

α -Subunit of β -Conglycinin, an Allergenic Protein Recognized by IgE Antibodies of Soybean-sensitive Patients with Atopic Dermatitis

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One of the major allergenic proteins in the soybean 7S-globulin fraction, which was recognized by sera of about 25% soybean-sensitive patients with atopic dermatitis, was identified as α -subunit of β -conglycinin. The IgE antibodies recognizing the α -subunit did not show a cross-reactivity against both α' - and β -subunits known to be highly homologous to α -subunit, and also against other allergenic components identified in soybeans [Ogawa *et al.*, *J. Nutr. Sci. Vitaminol.*, 35, 555–565 (1993)]. The IgE-binding site(s) was estimated to be located on the peptide 232–383 of α -subunit.

In our previous paper,¹⁾ we showed that about 30% of the patients with atopic dermatitis were sensitive to soybean proteins and about 15 protein components were found to be recognized by sera of the patients. Of these proteins, three components with molecular masses of about 70, 34, and 28 kDa were fractionated into the 7S-globulin fraction and identified as major allergenic proteins. Purification and characterization of allergenic components are essential for the studies developing hypoallergenic soybean proteins and products. The 34 kDa component, *Gly m Bd 30 K*, was isolated and assigned to the soybean 34 kDa oil-body-associated protein,²⁾ which was shown to be classified as a thiol proteinase of the papain superfamily, highly homologous to the house dust mite allergen, *Derp I*. Another allergenic protein with a molecular mass of about 70 kDa, which was recognized by about 25% of soybean-sensitive patients, was assigned to one of the major components of 7S-globulin fraction such as α - or α' -subunit of β -conglycinin.

In this report, the isolation and characterization of the 70 kDa allergenic protein component in 7S-globulin fraction by means of sera of the soybean-sensitive patients with atopic dermatitis is described.

Materials and Methods

Materials. A kit of Phadebas RAST (radioallergosorbent test; ¹²⁵I-labeled antihuman IgE antibodies, 1.7 × 10⁵ Bq/5 ml) was obtained from Pharmacia Diagnostics (Uppsala, Sweden). Sepharose 6B, DEAE-Sephacel, and Con A-Sepharose 4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and α -chymotrypsin (Type II, 4 cryst.) was from Sigma Chemical Co. (St. Louis, MO). Defatted soybean flakes (*Glycine max.*, mixture of IMO varieties) were generously supplied from Fuji Oil Co. (Izumisano, Osaka). Other reagents used were of analytical grade as described in our previous paper.^{1,2)}

Patient's sera. Serum samples were prepared from 69 soybean-sensitive patients with atopic dermatitis (mean age: 6.1 years old, ranging from 3 months to 21 years old) who were asked to donate blood samples for RAST at National Kagawa Children's Hospital.¹⁾ The sera of 6 patients carrying IgE antibodies against the 7S-globulin components were screened by immunoblotting analyses using the fractionated soybean proteins (purified 7S-globulin fraction) described previously.¹⁾ The control sera were obtained from 5 healthy adult volunteers who have no clinical history of allergic diseases.

Isolation of the subunit components of β -conglycinin. Major subunit components, α -, α' -, and β -subunits of β -conglycinin, were purified from crude 7S-globulin fraction³⁾ by the method of Coates *et al.*⁴⁾ The 7S-globulin fraction (protein, 680 mg) was chromatographed on a 3.7 × 75 cm column of Sepharose 6B equilibrated with 35 mM sodium phosphate buffer, pH 7.6, containing 0.4 M NaCl and 10 mM 2-mercaptoethanol (buffer A). The fractions containing β -conglycinin were pooled and dialyzed against the buffer A without 2-mercaptoethanol (buffer B). The dialyate (protein, 569 mg) was put on the 1.4 × 20 cm column of Con A-Sepharose 4B. After washing the column with buffer B, β -conglycinin adsorbed on the column was eluted with 0.1 M α -methylmannose in buffer B. The eluate was dialyzed against 19 mM potassium phosphate buffer, pH 7.0, containing 6 M urea and 1 mM EDTA. The dialyate was chromatographed on DEAE-Sephacel equilibrated with the above buffer. The each subunit adsorbed was eluted with a linear gradient of 0–0.3 M NaCl in the same buffer. The fractions containing each subunit component were separately pooled, dialyzed against water, and lyophilized (see Fig. 1).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot. SDS-PAGE was done on a 12.5% gel by the method of Laemmli.⁵⁾ The proteins separated on the gels were stained with Coomassie Brilliant Blue R-250 (CBB R-250). On the other hand, the proteins separated on the gels were also transferred electrophoretically onto nitrocellulose membranes as described previously.²⁾ One part of the membrane was stained with Amide Black 10B and the other part was treated with the patient's sera, and allergens specifically bound with IgE antibodies were stained with ¹²⁵I-labeled anti-human IgE antibodies. The immunostained proteins on the membrane were detected by autoradiography on a X-ray film.¹⁾

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). 2D-PAGE (first dimension by isoelectric focusing and 2nd dimension by SDS-PAGE in the presence of 2-mercaptoethanol) was done by the method of Hirano.⁶⁾ Proteins on the gel were stained with CBB R-250 and glycoprotein were detected by staining with periodate-Fuchsin reagent.⁷⁾

Cleavage of peptide bonds with CNBr and chymotrypsin. Cleavage of peptide bonds by CNBr was done by the method of Beerley.⁸⁾ Chymotrypsin digestion of the peptides was done on the polyacrylamide gel during SDS-PAGE as described by Cleaveland *et al.*⁹⁾ Bands cut from SDS-PAGE stained with CBB R-250 were digested by placing gel slices containing the peptide fragments in the sample wells of a second SDS gel and then overlaying each slice with chymotrypsin. Digestion proceeded directly in the stacking gel according to the method of Cleaveland *et al.*⁹⁾ The CNBr-cleaved and chymotrypsin-digested fragments were separated on SDS-PAGE, blotted onto a polyvinylidene difluoride membrane, and stained with CBB R-250. The N-terminal amino acid sequences of the stained peptide bands on the membrane were analyzed by a gas-phase protein sequencer (477A, Applied Biosystems).⁶⁾

Results

Identification of the 70 kDa allergenic protein in 7S-globulin fraction

It has been demonstrated that sera of about 25% of the patients recognized a soybean protein component with a molecular mass of about 70 kDa in the 7S-globulin fraction. The 2D-PAGE pattern of 7S-globulin fraction and the autoradiogram of its immunoblot with patient's sera are shown in Fig. 1. The radioactive allergenic component with *pI* of about 5.0–5.2 and molecular mass of about 68 kDa can be assigned to the α -subunit of β -conglycinin. Moreover, this protein component was positive to periodate-Fuchsin reagent, suggesting that it belongs to the 7S-globulin components as a typical glycoprotein (data not shown). To confirm this allergenic protein as the α -subunit, three major subunit components of β -conglycinin were purified using DEAE-Sephacel column (Fig. 2) and each subunit isolated was tested by SDS-PAGE and immunoblotting analyses with pooled sera of the patients who have IgE antibodies specifically recognizing the 7S-globulin components (Fig. 3). It is clearly shown that the allergenic component recognized by the patients' sera is the α -subunit.

Characterization of CNBr-cleaved and chymotrypsin-digested peptide fragments

As shown in Fig. 4-A, SDS-PAGE pattern of the products derived from α -subunit by CNBr-cleavage gave two major

peptide bands with molecular masses of about 54 kDa (band LF) and 20 kDa (band SF). The analyses of N-terminal amino acid sequences of these peptides showed VEK--- for LF and NEG--- for SF, respectively. This results and the molecular masses of these peptides supported the fact that the fragments were derived by the cleavage of the peptide bond between methionine 383 (the only methionine residue in the α -subunit) and asparagine 384 residues.¹⁰⁾ Location of the epitope(s) on the peptide fragments was estimated from their binding ability to IgE antibodies. Figure 4-B shows that the fragment LF strongly binds to IgE antibodies, indicating that the epitope(s) occurs in this region. The fragment LF was stained with CBB R-250, removed from the gel and further digested by chymotrypsin in the stacking gel during the gel-electrophoresis as described in Materials and Methods. The electrophoretic pattern of the chymotryptic peptides derived from the CNBr-fragment LF is shown in Fig. 5-A. Although several trace fragments were detected, there appeared the two major fragments with molecular masses of about 36 kDa (band C-1) and 18 kDa (band C-2) on the gel. The immunoblotting pattern (Fig. 5-B) showed only the small fragment C-2 could be recognized by patients' sera. The N-terminal amino acids of these peptides were sequenced. The C-2 peptide gave

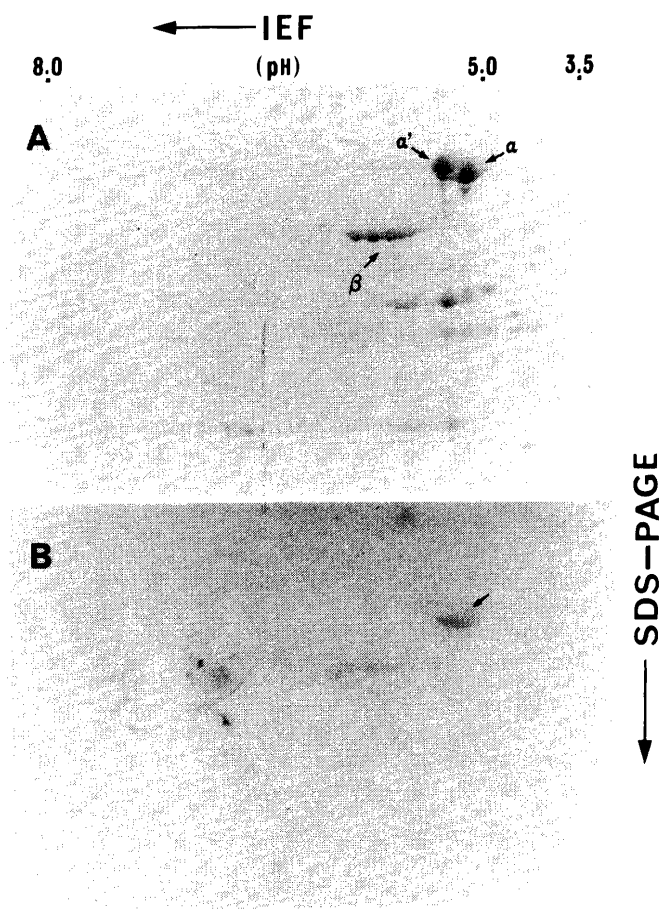


Fig. 1. Two-dimensional Gel-electrophoresis of 7S-Globulin Fraction. The proteins in 7S-globulin fraction were separated by isoelectrofocusing (IEF) in the first dimension and by SDS PAGE in the 2nd dimension. A, Proteins stained with CBB R-250; B, Proteins immunostained with patient's serum and ¹²⁵I-anti-human IgE antibodies after blotted onto nitrocellulose membrane (autoradiogram). Arrow in figure B indicates the position of the allergenic protein.

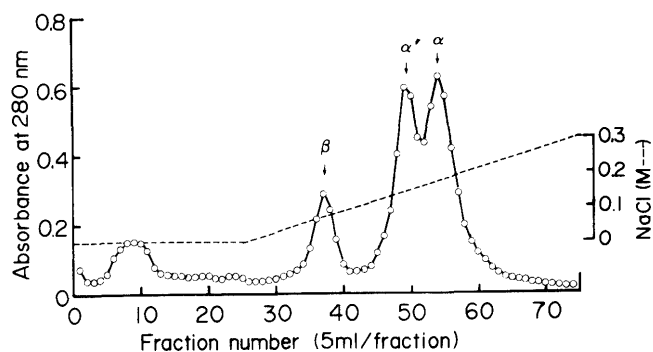


Fig. 2. Chromatogram of the 7S-Globulin Fraction on DEAE-Sephacel Column.

Peaks α' , α , and β represent the elution positions of the respective subunit components of β -conglycinin.

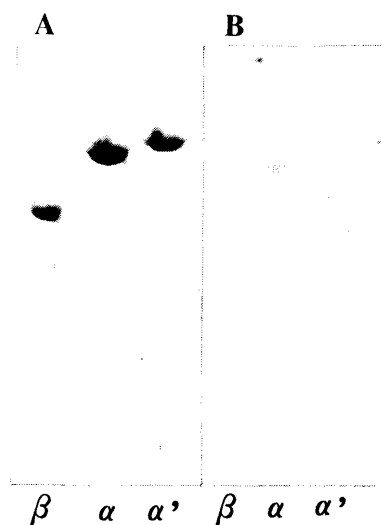


Fig. 3. SDS-PAGE and Immunoblotting Patterns of the Subunit Components of the β -Conglycinin.

A, Proteins stained with Amido Black 10B. B, Proteins immunoblotted with patient's sera. Letters α , α' and β in figures indicate the respective subunit components of β -conglycinin.

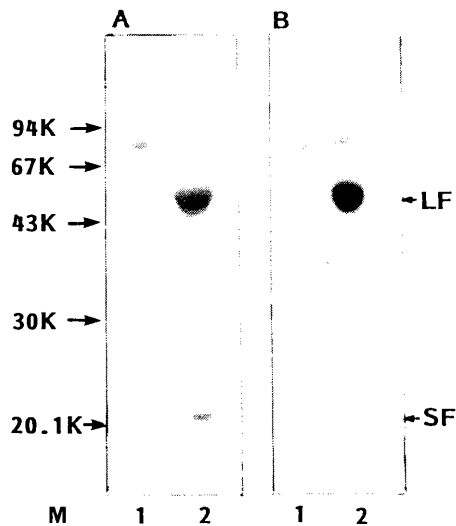


Fig. 4. SDS-PAGE and Immunoblotting Patterns of the CNBr-cleaved Products of α -Subunit.

A, stained with Amido Black 10B; B, immunoblotted with patient's sera. M, molecular mass marker (Da); lane 1, authentic α -subunit; lane 2, CNBr-cleaved products (see the text).

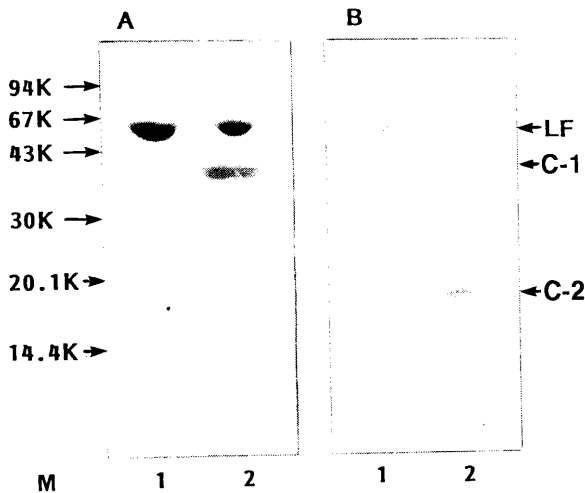


Fig. 5. SDS-PAGE and Immunoblotting Patterns of the Chymotryptic Peptides Derived from LF (see Fig. 4).

A, Proteins stained with Amido Black 10B; B, Proteins immunoblotted with patient's sera. M, molecular mass marker (Da); lane 1, intact LF fragment; lane 2, chymotryptic products of LF (see the text).

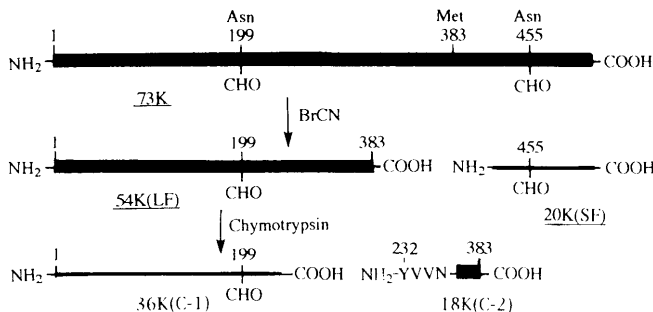


Fig. 6. Schematic Representation of the IgE-recognizing Epitope Region on α -Subunit of β -Conglycinin.

CHO represents the putative position of sugar moiety.⁴¹

YVVN--- as its N-terminal amino acid sequence to be assigned to the amino acid residues 232, 233, 234, and 235 of mature α -subunit.^{4,10} This showed that limited proteolysis of the peptide bond between residues Tyr-231 and Tyr-232 had occurred. From these results, it is evident

that the epitope(s) of the α -subunit is located on the peptide 232–383, as summarized in Fig. 6. The periodate-Fuchsin staining demonstrated that this fragment had no sugar moiety, suggesting the sugar moiety of α -subunit is not essential for the binding of IgE antibodies.

Discussion

In this experiment, it was shown that IgE antibodies of the soybean-sensitive patients recognized only the α -subunit among the three major subunit components of β -conglycinin. It has often been demonstrated that the 7S-globulin fraction is the most allergenic among soybean protein fractions for soybean-sensitive patients.^{11,12} However, this is the first evidence that the α -subunit of β -conglycinin is the major allergenic component in the 7S-globulin fraction recognized by at least the patients with atopic dermatitis. The structural analyses of β -conglycinin showed that three major subunits, α -, α' -, and β -subunits, were highly homologous to each other.^{10,13,14} Especially, it is shown that there is over 90% similarity between α - and α' -subunits.¹³ The predicted epitope region, peptide 232–383, of α -subunit is shown to correspond to the peptide 268–417 of α' -subunit.^{4,10} Seventeen residues of 160 of the sequence of the α -subunit peptide are different from those of α' -subunit. Epitope prediction may be helpful in identification of immunogenic sites and may also provide a strategic information about development of hypoallergenic soybean proteins and soybean products. Further investigation on the question of whether special structural features are responsible for allergenicity of α -subunit is in progress.

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