

# Identification of Sequential IgE-Binding Epitopes on Bovine $\alpha_{s2}$ -Casein in Cow's Milk Allergic Patients

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## Key Words

Cow's milk allergy · Epitope · Sequential,  
conformational ·  $\alpha_{s2}$ -Casein · SPOTs membrane

## Abstract

**Background:** Caseins are the major allergens responsible for cow's milk allergy (CMA). We have previously identified the IgE-binding epitopes of the major cow's milk (CM) proteins except for  $\alpha_{s2}$ -casein. **Methods:** Overlapping decapeptides representing the entire length of  $\alpha_{s2}$ -casein were synthesized on a cellulose-derivatized membrane. Sera from 13 CM-allergic children, 4–15 years of age, with a median level of CM-specific IgE >100 kU/l (range 33.7 to >100 kU/l) were used to identify IgE-binding epitopes. **Results:** Four major and six minor sequential IgE-binding regions were identified on  $\alpha_{s2}$ -casein. The first major region is located in the middle of the protein at amino acids (AA) 83–100, and the other three major regions are located in the carboxy terminal portion of the protein at AA 143–158, 157–172 and 165–188. The minor IgE-binding regions were identified at AA 31–44, 43–56, 93–106, 105–114, 117–128, and 191–200. **Conclusion:** We identified 10 sequential IgE-binding regions on  $\alpha_{s2}$ -casein and performed the first crucial step in the development of immunotherapeutic interventions for CMA.

## Introduction

Cow's milk allergy (CMA) is one of the major causes of allergy in childhood. Cow's milk (CM) has about 30–35 g of proteins per liter, of which 80% are comprised of caseins, and the remaining 20%, of whey proteins. The four casein fractions,  $\alpha_{s1}$ -,  $\alpha_{s2}$ -,  $\beta$ -, and  $\kappa$ -casein, comprise 32, 10, 28 and 10% of the total milk proteins, respectively [1]. Caseins appear to be the major allergens responsible for CMA. Although the four caseins have little primary structural homology, many patients with CMA have specific serum IgE to all of them [2].  $\alpha_{s2}$ -Casein (25.2 kD) is comprised of 207 amino acids (AA) and has one disulfide bond per molecule [1]. Four genetic variants are known: A, B, C, D. Variants A and D are the most common types observed in European breeds [3]. Variant D differs from variant A in that it has a deletion of nine residues, including three phosphoserine residues, which changes the structural characteristics [4].

Previous work done by our group has identified the IgE-binding regions on  $\alpha_{s1}$ -,  $\beta$ -, and  $\kappa$ -casein [5, 6] as well as on two major whey proteins [7]. In this study, overlapping synthetic peptides were used to identify IgE-binding regions of  $\alpha_{s2}$ -casein in CMA patients.

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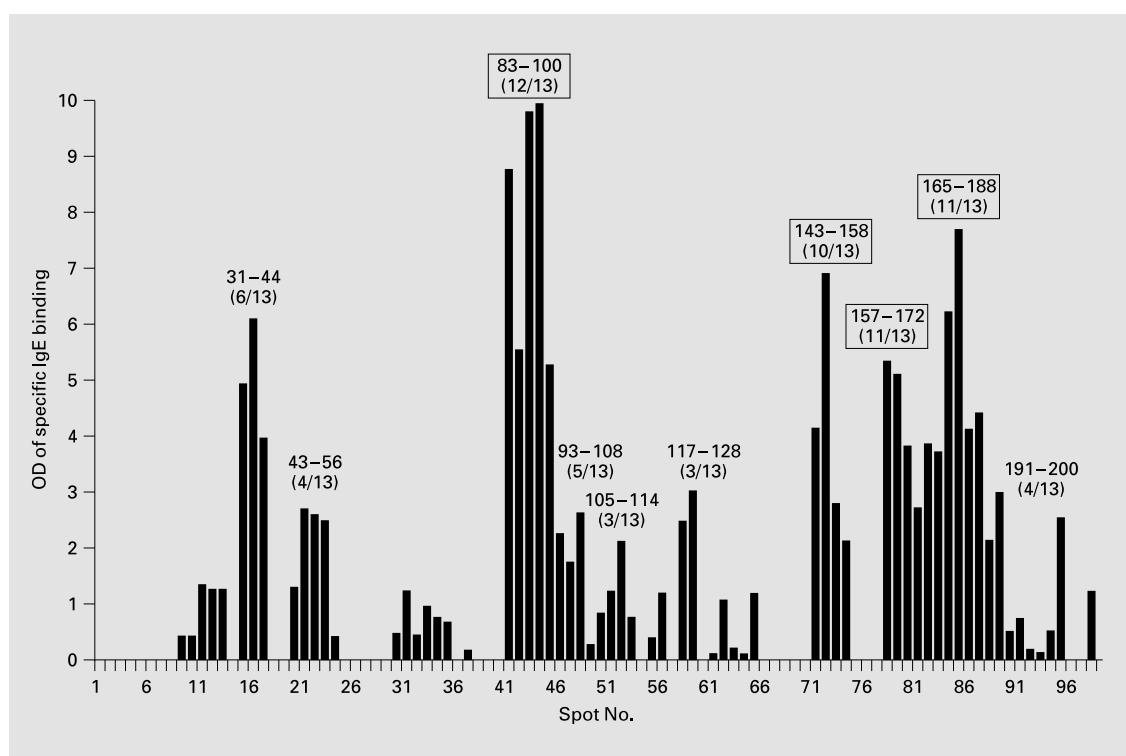


Fig. 1. Cumulative OD scores for specific IgE to each of the synthetic decapeptides of  $\alpha_2$ -casein. According to the known AA sequence, 99 overlapping decapeptides were synthesized on a cellulose-derivatized membrane. Individual sera from 13 milk-allergic children were used to identify IgE-binding epitopes. The IgE-binding epitopes and their corresponding AA sequence are presented above the bars. The major IgE-binding sites are shown in the boxes. The number of patients recognizing the epitope is shown in parentheses.

## Materials and Methods

Sera from 13 patients (4 females, 9 males) whose ages ranged from 4 to 15 years (median 8 years) with IgE-mediated CMA and atopic dermatitis were used. In 5 children the diagnosis was confirmed by double-blind, placebo-controlled food challenge (DBPCFC). In the challenge, 4 of the children developed cutaneous symptoms and 1 had gastrointestinal symptoms. Of the remaining 8 children, 3 had a history of anaphylaxis and 5 experienced gastrointestinal symptoms (vomiting) combined with wheezing/difficulty breathing and were therefore excluded from food challenges. Twelve patients had a CM-specific IgE >100 kU/l and 1 child of 33.7 kU/l measured by the CAP System FEIA<sup>TM</sup> (Pharmacia Diagnostics, Uppsala, Sweden) – a level previously shown to represent a greater than 95% positive predictive value for a positive food challenge [8]. Serum from an atopic, non-milk-allergic individual with CM-specific IgE <0.35 kU/l was used as a control. Informed consent was obtained, and the study was approved by the Mount Sinai School of Medicine Institutional Review Board.

SPOTs<sup>TM</sup> membrane (Genosys Biotechnologies, Woodlands, Tex., USA), a derivatized cellulose membrane, was used to generate 99 decapeptides, overlapping by 8 AA and covering the entire sequence of variant A. Peptides were synthesized as described earlier [5], blocked and incubated with individual sera diluted 1:30. IgE

antibody binding was detected as described earlier [5] using biotinylated monoclonal mouse anti-human IgE (PharMingen, San Diego, Calif., USA). The optical density (OD) of each peptide spot was measured by reflection densitometry and assigned a 'cumulative score' [5]. Major IgE-binding regions are defined as sites bound by IgE antibodies from at least 50% of the patients. The cut-off level for significant binding was an OD of 0.1 for individual sera.

## Results

Ten IgE-binding regions could be identified; the cumulative ODs for the total study population are shown in figure 1. Using individual ODs, 4 of them were recognized by the majority of patients. Their locations throughout the protein are shown in figure 2. The last 3 regions were located close to each other and seemed to harbor multiple epitopes. The other 6 were minor IgE-binding regions (fig. 1, 2). The median number of IgE-binding regions, both major and minor, recognized per individual was 5 (range 1–8). Sera from the control patient showed no IgE binding to the peptides (data not shown).

|                     |                             |                                    |
|---------------------|-----------------------------|------------------------------------|
| 1                   | 20                          | 40                                 |
| KNTMEHVSSSEESIISQET | YKQEK                       | NMAINP <u>SKENLCSTFC</u>           |
| 41                  | 60                          | 80                                 |
| KEVVRNANEE          | EYSIGSSSEESA                | EVATEEVKITVDDKHYQK                 |
| 81                  | 100                         | 120                                |
| ALNEINQFYQKF        | <u>PQYLQYLYQGPIVLNPWDQV</u> | <u>KRNAVPIT</u>                    |
| 121                 | 140                         | 160                                |
| PTLNREQL            | STSEENSKKTVDME              | <u>STE</u> VFTKKT <u>KLTEEEKNR</u> |
| 161                 | 180                         | 200                                |
| LNFLKKISQRYQKF      | ALPQYLKTVYQH                | <u>QKAMKPW</u> IQPKTKV             |
| 301                 | 207                         |                                    |
| IPYVRYL             |                             |                                    |

Fig. 2. AA sequence of  $\alpha_{s2}$ -casein. Major IgE-binding epitopes are in bold face, and minor ones underlined.

## Discussion

With the identification of 10 IgE-binding regions in  $\alpha_{s2}$ -casein, this report completes the B-cell epitope mapping of the major milk proteins. The patients in this study generally represent an older group of CMA patients, the majority of whom have persistent CMA and high concentrations of milk-specific IgE antibodies. Interestingly, 2 of the major IgE-binding regions (AA 83–100 and AA 165–188) share 50% AA sequence homology. The second major IgE-binding region (AA 143–158) contains a phosphorylation site at its amino terminal portion, which may increase its allergenicity [4]. Another phosphorylation site is at AA 168. Dephosphorylation or a natural deletion of a multi-phosphorylation site that occurs in the D variant (AA 51–59) has been shown to significantly diminish the allergenicity of  $\alpha_{s2}$ -casein [4]. This sequence was found to be a minor IgE-binding site in the variant A in our study.

The allergenicity of most proteins seems to depend upon primary, secondary, and tertiary structures. Previously modifications of  $\alpha$ -casein prepared by treatment with urea, hydrochloric acid, sodium hydroxide, sodium dodecyl sulfate, or heating, which result in the loss of secondary and tertiary structures, did not significantly affect the allergenicity of  $\alpha$ -casein in most of the patients studied [9]. These findings suggest that the primary structure (sequential epitopes) of  $\alpha$ -caseins is most important for their allergenicity.

In conclusion, this report completes the mapping of the sequential B-cell epitopes of the major CM proteins [5–7]. The next steps will include the determination of the AA crucial for IgE binding. The identification of immunodominant epitopes is important in understanding the pathophysiology of CMA. In addition, epitope mapping fulfills the first crucial step in future attempts to develop immunotherapeutic interventions for CMA, such as immunotherapy with engineered hypoallergenic proteins or peptides. Furthermore, the knowledge of the B-cell epitopes will enable us to study differences in epitope recognition between different patient groups (e.g. persistent versus transient CMA).

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