

Allergenicity of Proteolytic Hydrolysates of the Soybean 11S Globulin

H.-W. LEE, E.-H. KEUM, S.-J. LEE, D.-E. SUNG, D.-H. CHUNG, S.-I. LEE, AND S. OH

ABSTRACT: A substantial portion of the human population has immune hypersensitivities to various food materials. Soybean is one of the most common foods involved in such hypersensitivity reactions, especially in younger children. In this study, we investigated the effect of peptic and chymotryptic hydrolysis on the allergenicity of the 11S soybean globulin, which is the primary soybean allergen. The 11S globulin is composed of both acidic and basic polypeptides, and we found that the acidic polypeptide was effectively hydrolyzed, while basic polypeptide was more resistant to hydrolysis. The 11S globulin hydrolysate was size-fractionated by gel filtration, and 9 of the fractions obtained were tested for allergenicity against sera from 6 soybean-allergic patients. The overall allergenicity of soybean 11S globulin was reduced by peptic and chymotryptic hydrolysis, although a gel filtration fraction with a major peptide of 20 kDa was highly immunoreactive. Hydrolyzed fragments of less than about 20 kDa were not immunoreactive.

Keywords: allergenicity, glycinin, 11S globulin, peptic and chymotryptic hydrolysis, soybean

Introduction

Storage globulins in soybean seeds make up the bulk of the protein in soybeans. There are 2 primary soybean globulins, and they are differentiated by their sedimentation coefficients: β -conglycinin is known as the 7S globulin, and glycinin is known as the 11S globulin. Glycinin is a hexamer composed of 2 trimers, each consisting of 3 nonidentical subunits formed by an approximately 40 kD acidic(A) and approximately 20 kD basic(B) chain linked by a single disulfide bond (Christensen and others 2003). Only 5 major subunits, G1, G2, G3, G4, and G5, have been identified: A_{1a}B_{1b}, A₂B_{1a}, A_{1b}B₂, A₃B₄, and A₅A₄B₃ (Utsumi and others 1997). Soybean is one of the major allergens to which children under 3 y of age generate food hypersensitivity reactions, although the prevalence of soybean hypersensitivity in the general population is probably below 0.5% (Besler and others 2000). However, because soy protein is increasingly being used in a variety of food products, some predict that this increased exposure will result in increased hypersensitivity in the general population (Breiteneder and Ebner 2000). At least 16 potential soy protein allergens have been identified. The major ingestive allergens appear to be Gly m Bd 30 k (soybean vacuolar protein), the storage proteins glycinin and β -conglycinin, and profilin (Gly m 3) (Christopher 2004). Recent work has shown that the acidic subunit of glycinin G1 and the basic subunit of glycinin G2 are important allergens in patients who have a food allergy to soybeans (Beardslee and others 2000; Helm and others 2000). Acidic glycinin chains of soybean were found to be the binding site of IgE from soy allergic individuals (Pedersen and Djurtoft 1989).

Solubility, stability, size, and compactness of the overall protein fold are aspects of protein structure that are related to allergenicity (Aalberse 2000). Glycinin, one of soy protein allergens, is thermostable up to 95 °C; heating above 95 °C forms large aggregates that retain secondary structure (Mills and others 2003b), which may

contribute to the reduction of immunogenicity (Demonte and others 1997). Extreme pH such as when glycinin was taken to pH 2.0 or pH 11 gave also reduction in immunogenicity in the presence of antiglycinin serum (Demonte and others 1997). It is features such as those discussed above that allow a number of food allergens to bind various types of ligands as well as large numbers of disulfide bonds (Breiteneder and Ebner 2000). The epitope types of native proteins might also determine aspects of allergic hypersensitivity: Individuals who possess IgE antibodies to sequential, linear epitopes react to a food allergen in any form (for example, extensively cooked or partially hydrolyzed) because the linear sequence of the amino acids is generally unaltered by denaturation. In contrast, those with IgE antibodies to discontinuous conformational epitopes appear to have a lesser reaction to small amounts of a food allergen after the food has been extensively cooked or partially hydrolyzed: they are then able to partially tolerate it because the tertiary structure of the protein is altered and the conformational epitopes are destroyed (Sampon 2004). The characterization of IgE epitopes of important food allergens is of fundamental importance for understanding mechanisms of food allergy and for designing safe immunotherapeutics (Shreffler and others 2004). Information about the digestibility of food proteins and about the residual antigenicity/allergenicity of absorbed food fragments and IgE epitopes is essential because the denaturation of food by gastric acids and the digestibility of food protein in the gastrointestinal tract are likely to be factors that play some part in determining the allergenic potential of a particular food (Houben and others 1997). We undertook this study to investigate how the allergenicity of the 11S globulin, which is the primary allergen of soybean, is affected by peptic and chymotryptic hydrolysis, and to identify allergenic fragments of the 11S globulin using SDS-PAGE.

Materials and Methods

Patient sera

Sera were arbitrarily obtained from 6 soybean-sensitive patients with atopic dermatitis who were treated at Samsung Medical Center (Seoul, Korea). Specific IgE levels were measured by the CAP IgE FEIA method (Pharmacia, Uppsala, Sweden) and were between 0.35 and 18.4 kUA/L (Table 1). These patient sera were not further

MS 20060514 Submitted 9/16/2006, Accepted 2/4/2007. Authors Lee, Keum, Lee, Sung, and Oh are with Dept. of Food Science and Technology, Ewha Womans Univ., Seoul, Korea. Author Chung is with Dept. of Food Science and Technology, Gyungang Natl. Univ., Korea. Author Lee is with Dept. of Pediatrics, Sungkyunkwan Univ. School of Medicine, Suwon, Korea. Direct inquiries to author Oh (E-mail: ssoh71@ewha.ac.kr).

characterized to determine whether they are sequential or conformational epitopes.

Extraction, purification, and concentration of the 11S globulin

Defatted soybean was obtained from Sigma Chemical Co. (St. Louis, Mo., U.S.A.), and the 11S-rich soybean globulin was prepared by the method of Nagano and others (1992). Extracted 11S-rich globulin was purified using Con A-Sepharose 4B affinity chromatography (Amersham BioSciences Piscataway, N.J., U.S.A.). Binding buffer was 20-mM Tris-HCl, 0.5-M NaCl, 1-mM MnCl₂ at pH 7.4; elution buffer was 0.5-M methyl- α -D-glucopyranoside, 20-mM Tris-HCl, 0.5-M NaCl at pH 7.4. The purified 11S globulin was concentrated by Centriprep (Millipore Co., Billerica, Mass., U.S.A.) (Kitamura and others 1974).

Hydrolysis of the 11S globulin

Hydrolysis of extracted 11S globulin was initiated with 2340 units of pepsin (Sigma) at pH 2, 38 °C for 60 min, after which 82.5 units of chymotrypsin (Sigma) at pH 8 was added and the mixture was incubated at 38 °C for 60 min in water bath (Eyela, Tokyo, Japan). The hydrolysis was stopped by adjusting the pH to 7 and heating at 80 °C for 15 min (Joo and others 2004).

Determination of degree of hydrolysis

The degree of hydrolysis (DH) values for hydrolyzed 11S globulin were determined by the TNBS method (Nissen 1979), which measures the α -amino group of hydrolyzed 11S globulin; L-leucine was used as a standard. The h_{tot} of soy protein was 8.8 meq/g (see equation below).

$$\text{DH}(\%) = h/h_{\text{tot}} \times 100$$

h : concentration of α -amino groups in the protein (milliequivalent/g protein); h_{tot} : concentration of amino acid in the intact protein (milliequivalent/g protein).

Fractionation of the 11S globulin by gel filtration

The peptic and chymotryptic hydrolysate of 11S globulin was fractionated using Sephacryl S-100 (Amersham Biosciences, Uppsala, Sweden). The column was equilibrated with 50 mM sodium phosphate buffer containing 0.15 M NaCl, and 6 mL (25 mg/mL) of the sample was loaded onto the column. The gel filtration was run at 4 °C at a flow rate of 0.5 mL/min. After 4 h of injecting hydrolysate of 11S, its fraction was gathered at the rate of 5 mL. The eluate was monitored by measuring the absorbance at 280 nm (Spectronic Unicam, Rochester, N.Y., U.S.A.) (Allaoua and others 1998).

Enzyme-linked immunosorbent assay (ELISA)

The protein concentration was measured by BCA Protein Assay Reagent Kit (Pierce, Ill., U.S.A.). Standard material was bovin serum albumin (BSA). For ELISA analysis, a 96-well microplate was coated

overnight at 4 °C with 100- μ L protein (10-ng protein per μ L) diluted in PBS. After washing (0.05% Tween in PBS), the plate was blocked by adding 100 μ L/well of 1% BSA in PBS and incubating for 1 h at room temperature. The plate was then incubated for 1 h at room temperature with 100 μ L/well of a patient's sera diluted 1:20 in 1% BSA (Sigma), washed again, and incubated with peroxidase-labeled goat antihuman IgE (Kirkegaard & Perry Laboratories, Md., U.S.A.) diluted 1:1000 in 1% BSA for 1 h. The plate was then developed by the addition of 100 μ L/well of a solution of 3,3',5,5'-tetraethylbenzidine (Sigma). The reaction was stopped after 30 min by addition of 1 M H₂SO₄. Optical density was measured at 450 nm using an ELISA reader (GMI, Ramsey, Minn., U.S.A.).

Electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (1970). Electrophoresed proteins were stained with Coomassie Brilliant Blue R-250. The pick fraction of a portion of the gel filtration eluate was analyzed using a silver staining kit (Bio-Rad, Hercules, Calif., U.S.A.).

Statistical analysis

The data were analyzed using a general linear model (GLM), and the significance between means was tested using Duncan's multiple range test (Steel and Torrie 1984).

Results and Discussion

Extraction and purification of the soybean 11S globulin

The 2 major components of soybeans, 7S globulin and 11S globulin (glycinin max.), make up about 80% of the total storage proteins in soybeans, and both the 7S and 11S globulins have been identified as soy allergens (Maruyama and others 1998). The 7S globulin is a complex protein mixture that includes α -conglycinin, β -conglycinin, and γ -conglycinin. In a previous analysis of the isolated 7S globulin, we found that β -conglycinin was a trimeric protein composed of 3 major subunits (α' = 76 kDa, α = 72 kDa, and β = 54 kDa) (Keum and others 2006). The 11S globulin included 5 glycinins (G1, G2, G3, G4, and G5), and the polypeptide constituents of these 5 major subunits have been identified as A_{1a}B_{1b}, A₂B_{1a}, A_{1b}B₂, A₃B₄, and A₅A₄B₃, respectively (Utsumi and others 1997). G1 and G2 glycinin are known to be the primary allergens of the 11S globulin (Beardslee and others 2000; Helm and others 2000). In this study, we extracted an 11S globulin-rich fraction from defatted soy protein, and further purified the 11S globulin using a Con A-Sepharose 4B affinity resin, which binds glycoproteins, including the 7S globulin. The 11S globulin, which was in the flow-through, was concentrated to about 25 mg/mL and 20 μ g of protein were loaded and electrophoresed (Figure 1) to confirm the presence of the acidic (approximately 37 to 43 kDa) and basic (approximately 17 to 20 kDa) polypeptides by estimating the molecular weight of the protein bands on the gel compared with electrophoresis standards. Sathe (1991) reported that acidic polypeptide, A_{1a}, A_{1b}, A₂, A₃, and A₄, is 37 to 43 kDa and basic polypeptide, B₃, B_{1a}, B_{1b}, B₂, and B₄, is 17 to 20 kDa. And the upper direction of the band, A_{1a}, A_{1b}, A₂, and A₄, is A₃ polypeptide. It correlates well with the data presented in this study.

Peptic and chymotryptic hydrolysis of the 11S globulin

The purified 11S globulin was analyzed by SDS-PAGE in the absence of the reductant 2-mercaptoethanol in SDS-PAGE (Figure 2).

Table 1—Biological characteristics of sera from 6 soybean-sensitive patients

Patient number	Age (y)	Sex	CAP IgE FEIA soybean IgE (kU/L)
1	9	M	0.35
2	2	M	0.36
3	9	F	18.4
4	4	F	12
5	4	M	3.7
6	1	F	2.39

Under these conditions, the individual acidic (approximately 37 to 43 kDa) and basic (approximately 17 to 20 kDa) polypeptide bands disappeared and a new band of about 60 kDa appeared. This appearance of about 60-kDa band suggests that the acidic and basic polypeptides are most likely linked by disulfide bonds. The polypeptides might migrate together. These disulfide bonds may explain some of the properties of the 11S soybean globulin, namely, its high thermostability, and resistance to proteolysis, and resistance to conformational changes induced by extreme pH values (Breiteneder and Clare 2005). We carried out peptic and chymotryptic hydrolyses of the 11S globulin in order to assess the effect of enzymatic

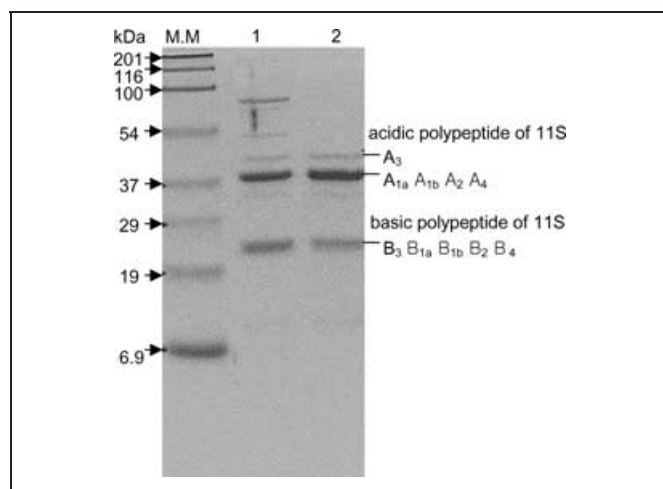


Figure 1—SDS-PAGE analysis of 11S globulin purification fractions: M.M = molecular weight markers; 1 = 11S globulin-rich fraction; 2 = purified 11S globulin eluted from Con A sepharose affinity chromatography. Acidic polypeptide of 11S (A_{1a} , A_{1b} , A_2 , A_3 , and A_4); basic polypeptide of 11S (B_3 , B_{1a} , B_{1b} , B_2 , and B_4).

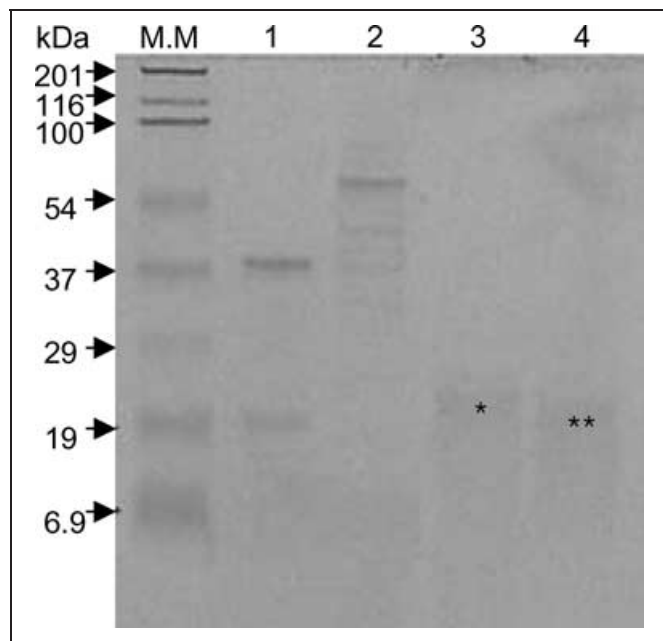


Figure 2—SDS-PAGE analysis of intact 11S globulin and of the peptic and chymotryptic hydrolysates of 11S globulin. M.M = molecular weight marker; 1 = 11S globulin; 2 = 11S globulin without 2-mercaptoethanol; 3 = 11S globulin peptic hydrolysate; 4 = 11S globulin peptic and chymotryptic hydrolysate. *Smearing band with MW about 23 kDa. **Smearing band with MW about 20 kDa.

hydrolysis on the allergenicity of the hydrolyzed fragments. When subjected to peptic hydrolysis, a new band of about 23 and 20 kDa appeared. When subjected to peptic and chymotryptic hydrolysis, the band of about 20 kDa remained. Unlike the basic polypeptide (approximately 17 to 20 kDa), So and Kim (2001) reported that the acidic polypeptide (approximately 37 to 43 kDa) of the 11S globulin was effectively hydrolyzed by pepsin. And the basic polypeptide (approximately 17 to 20 kDa) of 11S globulin is markedly resistant to enzyme hydrolysis. This differential susceptibility to hydrolysis of the acidic compared with basic polypeptides was probably due to the greater hydrophobicity and compactness of the basic polypeptides of the 11S globulin compared with that of the acidic polypeptides (Kang and others 1988). According to the result of these studies, the band of about 20 kDa is thought to be a basic polypeptide and the smaller band is thought to be a hydrolysate of acidic polypeptide.

We also analyzed the final degree of hydrolysis (DH) value for the hydrolysis of the 11S globulin using a 2-step incubation in which the 11S globulin was treated with pepsin for 60 min, after which chymotrypsin was added and incubation continued for another 60 min (Figure 3). The DH value for hydrolysis by pepsin was about 8%; the rate of hydrolysis rose steeply for the 1st 15 min of incubation and then reached a plateau after about 30 min of incubation. Chymotrypsin showed a similar profile in that the rate of hydrolysis rose steeply for 15 min before reaching a plateau at the final DH value of about 13%.

Allergenicity of 11S globulin and its hydrolysates

We carried out an enzyme-linked immunosorbent assay (ELISA) in order to examine the reactivity between sera from 6 patients with soybean allergies and soybean protein fragments that had been hydrolyzed by pepsin and/or chymotrypsin.

Shibasaki and others (1980) reported that 11S and 7S globulins are the major allergen components of soybean, and that the allergenicity of the 7S globulin was higher than that of 11S globulin. In a separate study, Keum and others (2006) later confirmed that the 7S globulin was more allergenic than the 11S globulin. ELISA analysis for the 11S globulin hydrolysates (Figure 4) showed that peptic hydrolysis substantially reduced the interaction of the 11S globulin with sera from patients of soybean allergies. Interaction of the 11S globulin that had been subjected to sequential peptic and chymotryptic hydrolysates also decreased when they were treated with patients sera, although to a somewhat lesser extent than that of the peptic hydrolysate (Figure 4). Significant difference was found among these 3 samples—11S globulin, peptic hydrolysates, and sequential hydrolysates with pepsin and chymotrypsin—when

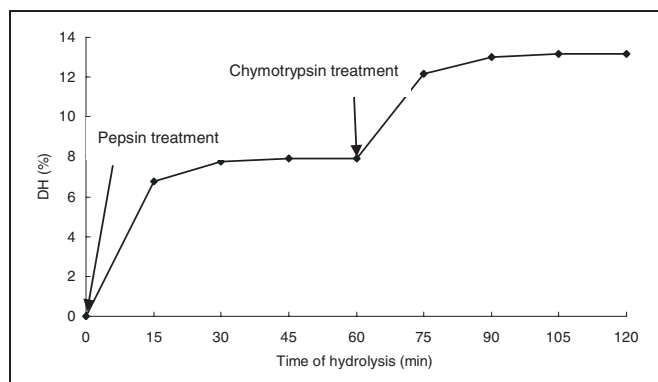


Figure 3—Degree of hydrolysis (DH) of 11S globulin. 11S globulin was incubated with pepsin for 60 min after which chymotrypsin was added and incubation was continued for another 60 min.

Duncan's multiple range test was performed. The decreased allergenicity may result from the proteolytic destruction of the conformational epitopes of major allergens (Mills and others 2003a). In contrast to the behavior of the 11S globulin, a previous study (Keum and others 2006) has shown that the allergenicity of the 7S globulin is actually higher after sequential peptic and chymotryptic treatment; this may be due to the exposure of a previously inaccessible epitope by the enzymatic treatment. Burks and others (1992) similarly treated soybean protein with digestive enzymes, using 2 successive steps of hydrolysis: either pepsin and trypsin, or chymotrypsin and an intestinal mucosa peptidase. The authors found that when the hydrolyzed soybean protein was ingested, there was a notable decrease in IgE binding (which is a rough measure of antigenicity) as compared to the IgE binding resulting from ingestion of the intact, nonhydrolyzed soybean protein. These data correlate well with the data presented in this study.

Allergenicity of hydrolysis fragments of the 11S globulin

After sequential peptic and chymotryptic treatment of the 11S globulin, we carried out gel filtration to separate the hydrolysate by size, and obtained 9 fractions (A through I) (Figure 5). Fraction A on the silver staining SDS-PAGE did not show a significant band (Figure 5), and its reactivity with patient sera was less than fraction B, probably due to the small amount of reaction peptides and/or unopened epitope (Figure 6). Silver staining SDS-PAGE analysis of fraction B showed that this fraction contained peptide(s) of about 20 kDa (Figure 5), and we found that these peptides reacted with sera from patients with soybean allergies (Figure 6). Fraction C showed that this fraction contained peptide(s) of about 15 kDa (Figure 5), and we found that these peptides have no interaction with sera from patients with soybean allergies (Figure 6). We also analyzed fractions D through I by silver-stained SDS-PAGE, but were not able to see any obvious peptide components in these fractions. Previous work has shown that most allergens are ≥ 10 kDa to show allergenicity (Lee and others 1994). Smaller polypeptides than 10 kDa, however, showed allergenicity (Gonzalez and others 1992; Garcia-Ortega and others 1998). In the case of the 11S globulin hydrolysate, we found reactivity with sera from soybean-allergic patients only for the about 20 kDa polypeptides in fraction B. Fractions C through I, which presumably contained polypeptides < 20 kDa, did not appear to be immunoreactive in the ELISA assay. Taken together, these data suggest that immunoreactivity of the intact 11S globulin in the ELISA assay is likely to be largely due to conformational (discontinuous) epitopes rather than to sequential (linear) epitopes. Since conformational epitopes are likely to be discontinuous in the linear amino acid sequence (but

contiguous in the folded 3-D sequence), proteolysis would be to destroy epitopes by disrupting the complex protein structure, whereas linear epitopes would have a good chance of remaining intact even after proteolysis.

Conclusions

Soybeans represent an importance food source in practically all regions of the world, and are commonly used in daily meals of all sorts. Soybeans are also often used as a substitute food for young patients who are allergic to milk, so younger children are often exposed to soybeans even in countries in which soybeans are

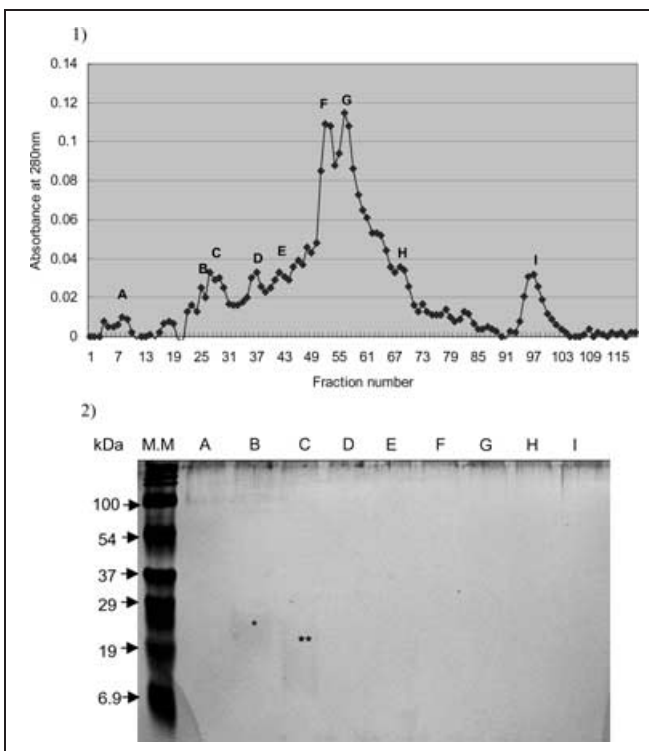


Figure 5—1) Sephacryl S-100 fractionation of a sequential peptic and chymotryptic hydrolysate of 11S globulin, 2) silver-stained SDS-PAGE gel of Sephacryl S-100 fractionation of an 11S globulin peptic and chymotryptic hydrolysate. M.M = molecular weight markers; lanes A through I indicate the pooled gel filtration fractions A through I. *Smear band of MW about 20 kDa. **Smear band of MW about 15 kDa.

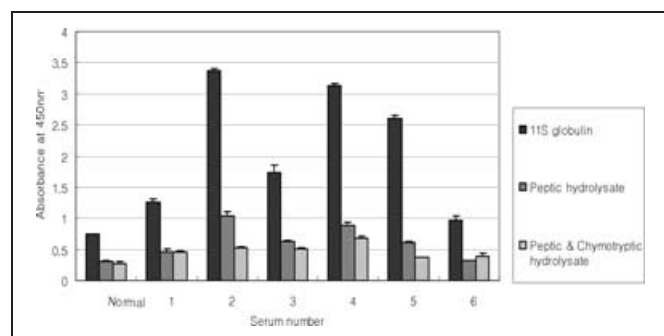


Figure 4—ELISA analysis of immunoreactivity of soybean-allergic patient sera against intact or hydrolyzed 11S globulin. Serum from 6 patients (serum numbers 1 to 6) was tested (Duncan's multiple range test, $P < 0.05$, $n = 3$, mean $\pm$ standard deviation).

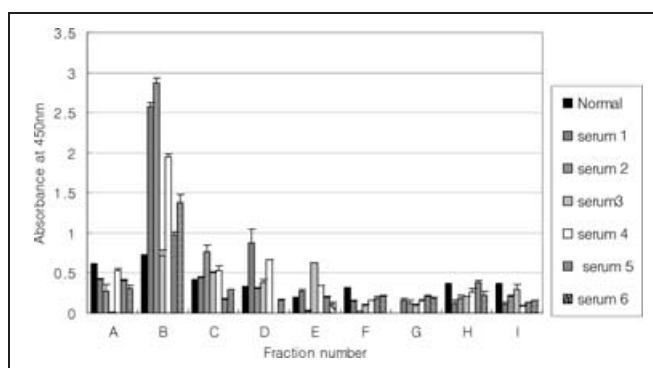


Figure 6—ELISA analysis of immunoreactivity of Sephacryl S-100 fractions A through I of the 11S globulin peptic and chymotryptic hydrolysate. Fractions were tested against sera from 6 patients with soybean allergies (1 to 6) and also against normal serum as a control. Duncan's multiple range test, $P < 0.05$, $n = 3$, mean $\pm$ standard deviation.

not normally a staple food. Since the young are more likely to develop food hypersensitivities, it is important to analyze what components of soybeans are responsible for allergenicity in order to try to mitigate these allergenic effects.

In this study, we confirmed that sequential peptic and chymotryptic hydrolysis of the 11S soybean globulin resulted in a decrease in its interaction with sera from patients of soybean allergies compared to that of the intact protein. The 11S globulin is one of the major allergens of soybean, so it is notable that although the interaction with sera from patients of soybean allergies was decreased by this proteolytic treatment, a certain level of immunoreactivity still remained even in the hydrolyzed 11S protein fraction. This suggests that when soybeans are digested in the body and nutrients are absorbed, the epitope recognized by IgE still remains intact. Consequently, it is likely that at least a substantial portion of the allergenicity of soybeans is maintained during the process of ingestion and digestion. However, because we focused this study only on the enzymes pepsin and chymotrypsin, additional research is needed to elucidate the complex effect of digestive hydrolysis on the allergenicity of soybeans.

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