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Abbreviations: GM: Goat's Milk, **CM**: Cow's Milk, **CMA**: Cow's Milk Allergy, **GMA**: Goat milk Allergy, **CMP:** Cow's milk protein, **GMP:** Goat's milk protein, **CN**: Casein.

Keywords: Allergy, Casein, Epitope, Goat's milk, IgE

Abstract

Scope: Despite a sequence homology of 90% between bovine and caprine β-caseins (CN), IgE antibodies from patients allergic to goat's milk (GM), but tolerant to cow's milk (CM), recognize caprine β-CN without cross-reacting with bovine β-CN. We investigated this lack of cross-reactivity by evaluating the IgE-reactivity towards peptides isolated from plasmin-hydolysates of bovine and caprine β-CN.

Methods and Results: The IgE-binding capacity of plasmin-derived peptides was evaluated with sera from 10 CM-allergic patients and 12 GM-allergic/CM-tolerant patients. In CM-allergic patients, IgE-reactivity of caprine fragments (f29-107) and (f108-207), but not (f1-28), was similar to that of the bovine counterparts. In contrast, all bovine fragments were poorly recognized by IgE antibodies from GM-allergic/CM-tolerant patients. The peptide (f29-107) was generally the most immunoreactive fragment of caprine β-CN. By using synthetic peptides, the immunodominant IgE-binding epitope recognized by most GM-allergic/CM-tolerant patients was located in the caprine domain 49-79.

Conclusion: The restricted specificity of the IgE response toward the caprine β-CN in GM-allergic/CM-tolerant patients is mainly directed against the domain 49-79, which differs from its bovine counterpart by only 3 amino acid substitutions.

1 Introduction

Cow's milk allergy (CMA) affects 1.8 to 3% of children during the first year of life and is thereby the most frequent cause of food allergy in infants [1-3]. CMA was usually considered to resolve in the first three years of life but recent studies reported that children with IgE-mediated allergy may acquire tolerance to CM at older age [1, 4-7]. Because of the high degree of sequence homology between cow's milk proteins (CMP) and goat's milk proteins (GMP), CMA is almost always associated with goat's milk allergy (GMA) [8]. Consequently, when tolerance to CM is acquired, it would be expected that this tolerance extends to GM. However, numerous cases of GMA without CMA have been reported since the first observations made by Wuthrich *et al*. [9-14]. With a mean age of six years, GM-allergic/CM-tolerant patients are generally older than those presenting CMA and their symptoms are more frequently severe [9]. A recent study reported a high prevalence (26%) of allergy to goat's and/or sheep's milk (SM) in a population of CM-allergic children treated with CM-oral immunotherapy, as confirmed by a controlled positive oral challenge, which led to an anaphylactic reaction for almost half of the GM/SM-allergic patients [15].

Whole casein (CN) accounts for 80% of CMP and is a major allergen responsible for CMA [16-19]. The IgE response to whole CN is particularly predominant in adults or children with persistent CMA [18, 20]. In a previous study, we reported, among other observations, that 90% of 58 patients sensitized to bovine whole CN had specific IgE antibodies against β-CN, one of the four constituents of whole CN with αS1-, αS2- and κ-CN [21]. In contrast to CMA, IgE responses of GM-allergic/CM-tolerant patients were weak against whey proteins, i.e. β-lactoglobulin (BLG) and α-lactalbumin, and mostly directed against αS1-, αS2- and β-CN. As observed with CM-allergic patients, 90% of 18 GM-allergic/CM-tolerant patients exhibited IgE antibodies specific of the caprine β-CN [9].

The β-CN fraction constitutes approximately 36% of the bovine whole CN and up to 60% of the caprine whole CN [22, 23]. Despite some contradictory studies, β-CN is generally considered to possess no or little regular secondary structure and no rigid tertiary structure, thus suggesting that the major IgE-binding epitopes are mainly sequential [24-26]. Actually, immunodominant IgE-binding domains of bovine β-CN have been mostly identified by means of synthetic peptides [24, 27-29]. The IgE epitopes have been located all along bovine β-CN molecule. Surprisingly, despite a sequence homology of 90% between bovine and caprine β-CN, GM-allergic/CM-tolerant patients display an IgE response to the caprine β-CN without significant cross-reactivity with the bovine β-CN [9].

In the present work, we compared the distribution of IgE-binding epitopes along bovine and caprine β-CN when GMA was associated or not with CMA. For this purpose, the IgE-reactivity of different fragments covering the full-length allergen molecules was evaluated with sera from CM-allergic and GM-allergic/CM-tolerant patients. As post-translational phosphorylation of seryl residues may be critical for the recognition of β-CN by IgE [30], fragments were generated from purified β-CN by performing digestion with plasmin, the main endogenous proteinase present in milk. Plasmin hydrolyzes β-CN into several fragments, including the hydrophilic fraction of proteose-peptones and the hydrophobic fraction of γ-caseins [23, 31-33]. Plasmin cleavage of β-CN naturally occurs in milk and its action contributes to cheese ripening [34, 35]. The IgE-binding capacity of plasmin-derived peptides and their relative affinity to IgE antibodies from CM-allergic and GM-allergic/CM-tolerant patients were compared. The restricted IgE specificity of GM-allergic/CM-tolerant patients toward the most immunoreactive fragment of caprine β-CN was further investigated with synthetic peptides that covered the different amino acid substitutions occurring between bovine and caprine β-CN sequences.

2 Materials and Methods

2.1 Human sera

Sera from 22 children recruited in two different paediatric allergy units were collected after informed consent from patient's parents. Ten patients were suffering from CMA and 12 patients were allergic to GM and tolerant to CM (Table 1). The allergy to GM was confirmed by clinical manifestations observed in less than one hour after ingestion of GMP. The absence of CMA was confirmed by routine ingestion of CM or derived products without any adverse reaction.

2.2 Purification of milk proteins and plasmin hydrolysis of β-CN.

Bovine and caprine BLG, whole CN and β-CN fractions were prepared with milk from one cow and one goat as previously described [36]. Each β-CN was dissolved to a final concentration of 10 mg/ml in 0.05 M sodium phosphate (pH 8) and was incubated at 37°C with plasmin (1/200 w/w) from bovine plasma (EC 3.4.21.7 from Roche Applied Science, France). Samples were collected at 0, 30, 120, and 240 min, heated 95°C for 15 min to inactivate plasmin and then cooled to -80°C. SDS PAGE analysis of the sample was performed with 15 % polyacrylamide gels.

Bovine and caprine β-CN were finally subjected to plasmin hydrolysis during 60 min in order to limit the cleavage of the allergen at Lys^{28}, Lys^{105} and Lys^{107}. Proteose-peptones were separated from γ-CN by performing mild acidic precipitation at pH 4.6. After centrifugation at 5000 g for 20 min at 4°C, soluble bovine fragments (f1-28), (f29-105) and caprine fragments (f1-28) and (f29-107) were purified by RP-HPLC using a 300 Å C4 Vydac column (250 X 10 mm) and a linear gradient from 0 to 50% of solvent B (Acetonitrile/ TFA 0.04%) in solvent A (H_2O/TFA 0.1%) at a flow rate of 2 mL/min. The pellet of pH4.6-insoluble peptides was resuspended in buffer A (Urea 4 M, Tris 5 mM pH 8.0). The C-

terminal fragments, (f106-209) of bovine β-CN and (f108-207) of caprine β-CN, were then isolated using a AKTA purifier system (GE Healthcare, France) and a Mono Q 5/50 Gl column. After a step at 100% of buffer A for 7 minutes, elution was performed using a 35 min linear gradient from 0 to 35% of buffer B (Urea 4 M, Tris 5 mM and 1 M NaCl pH 8.0). Collected fractions were further purified by RP-HPLC in the conditions described above.

2.3 Peptide synthesis

Peptides (f49-107), (f49-79) and (f80-107) corresponding to caprine β-CN were synthesized using a standard solid-phase synthesis by the Fmoc (9-fluorenyl-methoxycarbonyl) continuous-flow method on the Applied Biosystems peptide synthesizer model 433A. For peptides containing seryl residues which has been reported to be systematically phosphorylated, phosphoserine was incorporated as Fmoc-Ser(PO3HBzl)-OH. After standard procedure including TFA cleavage and ether precipitation, crude peptides were purified by RP-HPLC as described above.

2.4 Characterization of proteins and peptides by mass spectrometry

The purified proteins and peptides were characterized by MALDI-TOF MS analysis using a Voyager-DE STR spectrometer (Applied Biosystems, Foster city, CA). Samples were prepared according to the dried droplet method using sinapinic acid as matrix. The matrix solution was prepared by dissolving α-cyano-4-hydroxycinnamic acid or sinapinic acid in CH_3CN/H_2O, 0.1% TFA (60/40 v/v) at a concentration of 10 mg/mL. Mass assignments were obtained using Bovine Serum Albumin and calibration mixtures containing Apomyoglobin, Thioredoxin, Insulin and ACTH fragments (AB Sciex, France) as external standards.

2.5 IgE quantification by direct Enzyme Allergo Sorbent Test (EAST)

IgE responses to milk proteins were quantified using a direct EAST in which purified GMP, CMP and peptides were passively adsorbed on microtiter plates as previously described [21]. Tracers were prepared by covalent linkage of the purified proteins to the tetrameric form of AChE according to Bernard et al. [37]. After washing, Ellman's reagent was used as the enzyme substrate and absorbance was measured at 414 nm.

2.6 IgE-binding capacity as determined by reverse EAST inhibition

Plates were coated with anti-human IgE monoclonal antibody LE27 [37]. Fifty µL/well of serum from each patient at appropriate dilutions (1/20 to 1/500) were incubated overnight at 4°C. After washing, 50 µL of inhibitors (i.e. increasing concentrations of β-CN or fragments) and 50 µL of AChE-labelled β-CN, prepared as previously described [37], were mixed and incubated for 4 h at room temperature. Results were expressed as B/B0, where B0 and B represent the amount of labelled β-CN bound to immobilised IgE antibodies in the absence or presence of a known concentration of inhibitor.

2.7 Statistical analysis

Data were analyzed using the non-parametric Wilcoxon matched pairs signed rank test. Statistical analyses were performed with GraphPad Prism 5.01 software and a $P<0.05$ was considered significant (* $P<0.05$, ** $P<0.01$ and *** $P<0.001$).

3 Results

3.1 IgE specificity of sera from CM-allergic and GM-allergic/CM-tolerant patients.

All sera from both groups displayed a significant IgE response to caprine whole CN and to caprine β-CN (Table 1). However, two different patterns of IgE-specificity were evidenced between CM-allergic and GM-allergic/CM-tolerant patients. IgE antibodies from CM-allergic patients recognized similarly CMPs and GMPs and IgE responses against BLG were generally much lower than those against CN. In contrast, most of GM-allergic/CM-tolerant patients showed very weak IgE responses to caprine BLG and to all bovine CMPs.

3.2 Plasmin hydrolysis of β-CN

Bovine and caprine β-CN were subjected to plasmin hydrolysis in order to produce large fragments covering their entire sequence. As shown on Fig. 1, full-length bovine and caprine β-CN were still detectable after 30 min of hydrolysis but disappeared completely after 120 min. Among the different bovine breakdown products, presence of the γ-caseins (f106-209) and (f108-209), and proteose-peptones (f1-28) and (f29-105/7) was confirmed by MS analysis (Fig. 2A). Caprine β-CN was also rapidly and extensively degraded as revealed by the presence of additional degradation products such as peptides (f49-105) and (f29-97) (Fig. 2B).

3.3 Impact of plasmin hydrolysis on β-CN IgE-reactivity

Considering that plasmin hydrolysis could affect immunodominant IgE-binding epitopes, IgE-reactivity of total hydrolysates was evaluated. Reverse EAST inhibition tests were performed with sera from two CM-allergic patients and two GM-allergic/CM-tolerant patients. For all patients, bovine and caprine digesta resulting from plasmin hydrolysis of β-CN for 240 min displayed an IgE-binding capacity similar to that of the full-length proteins. Bovine and caprine hydrolysates inhibited the binding to bovine β-CN of IgE antibodies from both CM-

allergic patients, as illustrated with patient 135 (Fig. 3A). In contrast, the bovine digesta failed to inhibit the binding to caprine β-CN of IgE antibodies from both GM-allergic/CM-tolerant patients, as illustrated with patient 64 (Fig. 3B).

3.4 IgE responses to the plasmin-derived peptides from bovine and caprine β-CN

IgE responses to each purified peptide were first quantified using direct EAST on immobilized fragments. In this assay, all the bovine β-CN peptides were efficiently recognized by IgE antibodies from CM-allergic patients (Fig. 4A). The IgE response toward the bovine β-CN peptide (f29-105) was nevertheless significantly higher than those against peptides (f1-28) and (f106-209). Moreover, IgE responses to caprine β-CN peptides were not significantly different from those against the corresponding bovine peptides, except for the caprine N-terminal fragment (f1-28) which displayed an IgE-reactivity significantly lower than that of the bovine counterpart.

A different profile of IgE responses toward bovine and caprine β-CN fragments was observed with sera from GM-allergic/CM-tolerant patients (Fig. 4B). Indeed, only caprine β-CN peptides exhibited a significant IgE-reactivity whereas all bovine β-CN fragments were poorly recognized. Among the caprine peptides, the central fragment (f29-107) was again the most IgE-reactive peptide.

3.5 IgE-binding capacity of β-CN fragments using reverse EAST inhibition

The IgE-binding capacity of β-CN fragments was further investigated by performing reverse EAST inhibition on sera from four CM-allergic patients (77, 83, 84 and 116) and six GM-allergic/CM-tolerant patients (8, 16, 50, 64, 86 and 96).

Bovine β-CN exhibited a slightly higher IgE-binding capacity than caprine β-CN but both β-CN were efficiently recognized by CM-allergic patients' IgE antibodies, as illustrated

with sera 77 and 116 (Fig. 5A and B). For all CM-allergic patients, the highest IgE-binding capacity was displayed by the bovine fragment (f29-105), as observed with direct EAST. Unexpectedly, the N-terminal fragment (f1-28) exhibited a very weak IgE-binding capacity although the corresponding IgE response measured by direct EAST was relatively high. The IgE-binding capacity of the C-terminal fragment (f106-209) varied significantly between the different sera, as illustrated with sera 77 and 116 (Fig. 5A and B).

As expected, only caprine β-CN peptides inhibited the binding of IgE antibodies from GM-allergic/CM-tolerant patients to the full-length caprine β-CN (Fig. 5C and D). The predominant role of fragment (f29-107) was again observed while no IgE-binding inhibition was observed with the N-terminal fragment (f1-28). The IgE-binding capacity of the fragment (f108-207) was limited, as illustrated with serum 64 (Fig. 5C and with sera 8, 50 and 96, data not shown) but was more significant with serum 86 (Fig. 5D and with serum 16, data not shown).

The IgE response of GM-allergic/CM-tolerant patients to the most immunoreactive caprine fragment (f29-107) was then further investigated by using synthetic peptides covering the domain 49-107, which contains all the residue divergences occurring between the bovine and caprine (f29-107) fragments (Fig. 6). The synthetic peptides (f49-107) and (f49-79) inhibited the binding of IgE antibodies from serum 64 (and sera 8, 50 and 96, data not shown) whereas the peptide (f80-107) displayed no significant IgE-binding capacity (Fig. 7A). However, with serum 86, none of the short peptides (f49-79) and (f80-107) displayed any significant IgE-binding capacity although peptide (f49-107) could partially inhibit the IgE-binding to the full-length caprine β-CN (Fig. 7B).

4 Discussion

In the present study, we investigated the structural determinants that account for the lack of immunological cross-reactivity between the highly homologous bovine and caprine β-CN, in patients suffering from GMA without associated CMA. For this purpose, large peptides of β-CN were generated by plasmin hydrolysis in order to compare their IgE-binding capacity between GM- allergic/CM-tolerant and CM-allergic patients. It is noteworthy that bovine and caprine β-CN kept their IgE-reactivity after plasmin hydrolysis. Thereby, the sole action of endogenous plasmin is not expected to reduce the allergenicity of fermented dairy products.

All bovine β-CN plasmin fragments were recognized by IgE antibodies from CM-allergic patients thus confirming the heterogeneity of the IgE-binding epitopes that are spread all over the bovine β-CN molecule as previously reported [24, 27, 28]. However, both direct EAST and reverse EAST inhibition indicated that the fragment (f29-107) is the most IgE-immunoreactive, thus suggesting that some of the IgE-binding epitopes previously reported in regions 43-54, 55-74 and 83-92 are particularly immunodominant [24, 27, 28].

Except for the fragment (f1-28), bovine and caprine peptides were similarly recognized by IgE from CM-allergic patients using direct EAST. This is in agreement with the immunological cross-reactivity observed between bovine and caprine β-CN. However, the caprine N-terminal fragment (f1-28) displayed a significantly decreased IgE-binding capacity compared to the bovine counterpart. Alignment of primary structures of bovine and caprine β-CN shows a gap of two amino acid residues and 17 substitutions scattered along the molecules (Fig. 6). Interestingly, none of the substitutions involves residues critical for the recognition of bovine β-CN epitopes, except for residues Leu^3 and Pro^9 [24]. These two substitutions with Gln^3 and Val^9 in caprine β-CN may thus account for the weak IgE response of CM-allergic patients towards caprine peptide (f1-28). Accordingly, Pizzano et al. suggested that the substitution of Pro^9 by Val was likely responsible for the restricted specificity of

polyclonal antibodies raised against the bovine fragment (f1-28) that could not recognize the caprine counterpart [38]. In reverse EAST inhibition, bovine and caprine N-terminal fragments (f1-28) were not able to inhibit IgE-binding to the full length protein, thus suggesting that this fragment contains only low IgE affinity epitope(s) or that the presentation of these epitopes on the short fragment (f1-28) is not appropriate for an efficient recognition by IgE antibodies in a fluid-phase assay. Reverse EAST inhibition revealed also that IgE from CM-allergic patients displayed a higher affinity to bovine β-CN than to caprine β-CN. This higher affinity could reflect the fact that CM-allergic patients were initially sensitized to CMP rather than to GMP.

As evidenced in bovine β-CN, the IgE-binding epitopes were also evenly distributed along the caprine β-CN since all fragments were recognized by IgE from GM-allergic/CM-tolerant patients. In this case, the bovine fragments were poorly recognized, in agreement with the absence of immunological and clinical cross-reactivity between bovine and caprine proteins. The caprine fragment (f29-107) also exhibited the highest IgE-binding capacity compared to fragments (f1-28) and (f108-209). In 4 out 6 tested sera, the immunodominant IgE-binding epitope was further located in the domain 49-79, which corresponds in bovine β-CN to a major IgE-binding epitope, between Phe^{52} and Ile^{74} [27, 28]. The primary structure of the caprine fragment (f49-79) displays 3 amino acid substitutions with the bovine sequence at positions 55 (Ala/Thr), 63 (Thr/Pro) and 75 (Leu/Pro). Interestingly, two substitutions involve proline residues, which introduce turns into the structure of β-CN and interrupt 2D structure sequences [39]. Thereby, substitutions of these proline residues may significantly affect the conformational arrangement of this domain and could support the generation of IgE antibodies specific of the caprine domain without cross-reacting with the bovine counterpart.

Considering patient 86, short synthetic peptides (f49-79) and (f80-107) did not exhibit any significant IgE-binding capacity compared to that of the larger plasmin-derived fragment

(f29-107). A recent study from Johansson *et al.* reported that several monoclonal antibodies were able to bind a peptide obtained by tryptic hydrolysis of native β-CN whereas they did not recognize any of the synthetic peptides covering the β-CN sequence [25]. In that case, conformational features present in more structured short synthetic peptides have been proposed to explain the differences of binding capacity between synthetic peptides and proteolytic fragments. Although the occurrence of conformational epitopes in β-CN and their role in β-CN allergenicity has been suggested [40, 41], β-CN is generally considered to not have a well defined 3D structure, with several rigid parts and more flexible domains [25, 39]. However, β-CN fragments, compared to the full-length protein, have been described to exhibit specific 2D-structures and particular physical properties [26, 42-44]. We thus suggest that structural features of the synthetic peptides prevented their recognition by IgE antibodies from patient 86.

In conclusion, our results showed that the absence of cross-reactivity between the bovine and caprine β-CN observed in GM-allergic/CM-tolerant patients is accounted for by structural determinants located, in most cases, in the domain 49-79. The lack of cross-reactivity between these highly homologous allergens can be thus explained by few modifications in their primary structure. The relative role of each substitution occurring in domain 49-79 is currently under investigation.

The authors have declared no conflict of interest.

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Table

Table 1. Clinical features and IgE responses to bovine and caprine milk proteins of CM-allergic patients and GM-allergic/CM-tolerant patients.

Patients	Symptoms	Age (year) /sex	Specific IgE levels to milk proteins (IU/ml)					
			Bovine BLG	Caprine BLG	Bovine whole CN	Caprine whole CN	Bovine β-CN	Caprine β-CN
CM-allergic patients								
4	GU, A	3/M	1	0.6	20	10	4	3
18	AS	3.5/F	0.4	0	10	9	3.5	3.5
77	GU	2/F	6	1.5	15	25	22	21
83	AD, U	3.5/M	12	12	84	83	40	35
84	GU, A	7/M	5	6	15	11	10	7
116	AS	14/F	11	12	40	85	60	78
135	AS	13/F	3.5	4	8	14	18	15
138	U	2.5/M	84	67	28	18	15	14
185	U, V	5.5/F	105	61	330	220	150	130
190	U, V, AO	5/F	32	18	130	90	44	37
GM-allergic/CM-tolerant patients								
8	U, V	11/M	1.3	1	1.5	17	1	9
15	U, V	10/M	5	6	2	22	1	14
16	GU, LO	9/M	1	2	1	20	2	17
50	A, U, RC	5/F	1.5	1.4	0.1	60	0.5	19
53	AO, LO, V, RC, GU	4/M	0	0	0	40	0	28
64	AO	2.5/M	0.7	0.1	0	10	1.5	10
86	OS	9/M	0.6	0.2	1.4	15	1	9
88	AO	4/F	0	0	0	15	0.3	2
96	AS	8/M	3.5	6	3	50	5	40
97	AO	5.5/M	0	0.1	0	4	0.1	2
183	A, U, RC	5/F	0	8	0	95	0	50
193	GU, V, A	4.5/F	0	6	17	90	6	50

AD, atopic dermatitis; AO, angio-oedema; A, asthma; OS, oral allergy syndrome; RC, rhino-conjunctivis, GU, generalized urticaria; LO, laryngeal oedema; U, urticaria; V, vomiting; AS, anaphylactic shock; M, male; F, female

Figures legends

Figure 1. SDS-PAGE analysis of bovine (A) and caprine (B) β-CN hydrolyzed by plasmin.

Figure 2. Mass spectrometry analysis of bovine (A) and caprine (B) β-CN digesta after 120 min of plasmin hydrolysis. The β-CN peptides corresponding to the numbered peaks were identified by taking into account the phosphorylation of seryl residues (*).

Figure 3. Impact of plasmin hydrolysis during 240 min on the IgE-reactivity of bovine and caprine β-CN. Competitive binding-inhibition (reverse EAST inhibition) of IgE antibodies from CM-allergic patient (serum 135) to bovine β-CN (A) and of IgE antibodies from GM-allergic/CM-tolerant patient (serum 64) to caprine β-CN (B) by increasing concentrations of native (○) or hydrolyzed (◇) bovine β-CN and native (●) or hydrolyzed (◆) caprine β-CN.

Figure 4. IgE responses (IU/mL) to plasmin-derived fragments of bovine and caprine β-CN quantified by direct EAST in CM-allergic patients (A, n=10) and in GM-allergic/CM-tolerant patients (B, n=12). Statistical analysis was performed with the non-parametric Wilcoxon matched pairs signed rank test. Significant differences between IgE responses to bovine and caprine β-CN fragments are indicated (** $P<0.01$ and *** $P<0.001$).

Figure 5. Competitive binding-inhibition (reverse EAST inhibition) of IgE antibodies from CM-allergic patients (sera 77 (A) and 116 (B)) to bovine β-CN and of IgE antibodies from GM-allergic/CM-tolerant patients (sera 64 (C) and 86 (D)) to caprine β-CN by increasing concentrations of full-length bovine (○) and caprine (●) β-CN, bovine β-CN peptides ((f1-

28): □, (f29-105): △ and (f106-209): ▽) and caprine β-CN peptides ((f1-28): ■, (f29-107): ▲ and (f108-209): ▼).

Figure 6. Alignment of bovine (Bov, Swiss-Prot accession number P02666) and caprine (Cap, Swiss-Prot accession number P33048) β-CN. Numbering of the bovine β-CN is shown and only amino acid substitutions are indicated in the caprine β-CN. The major plasmin cleavage sites are indicated by arrows after Lys^{28}, Lys^{106} and Lys^{108}. The caprine peptides synthesised in this work are shown in grey.

Figure 7. Competitive binding-inhibition (reverse EAST inhibition) of IgE antibodies from GM-allergic/CM-tolerant patients (sera 64 (A) and 86 (B)) to caprine β-CN by increasing concentrations of caprine plasmin-derived fragment (f29-107): ▲ or of caprine synthetic peptides (f49-107): ✚, (f49-79): ◆ and (f80-107): ✱.

Figure 1.

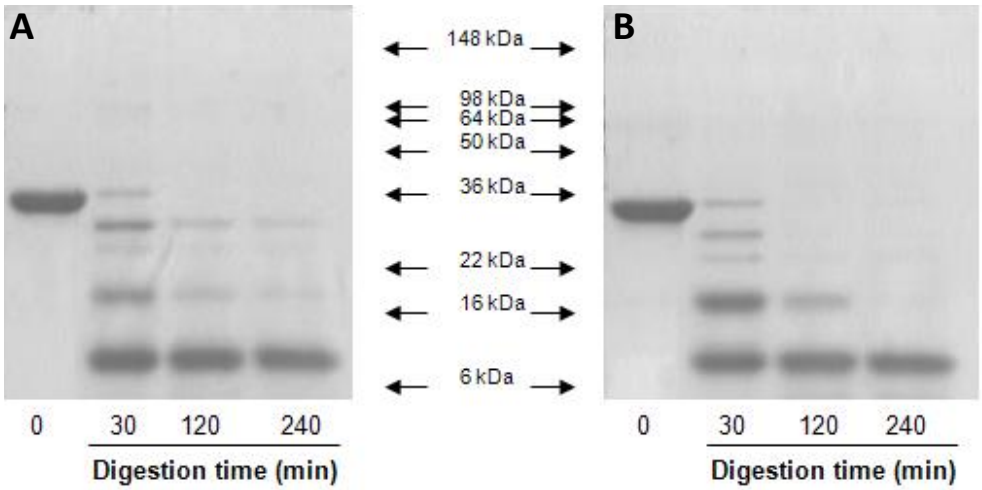
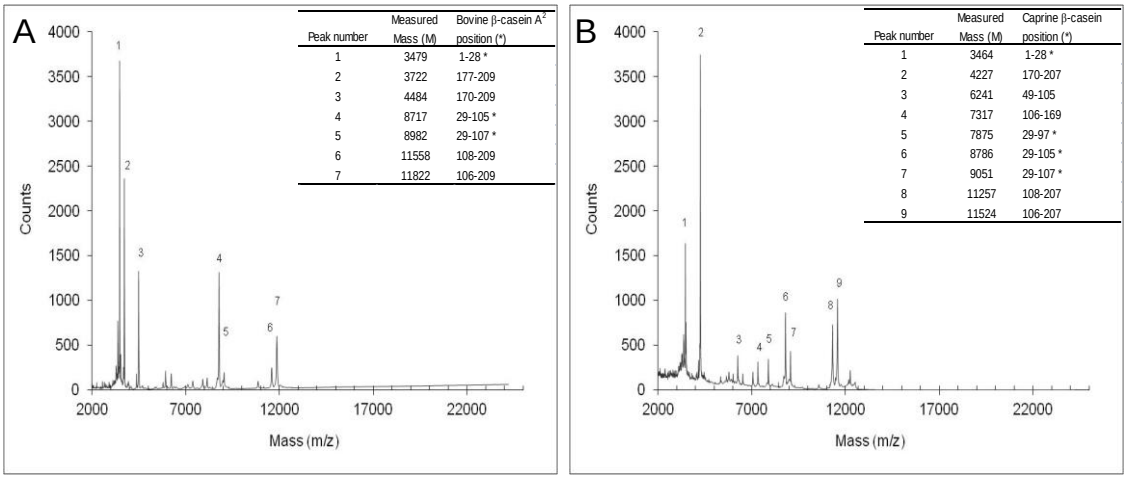

A
B
148 kDa
98 kDa
64 kDa
50 kDa
36 kDa
22 kDa
16 kDa
6 kDa
0
30
120
240
Digestion time (min)
0
30
120
240
Digestion time (min)

Figure 2.



A

Peak number	Measured Mass (M)	Bovine β-casein A[2] position (*)
1	3479	1-28 *
2	3722	177-209
3	4484	170-209
4	8717	29-105 *
5	8982	29-107 *
6	11558	108-209
7	11822	106-209

B

Peak number	Measured Mass (M)	Caprine β-casein position (*)
1	3464	1-28 *
2	4227	170-207
3	6241	49-105
4	7317	106-169
5	7875	29-97 *
6	8786	29-105 *
7	9051	29-107 *
8	11257	108-207
9	11524	106-207

Figure 3.

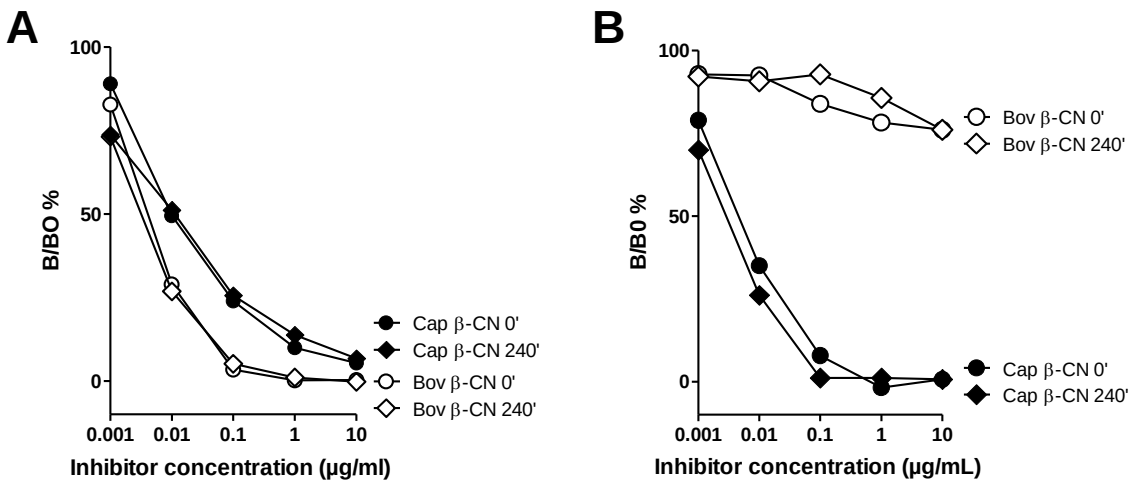

A
100
50
0
B/BO %
0.001
0.01
0.1
1
10
Inhibitor concentration (µg/ml)
Cap β-CN 0'
Cap β-CN 240'
Bov β-CN 0'
Bov β-CN 240'
B
100
50
0
B/B0 %
0.001
0.01
0.1
1
10
Inhibitor concentration (µg/mL)
Bov β-CN 0'
Bov β-CN 240'
Cap β-CN 0'
Cap β-CN 240'

Figure 4.

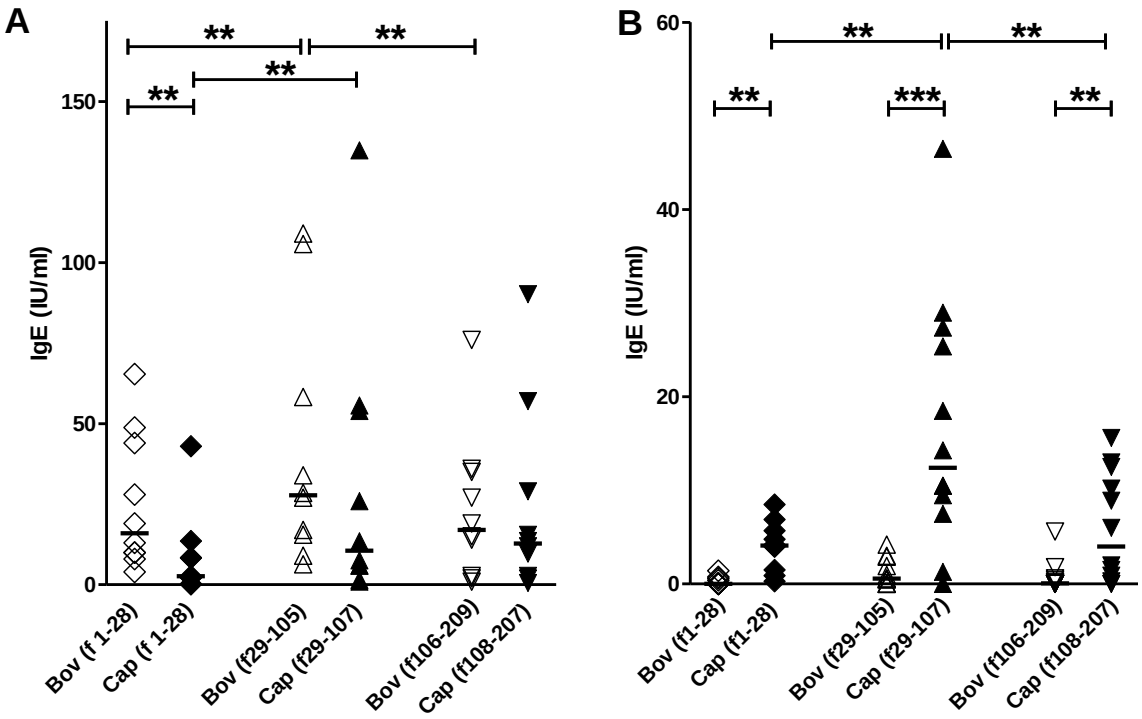

A
B
IgE (IU/ml)
150
100
50
0
60
40
20
**
***
Bov (f 1-28)
Cap (f 1-28)
Bov (f29-105)
Cap (f29-107)
Bov (f106-209)
Cap (f108-207)
Bov (f1-28)
Cap (f1-28)

Figure 5.

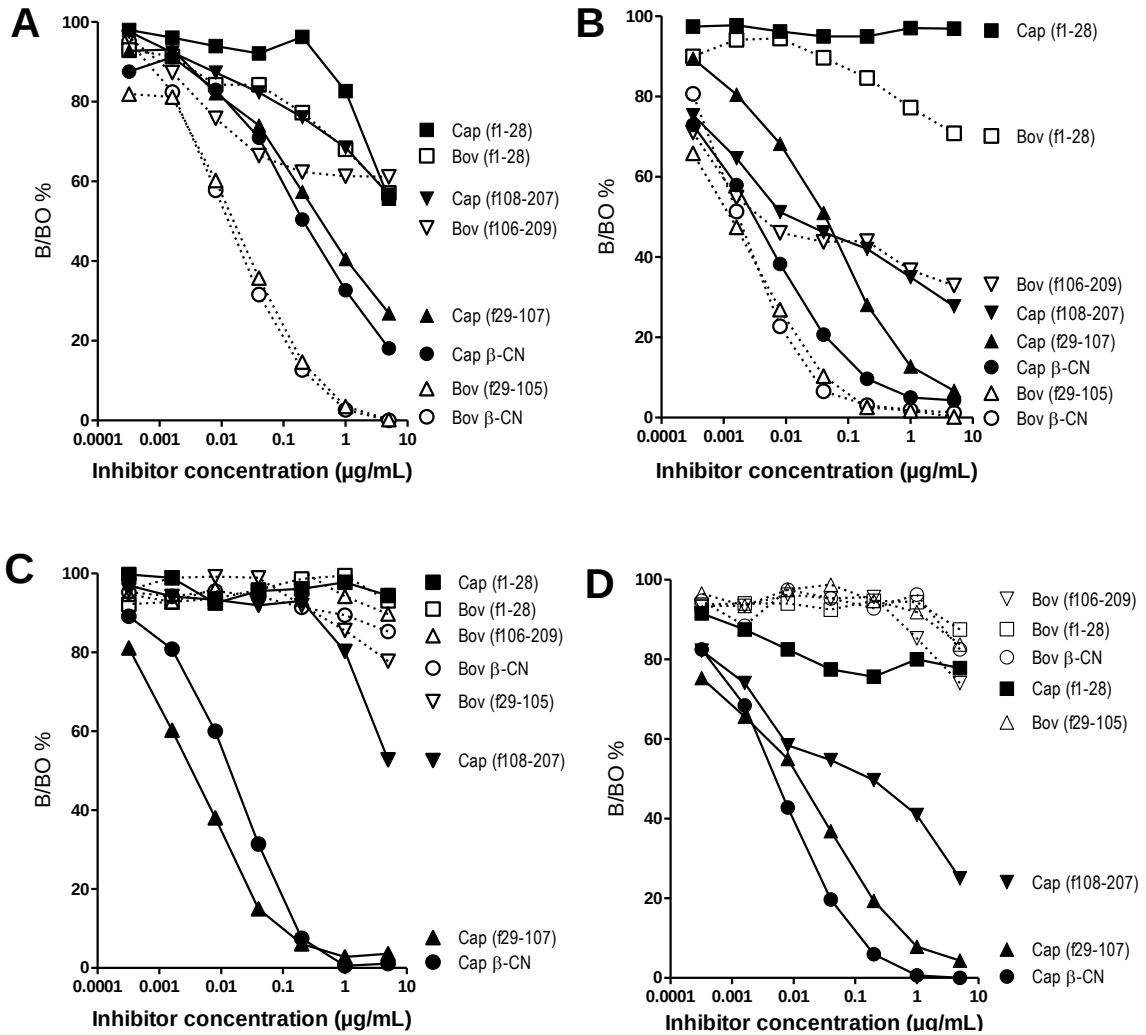

A
B
C
D
B/BO %
Inhibitor concentration (µg/mL)
Cap (f1-28)
Bov (f1-28)
Cap (f108-207)
Bov (f106-209)
Cap (f29-107)
Cap β-CN
Bov (f29-105)
Bov β-CN

Figure 6.

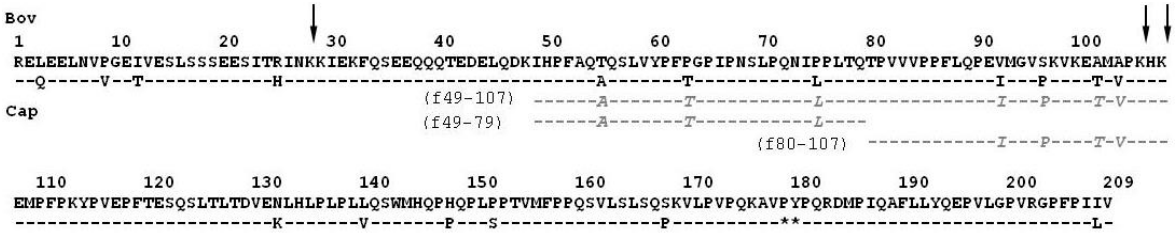
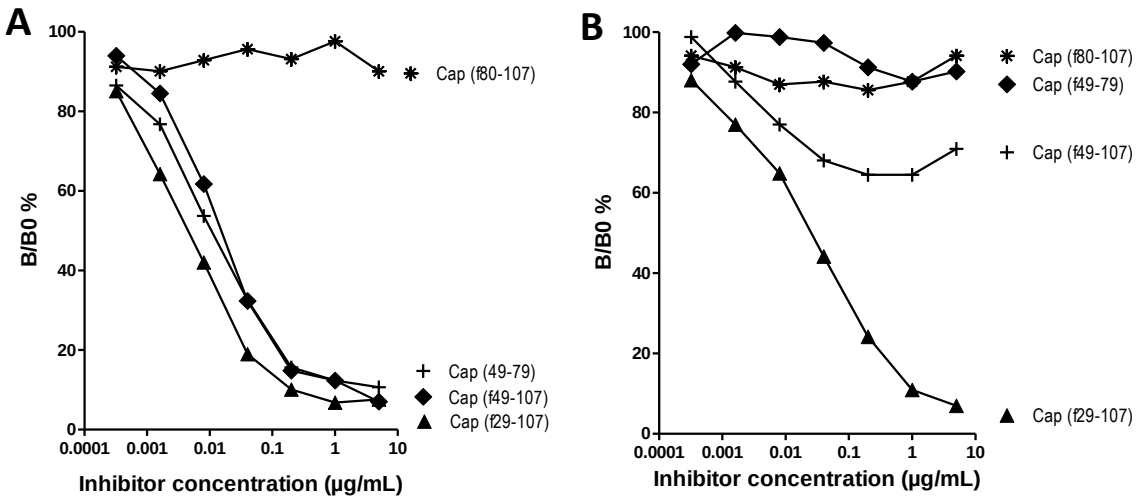

Bov
1 10 20 30 40 50 60 70 80 90 100
RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKHK
--Q-----V--T----------H----------A-------T----------L----------I---P----T-V----
Cap
(f49-107) ------A-------T----------L----------I---P----T-V----
(f49-79) ------A-------T----------L----
(f80-107) ----------I---P----T-V----
110 120 130 140 150 160 170 180 190 200 209
EMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV
----------K-------V-------P---S----------P----------**----------L-

Figure 7.


A
100
80
60
40
20
0
B/B0 %
0.0001
0.001
0.01
0.1
1
10
Inhibitor concentration (µg/mL)
Cap (f80-107)
Cap (49-79)
Cap (f49-107)
Cap (f29-107)
B
100
80
60
40
20
0
B/B0 %
0.0001
0.001
0.01
0.1
1
10
Inhibitor concentration (µg/mL)
Cap (f80-107)
Cap (f49-79)
Cap (f49-107)
Cap (f29-107)