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

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June 25, 2020

Abstract

Background: We previously reported that the specific IgE levels to αs1-casein (CN) and β-CN in patients with cow's milk allergy decreased with similar dynamics during oral immunotherapy. Therefore, we hypothesized that αs1- and β-CN have strong cross-reactivity among CN components, despite the low similarity in the full-length amino acid sequences. Methods: The αs1-, 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 αs1-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 αs1-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 αs1-CN, suggesting the presence of common epitopes between them. In silico analysis detected 24 peptide sets with PD values lower than 10 between αs1- and β-CN, and 14 sets between αs1- and κ-CN. The amino acid sequences of αs1- (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.

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, αs1-, αs2-, 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] αs-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 αs1-, αs2-, 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 homology of amino acid sequences among CN components is extremely low (Table S1).

We previously reported that specific IgE levels to purified αs1-CN and β-CN, but not κ-CN, showed simultaneous decreases during CM oral immunotherapy.[4] Therefore, we hypothesized that αs1- 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

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also employed an *in silico* analysis at the peptide level using the peptide similarity tool on the Structural Database of Allergenic Proteins (SDAP).

METHODS

2.1 Materials

Each CN component (αs1-, β-, and ϰ-CN) was prepared following the previously described methods.[4] Protein-Free Blocking Buffer (PFBB) was purchased from Thermo Fisher Scientific (Roskilde, Denmark). Alkaline phosphatase (AP)-conjugated goat anti-human IgE was purchased from Bethyl Laboratories (Montgomery, AL, USA). Anti-α-casein mouse monoclonal antibody was purchased from Cosmo Bio (Tokyo, Japan; out of production). Peroxidase-conjugated anti-mouse IgG was purchased from Jackson ImmunoResearch (PA, USA).

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.[4,7] 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 to CM and whole CN by ImmunoCAP®.

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

The αs1-, 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 αs1-CN-specific immunoglobulin levels. These measured values were used in this study.

2.4 Competitive ELISA

The inhibition ratios of β- and ϰ-CN against αs1-CN-specific IgE were evaluated by competitive ELISA. The 96-well microtiter plates (Nunc-ImmunoTM Plate II, Thermo Fisher Scientific) were coated with αs1-CN (5 μg/mL in PBS) and incubated overnight at 4. After washing the plates with PBS containing 0.05% Tween 20 (PBS-T), the plates were blocked with PFBB containing 0.05% Tween 20 (PFBB-T) for 2 h at 4. Patient sera (n = 39) were diluted 1:5 in PFBB-T and pre-incubated with inhibitor (αs1-, β-, and ϰ-CN diluted in PBS at the final concentrations of 0.001–100 μg/mL). The pre-incubated sera were added to each well and incubated overnight at 4. After washing each well with PBS-T, bound antibodies were detected with AP-conjugated goat anti-human IgE (diluted 1:1,000 in PFBB-T) for 1.5 h at 37. 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. The inhibition ratio (%) was calculated using the following formula:

Inhibition ratio (%) = 100 - (Inhibitor - NC) / (PC - NC) × 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 αs1-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), αs1-CN was detected by anti-α-casein mouse monoclonal antibody and peroxidase-conjugated anti-mouse IgG.

2.5 Bioinformatics analysis

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To quantify the similarity between αs1-CN and the other CN components, the Property Distance (PD) value was calculated using the Property-Based Peptide Similarity Index PD for Two Sequences tool (http://Fermi.utmb.edu/ SDAP/sdap_pdi.html).[8] In brief, the sequential peptides consisting of ten amino-acids, overlapping by seven (three-offset), in the primary sequence of αs1-CN were applied to the amino-acid sequences of β- and ϰ-CN. Peptides with PD values lower than 10 indicated significant physicochemical similarities, whereas much higher PD values implied unrelated peptides. In addition, to identify the putative cross-reactive peptides, αs1-CN-specific IgE epitopes reported by the previous studies were also applied to the amino acid sequences of αs2-, β-, and ϰ-CN.

2.6 Statistical analysis

Correlations among αs1-, 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 ± 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).

RESULTS

3.1 Correlations between casein components-specific immunoglobulins

Correlations between immunoglobulin levels of each CN component are shown in Figure 1. The αs1-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 αs1-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 sensitized to αs1- and β-CN at the same time, and/or a cross-reactive epitope exists between these two CNs.

Moreover, αs1-, 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 αs1-CN.

3.2 Cross-reactivity of αs1-casein with casein components

To confirm whether a cross-reactive epitope exists between the αs1- and β-CN components, we performed a competitive ELISA of β-CN against αs1-CN-specific IgE (Figure 2). Complete inhibition was observed at high concentrations of β-CN (10–100 μg/mL, Figure 2A). The IC_{50} of the β-CN (0.160 μg/mL) was about 10 times higher than that of αs1-CN itself (0.0139 μg/mL), suggesting differences in the number or the affinity of IgE epitopes. On the other hand, the IC_{50} of ϰ-CN (13.6 μg/mL) was about 1,000 times higher than that of αs1-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 exists between αs1- and β-CN.

To exclude the possibility that αs1-CN was contaminated with the purified β-CN, we performed SDS-PAGE and immunoblotting using α-casein-specific monoclonal antibody. Even in the ten times more protein supplementation of β-CN by SDS-PAGE, no bands detected by the αs1-CN-specific immunoblotting (Figure S2). Therefore, the possibility of protein contamination was unlikely.

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

Using the Property-Based Peptide Similarity Index PD for Two Sequences tool, we detected 24 peptide sets between αs1- and β-CN and 14 sets between αs1- 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 αs1-CN (E61–E70) and β-CN (I12–E21). On the other hand, the lowest *PD* value between αs1-CN and ϰ-CN was 8.47. Furthermore, we found 38 peptide sets with *PD* values lower than 10 between αs1- and αs2-CN, 34 sets between αs2- and β-CN, 19 sets between αs2- 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, αs1-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 αs2-CN against αs1- 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 the known IgE epitopes of CN components

To identify the putative cross-reactive epitope peptides in αs1-CN, we also applied *in silico* analysis on the previously reported IgE epitopes of αs1-CN[10-13] against the amino acid sequences of αs2-, 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 αs2-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, known as the core motif of casein phosphopeptide (CPP), are shown in bold characters, but the reported IgE epitopes were scarcely located on the "SSSEE" motif except one located at the N-terminus of αs2-CN.

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 αs1- 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 αs1-CN and ϰ-CN. Unfortunately, we could not examine αs2-CN due to purification difficulties.

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 αs1-, αs2-, 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 "SSSEE". 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 strong allergenic activity. CPP derived from αs- and β-CN. CPP identified in αs1-CN (D43–K79), αs2-CN (K1–K32 and N46–K70) and β-CN (R1–K28) (Figure 3).[22,23]

In this study, we did not find strong cross-reactivity and detect a highly homologous amino acid sequence between αs1-CN and ϰ-CN. The CPP motif does not exist in ϰ-CN, and the C-terminal half of ϰ-CN consists of

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a unique water-soluble glycoprotein designated as a glycomacropeptide.[24] Epitope-mapping of κ-CN reveals many IgE binding sites, which might have individual allergenic properties with low cross-activity between αs- 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.[25-29] 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 supposed 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 αs1-CN was lower than that to native αs1-CN.[30] 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 is that we suggest the presence of cross-reactive epitopes between αs1-CN and β-CN based on bioinformatics finding of the presence of highly homologous amino acid sequences in the CPP regions; however, we need to combine these findings with direct experimental data. Therefore, future experiments are expected to define the IgE-binding capacity of highly purified CPPs from natural CN and evaluate the effect of dephosphorylation. If this agenda were achieved, this would suggest the following: (i) CPP can induce milk allergy, (ii) possibility of cross-reaction caused by the similarity of CPP motifs on the different CN components, (iii) change in IgE binding ability by dephosphorylating the side chain of an amino acid, and (iv) partial negation of epitope analysis using the conventional overlapping peptides.

In conclusion, despite the homology among CN components being extremely low, αs1-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.

Acknowledgements

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 inhibition assay. MN performed *in silico* analysis and statistical analysis. TM and KI reviewed the manuscript. All authors approved the final submitted manuscript.

Impact Statement

The homology among casein (CN) components is extremely low, but αs1-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 αs1-CN and β-CN.

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Table 1. Putative peptides similarity between αs1-casein and β-casein and between αs1-casein and ϰ-casein (*in silico* analys

Amino acid

αs1-Casein

7 - 16

7 - 16

22 -31

28 - 37

31 - 40

34 - 43

40 - 49

46 - 55

46 - 55

46 - 55

49 - 58

55 - 64

61 - 70

61 - 70

64 - 73

79 - 88

94 - 103

97 - 106

109 - 118

112 - 121

115 - 124

118 -127

124 - 133

139 -148

performed by Structural Database of Allergenic Proteins (SDAP, http://Fermi.utmb.edu/SDAP/). The each peptide has *P.*

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

Table 2. The

αs2-Casein

αs1-Casein

αs1

αs2

PD

7

αs2-Casein

β-Casein

The matched amino acids were shown by under line.   The matched a

Table 3. Identification of putative cross-reactive peptides of αs1-casein-specific IgE epitopes
Ref.

10

11

12

13

performed by Structural Database of Allergenic Proteins (SDAP, http://Fermi.utmb.edu/SDAP/). The αs1-CN-sIgE epitop

Figure Legends

Figure 1

Specific-IgE levels (%) to αs1- and β-casein (A), to αs1- 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 αs1-casein was determined by competitive ELISA (A). The αs1-casein (circle) was used as the control, and β-casein (triangle) and κ-casein (square) were used as inhibitors (n = 39). The IC_{50} is the concentration of inhibitor that causes a 50% inhibition of antibody binding (B).

Figure 3

Schematic representation of each casein's amino acid sequence and determined peptides. The 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. Amino acid sequence, SSSEE, is shown in bold.

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