

Alteration of Allergen Fold of Bos d 5 into a Hypoallergenic Vaccine for Immunotherapy of Cow's Milk Allergy

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Keywords

Cow's milk allergy · Bos d 5 · Epitope · Allergen-specific immunotherapy · Genetic engineering · Bioinformatics

Abstract

Background: Cow's milk allergy (CMA) is the most common IgE-mediated food allergy and Bos d 5 is the major allergen in cow's milk proteins. More than 60% of the patients with CMA are sensitized to this protein. **Methods and Results:** A recombinant protein, encoded by a synthetic gene and consisting of reassembled Bos d 5 fragments, was expressed in *E. coli* strain BL21 (DE3) cells and purified to homogeneity. The B5M lacked relevant IgE-reactivity and allergenic activity compared with Bos d 5 in dot-blot and basophil activation assays. T-cell proliferation experiments demonstrated that B5M preserved the main T cell epitopes of Bos d 5. Immunization of rabbits with B5M induced protective IgG antibodies that blocked the binding of patients' IgE antibodies to the wild-type allergen and inhibited the degranulation of basophils induced by Bos d 5. **Conclusion:** Thus, we developed a new strategy, which was based on rational molecular reassembly for allergen-specific immunotherapy (AIT) of CMA and food allergy.

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Introduction

Cow's milk allergy (CMA) affects 2.5% of infants and children and is the most common IgE-mediated food allergy [1, 2]. The symptoms of CMA range from allergic asthma, diarrhea, cystitis to other severe and life-threatening symptoms [3, 4]. Bos d 5, also named as β -lactoglobulin, is the main cow's milk protein (CMP) and makes up approximately 10% of the CMPs [5]. More than 60% of the patients with CMA are sensitized to this protein [6]. In a study performed with 470 children, half of the samples (233/470) were sensitized to cow's milk in the ImmunoCAP test and 79% of the cow's milk allergic children were sensitized to Bos d 5 [7]. Bos d 5, a small soluble protein, naturally exists as a 36-kDa dimer. It belongs to the lipocalin-proteins family, which is characterized by β -barrel structures composed of 8 anti-parallel β -strands [8]. The molecule possesses 2 disulfide bridges and 1 free cysteine, which is responsible for the main physicochemical properties and interaction with casein during heat treatments [9]. The relative resistance of Bos d 5 to acid hydrolysis and gut proteases allows part of the protein to be absorbed intactly

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through the intestinal mucosa. Therefore, besides short linear sequences, some of the IgE-epitopes of Bos d 5 appear to be conformational [10].

Allergen-specific immunotherapy (AIT) is an allergen-specific treatment for allergy, which modifies the allergen-specific immune response of the patients and has a long-lasting effect [11–13]. It was first used in the therapy of grass pollen-induced hay fever in 1911 by Noon [14]. AIT is based on the administration of therapeutic vaccines that contain the disease-eliciting allergens and has persistent effects both during the treatment stage and after its discontinuation [15]. However, the application of natural allergen extracts as allergy vaccines has induced many side effects because of its unclear components [16]. Problems associated with allergen extracts include the quality and stability of important allergens in natural source materials and the presence of other unwanted substances. Several strategies have been applied for the development of hypoallergenic allergy vaccines [17–20]. The application of genetic engineering (including protein engineering) has improved the efficacy, safety and convenience of AIT.

In this study, we aim to construct a hypoallergenic molecule for the AIT against Bos d 5, the most important component of cow's milk allergen source. Four fragments were produced by disrupting the 3-dimensional structure and conformational IgE-epitopes of Bos d 5 [21, 22] but preserving its T-cell epitopes [23, 24]. Thus, the hypoallergenic fusion protein could reduce its allergenic activity, retain the immunogenicity and induce less side effects than wild-type allergen extracts.

Materials and Methods

Patients' Sera, Plasmids, E. coli Strains and Recombinant Allergen

Serum and blood samples involved in our research were obtained from patients with a case history indicative of CMA (i.e., diarrhea, emesis, asthma) and a positive IgE serology to milk allergens (ImmunoCAP, Thermo Fisher Scientific, Sweden). The enrolled patients were from Tianjin Children's Hospital and the second affiliated hospital of Tianjin Medical University. Of the patients, the median age at disease onset was 7 (range from 1 to 60) years and the ratio of male to female was 1.0:1.0. Detailed demographic information and the levels of cow's milk-specific IgE antibody were obtained from 45 patients (shown in see online suppl. Table 1; see www.karger.com/doi/10.1159/000517998 for all online suppl. material). The PET-42a plasmid, containing a kanamycin resistance gene was obtained from Sangon (Shanghai, China). The *E. coli* strain BL21 (DE3) was obtained from Genscript (Jiangsu, China). Natural Bos d 5 (nBos d 5) was purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Construction of the Hypoallergenic Protein-B5M

For construction of B5M, Bos d 5 was divided into 4 parts as follows (aa = amino acids): A: aa1–13; B: aa14–90; C: aa91–117; D: aa118–162 (Fig. 1a). The bioinformatics software-ProtScale (<https://web.expasy.org/cgi-bin/protscale/protscale.pl>), SOMPA (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html) and DNASTAR were used to predict hydrophilia and the secondary structure of the recombinant protein [25, 26]. The amino acid sequence of the hypoallergenic molecular was determined and the cDNA encoding was assembled using the PCR-based gene-SOEing [27]. The PCR products were digested with BamHI/HindII, gel-purified and inserted into the plasmid PET-42a (+). The correct sequence of the cDNA was verified by double stranded DNA sequencing (The Beijing Genomics Institute, Beijing, China).

Expression and Purification of B5M

The resulting plasmid was transformed into *E. coli* BL21 (DE3) cells and expressed in LB medium supplemented with 50 µg/mL kanamycin (Solarbio, Beijing, China). The culture was grown at 37°C to an OD₆₀₀ = 0.6–0.8, then incubated with 1 mM Isopropyl-β-thiogalactopyranoside (Solarbio, Beijing, China) for 4 h under the same condition. The cell culture was then centrifuged to collect the pellet (10 min at 9,000 rpm). The bacterial lysate was resuspended in PBS (0.01 M, pH 7.4) and sonicated on ice. Supernatant was collected and B5M was identified in the insoluble pellet fraction by SDS-PAGE. The B5M molecule was extracted from insoluble pellet and purified by Ni-NTA resin affinity columns (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The insoluble pellet was washed with 1 mM DTT, 1% Triton-X100 and 1 M β-mercaptoethanol (BME), successively. After centrifuging for 20 min at 15,000 g, the insoluble fraction was dissolved in 10 mM Tris, 0.1 M NaCl, 8 M urea (pH 8.0). The supernatant was collected by centrifugation for 30 min at 15,000 rpm and the urea was removed by stepwise dialysis. Then the dialyzate was centrifuged for 30 min at 12,000 rpm [28]. B5M was eluted with a 50–300 mM imidazole gradient in native wash buffer and concentrated using an ultrafiltration centrifuge tube with a molecular weight cut of 30 kDa. The Purity of the recombinant protein was confirmed by SDS-PAGE. The concentration of B5M was determined with a Micro BCA kit (Thermo Fisher Scientific, Sweden).

Circular Dichroism (CD) Analysis

The CD spectra of the wild-type allergen and its derivatives were measured on a JascoJ-810 spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan) at room temperature [29]. nBos d 5 and B5M were diluted to 1 mg/mL with PB buffer. After adjusting the instrument parameters, nBos d 5 and B5M were scanned in the far-ultraviolet region of 190 nm–250 nm according to the above parameters and data were collected. Jwstda32 software was used to process the images and data. JWSSE32 software was used to conduct the fitting calculation of the secondary structure of the samples. The standard curve was referred the standard α-helix, β-pleated sheet, turn and Random coil curve, which were included in the software. Results were expressed as the mean residue ellipticity (θ) at a given wavelength and the program JWWSE32 was used to estimate the secondary structure of B5M.

IgE-Binding Activity

The IgE-reactivity of B5M was estimated by dot-blot experiments. For this purpose, aliquots (2 µL) containing 2 µg of nBos d 5, B5M, goat anti-mouse IgG (1:200, positive control) or TBS con-

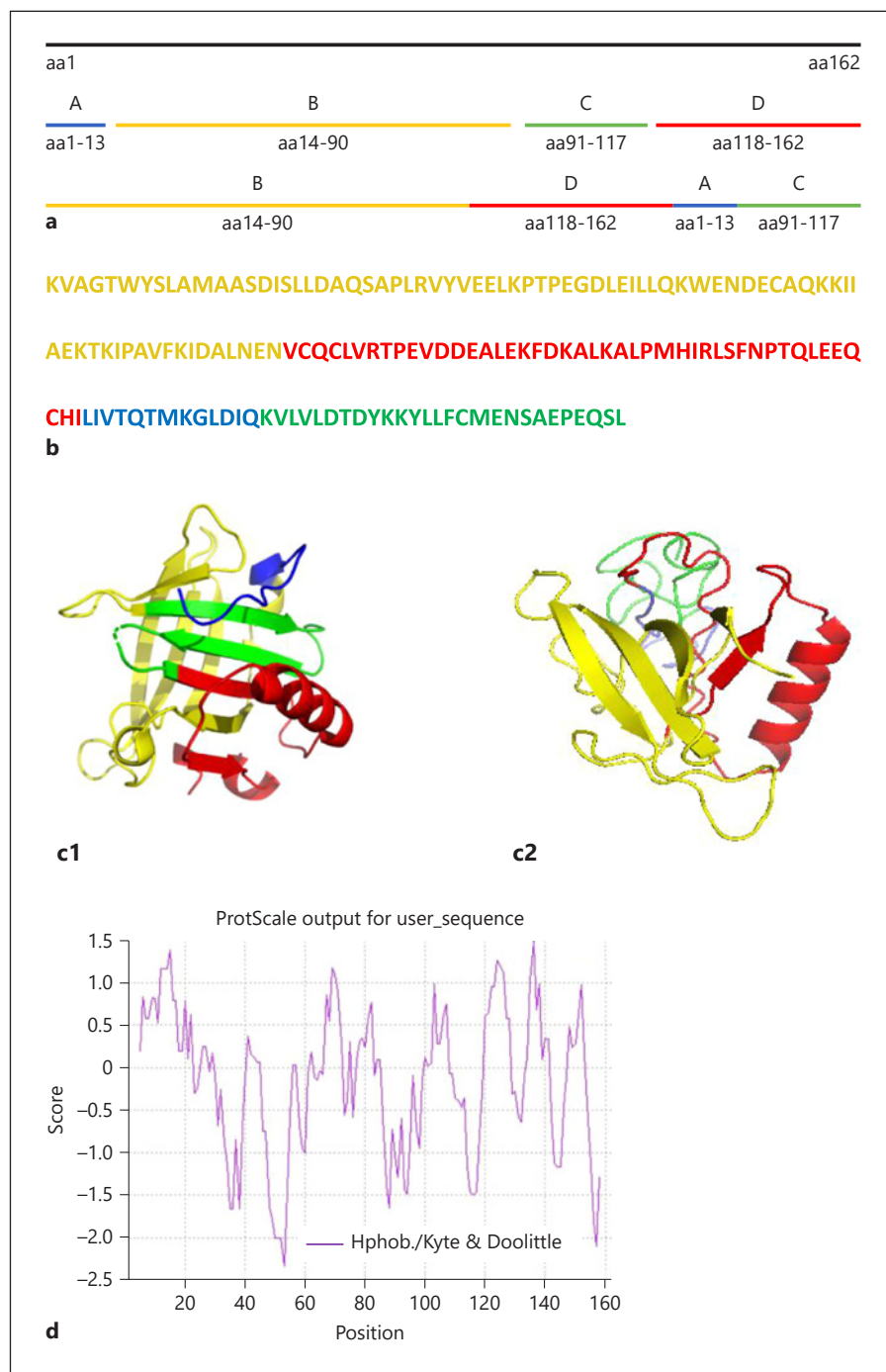


Fig. 1. Construction of B5M and the predicted results by bioinformatics softwares. **a** Schematic representation of construction of Bos d 5 and the recombinant protein-B5M. Four fragments (A, B, C, D) of Bos d 5 were painted in different colors and assembled in the order B-D-A-C. **b** The amino acid sequence of B5M. **c** The Ribbon representation of the 3-dimensional structure of Bos d 5 (**c1**) and B5M (**c2**). Four portions (A, B, C, D) were painted with the same colors used in Figure a. **d** Hydropathy profile of B5M obtained by ExPASy protscale. Hydrophilicity was expressed in negative values, hydrophobicity was indicated by positive values.

taining 0.05% v/v Tween-20 (TBST, negative control) were dotted onto nitrocellulose membrane strips (Solarbio, Beijing, China) and blocked with 3% (m/v) HSA. Then, the dot-blotted proteins were incubated with sera from 10 patients and a non-milk allergic donor (diluted 1:10 in TBST). The specific IgE antibodies binding on the proteins were detected by addition of alkaline phosphatase (AP)-labeled mouse anti-human IgE antibodies (1:1,000; Sigma-Aldrich, Saint Louis, MO, USA).

The IgE reactivity of additional 20 patients to B5M were determined by ELISA. Briefly, ELISA plates (Beyond, Shanghai, China), which had been coated with 125 μ L of nBos d 5 ($c = 5 \mu$ g/mL) or Gal d 1 (negative control, $c = 5 \mu$ g/mL) and blocked with polyvinyl alcohol (3% [w/v]), were incubated with 100 μ L sera (1:10) from 20 donors for 2 h at 37°C. After washing 5 times with PBST, the plates were incubated with 100 μ L horseradishperoxidase (HRP)-labeled goat anti-human IgE antibodies (1:1,000; Sigma-Aldrich,

Saint Louis, MO, USA) for 1 h at 37°C. The reactive substrate was added and kept in dark place for 15 min. The OD value for each well was read at 450 nm in the microplate reader.

Basophil Activation Assays (BAT)

Leukocyte from healthy volunteers were separated using human peripheral blood leukocyte separation kit (Solarbio life science, Beijing, China) according to the manufacturer's instructions. To shift the receptor-bound IgE, leukocyte was incubated with lactic acid (13.4 mM lactate, 140 mM NaCl, and 5 mM KCl [pH 3.9]) for 5 min at 4°C, then neutralized with RPMI (0.5% HSA, 12% Tris) [30, 31]. The cell pellet was incubated with sera from 3 patients with CMA and 2 healthy donors. Resensitized basophils were incubated in triplicate with serial dilutions of antigens (0.5–8 ng/mL), buffer (negative control), or anti-IgE antibody (positive control, Thermo Fisher Scientific, Sweden) for 1 h at 37°C. The cells were washed twice with RPMI (0.5% HSA) to terminate the degranulation of activated basophils and then incubated with 5 μ L PerCP-labeled HLA-DR, 20 μ L FITC-labeled CD63 and 5 μ L PE-labeled CD123 antibodies (Thermo Fisher Scientific, Sweden) for 30 min at RT. After washing, the cells were analyzed by 3-colour flow cytometry on a FACSCantoII (Becton Dickinson, Franklin Lakes, NJ, USA). The activation of basophils was calculated as the percentage of activated cells. The result was expressed as the stimulation index (SI):

$$SI = PAC_{\text{stimulated}}/PAC_{\text{control}}$$

Where $PAC_{\text{stimulated}}$ and PAC_{control} represent the percentage of activated cells obtained from stimulated and unstimulated basophils.

T-Cell Proliferation

To demonstrate that the construction of B5M maintained the T cell epitopes of Bos d 5, 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed with PBMCs from 3 Bos d 5-allergic patients and a non-allergic volunteer [32]. Briefly, human PBMCs were cultured in triplicate (5×10^5 cells/well) in the presence of different doses of nBos d 5, B5M or medium alone at 37°C with 5% CO₂ for 24 h. Then, the supernatant was removed and MTT (1 mg/mL in DMEM medium) was added. After incubation for 4 h, the MTT solution was removed and 50 μ L DMSO was added to stop tinting the cells. The results were analyzed by microplate reader at 595 nm and expressed as stimulation indices (SI):

$$SI = OD_s/OD_{us}$$

Where OD_s and OD_{us} represent the resulting OD values from stimulated and unstimulated cultures, respectively.

Immunization of Rabbits and Measurement of Specific IgG Antibody Responses

Rabbit anti-sera was obtained by immunizing New Zealand white rabbits with nBos d 5 or B5M [33]. 1–2 mL blood was collected from the ear vein of rabbits before each injection and the sera was obtained by centrifugation (3,000 rpm, 10 min). For the first injection, 500 μ g of the immunogens were mixed with Freund's complete adjuvant (Sigma-Aldrich, Saint Louis, MO, USA), respectively. The following 3 booster injections were with 500 μ g immunogens using Freund's incomplete adjuvant.

Rabbit IgG antibodies induced by nBos d 5 or B5M were determined by ELISA. ELISA plates, which had been coated with 125 μ L

nBos d 5 ($c = 2 \mu\text{g/mL}$), were incubated with 100 μ L rabbit anti-sera in serial concentrations ($1:20$ – $1:2 \times 10^8$) for 2 h at 37°C. Plates were washed 5 times with PBST. Then HRP-labeled goat anti-rabbit IgG antibody ($1:2,000$; R&D systems, Minneapolis, MN, USA) was added at 100 μ L/well and measured as above described.

ELISA Competition Assay

ELISA competition assay was performed to test the ability of B5M-induced rabbit anti-sera to inhibit the binding of allergic patients' IgE antibodies to the wild-type. ELISA plates coated with nBos d 5 ($5 \mu\text{g/mL}$) were preincubated with the rabbit anti-nBos d 5, anti-B5M sera and the corresponding preimmune sera ($1:200$) overnight at 4°C. After washing, the plates were incubated with sera ($1:10$) from patients with CMA. Bound human IgE antibodies were detected with HRP-labelled goat anti-human IgE antibodies (Sigma-Aldrich, Saint Louis, MO, USA), diluted $1:2,000$ in PBST. The inhibition ratio of IgE binding was estimated as follows:

$$(OD_{\text{Ast}}/OD_{\text{Pre}}) \times 100$$

Where OD_{Ast} and OD_{Pre} represent the OD values obtained by preincubated with the immune and preimmune sera of rabbits, respectively.

BAT Inhibition Assay

The BAT inhibition assay was performed as follows: 100 μ L nBos d 5 of different concentrations (0.5 – $8 \mu\text{g/mL}$) were preincubated with rabbit anti-nBos d 5, anti-B5M sera and the corresponding preimmune sera ($1:200$) in 96-well plates. Fresh leukocyte separated from 4 patients with CMA was adjusted to 1×10^6 /well and incubated with different rabbit sera-protein mixtures for 1 h at 37°C. After washing, the cells were incubated with different fluorescein-labeled antibodies as mentioned above. The activation of basophils was detected as the up-regulation of CD63.

Statistical Analysis

The data were analyzed using Sigmaplot and GraphPadPrism5 software. The 2-tailed student's *t*-test was used to determine the statistical significance of differences between groups. We considered *p* values <0.05 to be statistically significant. *p* values <0.05 were denoted with *, $p < 0.01$ **, $p < 0.001$ ***.

Results

Construction of B5M

With the aim to destroy the IgE epitopes of Bos d 5 while preserve major T-cell epitopes, 4 fragments of Bos d 5 were reassembled in the order B-D-A-C as a recombinant protein of Bos d 5 by using the previously mapped IgE and T-cell epitopes as reference (shown in Fig. 1a). The amino acid sequence of B5M was shown in Figure 1b. The hydrophilia and secondary structure of the recombinant protein were predicted, respectively. We found that B5M was a hydrophilic protein and its secondary structure changed compared with nBos d 5 (shown in Fig. 1c, d, online suppl. Fig. 1, 2).

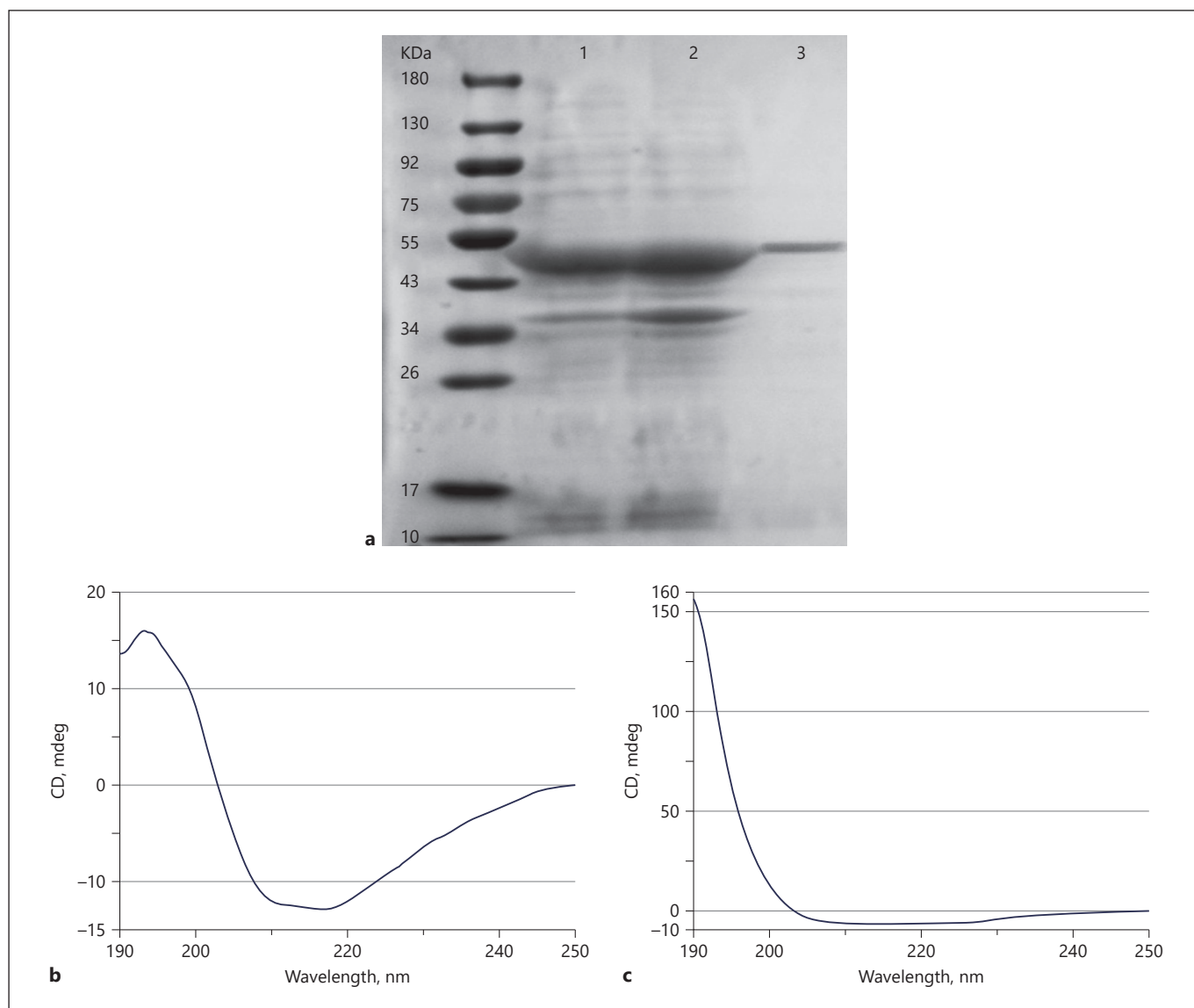


Fig. 2. a The recombinant protein B5M and a molecular mass marker were separated by SDS-PAGE and stained with Coomassie brilliant blue. lane 1, lysate from induced bacteria; lane 2, inclusion body from induced bacteria; lane 3, the purified recombinant protein B5M. The molecular marker (kDa) were shown on the left margin. **(b, c)** Far-UV CD spectra of B5M and nBos d 5. The mean residue ellipticity (y -axis) at a given wavelength (x -axis) was exhibited for the 2 proteins.

Expression, Purification and Physicochemical Structure of B5M

B5M was expressed in *E. coli* and purified to homogeneity by Ni-affinity chromatography. SDS-PAGE analysis demonstrated that B5M had been purified to >90% purity and migrated as a single band of 52 kDa (shown in Fig. 2a). The far UV spectra of purified B5M showed a minimum at 210 nm, indicating a α -helical conformation (shown in Fig. 2c). Secondary structure of B5M was ana-

lyzed using the program JWSSE32, yielding 52.8% α -helices, 25.7% β -sheet, 0.0% turns and 21.5% random coil structures. While, the CD spectrum of nBos d 5 exhibited a maximum at 192 nm and a minimum at 215 nm, which demonstrated a high content of β -sheet and α -helices (shown in Fig. 2b). The secondary structure analysis showed 18.2% α -helices, 53.7% β -sheet, 8.1% turns and 20.0% random coil structures.

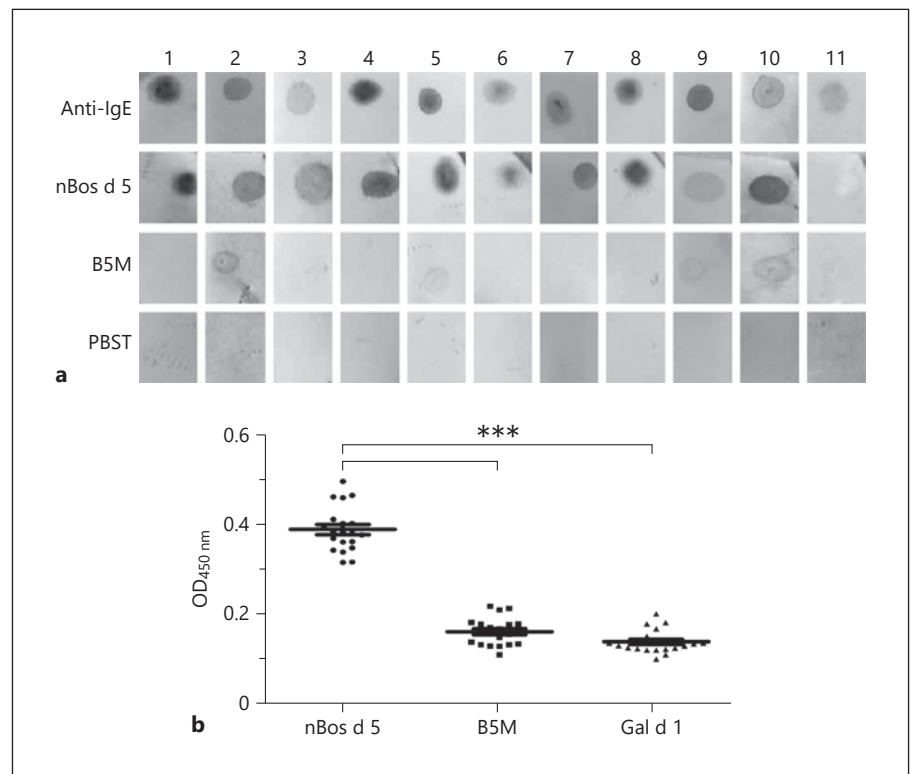


Fig. 3. The IgE reactivity of nBos d 5 and B5M. **a** Anti-IgE (positive control), nBos d 5, B5M and PBST (negative control) were dot-blotted with the aim to test for the IgE reactivity with sera from 10 milk allergic patients' sera and a healthy volunteer (11). **b** ELISA were also performed with 20 patients with CMA to quantify the IgE binding activity of nBos d 5, B5M and negative control (Gal d 1).

B5M Displayed Decreased IgE-Reactivity

The IgE-binding activity of B5M was compared with that of nBos d 5 in a nondenaturing dot-blot experiment performed with sera from 10 milk-allergic patients and a non-allergic individual. The results showed that B5M exhibited very weak or no IgE-binding capacity compared with nBos d 5. Serum from the nonallergic person showed no IgE antibody reactivity (shown in Fig. 3a). A similar result was obtained in ELISA using sera from 20 milk-allergic patients ($p < 0.001$; shown in Fig. 3b).

B5M Showed Weak Allergenic Activity

The allergenic activity of B5M and nBos d 5 was analyzed by allergen-specific release of CD63 on basophils. The CD63 expression of basophils from the volunteer's blood, which had been preincubated with sera from 3 Bos d 5-allergic patients was upregulated by incubating with nBos d 5 in a 0.5–8 ng/mL gradient (mean SI >6 in 8 ng/mL). While the expression of CD63 was significantly decreased by incubation with B5M (0.5–8 ng/mL, mean SI <2 in 8 ng/mL) ($p < 0.05$). The basophils preincubated with sera from 2 nonallergic donors induced little expression of CD63 in the presence of nBos d 5 or B5M. Anti-human IgE antibodies was used

as positive control and the buffer was negative control (shown in Fig. 4).

B5M Preserved T-Cell Epitopes

The results of MTT assay performed with PBMCs extracted from milk allergic patients were shown in Figure 5. Both nBos d 5 and B5M induced T-cell proliferation in 3 patients (mean SI_{nBos d 5} >6 at 8 μ g/mL and mean SI_{B5M} >5 at 8 μ g/mL, respectively), suggesting that B5M retained the relevant specific T-cell epitopes of Bos d 5. Whereas, the non-allergic person's PBMCs exhibited little response with nBos d 5 or B5M (SI_{nBos d 5} = 2 at 8 μ g/mL; mean SI_{B5M} = 1.8 at 8 μ g/mL).

Immunizing Rabbits with B5M Induced the Production of Bos d 5-Specific IgG Antibodies

Bos d 5-specific IgG antibody levels were estimated in the sera of rabbits immunized with nBos d 5 or B5M. nBos d 5 as well as B5M started to induce Bos d 5-specific IgG antibody response after the first immunization (shown in Fig. 6 a). The specific IgG antibody level obtained by immunizing rabbits with nBos d 5 was higher than that induced by B5M, even though the difference was not statistically significant (shown in Fig. 6 b).

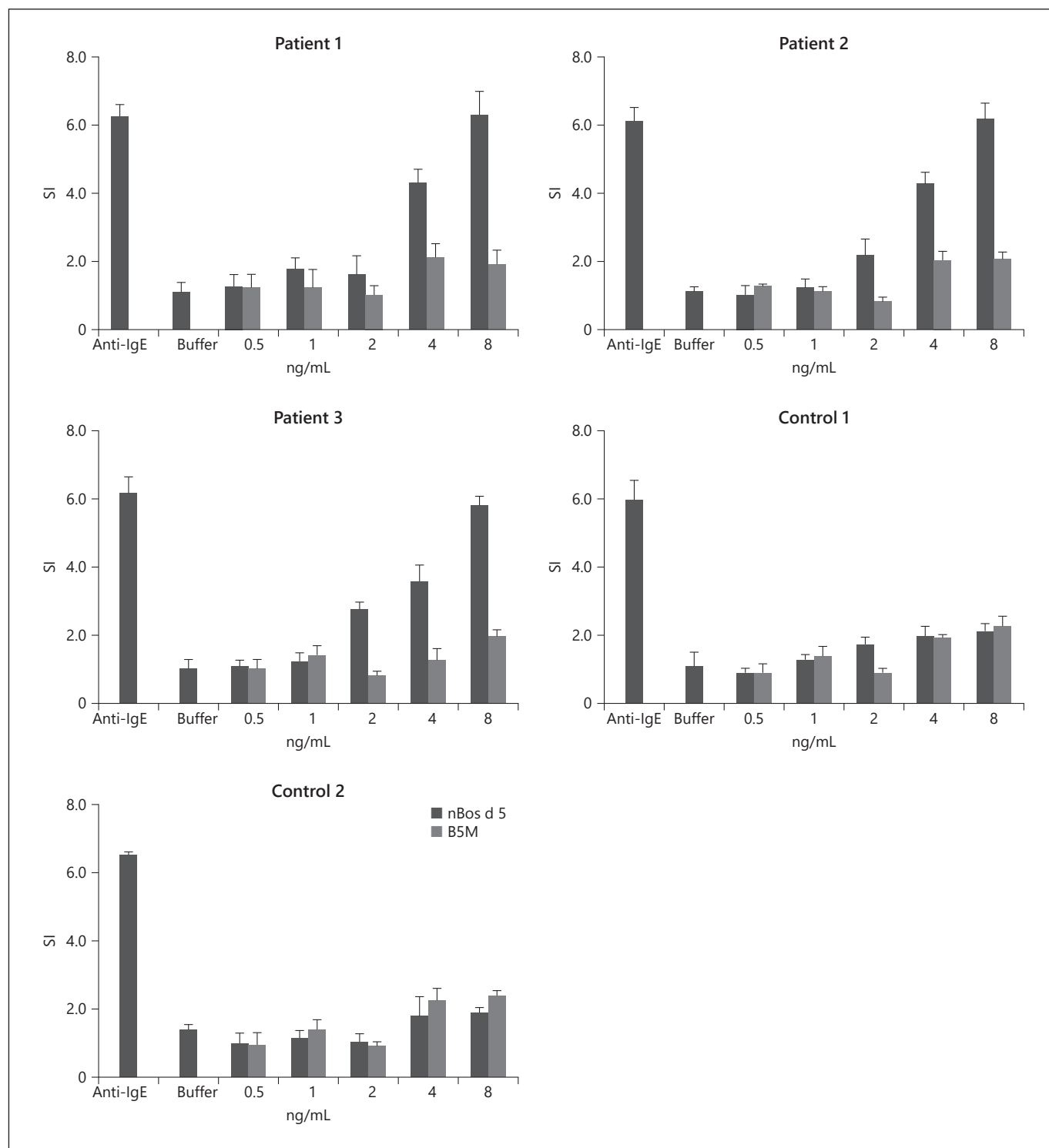


Fig. 4. CD63 expression on basophils induced by nBos d 5 and B5M. The up-regulation of CD63 was obtained from non-allergic people's preprocessed basophils, which had been incubated with anti-IgE, nBos d 5, B5M (0.5–8 ng/mL) and Buffer alone (x-axis). Allergen-induced up-regulation of CD63 was calculated by flowcytometry and expressed as stimulation indices: SI (y-axis).

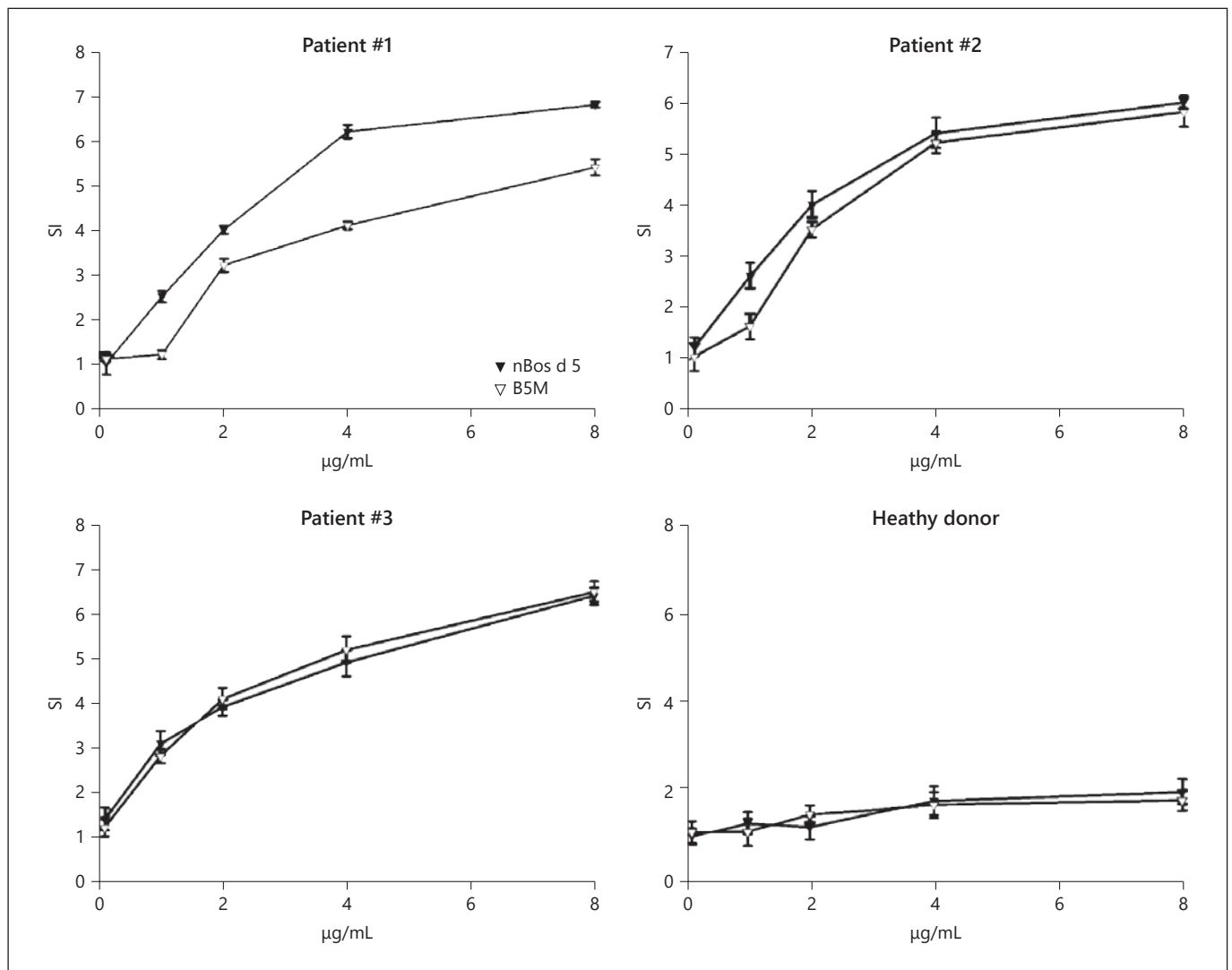


Fig. 5. PBMCs from 3 allergic patients and a healthy volunteer were stimulated with serial dilutions of nBos d 5 (0.5–8 $\mu\text{g/mL}$), B5M and buffer alone (x-axis) to test the integrity of T cell epitopes. The results of T-cell proliferation were exhibited as stimulation indices: SI (y-axis).

Rabbit Anti-Sera Induced by Immunizing with B5M Inhibited Milk Allergic Patients' sIgE Binding to nBos d 5

The ability of rabbit anti-sera, which were induced by immunizing with nBos d 5 and B5M, to inhibit the binding of nBos d 5 to patients' sIgE was analyzed for 45 patients with CMA (shown in Fig. 7). We found that the inhibition of rabbit anti-Bos d 5 sera against nBos d 5 binding to sIgE in allergic patients' sera was from 51.53% to 75.43% (mean inhibition = 65.95%). The inhibition raised by rabbit anti-B5M sera ranged from 42.24% to 66.67% (mean inhibition = 56.12%, shown in online suppl. Table. 2).

B5M-Induced Rabbit Anti-Sera Inhibited Allergen-Specific Release of CD63 on Basophils

The potential protective effect of B5M-induced anti-sera on IgE-mediated CD63 up-regulation was investigated in 4 patients' blood samples. For 4 allergic patients, a significant decline in the IgE-induced release of CD63 on basophils was obtained with rabbit anti-sera immunized with nBos d 5 or B5M in a dose-dependent manner ($p < 0.05$, shown in Fig. 8).

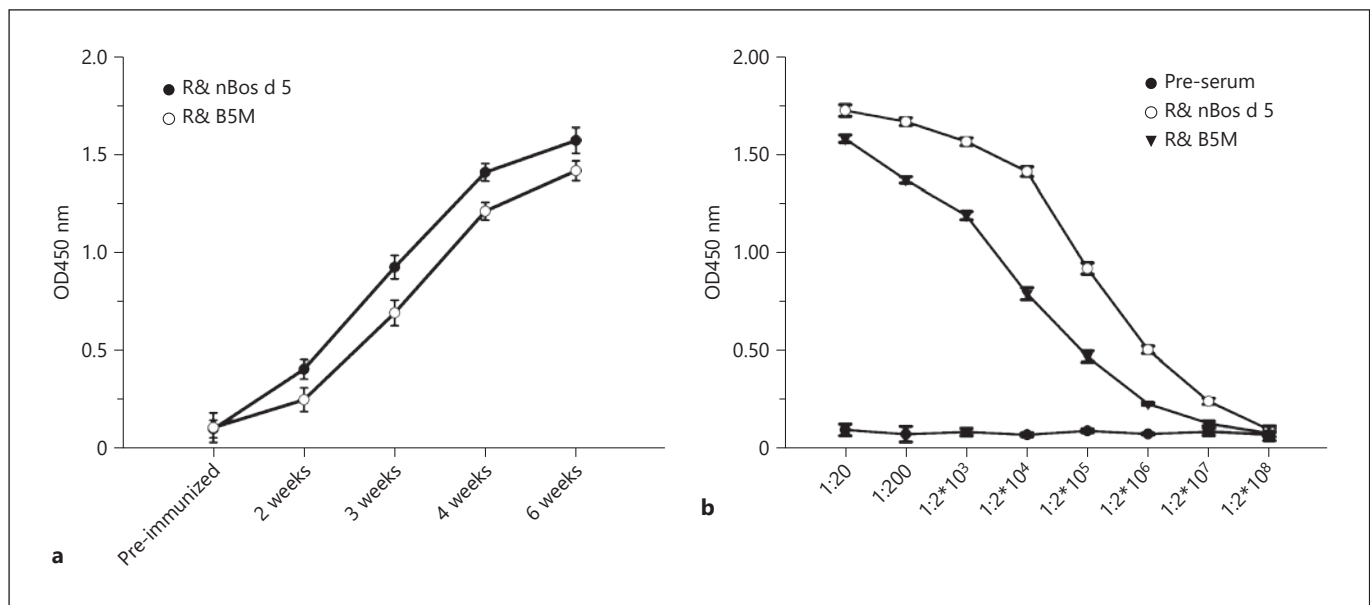


Fig. 6. a The levels of Bos d 5-specific IgG antibody were measured in sera obtained from rabbits before (pre) or after (immune) immunization with Bos d 5 or B5M (y axis: OD values). **b** Titers of Bos d 5-sIgG in rabbits' sera were measured by ELISA assay. The sera was collected after last immunization with Bos d 5 or B5M (x axis: serial dilution of 1:20–1:2 × 10⁸).

Discussion

As for AIT with natural allergens may induce IgE- and allergic sensitization, allergy vaccines based on molecular forms was raised up after the first allergen-encoding DNA sequences were analyzed by molecular cloning and the recombinant allergen proteins available [34, 35]. One approach for molecular forms is based on the generation of recombinant hypoallergenic allergen-derivatives which exhibit reduced IgE reactivity, preserved allergen-specific T cell epitopes, while is able to induce robust allergen-specific blocking IgG antibody responses [36]. In addition, the allergen specific immunoglobulins blunt the secondary IgE responses that played a role in the augmentation of allergen-specific IgE in allergic sufferers [36, 37]. Another approach is based on synthetic allergen-derived peptides targeting the receptors of allergen-specific T cells with the goal to induce T cell tolerance. In recent years, B cell epitope-based allergy vaccines are developed. This approach usually needs to obtain peptides derived from the IgE binding sites with a length of approximately 30 amino acids, which do not have reactivity with IgE antibodies due to lack of fold, then couple this peptides to a carrier protein. The carrier protein, containing no allergen-specific T cell epitopes is used to reduce the activa-

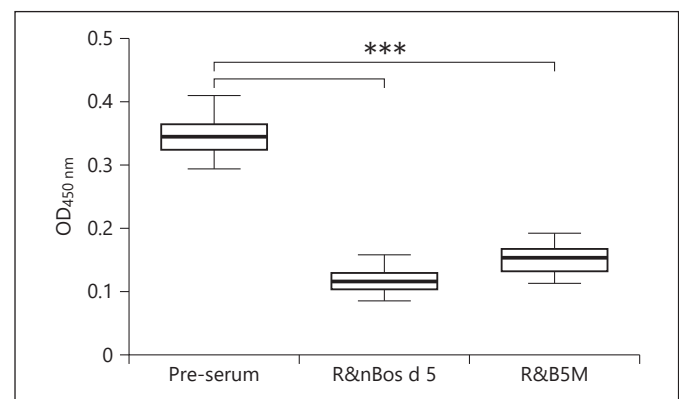


Fig. 7. Rabbit anti-sera obtained by immunizing rabbits with nBos d 5 and B5M were able to inhibit the binding of nBos d 5 to sIgE in allergic patients' sera. The results were expressed as the OD values (y axis) obtained by preincubated with different rabbits' sera (x axis).

tion of allergen-specific T cells and thus of late phase side effects [38].

In this study, we developed a hypoallergenic variant for AIT against CMA. Bos d 5 is one of the most important allergens in CMPs and can resist acid hydrolysis and proteases. Thus, it may keep its original 3-dimensional

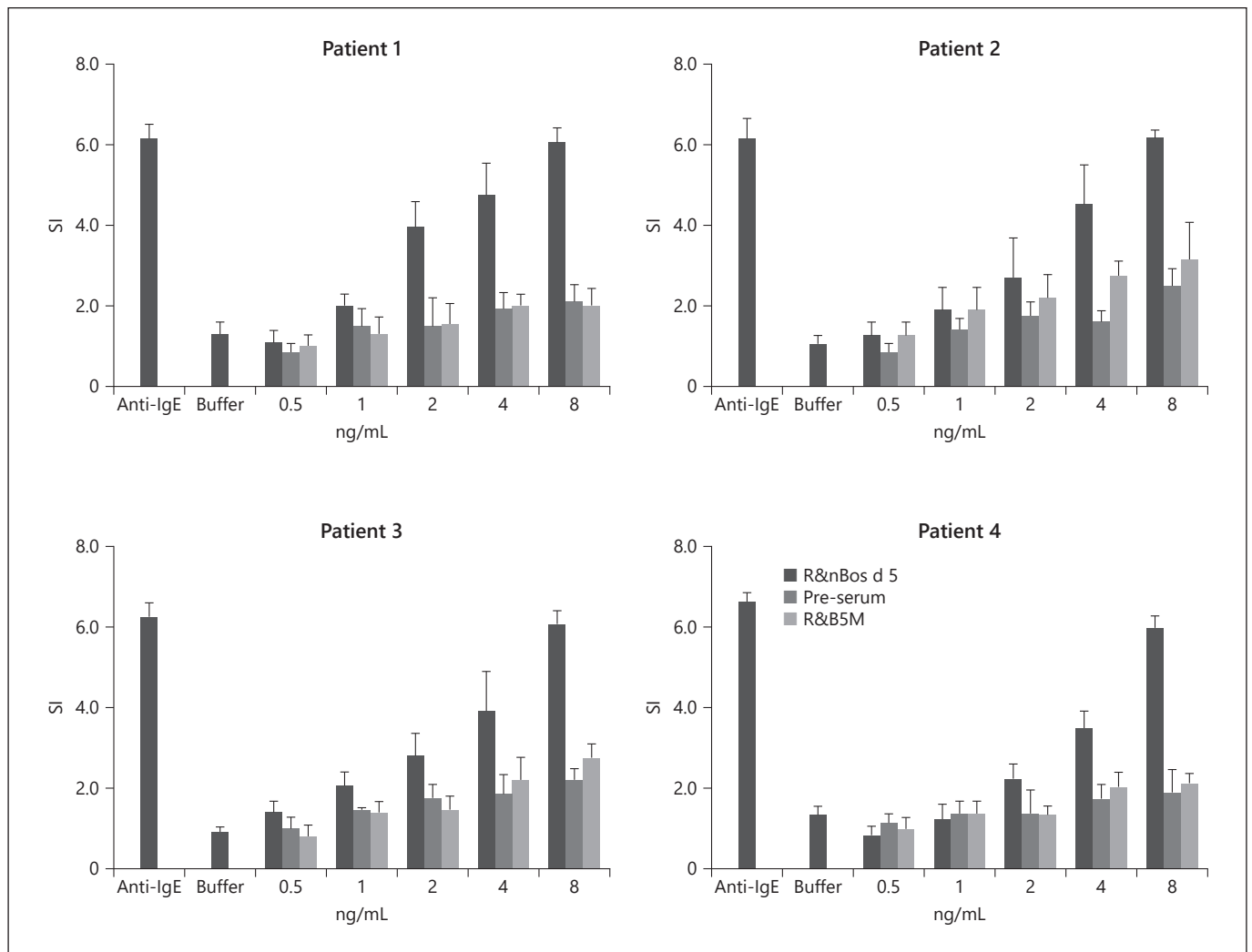


Fig. 8. B5M-induced rabbit anti-Bos d 5 sIgG inhibited the degranulation of basophils. PBMCs from 4 allergic patients, which had been preincubated with rabbit anti-sera before and after immunization were incubated with different concentrations of nBos d 5 (x-axis; 0.5–8 ng/mL), the activation of basophils was shown as stimulation indices: SI (y-axis).

structure and conformational epitopes. Based on these features, we have adopted a rational strategy to reassemble Bos d 5 and disrupted the conformational epitopes, which may reduce its allergic activity. As a consequence, B5M, a hypoallergenic derivative, was obtained in large amounts by expression in *E. coli* and purification by affinity chromatography. From the results of bioinformatics prediction and CD spectrum, we found that the secondary structure of B5M was relatively simple. In vitro experiments, B5M strongly reduced IgE-reactivity and allergenic activity, which was proven by dot-blot and basophil activation tests. Furthermore, B5M induced similar lymphoproliferative responses compared with nBos d 5

in T-cell proliferation experiments, which indicated that B5M maintained the Bos d 5-specific T-cell epitopes. The reserved T cell epitopes in the B5M aimed to induce T cell tolerance with AIT approach [39, 40]. Compared with rabbit anti-sera induced by nBos b 5, rabbit anti-sera induced by B5M had the similar ability to inhibit allergic patients' sIgE antibodies from binding with the wild type allergen. B5M-induced IgG antibodies also inhibited the specific activation of Bos d 5-allergic patients' basophils.

In summary, B5M showed strongly reduced IgE reactivity and allergenic activity. Furthermore, the specific IgG induced by B5M exhibited robust blocking against the allergen, which is crucial for an AIT vaccine. By this study,

we developed a strategy, which based on rational molecular reassembly to convert one of the most relevant allergen into a hypoallergenic derivative for immunotherapy of cow's milk allergy. However, a vaccine which only contains a hypoallergenic derivative of Bos d 5 will not be sufficient for treatment of CMA. Integrating multiple allergic components of cow's milk into a allergy vaccine needs further research.

Statement of Ethics

Ethical approval was obtained from the medical ethics committee of Tianjin Medical University (TMuhMEC2017008). Informed consent was obtained.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Study idea and design: Huiqiang Li, Chunquan Cai, Yaqiong Cui; clinical samples: Shuxiang Lin, Li Xu, Chunquan Cai, Yang Yu; experiment: Yaqiong Cui, Liuxu Li; data analysis: Yaqiong Cui, Yang Yu; manuscript: Huiqiang Li, Yaqiong Cui; critical review of the manuscript: Yang Yu, Huiqiang Li, Chunquan Cai; final approval: all authors.

Data Availability Statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Table. S1. Demographic and the level of serologic sIgE of 45 cow-milk allergic patients included in our study.

Patients	Gender ^a	Age	SIgE to milk	
			(kU _A /L)	Symptoms ^b
1	F	2	1.65	V,D
2	F	3	1.20	F,N
3	M	5	0.93	V,C
4	F	10	10.12	U,F
5	F	8	0.88	F,NC
6	M	40	12.72	U,C,W
7	M	9	2.82	D,C
8	M	55	15.00	U,D
9	M	5	0.54	F,C
10	F	2	0.73	V,C
11	F	4	1.56	U,NC
12	M	12	3.47	P,LE
13	F	39	14.98	AP,W
14	F	15	2.42	U,LE
15	F	3	1.47	D
16	M	8	2.87	U,P
17	M	46	1.70	LE,W
18	M	3	5.45	F,V,D
19	F	9	1.56	AP,N,C
20	M	5	4.13	P,C,W
21	M	7	0.80	D
22	F	6	3.92	OI,NC
23	F	12	1.03	R,N
24	M	9	1.18	P,OI,C

25	M	7	0.65	F,N
26	M	45	5.72	V,D,N
27	M	60	8.03	R,LE,OS
28	F	3	1.78	D,AP
29	F	7	0.79	R,W,C
30	M	1	0.42	V,OS
31	M	6	2.58	LE
32	F	9	4.76	U,NC
33	F	11	20.58	V,D,C
34	F	3	0.38	R,P
35	F	5	0.59	F,C
36	M	2	0.62	F,NC
37	M	10	3.83	OI,LE
38	M	5	1.64	P,D
39	F	9	4.87	AP,C
40	M	8	0.87	P,LE
41	M	35	2.40	V,D,C
42	F	4	1.27	U
43	F	1	0.56	F,C
44	F	6	4.56	AP,N
45	F	7	0.93	R,D

^a M: male; F: female; ^b U: urticaria;F: flushing; P: puritus; R:rashes; V: vomiting; OI: oral itching;D:diarrhea;N:Nausea;AP:abdominal pain;NC:nasal congestion;LE:laryngeal edema;C:coughing;W: wheezing; OS: other symptoms.

Table. S2. Inhibition of CMA-allergic patients' IgE binding to nBos d 5 with rabbit anti-sera. IgE binding was expressed as OD values. Inhibition to nBos d 5 was shown. Pre: the preimmune sera; r&nB5: rabbit anti- nBos d 5 sera; r&B5M: rabbit anti-B5M sera.

Patients	OD values			% Inhibition to Bos d 5	
	Pre	r & nB5	r & B5M ^a	r & nB5	r & B5M
1	0.367	0.151	0.158	58.86	56.95
2	0.348	0.14	0.17	59.77	51.15
3	0.335	0.131	0.138	60.90	58.81
4	0.35	0.115	0.131	67.14	62.57
5	0.376	0.124	0.159	67.02	57.71
6	0.347	0.126	0.168	63.69	51.59
7	0.337	0.128	0.166	62.02	50.74
8	0.341	0.132	0.174	61.29	48.97
9	0.322	0.155	0.186	51.86	42.24
10	0.332	0.099	0.113	70.18	65.96
11	0.35	0.121	0.15	65.43	57.14
12	0.319	0.103	0.122	67.71	61.76
13	0.326	0.158	0.152	51.53	53.37
14	0.362	0.111	0.192	69.34	46.96
15	0.304	0.143	0.168	52.96	44.74
16	0.341	0.119	0.178	65.10	47.80
17	0.41	0.107	0.153	73.90	62.68
18	0.302	0.115	0.155	61.92	48.68
19	0.381	0.13	0.149	65.88	60.89
20	0.345	0.089	0.13	74.20	62.32
21	0.368	0.120	0.133	67.39	63.86
22	0.353	0.125	0.135	64.59	61.76
23	0.389	0.132	0.171	66.07	56.04

24	0.388	0.116	0.159	70.10	59.02
25	0.342	0.116	0.178	66.08	47.95
26	0.331	0.118	0.162	64.35	51.06
27	0.365	0.129	0.142	64.66	61.10
28	0.294	0.105	0.113	64.29	61.56
26	0.373	0.11	0.132	70.51	64.61
29	0.346	0.085	0.145	75.43	58.09
30	0.317	0.128	0.172	59.62	45.74
31	0.34	0.102	0.133	70.00	60.88
32	0.339	0.1	0.153	70.50	54.87
33	0.314	0.085	0.117	72.93	62.74
34	0.294	0.103	0.13	64.97	55.78
35	0.296	0.089	0.113	69.93	61.82
36	0.304	0.105	0.166	65.46	45.39
37	0.347	0.09	0.12	74.06	65.42
38	0.345	0.131	0.159	62.03	53.91
39	0.364	0.105	0.138	71.15	62.09
40	0.318	0.14	0.168	55.97	47.17
41	0.39	0.108	0.13	72.31	66.67
42	0.328	0.089	0.155	72.87	52.74
43	0.403	0.112	0.163	72.21	59.55
44	0.345	0.103	0.162	70.14	53.04
45	0.34	0.12	0.15	65.35	55.41
Mean				65.95	56.12

a pre: the rabbit sera before immunized with the proteins; r&nB5: the rabbit sera obtained by immunized with nBos d 5; r& B5M: the rabbit sera by immunized with B5M.

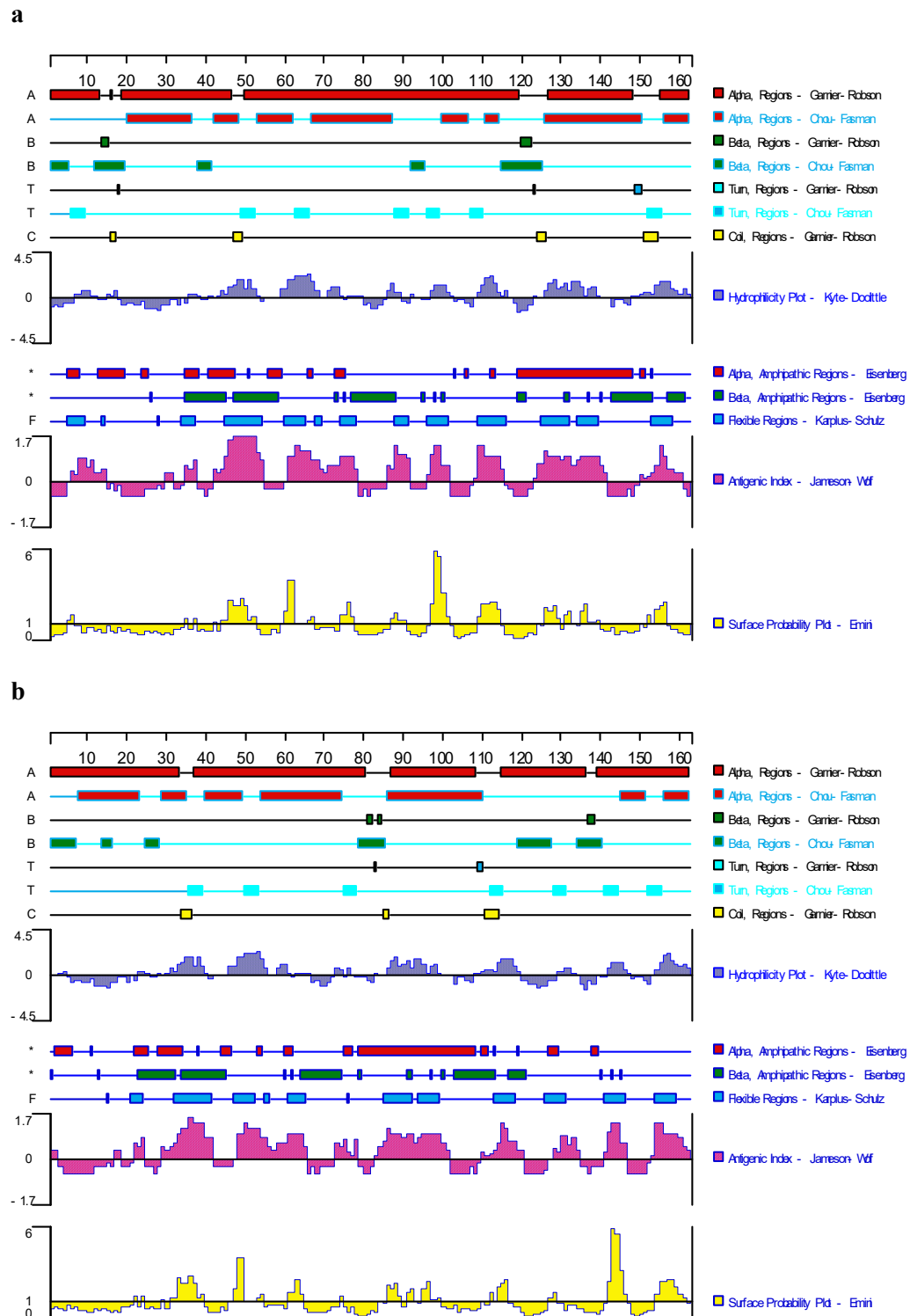


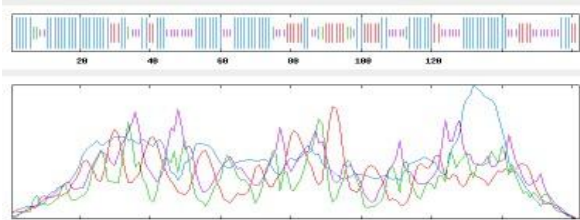
Fig S1. The secondary components of Bos d 5 (a) and B5M (b) were predicted by DNASTar.

LIVTQTMKGLDIIQKVAGTWYSLAMAAASDTSLLDAQSAPLRVYVEELKPTPEGDL EILLQKWENDECAQKK
eehhhhhhhtccchhhhhhhhhhhhhhhhhhhhhheehhtccchheehhhccccccccchhhhhhhhhccchhhhh
IIAEKTKIPAVFKIDALNENKVLVLDTDYKKYLLFCMENSAEPEQSLVCQCLVRTPEVDDEALEKFDKAL
hhhhhhhtccccceeeehhctttteeeeettchheeeehhcccccthhhhhhheecccccchhhhhhhhhhh
KALPMHIRLSFNPTQLEEQCHI
hhccccccccccccchhhee

Sequence length : 162

SOPMA :

Alpha helix	(Hh) :	78 is	48.15%
3 ₁₀ helix	(Gg) :	0 is	0.00%
Pi helix	(Ii) :	0 is	0.00%
Beta bridge	(Bb) :	0 is	0.00%
Extended strand	(Ee) :	31 is	19.14%
Beta turn	(Tt) :	9 is	5.56%
Bend region	(Ss) :	0 is	0.00%
Random coil	(Cc) :	44 is	27.16%
Ambiguous states (?) :		0 is	0.00%
Other states	:	0 is	0.00%



20 40 60 80 100 120

20 40 60 80 100 120

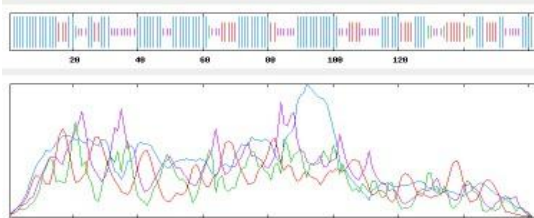
10 20 30 40 50 60 70

KVAGTWYSLAMAASDISLDAQSAPLRVYVEELKPTPEGDLLEILLQKWENDECAQKKIIAEKTKIPAVFK
c h h h h h h h h h h h h h e e e h t c c c h h e e h h h c c c c c c c h h h h h h h c c c h h h h h h h h h t c c c e e e e
IDALNENVQCLVRTPEVDDEALEKFDKALKALPMHIRLSFNPTQLEEQCCHILIVTQTKMGKLDIQKVLVL
e h h h h h h h h h h e e c c c c c c h h h h h h h h h h h c c c e e e e c c c c c c h h h h h h e e e e h h h t t c c c t e e e e
D TDYKKYLLFCMENSAPAEQSL
e t t c h h h e e e e h h c c c c c h h h h

Sequence length : 162

SOPMA :

Alpha helix (Hh) :	83 is	51.23%
3 ₁₀ helix (Gg) :	0 is	0.00%
Pi helix (Ii) :	0 is	0.00%
Beta bridge (Bb) :	0 is	0.00%
Extended strand (Ee) :	30 is	18.52%
Beta turn (Tt) :	7 is	4.32%
Bend region (Ss) :	0 is	0.00%
Random coil (Cc) :	42 is	25.93%
Ambiguous states (?) :	0 is	0.00%
Other states :	0 is	0.00%



20 40 60 80 100 120

20 40 60 80 100 120

6