

Evaluation of the Allergenicity and Antigenicity of Bovine-Milk α s1-Casein Using Extensively Purified Synthetic Peptides

S. Elsayed*, D. J. Hill† & T. V. Do*

*Allergy Research Group, Laboratory of Clinical Biochemistry, Haukeland University Hospital, Institute of Medicine, University of Bergen, Bergen, Norway; and †Department of Allergy, Royal Children's Hospital North Melbourne, Australia

Received 22 April 2004; Accepted in revised form 17 June 2004

Correspondence to: Dr S. Elsayed, Allergy Research Group, Laboratory of Clinical Biochemistry, Haukeland University Hospital, 5021 Bergen, Norway. E-mail: said.elsayed@helse-bergen.no

Abstract

α s1-Casein (CAS1_BOVIN), the major allergen of cow's milk (CM), is widely used as hydrolysates in infant diet formulae and additive to other processed food items. To date, most of the reported B-cell epitope mapping were performed on polyethylene pins or cellulose-derivative membrane. We sought to locate the motifs critical for human-specific IgE and rabbit polyclonal IgG binding using extensively purified CAS1_BOVIN, synthetic peptides and derivatives. Thirteen overlapping peptides covering the whole CAS1_BOVIN encompassing 17 : 20 amino acid (AA) were synthesized by f-moc AA solid-phase polyamide peptide synthesis. In addition, six cyanogen bromide (CNBr) cleavage fragments were prepared. Limited hydrolysis, oxidized and reduced/alkylated derivatives were also produced. The preparations were purified by ion exchange, gel filtration chromatography, reversed phase and high-performance liquid chromatography. The homogeneity was visualized by sodium dodecyl sulfate (SDS) and poly acryl amide gel electrophoresis (PAGE) followed by IgE and IgG immunoblotting. IgE binding was measured by Biotin Streptavidin (Bio/strep) fluoro enzyme immuno assay (FEIA) or ELISA-inhibition. Eighteen CM allergy (CMA) sera from 45 clinically examined children (Melbourne) and five adults (Bergen) were selected. Individual sera and pools were used for mapping IgE-binding epitopes. Rabbit IgG sera and pools were used for locating the antigenic sites of the molecule. Results indicated that all the individual CMA sera and pools recognized the intact molecule and three of the CNBr fragments as major antibody-binding allergens. The N- and C-terminal peptides (CAS 16-35; CAS 136-155) showed high IgE-binding affinity. CAS 1-18 and CAS 181-199 showed high IgG bindings. Considering the diversity of the antibody specificities, a reasonable agreement between IgE and IgG epitopes were found at the N- and C-terminals of CAS1_BOVIN. Mapping IgE B-cell epitopes by direct Bio/strep FEIA allowed the development of a sensitive modified technique for detecting unlabelled, casein immune dominant peptides in food products.

Introduction

It is generally accepted that the incidence of allergic diseases has increased during the last two decades and mostly in industrialized countries [1]. Cow's milk proteins (CMP), chicken egg and peanut are the most frequent food allergens world over [2]. CMA is often encountered during the first months of life; one-third already during lactation and mostly within a week after the introduction of CM [3]. Approximately 2–7.5% of new born infants get

CMA; about half of those with signs of immediate reactions. The majority of those developing CMA (87%) tolerate milk by 3 years age although this can lead to other hypersensitivity symptoms in older age [4–6]. CMP are among the best characterized food allergens. The whole casein appears to be an association of four different proteins, α s1-, α s2-, β - and κ -casein, in approximate proportions of 40 : 10 : 40 : 10%, respectively. Statistically higher amounts of specific IgE were found to be

directed against α s1- and β -casein [7]. CAS1_BOVIN is a single-chain phosphoprotein of 199 residues. It constitutes more than 40% of the major CM casein. The primary structures are known for most genetic variants of α s1-, α s2-, β - and κ -caseins, β -lactoglobulin and α -lactalbumin [8, 9]. α s1-casein is not crystallizable and the tertiary structure has not been reported.

Some CMA's immunodominant epitopes have been proposed as good markers of persistent allergy to cow's milk protein (CMP) while other linear epitopes were associated with transient CMA [10–12]. B-cell epitopes were shown to be mostly of the sequential or continuous type, relatively short (12–16 AA) and located in hydrophobic parts of the molecule [2]. T-cell recognition pattern of α s1-casein have been studied and five large peptides and seven smaller have been recognized by T cells generated from CMA patients [13].

Hypoallergenic hydrolysates are widely used for the prevention and treatment of children CMA. Despite being marketed as low allergen content diet, some anaphylactic reactions have been experienced [14–16]. Nonetheless, cross reactivity between IgE epitopes from phylogenetically nonrelated allergens is an important problem and probably the direct cause of multiple food allergy [17–20]. In the present study we tested the potential allergenicity and antigenicity of 13 overlapping, synthetic peptides covering the intact molecule. Sera from 18 CMA and five healthy volunteers in addition to six rabbits polyclonal IgG sera were used.

Materials and methods

Purification. CAS1_BOVIN was provided by the purification of α -casein from bovine milk, essentially vitamin free, C5679 from Sigma Chemical Co, St Louis, MO, USA, with 85% α s-casein, was the initial reagent used through the purification procedures. Purification was performed by IEC, GFC and reversed phase (RP)/high-performance liquid chromatography (HPLC) [21].

RP/HPLC. RP/HPLC was performed using 130A Separation System, BrownleeTM C18, Aquapore RP-300, 7- μ m 30 $\times$ 2.1 mm cartridges (Applied Biosystems, Foster City, CA, USA). Solvent gradient used was 0.1% trifluoroacetic acid in 70% acetonitrile for 30 m at flow rate of 200 μ l/m [22, 23].

Oxidation, CNBr – cleavage, reduction and alkylation. CAS1_BOVIN (5 mg/ml) was oxidized by treatment with 70% formic acid to give 1% (w/v) 10 ml solution, for 15 m. H₂O₂ (70 ml) was then added, and the solution was lyophilized and stored until used.

Cyanogen bromide (CNBr) cleavage was done by the addition of CNBr in acetonitrile, 50–100-fold molar excess over methionine residues (200 μ g/100 μ g protein) in a small volume of 70% formic acid. The sample was

kept in dark under N₂, at RT for 24 h. The sample was then 10-fold diluted and lyophilized.

Reduction and alkylation was performed using 6 M guanidine-HCl (Fluka, 50940 Buchs, Switzerland) adding 1680 ml, 500 mM Tris-HCl (Sigma T-3253) and 10 mM ethylenediaminetetraacetic acid pH 8.50 (Sigma EDSS). DL-dithiothreitol, Cleland's reagent (DDT) (4.6 mg, DDT 99% Sigma D-0632), iodoacetic acid (Sigma I-6250) and 2-mercaptoethanol (Merck 805740, Darmstadt, Germany) by the previously described method [21].

Limited hydrolysis. CAS1_BOVIN was hydrolyzed by pepsin P 7000 from porcine gastric mucosa (Sigma Aldrich, St Louis, MO, USA). Limited cleavage was obtained by an E/S1/100 in 100 mM glycine buffer pH 4.0. The hydrolysis proceeded at different interval (15 : 60 m at RT), and non-optimal hydrolysis pH was used to avoid obtaining large number of short peptides.

SDS/PAGE and immunoblotting. Proteins were separated by SDS/PAGE according to early-described procedures [24]. The samples, together with molecular weight standard (Bio-Rad low range SDS/PAGE standards and prestained standards) were resolved in 15% separating gel at 200 V. Immunoblotting was carried out as described previously for 1 h at 100 V [25].

SPPS. Thirteen overlapping peptides covering the whole CAS1_BOVIN were synthesized using f-moc (AA) Solid phase peptide synthesis (SPPS) and a semiautomatic outfit (BiolynxTM 4175, LKB, Sweden). Eleven peptides overlapped at 5 AA-windows while two peptides 1–18 and 16–35 with only 2 AA-overlaps. The peptides were purified and their N-terminal AA-sequence were ascertained (Applied Biosystem, Foster City, CA, USA). The primary structure of CAS1_BOVIN peptides are shown in Table 1.

Patients. CMA diagnosis was based on family history, skin prick test (SPT), assay of serum total and specific IgE and CMP elimination [5, 6, 26]. Forty children were recruited from Department of Allergy, Melbourne, Australia. Patients had histories of anaphylactic or immediate responses to CMP with reactions such as asthma, gastrointestinal, urticaria or other symptoms. All patients have oral positive provocation with CM, positive SPT and score 3 : 20 mean 7. Classic radio allegro sorbent test values were 7.08 : >>100 mean 17.65 kU_A/l and s-total IgE mean 3079 KU_A/l, range 187–8932 KU_A/l. CMA adults' patient's sera (CAP/FEIA class > 2) were selected from routine laboratory diagnosis at the Laboratory of Clinical Biochemistry, University Hospital Bergen. Two nonallergic controls were included (s-IgE < 26 KU_A/l and class 0 CAP/FEIA) to common food allergens (egg, fish, milk and soy).

CAP/FEIA IgE and Biotin–Streptavidin coupling. Biotinylated CAS1_BOVIN and fragments were coupled to CAPs using Bio/strep Immuno CAPTM and assayed by UniCAP[®] 100/AutoCAPTM outfit, provided by Pharmacia

Table 1 Upper section showed the locations and amino acid (AA) sequence of the thirteen synthetic peptide covering CAS1_BOVIN; Lower section showed the CNBr fragments as enumerated 1 to 6 according to their elution profile

CAS1_BOVIN and peptides	AA sequence	CAP FEIA class								Percentage recognition and grade
		Number of sera reacted/class								
		6	5	4	3	2	1	0		
CAS1_BOVIN	RPKH—————TTMPLW	0	2	3	10	3	0	0	100	III
CAS 1-18	RPKHPIKHQGLPQEVLE	0	0	0	2	4	4	8	44.4	II
CAS 16-35	LNENL LRFFVAPFPEVFGKE	0	0	0	3	5	4	6	55.5	III
CAS 31-50	VFGKEKVNELSKDIGSESTE	0	0	0	2	3	3	10	33.3	II
CAS 46-63	SESTEDQAMEDIKQM EAE	0	0	0	0	5	1	12	16.6	I
CAS 61-77	EAESISSEIIVPNSVE	0	0	0	1	2	3	12	16.6	I
CAS 76-95	VEQKHIQKEDVPSERYLGYL	0	0	0	1	4	1	12	16.6	I
CAS 91-110	YLGYLEQLRLKKYKVPQLE	0	0	0	0	2	1	15	27.7	II
CAS 106-125	VPQLEIVPNSAEERLHSM KE	0	0	0	1	1	1	15	33.3	II
CAS 121-140	HSMKEGIHAQQKEPMIGVNQ	0	0	0	0	2	1	15	22.2	I
CAS 136-155	IGVNQELAYFYPELFRQFYQ	0	0	0	1	3	3	11	66.6	III
CAS 152-169	QFYQLDAYPSGAWYYVPL	0	0	0	1	3	0	14	33.3	II
CAS 166-183	YVPLG TQYTD APSFSDIPN	0	0	0	1	2	2	13	27.7	I
CAS 181-199	DIPNPIGSENSEKTTMPLW	0	0	0	2	2	1	13	27.7	I
CNBr fragments										
CNBr I 1–54	RPK—————QAM	0	0	2	3	11	1	1	94.4	III
CNBr III 61–123	EAE—————HSM	0	2	3	8	5	0	0	100	III
CNBr IV 124–135	KEG—————EPM	0	0	0	3	5	3	7	55.5	III
CNBr V 136–194	IGV—————TTM	0	1	1	4	11	0	1	94.4	III
CNBr II 55–60	EDIKOM	0	0	0	2	2	2	12	33.3	II
CNBr VI 195–197	PLW	0	0	0	0	0	0	18	0	0

The AA sequences of the peptides and CNBr-fragments were ascertained by automatic N-terminal sequence analysis. The number of sera were depicted under their class. The percentage recognition = the number of reacting sera divided by total number of sera, 18. Accordingly, the peptides were graded into three categories major, moderate and minor (I, II and III) where III was the highest binding

Diagnostics, Uppsala, Sweden. Biotinylated α -aminocaproic acid-*N*-hydroxy-succinimide ester (Biotin 7-NHS) coupling using Biotin Labelling Kit, Roche Diagnostics GmbH, Roch Molecular Biochemicals, D-68305 Mannheim, Germany, Cat. No.1418165. Pilot Bio/strept coupling was run on 17 birch pollen allergic patients' sera and excellent correlation was found between the new coupling method and the conventional CAP/Fluoro enzyme immunoassay (FEIATM). Bovine CAS1_BOVIN, 13 synthetic peptides and seven CNBr-cleavage peptides were biotinylated and the derivatives were purified according to the producer's instructions.

IgG antibodies. Rabbit immunization was approved by the Norwegian Experimental Animal Committee. Six rabbits were immunized with CAS1_BOVIN, oxidized, reduced/alkylated and 1 h pepsin-digested casein. Immunization was generally done using as follows; 0.5 ml of antigen (1 mg/ml sterile NaCl, 0.9%) in suspension with 0.5 ml Freund's Complete Adjuvant was injected in each animal at week 1. In weeks 3 and 5, each animal was injected with booster dose 0.5 ml (1 μ g/ μ l) in suspension with 0.5 ml Freund's Incomplete Adjuvant. The rabbit sera were tested for antibody production before each and after the third immunization. The animals were bled about 14 days after the last immunization. Sera were collected and stored at -80°C [27].

ELISA inhibition. IgE and IgG ELISA was performed as described earlier [28]. Ninety-six wells, flexible round-bottom, microtitre plates (Becton Dickinson Laboratory ware, Le Pont De Claix, France) were coated with CAS1_BOVIN, modified molecule or the 13 peptides were added in excess to the purified rabbit IgG sera or patients sera. Anti-rabbit IgG or Anti-human IgE alkaline phosphatase conjugates (Sigma A8025 or A-3525), respectively, were added to each well. Substrate *p*-nitro phenyl phosphate, tablets 5 mg, N-9389 (Sigma Chemical Co.) was added to each well before recording the absorbency ($\lambda = 405\text{ nm}$) using spectrophotometer (Labsystem Multiscan Bichromatic Type 348).

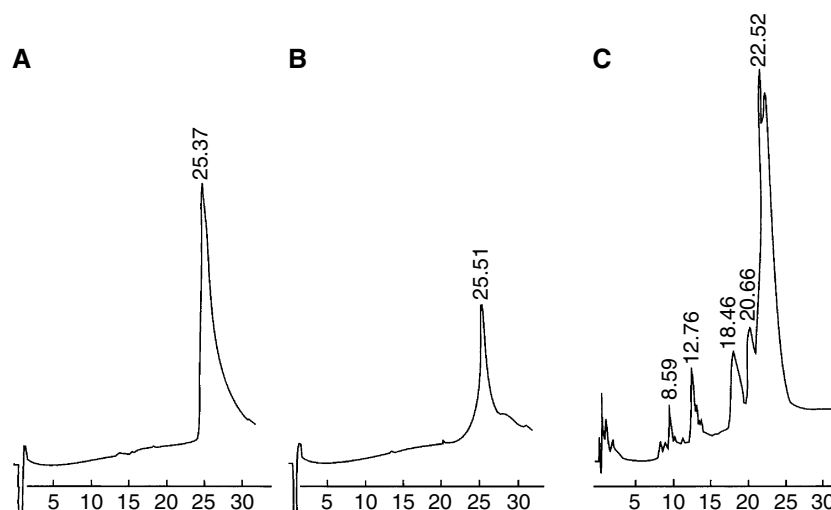
ELISA inhibition was expressed as: (Absorbency of control – Absorbency of Ag/Ab complex/Absorbency control) $\times 100$.

Results

GFC and RP/HPLC purification

Purification of CAS1_BOVIN by GFC, removed proteins with $M_r < 25\text{ kDa}$ and $>45\text{ kDa}$. Void volume peak from GFC was purified by RP/HPLC. Peaks with retention time RT. 25.37, 25.51 represented the highly purified CAS1_BOVIN (Fig. 1).

Figure 1 RP/HPLC of CAS1_BOVI and cyanogen bromide cleavage (CNBr)-fragments. (A) RP-HPLC of CAS1_BOVIN; (B) CAS1_BOVIN purified by Sephacryl 100; (C) CNBr cleavage showing six peaks which were designated from I to VI (R_t 22.52, 20.66, 18.46, 12.7 and 8.59, respectively).



The partially purified intact molecule showed two bands by SDS/PAGE, also when stained by blotting showing that both bind specific IgE from CMA patients and rabbit

IgG (Fig. 2). This was re-run on RP/HPLC and one symmetrical peak was preparative pooled Fig. 1(B). CNBr cleavage showing six peaks on RP/HPLC were shown in Fig. 1(C).

Hydrolysis of CAS1_BOVIN for 1 h allowed two populations detected on RP/HPLC. Two hours hydrolysis and longer periods showed no RP/HPLC signal differences.

Reduced alkylated and oxidized products of casein showed that the chemical modifications did not alter the R_t but resulted in broader denatured protein peaks (R_t 25.92 for the reduced; R_t 25.3 for the oxidized R_t 25.72 for the native). The N-terminal sequence analyses of the peaks ascertained their locations summarized in Table 1.

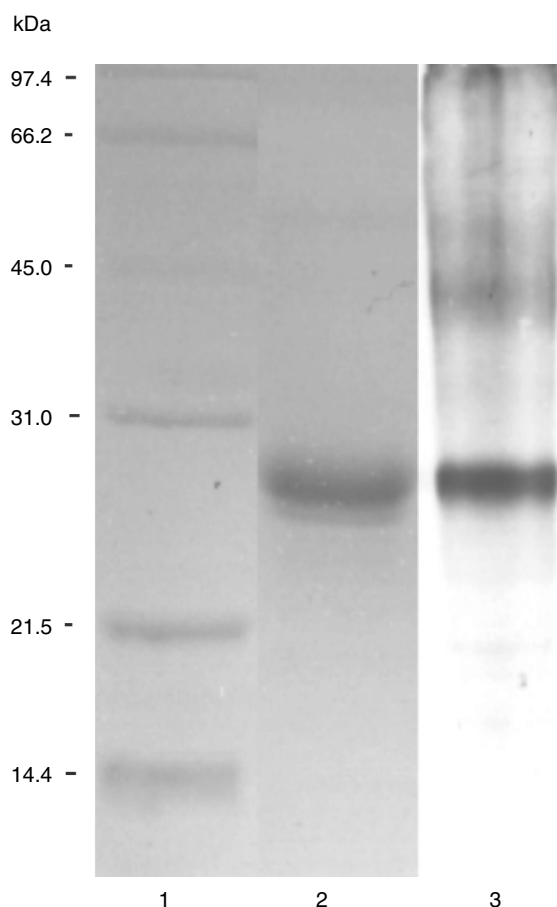


Figure 2 Sodium dodecyl sulfate (SDS) PAGE followed by immunoblotting. Lane 1 molecular weight marker; Lane 2 RP/HPLC-purified CAS1_BOVIN; Lane 3 rabbit serum pool, IgG antibody blot; Lane 4 CMA patient's serum pool-specific IgE antibody blot.

Peptide synthesis and homogeneity criteria

Thirteen synthetic overlapping peptides (with 3–5 AA-windows) covering the whole CAS1_BOVIN molecule were synthesized and cleaved from the resin. The homogeneity was judged by automatic N-terminal AA sequence analysis and RP/HPLC. Their NH₂-terminal AA sequence has also ascertained their locations as depicted in Table 1.

The allergenicity

IgE recognition pattern of CAS1_BOVIN and the synthetic peptides showed variability among the peptide as demonstrated by the numbers of IgE sera recognitions (Table 1). The results of FEIA showed that CAS 1-18, CAS 16-35, CAS 31-55 and CAS 136-155 encompassed the highest IgE-binding epitopes of the N- and C-terminal regions, respectively (Table 1) (Fig. 3). Larger peptides (CNBr I, III, IV and V) with AA residues 54, 63, 12 and 61, respectively, showed very high affinity for IgE (94.4, 100, 61.1 and 94.4%). CNBr II (55, 56, 57, 58, 59 and 60) was less active (33.3%) while CNBr VI (197, 198 and 199) showed no binding. The affinities of the

peptides for IgE binding was graded in three categories: (a) High III; (b) Moderate II- and (c) minor I-epitopes depending of the number of sera that recognized the epitope (Table 1).

The results of IgE ELISA inhibitions were illustrated in Fig. 4. Using a serum pool, the intact molecule and CAS 181-199 showed highest inhibition percentage. Particularly, CAS 166-183 and CAS 181-199 showed remarkable affinities (40.9–55.6%). The CNBr fragments I–IV have high inhibitions (58.4–72.2%).

The partial pepsin hydrolysates results showed that extensive hydrolysis totally abolished the allergenicity while limited hydrolysis for <60 m did not eliminate binding to IgE and could elicit allergic reactions.

The antigenicity

The results of IgG ELISA inhibition (Fig. 5) showed four epitope groups: (a) N-terminal with major IgG recognition, CAS 1-18 and CAS 31-50 recognized by 62.2 and 43.0%, respectively; (b) C-terminal with high recognition CAS 166-183 and CAS 181-199 (49.4 and 50.5%); (c) Core moderate recognition CAS 46-63 (36.5%), CAS 61-77 (31.9), CAS 76-95 (32.5), CAS 106-125 (38.4) and CAS 121-140 (39.2%); (d) Minor epitopes CAS 16-35, 91-110, 136-155 and 152-169, with inhibition range between 20.2 and 28%.

CAS1_BOVIN was recognized at high binding affinity as shown by IgG ELISA inhibition >90% (Fig. 5).

The average antigen concentrations necessary for achieving 50% ELISA inhibition (C-50) for the different preparations were as follows: CAS1_BOVIN (0.057–

0.07 µg); the limited pepsin hydrolysates (0.180 µg), CNBr cleavage preparation (0.350 µg).

The reduction and alkylation did not alter the antigenicity (similar inhibition to the nontreated CAS1_BOVIN).

Discussion

We examined the allergenic and antigenic epitopes of the intact CAS1_BOVIN, overlapping synthetic peptides, CNBr-cleavage fragments and the chemical derivatives. The unique conformation of CAS1_BOVIN permitted to investigate IgE and IgG epitopes without destruction of the reactivity [29]. Most of the reported B- and T-cell epitopes of different proteins have been done using polyethylene pins or polyethylene derivative membrane [30–32]. Although those methods were rapid, they could neither ascertain the correctness of the AA sequence nor the homogeneities of the peptides. Truncated or heterogeneous peptides missing 1–3 AA could easily pass unobserved. In this study, we have chosen the laborious and tedious SPPS purification using several techniques ending with RP/HPLC. The synthetic peptides AA sequence was analysed by automatic protein/peptide sequence analyzer.

Specific IgE recognition of the peptides allowed dividing the molecule into three regions: high, moderate and minor-binding regions (Table 1). CAS 1-18, CAS16-35, CAS 31-50 and CAS 136-155 could be recognized by most of the sera used. Three peptides (CAS 91-110, 106-125 and 121-140) were recognized by only three of the 18 sera. All the rest had moderate or minor recognition (22.2–33.3%) (Table 1). The epitopes that react with most of the CMA patients sera were at N- and C-termini of the molecule (CAS 1-18, CAS 16-35 and CAS 136-155).

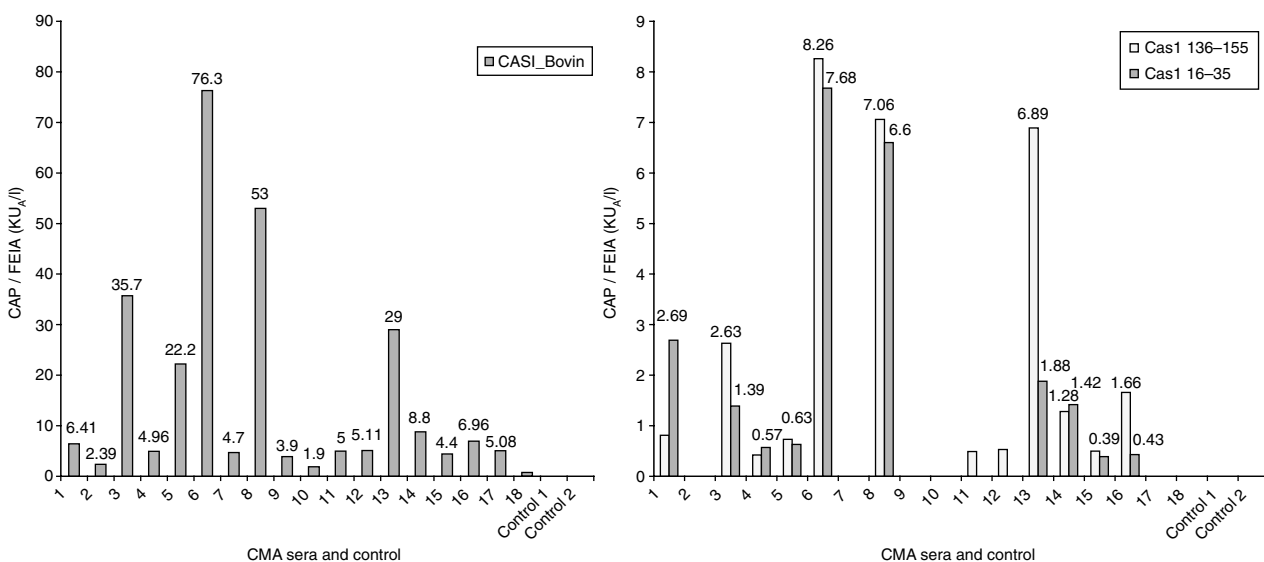


Figure 3 IgE ELISA inhibition showing that CAS1_BOVIN has large inhibition percentage (>90%), followed by the NH₂ terminal peptide CAS1-18 (>60%). Peptides at molecular core were mostly moderate epitopes.

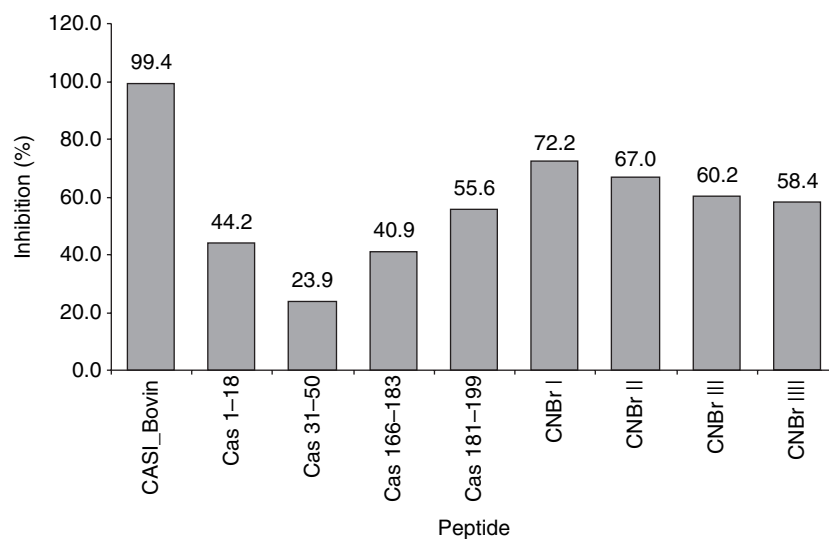


Figure 4 IgE ELISA inhibition α S1-CAS1_BOVIN and the peptides 1-18, 31-50, 166-199, 181-199 as well as the CNBr fractions. Eighteen CMA sera showed variable specificities. Only peptides with inhibition >20% were considered relevant.

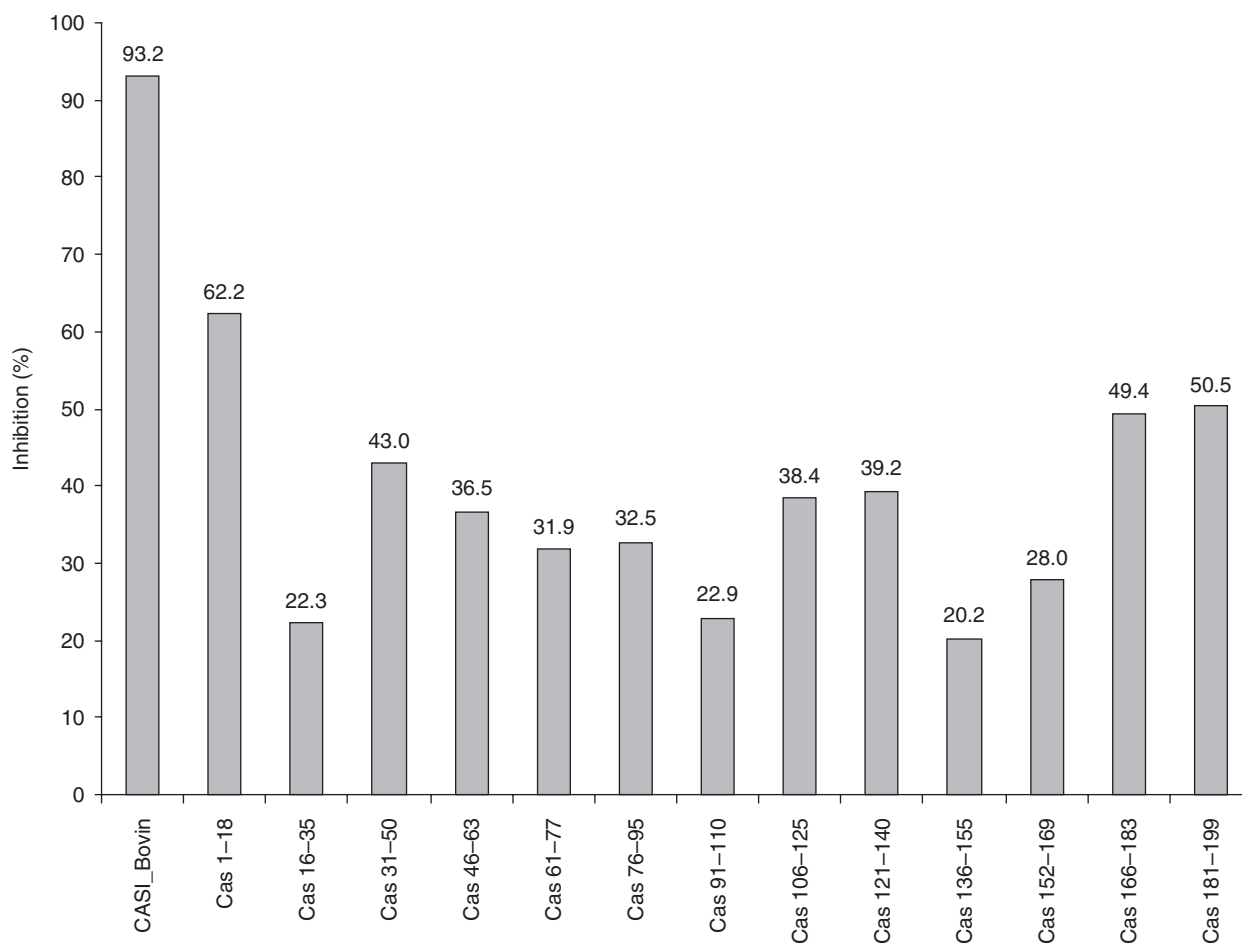


Figure 5 IgG ELISA inhibition using anti CAS1_BOVIN. The antigenicity of the different derivatives were compared by their C-50 inhibition values (0.057 ng for the reduced alkylated; for CAS1_BOVIN 0.07 μ g; for the limited hydrolysis 0.180 μ g and for the whole CNBr cleavage preparation 0.350 μ g. The reduction and alkylation yielded the highest antigenicity of sera, 18. Accordingly, the peptides were graded into three categories (I, II and III) where III was the highest binding.

Both peptides at the NH₂- and COOH-termini CAS 1-18 and CAS 166-183, CAS 181-199, respectively, were major inhibiting epitopes (>60% and approximately 50%, respectively). CAS 31-50; 46-63; 61-77; 76-96; 106-125; 121-140 moderately inhibited specific IgG (mean 37%). None of the peptide showed inhibition below 20%, which was the cut-off limit (Fig. 5).

Four of the CNBr cleavage peptides, with AA > 12 residues showed very high affinity for IgE. Two small peptides with 6 and 3 AA residues were nonbinding peptides.

Limited hydrolysis (<60 m) of different periods did not abolish the molecular immunogenicity explaining the residual activity in commercial CM hydrolysates.

Oxidation enhanced the allergenicity, suggesting that denaturation and unfolding the molecule exposed masked sites for binding the antibodies.

Considering the diversity of the antibody specificities, a reasonable agreement between IgE and IgG epitopes were mostly found at the N- and C-terminals of CAS1_BOVIN.

Recent study on T-cell recognition showed that seven motifs were recognized by CMA patients T cells. Both CAS 16-35 and 136-155 were recognized at both at the cellular and humoral levels. Likewise, three of the CNBr fragments (1-54, 61-123 and 136-194) were recognized by more than 94% of the sera could stimulate allergen specific T-cell lines [13]. CAS1_BOVIN minor IgE epitopes that stimulated allergen-specific T cells, could also be beneficial tool in studying CM tolerance in murine experimental model (Sun *et al.*, unpublished observations).

Peptide 152-169 encompassing the casomorphine (158-162) isolated from CAS1_HU was among the minor IgE-binding peptides. The metabolites containing this peptide or other exorphine AA sequences were suggested to affect the behaviour and mood of some of the hypersensitive patients [33].

CMA consumers rely on ingredients declaration of products. Complex ingredient terminology as for casein and labelling ambiguities could compromise the ability of the patients/parents to determine the safety of particular product. The present study allowed developing sensitive modified technique for detecting masked CMP epitopes in processed food.

Acknowledgments

We appreciate the excellent technical assistance of Judit Eriksen, Liv Kristine Øysæd, Randi Idsøe and Ann Kristin Gulliksen. The N-terminal AA sequence analysis was performed at the Biotechnology Centre of Oslo, University of Oslo, Professor Knut Sletten. We thank the Medical Research centre, University of Bergen for the facilities they provided.

References

- Kay A. Overview of "allergy and allergic diseases: with a view to the future". *Br Med Bull* 2000;56 (4):843-64.
- Elsayed S. Allergens. In: Van Regenmortel MHV, ed. *Structure of Antigens*. London, Tokyo: CRC Press Inc., 1993: 293-316.
- Jarvinen K, Suomalainen H. Development of cow's milk allergy in breast-fed infants. *Clin Exp Allergy* 2001;31 (7):978-87.
- Eggesbo M, Botten G, Halvorsen R, Magnus P. The prevalence of CMA/CMPI in young children: the validity of parentally perceived reactions in a population-based study. *Allergy* 2001;56 (5):393-402.
- Host A, Halken S. A prospective study of cow milk allergy in Danish infants during the first 3 years of life. *Allergy* 1990;45:587-96.
- Host A. Frequency of cow's milk allergy in childhood. *Ann Allergy Asthma Immunol* 2002;89 (6 Suppl. 1):33-7.
- Bernard H, Creminon C, Yvon M, Wal JM. Specificity of the human IgE response to the different purified caseins in allergy to cow's milk proteins. *Int Arch Allergy Immunol* 1998;115 (3):235-44.
- Nago M, Maki M, Sasakir R, Chiba R. Isolation and sequence analysis of bovine alpha-S1-casein cDNA clone. *Agric Biol Chem* 1984;48:1663.
- Chen R, Wang B, Zhang W, Liu W, Zhang J, Lao W. Cloning, mapping, and sequencing of 3' and its flanking region of bovine alpha-s1 casein gene. *Clin Biotechnol* 1992;18:235-45.
- Jarvinen KM, Beyer K, Vila L, Chatchatee P, Busse PJ, Sampson H. B-cell epitopes as a screening instrument for persistent cow's milk allergy. *J Allergy Clin Immunol* 2002;110 (2):293-7.
- Chatchatee P, Jarvinen K-ML, Bardina L, Beyer K, Sampson HA. Identification of IgE and IgG binding epitopes on α s1 casein: a difference in patients with persistent and transient cow's milk allergy. *J Allergy Clin Immunol* 2001;107 (2):379-83.
- Spuergin P, Mueller H, Walter M, Schiltz E, Forster J. Allergenic epitopes of bovine a s1-casein recognized by human IgE and IgG. *Allergy* 1996;51 (5):306-12.
- Elsayed S, Eriksen J, Øysæd LK, Idsøe RNB, Hill DJ. T cell recognition pattern of bovine milk a s1-casein and its peptides. *Mol Immunol* 2004, in press.
- Koppelman SJ, Wensing M, deLong GA, Knulst AC. Anaphylaxis caused by the unexpected presence of casein in salmon. *Lancet* 1999;354 (9196):2136.
- Walker-Smith J. Hypoallergenic formulas: are they really hypoallergenic? *Ann Allergy Asthma Immunol* 2003;90 (6):112-4.
- Houben GF, Knippels LMJ, Penninks AH. Food allergy: predictive testing of food products. *Environ Toxicol Pharmacol* 1997;4 (1-2):127-35.
- Jarmoc LM, Primack WA. Anaphylaxis to cutaneous exposure to milk protein in a diaper rash ointment. *Clin Pediatr (Phila)* 1987;26 (3):154-5.
- Werfel SJ, Cooke SK, Sampson HA. Clinical reactivity to beef in children allergic to cow's milk. *J Allergy Clin Immunol* 1997;99 (3):293-300.
- Ylitalo L, Makinen-Kiljunen S, Turjanmaa K, Palosuo T, Reunala T. Cow's milk casein, a hidden allergen in natural rubber latex gloves. *J Allergy Clin Immunol* 1999;104 (1):177-80.
- Martelli A, Chiara AD, Corvo M, Restani R, Fiocchi A. Beef allergy in children with cow's milk allergy; cow's milk allergy in children with beef allergy. *Ann Allergy Asthma Immunol* 2002;89 (6 Suppl. 1):38-43.
- Elsayed S, Hammer AS, Kalvenes MB, Florvaag E, Apold J, Vik H. Antigenic and allergenic determinants of ovalbumin. I. Peptide mapping, cleavage at the methionyl peptide bonds and enzymic hydrolysis of native and carboxymethyl OA. *Int Arch Allergy Appl Immunol* 1986;79 (1):101-7.
- Elsayed S, Stavseng L. Epitope mapping of region 11-70 of ovalbumin (Gal d1) using five synthetic peptides. *Int Arch Allergy Immunol* 1994;104 (1):65-71.

- 23 Eriksson TL, Johansson E, Whitley P, Schmidt M, Elsayed S, van Hage-Hamsten M. Cloning and characterisation of a group II allergen from the dust mite *Tyrophagus putrescentiae*. *Eur J Biochem* 1998;251 (1-2):443-7.
- 24 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227 (259):680-5.
- 25 Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* 1979;76 (9):4350-4.
- 26 Hill DJ, Hosking CS, Reyes-Benito LV. Reducing the need for food allergen challenges in young children: a comparison of *in vitro* with *in vivo* tests. *Clin Exp Allergy* 2001;31 (7):1031-5.
- 27 Elsayed S, Apold J. Effect of polymerization and modification on the immunochemical reactivity of cod allergen M. *Allergy* 1980;35 (3):202-3.
- 28 Holen E, Bolann B, Elsayed S. Novel B and T cell epitopes of chicken ovomucoid (Gal d1) induce T cell secretion of IL-6, IL-13, and IFN-gamma. *Clin Exp Allergy* 2001;31 (6):952-64.
- 29 Kumosinski T, Brown EM, Farrell HM Jr. Three-dimensional molecular modelling of bovine caseins: α_1 -casein. *J Dairy Sci* 1991;74:2889-95.
- 30 Viudes A, Perea S, Lopez-Ribot JL. Identification of continuous B-cell epitopes on the protein moiety of the 58-kilodalton cell wall mannoprotein of *Candida albicans* belonging to a family of immunodominant fungal antigens. *Infect Immun* 2001;69 (5): 2909-19.
- 31 Cianfriglia M, Willingham MC, Tombesi M, Scagliotti GV, Frasca G, Chersi A. P-glycoprotein epitope mapping. I. Identification of a linear human-specific epitope in the fourth loop of the P-glycoprotein extracellular domain by MM4.17 murine monoclonal antibody to human multi-drug-resistant cells. *Int J Cancer* 1994;56 (1):153-60.
- 32 Tribbick G, Triantafyllou B, Lauricella R, Rodda S, Mason T, Geysen H. Systematic fractionation of serum antibodies using multiple antigen homologous peptides as affinity ligands. *J Immunol Methods* 1991;139 (2):155-66.
- 33 Kampa M, Loukas S, Hatzoglou A, Martin P, Martin P, Castanas E. Identification of a novel opioid peptide (Tyr-Val-Pro-Phe-Pro) derived from human α 1 casein (α 1-casomorphin, and α 1-casomorphin amide). *Biochem J* 1996;1 (319 Pt 3): 903-8.