contained within the design space. Analysis of variance (ANOVA) was carried out to assess model fit of the experimental data obtained from the serum of each participant using SPSS 15 for Windows. Additionally, ANOVA was carried out to determine the effect of different hydrolysis conditions and of glycation on %reduction in immunoreactivity within each serum used; and to determine the effect of glycation time on Amadori compound formation, lysine blockage and browning. When a factor or interaction was found significant ($P \leq 0.05$), differences between means was determined using Tukey-Kramer multiple means comparison test.

3. Results and discussion

3.1. Protein/peptide molecular weight distribution of produced hydrolysates

Upon hydrolysis of SPI, the bands corresponding to α' and α subunits of β -conglycinin, identified based on molecular weight distribution (Wang, Bring, Berhow, & Gonzales de Mejia, 2008), disappeared while that corresponding to the β subunit, for the most part, became fainter as the %DH increased (Fig. 1A). Up to 93.3% and 94.2% reduction (Table S2) in the band intensities of α' and α subunits of β -conglycinin, respectively, were observed in SPH 15 with the highest DH (Table 1). The %reduction (~90%) in band intensity of α' and α subunits of β -conglycinin for SPH 9 with a moderate DH (Table 1), was not much different than that of SPH 15. On the other hand, 46.2% reduction in band intensity of the β subunit of β -conglycinin was observed for SPH 15 compared to 30.6% reduction for SPH 9. Alcalase has been shown to be specific toward β -conglycinin subunits (Lee, Park, & Rhee, 1990; Tsumura et al., 1999). Tsumura et al. (1999) who utilized Alcalase for the production of hypoallergenic soy protein, found that it had equal specificity to all three β -conglycinin subunits; however, the DH (28%) of their product was much greater than what is observed in this study.

The protein band corresponding to glycinin A3 acidic chain became fainter as the DH increased (Fig. 1A), with few exceptions. SPH 15 had 60.1% and 45.1% reduction in band intensity of the A3 acidic chain and of the A1, A2, and A4 acidic chain, respectively (Table S2). On the other hand, SPH 9 had only 33.7% and 23.1% reduction in band intensity for the A3 acidic chain and for A1, A2, A4 acidic chain, respectively. Similar to what was reported by other researchers (Lee, Keum, Lee, Sung, & Chung, 2007), glycinin basic subunit (20 kDa) resisted enzymatic degradation, with %reduction in band intensity not exceeding 11% (Table S2).

3.2. Effect of hydrolysis conditions on immunoreactivity

Major allergens in SPI, α' , α , and β subunits of β -conglycinin, and glycinin acidic and basic chains demonstrated

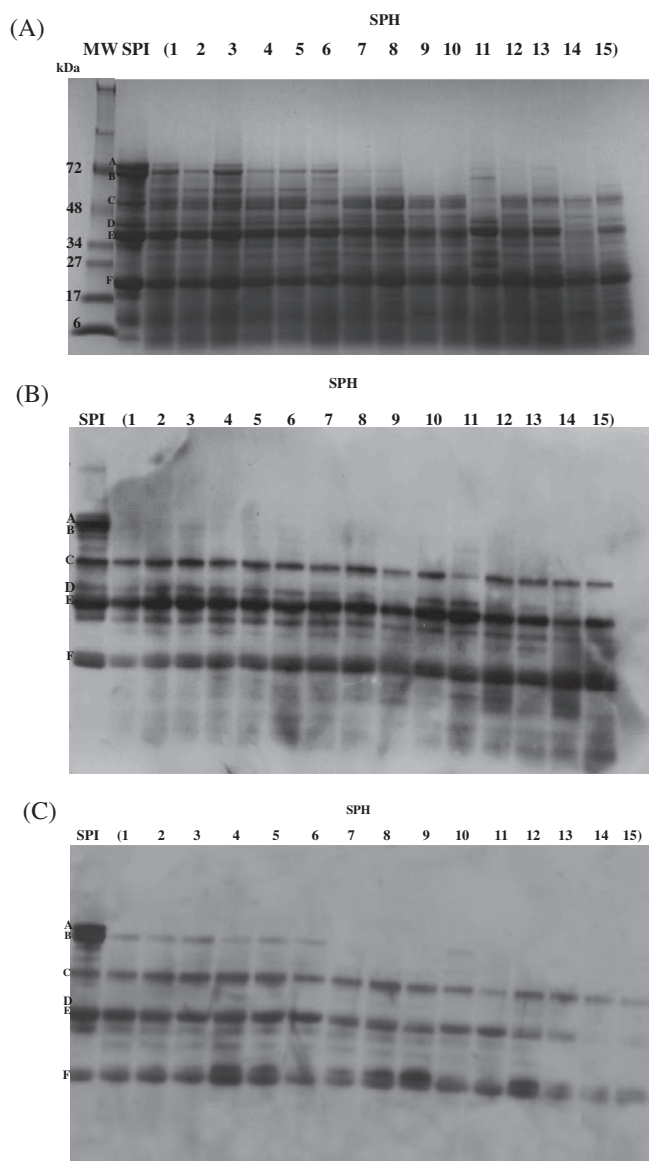


Fig. 1. A) SDS-PAGE visualization of soy protein isolate (SPI) and various soy protein hydrolysate (SPH) samples of varying degree of hydrolysis (DH). Lane 1: molecular weight marker (MW); Lane 2: SPI; Lanes 3–18: hydrolysates, numbered 1–15 corresponding to the SPH numbers found in Table 2. B) Western blot generated using sera from participant 2 (soy specific IgE 20.90 kU/L); C) Western blot using sera from participant 4 (6.96 kU/L soy specific IgE); A: α' (72 kDa); B: α (68 kDa) of β -conglycinin; C: β (48 kDa) from β -conglycinin; D: glycinin A3 acidic chain (42 kDa); E: glycinin A1, A2, A4 acidic chain (33.6–37.0 kDa); F: glycinin basic chain (20 kDa).

immunoreactivity, as shown in two sample western blots obtained using sera from participants 2 and 4 (Fig. 1B and C, blots obtained using sera from participants 3 and 5 are shown in Fig. S1). No immunoreactivity for the α' and α subunits of β -conglycinin was detected in any of the SPH samples when using serum from participant 2 (Fig. 1B) and participants 1, 3 or 5. In comparison, when using serum from participant 4 (Fig. 1C), immunoreactivity of the α subunit of β -conglycinin was detected for SPH 1–6 (DH < 3.7%), and was significantly reduced by 93.3% (Table S3) for SPH 10 (DH = 6.77%). On the other hand, the immunoreactivity of the β subunit was only slightly reduced. While the combination of variables can result in similar DH, values, each independent variable may uniquely contribute to the protein/peptide distribution. For example, when comparing SPH 11–13, having less than 1% difference in DH values, the higher temperature and longer time employed to produce SPH 11 compared to SPH 13, may have

caused increased access to the β -subunit cleavage sites, resulting in a fainter band (Fig. 1A) and a consequently lower apparent immunoreactivity (Fig. 1B).

While A3 acidic subunit of glycinin in general became less immunoreactive as the DH increased, using serum from participant 2 (Fig. 1B), it was not immunoreactive when using serum from participant 4 (Fig. 1C), demonstrating that individuals with soy allergy have varying immunoreactivity toward different soy protein subunits. Changes in immunoreactivity of the A1, A2, A4 acidic chains of glycinin also varied among the sera used. For example, % reduction in band intensity corresponding to these subunits when using serum from participant 4, in contrast to participant 2, increased with increasing DH reaching up to 90.5% reduction for SPH 15 (Table S3). For most of the SPH samples, marginal reductions in the immunoreactivity of the basic subunit from glycinin were observed regardless of the sera used. The greater residual immunoreactivity of glycinin subunits post-hydrolysis compared to that of β -conglycinin subunits is mostly attributed to the resistance of glycinin to enzymatic hydrolysis by Alcalase. The resistance to enzymatic hydrolysis exhibited can be attributed to the disulfide linkages (Breiteneder & Ebner, 2000), high hydrophobicity and compact structure of glycinin (Khang, Rhee, & Park, 1988).

The observed differences in immunoreactivity when using sera from different participants can be attributed to variations in protein epitopes that soy specific IgE from different sera may react with. Different enzymatic conditions may have altered the structure of certain sequences, which resulted in reducing IgE binding for some sera. Pedersen and Djurtoft (1989) reported that only the acidic subunits can illicit IgE binding, while Helm, Cockrell, Connaughton, Sampson, and Bannon (2000) observed that both the acidic and basic subunits of glycinin are immunoreactive. Immunoreactivity was detected for all three subunits of β -conglycinin in our study confirming the findings of Burks, Williams, Thresher, Connaughton, and Cockrell (1992). However, Ogawa et al. (1991) reported that the α' does not exhibit immunoreactivity. Differences in the immunoreactivity observed in our study and others' is attributed to number of reasons including patient age and sensitization history, and most specifically the soy protein specific IgE present in each serum.

Immunoreactivity of SPH samples shifted toward lower molecular weight with increasing DH (Fig. 1B and C). As the DH increased, epitopes found within the interior of the protein may have been exposed or new epitopes formed. Dark bands below 20 kDa were observed for SPH 6, and appeared more prominent with increasing DH (Fig. 1B). While reduced immunoreactivity was noted in the molecular weight region of the original subunits, some of the resulting peptides post-hydrolysis remained immunoreactive. This observation indicated that some epitopes may not have been cleaved and remained present in the smaller molecular weight peptides.

ELISA data showed significant reductions in immunoreactivity upon hydrolysis of SPI, regardless of the serum used (Fig. 2A and B, data using sera from participants 1, 2 and 4 are not shown). Low DH resulted in lowest %reduction in immunoreactivity with few exceptions such as the case for SPH 5 and 6, which showed lower %reduction than SPH 3 and 4 (Fig. 2A). This observation can be attributed to variation in soy specific IgE among the different sera coupled with variation in the hydrolysis conditions, which in turn resulted in different protein/peptide distribution. On the other hand, the hydrolysis conditions that yielded the greatest reduction in immunoreactivity were similar across the different sera, with SPH 10 (DH = 6.77) showing the most pronounced reduction. However, maximum %reduction in immunoreactivity varied across the sera used. Additionally, as the DH increased beyond that of SPH 10, %reduction in immunoreactivity remained the same or decreased (Fig. 2A and B). Exposure of epitopes buried within the

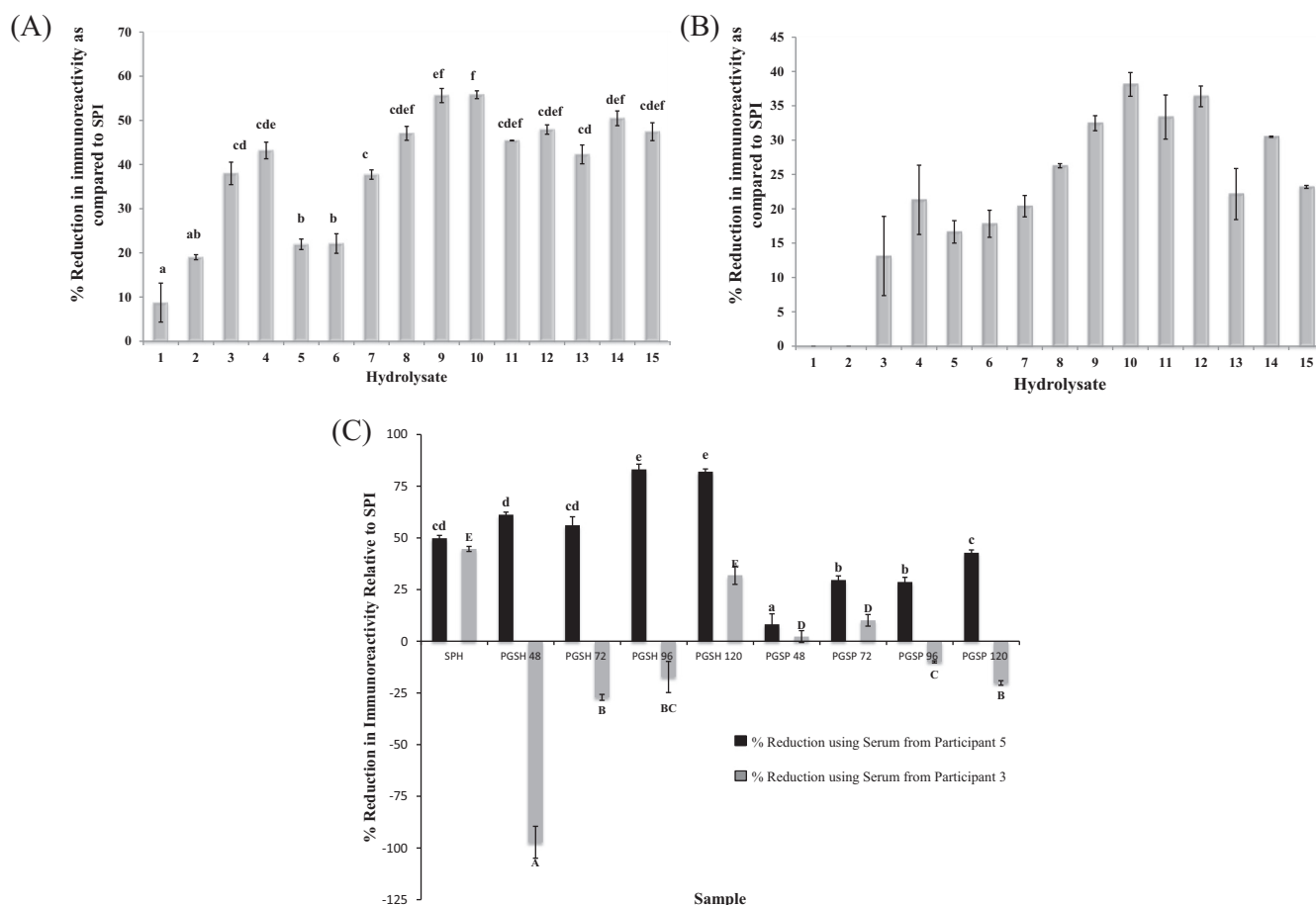


Fig. 2. %Reduction in immunoreactivity of SPH samples 1–15 following ELISA using sera from A) participant 3 (soy specific IgE of 4.16 kU/L) and B) participant 5 (soy specific IgE of 45.90 kU/L). Different lower case letters above the bars indicate significant differences between the means according to Tukey-Kramer multiple means comparison ($P \leq 0.05$). C) Percent reduction in immunoreactivity of SPH produced under optimized conditions, PGSPH, and PGSP relative to SPI using sera from participants 3 and 5. Different lower case letters and upper case letters indicate significant differences in %reduction in immunoreactivity, using serum from participant 5 and 3, respectively, among PGSP incubated with dextran for 48, 72, 96, and 120 h at 60 °C and a_w of 0.49, SPH, and PGSPH incubated under the same conditions, according to Tukey-Kramer multiple means comparison test ($P < 0.05$). Error bars represent the standard errors; $n = 3$.

interior of the protein may occur simultaneously as epitopes are destroyed upon hydrolysis. Depending on the sensitivity of the sera to specific epitopes exposed, one component of the two outcomes may dominate upon protein unfolding due to hydrolysis. The significantly lower %reduction in immunoreactivity observed for SPH 15 compared to that of SPH 10 (Fig. 2B) suggested that the effect of exposure of previously inaccessible epitopes was greater than the extent of epitope breakdown. This could mostly be attributed to the difference in enzyme: substrate ratio, which likely influences the effect of exposure to proteolytic digestion. Using sera from other participants, such as that of participant 3, the breakdown and exposure of specific epitopes appeared to be balanced upon hydrolysis (Fig. 2A). These observations and variations among IgE reactivity from various sera signifies the importance of optimizing hydrolysis conditions that lead to maximal reduction in immunoreactivity, while controlling the DH, in order to develop a hypoallergenic protein ingredient. Accordingly, optimization of hydrolysis conditions was conducted following RSM as discussed below.

3.3. Response surface modeling for DH

The DH was fit as a second order model, with the greatest number of significant model terms as demonstrated by the equation in Table S4. Lack of fit p-values for first order, first order with interactions, and second order were all significant (Table S5). Thus, selection of the second order model was justified by analysis of the

sequential model sum of squares p-value. Significant model terms included time, temperature, E:S, temperature and E:S interaction, and E:S squared. The second order model also had the highest adjusted R^2 , 0.95 (Table S5), and hence was used to generate RSM plots (Fig. S2). The median of the absolute residual differences between the experimental and predicted values (Table 1) was 0.24%; in terms of DH this is a very small difference. This observation further confirmed the validity of the prediction model that had a high adjusted R^2 (Table S5).

3.4. Response surface modeling for %reduction in immunoreactivity per serum used

Individual analysis was conducted on the data obtained using different sera, in order to take into account variation among sera. For the %reduction in immunoreactivity data obtained using serum from participant 1, the lack of fit p-value for all models was significant (Table S5). Hence, model selection was based on analysis of the sequential model sum of squares p-values, which indicated that significant ($P \leq 0.05$) model terms (all three terms) were only present in the linear model. Although, this model had the lowest adjusted R^2 in comparison to the other models, the significant sum of square p-value for the linear model implied that the model is appropriately fit, and hence RSM plots were generated using this model (Fig. S3A). When fitting responses obtained using sera from participants 2, 3, 4 and 5, models had insignificant lack of fit

(Table S5). Thus, analysis of the sequential model sum of squares was done to select the appropriate fit. The data obtained using sera from participants 2 and 3 were best fit using second order models with several model terms being significant and with the highest

adjusted R^2 (Table S4). Since the fit was a second order model, predictions of hydrolysis conditions that result in a maximum reduction in immunoreactivity were possible. Strong curvature was seen (Fig. 3A and B), allowing for the determination of a maximum

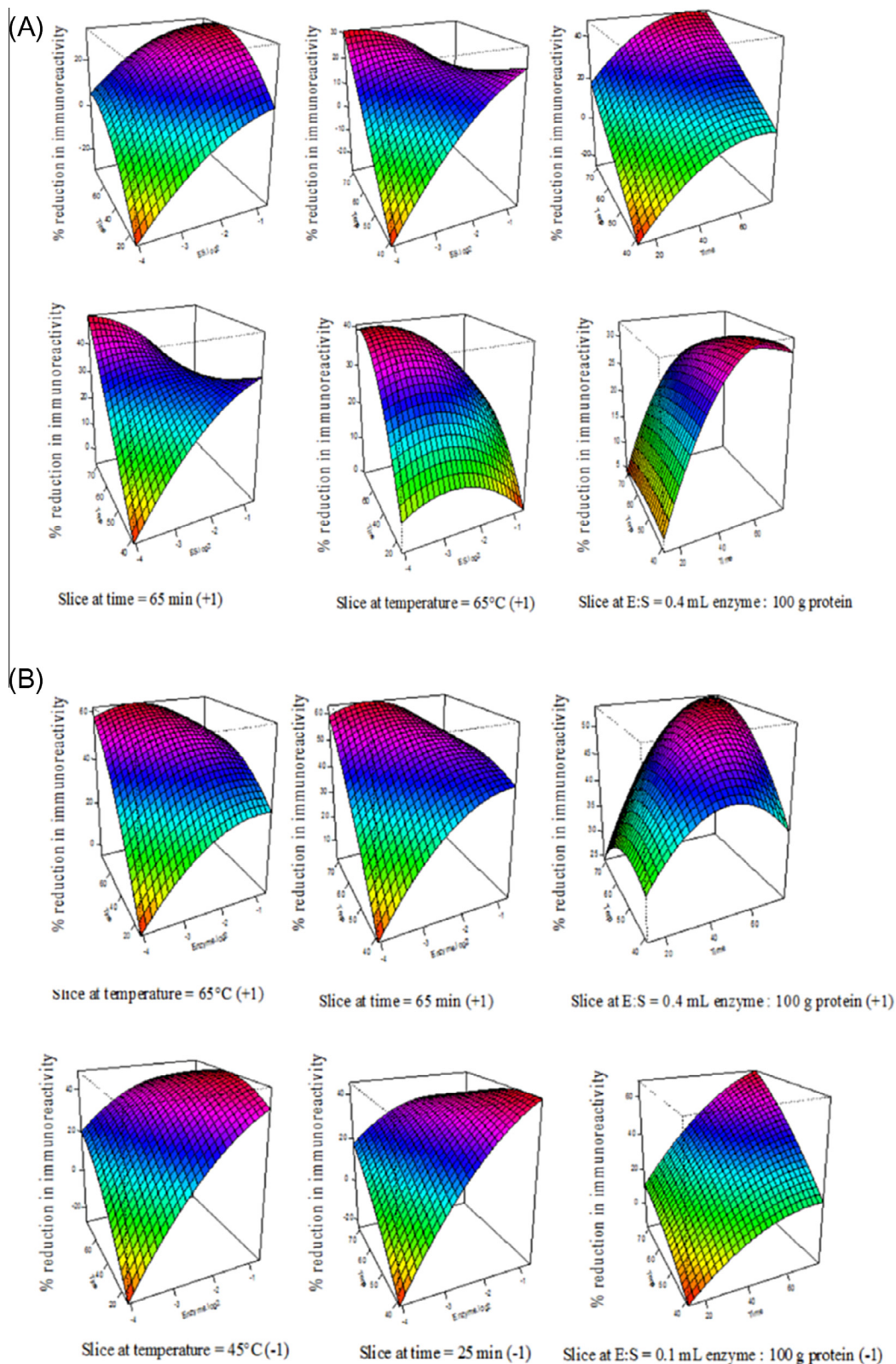


Fig. 3. RSM plots representing the effect of temperature, time, and enzyme to substrate ratio (E:S) on the % reduction in immunoreactivity using different sera A) from participant 2, and B) from participant 3. Each individual plot shows the effect of two variables while the other variable is held constant. Enzyme.log2 represents the transformation of the levels of E:S to the log base 2 scale used to create equal numerical movement from the 0 point to the ± 1 levels.

response. Based regression analysis, the hydrolysis conditions that would yield the greatest reduction in immunoreactivity were 65 min of incubation, 65 °C, and E:S of 0.1 and 0.14 mL of enzyme to 100 g protein, respectively. Analysis of the sequential sum of squares showed that for the responses obtained using sera from participants 4 and 5 the linear model was the best model with the greatest number of significant model terms (Table S4). Although the linear models did not have high adjusted R^2 , significance of model terms confirmed that the model was appropriately fit, and hence these models were used to generate the RSM plots (Fig. S3B and C). Since the data obtained fit a linear model, as was the case for the data produced using serum from participant 1, conclusions can only be drawn within the levels that were tested. The %reduction in immunoreactivity continued to increase linearly with the increase in the levels of each factor. Consequently, optimum conditions that elicit the maximum response probably lies outside of the levels tested, and hence could not be detected for this set of data. For future work, more levels should be tested.

Using the best fitted models, predicted responses were generated for the different hydrolysates (Table 2). For the most part, predicted responses were comparable to the actual responses. Deviations between actual and predicted values could be attributed to the adjusted R^2 of the various models obtained for the different sera used.

When the DH exceeds 8%, protein functionality and sensory quality can be negatively affected. Thus, constraint optimization, based on modifications to the model fit analysis, was performed to ensure that the DH did not exceed 8%. Predicted hydrolysis conditions that would result in the maximum %reduction in immunoreactivity with a DH of 7.5% were the same (65 °C, 65 min and 0.12 E:S) for all sera used with one exception, serum from participant 2 (only differed in E:S ratio, which was 0.1 compared to 0.12). Given this minor difference, these conditions were considered as the optimized hydrolysis condition.

A hydrolysate was produced under the optimized hydrolysis conditions and ELISA was conducted to compare the actual %reduction in immunoreactivity to the predicted %reduction in immunoreactivity (Table 2). Noted variation between the predicted and actual responses may be partially attributed to the level of the adjusted R^2 . The actual and predicted DH values were comparable given that the adjusted R^2 (0.95) of the model was high, which was not always the case for the %reduction in immunoreactivity. Another possible explanation for the observed variation

between the expected and predicted responses is differences in protein conformation. After hydrolysis the conformation of a protein is altered, which may expose or hide epitopes. The conformation of a protein coupled with variation in sera, and hence the different epitopes recognized by the IgE of different sera, can further complicate predictions.

The %reduction in immunoreactivity was 40–50% (Table 2). In order for a protein ingredient to be classified as hypoallergenic, a 90% reduction in immunoreactivity has to be achieved. The next phase of this project focused on determining the effect of Maillard-induced glycation on further reduction in the immunoreactivity of the SPH produced under optimized conditions.

3.5. Characterization of PGSP and PGSPH produced

The extent of glycation of SPI and SPH with dextran was assessed by determining amine blockage. The % free amine groups in SPI prior to glycation was 5.9%, which is comparable to the reported average value of 5.4% (Van de Lagemaat, Manuel Silván, Javier Moreno, Olano, & Dolores del Castillo, 2007). The % free amine groups (12.1%) in SPH prior to glycation was higher than that of SPI due to peptide bond breakage upon hydrolysis causing the release of free amine groups. A significant decrease ($P < 0.05$) in % amine groups was noted after 24 h of incubation with dextran for both PGSPH and PGSP. No significant decrease in % amine groups, however, was observed beyond the 24 h time point for both PGSPH and PGSP, an observation that complemented the DUV results (data not shown), confirming a slow rate of the reaction. Percent amine blockage after 120 h of incubation, however, was more pronounced for SPH (~22%) than SPI (~17%). This difference could be partially attributed to denaturation of the protein structure upon hydrolysis, which could have led to the exposure of additional ϵ -amines of the lysine residues. Additionally, compared to SPI, SPH had higher % free amine groups that can potentially be more readily glycosylated. Nevertheless, 22% reduction in amine groups is considered a moderate level compared to 75% reduction observed under more adverse Maillard reaction conditions, such as 19 days of incubation at higher a_w (0.65) (Van de Lagemaat et al., 2007). To further characterize the progression of the Maillard reaction, fluorescence index (FI) was evaluated to assess intermediate products' formation as indicators for the propagation of the reaction (Friedman & Kline, 1950). Advanced glycation end products (AGE) formed during the advanced stage

Table 2

Actual (A) and predicted (P) responses for SPH samples (1–15) for %reduction in immunoreactivity obtained using sera from participants 1, 2, 3, 4 and 5.

SPH	%Reduction in Immunoreactivity using Sera from									
	Participant 1		Participant 2		Participant 3		Participant 4		Participant 5	
	A	P	A	P	A	P	A	P	A	P
1	12.2	18.8	0	−3.64	8.74	5.79	0.491	3.79	0	4.59
2	24.3	26.9	0	4.93	19.0	21.6	10.0	15.3	0	9.08
3	28.5	29.3	15.8	17.1	38.0	22.1	8.83	11.8	13.1	15.8
4	27.1	29.8	11.8	18.1	19.0	30.4	4.93	9.17	7.01	13.3
5	36.4	35.3	18.3	16.0	21.9	28.6	12.0	11.9	16.6	21.0
6	39.0	31.8	23.0	22.2	22.1	24.1	23.9	19.5	17.8	15.8
7	42.8	32.4	22.6	17.5	37.7	40.4	21.9	16.3	20.3	12.7
8	38.2	48.8	32.1	31.9	22.1	46.0	21.4	24.4	32.4	29.1
9	44.7	40.3	28.2	28.1	45.1	45.4	21.5	22.0	26.3	22.4
10	49.2	48.2	36.1	40.0	55.8	56.2	37.8	27.7	38.1	32.2
11	47.1	50.8	41.0	36.2	47.9	51.4	24.4	34.8	33.3	31.6
12	58.0	53.7	33.4	30.4	45.4	52.3	40.2	28.6	36.3	35.8
13	51.3	45.3	14.5	15.9	55.6	38.7	43.5	32.0	22.1	23.9
14	59.8	51.3	24.9	25.0	50.4	48.1	37.1	32.0	30.5	29.0
15	43.5	61.7	25.5	28.2	47.4	53.0	24.0	40.2	23.1	40.3
Optimized	52.5 ± 1.34	51.0	19.8 ± 1.51	40.1	44.7 ± 1.24	58.2	43.0 ± 2.10	30.2	49.5 ± 1.41	33.9

Refer to Table 1 for hydrolysis conditions and coded SPH samples.

of the Maillard reaction can also fluoresce (Tessier, Monnier, & Kornfield, 2002). Marginal increase in FI was observed for PGSPH and PGSP over the course of incubation (data not shown), confirming that the reaction was properly controlled and limited to the early stage. Since no changes in browning were observed based on 420 nm measurements (data not shown), it can be concluded that the observed marginal increase in fluorescence is attributed to limited breakdown of the Amadori compounds at the beginning of the intermediate stage and is not a result of AGE formation.

Limited formation of Amadori compounds could have been the result of steric hindrance or slow progression to the intermediate and advanced stages. First, steric hindrance caused by the attachment of dextran could limit the extent of protein carbohydrate interaction by limiting the accessibility of amine groups (Choi, Kim, Park, & Moon, 2005). The attachment of a polysaccharide can also prevent the progression of the Maillard reaction by imparting partial stability to the Amadori compound, preventing side reactions and color development (Kato, 2002).

Glycation of different soy protein subunits was visualized using SDS-PAGE (Fig. S4). Upon glycation with dextran, α , α' , and β sub-

units of β -conglycinin of SPI shifted to a higher molecular weight distribution as indicated by the longitudinal smearing observed in lanes 3–7 (Fig. S4). The longitudinal smearing, which indicated a wide spread of molecular weight, is attributed to the heterogeneous distribution of glycosylated subunits, i.e. each subunit might have been glycosylated with a different number of dextran molecules (~10 kDa each). The intensity of the protein bands corresponding to the acidic subunits of glycinin became fainter for SPI and SPH samples (Fig. S4, Lanes 3–7, and 9–12), indicating that acidic subunits were partially glycosylated. Thus the bands shifted to a higher molecular weight distribution further contributing to the observed smearing. Change in the intensity of the glycinin basic subunit was not pronounced. The two bands observed around 27 kDa (Lanes 8–12), not present in SPI (Lane 1), are most likely peptides released upon hydrolysis of β -conglycinin. Upon incubation with dextran, these bands appeared much fainter suggesting that they were glycosylated as well (Fig. S4, Lanes 9–12). Diftis and Kiosseoglou (2006) confirmed that compared to glycinin subunits, β -conglycinin subunits more readily participate in Maillard-induced glycation with dextran.

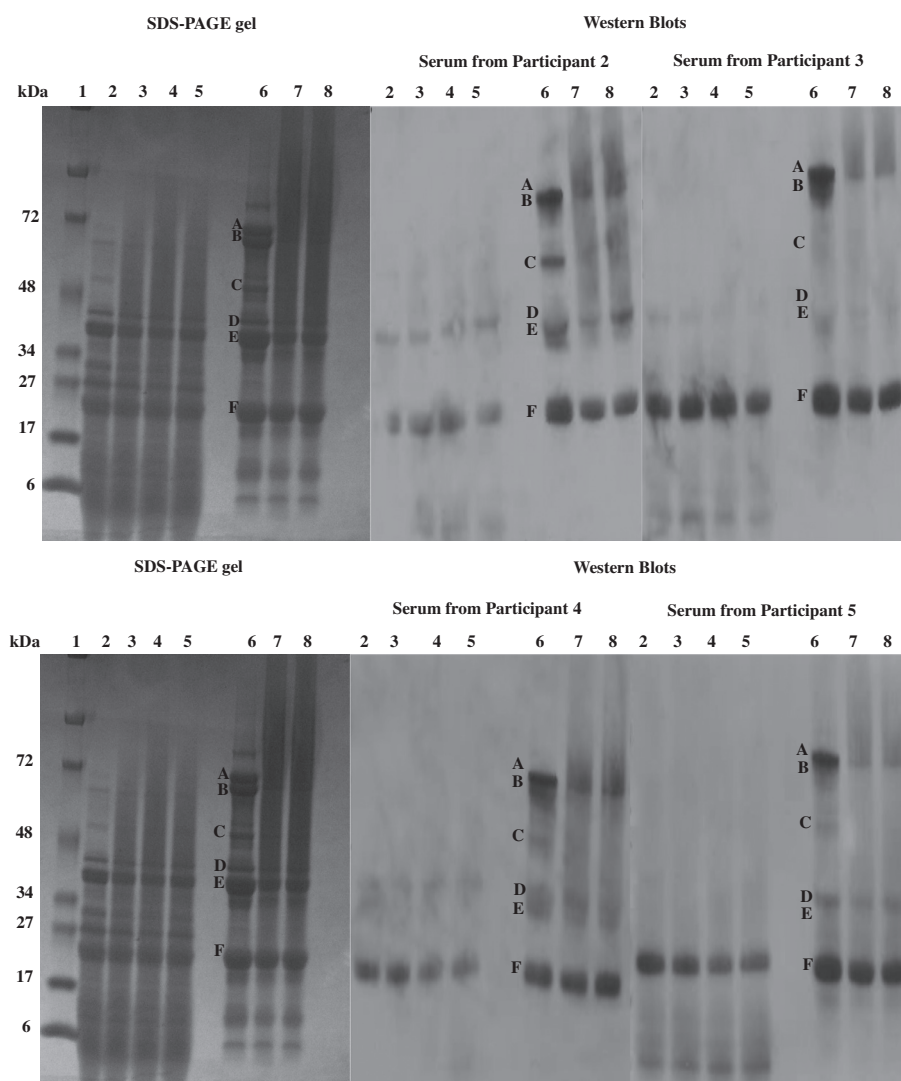


Fig. 4. SDS-PAGE gel and corresponding Western blots developed using sera from participants 2, 3, 4 and 5. Lane 1: molecular weight marker; Lane 2: soy protein hydrolysate (SPH) produced under the optimized hydrolysis conditions of time = 65 min, temperature = 60 °C, and enzyme to substrate ratio (E:S), 0.12 mL enzyme: 100 g protein; Lanes 3–5: partially glycosylated soy protein hydrolysate (PGSPH) incubated with dextran for 72, 96, and 120 h at 60 °C and a_w of 0.49; Lane 6: soy protein isolate (SPI); Lanes 7 and 8: partially glycosylated soy protein (PGSP) incubated or 96 and 120 h under the same conditions listed above; A: α' subunit of β -conglycinin (72 kDa); B: α subunit of β -conglycinin (68 kDa); C: β subunit of β -conglycinin (48 kDa); D: A3 acidic subunit of glycinin (42 kDa); E: A1, A2, A4 acidic subunit of glycinin (33.6–37); F: basic subunit of glycinin (20 kDa).

3.6. Effect of controlled and limited glycation on the immunoreactivity of SPI and SPH

Although α' , α , and β subunits of β -conglycinin have been glycated, immunoreactivity, using the different sera, was still apparent around 72 kDa for PGSP, but was not as intense as that of SPI (Fig. 4, lanes 6–8). However, the immunoreactivity shifted toward a higher molecular weight as noted by the longitudinal smearing, which indicated that even when glycated, the proteins remained immunoreactive. Similarly, the acidic and basic subunits of glycinin remained immunoreactive post-glycation, with slight differences across the sera used.

Alcalase targeted mostly α' , α , and β subunits of β -conglycinin. Consequently, the immunoreactivity of β -conglycinin was not apparent in all of the Western blots for SPH and PGSPH samples (Figs. 4 and S5). Not much of a difference was noted in the immunoreactivity of glycinin upon glycation of SPH. For the western blots developed using sera from participants 2, 3, and 4, a slight immunoreactivity of the acidic subunit of glycinin could be observed, which was reduced even further upon glycation for longer incubation times. Glycinin has fewer reactive lysine residues, especially on the basic subunit (Utsumi, Matsumura, & Mori, 1997), consequently had been minimally glycated if at all. This observation may explain the minimal reduction in immunoreactivity of the basic chain. Based on these observations, it would be worthwhile targeting the hydrolysis of glycinin using another enzyme such as pepsin, which has been shown to have specificity toward glycinin and not β -conglycinin (Margatan et al., 2013), followed by Maillard-induced glycation.

Glycation of SPI was performed under the same conditions used to glycate SPH, in order to single out the effect of glycation on immunoreactivity of intact soy protein. Based on ELISA data, glycation of SPI did not always result in reduced immunoreactivity, instead an increase in immunoreactivity was noted depending on the sera used, as illustrated by the data produced using sera from participants 3 and 5 (Fig. 2C). For example, ~42% reduction in immunoreactivity vs. ~20% increase in immunoreactivity was observed upon glycation of SPI (PGSP 120, SPI incubated with dextran for 120 h), when using sera from participant 5 and participant 3, respectively. On the other hand, glycation of SPH resulted in an additional %reduction in immunoreactivity when using sera from participant 5 (having the highest soy specific IgE level, Table S1), increasing from ~50% reduction for SPH to ~83% reduction for PGSPH (PGSP 96 and PGSPH 120, SPH incubated with dextran for 96 h or 120 h). However, when using sera from participant 3, glycation of SPH had a negative effect.

Variability in specificity to different soy protein epitopes, could have contributed to the observed differences in immunoreactivity upon hydrolysis and glycation when using different sera. Upon hydrolysis, sequences are cleaved while others are exposed. Similarly, upon glycation, sequences are blocked, while others are exposed due to partial denaturation (Wang, He, Labuza, & Ismail, 2013). Consequently, depending on the specificity of IgE's in the sera used, immunoreactivity may either get reduced, increased, or remain unaffected. Using sera from participants 1, 2 and 4 similarly resulted in mixed outcomes (data not shown). Similar to our findings, Panda et al. (2015) determined that the immunoreactivity of soy protein hydrolysates varied based on the sera used. Accordingly, the variability among sera needs to be addressed carefully when developing hypoallergenic proteins.

The use of individual sera provides a better estimate of the degree of variability in IgE that can bind to different epitopes. Usui et al. (2004) evaluated the effect of glycation of soy protein with chitosan on the conformation of the protein and thus epitopes available for IgE binding using a monoclonal antibody specific for P34. As observed in this work and by Diftis and Kiosseoglou

(2006), different soy protein subunits were glycated, not just the P34 subunit, potentially resulting in changes to multiple epitopes. Thus, in order to assess the efficacy of protein modification, the use of polyclonal antibodies from sera, provides more inclusive information.

4. Conclusions

RSM allowed for fewer experimental conditions and provided optimized hydrolysis conditions that maintained a limited DH (7.5%) and resulted in substantial reductions (up to ~50%) in the immunoreactivity of soy protein. On the other hand, results showed that limited Maillard-induced glycation may further reduce or increase the immunoreactivity of SPH, depending on the serum used. Further optimization of Maillard-induced glycation conditions using RSM is warranted to determine whether or not a dual approach will achieve a greater reduction (>90%) in immunoreactivity, across different sera, in order to claim a hypoallergenic product. Results of this work highlight the complication in terms of variation among IgEs specific to different epitopes, obtained from different individuals. What might prove to be hypoallergenic for one individual might not be so for another. Nevertheless, this work is a step toward understanding complications and limitations, and provides premises for future studies intended to prove the potency of the combined methodologies to produce a hypoallergenic protein ingredient, while potentially maintaining functionality, flavor, and nutritional quality.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2016.07.012>.

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Supporting Material

Table S1. Values of IgE total, IgG and IgE specific to soy in the sera of five selected study participants.

Participant	IgE Soy Specific (kU/L)	IgE Total (kU/L)	IgG Total (mg/dL)
1	5.61	606	840
2	20.9	1225	736
3	4.16	1728	1224
4	6.96	245	794
5	45.3	7289	1100

Table S2. Percent reduction in band intensity of several soy protein subunits upon hydrolysis by Alcalase as determined using densitometry.

% Reduction in Band Intensity ^A ± SD ^B			
β-conglycinin			
SPH	α'	α	β
1	51.0 ± 12.9	37.6 ± 2.05	0
2	66.1 ± 13.6	56.8 ± 2.74	0
3	53.4 ± 8.73	38.9 ± 0.448	0
4	65.7 ± 7.49	60.8 ± 1.46	0
5	46.0 ± 6.05	43.5 ± 11.9	0
6	63.2 ± 3.97	57.9 ± 6.16	21.4 ± 2.50
7	74.1 ± 3.71	73.9 ± 0.116	6.50 ± 1.13
8	84.5 ± 1.25	84.4 ± 2.68	5.57 ± 0.338
9	89.7 ± 1.25	89.3 ± 2.98	30.6 ± 3.78
10	77.6 ± 0.391	79.5 ± 1.33	44.2 ± 0.713
11	80.1 ± 1.88	83.4 ± 1.77	52.7 ± 3.22
12	84.0 ± 0.153	85.7 ± 1.89	28.0 ± 2.56
13	84.9 ± 2.32	86.8 ± 0.885	33.7 ± 0.191
14	91.9 ± 0.798	93.2 ± 1.56	52.0 ± 1.32
15	93.3 ± 1.13	94.2 ± 0.071	46.2 ± 3.84
Glycinin			
SPH	A3	A1, A2, A4	Basic
1	9.41 ± 2.19	4.00 ± 3.17	5.06 ± 2.06
2	10.6 ± 3.13	7.08 ± 2.30	4.05 ± 2.12
3	8.56 ± 3.14	3.67 ± 3.30	4.38 ± 1.72
4	7.31 ± 4.91	6.66 ± 5.06	0
5	0	4.99 ± 4.70	0
6	13.8 ± 2.62	7.49 ± 4.41	0.677 ± 0 1.66
7	10.8 ± 2.10	12.1 ± 0.83	0.844 ± 0.890
8	19.9 ± 3.92	13.2 ± 2.38	1.73 ± 0.655
9	33.7 ± 0.744	23.1 ± 2.17	12.04 ± 1.19
10	31.4 ± 1.47	16.7 ± 2.26	6.63 ± 3.97
11	24.1 ± 0.343	11.7 ± 3.85	3.88 ± 2.91
12	28.2 ± 1.96	20.9 ± 4.06	4.81 ± 3.26
13	39.1 ± 0.768	25.6 ± 0.46	4.40 ± 4.26
14	56.5 ± 1.63	51.8 ± 0.250	11.2 ± 1.18
15	60.1 ± 0.337	45.1 ± 0.907	8.79 ± 1.73

^A Obtained from SDS PAGE gels

^BSD: standard deviation, n=3.

Table S3. % Reduction in band intensity from the Western blot developed using sera from Participant 2 (soy specific IgE 20.90 kU/L) and 4 (soy specific IgE 6.96 kU/L).

SPH		% Reduction in band intensity				
Serum from Participant 2						
β-Conglycinin				Glycinin		
1	α'	α	β	A3	A1, A2, A4	Basic
2	-	-	36.07	59.4	30.64	19.95
3	-	-	20.57	24.78	20.44	13.22
4	-	-	20.98	26.87	20.44	17.76
5	-	-	32.22	37.95	22.14	12.51
6	-	-	33.13	34.97	22.32	16.57
7	-	-	31.66	34.22	25.59	0
8	-	-	39.96	39.95	22.37	0
9	-	-	25.23	46.61	22.92	0
10	-	-	59.44	64.94	29.22	0
11	-	-	31.29	20.15	20.24	0
12	-	-	71.64	17.67	20.63	0
13	-	-	46.14	34.38	24.79	0
14	-	-	48.84	59.4	29.48	0
15	-	-	57.44	86.36	36.96	0
-						
Serum from Participant 4						
β-Conglycinin				Glycinin		
1	α'	α	β	A2, A3, A4		Basic
2	-	80.86	26.91	7.372		0
3	-	82.01	4.892	16.97		0
4	-	70.42	1.897	19.28		0
5	-	84.19	1.172	11.42		0
6	-	79.89	1.804	15.57		0
7	-	87.77	24.07	20.83		0
8	-	-	34.48	44.43		5.467
9	-	-	14.45	31.08		0
10	-	-	25.16	39.67		0
11	-	93.30	41.25	38.17		0
12	-	-	61.97	35.77		0
13	-	-	27.77	50.65		0
14	-	-	24.51	68.1		23.92
15	-	-	58.55	91.47		46.99
	-	-	77.15	90.50		41.58

Table S4. Equations derived from RSM models with the best fit for the prediction of the dependent variables (Y_1 - Y_6), with temperature as A, time as B, and enzyme to substrate ratio (E:S) as C on the log base 2 scale. All equations represent levels of the variables in the coded form.

Response	Fitted Equation	Adjusted R^2	p-value
DH (SO ^A)	$Y_1=6.23 + 2.16A + 1.66B + 2.77C + 0.48AB + 1.02AC + 0.27BC - 0.40A^2 - 0.41B^2 + 0.59C^2$	0.9454	0.0004
% reduction in immunoreactivity serum from participant 1 (FO ^B)	$Y_2=40.33 + 6.46A + 8.23B + 6.76C$	0.5034	0.0050
% reduction in immunoreactivity serum from participant 2 (SO ^A)	$Y_3=28.17 + 5.54A + 7.79B + 2.42C - 0.45AB - 6.95AC - 1.38BC - 0.38A^2 - 4.05B^2 - 2.66C^2$	0.7857	0.3021
% reduction in immunoreactivity serum from participant 3 (SO ^A)	$Y_4=45.40 + 6.46A + 9.43B + 7.97C + 2.30AB - 5.02AC - 4.32BC - 1.67A^2 - 3.17B^2 - 3.87C^2$	0.8467	0.1964
% reduction in immunoreactivity serum from participant 4 (FO ^B)	$Y_5=22.00 + 7.87A + 4.08B + 6.26C$	0.5444	0.0641
% reduction in immunoreactivity serum from participant 5 (FO ^B)	$Y_6 = 22.47 + 5.61A + 8.21B + 4.05C$	0.5601	0.3985

^ASO: second order; ^BFO: first order

Table S5. Analysis of Variance (ANOVA) for RSM data.

Response	Model	SS^D	DF^E	MS^F	F	Lack of Fit p-value	Adjusted R²
DH	FO ^A	26.68	11	2.43	86.39	<0.0001	0.8600
	FO + 2WI ^B	15.88	8	1.98	70.68	0.0001	0.8971
	SO ^C	6.400	5	1.28	45.60	0.0004	0.9454
1^G	FO ^A	1426	11	129.63	13.45	0.0050	0.5034
	FO + 2WI ^B	1031	8	128.91	13.37	0.0055	0.5541
	SO ^C	703.6	5	140.73	14.60	0.0053	0.5963
2^G	FO ^A	852.8	11	77.53	3.95	0.0705	0.5066
	FO + 2WI ^B	449.7	8	56.21	2.86	0.1307	0.6501
	SO ^C	160.0	5	32.01	1.95	0.3021	0.7857
3^G	FO ^A	921.4	11	83.77	4.70	0.0501	0.6663
	FO + 2WI ^B	528.2	8	66.03	3.70	0.0828	0.7491
	SO ^C	201.1	5	40.22	2.26	0.1964	0.8467
4^G	FO ^A	879.8	11	79.98	4.15	0.0641	0.5444
	FO + 2WI ^B	515.8	8	64.47	3.35	0.0996	0.6484
	SO ^C	391.5	5	78.24	4.06	0.0751	0.6359
5^G	FO ^A	673.7	11	61.25	1.33	0.3985	0.5601
	FO + 2WI ^B	322.1	8	40.27	0.87	0.5890	0.6691
	SO ^C	185.1	5	37.02	0.080	0.5920	0.6765

^A FO: First order; ^BFO + 2 WI: first order plus two way interaction; ^CSO: Second order; ^D SS: sum of squares; ^EDF: degrees of freedom; ^FMS: mean squares; ^G: % reduction in immunoreactivity using serum from participant 1, 2, 3, 4 and 5.

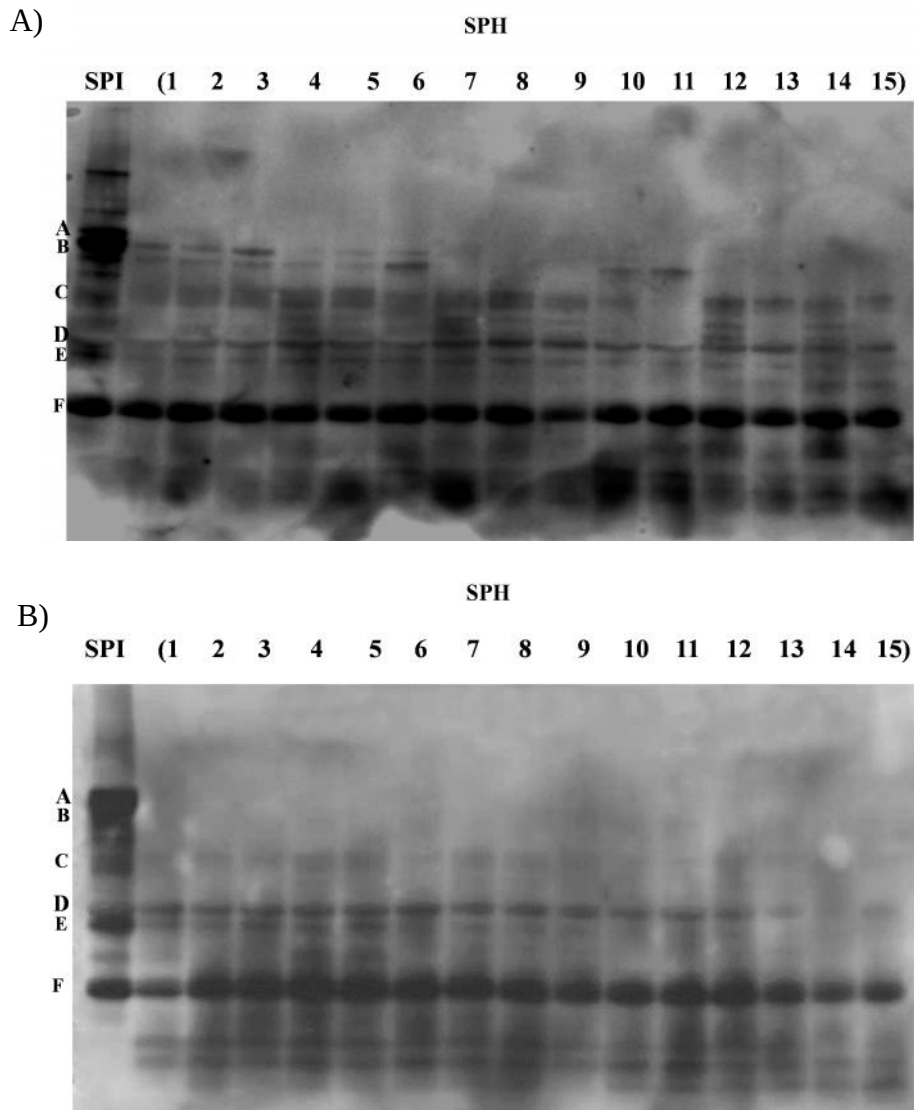
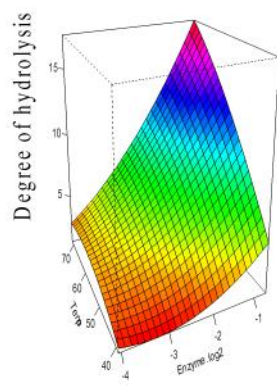
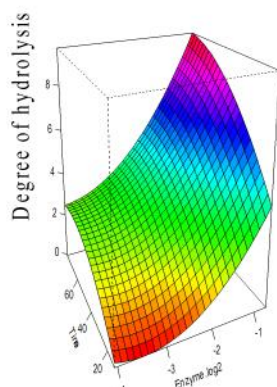


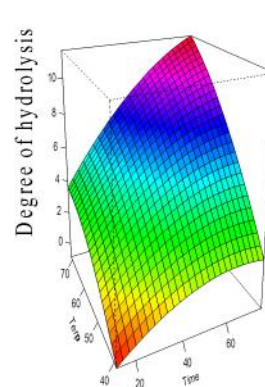
Figure S1. Western blots generated using different sera showing the immunoreactivity of SPI and SPH 1-15. The SPH numbering corresponds to those presented in Table 1. A) Western blot generated using sera from participant 3 (soy specific IgE 4.16 kU/L); B) Western blot using sera from participant 5 (45.3 kU/L soy specific IgE); A: α' (72 kDa); B: α (68 kDa) of β -conglycinin; C: β (48 kDa) from β -conglycinin; D: glycinin A3 acidic chain (42 kDa); E: glycinin A1, A2, A4 acidic chain (33.6-37.0 kDa); F: glycinin basic chain (20 kDa).



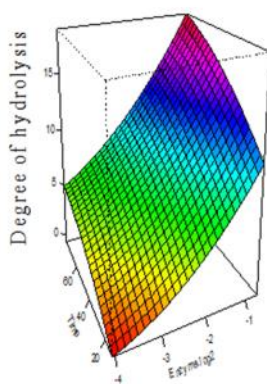
Slice at time = 25 min (-1)



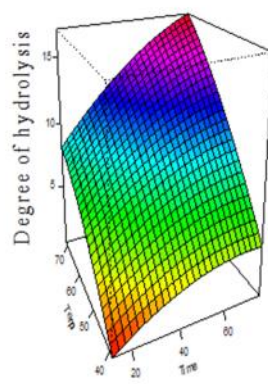
Slice at temperature = 45°C (-1)



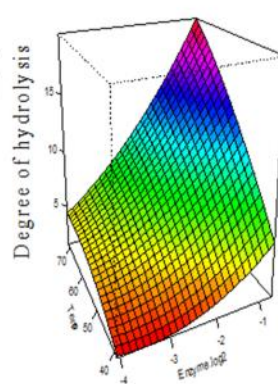
Slice at E:S = 0.1 mL of enzyme: 100 g protein (-1)



Slice at temperature = 65°C (+1)



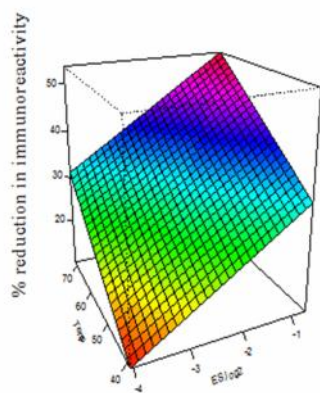
Slice at E:S = 0.4 mL enzyme: 100 g protein (+1)



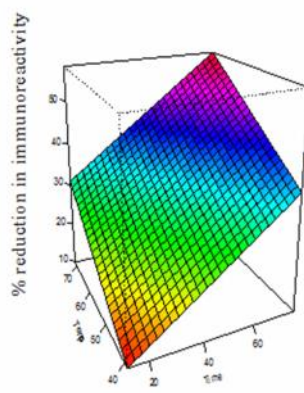
Slice at time = 65 min (+1)

Figure S2. RSM plots representing the effect of temperature, time, and enzyme to substrate ratio (E:S) on the DH using Alcalase. Each plot shows the effect of two variables while the other variable is held constant. Enzyme.log 2 represents the transformation of the levels of E:S to the log base 2 scale used to create equal numerical movement from the 0 point to the ± 1 levels.

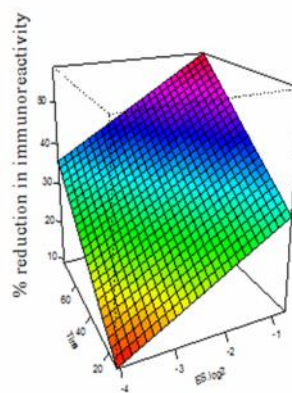
A)



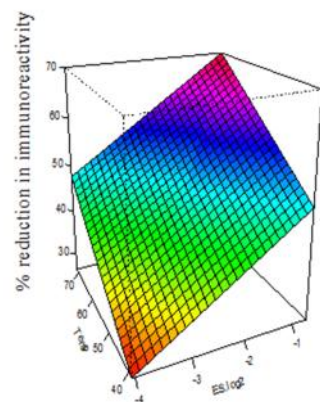
Slice at time = 25 min (-1)



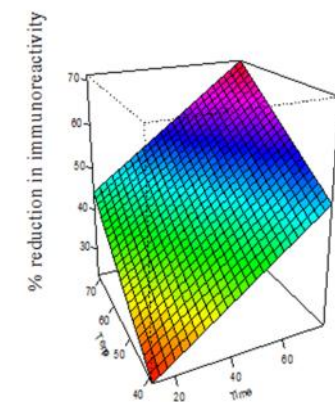
Slice at E:S = 0.1 mL of enzyme : 100 g protein (-1)



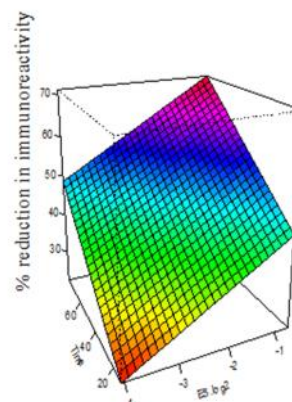
Slice at temperature = 45°C (-1)



Slice at time = 65 min (+1)

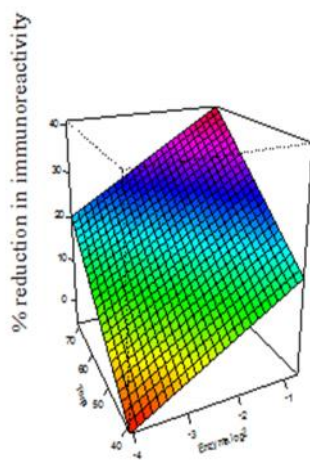


Slice at E:S = 0.4 mL enzyme : 100 g protein (+1)

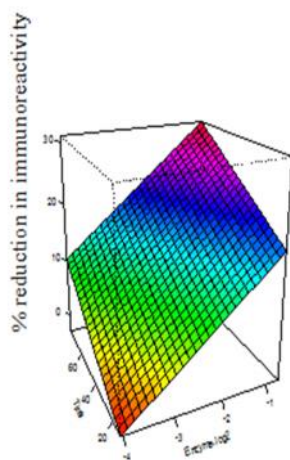


Slice at temperature = 65°C (+1)

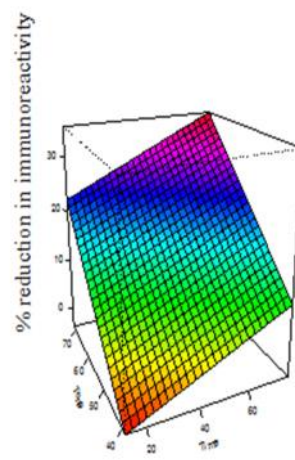
B)



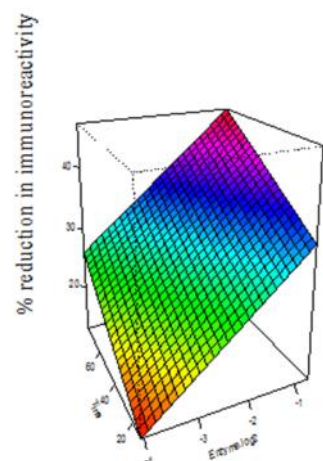
Slice at time = 25 min (-1)



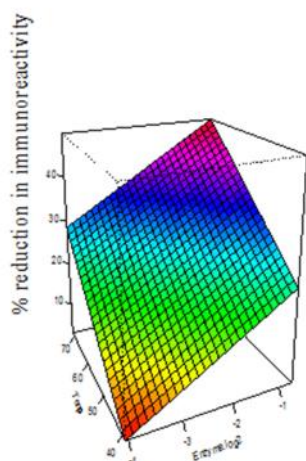
Slice at temperature = 45°C (-1)



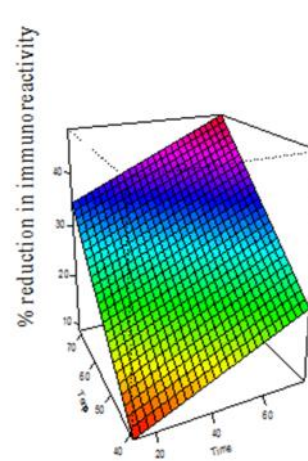
Slice at E:S = 0.1 mL enzyme : 100 g protein



Slice at temperature = 65°C (+1)



Slice at time = 65 min (+1)



Slice at E:S = 0.4 mL enzyme : 100 g protein (+1)

C)

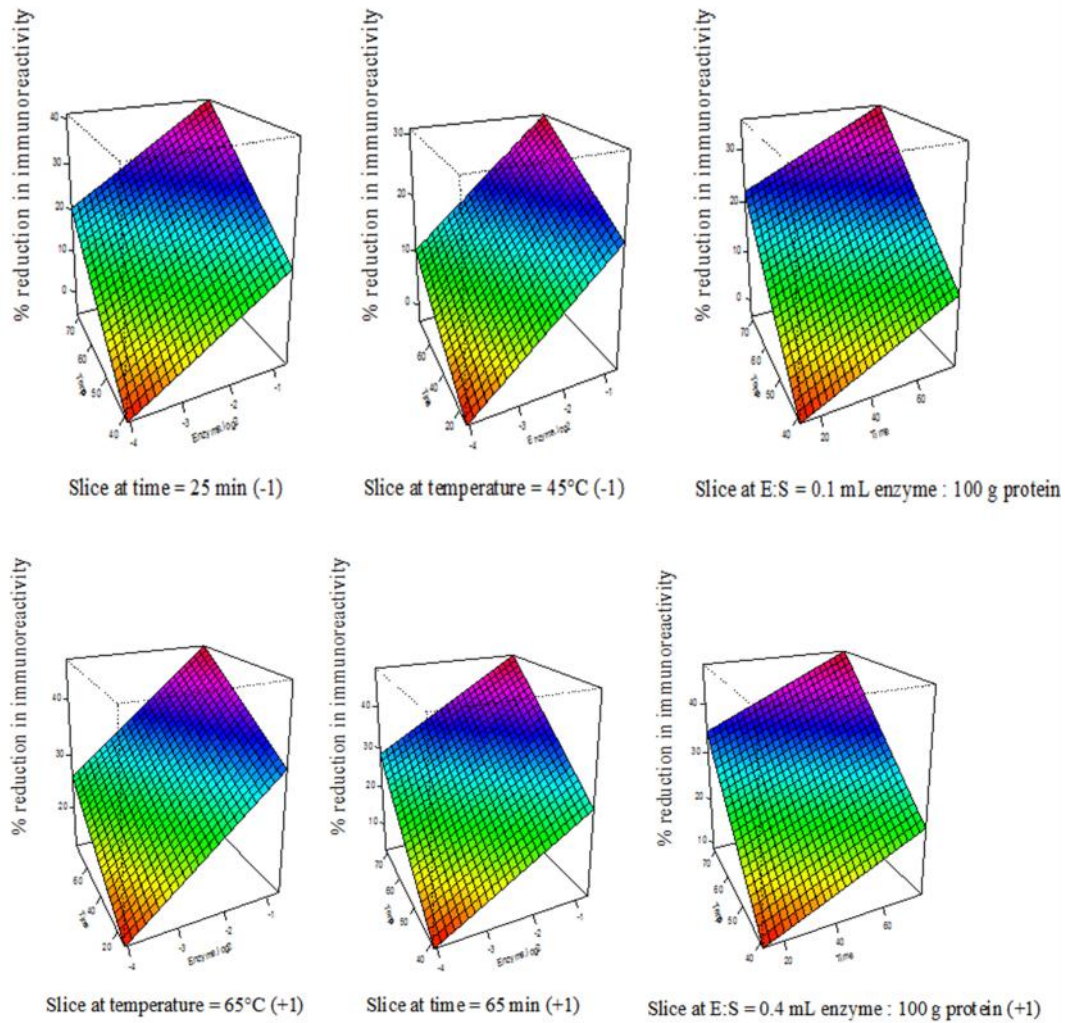


Figure 3S. RSM plots representing the effect of temperature, time, and enzyme to substrate ratio (E:S) on the % reduction in immunoreactivity using different sera A) from participant 1, B) from participant 4, and C) from participant 5. Each individual plot shows the effect of two variables while the other variable is held constant. Enzyme.log 2 represents the transformation of the levels of E:S to the log base 2 scale used to create equal numerical movement from the 0 point to the ± 1 levels.

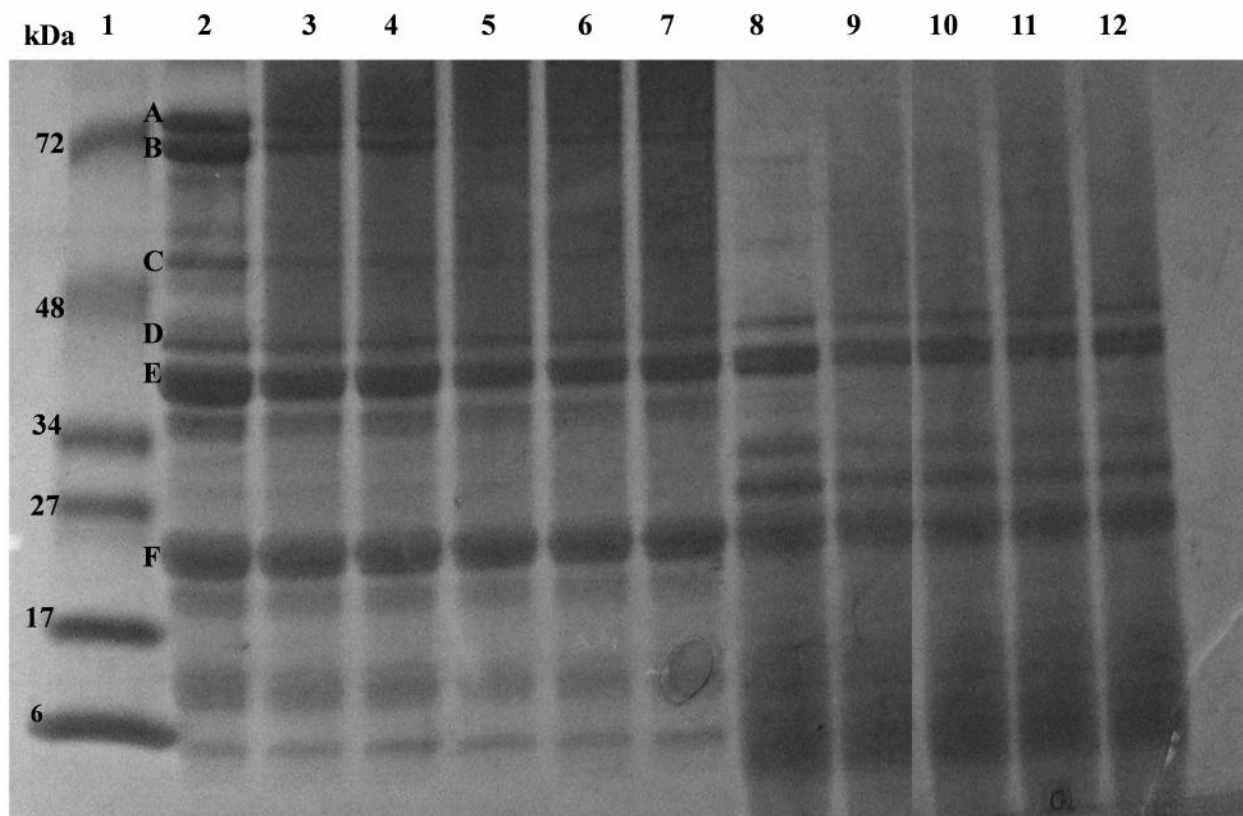


Figure S4. SDS-PAGE visualization of change in molecular weight distribution of protein subunits/peptides of SPI and SPH upon glycation with dextran at 60°C and 0.49 a_w . Lane 1: molecular weight marker; Lane 2: soy protein isolate (SPI); Lane 3-7: partially glycated soy protein (PGSP) incubated for 24, 48, 72, 96, and 120 h; Lane 8: soy protein hydrolysate (SPH) produced under the optimized hydrolysis conditions of time = 65 min, temperature = 60°C, and enzyme to substrate ratio (E:S), 0.12 mL enzyme : 100 g protein; Lane 9-12: partially glycated SPH (PGSPH) incubated for 48, 72, 96, and 120 h; A: α' subunit of β -conglycinin (72 kDa); B: α subunit of β -conglycinin (68 kDa); C: β subunit of β -conglycinin (48 kDa); D: A3 acidic subunit of glycinin (42 kDa); E: A1, A2, A4 acidic subunit of glycinin (33.6-37); F: basic subunit of glycinin (20 kDa).

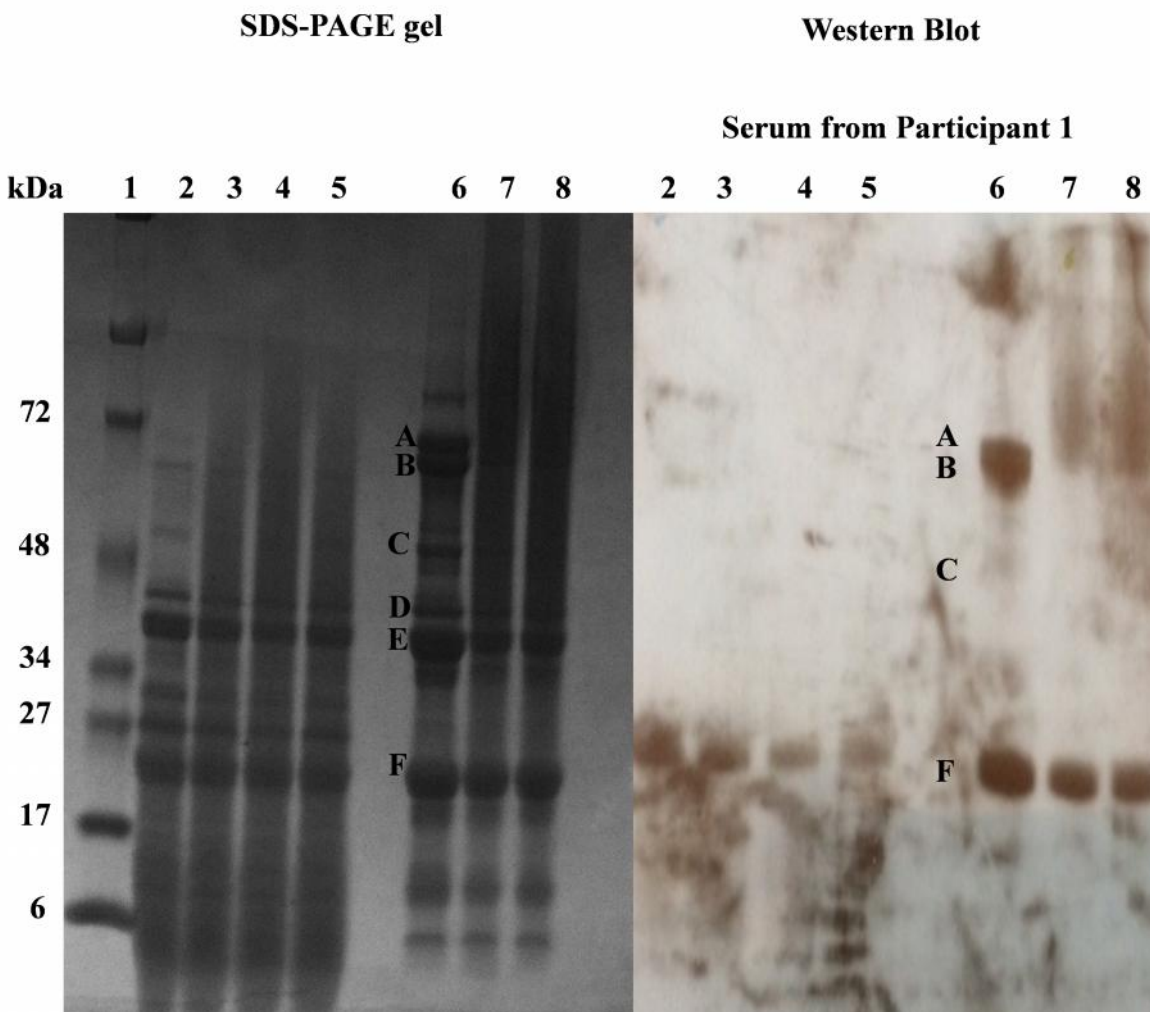


Figure 5S. SDS-PAGE gel and corresponding Western blot developed using sera from participants 1. Lane 1: molecular weight marker; Lane 2: soy protein hydrolysate (SPH) produced under the optimized hydrolysis conditions of time = 65 minutes, temperature = 60°C, and enzyme to substrate ratio (E:S), 0.12 mL enzyme : 100 g protein; Lane 3-5: partially glycosylated soy protein hydrolysate (PGSPH) incubated with dextran for 72, 96, and 120 h at 60°C and a_w of 0.49; Lane 6: soy protein isolate (SPI); Lane 7-8: partially glycosylated soy protein (PGSP) incubated for 96 and 120 h under the same conditions listed above; A: α' subunit of β -conglycinin (72 kDa); B: α subunit of β -conglycinin (68 kDa); C: β subunit of β -conglycinin (48 kDa); D: A3 acidic subunit of glycinin (42 kDa); E: A1, A2, A4 acidic subunit of glycinin (33.6-37); F: basic subunit of glycinin (20 kDa).