Food Sci. Biotechnol. Vol. 15, No. 1, pp. 128 ~ 132 (2006)

Food Science and Biotechnology

Effect of Enzymatic Hydrolysis of 7S Globulin, a Soybean Protein, on Its Allergenicity and Identification of its Allergenic Hydrolyzed Fragments Using SDS-PAGE

Eun-Hee Keum, Sang-Il Lee[1] and Sangsuk Oh*

Department of Food and Nutritional Sciences, Ewha Womans University, Seoul 120-750, Korea
[1]Department of Pediatrics, School of Medicine, Sungkyunkwan University Suwon, Kyonggi 440-746, Korea

Abstract This study was undertaken to investigate the effect of peptic and chymotryptic hydrolyses of 7S globulin, the major allergen of soybean protein, on its allergenicity, as measured by enzyme linked immunosorbent assay (ELISA), and to identify the allergenic hydrolyzed fragments of 7S globulin using SDS-PAGE. When 7S globulin was hydrolyzed by pepsin, the allergenicity was reduced by over 50%. However, the allergenicity of 7S globulin reduced by peptic hydrolysis was recovered in the sera from 5 out of 10 patients following sequential chymotryptic hydrolysis. Two fragments, with molecular weights 20-25 and 13-16 kDa, among the hydrolysate of 7S globulin by sequential pepsin and chymotrypsin showed reactivity with sera from 10 soybean-allergenic patients. As a result of the theoretical hydrolyses of β-conglycinin, which is a major protein of 7S globulin, it is suggested that the 20-25 kDa fragments were the fragments of the α-subunit of β'-conglycinin and that the 10-16 kDa fragments were from the α'-subunit.

Keywords: 7S globulin, allergenicity, peptic and chymotryptic hydrolyses, allergenic fragments, ELISA

Introduction

Soybean is one of the major allergens in children younger than 3 years of age, and its prevalence is probably below 0.5% in the general population (1). Furthermore, as soy protein enjoys widespread use in the food industry, its allergenic sensitivity has been predicted to increase (2). At least 16 potential soy protein allergens have been identified, and Gly m Bd 30K (thol protease P34), glycinin, and β-conglycinin of these have been identified as being related to food allergies causing the sensitization process which occurs in the gastrointestinal tract (2, 3).

Solubility, stability, size, and compactness of the overall fold are aspects of the protein structure related with allergenicity (4). In general, food allergens tend to be soluble glycoproteins with molecular weights of 10-70 kDa. They are usually stable to heat and acid, and relatively persistent to proteolytic digestion (5). This is because a number of food allergens are able to bind various types of ligands and large numbers of disulfide bonds, and induce the oligomerization or aggregation, glycosylation, and potential interaction with cell membranes or lipid structures (3). The epitope types of native proteins might also be responsible for allergic reactions. Individuals who possess IgE antibodies to sequential epitopes react to the food in any form, whereas those with IgE antibodies to conformational epitopes are tolerant of small amounts of the food after extensive heating or partial hydrolysis because the tertiary structure of the protein is altered and the conformational epitopes are destroyed (6).

For an understanding of the mechanisms of food allergy and for the safe design of immunotherapeutics, the characterization of IgE epitopes of food allergens is important (7). Acid-denaturation and digestibility of food protein in the gastrointestinal tract are likely to be important factors partly determining the allergenic potential. Accordingly, information about the digestibility of food proteins, the residual antigenicity, and the allergenicity of absorbed fragments, as well as IgE epitopes, is essential (8).

This study was therefore undertaken to investigate the effect of peptic and chymotryptic hydrolyses of 7S globulin, the major allergen of soybean, on its allergenicity and to identify the allergenic fragments of 7S globulin using SDS-PAGE.

*Corresponding author: Tel: 82-2-3277-3558; Fax: 82-2-3277-2862
E-mail: ssoh71@ewha.ac.kr
Received October 31, 2005; accepted December 19, 2005

Materials and Methods

Patient Sera Sera were obtained from 10 soybean-sensitive patients with atopic dermatitis who were treated at Samsung Medical Center (Seoul, Korea). Specific IgE levels measured by CAP IgE FEIA method (Pharmacia Bio-Tech. Ltd., Uppsala, Sweden) were between 0.63-101 kU/L (Table 1).

Extraction of 7S globulin from defatted soybean Defatted soybean was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The 7S globulin of soybean was prepared by the method of Nagano *et al.* (9).

Hydrolysis of 7S globulin The extracted 7S globulin was hydrolyzed by using 2,340 units pepsin (Sigma Chemical Co., St. Louis, MO, USA) at pH 2, followed by 82.5 units chymotrypsin (Sigma Chemical Co.) at pH 8 in a water bath at 38°C (EYELA, Tokyo, Japan). The hydrolysis was stopped by the adjustment to pH 7 and heating at 80°C for 15 min (10).

Table1. Biologic characteristics of sera from 10 patients

Patient No.	Age (yr)	CAP IgE FEIA soybean IgE (kUA/L)
1	1	0.63
2	2	1.01
3	3	101
4	8	2.06
5	3	17.6
6	3	0.52
7	2	22.2
8	2	16.5
9	3	101
10	4	7.84

Fractionation using gel filtration 7S globulin hydrolysate by peptic and chymotryptic hydrolyses was fractionated using Sephacryl S-100 HR (Amersham Biosciences, Uppsala, Sweden). The column was equilibrated with 50 mM sodium phosphate buffer containing 0.15 M NaCl and 3 mL of the sample was loaded onto the column. The gel filtration was run at cold temperature with a flow rate of 0.4 mL/min. The eluate was monitored by measuring the absorbance at 280 nm (Spectronic Unicam, Cambridge, UK).

Enzyme linked immunosorbent assay A 96-well microplate was coated overnight at 4°C with 1 ug protein (10 ng protein /mL) diluted in phosphate buffered saline (PBS). After washing (0.05% PBS-Tween), 100 ß°/well of 1% bovine serum albumin (BSA)-PBS (w/v) was added and the plate was incubated for an hour at room temperature to protect non-specific binding. Thereafter the washed plate was incubated for another hour at room temperature with 100 μL/well of patient sera diluted 1:20 in 1% BSA (Sigma Chemical Co.) and washed again. Next, the plate was incubated with peroxidase-labeled, goat anti-human IgE (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA) diluted 1:1000 in 1% BSA for 1hr and then was developed by incubating with 100 μL/well of a 3, 3', 5, 5'-tetraethyl-benzidine (Sigma Chemical Co.). The reaction was stopped after 30 min by 1 M H_2SO_4. Optical density was measured at 450 nm using enzyme linked immunosorbent assay (ELISA) reader (Spectra Max 340, GMI Inc., Ramsey, MN, USA).

SDS-PAGE SDS-PAGE was performed according to the method of Laemmli (11). The protein was resolved on 12 % separating and 5% stacking gels for 30 min at 40 V and 1 hr at 80 V, respectively. The proteins separated on the gels were stained with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Results and Discussion

Peptic and chymotryptic hydrolyses of 7S globulin The 7S globulin (β-conglycinin) and 11S globulin (glycinin), identified as soy allergens, are the two major components of soybeans, which comprise ~80% of storage proteins (12). The 7S globulin is a complex mixture of proteins, including β-conglycinin, γ-conglycinin and basic 7S. Of these, β-conglycinin is the most prevalent and accounts for 30-35% of the total soybean protein, which is used interchangeably with 7S protein because it is the major 7S protein (13). In the extracted 7S globulin (Fig. 1), β-conglycinin was found to be a trimeric protein composed of three major subunits (α'=76 kDa, α=72 kDa, β=53 kDa). For 7S globulin without 2-mercaptoethanol, SDS-PAGE indicated that the α-subunit of β-conglycinin had disappeared, to be replaced by a new band of about 140 kDa (Fig. 1). According to the amino acid sequence and secondary structure prediction of β-conglycinin, both α'- and α-subunits have one cysteine each. In contrast with the α-subunit, however, a cysteine of the α-subunit was revealed (14, 15). It is suggested that the α-subunit was bound by a disulfide bond. The acidic polypeptide (35-40 kDa) and basic polypeptide (20 kDa) of 11S globulin were linked by a disulfide bond as well (Fig. 1). These disulfide bonds are the cause for the high thermostability and resistance to extreme pH and proteolysis exhibited by soy globulin (3).

Peptic and chymotryptic hydrolyses of soybean 7S globulin (β-conglycinin) were performed to examine the effect of enzymatic hydrolysis on it. When pepsin was added to the 7S globulin solution, the β-subunit (72 kDa) of the β-conglycinin was effectively hydrolyzed, while the α'-(76 kDa) and β-(53 kDa) subunits were not. However, the β-subunit was hydrolyzed by chymotrypsin following peptic hydrolysis. The β-subunit of β-conglycinin was markedly resistant to sequential peptic and chymotryptic hydrolyses, possible because of the higher content of hydrophobic amino acid and β-sheet of the β-subunit's core, compared to those of the α'- and α-subunits (16, 17, 18). The major breakdown products of 7S globulin by pepsin were sized approximately 30 kDa, 20-25 kDa and ~6 kDa. When 7S globulin underwent sequential peptic and chymotryptic hydrolyses, the band of 30 kDa disappeared, to be replaced by new bands of 10-16 kDa. Other bands, however, remained without further hydrolysis (Fig. 1: lane 1, 5, and 6).

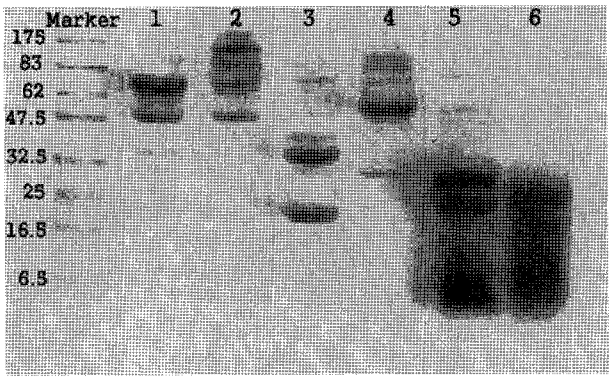

Fig. 1. SDS-PAGE gel of 7S- and 11S-globulin and hydrolysates of 7S globulin. 1. 7S globulin with 2-mercaptoethano (2-ME); 2. 7S globulin without 2-ME; 3. 11S globulin with 2-ME; 4. 11S globulin without 2-ME; 5. 7S globulin hydrolysate by peptic hydrolysis; 6. 7S globulin hydrolysate by sequential peptic and chymotryptic hydrolyses.

Allergenicity of 7S globulin and its hydrolysates ELISA was performed to determine the reactivity between sera from 10 soybean-allergenic patients and soybean protein fragments hydrolyzed by pepsin and/or chymotrypsin.

Burks *et al.* (19) reported that IgE and IgG specific to crude soy are elevated in patients with positive double-blind, placebo-controlled food challenges to soy but that no fraction is clearly more antigenic. In this study, however, it was shown that 7S globulin is more allergenic than 11S globulin in the sera from 6 out of 10 patients (data not shown). Shibasaki *et al.* (20) reported that 11S, 7S, and 2S globulins are the major allergens of soybean, that especially the latter had the highest reactivity, and that the allergenicity of 7S globulin was higher than that of 11S globulin.

It was shown that the allergenicity of 7S globulin was reduced by peptic hydrolysis according to the ELISA results for 7S globulin hydrolysates (Fig. 2). This may have occurred because the conformational epitopes were destroyed, together with Gly m Bd 30K and β-conglycinin, which are the major allergens of 7S globulin (21). However, its allergenicity was increased by sequential peptic and chymotryptic hydrolyses in the sera from 5 out of 10 patients (Fig. 2). It may be considered that chymotryptic hydrolysis leads to the exposure of closed sequential epitopes of 7S globulin. Although the allergenicity of 7S globulin was affected by peptic and chymotryptic hydrolyses in most of the patients' sera, sera with high IgE levels for soybean (Table 1: sera 3 and 9) were hardly influenced by enzymatic hydrolysis.

Identification of allergenic hydrolyzed fragments of 7S globulin When gel filtration of 7S globulin hydrolysate by sequential peptic and chymotryptic hydrolyses was performed, it was separated into 10 fractions (A-J) of which six were identified on SDS-PAGE: fraction A, several hydrolyzed fragments in the void volume of the column (void volume is fully accessible for all sizes of fragments) (22); fraction B, 20-25 kDa molecular weight fragments; fraction C, 13-16 kDa; fraction D, 10-12 kDa; fractions E and F, ~6 kDa (Fig. 3). Because the fractions G-J were not identified on SDS-PAGE, they were considered to be fragments of lower molecular weights.

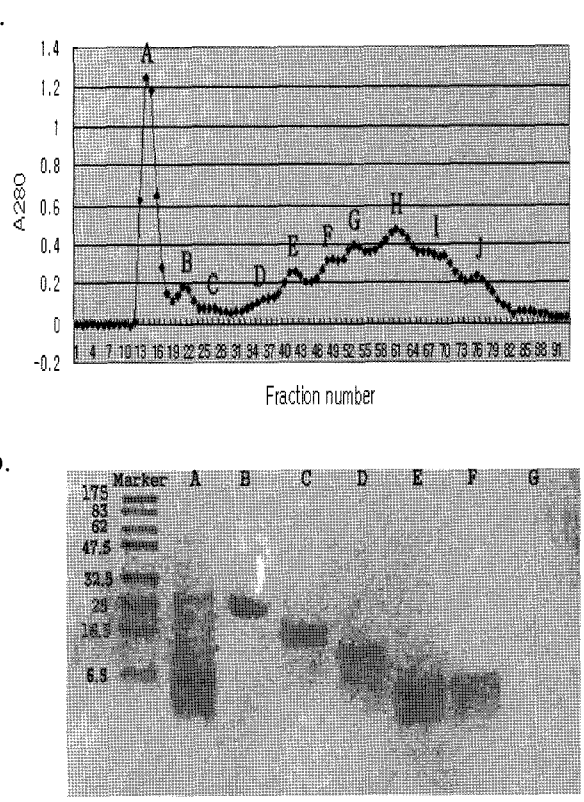

Fig. 3. Fractionation on Sephacryl S-100 HR of 7S globulin hydrolysate by (a) sequential peptic and chymotryptic hydrolyses and (b) SDS-PAGE.

Fractions B and C (13-25 kDa) showed an allergenic reaction with the sera of the soybean-sensitive patients (Fig. 4). In addition, in the sera with high IgE levels (sera 3 and 9) it reacted to the 10-12 kDa molecular weight fragments (fraction D) as well. The fragments with lower molecular weight had no effects on allergenicity (Fig. 4). This is a significant property of the allergen. The allergen must have a molecular weight of at least 10 kDa to show allergenicity in general (23).

By using the 'ExPASy Peptide Cutter' program, it was predicted that the hydrolyzed amino acid sequence of β-

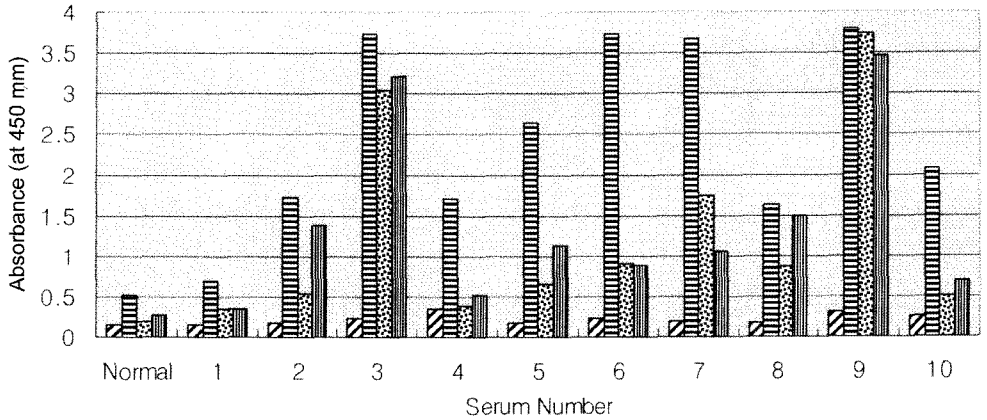

Fig. 2. Effects of peptic and/or chymotryptic hydrolysis for 7S globulin allergenicity (▨ Blank, ▤ 7S globulin, ▩ peptic hydrolysate, ▥ peptic and chymotryptic hydrolysate).

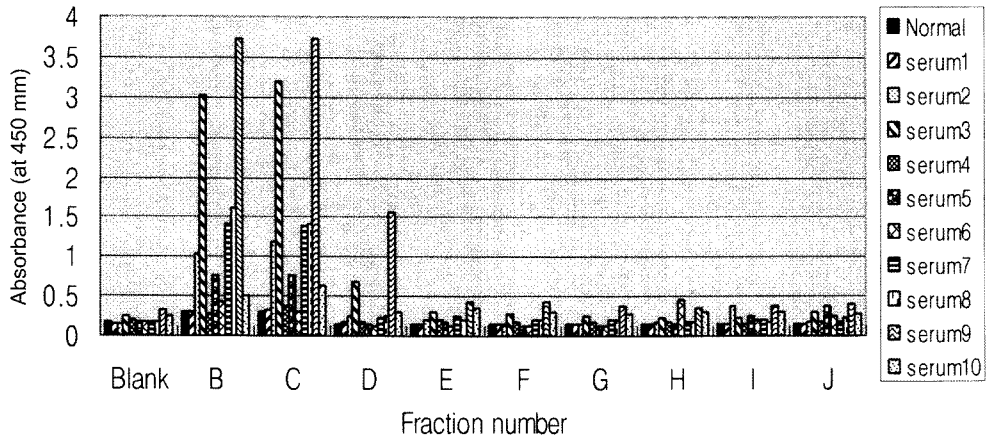

Fig. 4. Allergenicity of fractions by sequential peptic and chymotryptic hydrolyses of soybean 7S globulin.

conglycinin fragments would be obtained by peptic and chymotryptic hydrolyses (data not shown). Theoretically, the α'-subunit of β-conglycinin was hydrolyzed to fragments of about 9 kDa, 7 kDa, 5 kDa, and lower molecular weights. Fragments of about 17 kDa molecular weight and the residual fragments after sequential peptic and chymotryptic hydrolyses were obtained from the β-subunit. The β-subunit was broken into fragments of ~3 kDa and fragments of lower molecular weight. Therefore, these results indicated that the 20-25 kDa fragments identified on SDS-PAGE were from the α-subunit of β-conglycinin while the 10-16 kDa fragments were from the α'-subunit.

Ogawa *et al.* (24) reported that the α-subunit of β-conglycinin is a major allergenic component in 7S globulin, which is recognized by at least the patients with atopic dermatitis, while the α'-subunit, which is highly homologous to the α-subunit, had no allergenicity. In the present study, it was found that 10-16 kDa fragments from hydrolysate of the α'-subunit were obtained by sequential peptic and chymotryptic hydrolyses and that these fragments had an effect on allergenicity. The exposure of closed sequential epitopes of the α'-subunit by sequential peptic and chymotryptic hydrolyses may have increased the 7S globulin allergenicity.

Acknowledgments

This study was conducted as a part of the project "Life Technology Safety Evaluation Development" supported by The Ministry of Science & Technology, Korea.

References

1. Besler M, Helm RM, Ogawa T. Soybean (Glycine max). Internet Symposium on Food Allergens 2: 1-35 (2000)
2. Breiteneder H, Ebner C. Molecular and biochemical classification of plant-derived food allergens. J. Allergy Clin. Immunol. 106: 27-36 (2000)
3. Breiteneder H, Clare Mills EN. Molecular properties of food allergens. J. Allergy Clin. Immunol. 115: 14-23 (2005)
4. Aalberse RC. Structural biology of allergens. J. Allergy Clin. Immunol. 106: 228-238 (2000)
5. Kopper RA, Odum NJ, Sen M, Helm RM, Stanley JS, Burks AW. Peanut protein allergens: Gastric digestion is carried out exclusively by pepsin. J. Allergy Clin. Immunol. 114: 614-618 (2004)
6. Sampson HA. Update on food allergy. J. Allergy Clin. Immunol. 113: 805-819 (2004)
7. Shreffler WG, Beyer K, Chu TT, Burks AW, Sampson HA. Microarray immunoassay: Association of clinical history, in vitro IgE function, and heterogeneity of allergenic peanut epitopes. J. Allergy Clin. Immunol. 113: 776-782 (2004)
8. Houben GF, Knippels LMJ, Penninks AH. Food allergy: predictive testing of food products. Environ. Toxic. Pharma. 4: 127-135 (1997)
9. Nagano T, Hirotsuka M, Mori H, Kohyama K and Nishinari K. Dynamic viscoelastic study on the gelation of 7S globulin from soybeans. J. Agric. Food Chem. 40: 941-944 (1992)
10. Joo JH, Yi SD, Lee GH, Lee KT and Oh MJ. Antimicrobial activity of soy protein hydrolysate with Asp. Saitoi protease. J. Korean Soc. Food Sci. Nutr. 33: 229-235 (2004)
11. Laemmli UK. Cleavage of structural proteins during the assembly of the bead of bacteriophage T4. Nature 227: 680-685 (1970)
12. Maruyama N, Katsube T, Wada Y, Oh MH, Barba De La Rosa AP, Okuda E, Nakagawa S, and Utsumi S. The Roles of the N-linked glycans and extension regions of soybean β-conglycinin in folding, assembly and structural features. Eur. J. Biochem. 258: 854-862 (1998)
13. Hou HJ, Chang KC. Structural characteristics of purified β-conglycinin from soybeans stored under four conditions. J. Agric. Food Chem. 52: 7931-7937 (2004)
14. Rost B, Sander C. Prediction of protein secondary structure at better than 70% accuracy. J. Mol. Biol. 232: 584-599 (1993)
15. Rost B, Fariselli B, Casadio R. Topology prediction for helical transmembrane segments at 95% accuracy. Prot. Sci. 7: 1704-1718 (1996)
16. Catsimpoolas N, Meyer EW. Gelation phenomena of soybean globulins: Protein-protein interactions. Cereal Chem. 47: 559-570 (1970)
17. Doyle JJ, Schuler MA, Godette WD, Zenger V, Beachy RN & Slightom JL. The glycosylated seed storage proteins of Glycine max and Phaseolus vulgaris. J. Biol. Chem. 261: 9228-9238 (1986)
18. Thanh VH, Shibasak K. Beta-conglycinin from soybean proteins. Isolation and immunological and physicochemical properties of the monomeric forms. Biochim. Biophys. Acta 490: 370-384 (1997)
19. Burks AW, Brooks JR, Sampson HA. Allergenicity of major component proteins of soybean determined by enzyme-linked immunosorbent assay (ELISA) and immunoblotting in children with atopic dermatitis and positive soy challenges. J. Allergy Clin. Immunol. 81: 1135-1142 (1998)
20. Shibasaki M, Suzuki S, Tajima S, Nemoto H, Kuruomo T. Allergenicity of major component protein of soybean. Int. Arch. Appl. Immunol. 61: 441-448 (1980)
21. Astwood JD, Leach JN, Fuchs RL. Stability of food allergens to

digestion in vitro. Nat. Biotechnol. 14: 1269-1273 (1996)
22. Dongen WMAM, Berkel WJH, Franssen MCR. Gel filtration. Available from: http://gcg.tran.wau.nl/local/Biochem/educatio/Colleges/BSM/lectures/lecture_2/gelfitratrion.doc. Accessed Dec. 08, 2005.
23. Lee SW, Ha WK, Juhn SL, Kim JW, Shon DW, Lee JY. Antigenicity of whey protein hydrolysates against rabbit anti β-lactoglobulin antiserum. Korean J. Food Sci. Technol. 26: 532-538 (1994)
24. Ogawa T, Bando N, Tsuji H, Nishikawa K, Kitamura K. α-Subunit of β-conglycinin, an allergenic protein recognized by IgE antibodies of soybean sensitive patients with atopic dermatitis. Biosci. Biotechnol. Biochem. 59: 831-833 (1995)