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Modification of IgE binding to α_{S1} -casein by proteolytic activity of *Enterococcus faecium* isolated from Iranian camel milk samples

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Highlights

1. The proteolytic activities of LABs isolated from Iranian camel milk were investigated.
2. Proteolysis of casein fractions observed after SDS-PAGE, was confirmed by analysis of peptide profiles by RP- HPLC.
3. The strain with highest proteolytic activity showed high allergenicity reduction of α_{S1} -casein.
4. 16S rDNA fragment sequencing resulted in the identification of strain as *Enterococcus faecium*.

Abstract

Milk is a perfect source of nutrients for neonates. When breast feeding cannot be done, an infant's alimentation is usually initiated to cow's milk, among the primary foods. It has been reported that about 2.5% of juveniles under the age of 3 years manifest allergic reactions to cow's milk proteins. Among the cow's milk proteins, casein fractions are considered as the strongest allergenic proteins. The proteolytic enzymes of lactic acid bacteria (LAB), during fermentation of dairy products, can break down milk proteins especially caseins and subsequently reduce the immune reactivity of allergenic proteins. In this research, raw bovine and camel milk samples were screened for *cocci* LAB strains and after isolation, their proteolytic activity against bovine milk caseins were evaluated by SDS-PAGE and RP-HPLC. The potential of *cocci* LAB strains on α_{S1} -casein degradation and their potential to break down the principle allergenic epitopes of this protein was detected using indirect competitive ELISA. Molecular identification of the best proteolytic strain was fulfilled by 16S rDNA fragment sequencing with universal primers. The obtained results

demonstrated that *Enterococcus faecium* isolated from raw camel milk samples was the most efficient isolate in hydrolyzing Na-caseinate and α_{S1} -casein. Hydrolysed α_{S1} -casein by *Enterococcus faecium* were also less recognized by IgE of bovine milk allergic patients' sera in comparison with native α_{S1} -casein. It has been proposed that *Enterococcus faecium* could be an efficient strain in allergenicity reduction of cow's milk proteins. So it could be an excellent candidate to be potentially used in dairy industries.

Key words: Cow's milk allergy; Casein; Lactic acid bacteria; Protein hydrolysis; Competitive ELISA; *Enterococcus faecium*.

1. Introduction

Food allergy (FA) is an unfavorable reaction of the immune system to an otherwise harmless food (Hadley, 2006). According to the World Health Organization (WHO), FA is the sixth problem affecting human health and its prevalence in general population has been estimated around 1-3% in adults and 4-6% in children (Pereira et al., 2005; Pawankar et al., 2011; Tetens, 2007). Milk, egg, peanut, tree nut, wheat, soy, fish, and crustacean shellfish are considered to cause the most incidences of food allergies (Tetens, 2007; Tammineedi and Choudhary, 2014).

Breast milk has the complete source of nutrients, but bovine milk is incorporated into an infant's diet when breastfeeding cannot be performed (Gartner et al., 2005). Despite the nutritional values of cow's milk, it may induce immune response in some children (Høst et al., 2002). Cow's milk allergy (CMA), the most common confirmed food allergies, remains an increasing health concern because of the relatively immature immune system in infants and their susceptibility to sensitize by environmental antigens (Wood, 2003; Host and Halken, 2014). CMA may be classified as IgE-mediated or non-IgE-mediated reactions (Sampson, 1999). It is notable that most often allergic reactions to cow's milk proteins (CMP) are IgE mediated, causing immediate or life threatening symptoms including cutaneous, gastrointestinal, and respiratory symptoms (Fiocchi et al., 2010; Hadley, 2006; Host and Halken, 2014; Sampson and Anderson, 2000). Moreover, children with IgE-mediated CMA are more vulnerable of developing other food allergies, asthma and rhino conjunctivitis earlier than ten years of age (Meglio et al., 2004).

Cow's milk has about 30–35 g of proteins per liter (80% caseins and 20% whey proteins) (Hochwallner et al., 2014). Caseins, β -lactoglobulin, and α -lactalbumin proteins are the main allergen proteins in bovine milk (Wal, 2002). The whole casein fractions comprises four proteins

including α_{S1} -, α_{S2} -, β - and κ -caseins (Farrell et al., 2009; Holt et al., 2013; Livney et al., 2004; Stanic et al., 2010). Among the casein fractions, α_{S1} -casein is the most significant allergen (Cocco et al., 2003; Ruiter et al., 2006). This protein is composed of one phosphorous protein chain of 199 amino acids with molecular weight of 23.6 kDa (Kumosinski et al., 1991; Tsaouri et al., 2014). The phosphorylated areas of α_{S1} -casein play an important role in its antigenicity (Otani et al., 1987; Spuergin et al., 1996).

Various methods and strategies of processing have been applied to decrease or eliminate CMP allergenicity. These attempts include the application of heat treatment, ultra-violet light, high hydrostatic pressure, mutation, and lactic acid bacteria (LAB) fermentation (Taheri-Kafrani et al., 2009; Tammineedi and Choudhary, 2014; Kazem-Farzandi et al., 2015; Taheri-Kafrani et al., 2015; Kazemi et al., 2018). Among them, the best way to reduce the antigenic/allergenic capacity of bovine milk caseins is through bacterial fermentation by LAB (Bu et al., 2010; Cross et al., 2001; Fugl et al., 2017; Nentwich et al., 2004). The proteolytic enzymes of LAB strains enable them to hydrolyze milk proteins and produce essential amino acids for their growth (Abu-Tarboush, 1996; Chen and Steele, 1998; Kunji et al., 1996; Zourari et al., 1992). The proteolysis of milk proteins can reduce the amount of antigenic epitopes and subsequently decrease milk allergenicity (Bertrand-Harb et al., 2003; Cross et al., 2001; Nentwich et al., 2004; Tzvetkova et al., 2007).

There are many evidences show that some LAB strains isolated from various dairy products from different parts of the world can reduce the allergenicity of bovine milk proteins especially α_{S1} -casein (Ahmadova et al., 2013; Bu et al., 2013; Biscola et al., 2016; El-Ghaish et al., 2011). It has been observed that the decrease in milk protein antigenicity varies with the LAB species and the conditions of fermentation process (Bu et al., 2010; Kleber et al., 2006). So, the aim of this study

was screening and evaluating raw camel milk samples for novel proteolytic *cocci* LAB strains, targeting caseins specially α_{S1} -casein and identifying the most promising strains. Eventually their capability to break down the main allergenic epitopes of bovine milk caseins and subsequently the α_{S1} -casein immunoreactivity changes were evaluated.

2. Materials and methods

2.1. Reagents

Culture media were obtained from Difco Laboratories (USA). High quality reagents of significant purity were obtained from Merck and Sigma–Aldrich chemical companies. The experimental reference, β -LG variant A from homozygote cow's milk, was obtained according to the Maillart and Ribadeau Dumas method (Maillart et al., 1988). As withdrawn from high performance liquid chromatograms and polyacrylamide gel electrophoresis, the β -LG variant A was > 98% pure. A pool of 14 sera obtained from CMA patients with diverse symptoms was used in competitive enzyme-linked immunosorbent assay (ELISA) experiments. A concentration range of 18.6 to 49.1 kAUL⁻¹ of total milk protein-specific IgE was exhibited by all sera, as determined by The Phadia ImmunoCAP System. The sera collection was done in the Laboratory of Immunology of Academic Hospital (Tehran, Iran). The internal Laboratory of Immunology of Academic Hospital Ethical Committee approved the sera collection. The sera from nonallergic patients were used as the negative control.

2.2. Isolation of strains and culture conditions

Lactic acid bacteria were isolated from 12 camel raw milk samples from various local farms of Iran. Ten ml of any milk sample was homogenized with 90 mL of sterile saline water (8.5 g/L NaCl) to make a 10-fold serial dilution. Then 0.1 mL of each dilution was subcultured onto the

M17 and MRS (Man-Rogosa-Sharp) agar. Aerobic and anaerobic incubation of media was done at 30-35 °C for 2-3 days (Ali, 2011; Badis et al., 2004; Guessas and Kihal, 2004). Through random picking, bacterial colonies were transferred in 10 mL of appropriate broth medium (MRS or M17). The chosen colonies were purified by repeated streaking on the suitable agar media (MRS and M17 agar plates). After several steps of purification, gram positive and catalase negative *cocci* isolates were considered as *cocci* LABs. These isolates were finally used for further experiments and conserved in MRS broth supplemented with 25% (w/v) glycerol as cryoprotector prior to freezing (Mathara et al., 2004). Selected strains were maintained on MRS agar slant at 4 °C and were streaked every 4 weeks.

2.3. LAB proteolytic activity evaluation

Milk-citrate-agar (MCA) medium was used to investigate the production of the bacterial proteinases (Pescuma et al., 2007). In the preparation of MCA medium, 2.4 g Na-citrate, 0.3 g yeast extract, 1.5 g glucose and 4.5 g agar were dissolved in 160 ml distilled water and the resulting solution was sterilized at 118 °C for 15 minutes. After that, 132 mL pasteurized milk was added at 60 °C. Induction of proteolysis enzymes production was achieved by pouring a portion (0.2 mL) of the activated preculture on top of MCA plates and incubation was done for 24 h at 37 °C. Fresh cells were then collected from MCA media and washed twice with 0.85% (w/v) saline solution supplemented with 10 mM CaCl₂, and brought to a final optical density (OD₆₀₀) of 20-30 by 100 mM sodium phosphate buffer pH 7.0. To one suspension volume of the cells, an equal volume of purified fractions of milk proteins (10 mg/mL of sodium caseinate or α _{S1}-casein) in 100 mM sodium phosphate buffer pH 7.0 was added and incubated for 12 and 24 h at 37 °C. The control for the experiment was sodium caseinate or α _{S1}-casein (10 mg/mL) in 100 mM sodium phosphate buffer pH 7.0 without addition of any strain.

2.4. Analysis of the proteolytic activity of LAB by SDS-PAGE

At different time intervals, samples were withdrawn and mixed with the same volume of loading (sample) buffer 2X (50 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and 0.025 % (w/v) bromophenol blue) supplemented with 5% β -mercaptoethanol and heated at 60 °C for 5-10 min. After that samples were taken and cells were pelleted by centrifugation (3000 g, 4 °C for 10 min). The clear supernatants were prepared in the same denaturing buffer at a 1:1 volume ratio and heated at 60 °C for 5 min in order to analyze protein hydrolysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). By using 12% polyacrylamide gel, the hydrolysis of proteins and formation of peptides were analyzed using standard procedures (Kazem-Farzandi et al., 2015). The LAB strain with the best proteolytic activity was selected for further experiments.

2.5. Proteins hydrolysis and peptide profiles assessment in reverse phase-HPLC

Reverse phase-high performance liquid chromatography (RP-HPLC) was used to measure time course of sodium caseinate and α_{S1} -casein degradation and peptide profiles generated after incubation of proteins with the cell suspension of selected proteolytic LAB strain. After proteolytic strain incubation with sodium caseinate or α_{S1} -casein in a nonproliferative cell system (pH 7.0), the acquired supernatant was added (1/1, v/v) to 8 M urea. The resulting mixture was centrifuged (10,000 g, 5 min, 4 °C) and then injected directly to the column. To carry out RP-HPLC, Waters HPLC System (Alliance System, Milford, MA) was used. Solvent A (90% acetonitrile and 0.1% trifluoroacetic acid in DI water) was used to equilibrate the column (Symmetry C₁₈, 5 μ m, 2.1 mm, 150 mm) at a flow rate of 0.5 mL/min. After the injection of the sample, the column was washed 25 min with solvent B (20% acetonitrile and 0.1% trifluoroacetic acid in DI water). Observation

of the peaks was carried out at 220 nm with the aid of a diode array spectrophotometer (model 996, Waters).

2.6. Competitive ELISA (Enzyme Linked Immunosorbant Assay)

Competitive ELISA was used for evaluation of the binding strength of the intact α_{S1} -casein or its bacterial hydrolysates to the immunoglobulin E (IgE) in pool sera of the CMA patients. Therefore, the strains that showed a total degradation of sodium caseinate and α_{S1} -casein were selected for competitive ELISA test. Microtitre plates with 96 wells were coated using 100 μ l in each well of α_{S1} -casein diluted to 5 μ g/ml in 100 mM PBS pH 7.4 (0.136 M NaCl, 2.68 mM KCl, 1.76 mM KH_2PO_4 , and 10.1 mM $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$) for 3 h at 30 °C. By substituting α_{S1} -casein with the same volume (100 μ l) of PBS, the negative control (blank) was prepared. When the coating process was complete, PBS containing 0.1% (v/v) Tween-20 (PBS/T) was used to wash all wells. The wells were saturated with 250 μ l of the PBS/T solution containing 1% (w/v) polyvinyl alcohol (PBS/T/PVA) for 2 h at 37 °C. A 14 sera sample pool obtained from α_{S1} -casein allergic patients was used. After a 1:20 ratio dilution, the pool sera were combined with equivalent volumes of α_{S1} -casein or α_{S1} -casein hydrolysate in 1% PBS/T/PVA (0 to 250 μ g/ml) serial dilutions. A 100 μ l portion of the mixture was added to the wells and incubated overnight at 4°C. After three times washing with PBS/T, the plate was incubated with the secondary antibody (alkaline phosphatase-conjugated polyclonal antihuman-IgE) diluted 1:1000 on PBS/PVA, for 2 h at 37 °C. After washing with PBS/T, binding of the secondary antibody was quantified by adding 4-methylumbelliferyl phosphate (4-MUP) substrate mixed in the ratio of 1:5 with Tris HCl pH 9.8. After 90 min of incubation at 37 °C, the fluorescence emission was measured with the FL_X800 plate reader (Biotech Instruments, Inc., Winooski, VT) fitted with a 360 nm excitation filter and a 440 nm emission filter.

2.7. Identification of proteolytic strain by 16S rDNA amplification and sequencing

Genotypic and molecular identification of the best proteolytic LAB strain was fulfilled through 16S rDNA fragment sequencing with universal primers Fd1 (27F: 5'-AGA GTT TGA TCM TGG CTC AG -3') and Rd1 (1492R: 5'-CGG TTA CCT TGT TAC GAC TT-3'). Total genomic DNA was extracted from the selected sample using boiling method and used as a template for 16S rDNA gene amplification. The concentration and integration of extracted DNA were measured by determination of the ratio between the absorbance (A) at 260 and 280 nm (A_{260}/A_{280}), using a spectrophotometer. The reaction mixture (25 μ L as final volume) was composed of 2 μ L DNA template, 0.5 μ L of each primer and 23 μ L of master mix contained PCR buffer (2.5 μ L), $MgCl_2$ (1 μ L), dNTPs (0.5 μ L), Taq DNA polymerase (0.5 μ L) and distilled water. The amplification program included a first denaturation step at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min (denaturation), 47 °C for 45 sec (annealing) and 72 °C for 1 min (extension) with a final extension step at 72 °C for 10 min. Amplicons were analyzed on 1% (w/v) agarose gel with safe red view (1 μ L/mL) in 0.5x TAE buffer pH 8.2-8.4, for 30 min at 100 V and made visible by UV trans-illumination. DNA sequencing was carried out by SeqLab sequencing service. The blast algorithm (<http://blast.ncbi.nlm.nih.gov>) was used to ascertain the most related sequence relatives in the NCBI nucleotide sequence database.

3. Results and Discussion

3.1. Isolation and identification

A collection of 9 strains were confirmed as *cocci* LAB isolated from raw camel milk samples. The ability of these isolates to hydrolyze casein fractions was shown by reduced color intensity of the bands in SDS-PAGE and appearance of peptides profile in RP-HPLC. The best isolate was

selected for amplification and sequencing of 16S rDNA. The results showed 99% identity with the 16S rDNA sequencing reported for *Enterococcus faecium* strains, such as *E. faecium* NBRC100466 (accession number: NR 113904.1), *E. faecium* NBRC100485 (accession number: NR 113903.1) and *E. faecium* ATCC 19434 (accession number: NR 115764.1), indicating that this isolate was *E. faecium*. In fact, *Enterococcus* is the most abundant and proteolytic LAB genus in raw milk. Two species of this genus, *E. faecalis* and *E. faecium*, are the most common in raw milk especially camel milk and other dairy products (Quigley et al., 2013).

3.2. SDS-PAGE

The ability of the strains to hydrolyze caseinate and α_{S1} -casein was determined by the reduced color intensity of the bands in the SDS-PAGE analysis. According to the band color density, it has been observed that some strains displayed weak proteolytic activity against casein fractions after 12 and 24 h of incubation (data not shown). As it is shown in Fig. 1, strain I and II showed weak proteolytic activity against Na-caseinate and α_{S1} -casein after 24 h of incubation, however, among all isolated LABs from raw camel milk, strain III (*E. faecium*) showed the highest proteolytic activity against both sodium caseinate and α_{S1} -casein even after 12 h of incubation. Once hydrolysis occurred by *E. faecium*, the staining intensity of all bands representing Na-caseinate and α_{S1} -casein was reduced at 12 h of incubation and subsequently at the end of the incubation period (24 h) all protein fractions were intensively hydrolyzed. So this strain was considered as the most active strain among all isolated LABs and selected for further characterization of proteolytic activity by RP-HPLC. Also, the results demonstrated that the proteolysis was increased with increasing the time of incubation and the peptides observed after 12 h of incubation, disappeared after longer incubation and reaching to a maximum after 24 h. This finding supports the fact that these strains preserved their hydrolytic activity even after 24 h of incubation. The

appearance of high molecular polypeptide chains in the electrophoregrams is generally due to the impurities in sodium caseinate or α_{S1} -casein and also the presence of proteolytic enzymes in the sample solutions.

[Fig. 1 to be inserted here]

3.3. RP-HPLC analysis

The peptide profile analysis of RP-HPLC produced from Na-caseinate and α_{S1} -casein proteolysis with *E. faecium*, after 24 h of incubation under optimal conditions (pH 7.0 and temperature 37 °C) is shown in Fig. 2. These results were in agreement with SDS-PAGE results. As it is shown in Fig. 2, within the 24 h of cultivation, the peak of casein fractions disappeared almost entirely and casein fractions were cleaved in to a large amount of hydrophilic peptides, suggesting that the tested strain has the good proteolytic activity against both Na-caseinate and α_{S1} -casein. It can be speculated that during fermentation times, casein proteins were broken down into different peptides and free amino acids. This leads to cleavage or elimination of native protein profiles in RP-HPLC (Fig. 2).

[Fig. 2 to be inserted here]

3.4. Competitive ELISA

The consequence of protein breakdown of α_{S1} -casein by *E. faecium* on its binding affinity to IgE was investigated through a competitive ELISA experiment. These experiments were performed in triplicate and the fluorescence data were corrected through deduction of the fluorescence of the control with no antigen. Fig 3 shows binding ability of the IgE of CMA patients' pool sera to the native α_{S1} -casein and its hydrolysate. It can be noted that α_{S1} -casein hydrolysates' recognition and binding to the serum IgE was significantly weaker in comparison with the native α_{S1} -casein. Protein (inhibitor) concentration desired for 50% inhibition of IgE binding (IC_{50}) was computed from the inhibition curves by relating fluorescence intensity to inhibitor concentration. The IC_{50}

in this work denotes the α_{S1} -casein concentration binding 50% of the IgE in the sera of the CMA patients. Also, the inhibition rate (%) of the antigenicity of α_{S1} -casein by *E. faecium* fermentation was calculated according to Eq. 1:

$$\text{Inhibition rate (\%)} = [(B_0 - B)/B_0] \times 100 \quad (1)$$

where B is the antigenicity of α_{S1} -casein in fermented milk by selected strain and B_0 is the antigenicity of α_{S1} -casein in unfermented milk. The values attained through the division of the hydrolysed IC_{50} of α_{S1} -casein by IC_{50} of the native whole α_{S1} -casein indicates the consequences of the protein breakdown on the binding to IgE and hence of the immuno-reactivity of the protein. According to Fig.3, the IC_{50} value of intact α_{S1} -casein was calculated to about 0.4-0.5 $\mu\text{g/ml}$ whereas the α_{S1} -casein hydrolysates had an IC_{50} value of about 30 $\mu\text{g/ml}$ high. These results demonstrate that IgE binding to the hydrolysed protein is approximately 66 times inferior as compared to the intact protein. The results illustrated that the proteolytic activity of *E. faecium* isolated from camel milk can reduces α_{S1} -casein allergenicity.

[Fig. 3 to be inserted here]

4. Conclusion

During the two past decades, several research attempts are continuing to develop novel hypoallergenic dairy products. Hydrolysis of α_{S1} -casein by LAB is of great value for baby formula milks, in which this protein is one of the most abundant allergen protein in mammalian milk. In this study, Na-caseinate and α_{S1} -casein were hydrolyzed by nearly all isolated *cocci* form LAB strains, however, one of the camel milk isolates showed relatively the highest proteolytic activity toward casein proteins under optimal conditions. The results demonstrated that recognition and IgE binding to α_{S1} -casein reduced significantly after hydrolytic activity of *Enterococcus faecium*.

The results of this study showed that this strain can hydrolyze α_{S1} -casein in many sites including the main allergenic epitopes and can alleviate allergic manifestations in children with CMA. This work presented new insight to the proteolytic activity of *Enterococcus faecium* isolated from Iranian camel milk against caseins and interestingly, this strain is an excellent candidate to be potentially used in dairy industry by its specific activity to improve the quality of dairy products; however further *in vivo* trials are needed to support this claim.

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Figure legends

Figure 1. SDS–PAGE analysis of the hydrolysis profiles of (A) sodium caseinate and (B) α_{S1} -casein caused by 3 cocci LAB strains isolated from camel milk samples (37°C, 12 and 24 h). N lines show negative control (native sodium caseinate or α_{S1} -casein) without proteolytic strains. Lines III show sodium caseinate or α_{S1} -casein hydrolysates caused by *Enterococcus faecium* after 12 and 24 h; lines I and II show profile of sodium caseinate or α_{S1} -casein incubated in the presence of other isolated strains after 12 and 24 h (All isolates are not shown).

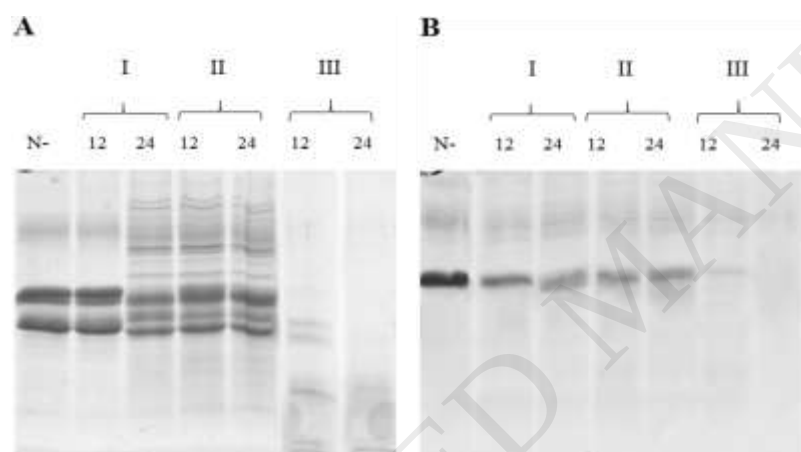


Figure 2. Reverse phase-HPLC of peptides released from hydrolysis of (A) Na-caseinate and (B) α_{S1} -casein after 24 h of incubation by proteolytic enzymes of *Enterococcus faecium* in comparison with native proteins profile.

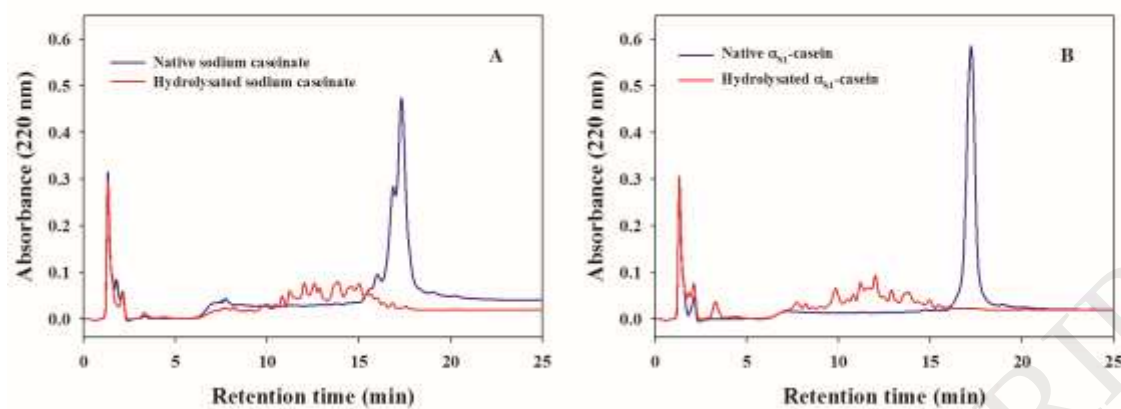


Figure 3. Comparison of IgE binding of native α_{s1} -casein and its hydrolysate (by *Enterococcus faecium*) in the sera pool of CMA patients.

