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Some human B and T cell epitopes of bovine serum albumin, the major beef allergen[☆]

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Abstract

Bovine serum albumin (BSA) is the major beef allergen. Since IgE and T cell recognitions are central to the specific immune response to allergens, the identification and immunologic characterization of B and T cell epitopes of BSA represent important steps in the development of treatments for beef allergy. Prior to our experiments, we hypothesized that BSA-specific antibodies and T cells react primarily with sequential epitopes in which the amino acid sequences differ greatly between bovine and human albumin. To clarify this hypothesis, 16 peptides corresponding to such regions were synthesized as candidate epitopes. Among them, at least two regions, aa336–345 and aa451–459, were found to be B cell (IgE-binding) epitopes. In inhibition ELISA experiments, EYAV (aa338–341) and LILNR (aa453–457) bound to patient IgE antibodies and were found to be the cores of the IgE-binding epitopes. Three regions, DDSPDLPKLPDPNTLC (aa107–123), PHACYTSVFDKHLVDEP (aa364–382), and LSLILNRLC (aa451–459), were found to induce T cell proliferation in more than half of the patients tested. Of interest was that these three regions were also recognized by B cells. Information concerning human B and T cells epitopes can contribute greatly to the elucidation of the etiology of beef allergy. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Beef allergy; Bovine serum albumin; Allergen; Epitope; IgE; T cell; Peptide

Epidemiologic studies suggest that between 10% and 20% of the world population exhibits some form of IgE-mediated hypersensitivity, which is manifested as asthma, atopic dermatitis, or allergic rhinitis [1]. Among them, allergic reactions to foods are important worldwide health problems. Hen's egg [2], cow's milk [3], wheat [4], and peanuts [5] are generally known allergens for food allergic patients. In contrast, beef allergies were poorly recognized before the 1990s. Since then, some papers [6–8] have appeared elucidating the nature, epidemiology, and symptoms of beef allergy in children allergic to cow's milk and children suffering from atopic

dermatitis. It is now clear that beef allergy is not an infrequent occurrence, with an incidence between 3.3% and 6.5% among children with atopic dermatitis [8].

The major beef allergen is bovine serum albumin (BSA) [6–9]. Fiocchi et al. [6] reported that all beef-allergic children tested reacted to BSA in a skin-prick test. Serum albumins are also the known causes of cat- and dog-dander allergies [10,11]. However, less detailed information about the epitope structure of BSA has been available until now.

Serum albumin is one of the most widely studied and applied proteins in biochemistry. It is also the most abundant protein in the circulatory system, accounting for 60% of the total serum protein with a concentration of approximately 40 mg/mL [12–14]. Serum albumins comprise about 580 amino acid residues and are characterized by a low content of tryptophan and a high content of cystine and charged amino acids, such as

[☆] Abbreviations: BrdU, 5-bromo-2'-deoxyuridine; BSA, bovine serum albumin; DMSO, dimethylsulfoxide; HSA, human serum albumin; PBMC, peripheral blood mononuclear cells.

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aspartic and glutamic acids, lysine and arginine [13,14]. The tertiary structure comprises three domains, I, II, and III (aa1–190, 191–382, and 383–581).

BSA and human serum albumins (HSA) display approximately 76% sequence homology and a repeating pattern of disulfides that is strictly conserved [14]. The fact that a major component of serum acts as an allergen is very surprising for the following reason: it is remarkable that albumins from animals, which are very similar in sequence, structure, and function to HSA, are recognized by the human immune system as allergens instead of tolerance being induced.

Allergen-specific T lymphocytes determine the quality of the subsequent antibody response by their pattern of cytokine production in response to specific activation. It is therefore necessary to analyze the immunoreactivity to antibodies at the level of T cells. Moreover, studies on T cell recognition of allergens could lead to allergen-specific immunotherapy [15–17]. The aim of this study was to characterize B as well as T cell responses to BSA in beef-allergic patients. Our group [9] and Berreta et al. [18] have previously reported that HSA fails to bind IgE antibodies in beef-allergic patients sensitive to BSA. Thus, it is highly likely that BSA-specific antibodies and T cells react primarily with sequential epitopes that differ greatly between BSA and HSA, since BSA and HSA have similar tertiary structures. We found 15 regions in which the amino acid sequences of BSA and HSA greatly differ (see Table 1). We therefore synthesized peptides corresponding to such regions as candidate epitopes and characterized the responses of B and T cells to these peptides.

Materials and methods

Patients. Peripheral blood samples were obtained from 12 patients (A–L; eight boys and four girls; age range, 4 months–4 years; mean, 2.3 years) allergic to beef with atopic dermatitis. Beef allergy was proven by clinical symptoms and positive radioallergosorbent test (CAP-RAST score: meat, 4–6; milk, 3–6). Serum from a nonallergic adult was also used as a negative control. Informed consent was obtained from all donors (their parents). Serum samples were used for immunoblot and ELISA after appropriate dilution with 0.1% HSA and 0.05% Tween 80 containing PBS. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples from four donors (A, J, K, and L) by density centrifugation using lymphocyte separation medium (ICN Biomedicals, USA) and used in the lymphocyte proliferation assays. We could not perform this assay using samples from the other eight patients due to the difficulty in obtaining a sufficient volume of blood from these patients, all of whom were infants or young children.

Immunoblots. Immunoblotting of patient sera was performed by the usual method. Briefly, BSA (Sigma, USA) was electrophoresed in 10% polyacrylamide gels and blotted onto PVDF membranes (Atto, Japan). Since patient IgE antibodies reacted strongly with BSA but not with HSA [9,18], the membrane was soaked in a 3% HSA (Sigma)–TBS solution for blocking, instead of using BSA, which is frequently chosen. The membrane was then incubated with 50-fold diluted patient sera for 2 h at room temperature. Bound IgE was detected using biotinylated anti-human IgE (Kirkegaard & Perry Lab, USA), streptavidin–peroxidase conjugate (Amersham Pharmacia Biotech, Sweden), and ECL (2,2'-azino-di-[3-ethylbenzthiazoline sulfonate, diammonium salt], Amersham Pharmacia Biotech).

Peptide synthesis. We compared the amino acid sequences of BSA and HSA by referring to the protein databank of the National Center for Biotechnology Information (NCBI). The accession numbers were 229552 and P02769 for BSA and 5821846 and P02768 for HSA. Then, as listed in Table 1, peptides corresponding to amino acid positions 57–65, 107–123, 123–137, 153–166, 178–191, 292–311, 336–345, 338–341, 364–382, 389–400, 431–444, 451–459, 453–457, 466–472, 497–506, 513–521, 545–552, 547–550, and 573–581 of BSA were synthesized as

Table 1
Amino acid sequence comparison^a of synthesized peptides derived from BSA with the corresponding sequences in HSA

Peptide no.	Sequence synthesized	Amino acid position in BSA	Corresponding sequences in HSA
1	ESHAGCEKS	57–65	ESAENCDKS
2	DDSPDLPKLPDPNTLC	107–123	DDNPNLPRLVRPEVDVMC
3	CDEFKADEKKFWGKY	123–137	CTAFHDNEETFLKKY
4	LLYANKYNGVFQEC	153–166	LLFFAKRYKAAFTFC
5	PKIETMREKVLTS	178–191	PKLDELRLDEGKASS
6	EKDAIPEDLPPLTADFAEDK	292–311	ENDEMPADLPPLAADFVESK
7	HPEYAVSVLL	336–345	HPDYSVVLLL
8	PHACYTSVFDKLLKHLVDEP	364–382	PHCEYAKVFDEFKPLMEEP
9	NCDQFEKLG	389–400	NCELFEQLG
10	VGTRCCTKPESERM	431–444	VGSKCKKHPEAKRM
11	LSLILNRLC	451–459	LSVVLNQLC
12	PVESKVT	466–472	PVSDRVT
13	PKAFDEKLFT	497–506	PKEFNAETFT
14	TLPDTEKQI	513–521	TLSEKERQI
15	VMENFVAF	545–552	VMDDFAAF
16	LVVSTQTAL	573–581	LVAASQAAL
7-2	EYAV	338–341	DYSV
11-2	LILNR	453–457	VVLNQ
15-2	ENFV	547–550	DDFA

Underlined residues represent amino acid differences between BSA and HSA.

Synthesized peptides were dissolved in DMSO at a stock concentration of 5 mM for storage.

^a NCBI accession numbers: BSA, 229552 and P02769; HSA, 5821846 and P02768.

candidate epitopes essentially according to the solid phase method using a peptide synthesizer (PSSM-8, Shimadzu, Japan). The synthesized peptides were applied to reversed-phase HPLC and the main peak was collected. The purified peptides were lyophilized, dissolved in dimethylsulfoxide (DMSO) to a concentration of 5 mM, and stored at -80°C until use.

ELISAs for specific IgE antibodies to the synthetic peptides. The peptide solutions were diluted 4-fold in 0.1 M carbonate buffer (pH 9.6) and aliquots (100 μL) were covalently immobilized in the wells of plates (AquaBind, M&E, Denmark) according to the method of Gregorius et al. [19]. After blocking with 200 μL of 1% HSA in PBS, 10-fold diluted pooled serum from ten BSA-sensitive patients was applied for assay. IgE antibodies to peptides were detected using biotinylated anti-human IgE (Kirkegaard & Perry Lab), streptavidin-peroxidase conjugate (Boehringer-Mannheim, Germany), and *o*-phenylenediamine (Wako Pure Chemical Industries, Japan). The values in Fig. 2A are expressed as relative ELISA values based on the absorption at 492 nm.

In addition, inhibition ELISAs were performed by the usual method [4,20]. For inhibition studies, peptides (0.5–50 μM at a final concentration) were added to serum samples diluted 5–20-fold in 0.05% Tween 80 containing PBS. The optimal serum dilution was determined in preliminary experiments for each patient. After 1 h of preincubation at 37°C , the peptide-serum mixture was added to polystyrene microtiter plates (Nunc, Denmark) coated with BSA (5 $\mu\text{g}/\text{well}$) and blocked with 1% HSA in PBS. Serum not treated with peptides was used as a control. The binding of IgE-antibody to BSA was determined by sequential incubation with biotinylated anti-human IgE, streptavidin-peroxidase conjugate, and *o*-phenylenediamine. DMSO (0.2% final concentration) had no effects on the inhibition ELISA. Percent inhibition in Figs. 2B and 3 was calculated as $(1 - A/B) \times 100$, where *A* is the ELISA value of serum preincubated with peptide and *B* is that of the control.

Preparation of beef protein hydrolysate. A beef protein hydrolysate (BPH) was prepared as follows. Minced beef (10 g) was incubated with Alcalase solution (70 μL , Novo Nordisk, Japan) for 4 h at 60°C . The heat-inactivated reaction product was freeze-dried and the resulting powder was extracted with PBS. The extract (1 mg/mL final concentration) was used as a positive control in the lymphocyte proliferation assays described below.

Lymphocyte proliferation assays. PBMCs from four patients were washed and resuspended in RPMI1640 medium containing 10% human AB serum, 3 mM glutamine, 50 μM 2-mercaptoethanol, 50 U/mL penicillin, and 50 $\mu\text{g}/\text{mL}$ streptomycin. The PBMCs ($1 \times 10^5/\text{well}$) were cultured in a 96-well cell culture plate (Nunc) for 72 h at 37°C under a humidified 5% CO_2 atmosphere, in the presence of 50 μM of synthesized BSA sequence peptides. Cultures of PBMCs alone were included as negative controls. Cultures of PBMCs in the presence of 10 $\mu\text{g}/\text{mL}$ phytohemagglutinin (PHA, Wako Pure Chemical Industries) or 1 mg/mL BPH were also assayed as positive controls. The cells were pulsed with 5-bromo-2'-deoxyuridine (BrdU) for the final 24 h of culture. The 96-well cell culture plate was centrifuged at 300g for 10 min and the BrdU incorporated by the pelleted cells was measured with an ELISA kit (Roche, USA). DMSO (1% final concentration) had no effect on PBMCs in the lymphocyte proliferation assay. In this study, peptides that induced 1.5 times more proliferation than the control culture (stimulation index >1.5) were considered to show increased proliferation. The assay was performed independently two times.

Results and discussions

With respect to the immunogenic determinants of BSA, it has been very recently reported by Beretta et al. [18] that the C terminal region of BSA may be an epitopic area for patients based on analyses of tryptic hy-

drolsates of BSA. Although this study is informative, it does not provide a precise location of the epitopes and does not fully identify those residues on BSA that contribute significantly to B and T cell recognition.

First, we evaluated whether the IgE antibodies in serum samples from 12 beef-allergic patients recognized BSA or not. The IgE-binding patterns of the patients are depicted in Fig. 1. Sera from 10 of the 12 patients reacted strongly with BSA. Thus, these 10 serum samples were used for further experiments. Serum samples from the two remaining patients (C and F) revealed little, if any, IgE binding to BSA.

BSA and HSA display approximately 76% sequence homology and their tertiary structures are very similar to one another [13,14]. Moreover, our group [9] and Berreta et al. [18] have previously reported that HSA fails to bind IgE antibodies in beef-allergic patients sensitive to BSA (see also Fig. 2A). Thus, it is highly likely that BSA-specific antibodies and T cells react primarily with sequential epitopes that differ greatly between BSA and HSA. Comparing the amino acid sequences of BSA and HSA, there are 15 regions in which the amino acid sequences greatly differ. We therefore synthesized peptides corresponding to these regions as candidate epitopes and characterized B and T cell responses to these peptides. One or two homologous amino acid residues were added to both ends of each peptide to mimic the conditions in which each peptide exists in its intact form. For example, since the HAGCE sequence in BSA (aa59–63) is quite different from the AENCD sequence in HSA, we synthesized ES-HAGCEKS (aa57–65, peptide no. 1) by attaching two homologous amino acids, ES and KS, to the N and C terminals of HAGCE, respectively, as in Table 1. Nineteen out of the 27 amino acid residues in the region aa109–135 differ between the two albumins. This sequence was divided at a C residue (aa123) near the

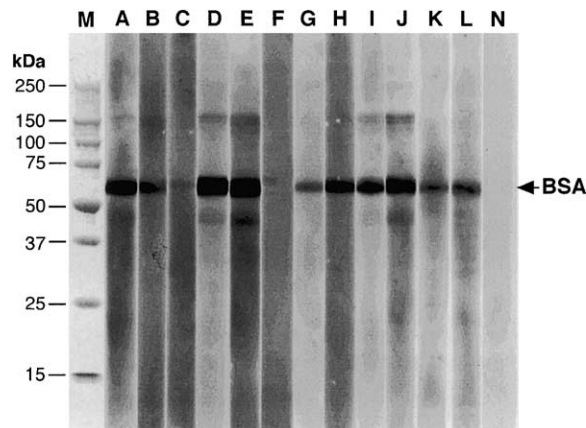


Fig. 1. Immunoblotting analysis of serum IgE-reactivity with BSA for beef-allergic patients A–L. M, molecular markers. N, nonallergic donor (control).

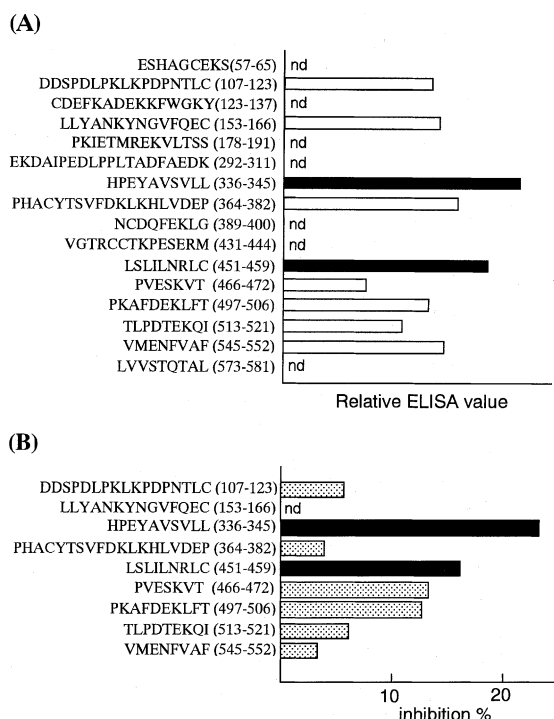


Fig. 2. IgE-binding abilities of synthetic peptides. (A) The synthesized peptides were covalently immobilized on the wells of immunoplates and applied to ELISA using diluted pooled serum. nd, not detected. (B) Inhibition ELISA of the nine IGE-binding epitopes of BSA. Nine synthetic BSA peptides, which were found to bind IgE-antibodies in (A), were further evaluated by inhibition ELISA using diluted pooled serum. Peptide no. 4 (LLYANKYNGVFQEC, aa153–166) failed to bind patient antibodies.

center of aa109–135, because the original sequence is too long to synthesize.

We examined the B-cell (IgE-binding) epitopes of BSA by two methods. In the solid phase method, the synthesized peptides were coated on a solid support and pooled serum was tested for IgE binding to the peptide fragments. (First of all, from this study, we could confirm that patients' IgE antibodies did not bind to HSA which was used in "blocking" step.) As expected, several antibody-binding regions for sera of beef-allergic patients were found among the synthesized peptides (Fig. 2A). Inhibition ELISAs of the nine peptides that found to bind IgE-antibodies (peptides nos. 2, 4, 7, 8, 11, 12, 13, 14, and 15) were also performed, since in the solid phase method, the ELISA value in Fig. 2A might be influenced by the binding affinity of the peptide to the plate. All peptides tested, except for peptide no. 4, had IgE-binding abilities. (An IgE-binding ability of peptide no. 4, which was shown in Fig. 2A, was concluded to be nonreproducible and false-positive.) Among them, peptide no. 7 (aa336–345) gave the highest ELISA inhibition value and peptide no. 11 (aa451–459) gave the second highest value. These two peptides were selected for further analysis.

Next, in inhibition ELISAs, each individual serum sample was used to clarify whether the epitopic peptides were recognized by individual patients and in what proportion. Since we also aimed to identify core sequences for IgE binding, we synthesized truncated versions of peptide nos. 7, 11, and 15. Comparing the amino acid sequences of peptide nos. 7 and 15, two putative IgE-binding motifs were found, EYAV and ENFV, within aa336–345 and aa545–552, respectively. Thus, peptide nos. 7-2 (EYAV, aa338–341) and 15-2 (ENFV, aa547–550) were applied to this assay. Peptide no. 11-2 (aa453–457), which has deletions of two homologous amino acid residues in both the N and C terminals of peptide no. 11, was also evaluated. As a result, it was proven that peptide no. 7-2 (EYAV) bound IgE antibodies in all serum samples (10/10), and peptide no. 11-2 (LILNR) bound IgE antibodies in half of the samples (5/10) (Fig. 3 left). Dose dependencies of IgE-binding abilities of EYAV and LILNR were shown in the IgE-binding profiles (Fig. 3 right). It appears that even four or five amino acid residues might be enough for an IgE binding. This is comparable to the results of our previous study on wheat allergen [4] in which the pentapeptide QQQPP was identified as the minimum IgE-binding epitope. However, peptide no. 15-2 (ENFV) failed to bind patient antibodies. This finding contradicts our hypothesis that the common EXXV sequence would be an IgE-binding epitope. Since peptide no. 15 (VMENFVAF) bound IgE antibodies in the pooled serum as shown in Fig. 2, amino acid residues other than ENFV must be essential for IgE binding; this point requires further study.

Very recently, Beretta et al. [18] prepared tryptic hydrolysates of BSA and found an IgE-binding polypeptide in the C-terminal region. In addition, they took interest in the same sequences as those of peptide nos. 13 (PKAFDEKLFT) and 14 (TLPDTEKQI) in this study. However, to our knowledge, a further immunoassay of these peptides has not been performed practically until the present study. As shown in Fig. 2, peptide nos. 13, 14, and 15 were able to bind IgE antibodies in pooled serum. Therefore there is no doubt that the C-terminal region of BSA plays an important role in IgE binding. However, it should be noted that middle regions of BSA, particularly aa338–341 (EYAV) and aa453–457 (LILNR), also have potent abilities to bind IgE antibodies (Figs. 2 and 3).

The production of allergen-specific IgE by B cells and the release of inflammatory mediators by mast cells result in the effector response in allergic disease. However, it is well established that these events are orchestrated by allergen-specific T cells [17]. Moreover, it has recently been demonstrated that the administration of peptide(s) containing immunodominant T cell epitopes from a certain allergenic protein can induce T cell nonresponsiveness to subsequent allergen challenge (specific immunotherapy)

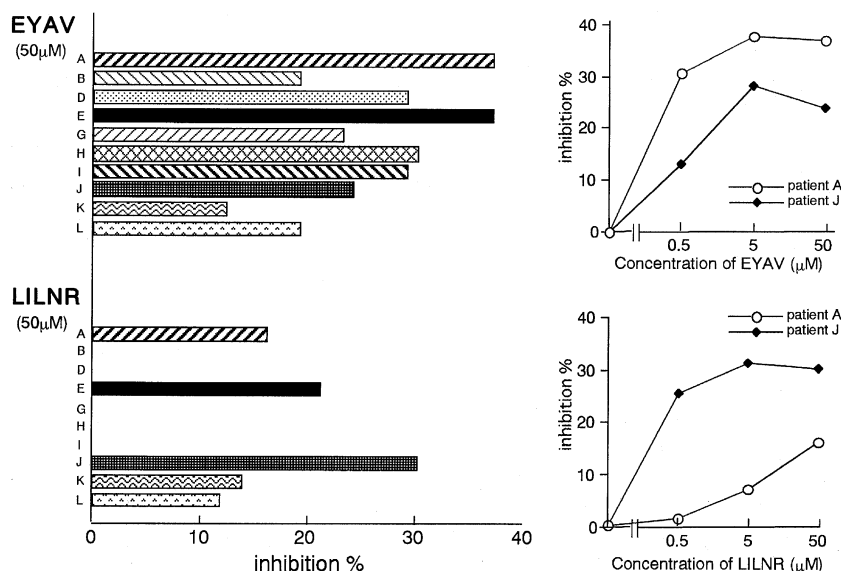


Fig. 3. Inhibition ELISA of the two IgE-binding epitopes of BSA. Three synthetic BSA peptides were evaluated using the sera of 10 individual patients. The assays using the sera of patients C and F were not performed, since their IgE titers were not high enough. (Left) Peptide 7-2 (EYAV) was recognized by all IgE-antibodies tested, while 11-2 (LILNR) was recognized by half of the patients. Peptide 15-2 (ENFV) unexpectedly failed to bind patient antibodies. (Right) IgE-binding profiles of EYAV and LILNR using the sera of patients A and J.

[15–17]. T cell epitope-containing hypoallergenic recombinant allergens have also been engineered for mite [21], birch [22], and latex [23] allergies for this purpose. Thus, the characterization of the T cell response to BSA is an important step in the development of a treatment for beef allergy.

Cultured PBMCs from four patients were stimulated with the synthesized peptides and the results of the lymphocyte proliferation assays are shown in Table 2. For positive controls in this experiment, phytohemagglutinin-P and beef protein hydrolysate (BPH) were selected, since BSA was found to be inapplicable for a positive control for the following reasons: (i) stimulation index of the culture even in the presence of 250 μM BSA was not high (SI = 1–2), probably because most of the T cell epitopic regions were embedded inside the molecule, (ii) it was difficult to prepare BSA hydrolysate with a lymphoproliferation activity, since intact BSA alone was rapidly degraded with proteases [18]. Proliferation was seen in more than half of the patient PBMCs tested when the cells were incubated in the presence of peptide nos. 2, 8, and 11. These three peptides were also recognized by B cells, although the ELISA values for peptide nos. 2 and 8 were not higher than those for peptide nos. 7 and 11. Thus, all of the major T cell epitopes found in this study are also concluded to be B cell epitopes. This is the first demonstration of the identification of human T cell epitopes of BSA. Studies of the T cell epitopes in the primary structure of other allergenic proteins [17,24–26] have revealed that, similar to BSA, two or more major T cell epitopes usually exist in the sequence of the allergen and disperse throughout the molecule.

Specific immunotherapy protocols involve the repeated administration of T cell epitope-containing allergens over prolonged periods of time, which induces relative immunologic tolerance. However, severe adverse reactions occasionally occur during specific

Table 2
Lymphoproliferation of PBMC in response to incubation with synthetic peptides

Samples	Patients			
	A	J	K	L
PHA	>10	>10	>10	>10
Beef protein hydrolysate	1.6	2.3	5.4	4.6
Peptides				
No. 1	–	–	–	2.6
No. 2	2.0	4.9	–	1.9
No. 3	–	–	–	–
No. 4	–	–	–	–
No. 5	–	–	–	–
No. 6	2.2	–	–	–
No. 7	2.2	–	–	–
No. 8	–	6.8	2.0	–
No. 9	–	–	–	–
No. 10	–	–	–	–
No. 11	2.1	6.2	2.0	–
No. 12	–	–	–	–
No. 13	–	–	–	–
No. 14	–	–	–	–
No. 15	–	–	–	–
No. 16	–	–	–	–

Values are given as stimulation index (SI) and (–) indicate SI values below 1.5. Phytohemagglutinin-P (PHA) and beef protein hydrolysate were used as positive controls.

immunotherapy with this approach, due to the binding of the administered allergen to allergen-specific IgE antibodies bound to mast cells. This can be avoided if allergen-derived synthetic peptides are used instead of whole allergenic proteins [17]. Among three T cell epitopes found in this study, peptide no. 11 (LSLILNRLC, aa451–459) is the shortest in length and best recognized by patients at high frequency. Since the nonapeptide LSLILNRLC might be unable to crosslink IgE antibodies and be unable to trigger the subsequent release of chemical mediators such as histamine [4,20], LSLILNRLC and/or its analog peptide(s) would be of interest for use in the treatment of beef allergy.

In conclusion, we have identified some IgE-binding and T cell epitope peptides in BSA, which is a major beef allergen and is also famous in biochemistry. This finding would contribute greatly to the elucidation of beef allergy.

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