Detection of Soybean Antigenicity and Reduction by Twin-Screw Extrusion

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Antiserum toward soybean meal was prepared by feeding a soybean meal diet containing large amounts of antigens to calves. The antiserum was used to develop a sensitive detection method for soybean meal antigens by competitive inhibition enzyme-linked immunosorbent assay. With this method, the effectiveness of twin-screw extrusion cooking in reducing antigenicity in soybean meal was examined. Antigenicity was reduced to 0.1% of the original activity by extrusion cooking with screws containing kneading-disc elements and die-end temperatures exceeding 66°C. Electrophoretic analysis of the cooked meal indicated that the reduction in antigenicity was due to degradation of protein structures, particularly those with molecular weights exceeding 40 kDa.

KEY WORDS: Antigenicity, antigens, antiserum, β-conglycinin, ELISA, glycinin, immunoblot, SDS-PAGE, soybean meal, twin-screw extrusion cooking.

Soybean meal is extensively used as a source of protein in livestock feed. Its usage is often limited due to antigenicity, which may cause severe diarrhea and growth stunting of young animals (1,2). Smith and Sissons (3) reported that soybean products caused digestive disturbance and induced antibody production in calves. Trypsin inhibitors and lectins are eliminated by ordinary toasting (4), but this process does not reduce antigenicity. Antigenic activities of soybean glycinin and β-conglycinin have been studied in animals (5,6). Happell *et al.* (7) determined antigenic glycinin and β-conglycinin in soybean-based infant formulas by using rabbit antisera against glycinin and β-conglycinin. However, no methods are presently available for evaluating soybean antigenicity.

Several procedures are available for reducing soybean protein antigenicity, such as aqueous alcohol treatment (5,8), but they are expensive. Mir *et al.* (9) found that calves fed twin-screw-extruded soybean meal gained more weight than those fed raw soybean meal. No immunological explanation was given for this difference in weight gain.

In the present study, a sensitive analytical method for assessing antigenicity in soybean was developed based on the enzyme-linked immunosorbent assay (ELISA), and twin-screw extrusion cooking was evaluated as a means to reduce antigenicity.

MATERIALS AND METHODS

Soybean products. Raw soybeans (S) (U.S., No. 2, yellow), hexane-defatted soybean meal (DSM-1) (Low-Pro) and hexane-defatted, dehulled and powdered soybean meal (DSM-2) (N-10) were obtained from Honen Corporation (Tokyo, Japan). Other commercial soybean products, indirectly steam-parched soybean powder (PS) (Kina-ko), single-screw extruded soybeans (SES) (Full Fat Ace), and alcohol-washed soybean protein concentrate (SPC) (Top Ace C), were obtained from Kawamitsu Co. (Chiba, Japan), Toyo Oil Mills Co. (Chiba, Japan) and Nisshin Oil Mills (Tokyo, Japan), respectively. S, DSM-1 and SES were ground in a hammer mill to pass through a 100-mesh screen. The others were used intact for analysis. Moisture, fat and crude protein (CP) (N × 6.25) contents, and nitrogen solubility indexes (NSI) were determined by AOCS Official Methods Bc 2-49, Bc 3-49, Bc 4-49 and Ba 11-65 (10), respectively (Table 1).

Other materials. Spray-dried skim milk and whey powder were obtained from Honen Corporation. Fetal calf serum was obtained from Nippon Bio Supplement Center Co. (Tokyo, Japan), and calf sera were obtained from Nippon Intermed Co. (Tokyo, Japan). Buckwheat flour was purchased at a local supermarket. Whole egg powder was obtained from Flemisch Egg Products (Flafo Bvba, Belgium); bovine serum albumin (BSA) was obtained from the Sigma Chemical Co. (St. Louis, MO).

Antiserum preparation. Three calves, each weighing about 40 kg, were fed an experimental diet (Table 2) containing 13% DSM-2. Before and during feeding, 2 mL of blood was taken from each animal at ten-day intervals to

TABLE 1

Characteristics of Soybean Products

Products	Moisture (%)	Fat (%)	CP (%)[a]	NSI[b]
Raw soybean (S)	12.2	19.5	35.8	90.3
Defatted soybean meal-1 (DSM-1)	12.8	1.0	45.7	22.9
Defatted soybean meal-2 (DSM-2)[c]	7.8	1.2	52.1	19.4
Parched soybean flour (PS)	6.7	19.2	38.3	12.9
Single-screw extruded soybean (SES)	9.1	20.7	35.8	15.6
Soybean protein concentrate (SPC)	7.1	—	68.4	4.2

[a]CP = crude protein.
[b]NSI = nitrogen solubility index.
[c]Dehulled and powdered soybean meal.

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TABLE 2

Diet Composition

Ingredient	%
Skim milk solids	53.0
Whey powder	10.0
Defatted soybean meal-2 (DSM-2)	13.0
Fish meal extract	1.0
Powdered shortening[a]	21.0
Vitamin and salt mixture	0.55
NaCl	0.3
Emulsifier[b]	0.3
Seasoning[c]	0.15

[a]R-35 (Riken Vitamin Co., Tokyo, Japan).
[b]MY-18 (Taiyo Kagaku Co., Mie, Japan).
[c]SFT68 (Soda Aromatic Co., Tokyo, Japan).

determine antibody titer. At 27 or 40 d, 50 mL of blood was taken from each animal from the jugular vein, and the serum was separated by centrifuging for 20 min at 3,000 × *g*. After addition of NaN_3 (0.1% wt/vol), the sera were subdivided and stored at −70°C.

Preparation of standard soybean protein solution (SSPS). DSM-2 (20 g) was suspended in 100 mL of phosphate-buffered saline (PBS; 8.2 mM $Na_2HPO_4 \cdot 12H_2O$, 1.8 mM KH_2PO_4, 137 mM NaCl, 3 mM KCl, pH 7.4) at room temperature for 30 min. All extracts were centrifuged at 3,000 × *g* for 10 min, and supernatants were filtered with a MILLEX-GV filtering unit (Millipore Products Division, Bedford, MA) to coat a microtiter plate and as the antigen standard for competitive inhibition ELISA. To measure the antigenicity of soybeans and their products, the same extraction procedure used for SSPS was carried out after grinding the samples. Soluble protein was measured by the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

ELISA. To determine changes in antibody titer, SSPS, diluted 100 times with PBS, was applied overnight to the wells of a microtiter plate (Nunc-Immuno Plate F; Nippon Intermed Co.) at 4°C. After washing the plate three times with PBS, containing 0.05% polyoxyethylene sorbitan monooleate, by using an Immuno Washer NK-100 (Nippon Intermed), the wells of the plate were desiccated *in vacuo* at room temperature for 60 min. One-hundred μL of antiserum, diluted 4^6 times, were injected into each well of the plate, and reactions of antibodies with antigens were allowed to proceed at 25°C for 30 min. After washing the plate with PBS containing 0.05% polyoxyethylene sorbitan monooleate, 100 μL of peroxidase-conjugated rabbit anti-cow immunoglobulins (Dako Japan, Tokyo, Japan), diluted 500 times with PBS containing 0.3% BSA, was added and allowed to react at 37°C for 30 min. The plate was washed again, and color was developed by adding 100 μL of 8 mM *o*-phenylenediamine in 0.008% H_2O_2. The plate stood at room temperature for 30 min, color development was terminated by adding 100 μL of 4 N H_2SO_4, and absorbance at 492 nm was measured with a MRP-A4 micro-plate reader (Tosoh Co., Tokyo, Japan).

For detection of antigens in soybean products, competitive inhibition ELISA was carried out. The microtiter plate was coated with SSPS as previously described. Fifty μL of the diluted (4^1–4^6) extract of the test materials and 50 μL of antiserum (previously diluted 4,000 times with PBS containing 0.3% BSA) were added to the plate, and antigen–antibody reactions were carried out at 25°C for 30 min. Subsequent procedures were the same as were used to measure the antibody titer. To determine maximum color development, PBS in place of the test extract was used, and the value obtained served as the control.

Extrusion cooking. DSM-1 was extrusion-cooked in a KEI-45 co-rotating twin-screw extruder (Kowa Kogyo Co., Osaka, Japan), which has a production capacity of 40–60 kg/h. The die with 10 ports (2-mm ϕ) was used in all experiments. Screw diameter was 46 mm, and it had a length/diameter ratio of 15. Two types of screws were used (Fig. 1). Screw speed and rate of water addition were 280 rpm and 12 L/h, respectively. The feeding rates and temperatures of the barrel ranged from 20 to 40 rpm and 66 to 134°C (temperature measured at the die end of the extruder), respectively. The barrel temperature was controlled by electric heating with a band heater and water-cooling. Extruded materials were dried at 80°C for 8 min and ground to pass through a 100-mesh screen.

Electrophoresis and immunoblotting. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out in the discontinuous buffer system of Laemmli (11) with a 5–20% SPG-520L gradient gel (Atto Corporation, Tokyo, Japan). Gels were stained with Coomassie Brilliant Blue R-250. For immunoblotting, electrotransfer of the proteins onto nitrocellulose (NC) membrane was conducted with a semi-dry electroblotter (Trans Blot; Nippon Bio-Rad Laboratories, Tokyo, Japan) for 10 min at 15 V in transfer buffer (25 mM Tris-HCl buffer, 192 mM glycine and 20% methanol, pH 8.3). Protein was also stained with Amide Black 10B. Antigens transferred onto the NC membrane were visualized by the indirect immunoperoxidase method (12). The NC membrane was first washed with PBS containing 0.05% polyoxyethylene sorbitan monolaurate for 10 min and then rocked in blocking buffer (PBS containing 3% BSA) at room temperature for 1 h with shaking. After washing the NC membrane three times, the membrane was covered with antiserum that was previously diluted 500 times by PBS containing 3% BSA and 0.05% polyoxyethylene sorbitan monolaurate, and reactions of antibodies with antigens were allowed to proceed at room temperature for 1 h. The membrane was then washed four times and covered with peroxidase-conjugated rabbit anti-cow immunoglobulins that were previously diluted 200 times by PBS containing

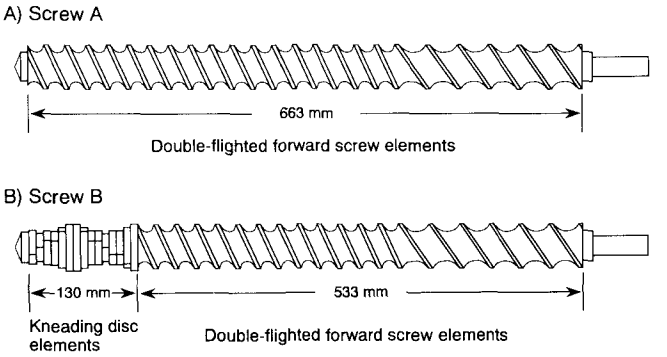

FIG. 1. Screw designs used in extrusion cooking.

3% BSA and 0.05% polyoxyethylene sorbitan monolaurate, and then the membrane was allowed to stand at room temperature for 1 h. The membrane was washed again, and color development was assessed with a POD Immunostain Set (Wako Pure Chemical Industries, Osaka, Japan).

Statistical analysis. ELISA data were analyzed by using the *t*-test ($P < 0.01$) with the Microsoft Excel 4.0 package (Microsoft, Redmond, WA).

RESULTS AND DISCUSSION

Development of ELISA for soybean antigenicity detection. For assessing soybean protein antigenicity, calf antiserum prepared by oral challenge is necessary, while intravenous antibody induction is generally carried out for antiserum preparation because immunoresponse of an intravenously induced antibody is slightly different from that of an orally-induced antibody (13,14).

A method for preparing calf antiserum by oral challenge has not been established to date. To start with, we fed a diet containing soybean meal to calves, and changes in antigen binding-capacities of the prepared antiserums during feeding were measured by noncompetitive ELISA. As shown in Figure 2, all three antisera reacted with SSPS in proportion to the feeding period. The intensity of the reaction of antiserum from one calf (calf serum No. 2) was significantly higher than those of two other calves. Ten days of induction were sufficient to increase antigen binding-capacity only in calf serum No. 2, thus indicating this calf was sensitive toward soybean antigens. The antiserum of this calf was used in subsequent experiments.

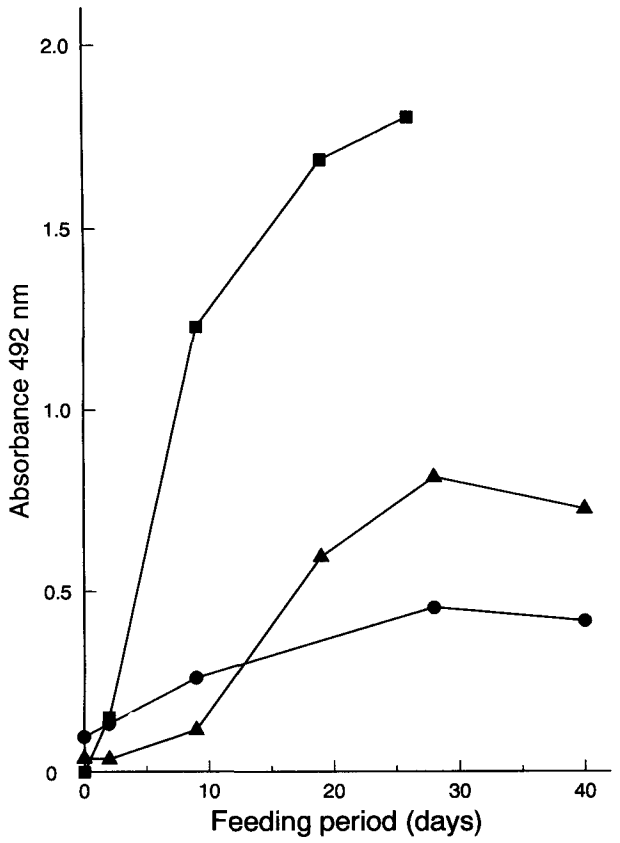

FIG. 2. Changes in antibody titers during feeding [Antiserum No. 1 (—●—), No. 2 (—■—) and No. 3 (—▲—) were diluted 4^6 times].

To confirm the reactivity of the prepared antiserum, reactivities toward soybean antigens of the prepared antiserum and commercial sera of calves fed milk or grass, but no soybean meals, were compared. Table 3 shows the reactivities of the prepared antiserum and commercial fetal calf and calf sera toward antigens in SSPS. Fetal calf serum did not react with SSPS, while commercial calf sera did so only slightly. In contrast, the prepared antiserum reacted strongly with SSPS, indicating that the calves fed the high antigenicity diet produced significantly large amounts of reactive antibodies toward soybean antigens.

Noncompetitive ELISA could be conveniently used for measuring antibody titers but not for detecting antigens in soybean meal products, including SPC (alcohol-washed) because of the test's low sensitivity. No clear differences in ELISA could be found for soybean meals that were twin-screw extruded under various conditions (data not shown). The method must be improved to measure antigenicity in soybean products. Based on the results of preliminary experiments, competitive inhibition ELISA was found suitable for antigen detection. In this method, color development was inhibited by antigens in the extract because antibody reactions with antigens adsorbed on the plate could not take place. By using the ELISA method, antigenicities of DSM-1 and other protein sources (Buckwheat, whole egg and BSA) were determined. As shown in Figure 3, DSM-1 strongly inhibited color development, compared with the control. Inhibition was still evident, even at 1/1,000 dilution. Other protein extracts had much less effect on color development, indicating that the prepared antiserum specifically reacted with antigens in soybean protein.

To assess the sensitivity of the present method, the antigenicities of various soybean products were determined by competitive inhibition ELISA. DSM-2 was used as a standard soybean antigen, and the antigen level of 10,000 units/10 mg DSM-2 was selected because of its high antigenicity. To determine the antigen level in a sample, regression analysis of ELISA values was conducted according to Rodbard and McClean (15). Antigenicities of S, DSM-1, PS, SES and SPC (alcohol-washed) were measured by competitive inhibition ELISA. As shown in Figure 4, S had the highest amount of antigen units, followed by DSM-1. PS or SES markedly decreased antigenicity, but not below the level of SPC.

This work presents the first evidence that the antigenicity of alcohol-washed SPC is about 0.1% that of soybean meal. The present method is adequately sensitive for assessing soybean products' antigenicity, even at low levels.

TABLE 3

Comparison of Reactivities Toward Soybean Antigens of Prepared Calf Antiserum and Commercial Calf Serum

Serum	Absorbance[a] (492 nm)
Prepared antiserum	1.083[a]
Fetal calf serum	0.001[b]
Calf serum	
No. 1	0.033[c]
No. 2	0.032[c]
No. 3	0.016[c]

[a]Means with different superscripts are significantly different ($P < 0.01$).

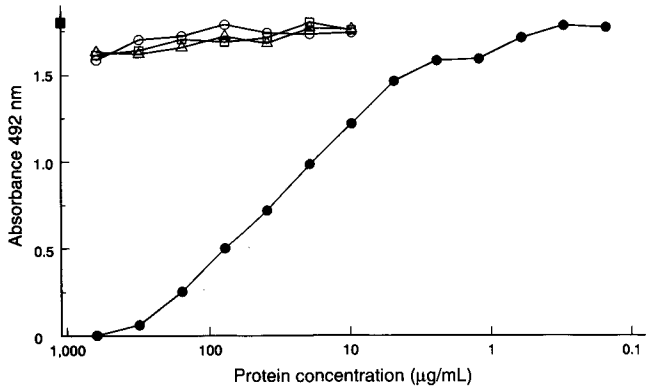

FIG. 3. Inhibition of protein extracts during color development in enzyme-linked immunosorbent assay [DSM-1 (—●—), buckwheat (—○—), whole egg (—□—) and bovine serum albumin (—△—) were compared with the control (■)].

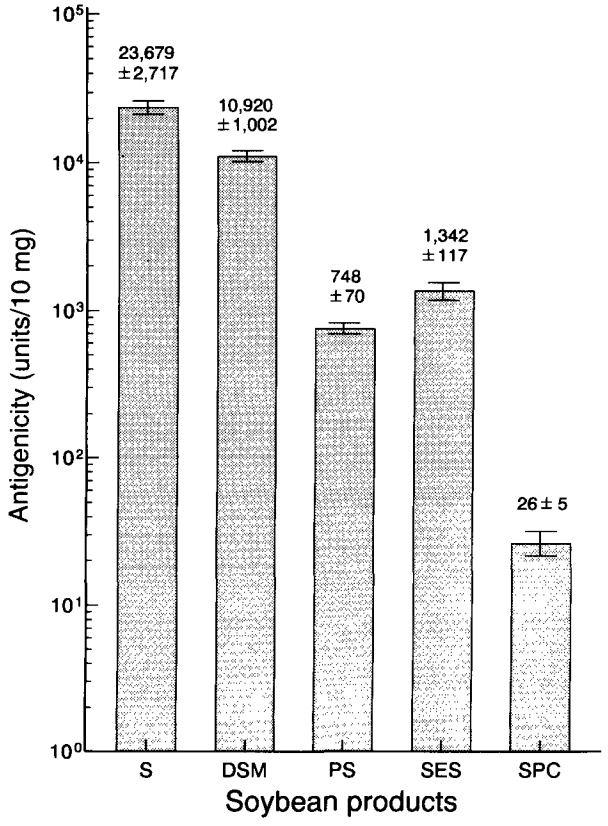

FIG. 4. Antigen units of raw soybeans (S), defatted soybean meal-1 (DSM-1), parched soybean powder (PS), single-screw extruded soybean (SES) and soybean protein concentrate (SPC). Values are means ± SD; values are significantly different ($P < 0.01$).

Reduction of soybean meal antigenicity by twin-screw extrusion cooking. The KEI-45 twin-screw extruder consisted of right-handed screws, which rotated in the same direction. Assuming that adequate reduction of soybean antigenicity is possible by twin-screw extrusion cooking, it should be possible to obtain low-antigenicity soybean products for feed that would be less expensive than those derived from alcohol-washing.

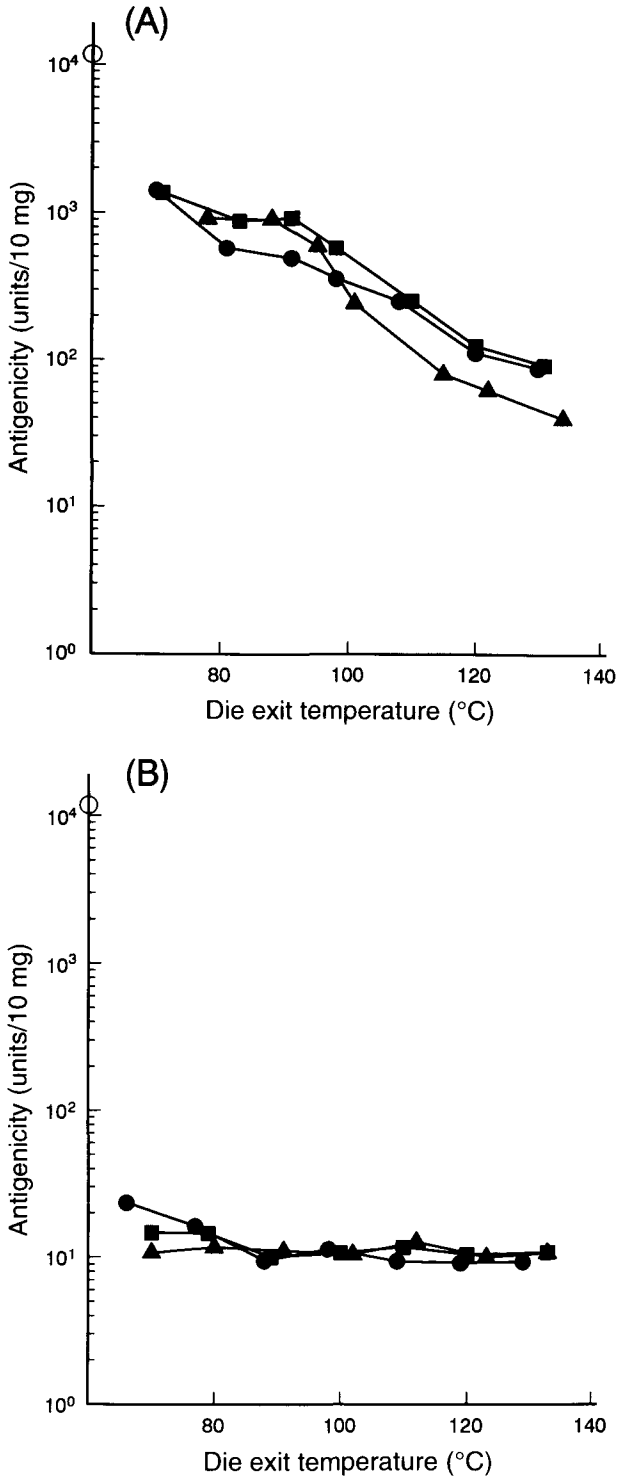

FIG. 5. Changes in DSM-1 antigen units (○) by twin-screw extrusion cooking with Screw A (A) and Screw B (B). Feed screw speeds were 20 rpm (—●—), 30 rpm (—■—) and 40 rpm (—▲—). Feed rates 23.7, 38.2 and 51.4 kg/h, respectively.

After extrusion cooking with forward screws (Screw A), antigenicity of DSM-1 decreased in proportion to the rise in the die exit temperature from 70 to 134°C (A in Fig. 5). After treatment with kneading-disc screws (Screw B),

antigenicity decreased markedly, even though product temperature at the die exit was 66°C (B in Fig. 5). Antigenicity was hardly affected by feeding rate of DSM-1 or die pressure.

Electrophoretic analysis of extrusion-cooked soybean meal. To determine why twin-screw extrusion cooking was effective in reducing soybean meal antigenicity, electrophoretic analysis of extrusion-cooked DSM-1 was carried out. Changes in the molecular structures of soybean proteins during cooking may have been a major factor in reducing antigenicity.

Figure 6 shows the electrophoresis patterns of proteins after treatment with Screw A. The amounts of the high-molecular-weight fractions, all exceeding 30 kDa, decreased when the product temperature at the die rose. Figure 7 shows the electrophoretic patterns of proteins from products treated with Screw B. The high-molecular-weight bands disappeared at low product temperature, indicating that the kneading disc screws with high mixing and kneading action effectively reduced the molecular weights of soybean proteins during extrusion cooking. Antigenicity would appear to be present mainly in high-molecular-weight fractions of soybean proteins.

Glycinin (11S-globulin) β-conglycinin (7S-globulin) and trypsin inhibitors (2S-globulin) have been shown to be potential antigens in animals (5,9,16,17) and humans (18,19). The structures of soybean antigens in calves have yet to be determined in detail. To clarify the relationship between soybean protein structure and antigenicity, immunoblot analysis of proteins in DSM-1 with the antiserum prepared in this study was carried out. As shown in Figure 8, antibodies in the antiserum reacted with most proteins with molecular weights of 40 kDA or more. Major reactive fractions were the subunits of β-conglycinin. The antiserum showed slight reaction with the subunits of glycinin and no reaction with the soybean trypsin inhibitor. The primary structures of β-conglycinin subunits appear to be important in antibody induction in calves. To determine the effects of steric structures of soybean proteins involving antigenicity will require further study because, as shown by immunoblot analysis, steric structures (tertiary or quaternary structures) were destroyed by denaturation with SDS. It is evident from the present results that twin-screw extrusion cooking with Screw B effectively decreased the antigenicity of soybeans through degradation of protein molecular structures (particularly high-molecular-weight fractions of soybean proteins).

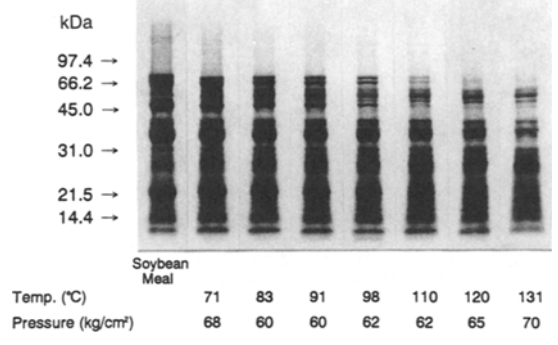

FIG. 6. Sodium dodecyl sulfate–polyacrylamide electrophoresis of DSM-1 extruded with Screw A at 38.2 kg/h feed rate.

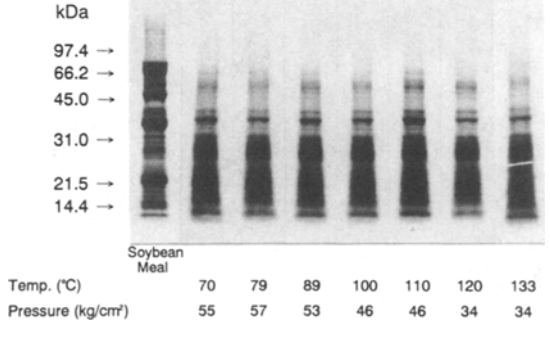

FIG. 7. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of DSM-1 extruded with Screw B at 38.2 g/h feed rate.

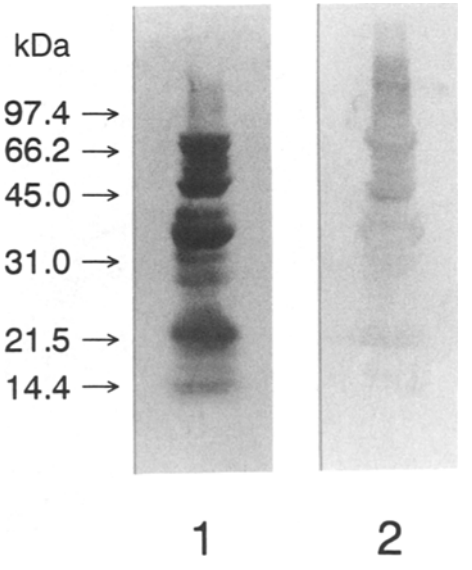

FIG. 8. Immunoblot of DSM-1 with the prepared antiserum. Lanes show stained proteins (1) and antiserum (2).

In conclusion, we have developed an effective method for assessing the antigenicity of soybean products in calves by using competitive ELISA with antiserum prepared in calves by highly antigenic soybean meal as feed. Twin-screw extrusion cooking effectively reduced the antigenicity of defatted soybean meal by degrading protein molecular structures.

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