



EXPRESSION, PURIFICATION AND IMMUNOCHEMICAL CHARACTERIZATION OF RECOMBINANT BOVINE BETA-LACTOGLOBULIN, A MAJOR COW MILK ALLERGEN

JEAN-MARC CHATEL,*[¶] HERVE BERNARD,* GILLES CLEMENT,*
YVELINE FROBERT,[†] CARL A. BATT,[‡] JERRIE GAVALCHIN,[‡]
GABRIEL PELTRE[§] and JEAN-MICHEL WAL*

*INRA-CEA, DRM-SPI, Bat 136, CE Saclay, 91191 Gif Sur Yvette, France; [†]CEA, DRM-SPI, Bat 136, CE Saclay, 91191 Gif Sur Yvette, France; [‡]Department of Food Science, Cornell University, Ithaca, NY 14853, U.S.A.; [§]Immunoallergie, Institut Pasteur, Paris, France

(Received 7 June 1996; accepted in revised form 2 August 1996)

Abstract—The immunological characteristics of a recombinant beta-lactoglobulin were studied using monoclonal antibodies, polyclonal antiserum and sera from allergic patients. Recombinant beta-lactoglobulin (rBLG) was expressed in *Escherichia coli* strain DH5 α and purified as described previously [Cho *et al.* (1994) *J. Biol. Chem.* **269**, 11 102–11 107]. The method has been modified by adding an immunoaffinity purification step. A quantity of 5–10 mg of purified rBLG per liter of medium culture can be produced. rBLG shared the same molecular weight as the natural BLG (nBLG) and also possessed at least one intrachain disulfide bridge. In HPLC, rBLG appeared as a single peak, and the purity was estimated to be greater than 95%. All the monoclonal antibodies (mAbs) used in this study recognized different epitopes of the BLG and presented compatible binding. No differences could be detected between rBLG and nBLG when tested in a Western blot with rabbit polyclonal antiserum or with three mAbs that bound preferentially the reduced and S-carboxymethylated form of BLG. In a competitive enzyme immunoassay (EIA) using either a rabbit polyclonal antiserum or four mAbs that recognized conformational epitopes, we could not distinguish between rBLG or nBLG. In direct ELISA using nBLG or rBLG as the immobilized allergen, we measured a similar concentration of specific anti-BLG IgE in five sera from allergic patients. The results of this study indicate that we have obtained a rBLG with biochemical and immunological properties very similar to nBLG. © 1997 Elsevier Science Ltd. All rights reserved

Key words: milk allergy, beta-lactoglobulin, recombinant protein, monoclonal antibodies.

INTRODUCTION

During the last few years, numerous proteins with allergic characteristics have been produced by recombinant methods (Scheiner and Kraft, 1995). Cloning and expression of major allergen genes in heterologous systems is expected to produce important advancements in the study, diagnosis and therapy of allergy. However, any highly purified recombinant allergen has to be shown to be identical to its natural counterpart with respect to physico-chemical and immunological parameters.

Until now, very few studies have dealt with recombinant food allergen (Batt *et al.*, 1990; Gonzalez de la Pena *et al.*, 1993; Leung *et al.*, 1994; Burks *et al.*, 1995; Vanek-Krebitz *et al.*, 1995), although food allergies become an increasing concern for public health. Cow milk allergy, and particularly the IgE mediated type, is known to affect 0.5–7.5% of infants (Gerrard *et al.*, 1973; Stintzing and Zetterstrom, 1979). Most milk proteins have been recognized as potential allergens (Otani *et al.*,

1989). Beta-lactoglobulin (BLG) is the most abundant component of the whey fraction of milk and is regarded as a dominant allergen. There are two main variants as a result of genetic differences, BLG A and B (Godovac-Zimmermann and Braunitzer, 1987). The molecular weight of bovine BLG is 18 000, which corresponds to 162 amino acid residues. It contains two disulfide bridges as well as one free cysteine. The existence of significant structural analogies between the BLG and retinol-binding protein suggests a possible physiological role for BLG involving the binding and transport of retinol (Papiz *et al.*, 1986). Bovine BLG A has been cloned and over-expressed in *Escherichia coli* (Batt *et al.*, 1990) and yeast (Totsuka *et al.*, 1990). Recently, Rocha *et al.* (1996) obtained a high yield of secreted ovine BLG in *Kluyveromyces lactis*. The recombinant BLG was used to study thermostable variants and to probe the retinol binding site (Cho *et al.*, 1994a,b).

In this paper, we describe the expression and purification of recombinant BLG. Using monoclonal antibodies, rabbit anti-serum and sera from allergic patients, we have compared the immunological properties of pur-

[¶] Author to whom correspondence should be addressed.

ified recombinant to those of BLG isolated and purified from bovine milk. We found that recombinant BLG and natural BLG had essentially the same biochemical and immunological characteristics.

MATERIALS AND METHODS

Purification of BLG from cow's milk

Natural BLG (nBLG) was purified from the milk of one single cow homozygous for the variant A of BLG, as described in Wal *et al.* (1995).

Expression and purification of recombinant proteins produced by pTTQ18 β lac.7.7.1

The plasmid pTTQ18 β lac.7.7.1 (Batt *et al.*, 1990) was transformed in *E. coli* strains. Different *E. coli* strains (DH5 α or JM109) growth temperatures, from 15°C to 37°C and concentrations of inductor isopropyl β -D-thiogalactopyranoside (IPTG), from 0.2 to 1 mM, were tested in an attempt to produce a soluble BLG. Finally, we decided to use the method described by Cho *et al.* (1994). *E. coli* DH5 α was grown at 37°C to an OD₆₀₀ of 0.5 and induced for 4 hr with 1 mM of IPTG. After induction, the insoluble proteins formed were solubilized in 8 M urea and 2 mM DTT. Refolding was avoided by dilution in a buffer containing 1 mM oxidized glutathione and 0.5 mM reduced glutathione. After overnight incubation, the pH of the solution was adjusted to 2.8 with HCl, in order to precipitate part of the contaminants. The supernatant was purified by affinity chromatography. The column was prepared by immobilizing a monoclonal anti-BLG antibody (mAb) on to CNBr-activated Sepharose 4B, as described by Pharmacia (Uppsala, Sweden). The mAb used was clone 117 (2 mg mAb/ml gel) which was prepared in the laboratory and was demonstrated to be specific for a conformational epitope of BLG. The gel was washed with 50 mM potassium phosphate buffer, pH 7.2, 150 mM NaCl (20 v/v gel). The elution buffer was a glycine buffer, 100 mM glycine-HCl, pH 2.5. The elution was immediately neutralized by a phosphate buffer, pH 7. The solution containing the purified recombinant BLG (rBLG) was dialysed against 50 mM Tris-HCl, pH 8. The total protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad, Munich, Germany). The concentration of purified nBLG or rBLG was measured using the same molecular absorption coefficient ($\epsilon_{278} = 17.6/\text{M}/\text{cm}$).

DNA manipulations

All routine DNA manipulations were carried out by the methods described by Sambrook *et al.* (1989).

Antibodies

Monoclonal antibodies were produced against nBLG using a method previously described (Grassi *et al.*, 1988). All the monoclonal antibodies used belong to different complementary groups, i.e. they were demonstrated to

recognize different parts of the protein and presented compatible binding. Some of them were found to be specific for conformational epitopes, while others recognized more specifically nBLG after reduction and s-carboxymethylation, RCM-BLG. In the different experiments, competition and Western blot, rabbit polyclonal anti-serum raised against nBLG was used. All these monoclonal antibodies will be described in greater detail in a manuscript in preparation.

SDS-PAGE and immunoblot

SDS-PAGE analysis was performed using a tricine buffer as described by Schagger and von Jagow (1987). Proteins were stained with Coomassie Blue G (Sigma, Saint Quentin Fallavier, France). For immunoblot analysis, proteins were separated by 12% SDS-PAGE and electroblotted (Towbin *et al.*, 1979) on to polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, U.S.A.). After blotting, nonspecific protein binding sites were blocked with 1% BSA in 50 mM Tris-HCl, pH 8, and 150 mM NaCl, 0.5% Tween 20. The nylon membranes were incubated overnight with a 1/2500 dilution of monoclonal antibody specific for RCM-BLG or a 1/100 000 dilution of a rabbit polyclonal anti-BLG anti-serum. After washing, the membranes were incubated for 1 hr with alkaline phosphatase conjugated anti-mouse or anti-rabbit antibody (1/7000) (Promega, Madison, WI, U.S.A.). Color development was obtained by adding 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro Blue Tetrazolium (NBT), according to the supplier's instructions.

IgE binding

Microtiter plates (96-well) were coated with natural or recombinant BLG (5 $\mu\text{g}/\text{ml}$) and an indirect ELISA was performed according to Wal *et al.* (1995). Two dilutions (1/4 and 1/20) of sera from BLG allergic patients or sera from nonallergic, birch or apple allergic patients (controls) were incubated overnight at room temperature. The IgE binding was revealed by incubation with an anti-human IgE mAb coupled to acetylcholinesterase (AChE). After washing, the AChE activity was measured by the colorimetric method of Ellman (Ellman *et al.*, 1961). Quantification as IU/ml was made by comparison with dose-response curves, obtained with a total IgE assay performed in identical conditions with known standards of human IgE (Pharmacia Prist test kit). The detection limit of specific anti BLG IgE, calculated as the dose corresponding to the average nonspecific binding (absorbancies measured with control sera) plus 3 S.D., is c. 0.1 IU.

Competitive enzyme immunoassay (EIA)

A one-step competitive enzyme immunoassay (EIA) was performed on microtiter plates coated with anti-mouse or anti-rabbit antibody. Anti-BLG mAb (50 μl), specific for conformational epitopes or rabbit polyclonal antibodies, was incubated overnight at 4°C with 50 μl of

a serial dilution of natural or recombinant BLG and with 50 μ l of nBLG-acetylcholinesterase (AChE) conjugate (1 Ellman Unit/ml). After washing, solid-phase bound AChE activity was measured using the Ellman method. Four anti-BLG mAbs, belonging to different complementary groups, were used in this experiment.

High performance liquid chromatography (HPLC)

HPLC experiments were performed using a photodiode array detector, Waters 996 (Waters, MA, U.S.A.) and a 300 A C-4 Vydac column (SFCC/Shandon, Eragry, France). All sample preparations, 100 μ l, were diluted in 100 μ l buffer A (H_2O -0.1% trifluoroacetic acid [TFA]). Elution was performed with a 25 min linear gradient of 20–80% buffer B (acetonitrile-0.1% TFA) in buffer A at a flow rate of 1 ml/min, followed by a 0.5 min linear gradient 80–95% buffer B and ended by 5 min of 95% buffer B. The effluent was monitored by scanning from 200 to 300 nm.

RESULTS

Expression and purification of recombinant BLG

The vector pTTQ18 β lac.7.7.1, described by Batt *et al.* (1990), has been used to produce a non-fusion rBLG. Whatever the conditions tested in this study, i.e. different types of *E. coli* strains, different temperatures and different concentrations of IPTG, rBLG has been expressed as inclusion bodies. Since we were unable to produce a soluble rBLG, we purified rBLG with a denaturation/renaturation method initially described by Cho *et al.* (1994). To purify rBLG further, an immunoaffinity column was prepared with a monoclonal anti-BLG antibody that recognized a conformational epitope. In SDS-PAGE analysis, after Coomassie Blue-staining, the purified rBLG appeared as a unique band with molecular weight similar to that of nBLG. The difference in mobility observed in reduced vs nonreduced conditions showed the existence of at least one intrachain disulfide bridge in the rBLG. For rBLG, as for nBLG, the dimer form was not detected (Fig. 1). A yield of 5–10 mg of purified rBLG

per liter of culture medium was obtained. Figure 2 shows the reversed-phase HPLC chromatographic pattern obtained from rBLG after affinity chromatography purification. Whatever the wavelength used for monitoring, the chromatogram shows a small peak near a major peak. The major peak has the same retention time as for nBLG. To estimate the purity of rBLG, we loaded the column with the same quantity of rBLG and nBLG. The quantity of rBLG was previously measured by a Bio-Rad Protein Assay. Assuming that rBLG and nBLG have the same molecular absorption coefficient, we estimated the purity of rBLG as at least 95%, by comparing the areas of both peaks obtained with nBLG and rBLG.

Antibody reactivity to purified natural and recombinant BLG

rBLG and nBLG could not be distinguished when a rabbit polyclonal anti-serum was used in a Western blot, as illustrated in Fig. 3. The same results were obtained with each of the three monoclonal antibodies directed against RCM-BLG. None of the four mAbs binding conformational epitopes provided any signal in the Western blot.

Competitive EIA were performed with one mAb that binds to conformational epitope, or with a rabbit polyclonal antiserum. As shown in Fig. 4, the competition curves were not significantly different. The same results were obtained with three other anti-conformational mAbs. This indicated that the epitopes recognized by these mAbs and by the polyclonal antiserum shared the same structure in nBLG and in rBLG. The immunological properties of the natural and rBLG were thus identical or very similar.

Direct IgE binding to purified natural and recombinant BLG

Direct ELISA assay was used to measure IgE binding from human BLG allergic patients to recombinant and natural BLG. The two BLG preparations were coated on microtiter plates (5 μ g/ml). None of the control sera from

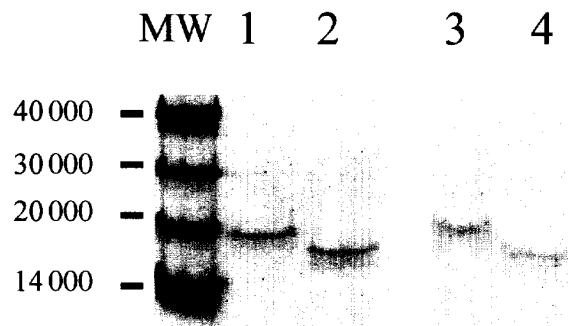


Fig. 1. SDS-PAGE in 12.5% polyacrylamide of rBLG and nBLG stained with Coomassie Blue G. Lane MW: molecular weight markers; lane 1, 2: rBLG; lane 3, 4: nBLG; lane 1, 3: reducing conditions; lane 2, 4: non-reducing conditions.

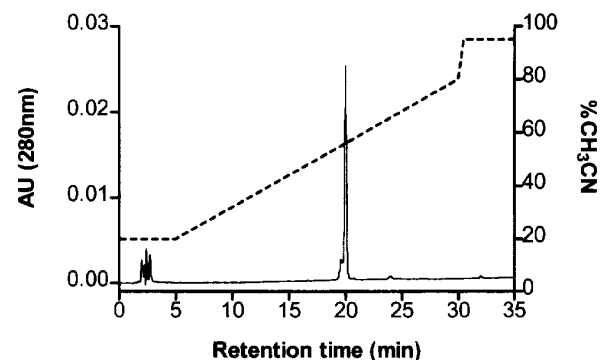


Fig. 2. RP-HPLC chromatogram of rBLG. Column Vydac 300 A C4; mobile phase: 0.1% trifluoroacetic acid/100% CH_3CN . The concentration of the mobile phase is shown by a dotted line. Proteins are monitored by UV absorption at 280 nm. The absorbance is expressed as Absorbance Units (A.U.).



Fig. 3. Western blotting experiments. Lane 1: 80 ng rBLG; lane 2: 80 ng nBLG. In this example, the rabbit polyclonal antibodies were used. The same result was obtained by using each of the three mAbs directed against RCM-BLG.

nonallergic patients gave any positive signal. The five different sera from BLG allergic patients tested provided the same signal, for a given dilution, when nBLG or rBLG was used as the immobilized allergen. For each serum we were able to calculate a concentration of specific anti-BLG IgE (IU/ml) by comparison with known standards of human IgE (Table 1). The epitopes recognized by the specific IgE in nBLG are similar in rBLG.

DISCUSSION

Recombinant proteins can be produced in large amounts, in a homogenous form, and their structure can be easily modified by site-directed mutagenesis. In the case of an allergen, they can be used as such or modified by site-directed mutagenesis to characterize the IgE binding epitopes and more generally to study their structure-immunoreactivity relationship. In this paper, we have described the production of recombinant BLG and its immunological properties using monoclonal antibodies, polyclonal antibodies raised against nBLG and sera from patient allergic to nBLG. There is an abundance of literature concerning recombinant allergens from pollen, arthropods or vertebrates but very few studies on food allergens (Matsuda and Nakamura, 1993; Scheiner and Kraft, 1995). We note, however, the work of Burks *et al.* (1995) on the allergens from peanuts; Vanek-Krebitz *et al.* (1995), allergen from apple; Gonzalez de la Pena *et al.* (1993), allergen from yellow mustard seeds and Leung *et al.* (1994), allergen from shrimp.

Totsuka *et al.* (1990) expressed bovine BLG in *Saccharomyces cerevisiae* and obtained a secreted rBLG with characteristics very close to the nBLG but with a low yield (1 mg per liter of culture). Recently, Rocha *et al.* (1996) published the expression and secretion of ovine BLG in *Kluyveromyces lactis* with a high yield, i.e. 40–

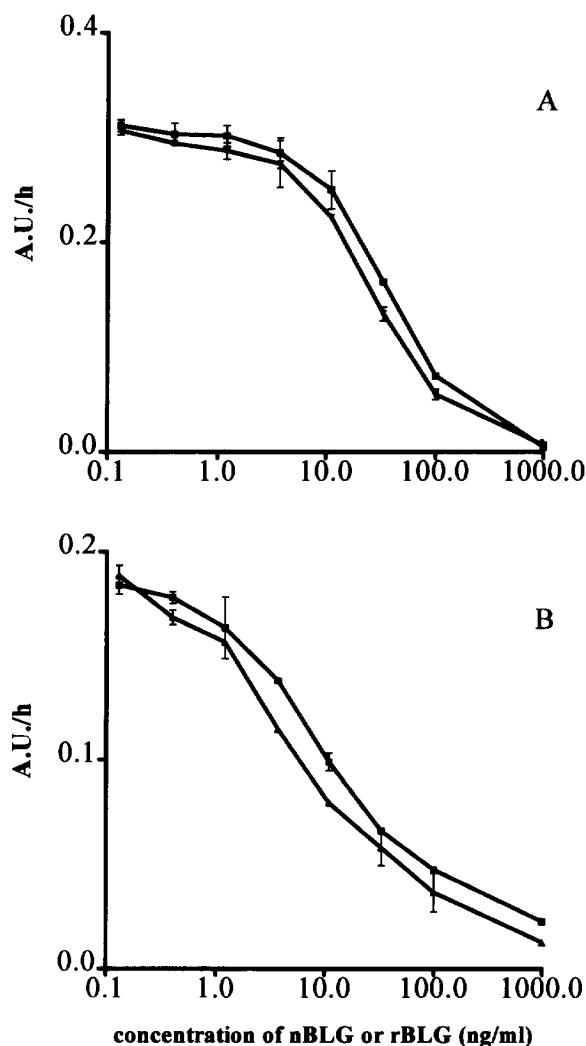


Fig. 4. Competitive EIA. Panel A: monoclonal antibody; Panel B: rabbit polyclonal antiserum; Competitor: rBLG (■); nBLG (▲). Monoclonal antibody, or rabbit polyclonal antiserum, nBLG-acetylcholinesterase conjugate and increasing concentrations of nBLG or rBLG were added in a microtiter plate previously coated with an anti-mouse or anti-rabbit antibody. The same result was obtained with each of the four mAbs that recognize the conformational epitope. Means of duplicate determinations. The absorbance was measured at 412 nm.

Table 1. Specific IgE titers in allergic patient sera

Patient	nBLG	rBLG
L92	4	4
L115	4	3
L67	6	4
L156	23	24
L198	2	2

Direct ELISA was performed as described in Materials and Methods, using nBLG or rBLG as immobilized allergen. Titers are expressed in IU/ml.

50 mg per liter of culture. Batt *et al.* (1990) described the expression of recombinant bovine BLG in *E. coli*. The rBLG was produced in an insoluble form, but it could be solubilized in urea. Four years later, Cho *et al.* (1994a) described a method to purify rBLG by denaturation in urea and renaturation in the presence of glutathione. Using this method, no difference between nBLG and rBLG can be found by CD and fluorescence studies, but no immunological characterization was shown. Hattori *et al.* (1993) showed, using monoclonal antibodies as probes, that a complete refolding of nBLG was never obtained after denaturation/renaturation. To avoid this denaturation/renaturation step, we have tried to produce soluble rBLG by using either the Thiofusion Expression System (Invitrogen, Leek, The Netherlands), the pEZZ18 vector (Pharmacia, Uppsala, Sweden) or the pET-26(b+) vector (Novagen, Madison, WI, U.S.A.). The Thiofusion Expression System expresses a fusion protein with the thioredoxin, which confers solubility to formerly insoluble proteins and is an easy purification method by osmotic shock (LaVallie *et al.*, 1992). Using this system, we were unable to produce soluble fusion protein, and it was not possible to purify the fusion protein by osmotic shock. BLG contains two disulfide bridges as well as one residue of free cysteine. Intrachain disulfide bridges are very important to stabilize the conformation of proteins. In *E. coli*, the formation of disulfide bridges is only possible in the periplasm. The vector pEZZ18 produces a fusion protein with the domain Z of the protein A, which allows the targeting to the periplasm and facilitates the purification of the recombinant protein (Nilsson *et al.*, 1987). The pET-26(b+) vector has the advantage of allowing expression of a fusion protein with the *pel B* sequence at the N-terminal extremity and a stretch of six histidines at the C-terminus (Studier *et al.*, 1990). The *pel B* sequence allows periplasmic localization and the stretch of histidines permits a single step purification method using metal chelation resin. Unfortunately, our attempts using these vectors were unsuccessful. In all cases, the fusion protein was not processed, even if located in the periplasm, indicating that the cleavage site is not accessible. It is well known now that the conformation of proteins is not solely determined by their amino acid sequence. The acquisition of the native conformation requires enzymes, like protein-disulfide-isomerase (PDI) or peptidyl-prolyl-isomerase (PPIase) and/or chaperones, like the heat-shock proteins (Hendrick and Hartl, 1993). It seems that, in the absence of an appropriate folding machinery, the BLG rapidly forms irreversible aggregates. In view of these results, we decided to adopt the expression vector pTTQ18 β lac.7.7.1. used by Batt *et al.* (1990) that produces a non-fusion protein. We introduced an additional immunopurification step at the end of the original protocol.

No differences between the two types of BLG could be detected by SDS-PAGE. They have the same molecular weight of approximately 18 000. Like nBLG, rBLG migrate faster in the nonreduced condition than in the reduced condition, showing the presence of at least one intrachain disulfide bridge. Like nBLG, rBLG is essen-

tially monomeric. After affinity chromatography purification, the HPLC chromatogram pattern shows a small peak near the major peak. The major peak has the same retention time as for that of the nBLG. The retention time of proteins is dependent on their conformation. We can consider that the rBLG has a conformation close to the nBLG. The small peak is recognized by the monoclonal antibody used in the affinity column. We cannot confirm whether it is an impurity or whether it can be considered as rBLG. It could correspond to a rBLG incorrectly refolded. In Western blot, three mAbs binding RCM-BLG and a rabbit polyclonal antiserum recognized the rBLG as well as the nBLG. We used four mAbs recognizing conformational epitopes and a polyclonal anti-serum to analyse the immunological reactivity of rBLG in solution. The four mAbs and the antiserum bound with the same affinity to rBLG or to nBLG. These results indicate that the rBLG purified by affinity chromatography has a similar conformation to that of the nBLG. Another aspect of the immunological characterization is the determination of whether IgE bind to rBLG as to nBLG. The five allergic patients' sera recognized both nBLG and rBLG identically. With regard to these results, we can state that we have obtained a rBLG of at least 95% purity, sharing essentially the same immunological properties as the nBLG. From these findings, we can conclude that the rBLG has the same epitopes as the nBLG. We cannot affirm whether the rBLG possess other epitopes that are not present in the nBLG. However, our goal was to obtain a rBLG which could not be distinguished from the nBLG, using monoclonal and polyclonal antibodies raised against nBLG and sera from patients allergic to nBLG.

In their article, Hattori *et al.* (1993) used four mAbs and found two epitopes which differed between the renatured and the native BLG. In our case, using four anti-BLG mAbs binding conformational epitopes, three anti-BLG mAbs specific for RCM-BLG and a rabbit polyclonal anti-BLG anti-serum, we could not distinguish between rBLG and nBLG. It is worth noting that the apparent discrepancies are probably due to the fact that we have characterized a recombinant BLG purified by immunoaffinity chromatography. During this step, we may have selected a subpopulation of rBLG with a conformation very close to that of the native protein.

Acknowledgements—The authors wish to thank P. Lamourette, L. Negroni and M. Plaisance for their technical assistance.

REFERENCES

- Batt C. A., Rabson L. D., Wong D. W. S. and Kinsella J. E. (1990) Expression of recombinant bovine β -lactoglobulin in *Escherichia coli*. *Agric. Biol. Chem.* **54**(4), 949–955.
- Burks A. W., Cockrell G., Stanley J. S., Helm R. M. and Bannon G. A. (1995) Isolation, identification and characterization of clones encoding antigens responsible for peanut hypersensitivity. *Int. Arch. Allergy Immun.* **107**(1–3), 248–250.
- Cho Y., Batt C. A. and Sawyer L. (1994) Probing the retinol-binding site of bovine β -lactoglobulin. *J. biol. Chem.* **269**(15), 11102–11107.

- Cho Y., Gu W., Watkins S., Lee S. P., Kim T. R., Brady J. W. and Batt C. A. (1994) Thermostable variants of bovine β -lactoglobulin. *Prot. Engng* **7**(2), 263–270.
- Ellman G. L., Courtney K. D., Andres V. and Featherstone R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmacol.* **7**, 88–95.
- Gerrard J. W., Mackenzie J. W. A., Goluboff N., Garson J. Z. and Maningas G. S. (1973) Cow's milk allergy: prevalence and manifestations in an unselected series of newborns. *Acta Paediatr. Scand.* **234**(suppl), 1–21.
- Godovac-Zimmermann J. and Braunitzer G. (1987) Modern aspects of the primary structure and function of β -lactoglobulin. *Milchwissenschaft* **42**(5), 294–297.
- Gonzalez de la Pena M. A., Villalba M., Garcia-Lopez J. L. and Rodriguez R. (1993) Cloning and expression of the major allergen from yellow mustard seeds Sin aI. *Biochem. biophys. Res. Commun.* **190**(2), 648–653.
- Grassi J., Frobert Y., Lamourette P. and Lagoutte B. (1988) Screening of monoclonal antibodies using antigens labeled with acetylcholinesterase: applications to the peripheral proteins of photosystem I. *Analyt. Biochem.* **168**, 436–450.
- Hattori M., Ametani A., Katakura Y., Shimizu M. and Kaminogawa S. (1993) Unfolding/refolding studies on bovine β -lactoglobulin with monoclonal antibodies as probes. *J. biol. Chem.* **268**(30), 22414–22419.
- Hendrick J. P. and Hartl F. U. (1993) Molecular chaperone functions of heat-shock proteins. *Ann. Rev. Biochem.* **62**, 349–384.
- LaVallie E. R., Di Blasio E. A., Kovacic S., Grant K. L., Schjendel P. F. and McCoy J. M. (1992) A thioredoxin gene fusion expression system that circumvents inclusion body formation in the *E. coli* cytoplasm. *Biotechnology* **11**, 187–193.
- Leung P. S., Chu K. H., Chow W. K., Ansari A., Bandea C. I., Kwan H. S., Nagy S. M. and Gershwin M. E. (1994) Cloning, expression, and primary structure of *Metapenaeus ensis* tropomyosin, the major heat-stable shrimp allergen. *J. Allergy Clin. Immunol.* **94**(5), 882–890.
- Matsuda T. and Nakamura R. (1993) Molecular structure and immunological properties of food allergens. *Trends Food Sci. Technol.* **4**, 289–293.
- Nilsson B., Moks T., Jansson B., Abrahamsen L., Elmlblad A., Holmgren E., Henrichson C., Jones T. A. and Uhlen M. (1987) A synthetic IgG-binding domain based on staphylococcal Protein A. *Prot. Engng* **1**, 107–113.
- Otani H., Dong X. Y., Hara T., Kobayashi M., Hayahara H. and Hosono A. (1989) Human serum antibodies to cow milk proteins in infants and children having clinically allergic symptoms. *Milchwissenschaft* **44**, 131–134.
- Papiz M. Z., Sawyer L., Eliopoulos E. E., North A. C. T., Findlay J. B. C., Sivaprasadarao R., Jones T. A., Newcomer M. E. and Kraulis P. J. (1986) The structure of β -lactoglobulin and its similarity to plasma retinol-binding protein. *Nature* **324**, 383–385.
- Rocha L. T., Paterson G., Crimmins K., Boyd A., Sawyer L. and Fothergill-Gilmore L. A. (1996) Expression and secretion of recombinant bovine β -lactoglobulin in *Saccharomyces cerevisiae* and *Kluyveromyces lactis*. *Biochem. J.* **313**, 927–932.
- Sambrook J., Fritsch E. F. and Maniatis A. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Edition. Cold Spring Harbor Press, Cold Spring Harbor.
- Schagger H. and Jagow G. von (1987) Tricine-SDS-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Analyt. Biochem.* **166**, 368–379.
- Scheiner O. and Kraft D. (1995) Basic and practical aspects of recombinant allergens. *Allergy* **50**, 384–391.
- Stintzing G. and Zetterstrom R. (1979) Cow's milk allergy. Incidence and pathogenic role of early exposure to cow's milk formula. *Acta Paediatr. Scand.* **68**, 383–387.
- Studier F. W., Rosenberg A. H., Dunn J. J. and Dubendorff J. W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes. *Meth. Enzym.* **185**, 60–89.
- Totsuka M., Katakura Y., Shimizu M., Kumagai I., Miura K. and Kaminogawa S. (1990) Expression and secretion of bovine β -lactoglobulin in *Saccharomyces cerevisiae*. *Agric. biol. Chem.* **54**, 3111–3116.
- Towbin H., Staehelin T. and Gordon J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350–4358.
- Vanek-Krebitz M., Hoffmann-Sommergruber K., Laimer da Camara Machado M., Susani M., Ebner C., Kraft D., Scheiner O. and Breiteneder H. (1995) Cloning and sequencing of *Mal dI*, the major allergen from apple (*Malus domestica*), and its immunological relationship to *Bet vI*, the major birch pollen allergen. *Biochem. biophys. Res. Commun.* **214**(2), 538–551.
- Wal J. M., Bernard H., Yvon M., Peltre G., David B., Creminon C., Frobert Y. and Grassi J. (1995) Enzyme immunoassay of specific human IgE to purified cow's milk allergens. *Food Agric. Immunol.* **7**, 175–187.