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Evaluation of cross-reactivity between casein components using inhibition assay and *in silico* analysis

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The datasets generated and analyzed during this study are not publicly available owing to privacy protection in relation to personal data; however, they are available from the corresponding author upon reasonable request.

ABSTRACT

Background: We previously reported that the specific IgE levels to α s1-casein (CN) and β -CN in patients with cow's milk allergy decreased with similar dynamics during oral immunotherapy. Therefore, we hypothesized that α s1- and β -CN have strong cross-reactivity among CN components, despite the low similarity in the full-length amino acid sequences.

Methods: The α s1-, β -, and κ -CN were purified from commercial cow's milk. We recruited 39 patients with cow's milk allergy and the serum IgE levels for each CN component were measured by enzyme-linked immunosorbent assay (ELISA). Cross-reactivity between CN components was investigated by competitive ELISA against α s1-CN. Sequence homology between CN components at the peptide level was calculated using *in silico* analysis and quantified by the Property Distance (*PD*) value.

Results: The α s1-CN-specific IgE levels exhibited a strong positive correlation with the β -CN-specific IgE ($r = 0.945$, $P < 0.001$). Complete competition was observed by β -CN against α s1-CN, suggesting the presence of common epitopes between them. *In silico* analysis detected 24 peptide sets with *PD* values lower than 10 between α s1- and β -CN, and 14 sets between α s1- and κ -CN. The amino acid sequences of α s1- (E61-E70) and β -CN (I12-E21) that showed the lowest *PD* value (5.30) were present in the characteristic sequence known as casein phosphopeptide (CPP).

Conclusion: We detected strong cross-reactivity between CN components. Furthermore, we found highly homologous sequences in the CPP region, which contains a core sequence of "SSSEE" with phosphorylated serine residues.

Key Message

The overall homology among casein (CN) components is extremely low, but α s1-CN and β -CN show cross-reactivity. Based on these results, there is a need for detailed examination of the

allergenicity of casein phosphopeptides, which includes common sequences of α s1-CN and β -CN.

Keywords: milk protein, casein, cross-reactions, enzyme-linked immunosorbent assay, *in silico* analysis

1. INTRODUCTION

Casein (CN), a major allergen for patients with persistent cow's milk (CM) allergy, comprises approximately 80% of CM proteins.¹ Bovine CN is constituted of four components, α 1-, α 2-, β -, and κ -CN, and exists as a micelle with a 30–300 nm diameter.² CN is an unfolded phosphoprotein and interacts with calcium phosphate in milk.³ α s-CN is defined as an insoluble fraction in 0.4 M calcium chloride, while κ -CN is the soluble fraction. β -CN is defined as soluble in 3.3 M urea and insoluble in 1.7 M urea. Bovine α 1-, α 2-, and β -CN are comprised of 199, 207, and 209 residues, respectively, and are calcium-sensitive.³ Bovine κ -CN is a glycoprotein comprised of 169 residues and is not calcium-sensitive. The overall homology of amino acid sequences among CN components is extremely low (Table S1).

We previously reported that specific IgE levels to purified α 1-CN and β -CN, but not κ -CN, showed simultaneous decreases during CM oral immunotherapy.⁴ Therefore, we hypothesized that α 1- and β -CN have cross-reactive epitopes, or alternatively, these two CNs might share remarkably similar antigenic properties. In this study, we investigated cross-reactivity among CN components.

Furthermore, Candreva et al. analyzed the *in silico* similarity between proteins at the peptide level.⁵ Cross-reactivity between milk and soy proteins has been suggested by other immunological studies.⁶ Thus, we also employed an *in silico* analysis at the peptide level using the peptide similarity tool on the Structural Database of Allergenic Proteins (SDAP)⁷.

2. METHODS

2.1 Materials

Each CN component (α 1-, β -, and κ -CN) was prepared following the previously described methods.⁴ However, α 2-CN could not be purified owing to its extremely low content in CM, and

purified α s2-CN was highly contaminated with other proteins.

2.2 Serum samples

This study used serum from 39 patients [median age 8 years (IQR 6–10 years)], from a previous study, with persistent CM allergy before oral immunotherapy.^{4,8} These patients were required to have retained their milk allergy over age 5 with the threshold dose less than 5 mL of CM confirmed by an oral food challenge test, and detectable specific IgE (>0.34 UA/mL) to CM and whole CN by ImmunoCAP®.

2.3 Enzyme-linked immunosorbent assay (ELISA) for CN component specific IgE levels

The α s1-, β -, and κ -CN-specific IgE levels were evaluated by indirect ELISA.⁴ The relative immunoglobulin levels (%) for each sample were calculated based on the reference serum with high α s1-CN-specific immunoglobulin levels. These measured values were used in this study.

2.4 Competitive ELISA

The inhibition rates of β - and κ -CN against α s1-CN-specific IgE were evaluated by competitive ELISA. The 96-well microtiter plates (Nunc-Immuno™ Plate II, Thermo Fisher Scientific) were coated with α s1-CN [5 μ g/mL in phosphate-buffered saline (PBS)] and incubated overnight at 4°C. After washing the plates with PBS containing 0.05% Tween 20 (PBS-T), the plates were blocked with Protein-Free Blocking Buffer (Thermo Fisher Scientific, Roskilde, Denmark) containing 0.05% Tween 20 (PFBB-T) for 2 h at 4°C. Patient sera ($n = 39$) were diluted 1:5 in PFBB-T and pre-incubated with inhibitor (α s1-, β -, and κ -CN diluted in PBS at the final concentrations of 0.001–100 μ g/mL). The pre-incubated sera were added to each well and incubated overnight at 4°C. After washing each well with PBS-T, bound antibodies were detected

with alkaline phosphatase-conjugated goat anti-human IgE (Bethyl Laboratories, Montgomery, AL, USA; diluted 1:1,000 in PFBB-T) for 1.5 h at 37°C. After washing each well with PBS-T and PBS, PNPP substrate (Thermo Fisher Scientific) was added. The reaction was stopped by the addition of 2 N NaOH and the absorbance was measured at 405 nm.

To confirm the absence of non-specific binding, the inhibition rates of α s1-, β -, and κ -CN were evaluated by competitive ELISA to egg white protein (EWP) using sera from patients (n = 5) with egg allergy. These sera have specific IgE to egg (median 87.5 UA/mL) and do not have specific IgE to CM (median 0.35 UA/mL) or CN (median 0.35 UA/mL). Ovalbumin was used as a control for specific binding.

The inhibition ratio (%) was calculated using the following formula:

$$\text{Inhibition rate (\%)} = 100 - [(\text{Inhibitor} - \text{NC})/(\text{PC} - \text{NC})] \times 100$$

Inhibitor: absorbance of inhibitor solution; NC: Negative control, absorbance of PFBB-T; PC: Positive control, absorbance of serum without inhibitor

2.5 Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

The purity of the isolated α s1-CN and contamination of isolated β -CN was confirmed by SDS-PAGE and immunoblotting. The gel (15% polyacrylamide) was stained with Coomassie Brilliant Blue R-250 (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan). For immunoblotting, proteins were transferred to polyvinylidene difluoride membranes. After blocking (1% w/v bovine serum albumin in Tris-buffered saline), α s1-CN was detected by anti- α -casein mouse monoclonal antibody (Cosmo Bio, Tokyo, Japan; out of production) and peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch, PA, USA).

2.5 Bioinformatics analysis

Sequence similarity was measured by the property distance (*PD*) value using the physicochemical properties of amino acids rather than their substitution frequencies in related proteins. Peptides with *PD* values lower than 10 are considered to have significant physicochemical similarities, whereas much higher *PD* values imply unrelated peptides. To quantify the similarity between α 1-CN and the other CN components, the *PD* value was calculated using the peptide similarity tool on SDAP (http://Fermi.utmb.edu/SDAP/sdap_pdi.html).⁷ In brief, the sequential peptides consisting of 10 amino-acids, overlapping by seven (three-offset), in the primary sequence of α 1-CN were applied to the amino-acid sequences of β - and κ -CN. In addition, to identify the putative cross-reactive peptides, α 1-CN-specific IgE epitopes reported by the previous studies were also applied to the amino acid sequences of α 2-, β -, and κ -CN.

2.6 Statistical analysis

Correlations among α 1-, β -, and κ -CN-specific IgE were analyzed by Pearson's correlation analysis. Differences among the inhibition rate of CN components were analyzed by one-way analysis of variance (ANOVA). Calculations were performed with EZR software produced by the Jichi Medical University Saitama Medical Center (Saitama, Japan).⁹ Data are expressed as the mean $\pm$ standard deviation (SD). Differences with *P* values of less than 0.05 were considered statistically significant.

2.7 Ethics statement

This study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents; patient's assent was also obtained at the time of oral food

challenge test, including the use of stocked sera for research purposes. This current study, which used the stocked sera, was again approved by the institutional ethical committee at Aichi Children's Health and Medical Center (#201669) and at Nagoya University of Arts and Sciences (#200).

3. RESULTS

3.1 Correlations between casein components-specific immunoglobulins

Correlations between immunoglobulin levels of each CN component are shown in Figure 1. The α s1-CN-specific IgE levels exhibited a strong positive correlation with the β -CN-specific IgE ($r = 0.945$, $P < 0.001$, Figure 1A). κ -CN-specific IgE levels showed relatively lower correlations between α s1-CN-specific IgE levels ($r = 0.57$, $P < 0.001$; Figure 1B) and β -CN-specific IgE levels ($r = 0.616$, $P < 0.001$; Figure 1C). These results suggested that the patients with CM allergy were independently sensitized to α s1- and β -CN, and/or a cross-reactive epitope exists between these two CNs.

Moreover, α s1-, β -, and κ -CN-specific IgG4 and IgA levels were also investigated (Figure S1). The specific IgG4 levels showed the same tendency with IgE. However, IgA levels showed that not only β -CN, but also κ -CN had a strong correlation with α s1-CN.

3.2 Cross-reactivity of α s1-casein with casein components

To confirm whether a cross-reactive epitope existed between the α s1- and β -CN components, we performed competitive ELISA of β -CN against α s1-CN-specific IgE (Figure 2). Complete inhibition was observed at high concentrations of β -CN (10–100 μ g/mL, Figure 2A). The half-maximal inhibitory concentration (IC_{50}) of the β -CN (0.160 μ g/mL) was about 10 times higher than that of α s1-CN itself (0.0139 μ g/mL), suggesting differences in the number or the

affinity of IgE epitopes. On the other hand, the IC_{50} of κ -CN (13.6 $\mu\text{g/mL}$) was about 1,000 times higher than that of $\alpha\text{s}1$ -CN, and the maximum inhibition rate remained 54.5% at the highest concentration (Figure 2B). These results suggested the possibility that a cross-reactive epitope existed between $\alpha\text{s}1$ - and β -CN.

To exclude the possibility that $\alpha\text{s}1$ -CN was contaminated with the purified β -CN, we performed SDS-PAGE and immunoblotting using α -casein-specific monoclonal antibody. Even with ten times more protein supplementation of β -CN by SDS-PAGE, no bands were detected by $\alpha\text{s}1$ -CN-specific immunoblotting (Figure S2). Therefore, the possibility of protein contamination was unlikely. Additionally, non-specific binding for CN components to EWP was not detectable (Figure 2C).

3.3 *In silico* analysis of the partial similarity of amino acid sequence

Using the SDAP tool, we detected 24 peptide sets between $\alpha\text{s}1$ - and β -CN and 14 sets between $\alpha\text{s}1$ - and κ -CN that showed PD values lower than 10 (Table 1). The lowest PD value, 5.30, was detected between amino acid sequences of $\alpha\text{s}1$ -CN (E61–E70) and β -CN (I12–E21). On the other hand, the lowest PD value between $\alpha\text{s}1$ -CN and κ -CN was 8.47. Furthermore, we found 38 peptide sets with PD values lower than 10 between $\alpha\text{s}1$ - and $\alpha\text{s}2$ -CN, 34 sets between $\alpha\text{s}2$ - and β -CN, 19 sets between $\alpha\text{s}2$ - and κ -CN, and 18 sets between β - and κ -CN (Table S2).

In the above results, the domains where PD values were low existed between αs - and β -CN. The peptide sets with the lowest PD values between each CN component are shown in Table 2. As a result, $\alpha\text{s}1$ -CN had a particularly low PD value against β - and κ -CN and the amino acid compositions in these peptides were identical in 7/10 and 3/10 residues, respectively. On the other hand, identical amino acid compositions of $\alpha\text{s}2$ -CN against $\alpha\text{s}1$ - and β -CN were 6/10 and 7/10 residues, respectively. Of note, these peptides included the particular "SSSEE" amino acid

sequence.

3.4 *In silico* analysis of known IgE epitopes of CN components

To identify the putative cross-reactive epitope peptides in α 1-CN, we also applied *in silico* analysis on the previously reported IgE epitopes of α 1-CN¹⁰⁻¹³ against the amino acid sequences of α 2-, β -, and κ -CN. Unexpectedly, we could only find six peptide sets that showed *PD* values lower than 10 (Table 3). Furthermore, the lowest *PD* value of 7.16 was detected in α 2-CN.

We arranged the amino acid sequences of casein components and filled the reported IgE epitopes in order to visualize the location (Figure 3).¹⁰⁻¹⁵ The four “SSSEE” motifs (bold in Figure 3), which is the core motif of casein phosphopeptide (CPP), were identified, but the reported IgE epitopes were scarcely located on the “SSSEE” motif except one located at the N-terminus of α 2-CN.

4. DISCUSSION

The purpose of this study was to investigate the cross-reactivity among CN components. The data clearly indicated that IgE (also IgG4 and IgA) specific to α 1- and β -CN were strongly associated, and the presence of strong cross-reactivity was confirmed using competitive ELISA. We also found lower cross-reactivity between α 1-CN and κ -CN.

Bernard et al. reported that the sum of the IgE levels to the different CN components was higher than the IgE levels to whole CN, suggesting the presence of cross-reactive epitopes between the CN components.¹⁶ Generally, cross-reactivity is expected between proteins with high sequence homology.¹⁷ However, an article which compiled cross-reactivities between non-homologous allergens was recently reported.¹⁸ With regard to CN, the homology of full-length amino acid sequences between CN components is extremely low (4–7%). To assess

this theoretical gap in knowledge, we employed *in silico* analysis to find the common peptide sequences in each CN component and found highly homologous amino acid sequences in α s1-, α s2-, and β -CN. The sequences commonly included the unique “SSSEE” motif, known as the basic structure of CPP.

During CN digestion, κ -CN, which covers the surface of CN micelles, is broken down and subsequently the other CNs too.¹⁹ CN is well known as a protein with high digestibility.²⁰ However, the fact that CN is a major allergen suggests that the major IgE epitope should be placed in the digestion-resistant structure.²¹ CPP, which results from the digestion of CN, has been identified as a trypsin-resistant peptide due to the highly phosphorylated serine residues in and around the core structure of the “SSSEE” motif. CPP plays a key role in the effective absorption of calcium due to the formation of micellar calcium phosphate at the phosphorylated serine residues. Based on this nutritional function, CPP is widely used medically to prevent osteoporosis and in dental care to ameliorate the calcium deposition on teeth. Remarkably, dental treatments using small amounts of CPP paste have the potential to be allergenic and cause anaphylaxis. This clinical fact suggests that CPP presents potential allergenic activity. CPP was identified in α s1-CN (E61–K79), α s2-CN (K1–Y20 and N46–K70), and β -CN (R1–R25) by tryptic hydrolysis (Figure 3).²²

In this study, we did not find strong cross-reactivity or detect a highly homologous amino acid sequence between α s1-CN and κ -CN. The CPP motif does not exist in κ -CN, and the C-terminal half of κ -CN consists of a unique water-soluble glycoprotein designated as a glycomacropeptide.²³ Epitope-mapping of κ -CN revealed many IgE binding sites, which might have individual allergenic properties with low cross-activity between α s- and β -CNs. Epitope-mapping of whole CN components has been extensively performed worldwide, using overlapping peptides and peptide microarray techniques.¹⁰⁻¹³ Nonetheless, the reported IgE-epitopes are different with each

other, and no consensus of the dominant IgE epitope has been established. Furthermore, prior to this study, cross-reactive epitopes among CN components have not been reported.

Recently, IgE- and IgG4-epitope mapping was carried out to develop a prediction tool for the prognosis of CM allergy.²⁴⁻²⁸ However, in these reports, there were few epitope sequences located in the CPP regions and no common epitopes were found in the CPP regions on the different CN components. We proposed the reason that IgE binding ability to synthetic peptide is low compared with natural CN is because serine residues were not phosphorylated in the synthetic peptides.

Generally, IgE-binding epitopes have hydrophilic properties and are present on the surface of a granular protein molecule. Phosphorylated serine residues comprise the water-soluble part, and low phosphorylated residues comprise the water-insoluble part of the CN molecule. Otani et al. reported that the IgG binding ability to dephosphorylated α s1-CN was lower than that to native α s1-CN.²⁹ Furthermore, while *O*-phospho-L-serine inhibited IgG binding, L-serine scarcely inhibited binding, thereby indicating the importance of the phosphorylated serine residue in the epitope. Therefore, phosphorylated serine residues in the CPP motifs might have the critical role in the formation of the dominant and cross-reactive IgE epitopes in the native CN components.

A major limitation of this study was that we could not examine cross-reactivity with α s2-CN due to difficulty in purification. Additionally, we suggest the presence of cross-reactive epitopes between α s1-CN and β -CN based on bioinformatics finding of the presence of highly homologous amino acid sequences in the CPP regions; the hypotheses we have generated from the *in silico* findings need to be tested experimentally.

In conclusion, despite the overall homology among CN components being extremely low, α s1-CN shows cross-reactivity with β -CN. The sequence of “SSSEE”, which is a basic structure of CPP, may have a potential of being the cross-reactive epitope.

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Author Contributions

MN, TM, KI, and HI designed the study and drafted the manuscript. TM, KT, and KI collected and sorted the serum samples. MN, CY, and HI performed ELISA and the inhibition assay. MN performed *in silico* analysis and statistical analysis. TM and KI reviewed the manuscript. All authors approved the final submitted manuscript.

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Table 1. Putative peptides similarity between α 1-casein and β -casein and between α 1-casein and κ -casein (*in silico* analysis)

Amino acid		PD value*	Amino acid		PD value*
α 1-Casein	β -Casein		α 1-Casein	κ -Casein	
7–16	147–156	8.46	13–22	1–10	9.89
7–16	169–178	9.98	40–49	79–88	8.94
22–31	113–122	9.85	67–76	134–143	9.63
28–37	89–98	7.72	70–79	77–86	8.47
31–40	92–101	8.23	73–82	80–89	9.27
34–43	20–29	8.94	79–88	112–121	8.63
40–49	70–79	9.75	88–97	139–148	9.12
46–55	33–42	9.80	94–103	4–13	9.74
46–55	35–44	9.96	100–109	113–122	9.88
46–55	38–47	8.80	103–112	116–125	8.98
49–58	41–50	8.08	106–115	73–82	9.40
55–64	28–37	9.77	172–181	115–124	9.77
61–70	12–21	5.30	175–184	118–127	9.67
61–70	29–38	9.63	190–199	113–122	9.46
64–73	15–24	5.38			
79–88	1–10	9.10			
94–103	19–28	9.01			
97–106	161–170	8.44			
109–118	13–22	9.85			
112–121	66–75	9.57			

115–124	18–27	9.58
118–127	14–23	7.87
124–133	99–108	8.51
139–148	27–36	9.67

*performed by Structural Database of Allergenic Proteins (SDAP,
<http://Fermi.utmb.edu/SDAP/>).

Each peptide has *PD* value lower than 10 is shown.

PD, property distance.

Table 2. The combination of partial amino acid sequence which has lowest *PD* value between each casein

	α s2-Casein	β -Casein	κ -Casein
α s1-Casein	α s1 61 EAESISSSEE 70	α s1 61 EAESISSSEE 70	α s1 70 EIVPNSVEQK 79
	α s2 3 TMEHVSSSEE 12	β 12 IVESLSSSEE 21	κ 77 QVLSNTVPAK 86
	<i>PD</i> 5.60	<i>PD</i> 5.30	<i>PD</i> 8.47
α s2-Casein		α s2 4 MEHVSSSEES 13	α s2 19 TYKQEKDMAI 28
		β 13 VESLSSSEES 22	κ 41 NYYQQKPFAL 50
		<i>PD</i> 3.74	<i>PD</i> 7.76
β -Casein			β 163 LSLSQSKVLP 172
			κ 48 VALINNQFLP 57
			<i>PD</i> 8.58

The matched amino acids are underlined.

PD, property distance.

Table 3. Identification of putative cross-reactive peptides of α s1-casein-specific IgE

epitopes				
Ref.	α s1-casein-specific IgE	<i>PD</i> value*		
	epitopes (amino acid)	α s2-Casein	β -Casein	κ -Casein
10	17–36	12.75	12.91	13.82
	39–48	9.50	10.52	10.20
	69–78	9.07	10.98	10.20
	83–102	11.90	10.05	11.86
	109–120	10.11	11.96	12.41
	123–132	10.57	9.82	11.89
	139–154	12.24	12.34	12.72
	159–174	14.45	12.48	13.99
	173–194	12.58	12.81	12.50
11	19–30	11.10	10.51	12.73
	93–98	7.16	7.88	7.63
	141–150	10.87	12.15	12.08
12	6–20	13.40	12.86	12.30
	11–25	13.21	12.03	11.56
	126–140	11.92	12.01	10.28
	171–185	12.90	11.98	10.19
13	28–50	12.44	13.04	14.21

*performed by Structural Database of Allergenic Proteins (SDAP, <http://Fermi.utmb.edu/SDAP/>).

The α s1-CN-sIgE epitopes were referred. Each peptide is shown on the smallest *PD* value.

PD, property distance.

Figure Legends

Figure 1

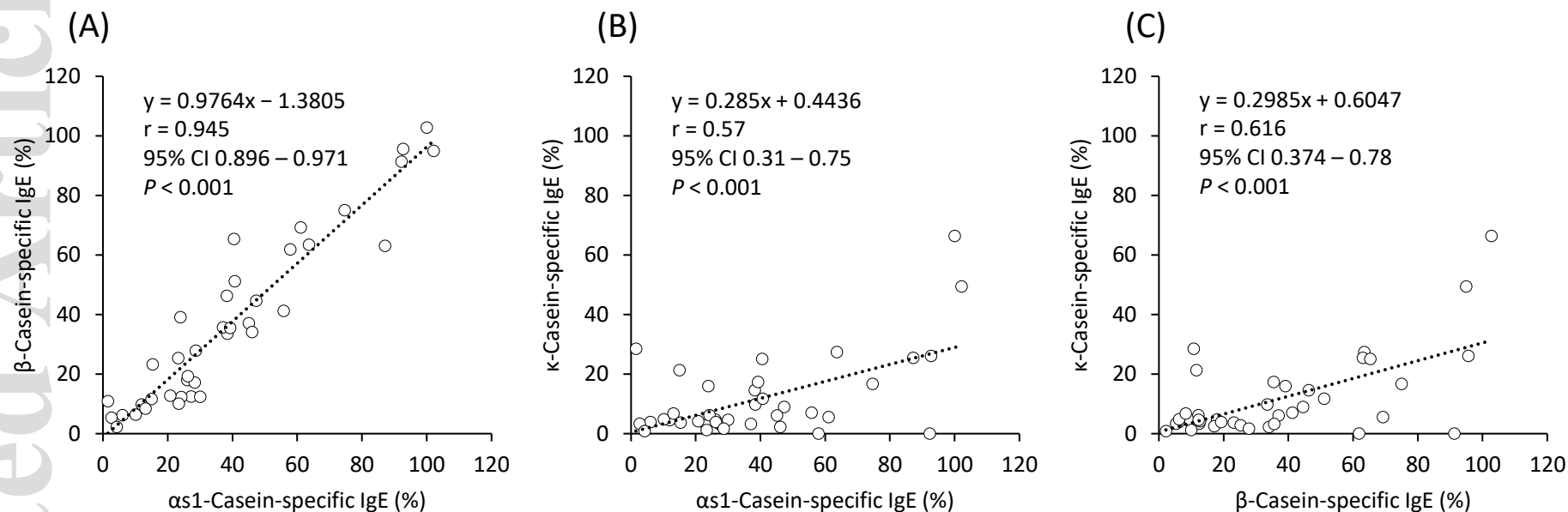
Specific-IgE levels (%) to α s1- and β -casein (A), α s1- and κ -casein (B), and β - and κ -casein (C) were plotted. Correlation analysis was performed by linear regression.

Figure 2

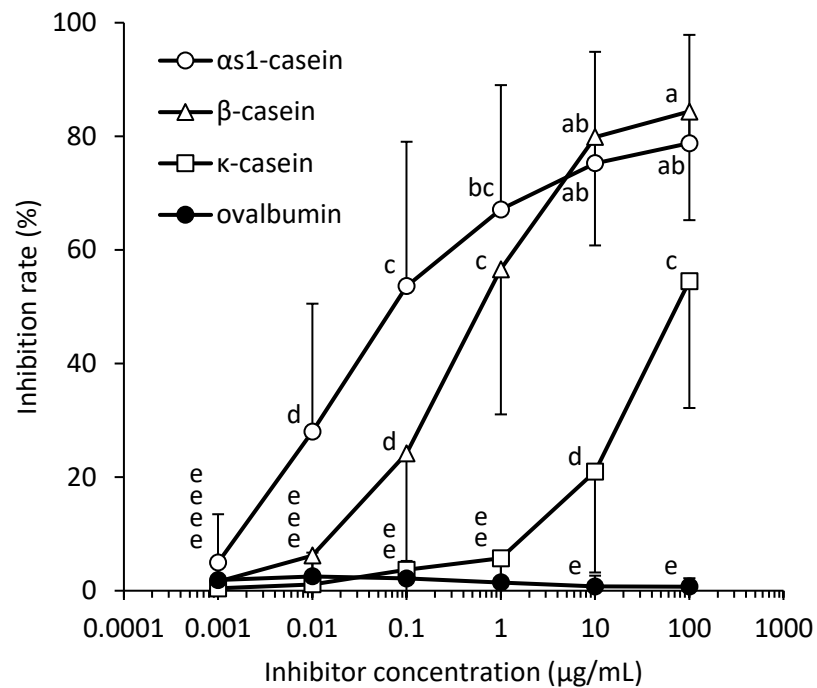
The cross-reactivity of α s1-casein was determined by competitive ELISA ($n = 39$) (A). IC_{50} indicates the concentration of inhibitor that causes a 50% inhibition of antibody binding (B). Non-specific binding of casein components was evaluated by competitive ELISA to egg white proteins using sera from patients with egg allergy ($n = 5$) (C). α s1-Casein (open circles), β -casein (triangles), κ -casein (squares), and ovalbumin (closed circles) were used as inhibitors. The right panel display corresponds to the area indicated on the left panel.

Figure 3

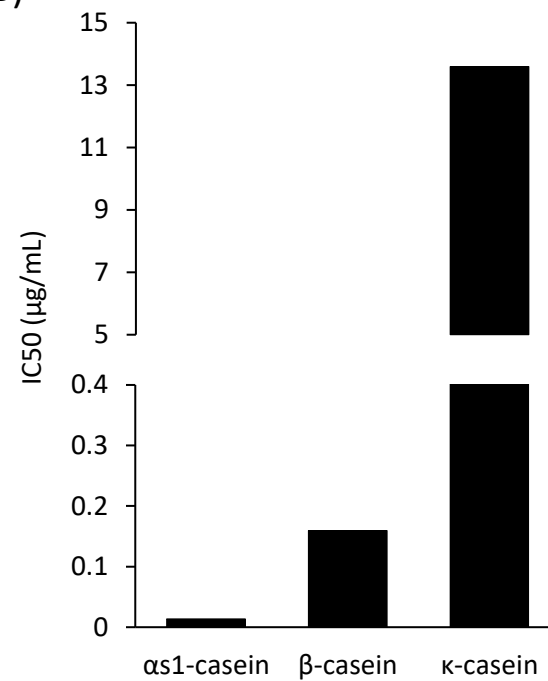
Schematic representation of the amino acid sequence of each casein component and determined peptides. Reported IgE epitopes are shown by the dotted line (ref. 10), broken line (ref. 11), solid line (ref. 12), dotted line and triangle (ref. 13), broken line and triangle (ref. 14), and solid line and triangle (ref. 15). Casein phosphopeptide (CPP), which is identified by trypsin digestion, is shown by the shaded region. The amino acid sequence, SSSEE, is shown in bold.



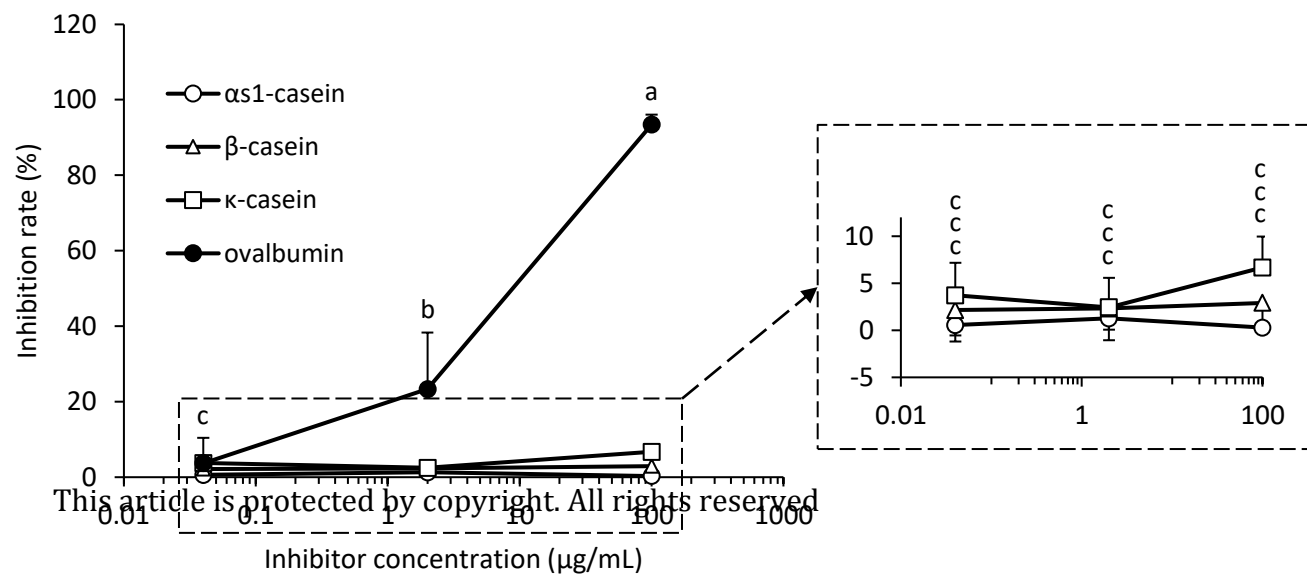
(A)



(B)



(C)



α1-Casein

RPKHPIKHQGLPQEVLNENLLRFFVAPFPPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSVEQKHIOKEDVPSELYLGTLEQLLR 100
LKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSGKTTMPLW 199

α2-Casein

KNTMEHVSSSEESIISOETKQEKMAINPSKENLCSSTFCKEVVRNANEEYSIGSSSEESAEVATEEVKITVDDKHQKALNEINQFYQKFPQYLQYLY 100
QGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVEFTKTKLTETEEKNRLNFLKKISQRYQKFALPQYLKTVYQHOKAMKPWIQPKTKV 200
IPYVRYL 207

β-Casein

RELEELNVPGEIVESISSSEESITRINKKIEKFQSEEQQQTEDELQDKIHQFAQTQSLVYPFPGPIPNLPPQNIPLTQTPVVVPPFLQPEVMGVSKVKE 100
AMAPKHKEMPFPKYPVEPFQTESQSLTLTDVENLHLLPLPLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGP 200
VRGPFPIIV 209

κ-Casein

QEONQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYAKPAAVRSPAQILQWQVLSNTVPAKSCQAQPTTMARHPH 100
PHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDSPEVIESPPEINTVQVTSTAV 169

Table S1. Comparisons of amino-acid sequence homology (%) of bovine caseins

	α 2-Casein	β -Casein	κ -Casein
α 1-Casein	7	4	4
α 2-Casein	—	5	5
β -Casein	—	—	7

performed by DNA Data Bank of Japan (DDBJ, <http://clustalw.ddbj.nig.ac.jp/>).

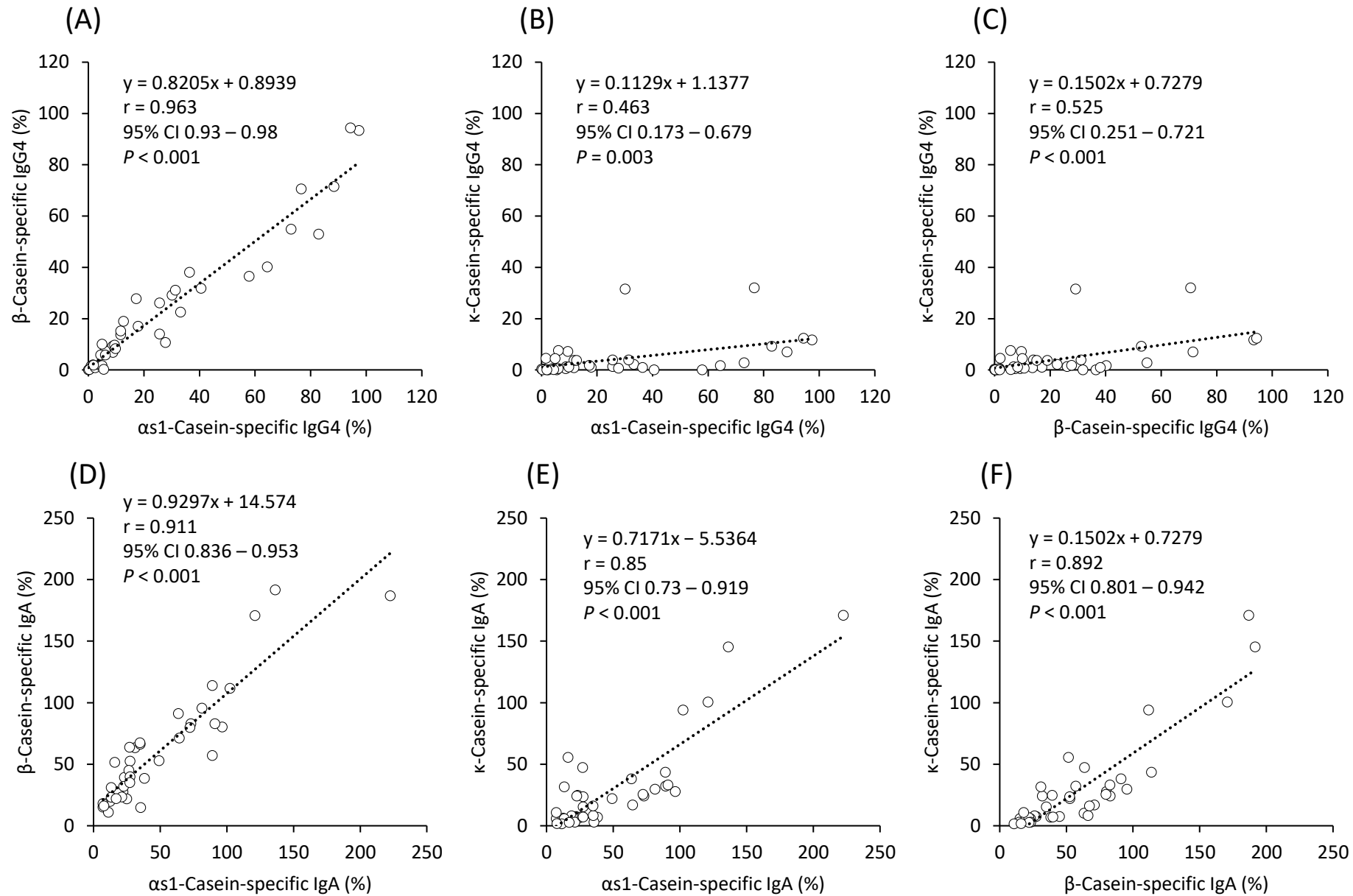


Figure S1

Specific-IgG4 (A, B, and C) and specific-IgA (D, E, and F) levels (%) to α 1- and β -casein (A and D), to α 1- and κ -casein (B and E), and β - and κ -casein (C and F) were plotted. Correlation analysis was performed by linear regression.

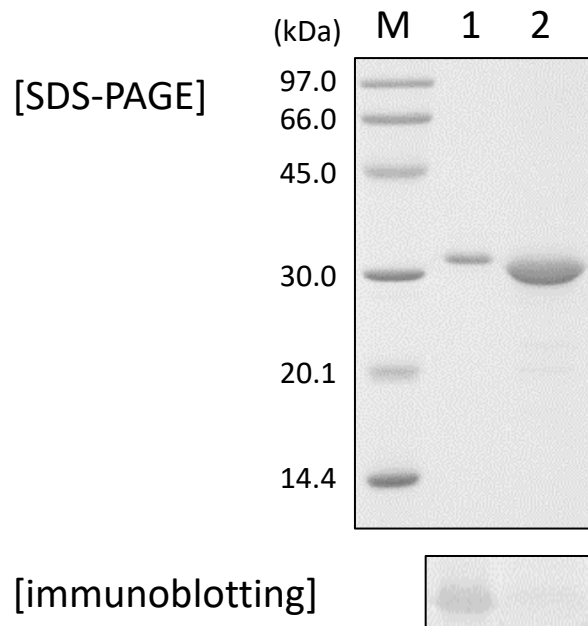


Figure S2

The purity of the isolated α s1-CN and contamination of isolated β -CN was confirmed by SDS-PAGE and immunoblotting. Lane M, molecular weight marker; lane 1, α s1-casein (1 μ g); lane 2, β -casein (10 μ g).

Table S2. Putative peptides similarity between α 1-CN and α 2-CN (*in silico* analysis)

Amino acid		PD value*	Amino acid		PD value*
α 1-Casein	α 2-Casein		α 1-Casein	α 2-Casein	
10–19	122–131	9.3	67–76	130–139	9.78
34–43	41–50	9.99	70–79	42–51	9.07
34–43	150–159	8.67	73–82	70–79	9.15
37–46	47–56	9.29	76–85	15–24	9.33
43–52	53–62	7.66	76–85	73–82	7.86
46–55	58–67	9.14	79–88	110–119	9.92
49–58	61–70	8.89	79–88	121–130	9.60
52–61	2–11	9.36	100–109	170–179	8.66
52–61	80–89	9.33	109–118	4–13	8.71
55–64	126–135	9.89	109–118	41–50	8.72
61–70	3–12	5.60	112–121	54–63	8.77
61–70	10–19	9.00	115–124	9–18	9.03
61–70	24–33	9.97	118–127	5–14	8.34
61–70	50–59	7.00	121–130	8–17	9.62
61–70	51–60	9.56	124–133	41–50	9.94
61–70	124–133	6.38	148–157	145–154	9.93
64–73	6–15	7.92	148–157	178–187	9.52
64–73	127–136	9.78	187–196	130–139	7.73
67–76	10–19	9.85	187–196	131–140	9.49

Table S2 (Continued). Putative peptides similarity between α 2-CN and β -CN (*in silico* analysis)

Amino acid		PD value*	Amino acid		PD value*
α 2-Casein	β -Casein		α 2-Casein	β -Casein	
1–10	10–19	9.72	112–121	173–182	9.39
4–13	13–22	3.74	118–127	65–74	9.63
7–16	16–25	4.55	124–133	12–21	7.18
10–19	19–28	9.15	124–133	34–43	9.77
16–25	35–44	8.48	124–133	35–44	8.63
28–37	16–25	8.88	127–136	15–24	6.82
52–61	13–22	8.75	136–145	28–37	9.74
55–64	16–25	9.02	145–154	117–126	8.31
58–67	35–44	8.48	148–157	35–44	8.06
82–91	23–32	9.71	151–160	31–40	8.29
85–94	23–32	9.52	166–175	25–34	7.26
85–94	26–35	9.35	166–175	44–53	9.27
94–103	56–65	9.95	169–178	47–56	9.80
94–103	72–81	9.47	181–190	169–178	9.77
97–106	75–84	8.18	181–190	176–185	9.42
100–109	78–87	6.70	196–205	104–113	9.83
103–112	81–90	9.09	196–205	166–175	8.86

Table S2 (Continued). Putative peptides similarity between α 2-casein and κ -casein (*in silico* analysis)

Amino acid		PD value*	Amino acid		PD value*
α 2-Casein	κ -Casein		α 2-Casein	κ -Casein	
1–10	133–142	8.97	88–97	18–27	9.46
1–10	140–149	9.99	97–106	41–50	9.15
16–25	5–14	9.61	115–124	24–33	9.47
19–28	41–50	7.76	115–124	160–169	9.08
22–31	44–53	9.41	118–127	27–36	9.71
25–34	105–114	8.03	121–130	157–166	9.10
37–46	132–141	9.71	133–142	3–12	9.34
64–73	134–143	9.25	136–145	159–168	9.92
79–88	116–125	9.89	198–207	53–62	9.81
85–94	28–37	9.98			

Table S2 (Continued). Putative peptides similarity between β -casein and κ -casein (*in silico* analysis)

Amino acid		PD value*	Amino acid		PD value*
β -Casein	κ -Casein		β -Casein	κ -Casein	
10–19	136–145	9.55	121–130	140–149	8.66
13–22	122–131	9.05	121–130	158–167	9.63
13–22	139–148	9.78	130–139	51–60	9.46
13–22	143–152	9.85	142–151	94–103	9.64
28–37	12–21	9.35	160–169	141–150	8.88
31–40	15–24	9.96	163–172	48–57	8.58
37–46	1–10	8.98	166–175	51–60	9.57
37–46	111–120	9.68	172–181	50–59	9.15
40–49	13–22	8.80	199–208	65–74	9.82

*performed by Structural Database of Allergenic Proteins (SDAP, <http://Fermi.utmb.edu/SDAP/>). Each peptide has PD value lower than 10 is shown. PD, property distance.