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Research Article

Update on optimized purification and characterization of natural milk allergens

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Highly purified allergens namely cow's milk α-lactalbumin (ALA). (Bos d 4), β-lactoglobulin (BLG) (Bos d 5) and casein (Bos d 8) and goat's milk casein were prepared from the raw milk from a single animal with a known genetic background. Consequently the natural isoforms are limited, constant and characterized. Purification included selective precipitations and chromatographical steps. Characterization of structure and allergenic activity assessment of milk allergens were carried out using physicochemical and immunochemical methods. Taken together data demonstrated the absence of impurities and of contamination by other milk allergens in each preparation. NMR and circular dichroism analyses confirmed the native conformation and proper folding of ALA and BLG and the expected absence of folding of bovine and caprine casein. Enzyme immuno assays confirmed the native conformation of BLG and the purity and immunoreactivity of all the proteins. The allergenic activity, *e.g.* the IgE binding capacity, of purified proteins was identical as that of those proteins when present in milk. The purified proteins also demonstrated the ability to provoke the degranulation of humanized rat basophilic leukaemia cells. All the data thus confirm the purity, identity, structural conformation and functionality of the prepared milk allergens.

Keywords: Cow's milk α-lactalbumin / Cow's milk β-lactoglobulin / Cow's milk casein / Goat's milk casein / Milk allergens

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1 Introduction

Milk allergy is an adverse reaction to milk proteins of different mammalian species including cow, goat and ewe. It is mainly mediated by immunological mechanisms that involve a particular class of Igs, namely IgE. It should be clearly distinguished from non immunological adverse reactions such as lactose-intolerance, which is due to lactase deficiency occurring in large sections of the general, predominantly adult, population.

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Abbreviations: AChE, acetyl cholinesterase; **ALA,** α-lactalbumin; **BLG,** β-lactoglobulin; **CAS,** casein fraction; **CMP,** cow's milk protein; **EAST,** enzyme allergo sorbent test; **EIA,** enzyme immuno assay

Milk is one of the very common and widespread allergen source that first affects atopic children in their early life. It is an allergenic food for most of the populations whatever their geographic origin is. In children the prevalence ranges from *ca.* 1–2% to 5% or even 7.5%. In unselected adults the prevalence among the general population has been reported to be around 1%. Most IgE-mediated milk allergy appears in young children in the first half year of life, a variable but large proportion of sensitive children spontaneously recover of their allergy between 2 and 5 years of age. The clinical picture can vary from mild to severe reactions involving the skin, respiratory tract, gastrointestinal tract or systemic reaction (anaphylactic shock); for review see [1]. Milk components responsible for allergy are proteins. Cow's milk contains about 30–35 g of proteins (CMPs) *per* litre. The action of chymosin (rennin), or the acidification of the milk to pH 4.6 enables two fractions to be obtained: lactoserum (whey), about 20% of the CMPs (*i.e. ca.* 6 g/L), and coagulum (curd), about 80% of the CMPs (*i.e. ca.* 28–30 g/L). Whey contains essentially globular proteins. The major

ones, β-lactoglobulin, Bos d 5 (BLG) (~3 – 4 g/L) and α-lactalbumin, Bos d 4 (ALA) (~1 – 1.5 g/L), are synthesized in the mammary gland, while others come from the blood. In the coagulum, the whole casein fraction, Bos d 8 (CAS), comprises four proteins coded by different genes carried on the same chromosome, namely α_{S1}-, α_{S2}-, β- and κ-caseins. All those proteins are involved in cow's milk allergy. Indeed, studies on large populations of allergic patients showed that most of the patients are sensitized to several proteins. A great variability is observed in IgE response but CAS, BLG and ALA are considered major allergens, *i.e.* more than 50% of the individuals in a milk allergic patient population are sensitized to those proteins [2, 3].

Multi sensitizations to the different caseins most often occur in patients sensitized to the whole CAS [4].

Goat's milk contains the same or at least very homologous proteins, sharing the same structural, functional and biological properties, and associated in more or less similar proportions. Whey protein concentration is *ca.* 4 g/L whereas whole casein concentration is *ca.* 25 – 30 g/L. Goat's milk casein contains essentially β-casein (~18 g/L); α_{S1}-casein concentration ranges from 0.6 to 3.6 g/L and its structural features are also highly variable depending on the genetic background of the animals.

A high IgE cross-reactivity between goat's and cows' milk casein occurs in most patients with cow's milk allergy [5 – 7]). However, IgE response and clinical reaction may also be quite specific and allergic reactions to goat's and ewe's milk without cow's milk allergy were described [8, 9].

Data available from validated studies, *e.g.* controlled food challenges, do not permit to establish reliable threshold doses. However it is very likely that the amount of CAS, BLG or even ALA present in a few mL of milk would be sufficient to trigger an allergic reaction in susceptible individuals.

BLG, Bos d 5, occurs naturally in the form of a 36 kDa dimer. It has no homologous counterpart in human milk. Each subunit corresponds to a 162 amino acid residue polypeptide. The molecule possesses two disulphide bridges and one free cysteine. This structure is responsible for the main physicochemical properties and also for interaction with casein during heat treatments. BLG belongs to the lipocalin family and is considered as a retinol-binding protein. Crystallography studies revealed very similar folding, called β barrel structure, with the same arrangements of eight (or ten) antiparallel β sheets [10 – 16].

ALA, Bos d 4, is a monomeric globular protein of 123 amino acid residues with 14.4 kDa molecular weight and has four disulphide bridges. It is a regulatory component of the enzymatic system of galactosyl transferase responsible for the synthesis of lactose. It possesses a high affinity-binding site for calcium, and this bond stabilizes its secondary structure. The complete amino acid sequence of ALA shows extensive homology with hen's egg white lysozyme but also with human ALA [17 – 20].

Whole CAS, Bos d 8, constitutes the coagulum, *i.e.* the solid fraction of proteins obtained after coagulation of milk. Each individual casein, α_{S1}-, β-, α_{S2}- and κ-casein, represents a well-defined chemical compound but they crosslink to form ordered aggregates: micelles, in suspension in lactoserum. Their proportion in the micelles is relatively constant *ca.* 37, 37, 13 and 13%, respectively. Their distribution is not uniform within these micelles which comprise a central hydrophobic part and a peripheral hydrophilic layer where major sites of phosphorylation containing phosphoserine residues are presented, in relation with the calcium binding and transfer properties of caseins. α_{S1}-, α_{S2}-, β- and κ-casein have little primary structure homology. Their functional properties also differ since three of them, α_{S1}-, α_{S2}- and β-casein, appear to be calcium-sensitive, while κ-casein is not. However, the four caseins display common features that differ greatly from other milk proteins. They are phosphorylated proteins, with a loose tertiary, highly hydrated structure [21]. CAS is often considered poorly immunogenic because of this flexible, non compact structure and because it is rapidly and extensively degraded by proteolytic enzyme during digestion. Caseins are not significantly affected by severe heat treatments but are indeed very susceptible to all proteinases and exopeptidases.

Milk proteins are very heterogeneous with very few structural or functional common features. This heterogeneity is complicated by their genetic polymorphism resulting in several variants for each protein. These variants are characterized by point substitutions of amino acids or by deletions of peptide fragments of varying size or by post-translational modifications such as phosphorylation, glycosylation. All of these modifications may affect the IgE binding capacity and allergenicity. Therefore, recombinant proteins would not reflect this heterogeneity and well-characterized batches of purified natural milk allergens are the material of choice for use in sensitive and specific diagnosis tests for milk allergy.

2 Materials and methods

2.1 Preparation of pure proteins from raw milk

Each CMP was prepared from the raw milk of a single well genetically characterized cow. Goat's milk whole casein was also obtained from the milk of a known individual goat.

The procedure used for the preparation of whole CAS and whey proteins has been previously described [2, 4, 22] and is summarized below. However the final step of purification was modified in order (i) to avoid contamination of each protein by minute residual amount of any other one and (ii) to obtain the different proteins under their native conformation and preserve their allergenic activity.

Defatted milk was precipitated at pH 4.6 using 1 M HCl. After centrifugation at 5000 rpm during 20 min, the super-

natant was collected and its pH adjusted to 7.5 using 1 M NaOH. This procedure was repeated twice. The pellet which contained the CAS was collected and stored at –30°C to avoid enzymatic hydrolysis by plasmin.

The pH of the supernatant which contained whey proteins was then adjusted to pH 2 with 6 M HCl and 7% NaCl was added which resulted in the formation of a precipitate. After centrifugation at 8000 rpm during 20 min, the supernatant and pellet were collected.

2.1.1 Purification of BLG

BLG was present in the supernatant which also contained trace amounts of ALA. This supernatant was precipitated by addition of 30% NaCl at pH 2 and then centrifuged at 8000 rpm during 20 min. The pellet was collected, redissolved in 50 mM phosphate buffer pH 7.4, dialysed against 20 mM Tris-HCl buffer pH 7.4 and then purified by anion exchange chromatography using an ÄKTA system (Amersham Biosciences) with a Source 30Q, XK26/10 column. Elution was achieved with a 130 min linear gradient from 0.15 to 0.35 M NaCl in 20 mM Tris-HCl buffer pH 7.4 at a 5 mL/min flow rate. The fractions corresponding to BLG were collected, then an additional purification step was applied, *i.e.* RP-HPLC using a C4 15 µm, 300 Å 22 × 250 mm, Vydac column and isopropanol–0.04% TFA as mobile phase in order to remove residual trace amounts of κ-casein (*i.e. ca.* 0.2%). Originally the separation used ACN–0.04% as eluent. However this resulted in a small part (*ca.* 2–5%) of the purified BLG which was obtained under a denatured form. Using isopropanol instead of ACN permitted to achieve the purification of BLG under its native conformation.

2.1.2 Purification of ALA

ALA was present in the pellet which also contained other whey proteins such as BSA and lactoferrin as well as trace amounts of BLG. The pellet was washed with 6% NaCl at pH 2 and centrifuged at 8000 rpm during 20 min. It was then dissolved in water and purified by anion exchange chromatography using an ÄKTA system with a Source 30Q, XK26/10 column. Elution was achieved with a 130 min linear gradient from 0 to 0.2 M NaCl in 20 mM Tris-HCl buffer pH 7.4 at a 5 mL/min flow rate.

2.1.3 Purification of bovine and caprine casein

CAS, *i.e.* cow's milk whole CAS was prepared from the raw milk of one cow homozygous for αs1-casein variant B, β-casein variant A1, αs2-casein variant A and κ-casein variant A. Goat's milk whole CAS was prepared from the raw milk of a goat which expressed the two variants B and F of αs1-casein. The pellet resulting from clotting of raw milk contained CAS and small amounts of whey proteins, *e.g.* BLG. It was dissolved and purified by affinity chromatography using a Sepharose 4B gel coupled with monoclonal antibodies raised against cow's milk or goat's milk BLG. The non retained fraction which contained CAS was dialysed and stored at –30°C.

2.2 Characterization of purified milk allergens

2.2.1 Assessment of structure

Characterization of structure and conformation of the prepared proteins was carried out using physicochemical and immunochemical methods.

2.2.1.1 HPLC

HPLC were performed in different conditions of those used for purification, using an ÄKTA purifier system with a Jupiter C4 column (10 µm, 300 Å 4.6 × 250 mm, Phenomenex) and elution by a 60 min linear gradient of 25–55% buffer B (ACN–0.04% TFA) in buffer A (H_2O–0.1% TFA) at a 1 mL/min flow rate. Peaks were detected by monitoring the absorbance at 220 and 280 nm.

2.2.1.2 Enzyme immuno assays (EIA)

Competitive and two-site immunometric (*i.e.* sandwich) EIA tests were performed to assess (i) the immuno reactivity of the purified allergens, (ii) the absence of cross-contamination by other CMPs and (iii) the structure of prepared BLG, *i.e.* native *versus* denatured. EIA inhibition tests used various commercial rabbit anti-whey proteins antibodies and different home made rabbit and chicken polyclonal anti-caseins antibodies as previously described for other antigens [23]. The sandwich EIA of native and denatured BLG used a set of anti-BLG monoclonal antibodies specific of conformational or linear epitopes [24]. EIA were performed in 96-well microtiter plates from Nunc (Roskilde, Denmark) using automatic Titertek microtitration equipment from Labsystem (Helsinki, Finland). All reagents were of analytical grade and obtained from Sigma (St. Louis, USA). Buffers and reagents as well as tracers, *e.g.* antibody and/or allergen–acetyl cholinesterase (AChE) conjugates, were prepared as described previously [2]).

2.2.1.3 MALDI-TOF MS

MALDI-TOF MS analyses were performed using a Voyager DE RP time of flight mass spectrometer (Applied Biosystems, Foster city, CA) equipped with a UV nitrogen laser (337 nm). Spectra were obtained in positive linear ion mode and were averaged from around 300 laser shots *per* spectrum to improve S/N level. Milk protein samples were prepared according to the dried droplet method using sinapinic acid as matrix. The matrix solution was prepared by dissolving sinapinic acid in CH_3CN, 0.1% TFA (60:40 v/v) at a concentration of 10 µg/µL. Mass assignments were made using BSA as external standard.

2.2.1.4 NMR analyses

The milk allergens were analysed by means of NMR, according to the following protocol. A solution of each pro-

tein was prepared in 0.45 mL H_2O plus 0.05 mL of D_2O. The concentrations were 0.28 mM for ALA, 0.22 mM for BLG and were estimated 0.08 mM for CAS and 0.19 mM for goat's milk casein. The solutions were put in high-quality NMR tubes with Ar as head-space gas.

Two high resolution NMR experiments were carried out using a Bruker Avance 700 spectrometer operating at a proton resonance frequency of 700 MHz (11.7 Tesla), at 298 K. The two experiments were different in the method to manage the water signal: the zgpr experiment minimizes it, while the zgesgp experiment suppresses the water peak. For each experiment 256 scans were programmed.

For CAS, a further ^1H spectrum (128 scans) with water suppression (zgesgp exp.) and a 2-D $^1H^1H$-NOESY spectrum (21 h) were scanned ($T = 298$ K) by means of a 900 MHz spectrometer equipped with a cryoprobe.

2.2.1.5 Circular dichroism

Circular dichroism spectroscopy was performed with purified ALA, BLG and CAS. The proteins were dialysed against 10 mM potassium phosphate, pH 7.4 and measured at concentrations of 0.1 mg/mL in a 0.1 or 0.2 cm quartz cuvette in a J-810S spectropolarimeter (Jasco, Easten, MD). Far UV spectra were recorded in the range between 190 and 260 nm at room temperature. Data of five measurements were accumulated.

2.2.2 Assessment of allergenic activity

The allergenic activity of the prepared milk proteins was assessed by IgE binding studies.

2.2.2.1 Enzyme allergo sorbent test (EAST) and EAST inhibition

EAST and EAST inhibition were performed as previously described [2]. Briefly microtiter plates were coated by passive adsorption with each of the prepared milk proteins at a concentration of 10 μg/mL in 50 mM phosphate buffer pH 7.4. After 24 h incubation, the plates were washed and HSA was used as saturating agent to avoid non specific binding. Plates were washed just before use. Fifty microlitre of individual serum at serial dilutions were dispensed *per* well. After 24 h incubation at room temperature and extensive washing, 50 μL of a solution of the antihuman IgE mAb BS17 conjugated to AChE (3 Ellman units/mL) were added *per* well. Following an overnight incubation, 200 μL of Ellman's reagent used as enzyme substrate were dispensed into each well and absorbance was measured at 414 nm.

Ten sera of allergic patients to cow's milk and/or to goat's milk, collected and characterized in previous studies, were used in IgE binding studies. The serum of an allergic patient to peanut but non allergic to milk was used as negative control and allowed to determine the non specific binding.

2.2.2.2 Humanized rat basophilic leukaemia cell degranulation test

Experiments were performed with rat basophilic leukaemia cells expressing the human FcεRI α, β and γ chains (RBL SX-38) [25]. The cells were obtained from J. P. Kinet. Cells were grown in EMEM (Eagle's minimum essential medium with Earle's salts) containing 10% decomplemented foetal bovine serum, 1% penicillin/streptomycin, 2 mM L-glutamine and 2.4 mg/mL geneticin G418 (all from Gibco).

For the release assay, cells were plated at a concentration of 1.2×10^5 cells/mL (100 μL/well) in 96-well culture plates and 100 μL/well of different dilutions of human serum from milk allergic or control patients were added to sensitize the cells. After incubation and washing, degranulation was induced by adding 50 μL/well of serial dilutions of each of the purified allergen. Release was measured by determining the enzymatic activity of β-hexosaminidase in the supernatant. Supernatant was transferred in a 96-well plate (30 μL/well) and 50 μL of 4-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma) (1.3 mg/mL in 0.1 M citric acid buffer pH 4.5) were added. After 1 h at 37°C, 100 μL of 0.2 M glycine solution pH 10.7 were added and absorbance was measured at 405 nm. Supernatant from cells incubated with the control serum of a non milk-allergic patient allowed to determine the background.

All results were expressed as the ratio (%) of the β-hexosaminidase released after activation of the sensitized cells with the allergen divided by the release measured after activation with anti-IgE antibodies.

3 Results

3.1 Purity and structure

3.1.1 HPLC

Figure 1A shows that both purified ALA and BLG eluted as a well defined single peak.

RP-HPLC profile of the whole casein of cow's milk appeared heterogeneous (Fig. 1B). This profile is characteristic of the four different caseins presenting for some of them, different levels of phosphorylation and glycosylation. β-Casein is associated with a minor peak at a retention time (RT) of 38 – 39 min. which corresponds to β-casein fragment (29 – 209) produced after natural hydrolysis by endogenous plasmin. Spiking CAS with 10% BLG resulted in an additional peak. The arrow indicates the RT of co-injected BLG. This chromatogram demonstrates that no detectable contamination of purified whole casein by BLG occurred.

RP-HPLC profile of the whole caprine casein showed similar characteristics (Fig. 1C). Goat's milk casein appeared as a multiplicity of peaks corresponding to the elution of the four different caseins including different variants for each component. Variants differ for amino acids

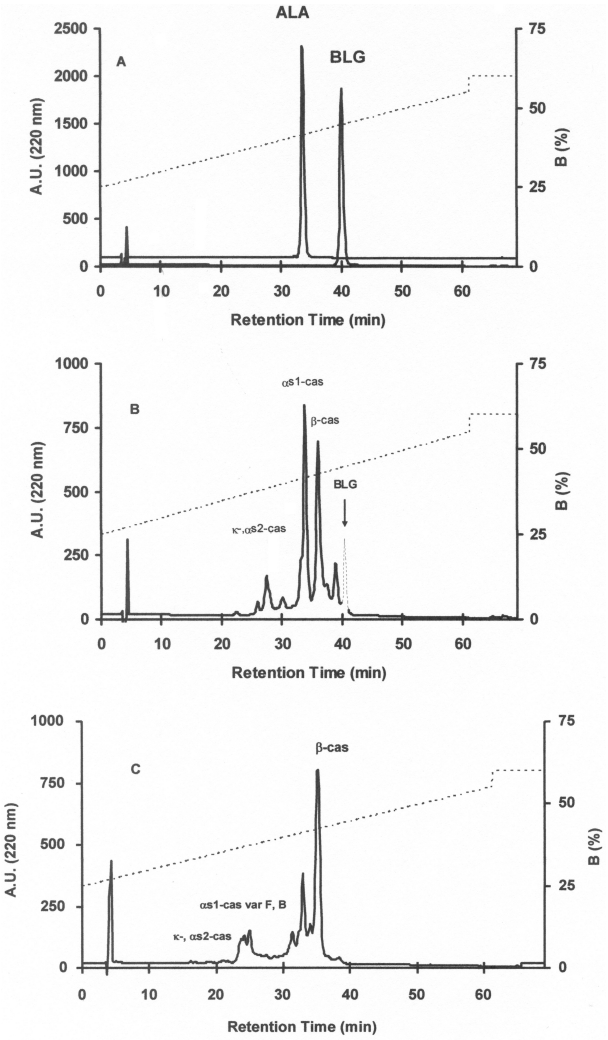

Figure 1. RP-HPLC of (A) ALA and BLG, (B) bovine whole casein and (C) caprine whole casein.

substitution or deletion and, in some cases, for the levels of phosphorylation and glycosylation. Peaks corresponding to αs1-caseins variants F and B appeared as minor peaks while β-casein was represented by a major one.

3.1.2 Enzyme immuno assays (EIA)

The sandwich ELISA tests using two sets of monoclonal antibodies specific of native or denatured BLG showed that the prepared BLG was only under its native conformation and that the purification process (*i.e.* RP-HPLC using isopropanol instead of ACN as mobile phase) did not lead to structural modification of the protein. Figure 2 shows the dose–response curves obtained for standard denatured BLG (*i.e.* reduced and *S*-carboxymethylated BLG) and for the present preparation of purified BLG, using the sandwich ELISA test of denatured BLG described in ref. [24]. It demonstrates that no denatured BLG is present in the preparation.

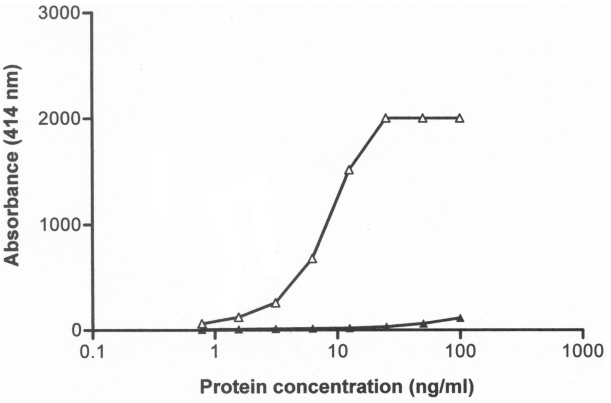

Figure 2. Dose–response curves of denatured BLG (△) and of the preparation of purified BLG (▲) in the sandwich ELISA test of denatured BLG according to [24].

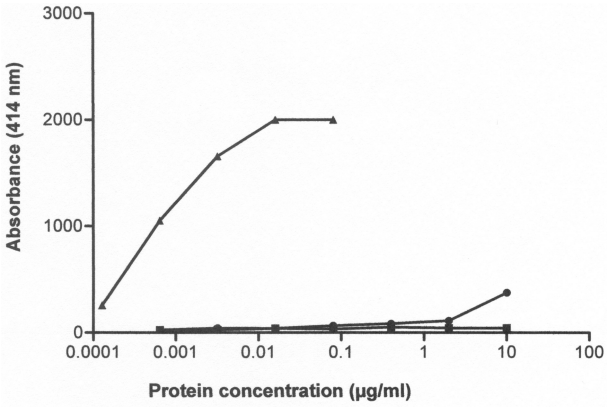

Figure 3. Dose–response curves of BLG (▲), ALA (■) and CAS (●) in the sandwich ELISA test of native BLG according to [24].

Combinations of different EIA inhibition studies using standard and labelled CMPs and various anti-CMP antibodies were used to assess the absence of cross-contamination of the CMP preparations. Figure 3 shows the dose–response curves obtained with the different CMP preparations in the sandwich ELISA test using monoclonal antibodies to native BLG. Figure 4 shows dose–response curves obtained in a competitive ELISA test using rabbit anti-whey proteins polyclonal antibodies and ALA labelled with AChE as tracer. Similar curves were obtained using the same antiserum and BLG labelled with AChE as tracer. In every case, the protein preparation totally inhibited the corresponding EIA but failed to significantly bind to any antibody specific of another protein even at high concentration. The absence of inhibition of any heterologous EIA showed that prepared CMPs contained less than 0.01% of any other protein including BLG.

The presence of immunoreactive individual caseins, namely α_{S1}-, α_{S2}-, β- and κ-casein in CAS was also demonstrated by EIA inhibition studies using specific hen anti-

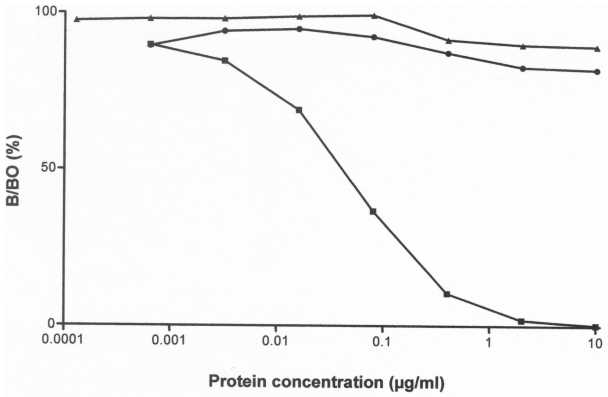

Figure 4. Competitive ELISA test: inhibition of the binding of rabbit antiwhey proteins polyclonal antibodies to AChE labelled ALA by increasing concentrations of ALA (■), BLG (▲) and CAS (●).

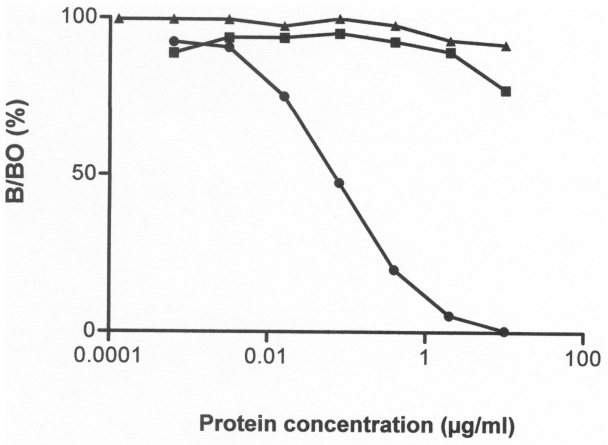

Figure 5. Competitive ELISA test: inhibition of the binding of hen anti-β-casein antibodies (IgY) to AChE-labelled β-casein by increasing concentrations of ALA (■), BLG (▲) and CAS (●).

α_{S1}-, α_{S2}-, β- and κ-casein antibodies (IgY) and standard/AChE-labelled α_{S1}-, α_{S2}-, β- and κ-casein. Figure 5 shows the inhibition of the binding of specific anti-β-casein hen IgY to AChE-labelled β-casein by increasing concentrations of BLG, ALA or CAS. It demonstrates the absence of contamination of the preparations of BLG and ALA by residual traces of CAS. Same results were obtained for α_{S1}-, α_{S2}- and κ-casein. All the data are presented in Table 1 in which the characteristics of the ELISAs in terms of sensitivity and specificity are summarized by the IC_{50} values (*i.e.* the concentration of protein which inhibits 50% of the binding of the antibodies to the corresponding tracer). The IC_{50} values reported in the table demonstrate the presence of each casein (*i.e.* α_{S1}-, α_{S2}-, β- and κ-casein) in the preparation of CAS and the absence of contamination of ALA and BLG preparations by traces of CAS. It is noteworthy that the specificity of the assay results from the quality of the antibodies and/or of the tracer used.

Table 1. Sensitivity and specificity of EIAs using various polyclonal antibodies raised against CMPs and purified proteins labelled with AChE as tracers

Inhibitor	Tracer (labelled CMP: P*)				
	ALA* IC_{50} (µg/mL)	κ-casein* IC_{50} (µg/mL)	αS_2-casein* IC_{50} (µg/mL)	αS_1-casein* IC_{50} (µg/mL)	β-casein* IC_{50} (µg/mL)
ALA[a)]	0.04	>10	>10	>10	>10
BLG[a)]	>10	>10	>10	>10	>10
CAS[b)]	>10	0.08	0.06	0.02	0.07

The IC_{50} value, *i.e.* the concentration of protein which inhibits 50% of the binding of the antibodies to the corresponding tracer characterizes both the sensitivity of the assay (a low IC_{50} of the homologous antigen means a high sensitivity) and its specificity (a high IC_{50} of a heterologous antigen reflects a low cross-reactivity). The tests were performed using:

a) Rabbit anti-whey polyclonal antibodies.
b) Hen anti-κ-, β-, αS_2- and αS_1-casein polyclonal antibodies (IgY).

Absence of BLG contamination in whole caprine casein was assessed by a specific immunometric assay of caprine BLG [24] with an LOD lower than 0.1 ng/mL. No significant signal was observed for whole caprine casein at concentration higher than 1 µg/mL which demonstrates that any contamination by BLG would be less than 0.01%.

3.1.3 MALDI-TOF MS

MALDI-TOF mass spectra of BLG and ALA in Figs. 6A and B show that both purified proteins were present as a unique compound with a molecular weight very close to that theoretically expected (18 366 *vs.* 18 362 and 14 181 *vs.* 14 186 Da for BLG variant A and ALA, respectively). No other mass was observed.

Molecular masses of some components purified from the whole casein were determined using MALDI-TOF MS. Due to the different phosphorylated and/or glycosylated forms, average masses were expressed *i.e.* 19.43, 25.6, 18.74, 23.35 and 23.85 kDa for κ-, αs2-, αs1F-, αs1B- and β-caseins, respectively. These values are very close to those described in different studies [26, 27].

3.1.4 NMR analyses

The dispersions of aromatic and amide protons above 9 ppm and below 7 ppm, of *alpha* protons above 5 ppm and of methyl peaks below 0.5 – 0 ppm or more, are reliable indexes of how much a protein is folded to form a tertiary structure.

The spectra of ALA (Bos d 4) in Fig. 7A were full of peaks from 0.8 to 10 ppm. The peaks were narrow and separated. The protein had an unquestionable tertiary structure in solution, in the experimental condition tested here.

The spectra of BLG (Bos d 5) in Fig. 7B shared many features with the spectra of Bos d 4, with some difference.

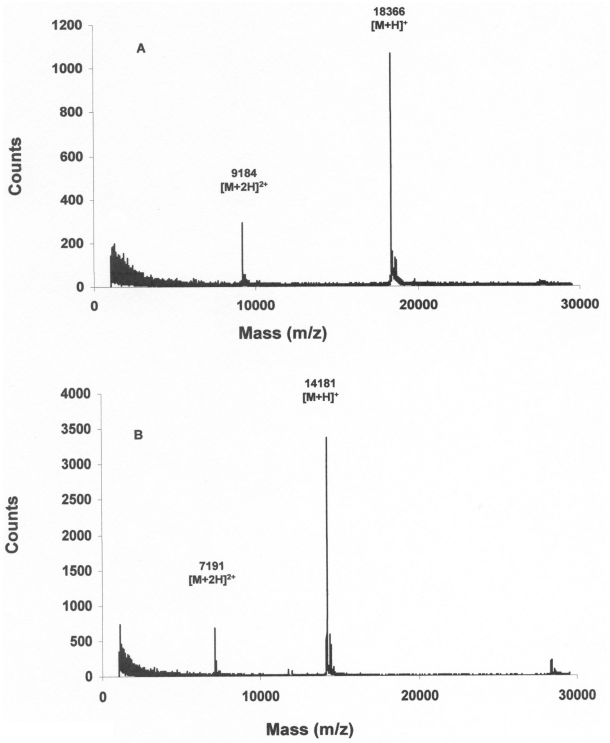

Figure 6. MS identification of (A) BLG variant A and (B) ALA.

There was less dispersion of the methyl signals: the last peak scores 0 ppm. All the peaks were slightly broader and less resolved, especially in the 5 – 10 ppm range. A portion, eventually very small of the protein could be unstructured and coexist with a structured part, whose presence was apparent anyway. Another hypothesis could involve, in the particular experimental condition adopted for the NMR, a mixture of folded and unfolded state of BLG.

The ^{1}H 900 MHz spectrum of CAS (Bos d 8) (23.6 + 24.0 + 25.2 + 19.0 kDa) 0.08 mM (very approx.), zgesgp experiment, 128 scans, 298 K and the 700 MHz spectrum of goat's milk casein are given in Figs. 7C and D, respectively.

The NMR analyses of whole cow's and goat's milk CAS showed very similar spectra and the same conclusion can be drawn regarding the absence of indication for folding of the samples which is quite confirmatory of what is known on the structure of caseins.

3.1.5 Circular dichroism

CD spectrosocopy analysis of purified ALA and BLG identified the proteins with a folded structure, in the case of ALA with α-helical structures being predominant whereas BLG for which the transition point was around wavelength 201.5 – 202, displayed rather β-sheet formations. In contrast the mixture of caseins, *i. e.* CAS, displayed no well defined structure but the data suggest a random coiled nature (Fig. 8).

Table 2. Determination of specific IgE to BLG, ALA, cow's milk CAS and goat's milk casein in ten allergic patients to milk and one allergic patient to peanut (negative control no. 263)

Patient no.	Specific IgE (IU/mL)			
	BLG	ALA	CAS	Goat's milk casein
10	0	0	6.9	–
28	4.9	2.8	33.0	–
29	4.7	5.6	6.0	–
69	9.0	31	180	202
74	0	0	0	6.2
76	2.2	27	157	203
77	2.7	0	14.8	–
78	0	0	0	5.5
81	0	0	0	29
83	3.8	6.0	85	–
263	0	0	0	0

– not done
Specific IgE concentrations are expressed as International Units *per* mL of serum.
Patients 10, 28, 29, 69, 76, 77 and 83 were allergic to cow's milk with a cross-reactivity to goat's milk while patients 74, 78 and 81 were specifically allergic to goat's milk without associated allergy to cow's milk [9].

It is noteworthy that the NMR and CD spectra can give different results because of the large differences in the experimental conditions, particularly the difference in the protein concentration in the solutions used in the two studies.

3.2 Allergenic activity

3.2.1 EAST and EAST inhibition

EAST determinations of specific IgE against raw cow's milk, CAS, BLG and ALA in ten milk allergic patients are reported in Table 2. Some of the patients were allergic to CMPs and presented a (high) IgE cross-reactivity to goat's milk casein whereas some others were only allergic to goat milk without associated cow's milk allergy [9].

EAST inhibition studies using sera of cow's milk allergic patients showed that in every case, increasing concentrations of the prepared pure CMPs could totally inhibit the binding of specific IgE to standard and labelled allergens and that the inhibition curves paralleled those obtained with raw milk. This indicates that the purified allergens bound to human specific IgE in the same manner and with the same apparent affinity as the allergens present in raw milk. Figures 9A and B show, as an example, the EAST inhibition curves obtained in one patient with BLG and CAS. Similar results were obtained with ALA and goat's milk casein. The IC_{50} values for BLG, CAS and raw milk (arrows) were in the same ratio as the concentrations of BLG in milk (Fig. 9A) and of whole casein in milk (Fig. 9B).

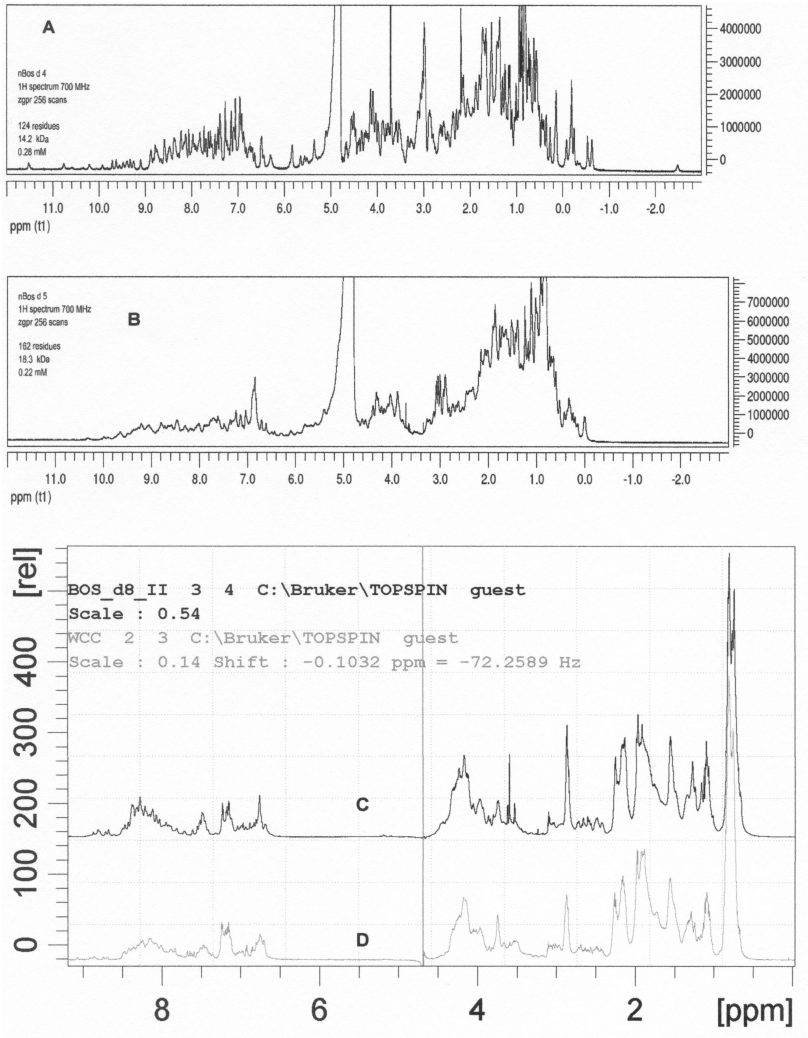

Figure 7. NMR 1H spectra (700 MHz) of (A) ALA and (B) BLG; (C) NMR 1H spectra (900 MHz) of bovine whole casein and (D) NMR 1H spectra (700 MHz) of caprine whole casein.

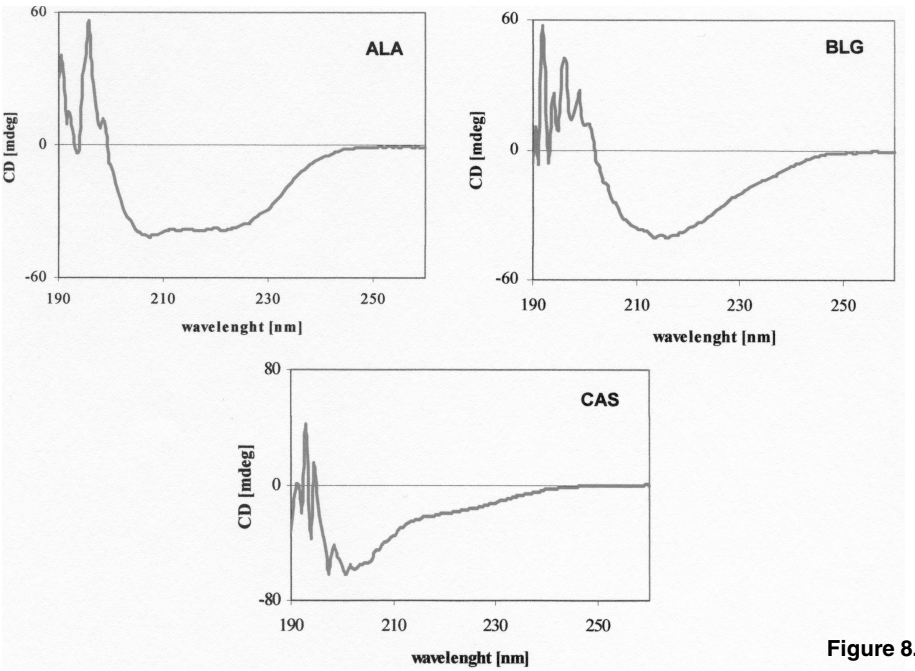

Figure 8. CD spectra of ALA, BLG and CAS.

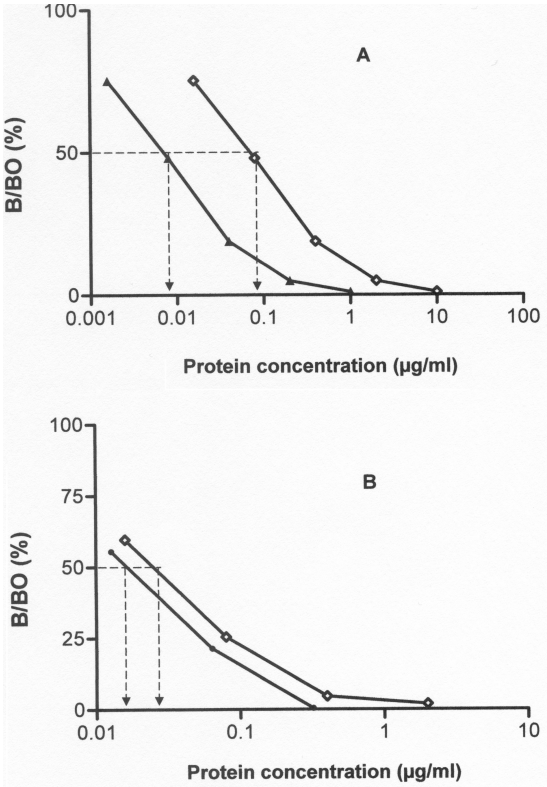

Figure 9. EAST inhibition of the binding of specific IgE to (A) immobilized BLG, by increasing concentrations of raw milk (⋄) and purified BLG (▲) and (B) immobilized CAS, by increasing concentrations of raw milk (⋄) and purified CAS (●). In both case the arrows indicate the IC_{50} values, *i.e.* the concentrations of raw milk and of the purified CMP that inhibit 50% of the binding of the IgE to the corresponding immobilized protein.

3.2.2 Humanized rat basophilic leukaemia cell degranulation test

The prepared CMPs were able to provoke the degranulation of rat basophilic leukaemia cells (RBL SX-38) sensitized using the serum of an allergic patient to cow's and goat's milk.

As shown in Fig. 10, degranulation obtained after activation by caseins reached a peak for very low amounts of allergens (1 – 10 ng/mL) with maximum release values of *ca.* 60% for bovine casein and 46% for caprine casein. For BLG and ALA maximum degranulation rates were obtained for higher amounts of allergens (1 µg/mL) and maximal degranulation were *ca.* 27 and 23% for BLG and ALA, respectively.

4 Conclusions

The milk proteins/allergens, namely ALA, BLG and whole CAS were prepared from the raw milk of a single well genetically characterized cow, homozygous for the major

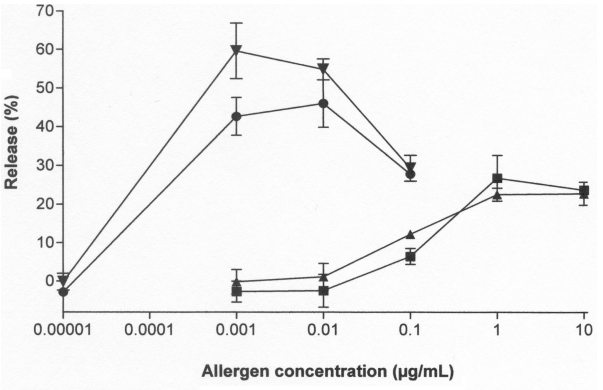

Figure 10. Humanized rat basophilic leukaemia cell degranulation test using purified milk allergens. BLG (■); ALA (▲); CAS (▼) and caprine casein (●). Results are expressed as the ratio (%) of the β-hexosaminidase released after activation of the sensitized cells with the allergen divided by the release measured after activation with anti-IgE antibodies.

protein variants. Goat's milk whole CAS was also prepared from the milk of a single genetically characterized goat. Consequently the number of natural isoforms that were present in the prepared fractions was limited, constant and the corresponding variants have been characterized. This strategy was finally preferred to the one which consisted to prepare the proteins from commercial batches of mixed milks coming from numerous cows of different origin and of different genetic background. This would have reflected the natural huge variability of milk protein isoforms that are consumed in everyday life. However, each purified protein would have consisted of a mixture of many natural variants the composition of which would have been neither completely characterized nor reproducible. Therefore, we prepared known variants of each pure protein that were all identified. On the other hand, recombinant proteins would not reflect the structural heterogeneity and presence of various post-translational modifications. Their use in diagnosis tests for milk allergy may therefore result in insufficient sensitivity and specificity.

The proteins were characterized using spectrometric analyses and the purity was checked using several techniques of different nature, including immunochemical studies. All demonstrated the absence of impurities but particularly the absence of cross-contamination of one protein preparation by other milk protein(s). High degree of purification may affect the structural features of a protein and then alter its biological activity and its allergenicity. Chromatographic conditions were developed in order to prepare BLG under its native conformation whereas a fraction is generally denatured by the conditions of elution usually applied. Analyses using NMR and circular dichroism confirmed the proper folding of ALA and BLG and the absence of folding of bovine and caprine whole CAS that are naturally unfolded and display little structural features. High degree of purification should also not alter the biological

activity of the prepared proteins. Indeed using a large set of monoclonal and polyclonal antibodies, including specific IgE of allergic humans, we showed that the IgG and IgE binding capacity of the prepared proteins were identical as those of the proteins present in the milk. The proteins were recognized by antibodies with a similar apparent affinity. In addition the purified proteins demonstrated the ability to provoke the degranulation and mediator release of passively sensitized humanized RBL cells *in vitro*. All the data thus confirm the purity, identity and structural conformation and functionality of the prepared milk proteins/allergens which use may then improve diagnostic tests for food allergy.

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