

Meat Allergy: Investigation of Potential Allergenic Proteins in Beef

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The potential allergenic proteins in beef were investigated. The sera of ten beef-allergic patients suffering from atopic dermatitis and having a positive RAST score to beef, aged 3–18 years, were obtained from Yoshida Hospital in Japan, and five non-allergic individuals were subjected to this study. The sera of the ten patients reacted strongly to a beef extract, but not to pork and chicken extracts by both ELISA and immunoblotting. The sera of the five control subjects did not react to any of these meat extracts. Three bands having molecular masses of ~200 kDa, ~67 kDa and ~60 kDa were observed by immunoblotting after SDS-PAGE. Two fractions of the beef extract from a Sephadex-gel (G-200) filtration column strongly reacted with the sera of the beef-allergic patients by ELISA and immunoblotting: one fraction had the ~67 kDa component and the other had the ~200 kDa and ~60 kDa components. One of them (~67 kDa) was confirmed to be bovine serum albumin (BSA) by an analysis of the N-terminal amino acid sequence. We could not identify the others by sequencing, but the ~200 kDa and ~60 kDa components were presumed to be glycoproteins. Bovine gamma globulin (BGG; M.W. ~160 kDa) is a glycoprotein and has several subunits. The beef-allergic patients showed strong reactivity to the ~200 kDa and ~60 kDa components of pure BGG by immunoblotting. Inhibition-ELISA showed that pure BGG preparations strongly inhibited the binding of sera from the beef-allergic patients to the beef extract. These results suggest that the ~200 kDa, ~67 kDa and ~60 kDa components in the beef extract had strong allergenicity: ~67 kDa was BSA, and ~200 kDa and ~60 kDa were presumably aggregated BGG and its heavy chain, respectively.

Key words: meat allergy; beef allergy; beef extract; BSA; BGG

The number of food-allergic patients has been increasing in recent years. Food allergy usually appears in infants or children, and it might often be outgrown with age.¹⁾ However, children diagnosed as having food allergies after 3 years old were found less likely to outgrow the allergy.²⁾ Food allergy is now prevalent not only in children but also in adults. Food allergy is usually thought to be the type I allergy of four types of allergic reaction.³⁾ The type I allergy is an IgE-mediated immediate allergic reaction.

The main food allergies caused by cow's milk, egg, shrimp, soybean, peanut, and rice have been studied by many groups,^{4–17)} cow's milk and chicken-egg allergy being the most common. Potent allergenic proteins have been identified as casein, beta-lactoglobulin and bovine serum albumin (BSA) in cow's milk, and ovalbumin and ovomucoid in egg.^{4–6,8,17)} Some research groups have exploited new technology to eliminate these allergenic substances from food. For example, Watanabe *et al.*^{18,19)} in Japan have succeeded in eliminating the 16 kDa allergenic component in rice by an enzymatic treatment and are now supplying hypoallergenic rice for rice-allergic patients.

On the other hand, meat allergy has been considered as a rare pathological problem, although recent reports demonstrate that meat allergy is a serious problem mainly in children that strikes unexpectedly. However, little information about the allergens in meat has been obtained. This may be partly due to the complexity of the components in meat. Patients reacting to meat (chicken or beef) are said to be sensitive to egg or cow's milk too, because their sera have shown cross-reactivity to egg or cow's milk proteins.^{7,20–23)} This fact makes the interpretation of information regarding meat allergens difficult. There are conflicting reports regarding beef-associated allergy. Ogle & Bullock¹⁾ have published a clinical

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report advising that, since beef was hypoallergenic, children with food sensitivity could be placed on an elimination diet including beef. In contrast, Fiocchi *et al.*^{24,25)} have reported that BSA was an important beef allergen. Werfel *et al.*²³⁾ have reported that the 17.8 kDa fraction of a beef extract also bound to beef-specific IgE-antibodies from cow's milk-allergic patients reacting to rare and well-cooked beef. According to the report of Restani *et al.*,²⁶⁾ actin and BSA were found to be beef-allergenic proteins by immunoblotting for beef-allergic patients, although the involvement of actin was not confirmed by a skin-prick test.

Meat is an important food because of its high nutritional and functional properties. It has been suggested, but not yet well proven, that potential allergenic proteins may be present in meat. Thus, the aims of our research are to identify the allergenic proteins in meat and establish the technology to eliminate meat-associated allergenic proteins in the near future. The present study describes the identification and determination of the allergenic proteins in beef by an immune-assay with the sera of beef-allergic patients and by a physico-chemical assay.

Materials and Methods

Sera from beef-allergic patients. We obtained the sera from 10 beef-allergic patients, whose ages were 3–18 years, from M. Matsuno, Medical Doctor of Yoshida Hospital in Yoshida, Niigata prefecture in Japan. They were suffering from atopic dermatitis and their RAST score ranged from 2 to 4 for beef. They did not show a positive RAST score with pork, chicken, chicken egg white, wheat or soybean. The sera of 5 non-allergic individuals were used in this study as a control.

Preparation of the beef, pork and chicken extracts. After removing the fat and connective tissue, 10 g of the shoulder part of a beef (Holstein) carcass (one day after slaughter and stored at -20°C) was finely cut by scissors and homogenized three times for 5 seconds with 100 ml of a 20 mM sodium phosphate buffer (pH 7.4). The homogenate was centrifuged at $20,000 \times g$ for 15 min. The supernatant was filtered through Whatman No. 2 filter paper; all procedures were conducted at 4°C . The protein concentration of the extract was determined by the biuret method standardized with BSA²⁷⁾ and adjusted to 5 mg/ml. The pork and chicken extracts were prepared by the same method.

Preparation of bovine myofibrillar protein. Myofibrillar protein was prepared from the residue of the beef extract. The residue of the beef extract was suspended in the same buffer as that already mentioned, and the suspension centrifuged at $20,000 \times g$

for 15 min. The supernatant was discarded, and the residue was suspended again in the same buffer. This procedure was conducted 3 times. The protein concentration was adjusted to 5 mg/ml.

Preparation of BSA, BGG and cow's skim milk solutions. Pure BSA and BGG (purchased from Sigma Chemical Co. Ltd., U.S.A.) and cow's skim milk (purchased commercially) were each dissolved in a 20 mM sodium phosphate buffer (pH 7.4). The protein concentration was adjusted to 2 mg/ml (BSA and BGG) and 5 mg/ml (cow's skim milk).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was carried out according to the method of Laemmli²⁸⁾ with a slight modification, using 4% stacking gel and 10% separating gel. The beef extract, pork extract, chicken extract, bovine myofibrillar protein, BSA, BGG, and cow's skim milk solutions and the eluates from chromatography were each suspended in an SDS-sample dilutor (0.25M Tris-HCl buffer at pH 6.8 containing 10% SDS, 4% 2-mercaptoethanol, 20% glycerol, 0.05% bromo-phenol blue (BPB), and 10 mM EDTA). Each sample was denatured by heating at 100°C for 2 min. Middle range SDS-PAGE protein standards (Promega Co. Ltd., Japan) were used as molecular weight markers. The gel was stained with a solution containing 0.025% coomassie brilliant blue (CBB) R-250, 50% methanol and 5% acetic acid, and destained with 7.5% acetic acid and 5% methanol. The gel was also stained with the periodic acid-schiff (PAS) reagent for detecting glycoprotein.^{29,30)}

Determination of the molecular weight of the allergenic protein. The molecular weight was determined according to the method of Weber & Osborn³¹⁾ with the following modifications. The molecular weight of each protein was determined by calculating the distance of migration after transferring from the SDS-PAGE gel to a PVDF (polyvinylidene difluoride) membrane. The relative mobility was calculated as follows:

$$\text{Mobility} = \text{distance of protein migration} / \text{distance of BPB migration}$$

Enzyme-linked immunosorbent assay (ELISA). ELISA was performed according to the method of Engval & Perlmann³²⁾ with slight modifications. The prepared beef, pork and chicken extracts were each diluted in a 50 mM sodium carbonate buffer (pH 9.6; 1:500 vol/vol), and 50 μl of each diluted extract (0.01 $\mu\text{g}/\mu\text{l}$) per well was coated on a 96-well ELISA plate (Costar Co. Ltd., U.S.A.). The plate was incubated overnight at 4°C , washed with phosphate-buffered saline (PBS; 20 mM sodium phosphate, 150 mM

NaCl, 2.7 mM KCl, pH 7.4), blocked for 1 hour with a 2% gelatin (Bio-Rad., U.S.A.)-PBS solution, and then washed three times with PBST (PBS + 0.05% Tween-20). The patient's serum diluted in 1% gelatin-PBS (1:5 vol/vol) was added to the plate (50 μ l per well). The plate was incubated for 2 hr at 25°C, and washed four times with PBST. Subsequently, the 2nd antibody (anti-human IgE peroxidase conjugate, Sigma Co. Ltd., U.S.A.) was added to the plate. The plate was washed five times with PBST, and developed with an ABTS Peroxidase substrate system (KPI Co. Ltd., U.S.A.) before being read with a plate reader (Bio-Rad., U.S.A.) at 405 nm.

Immunoblotting. Immunoblot analyses were carried out according to the method of Towbin³³ with slight modifications. After SDS-PAGE, the proteins were transferred from the gel on to a PVDF membrane (Bio-Rad., U.S.A.) with a transfer buffer consisting of 25 mM Tris, 192 mM glycine and 5% methanol. The PVDF membrane was blocked for 2 hours at 25°C in a 2% gelatin-PBST solution. The blocked membrane was rinsed with PBS and then incubated overnight in PBST containing the patient's serum (diluted 1:5 vol/vol) at 25°C. The membrane was then washed three times with PBST. Subsequently, the membrane was incubated in PBST containing a 1:500 dilution of alkaline phosphatase (AP)-conjugated goat anti-human IgE (Tagoimmunologicals Co. Ltd., U.S.A.) as the 2nd antibody for 1 hr 30 min at 25°C. After washing three times with PBST and once with PBS, AP was detected with an AP conjugate substrate kit (Bio-Rad., U.S.A.). In order to check the efficiency of the electrotransfer, the proteins on the PVDF membranes were stained with 0.1% CBB R-250 and 99% methanol, and destained with 50% methanol and 5% acetic acid.

Gel filtration chromatography. Chromatography was performed in a Sephadex G-200 super fine (Pharmacia Biotech) column (1.4 $\times$ 100 cm). The freeze-dried beef extract was dissolved in a 20 mM phosphate and 150 mM NaCl buffer (pH 7.5), and loaded into the column. A constant flow rate of 0.052 ml/min was used. The amount of loaded beef extract was 2 ml (10 mg/ml) per run, and fractions were collected in 2-ml aliquots. The eluate was detected by measuring the absorbance at 280 nm.

N-terminal amino acid sequence determination. The N-terminal amino acid sequence of the protein was determined with an N-terminal amino acid sequencer (PPSQ-21/23, Shimadzu Co. Ltd., Japan). After SDS-PAGE, the protein was transferred to a PVDF membrane with a buffer (0.1M Tris in 5% MetOH for the -pole, and 25 mM Tris and 40 mM ϵ -amino-N-caproic acid in 5% MetOH for + pole). The membrane was washed double distilled water (DDW) for 5 min. The band was stained with 0.1% CBB for 1 min and destained with 50% MetOH for 10 min. The membrane was dried, and the band of protein was cut from the membrane. The cut membrane was destained several times with 50% and 100% MetOH until the CBB color had disappeared. After drying, the membrane was subjected to an N-terminal amino acid sequence determination.

Inhibition-ELISA. Inhibition-ELISA was carried out with pure BSA and BGG which had been purchased from Sigma Chemical Co. Ltd., U.S.A. The serum 50 μ l (diluted 1:5 vol/vol) from a beef-allergic patient was incubated with various concentrations of BSA or BGG (0–500 μ g/ml) for 2 h at 25°C. The inhibited serum was then used as the antibody for ELISA as already described. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} = \frac{\text{OD}_{405\text{nm}} \text{ without BSA or BGG} - \text{OD}_{405\text{nm}} \text{ with BSA or BGG}}{\text{OD}_{405\text{nm}} \text{ without BSA or BGG}} \times 100$$

Results and Discussion

Ten beef-allergic patients identified by Yoshida Hospital and five non-allergic control individuals were subjected to this study. The sera of the beef-allergic patients and non-allergic control individuals were applied to ELISA. In Fig. 1, ten beef-allergic patients show high specific IgE binding activity to the beef extract, but not to the pork or chicken extract. These results are in accordance with the RAST scores at the hospital. It was thus determined that our patients had a specific reaction only to beef. The non-allergic control individuals did not show any specific

binding activity to any extracts as can be seen in Fig. 1. Immunoblotting of the beef-allergic patients and non-allergic control individuals is shown in Fig. 2. The beef-allergic patients reacted strongly to three components of the beef extract (Fig. 2B, C-1). Some tailing bands were also observed (Fig. 2C-1). The beef-allergic patients in this study didn't show any specific binding activity to bovine myofibrillar protein (Fig. 2B, C-2). On the other hand, Restani *et al.*²⁶ have reported actin to be the protein involved in the IgE-mediated response of beef allergic patients. A calculation of the distance of migration by SDS-PAGE enabled the molecular masses of the

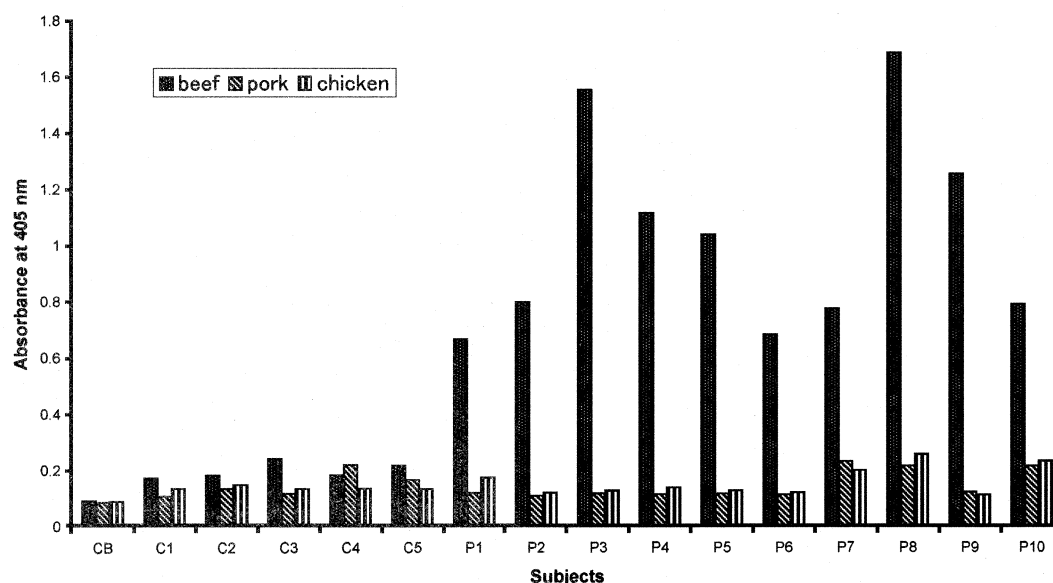


Fig. 1. ELISA with Sera of Beef-allergic Patients and Non-allergic Control Individuals to Beef, Pork and Chicken Extracts. CB, sodium carbonate buffer; C1-C5, sera of non-allergic control individuals; P1-P10, sera of beef-allergic patients

three bands to be estimated as ~ 200 kDa, ~ 67 kDa and ~ 60 kDa. As shown in Fig. 2, the beef-allergic patients could be classified into two groups according to their specific IgE-binding reactivity: one group had strong specific IgE-binding activity to ~ 67 kDa, and weak activity to ~ 200 kDa and ~ 60 kDa (type 1; Fig. 2B-1), and the other to ~ 200 kDa, ~ 60 kDa and some tailing components in beef extract (type 2; Fig. 2C-1). Most of our patients were classified into type 1 (7 out of 10 patients) in this study. The five non-allergic subjects did not react against either the beef extract or bovine myofibrillar protein (Fig. 2D-1, 2). These results suggest that the beef-allergenic proteins were mainly present in the beef extract and that their molecular masses were ~ 200 kDa, ~ 67 kDa and ~ 60 kDa.

To separate these allergenic proteins, the beef extract was subjected to Sephadex-gel (G-200) filtration column chromatography. Fig. 3 presents the chromatographic pattern of the beef extract and the corresponding ELISA pattern with the sera of the beef-allergic patients. The beef-allergic patients showed specific reactions to fractions 35 (Fr 35) and 43 (Fr 43) as can be seen in the first two main parts of the figure. However, the type 1 and 2 patients differed considerably from one another in the response of their sera to each fraction. The type 1 patients showed strong reactivity to both Fr 35 and 43, while the type 2 patients displayed strong reactivity particularly to Fr 35 and less to Fr 43. These two fractions were treated with SDS for immunoblotting. The immunoblotting result showed that the ~ 200 kDa and ~ 60 kDa components were present in Fr 35 while the ~ 67 kDa component was present in Fr 43, the type 1 patients reacting to 67 kDa in Fr 43 (Fig. 4B-2) and

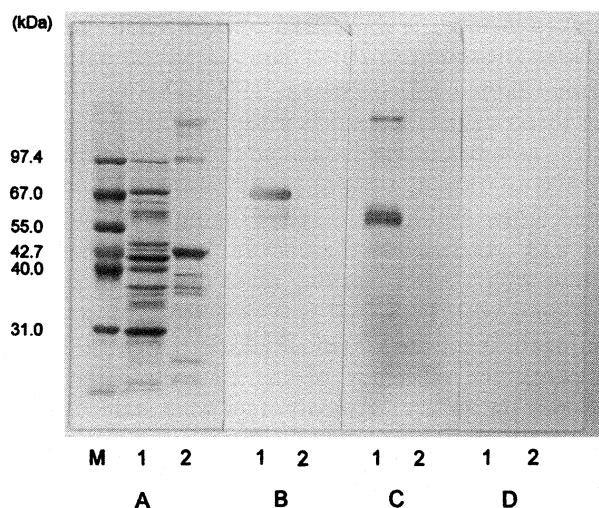


Fig. 2. Immunoblot Analyses (10% gel) to the Beef Extract and Bovine Myofibrillar Proteins.

(A) Coomassie brilliant blue stain; (B) and (C) immunoblotting with sera of beef-allergic patients; (D) immunoblotting with sera of non-allergic control individuals. Lane M shows molecular weight markers, and lanes 1 and 2 show the crude beef extract and bovine myofibrillar proteins, respectively.

the type 2 patients reacting to ~ 200 kDa and ~ 60 kDa in Fr 35 (Fig. 4B-1). However, this result contradicts the principle of gel filtration that the larger the molecular mass, the faster it is usually eluted. Therefore, ~ 60 kDa is only the molecular mass by SDS-PAGE, and its intact molecular mass seems to be much larger than ~ 67 kDa. We then tried to identify these components by their N-terminal amino acid sequences. As a result, the 15 N-terminal amino acid sequence of the ~ 67 kDa component was found to completely match that of BSA. On the other hand, we

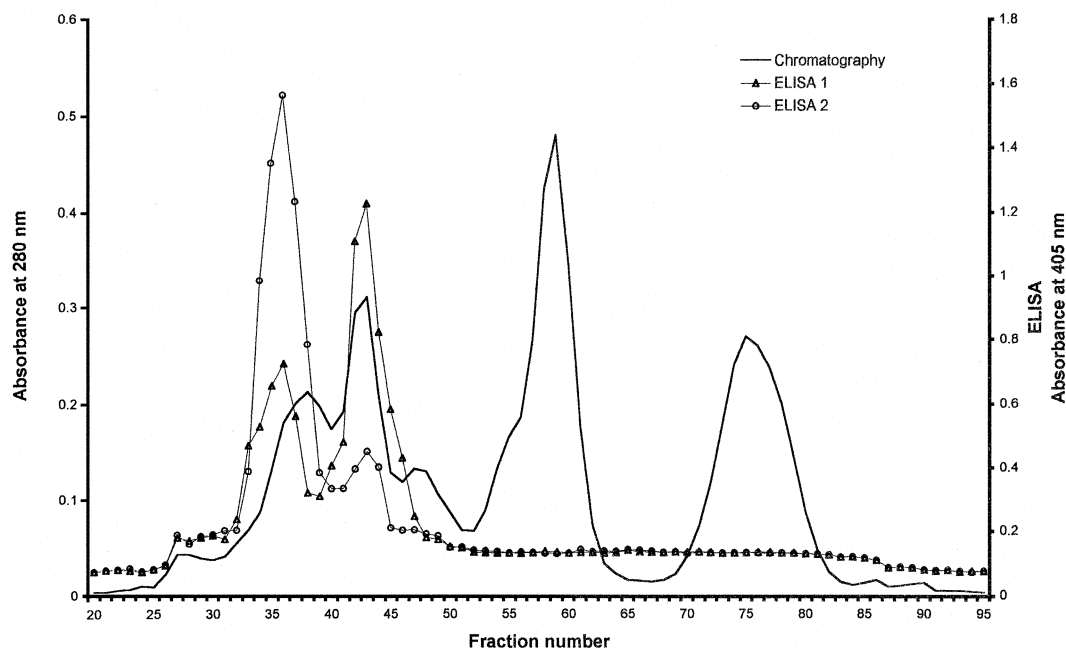


Fig. 3. Sephadex G-200 Chromatography of the Crude Beef Extract.

A Sephadex G-200 column (1.4 × 100 cm) was equilibrated with a 20 mM phosphate and 150 mM NaCl buffer (pH 7.5). Fractions were collected in 2-ml aliquots. ELISA 1, serum of a type 1 patient; ELISA 2, serum of a type 2 patient.

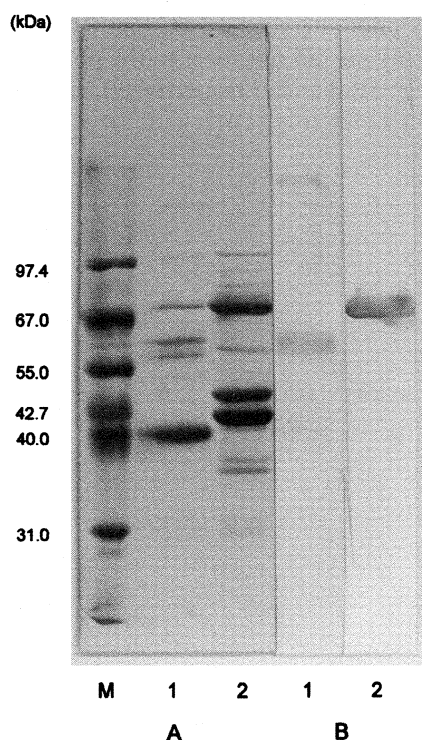


Fig. 4. Immunoblot Analyses (10% gel) Fraction Numbers 35 and 43 for Type 1 and Type 2 Patients, Respectively.

(A) Coomassie brilliant blue; (B) immunoblotting for type 1 and type 2 patients; Lane M shows molecular weight markers. Lane 1 shows the immunoblot of fraction number 35 for a type 2 patient; lane 2 shows the immunoblot of fraction number 43 for a type 1 patient.

could not identify the ~200 kDa and ~60 kDa components by their N-terminal amino acid sequences, probably because they comprised several subunits and were modified proteins that were unlikely to be easily degraded by the Edman method. Although data are not shown, the ~200 kDa and ~60 kDa bands by SDS-PAGE were dyed with the PAS (periodic acid-schiff reagent), suggesting that they might be glycoprotein. The ~60 kDa component was therefore presumed to be a heavy chain of bovine gamma globulin (BGG, M.W. ~160 kDa) which is a glycoprotein and comprises several subunits.

To confirm that BSA and BGG were beef allergens, immunoblotting and inhibition ELISA were performed with purified BSA and BGG purchased from Sigma Chemical Co. (U.S.A.) on the sera of the type 1 and type 2 patients (Fig. 5). As shown in Figs. 5B-1 and -2, the type 1 patients reacted strongly to ~67 kDa in both the crude beef extract and pure BSA. The types 1 and 2 patients reacted to ~200 kDa and ~60 kDa in both the crude beef extract and pure BGG (Figs. 5B-1 and -3, and C-1 and -3). However, we don't know why the patients reacted to the ~200 kDa band in BGG while the molecular mass is ~160 kDa. Its characteristic property of remaining unsolved in the presence of SDS might have influenced the behavior by SDS-PAGE. This fact suggests that the ~200 kDa component by SDS-PAGE was BGG and the ~60 kDa component was its heavy chain. Of course, the ~200 kDa component could be regarded as a different beef allergenic protein. In Figs. 6 and 7, pure BSA and BGG effectively prevented the sera of

the types 1 and 2 patients from binding to the crude beef extract as expected. However, in both cases, 100% inhibition was not achieved in the concentration ranges employed. Pure BSA strongly inhibited the binding of sera from the type 1 patients to the beef allergen, but it was less effective by inhibition-ELISA with the type 2 patients, indicating that the

type 2 patients had few IgE antibodies to BSA (Fig. 6). In contrast, pure BGG strongly inhibited the binding of sera from the type 2 patients to the beef allergen, and also considerably inhibited the binding of sera from the type 1 patients to the beef allergen, supporting the fact that the type 1 patients had a high level of IgE antibodies not only to BSA but also to BGG (Fig. 7). These results correspond well with the immunoblotting results (Fig. 5). In other studies with beef- or cow's milk-allergic patients whose ages ranged from 16 months to 14 years, the ~67 kDa component (BSA) was considered as one of the beef allergens.^{23,24,26} The present results clearly indicate that the ~67 kDa component was BSA, and it is presumed that the ~200 kDa and ~60 kDa components were BGG. Further studies on the latter two proteins are necessary to confirm this presumption.

We also investigated the cross-reactivity between beef and cow's milk, as cow's milk includes BSA and BGG. Immunoblotting for the type 1 patients to cow's milk indicated strong reactivity (Fig. 8B-3) in the same position as that of BSA and the beef extract (Fig. 8B-1 and -2). Reactivity to BGG in cow's milk was also found with the type 2 patients (data not shown). These results support the report of Werfel *et al.*²³ who suggested that cross-reactivity between beef and cow's milk allergens exists. Fig. 8 also indicates that our patients did not react to the pork or chicken extract (Fig. 8B-4 and -5 respectively), supporting the results of ELISA (Fig. 1).

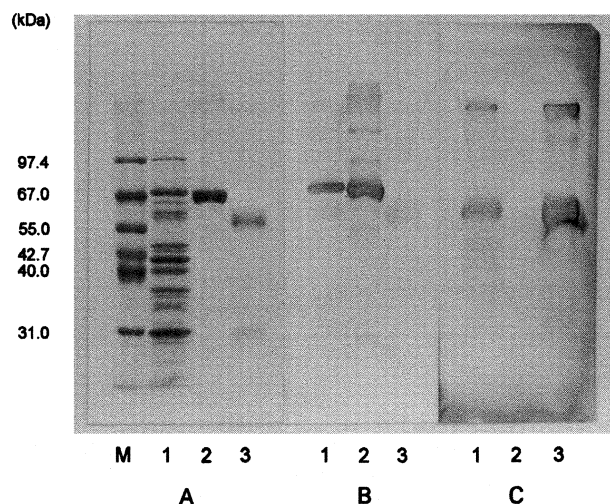


Fig. 5. Immunoblot Analyses (10% gel) of Pure BSA and BGG for Beef-allergic Patients.

(A) Coomassie brilliant blue; (B) immunoblot for a type 1 patient; (C) immunoblot for a type 2 patient; lane M shows molecular weight markers. Lane 1 shows crude beef extract; lane 2 shows pure BSA; lane 3 shows pure BGG.

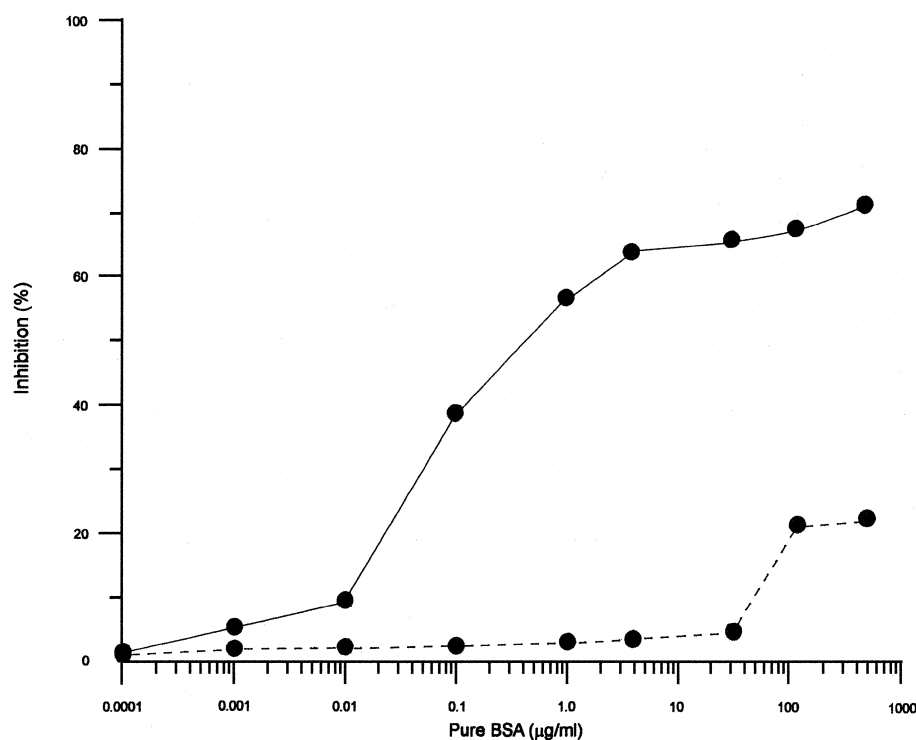


Fig. 6. Inhibition-ELISA with Pure BSA for the Beef Extract.

The sera of beef-allergic patients were incubated with various concentrations of BSA (0–500 µg/ml) for 2 hours at 25°C. — type 1 patient; - - - type 2 patient.

The sera of types 1 and 2 shown in Figs. 2–8 were obtained from the same patients. The same results were observed in the experiment with the sera of the other patients.

Meat allergy has long been considered to be a rare pathological condition. However, many people, par-

ticularly children who are in need of high-nutrition foods such as meat for their growth, are actually suffering from meat-associated atopic dermatitis. According to our investigation of the recent incidence of food allergy by questionnaires to 2,369 subjects aged 18 to 24 at the Health Administration Center of

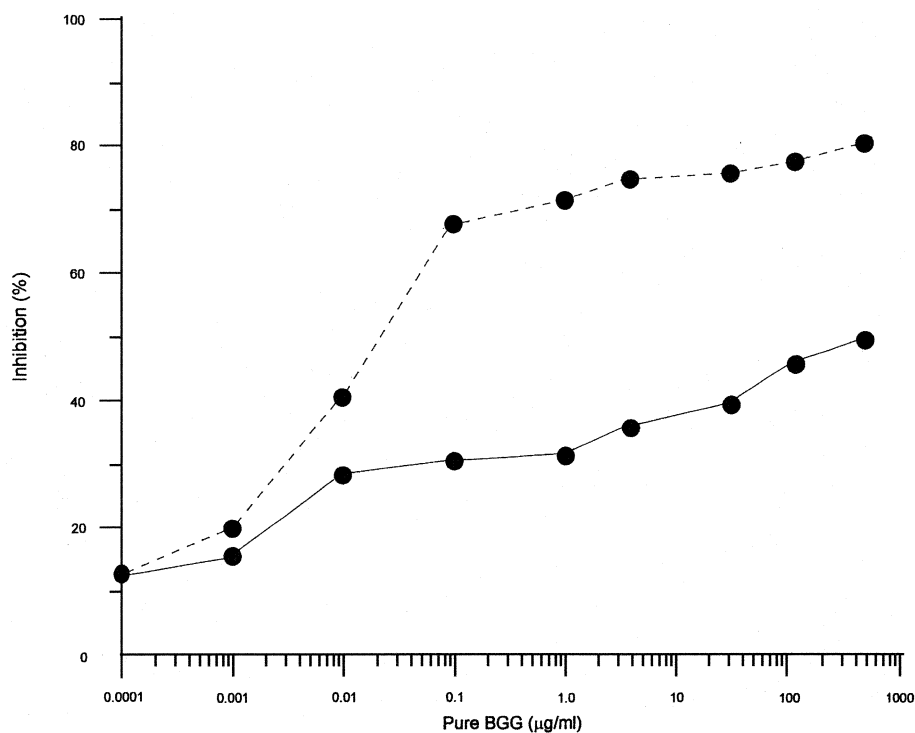


Fig. 7. Inhibition-ELISA with Pure BGG for the Beef Extract.

The sera of beef-allergic patients were incubated with various concentrations of BGG (0–500 μg/ml) for 2 hours at 25°C. — type 1 patient; - - - type 2 patient.

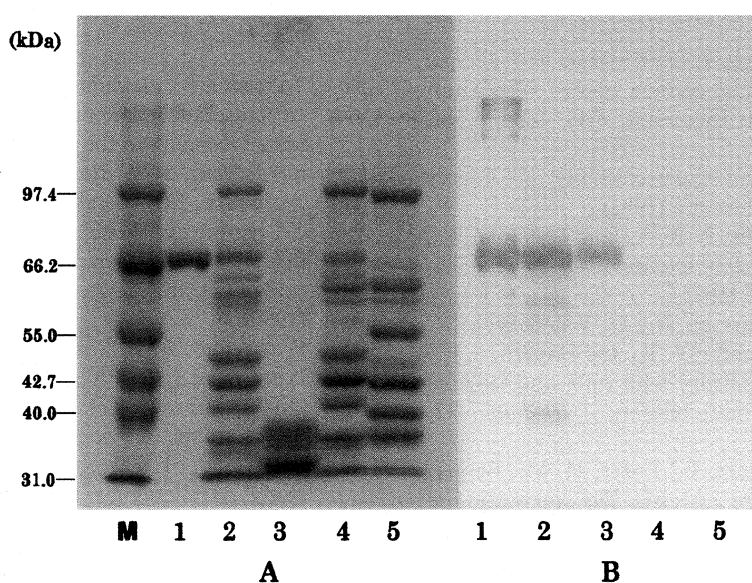


Fig. 8. Immunoblot Analyses (8% gel) of Pure BSA, Beef Extract, Cow's Skim Milk, Pork Extract and Chicken Extract for a Type 1 Patient.

(A) Coomassie brilliant blue; (B) immunoblot for a type 1 patient; lane M shows molecular weight markers. Lane 1 shows pure BSA; lane 2 shows the beef extract; lane 3 shows cow's skim milk; lane 4 shows the pork extract; lane 5 shows the chicken extract.

University of Niigata, 10.64% [252 of 2,369 subjects] had a history of food allergy, 10.7% [27 of these 252 subjects having a history of food allergy] had a meat allergy history, and 2.2% [6 of these 252 subjects having a history of food allergy] had a history of beef allergy. Sampson & McCaskill³⁴ have reported that 15.9% of 113 atopic children subjected to the skin-prick test showed a positive response to meat. Restani *et al.*²⁶ have reported an 8.96% incidence of beef allergy in a group of atopic children. Even if the methods of investigation were different, the incidence of meat allergy in Japan seems to be lower than that reported by the European researchers. We are not sure whether this is due to the differences in diet between Japanese and European people. These data seem to be first report about the incidence of meat allergy in Japan, based on their anamnesis about meat allergy.

The number of people plagued by meat allergy has recently been increasing. It is thus desirable to identify the allergens of meat and to elucidate their characteristics. One meat processing company in Japan is in fact trying to identify meat allergens and to make hypoallergenic products.³⁵ Our study would contribute to progress in such research. Studies on the characteristics and elimination methods for beef allergens are now underway in our laboratory.

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