Antigenicity in Soybean Hypocotyls and Its Reduction by Twin-Screw Extrusion

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ABSTRACT: The purpose of the present study was to develop a simple method to make a low-antigenicity food and/or feed rich in isoflavones from soybean hypocotyls. The antigenicity of soybean hypocotyls for bovine antisoybean sera was assessed by enzyme-linked immunosorbent assay. Immunoblotting demonstrated that the antigenicity was derived from storage proteins, which were present in hypocotyls as glycinin and β-conglycinin, and from unknown proteins. Ground soybean hypocotyls (32-mesh sieve size) were passed through a twin-screw extruder to reduce the antigenicity to 1% of the original activity. The degradation of antigen proteins in soybean products was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. Trypsin inhibitor and urease activity were also greatly reduced. The concentrations of isoflavones were unaffected.

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KEY WORDS: Antigenicity, antigens, ELISA, embryo, hypocotyl, isoflavone, protein, trypsin inhibitor, twin-screw extrusion, urease.

Soybean seeds consist of cotyledons, seed coats, and hypocotyls, comprising approximately 92, 6, and 2% of seed content, respectively. The hypocotyl is approximately 10% crude oil and 38% protein and contains high levels of phytochemicals, such as isoflavones, saponins, phytosterols, and phytic acid. In particular, the proportion of isoflavones is more than five times higher in soybean hypocotyls than it is in whole seed (1). Isoflavones show estrogenic, antibacterial, antifungal (2), and antioxidative activity (3). Isoflavone ingestion is related to a reduced cancer risk (4) and prevention of bone loss (5). Soybeans have much antigenicity derived from their proteins, which can induce atopic dermatitis, especially in children. This symptom, mediated by allergen-specific immunoglobulin E (IgE) antibodies or IgE-binding proteins, has been studied using sera from humans who are sensitive to soybean proteins (6,7). At least 16 human-IgE binding proteins in soybeans have been reported (8). These antigenic soybean proteins were mainly derived from the 2S, 11S (glycinin) and 7S (β-conglycinin), proteins. Similarly, studies have also been conducted on soybean hypocotyl proteins. The protein content of soybean hypocotyls is approximately 38%, with many storage proteins classified as 11S and 7S (9), and these are assumed to have antigenicity. The use of soybean meal for livestock feed, especially for infant animals, is limited by antigenicity, which disturbs digestion and stunts growth (10).

Ohishi *et al*. (11) reduced the antigenicity to about 0.1% of the original activity by twin-screw extrusion. Furthermore, a comparison of human IgE-binding soybean protein with the antigenicity profiles of calf antisoybean protein sera was investigated by Hessing *et al*. (12), who noted that the soybean antigenicity of human IgE-binding protein and calf antisoybean protein sera is similar.

The present report investigates the antigenicity of soybean hypocotyl protein and evaluates the use of twin-screw extrusion to reduce antigenicity. In addition, the influence of this treatment on isoflavones was measured.

MATERIALS AND METHODS

Soybean materials. Whole soybeans were obtained from Honen Corporation (Tokyo, Japan). To collect the hypocotyls, the soybeans were heated to 80°C for 1 min by using a rotary kiln and then cracked using a roller mill. The soybean hypocotyl was separated by using appropriately sized sieves. Hexane-defatted soybean meal also was obtained from Honen Corporation.

Analyses. Crude protein, fat, moisture, ash, crude fiber contents, and urease activity were determined by using AOCS Official Methods Bc 4-49, Bc 3-49, Bc 2-49, Ba 5a-49, Ba 6-84, and Ba 9-58 (13), respectively (Table 1). Trypsin inhibitor activity analysis was carried out according to the method of Peace *et al.* (14).

Antibodies. Antisoybean protein antiserum was obtained from a cow weighing about 40 kg, that had been orally sensitized by feeding it with the experimental diet as previously described (11). Peroxidase-conjugated rabbit antibovine immunoglobulins were purchased from Dako Japan (Tokyo, Japan).

Sample extraction. One gram of sample was homogenized with 5 mL phosphate-buffered saline (PBS; 8.2 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 containing 137 mM NaCl and 3 mM KCl, pH 7.4) at room temperature for 30 min, and

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TABLE 1
Characteristics of Whole Soybeans and Soybean Hypocotyls[a]

	Whole soybeans	Soybean hypocotyls
Crude protein (%)	35.13	37.75
Fat (%)	18.87	10.38
Moisture (%)	12.26	11.80
Ash (%)	5.00	3.81
Crude fiber (%)	4.50	7.26
Urease activity (pH units)	1.95	2.06
Trypsin inhibitor (U/mg)	57.3	66.9
Antigenicity (U/10 mg)	4.00×10^4	3.74×10^3

[a]AOCS methods (13) were used for crude protein (Bc 4-49), fat (Bc 3-49), moisture (Bc 2-49), ash (Ba 5a-49), crude fiber (Ba 6-84), and urease activity (Ba 9-58). Trypsin inhibitor was measured by the method of Peace *et al.* (14). Method of determination of antigenicity is described in the text.

the homogenate was centrifuged at 3,000 × g for 10 min. The supernatants were filtered with a Millex-GV filtering unit (Millipore Co., Bedford, MA).

Electrophoresis and immunoblotting. A 50-μL sample extract was suspended with 250 μL of 50 mM Tris-HCl containing 35 mM sodium dodecyl sulfate (SDS) and 20% glycerol (pH 6.8). Then, 20 μL of 10% glycerol solution containing 1% (w/w) of bromophenol blue was added, and the mixture was boiled for 5 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a PhastSystem (Pharmacia LKB Biotechnology, Uppsala, Sweden) with Gradient 10-15 PhastGel. The gels were stained with PhastGel Blue R (Pharmacia LKB Biotechnology) or subjected to Western blotting. The band patterns of SDS-PAGE were scanned by Flying Spot Scanning Densitometer, CS-9300PC (Shimadzu Co., Kyoto, Japan).

For immunoblotting, the sample gel was separated from the entire gel by PhastTransfer Gel Backing Remover (Pharmacia LKB Biotechnology). Electrotransfer of the proteins onto nitrocellulose membrane was conducted with a Semi-dry Transfer Kit with PhastTransfer (Pharmacia LKB Biotechnology) for 10 min at 25 mA in a transfer buffer (25 mM Tris-HCl containing 192 mM glycine and 20% methanol, pH 8.3). Blotted membrane was washed three times with 20 mM Tris-HCl buffer (pH 7.4) containing 0.5 M NaCl and 0.3% Tween 20 for 20 min, and three times with distilled water for 2 min. Blotted proteins were confirmed with Colloidal Gold Total Protein Stain (Bio-Rad Laboratories, Hercules, CA). Antigens on the nitrocellulose membrane were visualized by using the indirect immunoperoxidase method (11).

Twin-screw extrusion. Soybean hypocotyl, which initially passed through a 10-mesh screen, was crushed or pulverized by using a hammer mill to pass through 16- or 32-mesh as needed. Twin-screw extrusion was carried out in a KEI-45 corotating twin-screw extruder (Kowa Kogyo Co., Osaka, Japan) with the 10-port die (2 mmϕ). The screws were combined with six blocks of kneading discs in the heads, and the barrel temperature was controlled by electric heating with a band heater and water cooling. Screw speed, feed rate, and product temperature at the end of the barrel were 280 rpm, 51.4 kg/h, and 72–143°C, respectively.

Antigenicity. Antigenicity in several soybean materials and products was determined by competitive inhibition enzyme-linked immunosorbent assay (ELISA). A 50-μL volume of appropriately diluted sample extract and 50 μL of bovine anti-soybean protein antiserum, which was previously diluted 4,000 times with PBS containing 0.3% bovine serum albumin (BSA), were added to a microtiter plate coated with soybean antigen (11) and incubated at 25°C for 30 min. After the plate was washed three times with washing buffer (PBS containing 0.05% Tween 80), 100 μL of peroxidase-conjugated rabbit antibovine immunoglobulins, which was previously diluted 500-fold with PBS containing 0.3% BSA, was added and reacted at 37°C for 30 min. The plate was again washed with washing buffer, and the color was developed with 100 μL of 8 mM *o*-phenylenediamine in 0.026% H_2O_2 at room temperature for 20 min. Color development was terminated by adding 100 μL of 4 N H_2SO_4, and absorbance at 492 nm was measured with an MRP-A4 micro-plate reader (Tosoh Co., Tokyo, Japan).

Quantitative analyses of isoflavones. The reversed-phase high-pressure liquid chromatography (HPLC) method was carried out to determine isoflavone content. The samples (500 mg) were lyophilized and extracted with 20 mL of 70% ethanol with shaking for 30 min at ambient temperature. Extracts were centrifuged at 3,000 × *g* for 10 min, and the supernatants were filtered with a Millex-GV filtering unit. The filtrate was dried using a rotary evaporator with a water bath not exceeding 40°C. The evaporated samples were dissolved in 20 mL isopropyl alcohol, added to 20 mL of 4 N HCl, and refluxed for 4 h to achieve acid decomposition. The solutions were then adjusted to 50 mL volume by adding 70% isopropyl alcohol.

Quantitative HPLC analysis was performed on a Shimadzu 10Avp (Shimadzu Co.) equipped with Zorbax ODS column (250 × 4.6 mm; Du Pont, Wilmington, DE), using a linear gradient of aqueous acetonitrile from 15 to 35% against a background of 0.1% acetic acid over 50 min at 35°C. The solvent flow rate was 1 mL/min, and the absorption was detected at 254 nm. Quantitative data were calculated according to the method of Wang *et al.* (15). The isoflavone content was obtained for comparison with daidzin, genistin, daidzein, and genistein standards purchased from Nacarai Tesque (Kyoto, Japan).

RESULTS AND DISCUSSION

Immunoblotting. The SDS-PAGE transferred and immunoblotted patterns for hypocotyl proteins are shown in Figure 1. Six significant bands, which immuno-reacted with bovine antisera, were obtained by immunoblotting. These six bands were designated α, α′-subunit, and β-subunit of β-conglycinin, A_3-subunit, $A_1A_2A_4$-subunit, and B_{1-4}-subunit of glycinin by using the nomenclature of Nishinari *et al.* (16), and unknown 33 kD protein (P33), respectively. Neither *Gly m* I nor P34 allergen protein, which had reacted with human IgE, as described by Ogawa *et al.* (8) and Herman *et al.* (17), respectively, was observed. This result was consistent with the research into the immunoreaction between calf antisoybean protein sera and soybean meal reported by Hessing *et al.* (12).

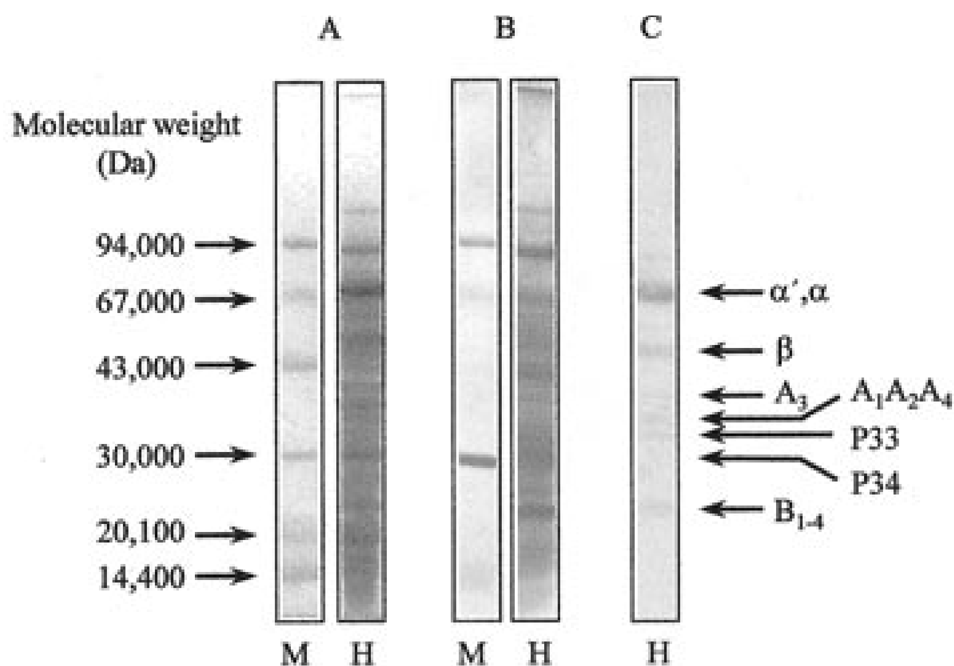

FIG. 1. Immunoblot profiles of soybean hypocotyl with the prepared antiserum. (A) Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), (B) transferred proteins stained with colloidal gold total protein stain, and (C) immunoblotted proteins. M, molecular marker; H, soybean hypocotyl. Nomenclature of Nishinari *et al.* (16) used to designate the α, α′-subunit and β-subunit of β-conglycinin, A_3-subunit, $A_1A_2A_4$-subunit, and B_{1-4}-subunit of glycinin. P33 and P34 were designated by Herman *et al.* (17).

The unknown allergenic P22-25 protein in soybean (11) was suspected to be the B_{1-4}-subunit of glycinin. Kunitz trypsin inhibitor, which showed antigenicity as described by Hessing *et al.* (12), exhibited no significant antigenicity in the present study. It was suggested that this discrepancy was attributable to differences in the antigen proteins between whole soybeans and hypocotyls, or to individual differences in the antiserum used in the two experiments.

Reduction of antigenicity. To reduce soybean hypocotyl antigenicity, twin-screw extrusion was applied. The process consists of mixing, shearing, and melting raw materials and then reforming them by means of a die. The materials are fed into one end of the barrel and carried inside by the action of the turning screws. As the materials are pushed through the die by the screws, they are melted and proteins are denatured due to the high temperature, pressure, and shearing action. The proteins are denatured to a greater extent than can be achieved by steam-parching or single-screw extrusion (11). Two parameters, temperature and screw shape, are important to reduce the soybean antigenicity in twin-screw extrusion treatment. As a consequence, the extrusion temperature was fixed at 120°C, and the screws that included six kneading-disc elements were used. These screws could reduce soybean meal antigenicity to 0.1% of the original activity (11).

Before twin-screw extrusion, the soybean hypocotyls had 3,700 U/10 mg of antigenicity (Table 1). Following the treatment, the antigenicity was reduced to 450 U/10 mg. This value was not low enough when compared with soybean meal, and this result is presumed to be due to the hypocotyl particles being too large to melt during extrusion. To reduce the antigenicity further, hypocotyls were ground by using hammer mill to pass through 16- or 32-mesh sieves and were

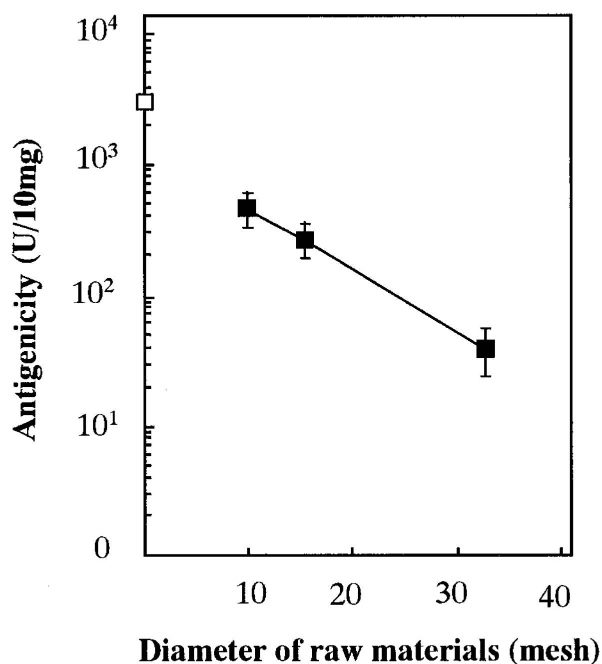

FIG. 2. Effects of precrushing on soybean hypocotyl antigenicity (■) by twin-screw extrusion treatment. □, Original activity of soybean hypocotyl. Values represent means and corresponding standard deviation of three replicates.

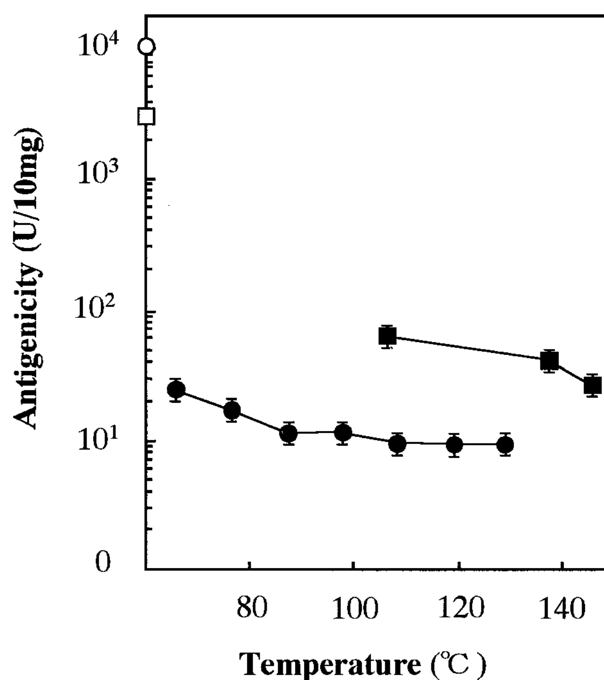

FIG. 3. Changes in soybean meal (●) and soybean hypocotyl (■) antigenicity by twin-screw extrusion treatment. ○, Original activity of soybean meal; □, original activity of soybean hypocotyl. Values represent means and corresponding standard deviation of three replicates.

passed through the twin-screw extruder. The results of these experiments are shown in Figure 2. The antigenicity of the twin-screw extruder-treated hypocotyl that was ground to pass through 16- and 32-mesh was reduced to 260 and 49 U/10 mg, respectively. Thus, grinding the materials to be passed through the twin-screw extruder was very important to reduce the antigenicity of soybean hypocotyls.

The influence of barrel temperature was tested with hypocotyls ground to 32-mesh (Fig. 3). The antigenicity of the products decreased slightly with rising temperature. Antigen units of soybean meal and soybean hypocotyls were decreased to 9.9 U/10 mg at 128°C, and to 40 U/10 mg at 143°C, respectively. For hypocotyls, controlling the machine to avoid blowing off or scorching was difficult. Below 107°C and above 143°C, the process was unstable. The difficulty with this treatment was likely due to the hardness of the soybean hypocotyl components. Nevertheless, 40 U/10 mg is less than 10% of the value obtained with popular edible steam-parched or single-screw extruded soybeans.

Degradation of proteins. The materials and products analyzed by SDS-PAGE and scanned by Flying Spot Scanning Densitometer are shown in Figure 4. The scanned band areas, which are involved in the antigenicity, are summarized in Table 2. For both soybean meal and hypocotyls, the twin-screw extrusion denatured the α,α'-subunit and β-subunit of β-conglycinin and A_3-subunit of glycinin completely. In comparison with whole soybean and extruded soybean meal, the band area of the $A_1A_2A_4$-subunit was reduced from 1429 to 118 (8.3% remaining), and that of P33 from 503 to zero, P34 from 715 to 348 (48.7% remaining), and B_{1-4} from 1358 to 257 (18.9% remaining). The A_{1-4}- and B_{1-4}-subunits of glycinin, β-conglycinin, and P33 were highly reactive with bovine antisoybean sera (Fig. 1), and reduction of these proteins was suspected to contribute profoundly to reducing antigenicity. Also in hypocotyls, the $A_1A_2A_4$-subunit was reduced from 678 to 151 (22.3% remaining), P33 from 421 to 278 (66.0% remaining), P34 from 689 to 430 (62.4% remaining), and the B_{1-4}-subunit from 822 to 538 (65.5% remaining). Although antigenicity was greatly reduced, quite a few antigen proteins of glycinin remained in the extruded hypocotyls. This result suggested that the reduction of antigen protein was closely related to the degradation of β-conglycinin.

Changes in trypsin inhibitor, urease activity, and isoflavone content. To confirm the usefulness of the twin-screw extruded soybean hypocotyls, the trypsin inhibitor, urease activity, and isoflavone contents in raw and treated soybean hypocotyls were analyzed (Table 3). Trypsin inhibitor and urease activity relate to the digestibility and feed efficiency in several species. Similar to the antigenicity, trypsin inhibitor and urease activity were also greatly reduced; however, the isoflavone was not degraded. According to Kudou *et al.* (3), 6″-*O*-malonyldaidzin, 6″-*O*-malonylglycitin, and 6″-*O*-malonylgenistin are thermally unstable and are converted into daidzin, glycitin, and genistin, respectively, when they are extracted at 80°C. Generally, twin-screw extrusion enables one to apply high pressurizing, heating, and shearing to the raw materials. Thus, the possibility of changing the glucoside moiety of isoflavone exists, but the total content of isoflavone aglycones was not changed. Soybean isoflavone aglycones were very stable.

TABLE 2
Antigenicity and Protein Content in the Subunit Composition of Soybean Products[a]

	Antigenicity (U/10 mg)	Peak area						
		$\alpha + \alpha'$	β	A_3	$A_1A_2A_4$	P33	P34	B_{1-4}
Whole soybean	4.00×10^4	1565	1111	886	1429	503	715	1358
Extruded soybean meal	9.86	ND[b]	ND	ND	118	ND	348	257
Raw hypocotyl	3.74×10^3	1585	438	789	678	421	689	822
Extruded hypocotyl	40.2	ND	ND	ND	151	278	430	538

[a]Values are the mean of three replicate analyses.
[b]ND, not detected.

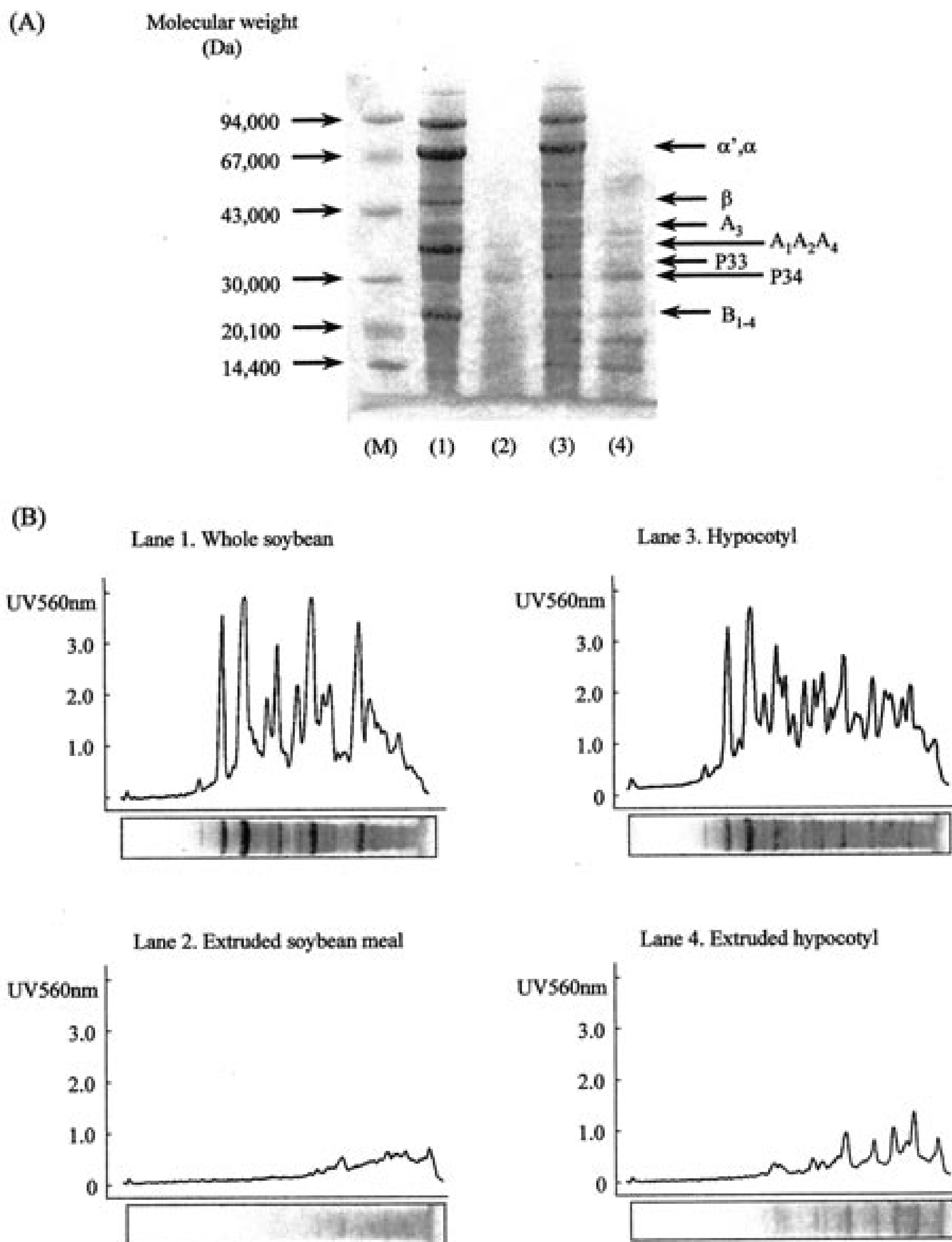

FIG. 4. SDS-PAGE profiles of soybean products (A) and their densitograms (B); whole soybean (lane 1), extruded soybean meal (lane 2), hypocotyl (lane 3), extruded hypocotyl (lane 4). (M), molecular marker. See Figure 1 for definition of bands and for abbreviation.

The present study demonstrates that raw soybean hypocotyls have 3,700 U/10 mg of antigenicity with bovine antisoybean sera, and the antigenicity can be reduced to about 1% by twin-screw extrusion if they are ground to 32-mesh size prior to extrusion. This reduction in antigenicity resulted from the degradation of antigen proteins. The trypsin

TABLE 3
Changes in Trypsin Inhibitor, Urease Activity, and Isoflavone Contents in Soybean Hypocotyls Caused by Twin-Screw Extrusion[a]

	Trypsin inhibitor (U/mg)	Urease activity (pH units)	Isoflavone (mg/g)		
			Daidzein	Glycitein	Genistein
Raw hypocotyls	66.9 ± 1.41	2.05 ± 0.02	10.57 ± 0.06	5.36 ± 0.06	1.30 ± 0.09
Extruded hypocotyls	1.8 ± 0.32	0.00	10.58 ± 0.04	5.45 ± 0.04	1.36 ± 0.08

[a]Values are means of three replicate analyses ± standard deviation.

inhibitor and urease activity also were greatly reduced, but the isoflavone content was unchanged. Twin-screw extrusion of soybean hypocotyl is a very simple and effective method of producing low-antigenicity food and/or feed rich in isoflavones.

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