

Two-site enzyme immunometric assays for determination of native and denatured β -lactoglobulin

L. Negroni ^a, H. Bernard ^a, G. Clement ^a, J.M. Chatel ^a, P. Brune ^c, Y. Frobert ^b,
J.M. Wal ^a, J. Grassi ^{b,*}

^a Laboratoire Associé INRA-CEA d'Immuno Allergie Alimentaire, SPI Bât 136, CE Saclay, 91191 Gif sur Yvette Cedex, France

^b CEA, Service de Pharmacologie et d'Immunologie, DRM, CE Saclay, 91191 Gif sur Yvette Cedex, France

^c SPI-BIO, 2 rue du buisson au fraise, 91741 Massy, France

Received 23 March 1998; revised 30 June 1998; accepted 16 July 1998

Abstract

Two enzyme immunometric assays suitable for measuring native and denatured β -lactoglobulin (BLg) have been developed. The assays were performed in 96-well microtitre plates and were based on the use of pairs of monoclonal antibodies specific to either the native form or the reduced and carboxymethylated form of BLg (RCM-BLg). Detection limits of 30 and 200 pg/ml were obtained for the native BLg and the RCM-BLg assay, respectively, with very low or negligible cross-reactivity of the other milk proteins and tryptic fragments of BLg. The validity of the assays in different media such as cow's milk and cow's milk products, saline buffer or serum was supported by recovery experiments. The assays were first applied to the determination of BLg and RCM-BLg in PBS and in raw skimmed milk. The ability of the RCM-BLg assay to detect heat-denatured BLg was confirmed by a kinetic study of BLg heat-denaturation in the two media. During heat treatment, the decrease in the concentration of native BLg was associated with an increase in denatured BLg specifically detected by the RCM-BLg assay. By selecting an appropriate monoclonal antibody which failed to recognize caprine BLg, we were able to establish a modified sandwich immunoassay permitting very sensitive detection of cow's milk in goat's milk. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: β -lactoglobulin; Thermal treatment; Enzyme immunometric assay; Monoclonal antibody; Acetylcholinesterase

1. Introduction

β -lactoglobulin is the major whey protein of the milk of ruminants. This globular protein is not present in human milk and is considered to be a major allergen of cow's milk (Koritz et al., 1987). Its impact on health and the corresponding conse-

Abbreviations: AChE, acetylcholinesterase; BLg, bovine β -lactoglobulin; EIA, enzyme immunoassay; HBS, HEPES buffer saline; mAb, monoclonal antibody; PBS, phosphate buffer saline; RCM, reduced and carboxymethylated; SPR, surface plasmon resonance

* Corresponding author. Tel.: +33-1-69-08-72-99; Fax: +33-1-69-08-59-07; E-mail: grassi@dsvidf2.cea.fr

quences for the milk industry led to a particular interest in its structure. β -lactoglobulin is an 18-kDa monomer of 162 amino acids which contains five cysteine residues (Godovac-Zimmermann and Braunitzer, 1987), four of which are involved in intra-chain disulphide bridges whose cleavage increases susceptibility to proteolysis (Reddy et al., 1988). Two variants of BLg (variants A and B) differing only by two substitutions in positions 64 (Asp/Gly) and 118 (Val/Ala) have been described. Crystalline β -lactoglobulin has nine strands of anti-parallel β -sheet and one short α -helix at the N-terminus. This three-dimensional structure looks like human retinol-binding protein and the other proteins of the lipocalin family (Papiz et al., 1986) which have the common property of binding small hydrophobic molecules within the central cavity of the β -barrel of calyx (for a review see Perez and Calvo, 1995).

Pasteurization, sterilization and spray drying are important technological processes in the milk industry. These heat treatments induce physicochemical changes of BLg which have been studied by using either biochemical methods (Iametti et al., 1995) or immunochemical methods (Levieux, 1980; Kamino-gawa et al., 1989). In this paper we describe one set of monoclonal antibodies (mAbs) directed against native BLg, some of which specifically recognize reduced and carboxymethylated BLg (RCM-BLg). Two pairs of these mAbs have been selected to establish two highly specific and sensitive enzyme immunometric assays for native and denatured BLg.

2. Materials and methods

2.1. Apparatus

Solid phase enzyme immunometric assays were performed using Labsystem (Helsinki, Finland) microtitration equipment which included an automatic plate washer, dispenser and multiscan spectrophotometer. The microtitration plates used were 96F Maxisorp Immunoplates (Nunc, Denmark). The high-performance liquid chromatography system (Waters Associated chromatograph) consisted of two M510 pumps and a 717 Autosample. Absorbance was measured with an SPD-10A UV-VIS detector (Shimadzu, Japan). The fast protein liquid chro-

matography system (FPLC, Pharmacia, Sweden) consisted of a GP-250 gradient programmer, two P500 pumps and a UV M II detector set at 220 nm. Surface plasmon resonance measurements were performed using a BIALITE™ apparatus from Pharmacia Biosensor. Sensor Chips CM5, rabbit anti-mouse IgG and the amine coupling kit were also obtained from Pharmacia Biosensor.

2.2. Reagents and buffers

Raw milk was obtained from a local dairy, pasteurized and sterilized milks were commercially available products. α -Lactalbumin and TPCK trypsin were from Sigma (St Quentin Fallavier, France). Bovine immunoglobulins were from Immunotech (Marseille, France). Bovine casein, bovine BLg and caprine BLg were produced as described by Wal et al. (1995). The BLg A variant was used in all experiments except for the determination of binding compatibility by SPR measurement (see below) which was performed with the BLg B variant. Reduced and carboxymethylated BLg (RCM-BLg) was prepared as described by McKenzie et al. (1972). Briefly, 100 mg of BLg (10 mg/ml in 0.2 M Tris/HCl pH 8.5, 0.027 M EDTA, 8 M urea) were reduced for 4 h by 0.1 ml of 2-mercaptoethanol. Carboxymethylation was performed for 20 min with 2 ml of a 135 mg/ml solution of iodoacetamide. The mixture was then dialyzed against water and lyophilized. Tryptic proteolysis (2.5% molar ratio of trypsin/BLg) was performed for 17 h at 37°C on a 10 mg/ml solution of BLg-B in 0.1 M Tris buffer pH 9, 0.1 mM CaCl_2 .

Buffers: (1) coating buffer; 50 mM phosphate buffer pH 7.4; (2) EIA buffer: 0.1 M phosphate buffer containing 1 g/l BSA, 0.15 M NaCl, 0.001 M Na_2EDTA , and 0.1 g/l sodium azide; (3) washing buffer: 0.01 M phosphate buffer pH 7.4 containing 0.05% Tween 20; (4) Ellman's medium: 7.5×10^{-4} M acetylthiocholine and 2.5×10^{-4} M 5,5'-dithio-bis-nitrobenzoic acid in 0.1 M potassium phosphate buffer pH 7.4 (Ellman et al., 1961). (5) HBS: 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3.4 mM EDTA, 0.005% surfactant P20.

Ninety-six-well microtitre plates were coated by adding 200 μl /well of a 5 $\mu\text{g}/\text{ml}$ solution of antibody or antigen diluted in 50 mM phosphate

buffer pH 7.4. After 18-h incubation at room temperature, plates were washed, saturated with EIA buffer (300 μ l/well), sealed and stored at 4°C.

2.3. Monoclonal antibody production

Anti-BLg monoclonal antibodies were produced by conventional techniques according to Fazekas de Saint Groth and Scheidegger (1980) and Grassi et al. (1988). Briefly, 0.03 mg of BLg emulsified in Freund's complete adjuvant was injected into female Biozzi high responder mice on day 0 and day 21. The mice producing antisera with the highest titre (see below) received an intravenous booster injection and 3 days later the spleen cells were fused with NS1 myeloma cells. The presence of anti-BLg antibodies in supernatants of hybridoma cells was tested ten days later by checking their capacity to bind enzymatic tracers composed of either BLg or RCM-BLg coupled to acetylcholinesterase (see below), as previously described for other antigens (Grassi et al., 1989; Frobert and Grassi, 1992). Positive hybridoma cells were cloned and expanded as ascitic fluids in nu/nu mice.

2.4. Preparation of BLg and RCM-BLg AChE conjugates

BLg and RCM-BLg were covalently coupled to acetylcholinesterase (AChE; EC 3.1.1.7) by the intermediaries *N*-succinimydyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) and *N*-succinimydyl-*S*-acetylthioacetate (SATA), as previously described (Caruelle et al., 1988). This method involved the reaction of thiol groups previously introduced by SATA into BLg or RCM-BLg with the maleimido groups, incorporated into the enzyme by treatment with SMCC.

2.5. Competitive EIA

The relative affinity of the monoclonal antibodies was estimated by competitive EIA in microtitre plates coated with goat anti-mouse antibodies (Jackson Immuno Research Labs., West Grove, PA). Before use, the plates were washed and each well was filled with 50 μ l of increasing concentrations of competitor (i.e., BLg or RCM-BLg in EIA buffer), 50 μ l of

enzymatic tracer (2 Ellman units/ml) and 50 μ l of a dilution of monoclonal antibodies. After overnight immunoreaction at 4°C, wells were washed and 200 μ l of Ellman's reagent were dispensed into each well. Absorbance at 414 nm of individual wells was measured after 1 h of enzymatic reaction. Standard curves were expressed in terms of B/B_0 (%) where B and B_0 represent solid-phase bound enzyme activity measured in the presence or absence of competitor, respectively. The relative affinity of BLg and RCM-BLg for antibodies was estimated by comparing $B/B_0 = 50\%$ values (the concentration of BLg or RCM-BLg that reduced B_0 by 50%).

2.6. Cross-reactivity with caprine BLg

Ninety-six-well microtitre plates were coated with either bovine or caprine BLg as previously described (see Section 2). Before use, plates were washed and each well was filled with 50 μ l of hybridoma supernatant (1/10 and 1/100) and 50 μ l of goat anti-mouse AChE conjugate (2 Ellman Units/ml). After overnight reaction at 4°C, wells were washed and filled with 200 μ l/well of Ellman's medium. Absorbance at 414 nm was measured after 1 h of enzymatic reaction.

2.7. Determination of binding compatibility for each pair of mAbs using surface plasmon resonance measurements

The compatible binding of two different mAbs for a single BLg molecule was determined using surface plasmon resonance methodology (Fägerstam et al., 1990) with a BIALITE apparatus (BIACORE, Sweden). Rabbit anti-mouse IgGs (RAMG1, BIACORE) were covalently coupled to Sensor Chips CM5 using a primary amine coupling procedure according to the manufacturer's recommendations. A first mAb, BLg, and a second mAb were successively injected in order to demonstrate possible additive binding. The successive injections were as follows: (1) 5 μ l of hybridoma supernatant containing the first mAb, (2) 5 μ l of non-specific mouse IgG (50 μ g/ml), (3) 5 μ l of BLg or RCM-BLg (10 μ g/ml in HBS), (4) 5 μ l of hybridoma supernatant containing the first mAb, (5) 5 μ l of another hybridoma supernatant containing a second mAb. If no significant binding

was observed, a third hybridoma supernatant was injected and so on until binding was achieved. In this case, 10 μ l of 100 mM HCl were injected in order to generate the RAMG1 sensor surface. Injections (2) and (4) were made to block all the sites available on BLg and to avoid reaction of RAMG1 with the second mAb. All samples were injected at a flow rate of 5 μ l/min with HBS as transport buffer. A washing step of 1 ml HBS buffer was included between each injection. MAbs applied in step (5) that were unable to bind the antigen were classified in the same group of binding compatibility as the first mAb. MAbs binding the antigen in the presence of the first mAb defined a new group of binding compatibility and were used as capture antibody for the following binding experiments.

2.8. Binding compatibility using EIA measurements

For each group of complementarity, the mAbs presenting the highest affinity as estimated by competitive EIA were expanded as ascitic fluid and purified using caprylic acid precipitation as previously described (Reik et al., 1987). Purified mAbs were coated in microtitre plates and were also biotinylated using activated *N*-hydroxysuccinimide ester of biotin as previously reported (Grassi et al., 1989). Binding complementarity observed in SPR experiments was confirmed in a sandwich immunoassay using microtitre plates with one of the selected mAbs as capture and others as tracer antibody in the presence of various concentrations of BLg (100, 10, 1 ng/ml) or RCM-BLg (100, 10 ng/ml) as follows: (1) 50 μ l of BLg or RCM-BLg were added to plates coated with one mAb together with 50 μ l of a biotinylated mAb and reacted for 2 h at room temperature; (2) After washing the plates, 100 μ l of streptavidin–AChE conjugate (2 Ellman Units/ml, Frobert and Grassi, 1992) were added for 2 h at room temperature; (3) After a second washing step, 200 μ l of Ellman's reagent were added to each well; (4) After 30 min of enzymatic reaction, absorbance at 414 nm was measured in each individual well. These experiments permitted the selection of pairs of mAbs providing more sensitive detection of BLg or RCM-BLg.

2.9. Labelling of mAbs with acetylcholinesterase

F(ab')₂ fragments were obtained from purified mAbs by pepsin treatment and the Fab' fragments were coupled with AChE using SMCC as previously described (Grassi et al., 1989).

2.10. Two-site enzyme immunometric assay for native and RCM-BLg

Ninety-six-well microtitre plates were coated with the capture antibody as previously described (see Section 2) and stored at +4°C until use. Plates were washed before use and 50 μ l of standard (BLg or RCM-BLg) or EIA buffer and 50 μ l of tracer (mAb–AChE conjugate) were added. After an 18-h immunoreaction at 4°C, plates were extensively washed and 200 μ l of Ellman's reagent were added to each well. After 1 h, enzymatic reaction absorbance at 414 nm was measured. Standard curves were fitted using a four logistic parameter transformation.

The detection limit was defined as the lowest concentration of standard inducing a statistically significant increase in the absorbance measured for non-specific binding (mean + 3 σ_{n-1}) as defined by the recommendations of the International Federation of Chemistry (Büttner et al., 1979). Precision was characterized by determining intra- and inter-assay coefficients of variation (CV). Intra-assay variation was estimated by measuring different concentrations of native and RCM-BLg standards eight times on a single plate. The working range was then defined as the concentration range for which CV < 10% was obtained. The inter-assay CV was calculated with two concentrations of standard (inside the working range) routinely assayed on different plates. Assay specificity was assessed by measuring BLg or RCM-BLg in a 0.1 to 10 μ g/ml solution of casein, α -lactalbumin, bovine immunoglobulins and tryptic fragments of BLg. Cross-reactivity coefficients (%) were calculated as the ratio of standard concentration divided by cross-reactant concentration providing the same signal in the two-site immunometric assays.

2.11. Heat treatment

Raw skimmed milk (0.5 ml) or purified BLg (2.5 mg/ml in PBS) was heated in polypropylene tubes

for 30 s, 1, 2, 5, 15, 30 min in a water-bath at 60°, 72°, 85°, 95° and 100°C, before being rapidly cooled in an ice–water mixture.

2.12. SDS-PAGE

SDS-PAGE of heated BLg was performed under reducing conditions and using a tricine buffer as described by Shagger and von Jagow (1987). Proteins were separated by SDS-PAGE (12% gel) and stained with Coomassie Blue G (Sigma).

3. Results

3.1. Selection of anti-BLg monoclonal antibodies suitable for establishing the sandwich immunoassays

Ten days after fusion (see Section 2), the capacity of hybridoma supernatants to bind BLg and RCM-BLg was tested using an enzyme immunoassay as described previously for other haptens and antigens (Grassi et al., 1988; Frobert and Grassi, 1992). In these tests, mouse antibodies in supernatants were successively reacted with BLg and RCM-BLg covalently coupled with the enzyme acetylcholinesterase (see Section 2). In a first screening test, 63 clones secreting antibodies recognizing the native BLg–AChE conjugate were identified, 11 of them also bound RCM-BLg–AChE conjugate in a second screening test (BLG-8R, BLG-21R, BLG-25R, BLG-31R, BLG-98R, BLG-74R, BLG-83R, BLG-89R, BLG-92R, BLG-100R, BLG-106R). It is worth noting that mAbs reacting with the RCM-BLg tracer only weakly recognized the native BLg conjugate. MAbs exclusively directed against native BLg were given a number followed by the letter N while those preferentially recognizing RCM-BLg were characterized by the letter R. All the positive clones were first expanded as culture supernatants (at least 50 ml of each) for subsequent characterization.

Since the aim of this study was to develop two-site immunometric assays for both native and RCM-BLg, we selected pairs of mAbs with optimal affinity and compatible binding for the antigens. MAbs were first characterized using a competitive enzyme immunoassay in order to determine their relative affinity for native or RCM-BLg (see Section 2). For the

52 mAbs of the N group, this led to the classification of antibodies into three categories with relative affinities ranging from 10^{-7} to 10^{-9} M (see Table 1). All the 11 mAbs of the R group had a low affinity ($> 5 \times 10^{-8}$ M, data not shown).

The binding compatibility of the 52 mAbs of the N group was established by means of SPR measurement using BLg as antigen (see Section 2). Using this iterative procedure, we roughly classified the 52 mAbs of the N group in six groups of complementarity (see Table 1). The same SPR procedure was applied to the 11 mAbs of the R group, replacing BLg by RCM-BLg. However, these experiments did not allow unambiguous classification of antibodies in complementary groups. This was due to lower binding of RCM-BLg to mAbs of the R group (probably related to the low affinity, see above). In addition we commonly observed additive binding when the same mAb was used as capture and tracer antibody. We thus decided to select arbitrarily three mAbs (BLG-74R, BLG-83R, BLG-92R) because they appeared to have the highest affinity for RCM-BLg. Their binding compatibility was tested later using EIA (see below).

Eleven mAbs were selected for expansion as ascitic fluids: 8 mAbs specifically directed against native BLg and presenting the highest affinity were chosen in each of the complementary groups (BLG-2N, BLG-69N, BLG-75N, BLG-82N, BLG-97N, BLG-107N, BLG-114N, BLG-117N), and the 3 mAbs directed against RCM-BLg (e.g., BLG-74R, BLG-83R, BLG-92R). All these 11 mAbs were purified, coated on 96-well microtitre plates and labelled with biotin (see Section 2). All possible combinations involving one mAb as capture antibody and a second mAb as tracer antibody were tested using either native BLg or RCM-BLg as sandwiched antigen. With a few exceptions, these tests confirmed the complementary groups defined by SPR measurement for the mAbs of the N group. The three mAbs of the R group (BLG-74R, BLG-83R and BLG-92R) also provided unambiguous results with EIA, demonstrating very clearly compatible binding allowing definition of 3 groups of complementarity.

Two pairs of mAbs providing more sensitive detection of native or RCM-BLg (i.e., producing the higher signal at low antigen concentration) were selected to establish the sandwich immunoassay.

Table 1

Classification of anti-native BLg mAbs according to their relative affinity ($B/B_0 = 50\%$) and complementarity groups

$B/B_0 = 50\%$	Complementary group					
	A	B	C	D	E	F
$> 5 \times 10^{-8} \text{ M}$	BLG-59N, BLG-105N	BLG-20N	BLG-38N, BLG-102N			BLG-114N
$5 \times 10^{-9} \text{ to } 5 \times 10^{-8} \text{ M}$	BLG-15N, BLG-35N, BLG-36N, BLG-44N, BLG-52N, BLG-67N, BLG-76N, BLG-79N, BLG-81N, BLG-89N, BLG-103N, BLG-104N, BLG-112N	BLG-30N, BLG-48N, BLG-70N, BLG-107N	BLG-1N, BLG-7N, BLG-9N	BLG-32N, BLG-54N, BLG-96N		
$< 5 \times 10^{-9} \text{ M}$	BLG-11N, BLG-13N, BLG-18N, BLG-24N, BLG-27N, BLG-43N, BLG-72N, BLG-88N, BLG-95N, BLG-99N, BLG-97N, BLG-101N	BLG-16N, BLG-37N, BLG-47N, BLG-69N, BLG-75N, BLG-80N, BLG-115N	BLG-117N	BLG-2N, BLG-23N	BLG-82N	

mAbs classified as belonging to the same complementarity group, as deduced by SPR measurement, were categorized in six groups (A–F). Within each group mAbs were classified according to their relative affinity, as determined with competitive EIA.

mAb BLG-97N (capture antibody) and BLG-117N (tracer antibody) were chosen for the native BLg assay and mAbs BLG-92R (capture antibody) and BLG-74R (tracer antibody) for the RCM-BLg assay.

3.2. Two-site enzyme immunometric assay for native and RCM-BLg

Fab' fragments of mAbs BLG-117N and BLG-74R were labelled by covalent coupling with enzyme acetylcholinesterase (see Section 2) and used as tracer antibodies for the immunometric assays. Fig. 1 shows standard curves obtained for BLg and RCM-BLg assay with detection limits of 30 and 200 pg/ml, respectively. The different characteristics of the two assays are summarized in Table 2. The intra-assay coefficients of variation were below 10% in the

range of 0.03 to 5 ng/ml and 0.2 to 20 ng/ml for the native and RCM-BLg assays, respectively, thus defining the working range of both assays (see Table 2). Inter-assay coefficients of variation calculated for different plates with different concentrations of standards (2, 0.05 and 20, 0.2 ng/ml for BLg and RCM-BLg, respectively, see Section 2) were 11% and 10% for the native and RCM-BLg assays, respectively.

The use of variant A or B for both native and RCM-BLg assays provided essentially identical results demonstrating that selected antibodies recognized the two variants similarly. Recovery experiments supported the validity of the assay in the different matrices such as Ringer's solution or serum (see Table 2). Lastly, α -lactalbumin, bovine immunoglobulins as well as the tryptic fragments of

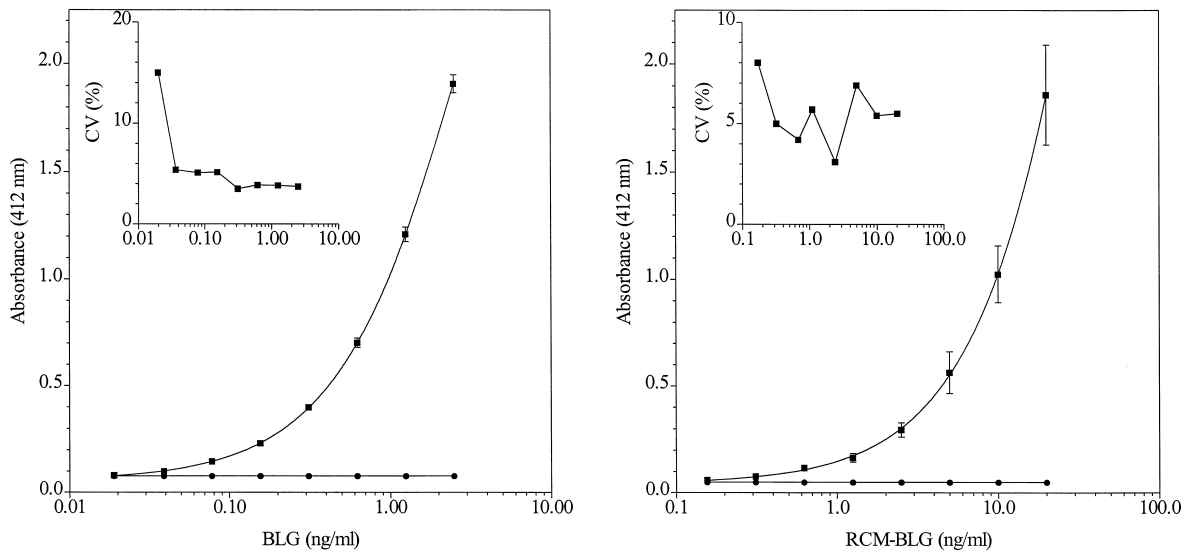


Fig. 1. Standard curves obtained for the immunometric assays of native and RCM-BLg. The assays were performed as described in Section 2. Detection limits (see Section 2) were 30 pg/ml for the native BLg assay (graph A) and 200 pg/ml for the RCM-BLg assay (graph B). It is worth noting that identical curves were obtained using either variant A or B of native and RCM-BLg. The error bars correspond to the SD of eight replicate measurements for each concentration of BLg (■) or RCM-BLg (●). The insets present the corresponding precision profiles.

Table 2
Characteristics of the immunometric assays for native and RCM-BLg

	Immunometric assay	
	Native BLg	RCM-BLg
Working range (ng/ml)	0.03–2	0.2–20
Inter-assay CV (%)	< 11	< 10
<i>Recovery (%)</i>		
In rabbit serum	99 ± 3	102 ± 6
In rat serum	105 ± 5	106 ± 5
In Ringer's solution	99 ± 4	103 ± 7
<i>Cross-reactivity (%)</i>		
Native BLg (A or B)	100	0.4
RCM-BLg (A or B)	0.018	100
Tryptic fragments of BLg	< 0.0005	< 0.002
Casein	0.01	0.1
α-Lactalbumin	< 0.0005	< 0.002
Bovine immunoglobulins	< 0.0005	< 0.002

The working range was defined as the range of concentration with an intra-assay CV < 10%. Intra- and inter-assay CVs were determined as described in Section 2. Recovery experiments were performed in rabbit or rat serum (1/5 dilution) or in Ringer's solution (1/2 dilution) spiked with 200 pg/ml or 2 ng/ml of native or RCM-BLg standard, respectively. The cross-reactivity coefficients for the different milk proteins (casein, α-lactalbumin, bovine immunoglobulins) and tryptic fragments of BLg were determined as described in Section 2.

BLg were not recognized by either of the assays. Casein exhibited low cross-reactivity (0.01% and 0.1% for native and RCM-BLg assays, respectively), as did RCM-BLg and native BLg which cross-reacted in native and RCM-BLg assays (0.018% and 0.4%, respectively).

3.3. Heat-denaturation of BLg

A first indication of the ability of the native BLg assay to detect specifically the intact protein was given by the measurement of BLg in raw milk (3.64 ± 0.06 mg/ml) compared with pasteurized milk (heat-treated for 15 s at 72°C; 0.380 ± 0.006 mg/ml) and sterilized milk (heat-treated for 1–2 s at 140–150°C; 0.084 ± 0.003 mg/ml).

The capacity of each assay to measure native or heat-denatured BLg was evaluated more closely by assaying heated BLg either as purified protein diluted in PBS buffer or in raw skimmed milk. The measurement of heat-denatured BLg was considered in the light of uncertainties concerning the protein structure following heat treatment. Indeed it is very likely that BLg forms various intermediate denatured

forms when heated. SDS-PAGE analysis of BLg heated for more than 1 min at 95°C clearly demonstrated the formation of BLg polymers (see Fig. 2) as previously described (Iametti et al., 1995). The presence of polymers in heated BLg solutions was also supported by measurements performed with a modified two-site immunometric assay in which antibody BLG-74R was used both as capture and tracer antibody (see Fig. 3). Under these conditions heated-BLg gave a very strong signal while RCM-BLg was not detected. These results clearly illustrate the difficulty of characterizing a complex mixture which cannot be reproduced by any standard solution. Despite these difficulties, we decided to use as a standard the only well-defined form of denatured BLg in our possession, i.e., RCM-BLg. The ability of this approach to allow relative measurement of denatured BLg was supported by the results of dilution tests demonstrating good parallelism between a standard curve established with RCM-BLg and dilution curves obtained with various solutions of heated BLg (see Fig. 4).

Results of tests performed either with purified BLg in PBS or with raw skimmed milk are presented in Figs. 5 and 6, respectively. Samples were heated in two ways: at various temperatures for 5 min (Fig. 5), or at a fixed temperature of 95°C for various periods of time (Fig. 6). These experiments showed that native BLg was quickly denatured at tempera-

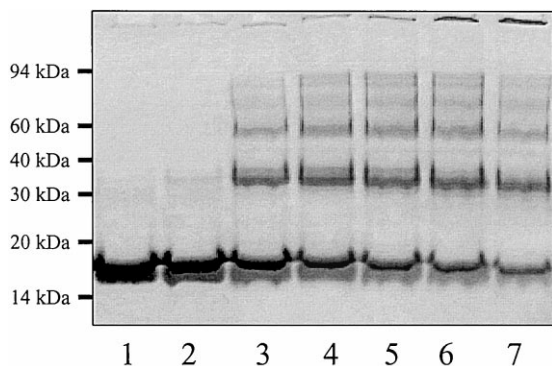


Fig. 2. SDS-PAGE analysis of heat-denatured BLg. Native and heat-denatured BLg (2.5 mg/ml in PBS) were analyzed by SDS-PAGE (see Methods). Lane 1: control, native BLg; lane 2: heated BLg 30 s at 95°C; lane 3: heated BLg 1 min at 95°C; lane 4: heated BLg 2 min at 95°C; lane 5: heated BLg 5 min at 95°C; lane 6: heated BLg 15 min at 95°C and lane 7: heated BLg 30 min at 95°C.

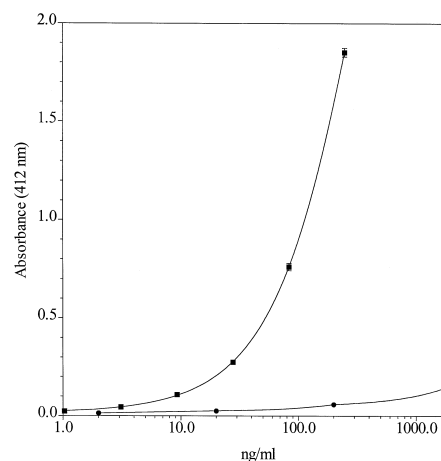


Fig. 3. Detection of polymeric forms of BLg. Polymeric forms of BLg were detected with a modified immunometric assay using antibody BLG-74R both as capture and tracer antibody. (■) Purified BLg (2.5 mg/ml in PBS) heated at 95°C for 5 min. (●) RCM-BLg.

tures above 72°C. Denaturation was almost total (> 95%) when BLg was dissolved in PBS buffer after 5 min of heat treatment at 95°C, while about 20% of the initial immunoreactivity was conserved in raw skimmed milk even after 5 min at 100°C (see Fig. 5). In every case, the drop in native BLg immunoreactivity was accompanied by a concomitant increase in RCM-BLg immunoreactivity. Denaturation

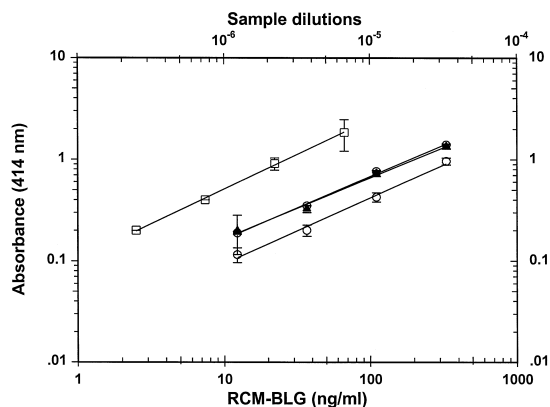


Fig. 4. Parallelism between standard curves obtained with RCM-BLg standard and dilution of heated BLg. Dilution curves of BLg (2.5 mg/ml in PBS) heated at 95°C for 1 min (○), 5 min (●) or 15 min (▲) and standard curve established with RCM-BLg (□) were determined in duplicate using the RCM-BLg assay.

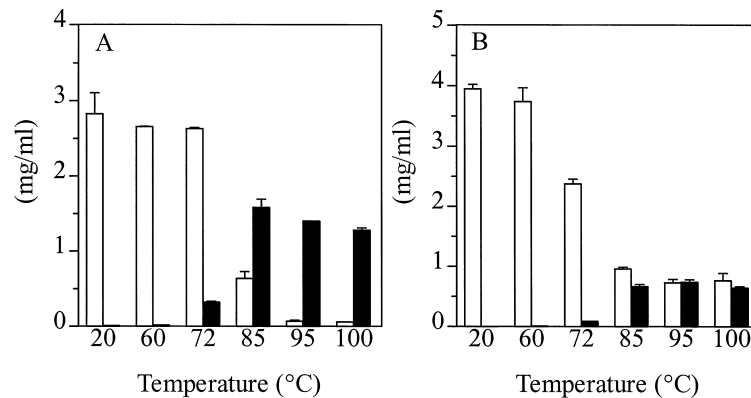


Fig. 5. Effect of temperature on denaturation of BLg in buffer or in raw skimmed milk. Results were obtained using either native (white) or RCM-BLg (black) immunometric assay performed on two series of samples heated for 5 min at various temperatures. (A) Purified BLg (2.5 mg/ml in PBS). (B) Raw skimmed milk.

tured BLg was undetectable in the BLg solution prepared in PBS buffer and represented less than 0.5% of initial immunoreactivity in raw milk. At temperatures above or equal to 95°C, we observed a significant time-dependent lowering of RCM-BLg immunoreactivity, suggesting a further degradation or denaturation of the protein.

A detailed analysis of these results confirms the difficulty of quantifying heat-denatured BLg even on a relative basis. It was apparent that the sum of native and denatured BLg detected separately by the two assays in heated samples was lower than the initial quantity of BLg in the unheated samples. This

was particularly true in raw skimmed milk where the sum never reached more than 50% of the initial quantity of BLg (see Fig. 5). In addition, the remaining immunoreactivity observed with the native BLg assay after prolonged heating, especially in milk, is unexplained. This may represent a small proportion of heat-resistant protein or more likely reflects the presence of incompletely denatured forms of BLg conserving significant immunoreactivity towards the antibodies used in the native BLg assay. In fact, it seems that BLg is more resistant to heat-denaturation in milk than in buffer probably because of interactions with other milk proteins. These results illustrate

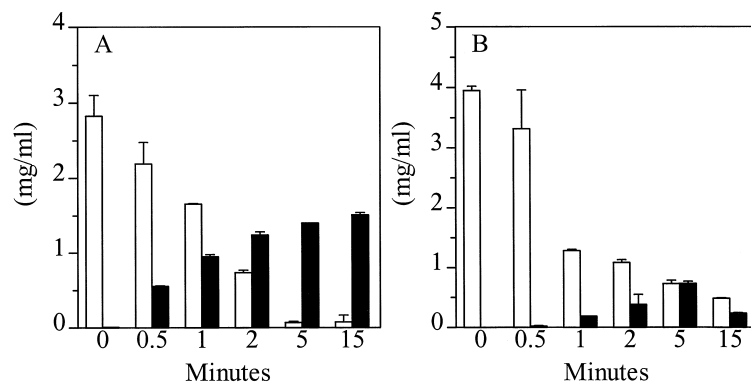


Fig. 6. Kinetics of heat denaturation of BLg in buffer or in raw skimmed milk. Results were obtained using either native (white) or RCM-BLg (black) immunometric assay performed on two series of samples heated at 95°C. (A) purified BLg (2.5 mg/ml in PBS). (B) Raw skimmed milk.

the complexity of the situation and it is clear that more detailed studies are required before we will completely understand the transformation of BLg under heat treatment.

Despite these difficulties and restrictions, we consider that the combined use of these two assays is a very convenient tool for quantitative or semiquantitative monitoring of the denaturation of BLg.

3.4. Cross-reactivity with caprine BLg: measurement of bovine BLg in goat's milk

In order to characterize anti-BLg mAbs further, with a view to developing an immunoassay allowing specific determination of bovine BLg in goat's milk, we examined cross-reactivity between bovine and caprine BLg, using solid-phase immobilized BLgs as described in the Section 2. Among the 31 mAbs tested, only three (BLG-25R, BLG-88N, BLG-95N) failed to react with caprine BLg. One of them, BLG-88N, was used as capture antibody in a two-site immunometric assay together with mAb BLG-117N as tracer. This modified assay allowed sensitive detection of bovine BLg with a detection limit of 0.2 ng/ml (see Fig. 7) without cross-reaction with

caprine BLg. This provided the basis for a test to detect the adulteration of goat's milk with cow's milk with a high sensitivity, i.e., less than 0.0001% of bovine BLg in goat's milk, corresponding to an 0.03% addition of cow's milk.

4. Discussion

Several reports describe the production and characterization of monoclonal antibodies directed against bovine β -lactoglobulin (Kaminogawa et al., 1987, 1989; Kuzmanoff and Beattie, 1991; Levieux and Venien, 1994; Venien et al., 1997). In addition, many enzyme immunoassays based on the use of monoclonal or polyclonal antibodies have been published in recent years (Mao and Bremel, 1991; Mäkinen-Kiljunen and Palosuo, 1992; Lovegrove et al., 1993; Levieux and Venien, 1994; Mariager et al., 1994). However, to date, no immunoassays suitable for measuring native and denatured BLg in the same sample have been described.

By immunizing mice with purified BLg, we could select and stabilize 63 hybridoma clones able to bind the same BLg preparation covalently coupled to the enzyme acetylcholinesterase. Surprisingly, 11 of these mAbs (R group) preferentially recognized the RCM-BLg-AChE conjugate and were finally found to be directed against a denatured form of BLg. This made it possible to establish an RCM-BLg assay exhibiting low cross-reactivity with native BLg (see Table 2 and Figs. 5 and 6). The same observation has been made by other groups (Kaminogawa et al., 1987, 1989; Venien et al., 1997) who also obtained monoclonal antibodies specifically recognizing denatured forms of BLg while immunization was done with a native BLg preparation. Different hypotheses can be proposed to explain this apparent contradiction. First, it may be due to the presence of small amounts of denatured BLg in the BLg immunizing preparation. This hypothesis is supported by the fact that mAbs of the R group weakly recognized the native BLg-AChE conjugate and by the fact that significant cross-reactivity (0.4%) was observed with native BLg in the RCM-BLg assay (Table 2). This last result suggests that the proportion of denatured BLg in the native BLg preparation would be < 1%.

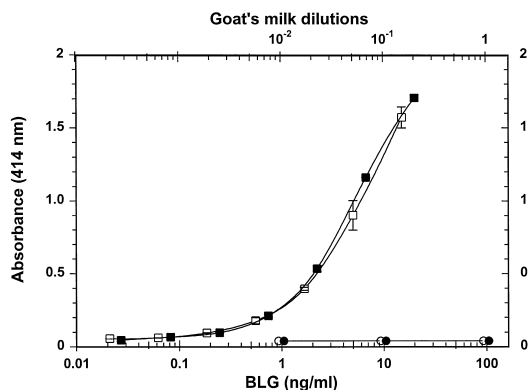


Fig. 7. Two-site immunometric assay of bovine BLg. MAb BLG-88N was used as capture antibody and mAb BLG-117N as tracer antibody. The assay was performed using sequential reaction of BLg (3 h) and tracer (2 h) as described in Section 2. The standard curve obtained with a dilution of purified bovine BLg diluted in EIA buffer ($\square$) is shown as strictly equivalent to the dilution curve of bovine BLg diluted in goat's milk ($\blacksquare$). No significant signal was produced with purified caprine BLg ($\bullet$) or goat's milk ($\circ$).

In addition it is worth noting that the way in which BLg was purified may influence the conformation of the protein as previously reported by Venien et al. (1997) who observed a difference in immunoreactivity when BLg was purified by gel filtration or by salt-fractionation. An alternative explanation is that native BLg has been partly modified and/or denatured (emulsification in Freund's adjuvant, *in vivo* proteolytic degradation for instance) during the immunization process, leading to the selection of antibodies recognizing preferentially denatured forms of the protein. So far we are not in possession of definitive experimental data which would permit us to choose between these two possibilities, which are not mutually exclusive. However, we consider this is a serendipitous situation since a single immunization procedure led to the production of two sets of mAbs with different specificities.

By selecting optimized pairs of mAbs inside the N and R groups we could establish two different sandwich immunoassays of good sensitivity (detection limit of 30 and 200 pg/ml for native and RCM-BLg assays, respectively) and precision (see Fig. 1 and Table 2). Both assays were suitable for measuring native or denatured BLg in various biological media, including raw skimmed milk, rat and rabbit serum. They proved to be very specific and presented negligible cross-reactivity with the other milk proteins and tryptic fragments of BLg. The native BLg assay is more sensitive than previously described enzyme immunoassays (Levieux and Venien, 1994; Lovegrove et al., 1993; Mariager et al., 1994), except for the assay established by Mäkinen-Kiljunen and Palosuo (1992) which was based on the use of polyclonal antibodies. It presented only slight cross-reactivity with RCM-BLg (0.018%) while the RCM-BLg assays appeared less specific with a cross-reaction of 0.4% with native BLg. The specificity of both assays was nonetheless sufficient for separate measurement of native and heat-denatured BLg in the same sample, as illustrated by heat-denaturation experiments performed in buffer or in raw milk (Figs. 5 and 6). Clearly the RCM-BLg assay cannot be considered as suitable for quantitatively measuring 'denatured BLg' since the 'denatured protein' is not a homogeneous entity which can be mimicked with a single standard preparation (here RCM-BLg). For instance, we observed polymeric

forms of BLg in heat-treated BLg solution (see Fig. 2) while our RCM-BLg preparation used as standard is entirely composed of monomers (see Fig. 3). At best, this assay provides a relative index allowing semiquantitative monitoring of the presence of 'denatured forms' of BLg. The situation is somewhat better for the native BLg assay since a more appropriate standard is available (purified BLg). However, its strict specificity for the native BLg is not fully demonstrated and we cannot exclude the possibility that it also detects partly denatured forms of BLg, as supported by the observation that significant immunoreactivity is still observed after prolonged heating of BLg solution (especially in raw milk).

Heat-denaturation is insufficient to abolish milk allergenicity; proteolytic degradation of the protein is also required (Zeiger, 1990). Thus, a method for measuring the presence of heat-denatured forms of BLg is needed in order to estimate the allergenic risk associated with hypoallergenic milk preparations. The native BLg assay appears suitable for measuring native BLg in various biological media including milk and serum, while the RCM-BLg assay provides a unique way to measure the relative concentration of 'denatured BLg'. The combination of the two assays constitutes a very useful tool for monitoring the conformational changes of BLg during technological or biological processes.

By screening 31 mAbs with solid-phase immobilized caprine BLg, we were able to select three antibodies specifically recognizing bovine BLg. One of them (BLG-88N) was used as the capture antibody in a modified sandwich assay allowing the establishment of a new assay suitable for detecting fraudulent addition of cow's milk to goat's milk. The strict specificity of mAb 88 for bovine BLg is very remarkable given that bovine BLg and caprine BLg only differ by five substitutions essentially located in the C-terminal part of the protein (130–162). However such a feature has already been observed using an immunoassay specifically detecting bovine BLg in goat's or ewe's milk (Levieux and Venien, 1994; Venien et al., 1997).

This peculiar application illustrates the value of having a large series of different mAbs of various specificities allowing detailed study of BLg immunoreactivity and epitopes. Much work remains to be done in order to characterize the present series fully

and take full advantage of its potential in studying the relationship between the structure and the activity of BLg in milk allergy.

Acknowledgements

This work received financial support from the Institut National de la Recherche Agronomique (INRA, France), the Commissariat à l'Energie Atomique (CEA, France) and the European Union (COPERNICUS project CIPA-CT-4-0190). The authors are greatly indebted to P. Lamourette and M. Plaisance for their expert technical assistance in monoclonal antibody preparation.

References

- Büttner, J., Borth, R., Boutwell, J.H., Broughton, P.M.G., Bowyer, R.C., 1979. Quality control in clinical chemistry. *Clin. Chim. Acta* 98, 145F–162F.
- Caruelle, D., Grassi, J., Courty, J., Groux-Muscattelli, B., Pradelles, P., 1988. Development and testing of ratio and enzyme immunoassays for acidic fibroblast growth factor. *Anal. Biochem.* 173, 328–339.
- Fägerstam, L.G., Frostell, A., Karlsson, R., Kullman, M., Larsson, A., Malmqvist, M., Butt, H., 1990. Detection of antigen–antibody interactions by surface plasmon resonance. *J. Mol. Recognition* 3, 208–214.
- Fazekas de Saint Groth, S., Scheidegger, D., 1980. Production of monoclonal antibodies: strategy and tactics. *J. Immunol. Methods* 35, 1–21.
- Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* 7, 88–95.
- Frobert, Y., Grassi, J., 1992. Screening of monoclonal antibodies using antigens labeled with acetylcholinesterase. In: Manson, M. (Ed.), *Methods in Molecular Biology*, Vol. 10. The Humana Press, Totowa, NJ, pp. 65–78.
- Godovac-Zimmermann, J., Braunitzer, G., 1987. Modern aspects of the primary structure and function of β -lactoglobulins. *Milchwissenschaft* 42, 294–297.
- Grassi, J., Frobert, Y., Lamourette, P., Groux-Muscattelli, B., Pradelles, P., 1988. Screening of monoclonal antibodies using antigens labeled with acetylcholinesterase: application to the peripheral proteins of photosystem 1. *Anal. Biochem.* 168, 436–450.
- Grassi, J., Frobert, Y., Pradelles, P., Chercuitte, F., Gruaz, D., Dayer, J.M., Poubelle, P.E., 1989. Production of monoclonal antibodies against interleukin-1 α and -1 β . *J. Immunol. Methods* 123, 193–210.
- Iametti, S., Cairolì, S., De Gregori, B., Bonomi, F., 1995. Modifications of high-order structures upon heating of β -lactoglobulin; dependence on the protein concentration. *J. Agric. Food Chem.* 43, 53–58.
- Kaminogawa, S., Hattori, M., Ando, O., Kurisaki, J.I., Yamauchi, K., 1987. Preparation of monoclonal antibody against bovine β -lactoglobulin and its unique binding affinity. *Agric. Biol. Chem.* 51, 797–802.
- Kaminogawa, S., Shimizu, M., Ametani, A., Hattori, M., Ando, O., Hachimura, S., Nakamura, Y., Totsuka, M., Yamauchi, K., 1989. Monoclonal antibodies as probes for monitoring the denaturation process of bovine β -lactoglobulin. *Biochim. Biophys. Acta* 998, 50–56.
- Koritz, T.N., Suzuki, S., Coombs, R.R., 1987. Antigenic stimulation with proteins of cow's milk via the oral route in guinea pigs and rats: 1. Measurement of antigenically intact beta lactoglobulin and casein in the gastrointestinal contents of duodenum, jejunum and ileum. *Int. Arch. Allergy Appl. Immunol.* 82, 72–75.
- Kuzmanoff, K.M., Beattie, C.W., 1991. Isolation of monoclonal antibodies monospecific for bovine β -lactoglobulin. *J. Dairy Sci.* 74, 3731–3740.
- Levieux, D., 1980. Heat denaturation of whey proteins comparative studies with physical and immunological methods. *An. Rech. Vet.* 11, 89–97.
- Levieux, D., Venien, A., 1994. Rapid, sensitive two-site ELISA for detection of cows' milk in goats' or ewes' milk using monoclonal antibodies. *J. Dairy Res.* 61, 91–99.
- Lovegrove, J.A., Osman, D.L., Morgan, J.B., Hampton, S.M., 1993. Transfer of cow's milk β -lactoglobulin to human serum after a milk load: a pilot study. *Gut* 34, 203–207.
- Mäkinen-Kiljunen, S., Palosuo, T., 1992. A sensitive enzyme-linked immunosorbent assay for determination of bovine β -lactoglobulin in infant feeding formulas and in human milk. *Allergy* 47, 347–352.
- Mao, F.C., Bremel, R.D., 1991. Enzyme-linked immunosorbent assays for bovine α -lactalbumin and β -lactoglobulin in serum and tissue culture media. *J. Dairy Sci.* 74, 2946–2951.
- Mariager, B., Sølve, M., Eriksen, H., Brogren, C.H., 1994. Bovine β -lactoglobulin in hypoallergenic and ordinary infant formulas measured by an indirect competitive ELISA using monoclonal and polyclonal antibodies. *Food Agric. Immunol.* 6, 73–83.
- McKenzie, H.A., Ralston, G.B., Shaw, D.C., 1972. Location of sulfhydryl and disulfide groups in bovine β -lactoglobulins and effects of urea. *Biochemistry* 11, 4539–4547.
- Papiz, M.Z., Sawyer, L., Eliopoulos, E.E., North, A.C.T., Findlay, J.B.C., Sivaprasadarao, R., Jones, T.A., Newcomer, M.E., Kraulis, J.P., 1986. The structure of β -lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* 324, 383–385.
- Perez, M.D., Calvo, M., 1995. Interaction of β -lactoglobulin with retinol and fatty acids and its role as a possible biological function for this protein: a review. *J. Dairy Sci.* 78, 978–988.
- Reddy, M., Kella, N.K.D., Kinsella, J.E., 1988. Structural and conformational basis of the resistance of β -lactoglobulin to peptic and chymotryptic digestion. *J. Agric. Food Chem.* 36, 737–744.
- Reik, L., Maines, S., Ryan, D., Levin, W., Bauderia, S., Thomas, P., 1987. A simple, non-chromatographic purification proce-

- dure for monoclonal antibodies. Isolation of monoclonal antibodies against cytochrome P450 iso-enzymes. *J. Immunol. Methods* 100, 123–130.
- Shagger, H., von Jagow, G., 1987. Tricine–SDS–polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166, 368–379.
- Venien, A., Levieux, D., Astier, C., Briand, L., Chobert, J.M., Haertle, T., 1997. Production and epitopic characterization of monoclonal antibodies against bovine β -lactoglobulin. *J. Dairy Sci.* 80, 1977–1987.
- Wal, J.M., Bernard, H., Yvon, M., Peltre, G., David, B., Creminon, C., Frobert, Y., Grassi, J., 1995. Enzyme immunoassay of specific human IgE to purified cow's milk allergens. *Food Agric. Immunol.* 7, 175–187.
- Zeiger, R.S., 1990. Prevention of food allergy in infancy. *Anal. Allergy* 65, 430–442.