

Allergenicity of an enzymatic hydrolysate of soybean 2S protein

Dongeun Sung,^a Kang Mo Ahn,^b Seung-Yong Lim^c and Sangsuk Oh^{a*}

Abstract

BACKGROUND: This study was performed to examine how the characteristics of soybean 2S protein influence allergenicity after enzymatic hydrolysis. Soybean 2S protein was extracted and enzymatic hydrolysis was performed using pepsin and chymotrypsin. Allergenicity was observed using soybean-sensitive patients' sera.

RESULTS: Only 13.3% (6/45) of soybean-sensitive patients reacted to soybean Kunitz trypsin inhibitor (SKTI), known as the major allergen of soybean 2S protein. After peptic hydrolysis for 90 min at pH 1.2, the intensity of SKTI decreased to 25% but was still visible on SDS-PAGE. Chymotryptic hydrolysis following peptic hydrolysis at pH 8 for 60 min showed a limited hydrolytic effect on soybean 2S protein. Peptic hydrolysis of soybean 2S protein partially reduced the allergenicity of soybean 2S protein, while chymotryptic hydrolysis following peptic hydrolysis increased slightly the allergenicity.

CONCLUSION: Food allergy caused by soybean 2S protein occurred in part of the soybean-sensitive patients. SKTI was partially digested after peptic hydrolysis for 90 min. The allergenicity was decreased with peptic hydrolysis, while subsequent treatment of chymotrypsin increased slightly the allergenicity.

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Keywords: soybean allergy; soybean 2S protein; soybean Kunitz trypsin inhibitor; enzymatic hydrolysis

INTRODUCTION

The use of soybean in the food industry has continuously increased due to its well-balanced amino acid composition and functional properties of soybean proteins. Nevertheless, soybean has been classified as one of the 'big eight' most allergenic foods, along with milk, eggs, peanuts, tree nuts, wheat, fish, and shellfish.¹ Eggs, cow milk, soybean, buckwheat, and wheat are also major food allergens in Korea, and the first four of these constitute over 95% of causative food of allergy.² Although the prevalence of soybean allergy in the general population is relatively low (0.5%), soybean is a major food allergen in children under the age of 3 years.³ A birth cohort study in Korea demonstrated that 6.5% of infants having trouble with food allergies reacted to soybean.⁴ Soybean exists as a 'hidden allergen' in various processed foods. It is difficult to find processed foods without soybean. Soy protein can appear even in highly refined oils, lecithin, margarine, cosmetics, pharmaceutical excipients, and infant formula.^{5–7}

Soybean 2S, 7S and 11S proteins have been found to be major allergens according to radioallergosorbent (RAST) and RAST inhibition tests with the sera of soybean-sensitive patients, and among them, the 2S fraction had the highest allergen potency.⁸ Another study has also advocated that the 2S fraction has a strong allergenicity on the basis of RAST testing.⁹ The soybean 2S protein is composed of the Bowman–Birk trypsin inhibitor (7.9 kDa), cytochrome C (12.5 kDa), Kunitz trypsin inhibitor (20.1 kDa), and enzymes (15–60 kDa).¹⁰ Soybean Kunitz trypsin inhibitor (SKTI) is a major allergen in the 2S fraction and is a globulin comprised of 181 amino acids and two disulfide bonds.^{11,12} Koide and Ikenaka¹³ determined its molecular weight (20 100 Da) and amino acid

sequence, and Brandon *et al.*⁷ demonstrated that it has multiple epitopes.

Food allergens are classified into two groups. Classical type 1 food allergens such as soybean can cause immunoglobulin E (IgE) sensitisation through the mucosa of the gastrointestinal tract. These allergens are hydrosoluble glycoproteins in the size range of 10–70 kDa and are quite resistant to heat, acid and proteases, whereas type 2 food allergens are easily degraded by thermal processing and gastrointestinal digestion.¹⁴ It has been considered that proteins that are easily digested in gastrointestinal digestion are less threatening as allergens than those that are resistant. SKTI also has been shown to be stable to heat, acid and peptic hydrolysis.^{15,16}

According to the IUIS Allergen Nomenclature Sub-committee, hydrophobic protein from soybean (Gly m 1), defensin (Gly m 2), profilin (Gly m 3), PR-10 protein (Gly m 4), β -conglycinin (Gly m 5, vicilin, 7S globulin), glycinin (Gly m 6, legumin, 11S globulin),

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and seed biotinylated protein (Gly m7) were officially registered as soybean allergens.¹⁷ SKTI has the potential to be an allergen because of its stability and, actually, the IgE binding has been shown in commercial soy lecithin including health supplements. It could exist as a hidden allergen even after processing but there is a lack of information.¹⁸

Food allergy is a notable public health problem, and the prevalence and economic cost of food allergy have been global issues. The US Food and Drug Administration (FDA) has classified publications related to food allergy, such as major food allergens, the effects of food matrix and processing on its allergenicity, and treatments to reduce allergenicity.¹⁹ Enzymatic hydrolysis of cow's milk protein showed a significant reducing effect on the level of both total and allergenic proteins, and cow milk hydrolysate has already been commercialised for children with cow milk allergy.²⁰ Studies on enzymatic hydrolytic properties are a prerequisite to reduce food allergens. The effects of sequential peptic and chymotryptic hydrolysis on soybean 7S and 11S globulins have been shown. The allergenicity of soybean 7S protein somewhat decreased, and that of soybean 11S protein was almost fully decreased.^{21,22} In this study, the hydrolysis of soybean 2S protein using pepsin and chymotrypsin was performed to analyse the allergenicity of the hydrolysate by using sera from soybean-sensitive patients.

MATERIALS AND METHODS

Patients' sera

Forty-five serum samples from soybean-sensitive patients (nine females and 36 males, ranging from 0 to 7 years of age) with atopic dermatitis who were treated at the Samsung Medical Center (Seoul, Korea) were used for this study. Total IgE and specific IgE levels were measured using the Phadia UniCAP-system (Pharmacia, Uppsala, Sweden), and the range of each concentration was 383–15 740 and 11.8–101 kUA L⁻¹, respectively (Table 1). Among the 45 samples, six were determined to have only IgE reactivity to SKTI as a major allergen of the soybean 2S protein and were used to evaluate the allergenicity of soybean 2S protein and its hydrolysates. After informed consent was received, blood samples were obtained, and the sera were frozen at -80 °C. This study was approved by the Samsung Medical Center Institutional Review Board.

Extraction of 2S protein from defatted soybean

Defatted soybean flour was obtained from Sigma (St. Louis, MO, USA), and soybean 2S protein was prepared following the method of Rao,²³ with minor modifications for further purification. The final solution of the Rao method was lyophilised, and distilled water was added to the powder in the proportion of 1:1 (w/v) and dialysed against distilled water for 48 h. This solution was filtered with a 100 kDa ultrafilter (Millipore, Cork, Ireland) to remove proteins having a molecular weight greater than 100 kDa and was then stored at -70 °C until use. Protein concentration was determined by a Pierce bicinchoninic acid protein assay kit (Rockford, IL, USA) with bovine serum albumin as the standard.

Hydrolysis of soybean 2S protein

Hydrolysis of soybean 2S protein with pepsin followed by chymotrypsin was performed following the method of Astwood *et al.*,¹⁵ with minor modifications of digestion time. Pepsin and chymotrypsin were purchased from Sigma, and other chemicals

Table 1. Biological characteristics of sera from six soybean-sensitive patients

Patient	Serum sample no.	Age (years)	Sex	Total IgE (kUA L ⁻¹)	Soybean-specific IgE (kUA L ⁻¹)
1	3480	1	M	5 001	81.5
2	3686	1	M	5 001	55.3
3	4145	2	M	5 001	101
4	8575	2	F	1 376	49.2
5	4571	2	F	5 460	38.1
6	4442	2	M	15 740	101
IgE, immunoglobulin E.					

were purchased from Duksan (Ansan, Korea). Simulated gastric fluid (SGF) was prepared to contain approximately 8140 U mL⁻¹ pepsin with 2 mg mL⁻¹ NaCl, and the pH was adjusted to 1.2 with 12 mol L⁻¹ HCl solution. To initiate the digestion, purified soybean 2S protein (5 mg mL⁻¹ in distilled water) was added to the pre-warmed (37 ± 2 °C) SGF (SGF:extracted soybean 2S protein = 6:1), which corresponds to a ratio of 10 U of pepsin activity per microgram of total protein. At each time point (0, 5, 10, 20, 30, 60 and 90 min), a 250 µL aliquot of the sample was collected, and the reaction was quenched by adding 50 µL of 0.7 mol L⁻¹ Na₂CO₃ and stored at -20 °C until further analysis. Chymotrypsin (66 U mg⁻¹) was added to the pepsin-hydrolysed sample after 90 min of hydrolysis and the pH was adjusted to 8. Chymotrypsin was added to the sample in the proportion of 1 to 10 (w/w) of protein. At each time point (0, 2, 5, 10, 20, 30 and 60 min), a 200 µL aliquot of the sample was collected. The hydrolysis was stopped by heating the samples at 80 °C for 15 min.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS-PAGE was performed according to the method of Laemmli.²⁴ The protein was pretreated for 5 min at 95 °C with sample buffer, followed by resolving on precast 4–12% Bis–Tris gradient gels (NuPAGE; Invitrogen, Carlsbad, CA, USA) for 35 min at 200 V, and then visualised by staining with Coomassie brilliant blue (Bio-Rad, Hercules, CA, USA). The Smart Color Protein Markers (ELPiS, Taejeon, Korea) were used as molecular weight markers.

Enzyme linked immunosorbent assay

ELISA was performed to evaluate the IgE reactivity of the patients' sera. A 96-well microplate (Costar, Corning, NY, USA) was coated with 100 µL of purified soybean 2S protein containing 50 µg mL⁻¹ of protein diluted in 50 mmol L⁻¹ carbonate–bicarbonate buffer (pH 9.6; Sigma), and incubated at 4 °C overnight. After washing the plate three times with 0.5 µL mL⁻¹ PBST [Tween20 (Sigma) diluted in phosphate-buffered saline (PBS) (Amersco, Solon, OH, USA)], the plate was blocked by adding 200 µL of 20 mg mL⁻¹ bovine serum albumin in 0.5 µL mL⁻¹ PBST (blocking buffer) to each well and incubating for 1 h at room temperature to prevent non-specific linking. Sera were diluted 1:20 in blocking buffer. The plate was washed three times and then incubated with 100 µL of diluted sera for 2 h at room temperature. After washing the plate three times, adsorbed IgE was detected with 100 µL of goat anti-human IgE-HRP (1 µg mL⁻¹) (KPL, Gaithersburg, MD, USA), diluted in blocking buffer (1 : 2500). The plate was further washed and developed with 100 µL of TMB peroxidase (3,3',5,5'-tetraethylbenzidine; KPL).

Absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA) at 595 nm in duplicate.

Immunoblotting

After performing SDS-PAGE, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes for 7 min at 20 V using an iBlot Dry Blotting System (Invitrogen). The membrane was blocked with 20 mg mL⁻¹ non-fat dry milk (Bio-Rad, Benicia, CA, USA) in 0.3 µL mL⁻¹ PBST overnight at 4 °C, after which it was reacted with an HRP-conjugated rabbit polyclonal antibody to soybean trypsin inhibitor (Abcam, Cambridge, UK) diluted in (1:20 000) for 30 min at room temperature. After washing three times, the membrane was reacted with chemiluminescence detection reagents (Amersham ECL western blotting system; GE Healthcare, Uppsala, Sweden) for 2 min. Then, the blotted membrane was exposed to high performance chemiluminescence film (GE Healthcare, Buckinghamshire, UK), after which the film was developed.

RESULTS AND DISCUSSION

Enzymatic hydrolysis of soybean 2S protein

Soybean 2S protein was purified following the method of Rao,²³ with minor modifications. The extracted soybean 2S protein was analysed using SDS-PAGE (Fig. 1). The major band (about 20 kDa) was identified as soybean trypsin inhibitor. The nominal mass was 19 853 Da and the sequence was similar to that of soybean trypsin inhibitor in previous published database of *Glycine max* (70% of sequence coverage) when it was analysed by matrix-assisted laser desorption/ionisation–time-of-flight–mass spectrometry (MALDI-TOF-MS).^{25,26} Moreover, according to amino acid sequencing after transferring the 20 kDa band from SDS-PAGE to a PVDF membrane, the five residues in the N-terminus (DFVLD) were in agreement with that of SKTI, as identified by Koide and Ikenaka.¹³ It was confirmed again by showing a distinct band in immunoassay with polyclonal antibody to soybean trypsin inhibitor (Fig. 2).

Peptic hydrolysis followed by chymotryptic hydrolysis was performed to examine the enzymatic hydrolysis of soybean 2S protein (Fig. 3). As shown in Fig. 3a, the smeared bands between 12 kDa and 20 kDa on the SDS-PAGE disappeared after hydrolysis with 90 min of pepsin treatment. The band of about 12 kDa was gradually hydrolysed and remained as a smeared band. Although the protein band of 20 kDa was still visible on SDS-PAGE after peptic hydrolysis for 90 min, the intensity of the band decreased to 25%. After hydrolysis with pepsin for 90 min, the pH of the sample solution was adjusted to pH 8, and hydrolysis with chymotrypsin was followed. As shown in Fig. 3b, new bands appeared above the band of 20 kDa. These bands seemed to be attributed to hydrolysed pepsin. The soybean 2S protein, including the 20 kDa protein, was hardly hydrolysed with chymotrypsin. It was demonstrated that SKTI can inhibit not only trypsin, but also chymotrypsin. Roychaudhuri *et al.*¹⁶ and De Vonis-Bidlingmeyer and Leary²⁷ reported that native SKTI is stable in chymotryptic digestion at pH 8.7. Immunoblotting using an antibody against soybean trypsin inhibitor was carried out to evaluate the hydrolysis of SKTI (Fig. 2). The intensity of the 20 kDa protein hydrolysed by pepsin decreased similarly to that of SKTI on SDS-PAGE (Fig. 2a). As shown in Fig. 2b, the intensity of the 20 kDa protein band on immunoblotting showed limited hydrolysis by chymotrypsin.

Roychaudhuri *et al.*¹⁶ have previously reported that SKTI was highly resistant during 60 min at pH 1.2. In this study, SKTI was

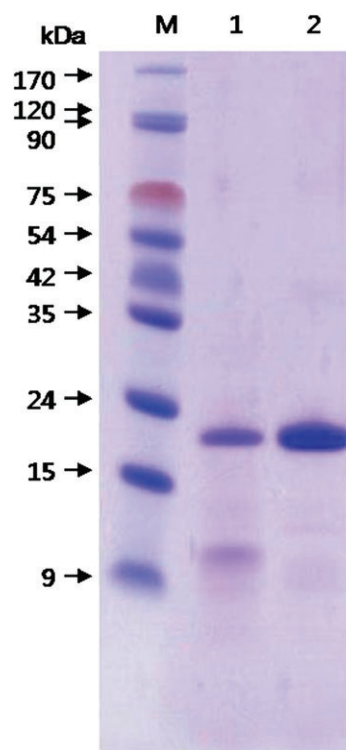


Figure 1. SDS-PAGE analysis of the soybean 2S protein. M: molecular weight markers; lane 1: purified soybean 2S protein; and lane 2: soybean Kunitz trypsin inhibitor standard.

partially digested after being treated for 90 min with pepsin. As shown by SDS-PAGE and immunoblotting (Fig. 2 and Fig. 3), the intensity of the 20 kDa band was lessened.

Allergenicity of soybean 2S protein and its hydrolysates

Allergenicity of soybean 2S protein

ELISA was performed to analyse the allergenic reactivity of soybean 2S protein against sera from soybean-sensitive patients. Sera from 45 soybean-sensitive patients were examined, and six samples reacted to SKTI as a major allergen in soybean 2S protein (13.3%). The six patients included four males and two females 1–2 years of age. Burks *et al.*²⁸ reported that only 20% of the soy challenge-positive patients reacted to the 20 kDa sized soybean trypsin inhibitor. Gu *et al.*¹⁸ also found that only one of six serum samples from soybean-sensitive patients reacted to SKTI (16.7%). The average total IgE and soybean-specific IgE of the six selected sera were 6083 kUA L⁻¹ (1376–15 740 kUA L⁻¹) and 69 kUA L⁻¹ (38.1–101 kUA L⁻¹), respectively. The mean total IgE and soybean-specific IgE of the others were 2690 kUA L⁻¹ (383–9930 kUA L⁻¹) and 55 kUA L⁻¹ (11.8–101 kUA L⁻¹), respectively. The six selected serum samples seemed to have higher total IgE and soybean-specific IgE values than others, although there was no significant correlation. In patients with a soybean-specific IgE level higher than 30 kUA L⁻¹, the possibility of experiencing an allergic response after eating soybeans is greater than 95%.²⁹ Bittencourt *et al.*³⁰ reported that, in an experiment with animals, soybean 2S protein did not cause any allergic reactions. In the present study, soybean 2S protein showed allergenicity in ELISA with the sera of soybean-sensitive patients. Some differences in the value of IgE reactivity among patients were observed. This is probably due to

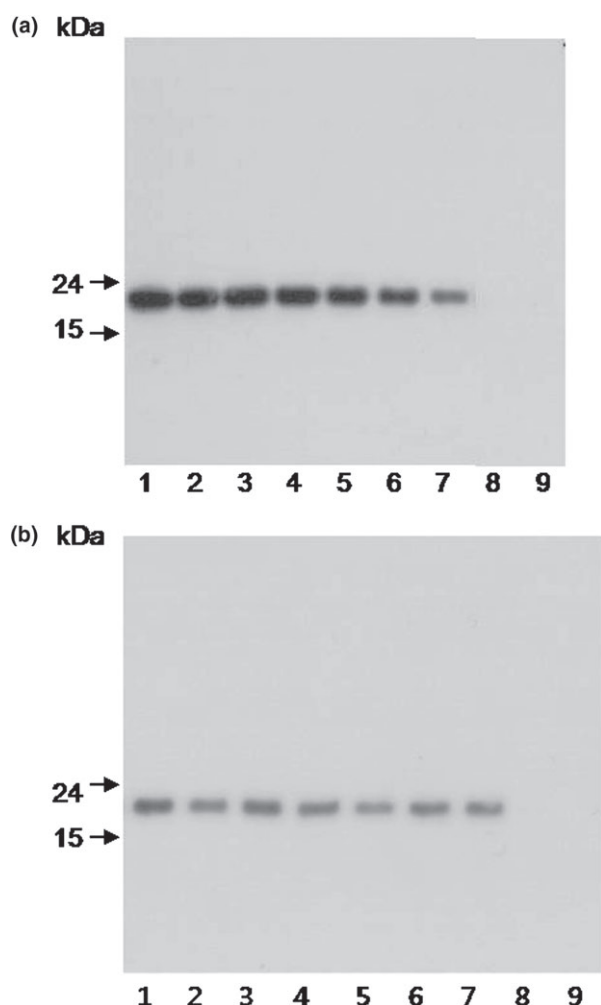


Figure 2. Immunoblotting analysis of peptic and chymotryptic hydrolysates of the soybean 2S protein with anti-trypsin inhibitor. Peptic hydrolysate (a) and chymotryptic hydrolysate following peptic hydrolysis (b). (a) Lanes 1–7: Peptic hydrolysate (0, 5, 10, 20, 30, 60, 90 min); and lanes 8 and 9: pepsin only (0, 90 min). (b) Lanes 1–7: peptic and chymotryptic hydrolysate (0, 2, 5, 10, 20, 30, 60 min); and lanes 8 and 9: pepsin and chymotrypsin only (0, 60 min).

the multiple epitopes of SKTI, and consequently each patient may respond to different epitopes of SKTI.

Allergenicity of peptic and chymotryptic hydrolysate

Pepsin could not completely eliminate the allergenicity of soybean 2S protein in 90 min (Fig. 4). Similar to the results of SDS-PAGE, the allergenicity of sera against soybean 2S protein slightly decreased but was still present. While the allergenicity of three serum samples (#3686, 4145 and 8575) against the peptic hydrolysates of soybean 2S protein decreased to the extent of that of negative serum, the other three samples (#3480, 4571 and 4442) interacted with the hydrolysates, even after a 90 min digestion with pepsin. A measured value below that of a normal patient was considered negative. The result implied that the allergenicity could remain after peptic hydrolysis for 90 min.

After the sequential peptic and chymotryptic hydrolysis of soybean 2S protein, five of six samples (#3480, 3686, 8575, 4571 and 4442) still showed allergenicity. Only one serum sample (#4145) showed a similar ELISA value to the negative serum. It seemed that

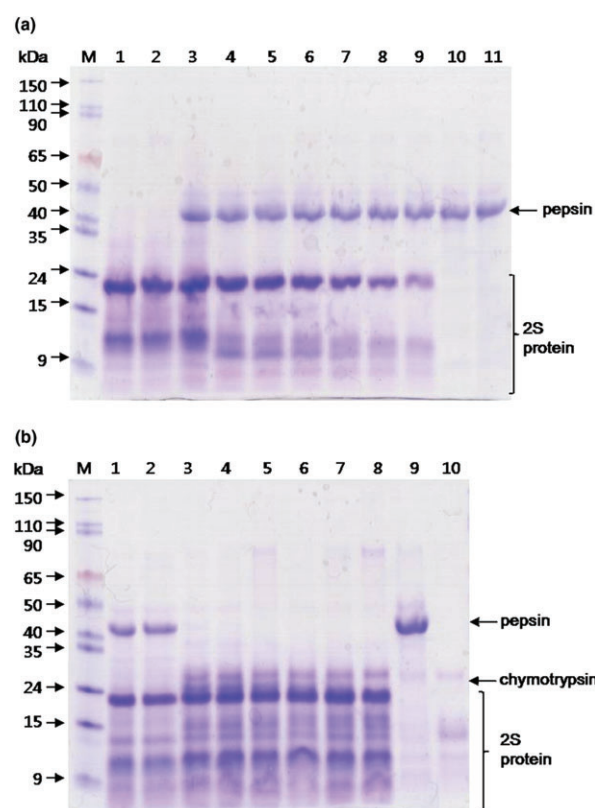


Figure 3. SDS-PAGE analysis of the peptic and chymotryptic hydrolysates of the soybean 2S protein. Peptic hydrolysate (a) and chymotryptic hydrolysate following peptic hydrolysis (b). (a) M: molecular weight marker; lanes 1 and 2: protein only (0, 90 min); lanes 3–9: peptic hydrolysate (0, 5, 10, 20, 30, 60, 90 min); and lanes 10 and 11: pepsin only (0, 90 min). (b) M: molecular weight marker; lane 1: peptic hydrolysate (90 min); 2–8: peptic and chymotryptic hydrolysate (0, 2, 5, 10, 20, 30, 60 min); and lanes 9 and 10: pepsin and chymotrypsin only (0, 60 min).

the allergenic epitopes of SKTI might not be destroyed by hydrolysis with pepsin followed by chymotrypsin. Interestingly, the allergenicity increased after chymotryptic hydrolysis, suggesting that limited chymotryptic hydrolysis might aid in the exposure of interior hidden epitopes of soybean 2S protein, resulting in increased allergenicity. This is similar to the result of Keum *et al.*²¹ who hydrolysed soybean 7S globulin with pepsin and found that the allergenicity decreased by 50%. However, after sequential hydrolysis with chymotrypsin, increment of the allergenicity was observed. The allergenicity of soybean 11S globulin, meanwhile, was significantly reduced by peptic and chymotryptic hydrolysis in an immunoassay with six soybean-sensitive patients.²² These findings coincide well with the findings of Nowak-Węgrzyn and Fioocchi,¹⁴ who reported that treatments such as heating and proteolysis used on food may result in the formation of new epitopes. This may promote the interaction of IgE with the immune system in the intestine and lead to a potential increase in the protein allergenicity. Shibasaki *et al.*⁸ also reported that the allergenicity of soybean 2S protein increased after heating at 80 °C for 30 min.

CONCLUSIONS

The results of this study suggest that sequential enzymatic digestion with pepsin and chymotrypsin cannot completely hydrolyse the allergens of soybean 2S protein, although pepsin may

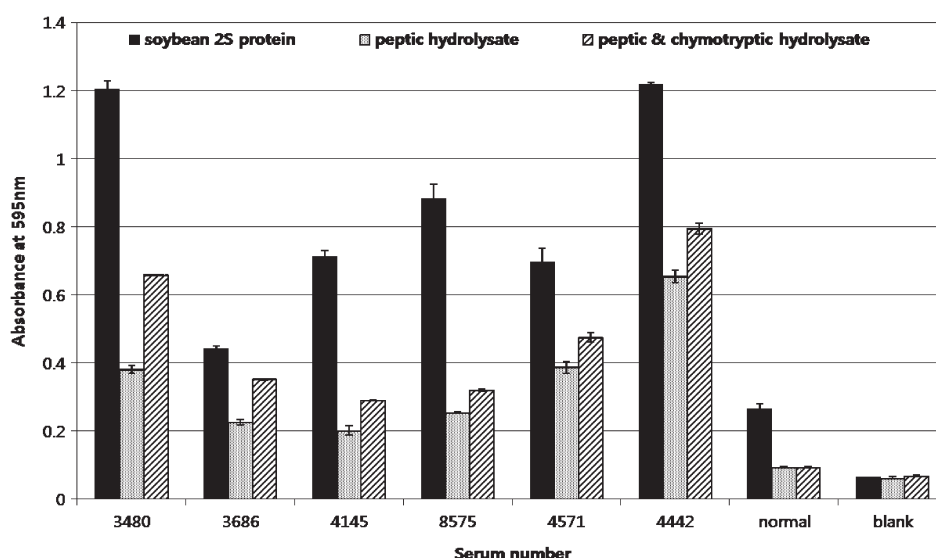


Figure 4. Effects of peptic and chymotryptic hydrolysis on soybean 2S protein. Error bars indicates standard deviations.

hydrolyse SKTI. Peptic hydrolysis partially decreased the allergenicity of soybean 2S protein, although subsequent chymotryptic hydrolysis actually increased the allergenicity. It seems that the hidden epitopes inside the protein may be exposed after treated with chymotrypsin. Soybean 2S protein still possesses allergenicity after sequential peptic and chymotryptic hydrolysis. This indicates that the allergenic epitopes of soybean 2S protein may not be fully destroyed after gastrointestinal digestion. Soybean 2S protein has been regarded as a minor allergen because only a minority of soybean-sensitive patients had a reaction to it. However, it is worthwhile to give it more attention because this protein sometimes causes severe reaction and has a high possibility to be a hidden allergen.

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