One-step method for isolation and purification of native β-lactoglobulin from bovine whey

Marija Stojadinovic[a], Lidija Burazer[b], Dilek Ercili-Cura[c], Ana Sancho[d †], Johanna Buchert[c], Tanja Cirkovic Velickovic[a] and Dragana Stanic-Vucinic[a]*

[a] University of Belgrade, Faculty of Chemistry, Department of Biochemistry, Studentski trg 16, 11 000, Belgrade, Serbia

[b] Institute of Virology, Vaccines and Sera – Torlak, Vojvode Stepe 458, 11152 Belgrade, Serbia

[c] VTT Technical Research Centre of Finland, P.O. Box 1000, FI-02044 VTT, Espoo, Finland

[d] Institute of Food Research, Norwich Research Park, Colney, Norwich NR4 7UA, UK

Correspondence to: Dragana Stanic-Vucinic, Department of Biochemistry, Faculty of Chemistry, University of Belgrade, Studentski trg 16, 11 000 Belgrade, Serbia,Tel: +381113336663, Fax: +381112184330, e-mail: dstanic@chem.bg.ac.rs

†*Current address:* University of Salzburg, Department of Molecular Biology, Christian Doppler Laboratory for Allergy Diagnosis and Therapy Hellbrunnerstrasse 34, 5020 Salzburg, Austria

Abstract

BACKGROUND: Major whey protein, β-lactoglobulin (BLG), has been widely studied for its functional properties. The scope of this paper is to develop an efficient, inexpensive and rapid one-step method for isolation and purification of BLG while preserving its native structure.

RESULTS: BLG was purified from defatted whey obtained from raw cow's milk by anion-exchange chromatography. Protein purity and identity was determined using reversed-phase HPLC and mass spectrometry. Total BLG yield was 80% with purity from 97-99%. BLG isoforms A and B were separated in some fractions with purity of 91% and 99 %, respectively. The structure and native conformation of the isolated BLG were compared to the standard commercial BLG by circular dichroism (CD) spectrometry, susceptibility to various cross-linking enzymes and by inhibition ELISA, respectively.

CONCLUSION: This method is very useful for rapid preparation of BLG suitable for studying antigenic and molecular characteristics of this protein, as well as the effect of food processing on these properties. This procedure requires only one day for the purification of about 300 mg BLG from single run while using small column (2.5 cm × 20 cm) of DEAE Sephadex and has potential for scaling up.

Running title: One-step method for isolation and purification of native β-lactoglobulin

Keywords: Native β- lactoglobulin; Isolation; Anion-exchange chromatography; Purification

INTRODUCTION

Beta-lactoglobulin (BLG) is the major whey protein in bovine milk, constituting approximately 50 % of total whey proteins [1] with a molecular weight around 18300 Da. [2] In cow's milk BLG appears in several genetic variants with A and B isoforms being the most prevalent (Asp_{64} and Val_{118} in A isoform are substituted with Gly_{64} and Ala_{118} in B isoform). [3] This protein is known for its high value as a food ingredient and techno-functional propreties. [4-6] BLG can, however, also present a significant health risk in patients allergic to milk. [7]

Various techniques have been used to isolate and purify BLG from other whey proteins, e.g. selective solubilization of BLG in the presence of 3% w/w trichloroacetic acid (TCA), [8-9] solubilization of BLG at low pH in the presence of salt, [10] peptic hydrolysis followed by selective membrane filtration, [9] followed by chromatographic techniques, [11, 12] ion-exchange chromatography, [13] affinity separation process. [14] However, these methods have several disadvantages in terms of harsh process conditions (high salt content, low pH or protease treatments) which would lower the product yield and/or quality (pure and native protein) or they include few steps and are time consuming or expensive.

When functional and antigenic properties of BLG are investigated, it is important to isolate completely native protein by a procedure that avoids all possible denaturing conditions. The main problem in whey protein isolation is the separation of its two main protein fractions, α-lactalbumin (ALA) from BLG, having close molecular weights, 14.4 and 18.3 kDa, and pI values of 4.8 and 5.3, respectively. [15] Gel-filtration is a choice only if the pH of buffer is adjusted so that BLG is in a polymerized form (e.g. pH 8.6). [12] On the other hand, ion-exchange chromatography has been widely used for

separation of proteins offering high resolution and high loading capacities. Gel filtration on the other hand has low loading capacities with the same matrix volume, while preserving proteins native state (depending on the pH and ionic strength of the eluting buffer). Whey proteins have been previously isolated by both strong and weak anion-exchange chromatography. [12, 13, 16]

In this work, a mild ~~and~~ one-step method, using ambient pressure anion-exchange chromatography, for isolation of native BLG from bovine milk is presented. The structure and native conformation of the isolated BLG were compared to the standard commercial BLG by circular dichroism (CD) spectrometry, susceptibility to various cross-linking enzymes and by inhibition enzyme-linked immunosorbent assay (ELISA), respectively.

MATERIALS AND METHODS

Materials

All regents were of analytical grade. Diethylaminoethyl (DEAE) Sephadex A-50 was purchased from GE Healthcare Bio-Sciences (Uppsala, Sweden). The standard BLG, 90% pure, was purchased from Sigma-Aldrich (Taufkirchen, Germany). Microbial transglutaminase from *Streptoverticillium mobaraense* (TG) was obtained from Ajinomoto Co. Inc., Japan and further purified according to Lantto et al. [1817] Laccase from fungus *Trametes hirsuta* (Lacc) and tyrosinase from *Trichoderma reesei* (TrTyr) were purified and characterized at VTT Technical Research Centre of Finland. [18, 19] Anti-rabbit IgG antibodies conjugated with alkaline phosphatase, para-nitrophenilphosphate, caffeic acid, galic acid, tyrosinase from *Agaricus bisphorus*

(AgaTyr), ABTS (2, 2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid), N-carbobenzoxy (CBZ)-glutaminyl-glycine/hydroxylamine and L-DOPA (3, 4-dihydroxy-L-phenylalanine) were also purchased from Sigma-Aldrich. De-ionized water used in the experiments was purified in a Milli-Q system (Millipore, Molsheim, France).

Whey preparation

Raw cCow's milk (physically and chemically untreated) was supplied by a local dairy. Skimmed milk was prepared by centrifugation (4,000 *g*) for 30 min at 4 °C. Casein was precipitated at pH 4.6 using 1 M HCl. After centrifugation (4,000 *g*) for 30 min, the supernatant, whey, was collected and additionally defatted by carbon tetrachloride (CCl_4) extraction. Whey and carbon tetrachloride were mixed in a 3:1 ratio, vortexed and centrifuged at 12,000 *g* during 15 min. Obtained whey was collected and dialyzed against 20 mM Tris buffer pH 7.5. BLG constituted 51% of all whey proteins as determined by reversed-phase HPLC (RP-HPLC).

Anion-exchange chromatography

Diethylaminoethyl (DEAE)-Sephadex A-50 was prepared for weak anion-exchange chromatography according to the manufacturer's instruction. Dry powder was equilibrated and swelled in 20 mM Tris pH 7.5 chromatography buffer. A 2.5 cm × 20 cm glass column with the 70 mL of the packed gel was used. Dialyzed whey (130 mL, containing 6 mg mL-1 of proteins) was loaded onto the column at 1 mL min^{-1} flow rate. Desorbed proteins were washed with 100 mL of the equilibration buffer. Adsorbed proteins were washed from the column using step elution, with 100 mL of the equilibration buffer containing increasing concentrations of NaCl (40-280 mM) for each step, at flow rate of 2 mL min^{-1}. Fractions of 20 mL were collected and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and

isoelectrofocusing (IEF). Fractions containing purified BLG were pooled, concentrated and desalted by ultrafiltration. Protein purity was determined by RP-HPLC.

Determination of protein concentration

Concentration of BLG was determined spectrophotometrically at 280 nm. Extinction coefficient for proteins under native conditions (ε) was calculated from the equation proposed by Pace et al. [20]:

$$\varepsilon\ (\text{mL mg}^{-1}\ \text{cm}^{-1}) = (5500 \times n_W + 1490 \times n_Y + 125 \times n_C) / M, \quad (1)$$

where n_W, n_Y, and n_C are the number of Trp, Tyr, and Cys per polypeptide chain and M is the molecular mass (Da). For BLG: M = 18285 Da, $n_W = 2$, $n_Y = 4$, $n_C = 2$ (19), from where $\varepsilon = 0.9412$ mL mg^{-1} cm^{-1}

SDS-PAGE and isoelectrofocusing

SDS-PAGE was carried out according to Laemmli [21] using a Hoefer scientific instrumentation apparatus (Amersham Biosciences, Uppsala, Sweden). Protein components were resolved on 14 % poly-acrylamide gels under reducing conditions using 5% β- mercaptoethanol. Coomassie Brilliant Blue R-250 (Sigma–Aldrich) was used to stain protein bands. Unstained molecular weight markers (#SMO431, Fermentas, Vilnius, Lithuania) were used.

Separation of protein's isoforms was achieved by isoelectrofocusing (5 % polyacrylamide gel, gradient of ampholytes pH 3.5-9.0, (Pharmacia, Uppsala, Sweden)) run on Multiphor II system (Pharmacia LKB, Uppsala, Sweden) according to the manufacturer's instructions. The pH gradient was determined by cutting the gel into

strips and incubating in 10 mM KCl for 30 min. Staining was done with Coomassie Brilliant Blue R-250 (Sigma–Aldrich).

Reversed- phase HPLC and electron spray ionization mass spectrometry (ESI MS)

Reversed-phase HPLC (RP-HPLC) and mass spectrometric analysis was done exactly as previously reported by Stanic et al. [22] Chromatograms were analyzed using UNICORN 4.0 computer software (Amersham Biosciences, Uppsala, Sweden). Total peak area was calculated by integration of the 280 nm absorbance curve using UNICORN 4.0 computer software. Protein purity was determined as the ratio of BLG peak area to the total peak area obtained by RP-HPLC; all measurements were done in duplicates.

Circular dichroism

CD spectra were recorded on a JASCO J-710 spectropolarimeter (JASCO, Japan). The system was calibrated with a 0.06 % solution of ammonium d-10-camphorsulfonat, which has known ellipticity of + 190.4 mdeg at 290.5 nm. The samples (1 mg mL-1 of BLG in 10 mM phosphate buffer pH 6.5) were analyzed at 25 °C in a 0.1 mm path length quartz cell for far UV and 10 mm path length cell for near UV spectra. The spectra were collected in 0.2 nm steps at a rate of 20 nm min^{-1} over the wavelength range 180-260 nm for far UV and 260-320 nm for near UV. Each spectrum was acquired four times, and the results were averaged. The reported spectra are the smoothed average of two experimental results performed independently. Results were expressed as residue-average molar ellipticity as follows:

$$[\theta] = \theta / (10 \times n \times C \times d), \quad (2)$$

where θ is measured ellipticity, n is number of BLG amino acid residues, C is molar concentration of BLG sample, d is pathlength of the cell.

Far UV spectra were analyzed by CONTIN software to determine the percentage of β-sheet, α-helical and random coil structures using CDPro software package (available on http://lamar.colostate.edu/~sreeram/CDPro/main.html). The program CONTIN includes the variable selection of reference proteins in the locally linearized model. The valid solutions from the variable selection are selected using the new selection rule (Helix Rule) proposed by Johnson. [23] For the calculations reference protein set SP29 was selected (29 soluble proteins).

Reactions with various cross-linking enzymes

Both purified and standard BLG were treated by TG, TrTyr, AgaTyr and lacc (with/without caffeic acid). Enzyme activities were assayed as previously reported by Stanic et al. [22] Cross-linking reaction mixtures contained BLG (1.7 mg mL-1) and TG or TrTyr or AgaTyr (1,000 nkat g^{-1} of BLG) in 50 mM sodium phosphate buffer pH at 8.0. Reaction mixtures for cross-linking with laccase contained BLG (1.7 mg mL^{-1}), laccase (1,000 nkat g^{-1} BLG) and 1 mM caffeic acid in 50 mM acetate buffer pH at 4.5. The enzymatic reactions were carried out at 40 °C for 20 h. The reaction mixture vessels were kept ~~remained~~ open and constant stirring was ensured. All reactions were stopped by addition of sample buffer (60 mM Tris, pH 6.8, 25 % glycerol, 2 % SDS, 14.4 mM β-mercaptoethanol 1% bromophenol blue) for SDS PAGE. Controls without enzyme addition were run in parallel. All reactions were run in duplicates. Aliquots from each reaction were analyzed by SDS-PAGE in reducing conditions. SDS–PAGE was performed according to Laemmli. [21] A Bio-Rad electrophoresis unit (Bio-Rad Laboratories, Richmond, CA, USA) and ready-made 12% Tris-HCl polyacrylamide gels (Bio-Rad, Hercules, CA, USA) were used. Protein bands were visualized by staining with Coomasie (Serva Blue R, Serva Electrophoresis, Heidelberg, Germany). Pre-

stained molecular weight markers (Pre-stained SDS-PAGE standards, broad range, Bio-Rad) were used.

Preparation of anti-BLG rabbit antibodies

Antibodies against pure BLG were raised in rabbits according to Harboe & Ingild [24] and partially purified by ammonium-sulphate precipitation. The high titer partly purified antibodies were used in the ELISA experiments described below.

ELISA inhibition assay

A 96-well microplate (Nunc, Maxi Sorb, Roskilde, Denmark) was used. Wells were coated with 100 µL of protein solution containing 5 µg mL^{-1} of standard BLG in carbonate-bicarbonate buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) at room temperature (RT, 24 ± 2°C) over night. After washing three times with TTBS (30 mM Tris with 0.9% (w/v) NaCl and 0.1% (w/v) Tween 20), the plate was blocked with 250 µL of 1% (w/v) solution of ovalbumin in TTBS at 37 °C for 1 h. Properly diluted rabbit anti-BLG antibodies in 0.1% (w/v) ovalbumin in TTBS were preincubated with different concentrations of purified and standard BLG solutions (100 pg-100 µg mL^{-1}) at RT for 1 h. Then 100 µL of the mixture were transferred to the wells in duplicate and incubated at 37 °C for 2 h. For ELISA inhibition experiments, rabbit anti-BLG antibodies were used in a dilution to achieve 80% of IgG binding as determined in a separate ELISA. The plate was washed three times with TTBS and 100 µL of goat anti-rabbit IgG antibodies conjugated with alkaline phosphatase diluted according to manufacturer's instructions in 0.1% (w/v) ovalbumin in TTBS were added to each well. The plate was again incubated 37 °C for 2 h. After washing the plate two times with TTBS and one time with TBS 100 µL of substrate solution, 1 mg mL^{-1} para-nitrophenylphosphate in 10 mM diethanolamine buffer pH 9.5, were added to each well.

Protein and antibodies dilutions were prepared in a diluting buffer consisting of 0.1% (w/v) ovalbumin in TTBS. After incubation at 37 °C for 30 min absorbance was measured at 405 nm using ELISA reader (LKB Micro plate reader 5060-006). Background was subtracted using negative control where 0.1% ovalbumin in TTBS was used instead of primary antibodies dilution. The percentage of inhibition was calculated as follows:

$$\text{inhibition (\%)} = (1 - (\text{OD of the inhibited sample} / \text{OD of the test sample})) \times 100, \quad (3)$$

where OD is optical density. The test sample (positive control) was the binding of the anti-BLG rabbit antibodies preincubated with 0.1% (w/v) ovalbumin in TTBS to the antigen coated in the well. Data were plotted using Origin Pro 8 package.

RESULTS AND DISCUSSION

Purification process

Our first experiments were based on the method of Ye et al., [13] where weak anion-exchanger was used to separate ALA from BLG. The majority of proteins in the whey were absorbed on DEAE-Sephadex. Whereas ALA was eluted as a dominant component with 120 mM and 160 mM NaCl, BLG eluted as two peaks with 200 and 240 mM NaCl, (Fig. 1(a)). BSA was also present in fractions corresponding to 200 mM NaCL elution buffer, however, this protein was absent from the fractions eluted with 240 mM as observed by SDS-PAGE (Fig 1b).

BLG eluted with 200 mM and 240 mM NaCl was analyzed by isoelectrofocusing (IEF) for further possible separation of BLG isoforms A and B (Fig. 1(c)). As suspected BLG isoforms A (pI 5.26) and isoform B (pI 5.34) [25] were separated in some fractions. At the beginning, with 200 mM NaCl, isoform B was desorbed from the column followed by

isoform A, and at the end of BLG elution, with 240 mM NaCl, only variant A was desorbed.

Analysis of purified BLG

Figure 2. shows the RP-HPLC chromatographic profiles and mass spectra (ESI MS) of total isolated BLG (BLG fractions eluted with 200 mM NaCl and 240 mM NaCl were pooled as separate pools), and fractions containing only A or B isoforms (fractions 26, 35, respectively, Fig. 1(c)). Two intense peaks can be observed with masses around 18363 Da and 18277 Da (Fig. 2(a)) corresponding to variants A and B, respectively, which is in accordance with mass spectra results obtained for standard BLG (Fig. 3). Mass spectra also provided information on the purity of the isolated genetic variants, with only one dominant peak of A (Fig. 2 (b)) or B isoforms (Fig 2 (c)).

From the RP-HPLC separation profiles protein purity has been determined being 97.41% (± 0.12) and 98.58% (± 0.21) for BLG preparations eluted with 200 mM and 240 mM NaCl (Table 1) and at 98.60% (± 0.14) and 90.60% (±0.85) for BLG variant A and B, respectively. Anion-exchange chromatography was repeated also with 250 mL of whey, obtaining similar protein yield (Table 1).

Secondary and tertiary structure patterns were obtained for the isolated BLG and the BLG standard, both in 10 mM phosphate buffer pH 6.5, by CD spectroscopy. Fig. 4(a) shows CD spectra in far UV from 180-260 nm, where the CD spectra for standard and isolated BLG overlapped and had a characteristic minimum at 218 nm, indicating that dominant secondary structure pattern is β-sheet. As calculated using CONTIN software, standard and isolated BLG had slightly different percent of β-sheet, 40.9% (±0.03) and 41.2% (±0.03), respectively, which is in agreement with previously published results. [11,]

[26] Similar profiles in far-UV spectra indicate that the isolated and standard BLG molecules have identical secondary structures.

Unlike to far-UV spectra, near-UV (250-320 nm) spectra of the isolated and standard BLG showed differences, mainly in the 270 – 290 nm region (Fig. 4) - decrease in intensity of the peak at 285 nm (ascribed to Trp 19), i.e. loss of aromatic dichroism that arises from non-rigid side-chain packing, in standard BLG spectrum indicates that standard BLG had slightly disrupted tertiary structure, compared to isolated BLG and therefore, a partial loss of the native structure. Accordingly, isolated BLG is native and has a more compact globular state than commercially available BLG.

Cross-linking of purified BLG

Enzymatic cross-linking was used as a method to analyze the effects of slight changes in tertiary structure between standard and isolated BLG. Cross-linking was carried out using microbial transglutaminase, tyrosinase of two different fungal origins and laccase. TG are acyl-transferase catalyzing formation of intra- and intermolecular cross-links *via* isopeptide bond between glutamine and lysine side-chains in proteins. [27] Laccases and tyrosinases are able to cross-link proteins by acting mostly on tyrosyl residues. [28] Addition of low molecular weight phenolic compounds appears to facilitate the cross-linking process by laccases and tyrosinases. [28] TG and tyrosinase reactions were carried out at pH 8, however, laccase reaction was performed at pH 4.5 since fungal laccases loose most of their activity at alkaline pH values. [29]

BLG susceptibility to cross-linking was estimated by the extent of protein polymerization as observed by SDS- PAGE in reducing conditions (Fig. 5). All experiments were done in the same way for both standard and isolated BLG (Fig. 5). Standard BLG was cross-linked the most with laccase, with caffeic acid as a mediator,

and to a lower extent with TG, giving dimmers (about 37 kDa), trimmers (about 55 kDa) and polymers (more the 200 kDa) (Fig. 5(a)). Both tyrosinases and laccase (without caffeic acid) led to formation of covalently cross-linked standard BLG dimers (Fig. 5(a)). No cross-linking was observed in the case of isolated BLG by lacc (with/without caffeic acid) or AgaTyr. TrTyr and TG cross-linked the isolated BLG, but to a smaller extent compared to standard BLG (Fig. 5(b)).

It is well known that BLG undergoes pH-dependent conformational changes. Between pH 4.5 and pH 6, BLG is converting from its expanded Q-form to the more compact native N-form. [30] At pH range 7 – 8, BLG undergoes the so-called Tanford transition, [31] entailing the modification of the closed dimer N-state into a dimer state with a more open conformation and loosening of the interior packing (the ‘‘R state’’). [30] Accordingly, at pH 8, it is not surprising to attain inter-molecular cross-linking as observed by both BLG samples, as amino acid residues may be more exposed facilitating increased reactivity of the molecule. At pH 4.5 isolated BLG is in its more compact form then at pH 8.0 and therefore less prone to crosslinking by laccase, comparing to other cross-linking enzymes. In contrast, it is likely that standard BLG, not having completely native conformation, at pH 4.5 is not in such compact / closed form thus being good substrate for laccase.

According to these results, purified BLG is more compact than standard BLG indicating that it retains more native protein conformation. Commercial BLG is probably partly unfolded, with amino-acid residues from the inner protein environment more exposed to the surface being more accessible to the enzymes.

Immunochemical characterization

Preservation of purified BLG epitopes was also examined. The degree of IgG cross-reactivity between isolated and standard BLG was determined in inhibition ELISA using anti-BLG rabbit antibodies for the detection of IgG binding to standard BLG adsorbed to the solid phase (Fig. 6). A similar inhibition profile by purified and standard BLG was observed as demonstrated by sigmoid dose-response curves, reaching 91.09% for maximum inhibitor concentration (100 g mL^{-1}) of purified BLG comparing to 92.31% of standard BLG. The median inhibition concentration (IC_{50}) was higher for standard BLG, 0.414 μg mL^{-1}, than for purified BLG, 0.163 μg mL^{-1}, thus showing that isolated protein binds IgG with higher affinity since it has a more native state. This is in concordance with previously mentioned results obtained with CD spectra and enzyme cross-linking assay.

Comparison with other methods

Many methods have been used for isolation and separation of β-lactoglobulin and other proteins from different whey preparations, some of them as mentioned in the Introduction along with their drawbacks and advantages. In our experiments, total BLG recovery was 80% with protein purity of 97-99% depending on the protein fraction (Table 1 and Table 2). A comparison of the proposed method with other BLG purification procedures reported in literature is shown in Table 2. Accordingly, protein yields of 47 – 70% are reported except for the method by de Jongh et al. [32] where 80% yield was obtained. Protein purity varied between 83-95% in different sources where the highest purity level of >98% was, again, obtained by the method proposed by the Jongh et al. [32] It is noticed that only the method of de Jongh et al. [32] has a slightly better efficiency than the method described here. Both methods are mild and result in isolation of BLG in its most native form, however the method reported by de Jongh et al. has two

steps, anion-exchange and gel chromatography, meaning it is more time consuming and more expensive.

In addition, we succeeded to separate BLG isoforms A and B in some fractions with a good purity of 99 and 91%, respectively. It should be emphasized that separation of BLG variants has been reported by analytical HPLC anion-exchange chromatography [9, 12, 33] which is expected because of excellent resolution, but the main disadvantage is low capacity. Ye et al. [13] separated BLG genetic variants by atmospheric pressure column anion-exchange chromatography on QAE-TP (Toyosoda, Tokyo, Japan) with electrophoretic patterns similar to ours but purity was not ~~n't~~ determined, therefore results cannot be compared appropriately. It was demonstrated that isolated protein was of high quality since it preserved nativity to a high degree, greater that commercially available protein preparation. This is one of the advantages of the proposed method comparing to others (Table 2).

CONCLUSION

A one-step method for isolation and purification of bovine β-lactoglobulin (BLG) presented here has several advantages over other methods: it is a cheap, efficient and reproducible method yielding milligrams/grams of highly purified native BLG. Also separation of A and B BLG isoforms, with a good purity, in some fractions was achived. Physicochemical analysis together with enzymatic cross-linking indicate that isolated BLG has a more native protein structure compared to the commercially available BLG standard, while preserving its IgG binding potential as showed by ELISA inhibition. Therefore this method is very useful for rapid preparation of BLG suitable for studying antigenic and molecular characteristics of this protein, as well as the effect of food processing on these properties. The overall yield of BLG is 80% with a purity of

between 97-99%. This method is suitable for laboratory or medium-scale isolation yet holding scale-up potential for industrial purposes.

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Table 1.

Table 1. Protein purity and yields for β-lactoglobulin (BLG) isolated from 130 (I) and 250 mL (II) of whey by proposed DEAE-Sephadex chromatography

	Protein preparation	Protein purity[a] (%)	Protein yield[b] (%)	Total protein yield (%)	Total yield (mg)
I	BLG eluted with 240mMNaCl	98.58 (± 0.12)	42	80	312
	BLG eluted with 200mM NaCl	97.41 (± 0.21)	38		
II	BLG eluted with 240mM NaCl	98.62 (± 0.16)	60	82	615
	BLG eluted with 200mM NaCl	96.95 (± 0.21)	22		

[a]Determined by reversed- phase HPLC. Values in the parenthesis represent standard deviation.

[b]BLG recovery is expressed relative to the amount of BLG from whey loaded onto the column

Table 2.

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Table 2. Comparison of protein yield and protein purity of the proposed method and several reported methods for the isolation of β-lactoglobulin (BLG)

Method for isolation of BLG	Protein yield (%)	Protein purity (%)
BLG purified according to the method described in this paper	80	97-99
Method based on the BLG solubility at low pH in the presence of salt [10]	47-69	84-95
DEAE batch followed by gel-filtration [33]	>80	>98
Peptic hydrolysis followed by membrane filtration [9]	67	94
Precipitation with 3% TCA: 1) [9] ; 2) [9]	1) 60; 2) 45	1) 95; 2) 92
Salting out: 1) [35] ; 2) [9]	1) 65; 2) 47	1) 95; 2) 87
Selective thermal precipitation [9]	50	83

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491 **Figure captions**

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493 **Fig. 1.** Fractionation of whey proteins by DEAE-Sephadex chromatography. a)
494 Chromatogram: BLG- β-lactoglobulin, ALA- α-lactalbumin. Electrophoretic patterns of
495 eluted fractions: b) SDS-PAGE under reducing conditions, 40 µL of each sample was
496 applied per well, Mm-molecular mass markers, W- total whey proteins; c) IEF, 20 µL
497 of each sample was applied per well.

498 **Fig. 2.** RP-HPLC chromatogram of β-lactoglobulin and ESI MS spectra (inserts) for: (a)
499 total isolated BLG; (b) isolated isoform A and (c) isolated isoform B.

500 **Fig. 3**. ESI MS spectra for standard β-lactoglobulin.

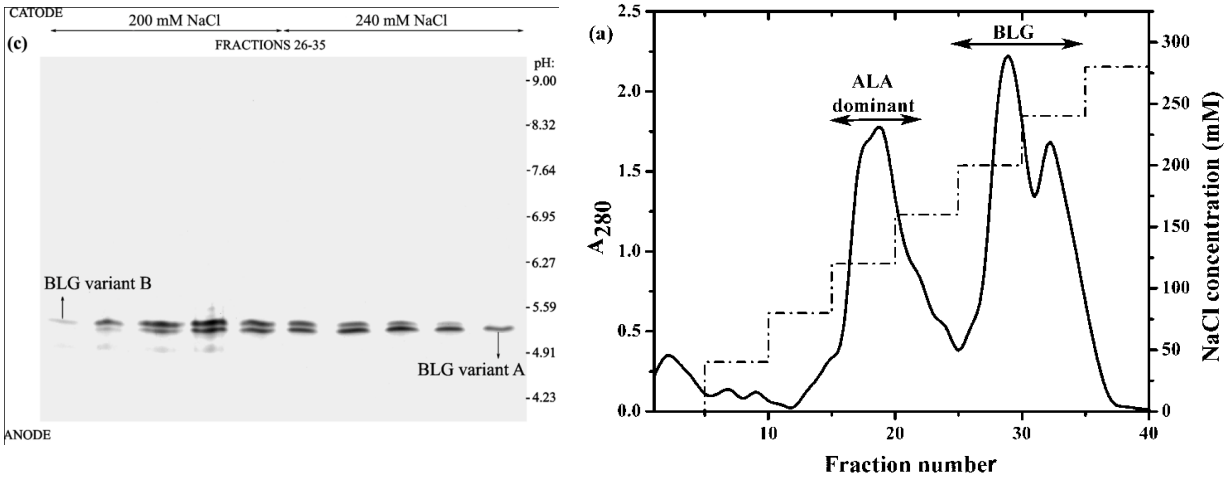
501 **Fig. 4.** Circular dichroism spectra of both isolated BLG (black) and BLG standard
502 (grey) in (a) far UV and (b) near UV spectral range at pH 6.5.

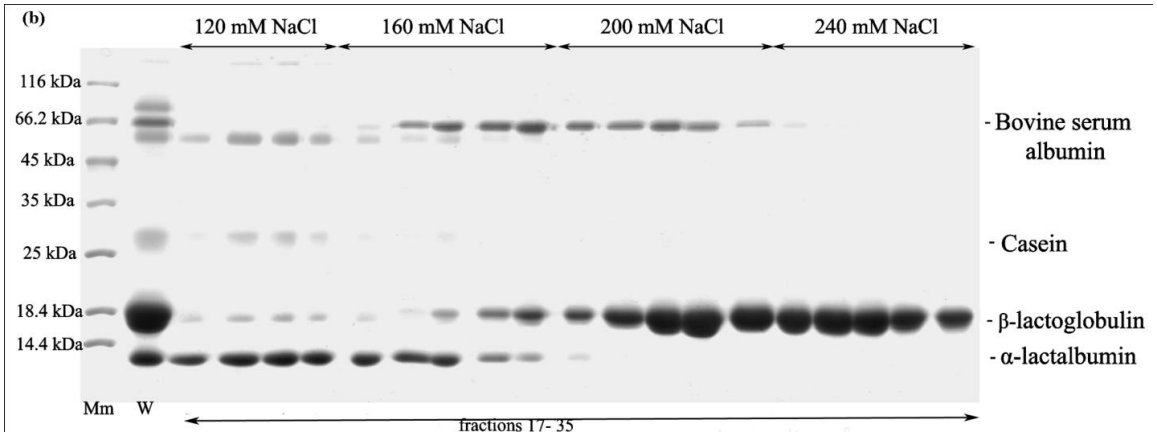
503 **Fig. 5.** SDS-PAGE under reducing conditions of cross-linking reaction mixtures of
504 standard (a) and isolated (b) β-lactoglobulin (BLG). Cont pH 8- untreated BLG in 50
505 mM sodium phosphate buffer pH 8, Cont pH 4.5- untreated BLG in 50 mM acetate
506 buffer pH 4.5, TG- BLG/transglutaminase, TrTyr- BLG/tyrosinase (*Trichoderma*
507 *reesei),* AgaTyr- BLG/tyrosinase *(Agaricus bisphorus),* Lacc- BLG/laccase (*Trametes*
508 *hirsuta),* Lacc+caff- BLG/ laccase (*Trametes hirsuta*)/caffeic acid , Mm- molecular
509 mass markers.

510 **Fig. 6.** IgG- ELISA inhibition curves using standard BLG adsorbed to the solid phase
511 and the following inhibitors: standard (grey) and isolated (black) BLG- (100 pg mL^{-1}-
512 100 µg mL^{-1}) expressed as – log (inhibitor concentration). Percentage of inhibition was
513 calculated by the residual antibody reactivity in relation to 100% reactivity (with no
514 inhibitors). Error bars represent the standard deviation from two different experiments
515 in triplicate.

Figures

Figure 1.





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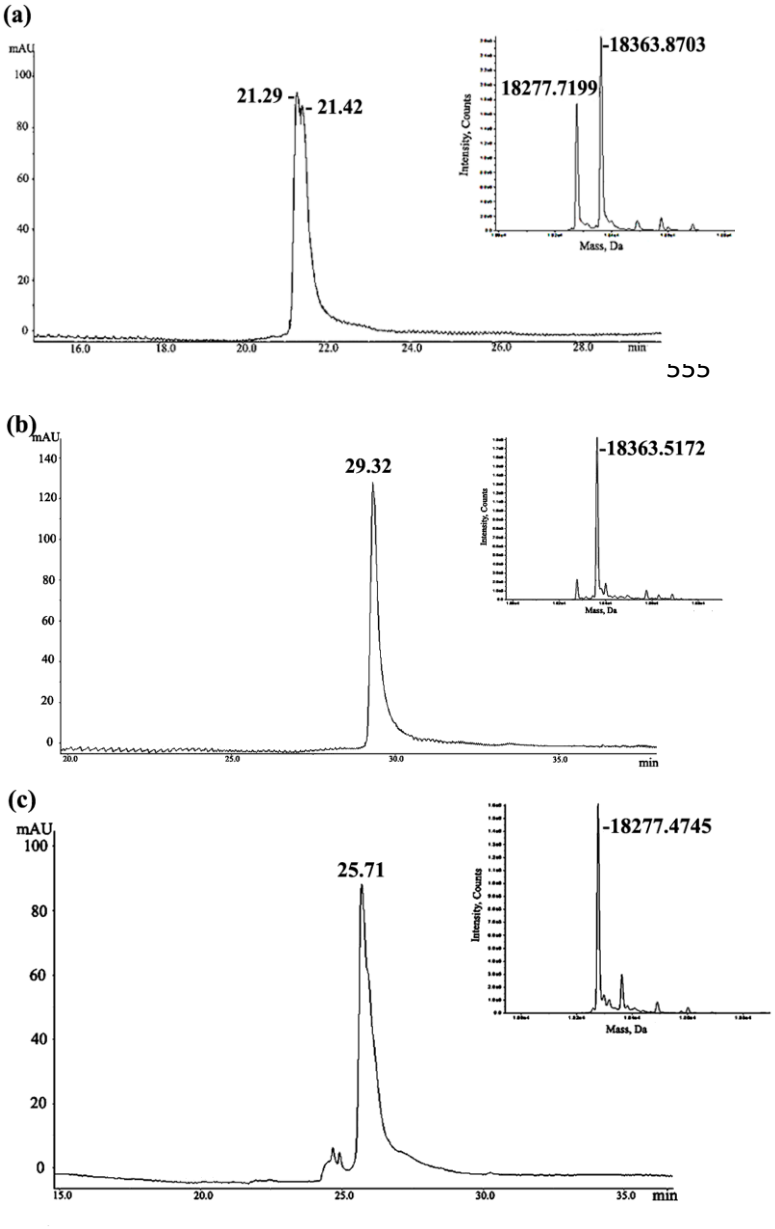
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Mass, Da
min
(b)
mAU
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Mass, Da
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(c)
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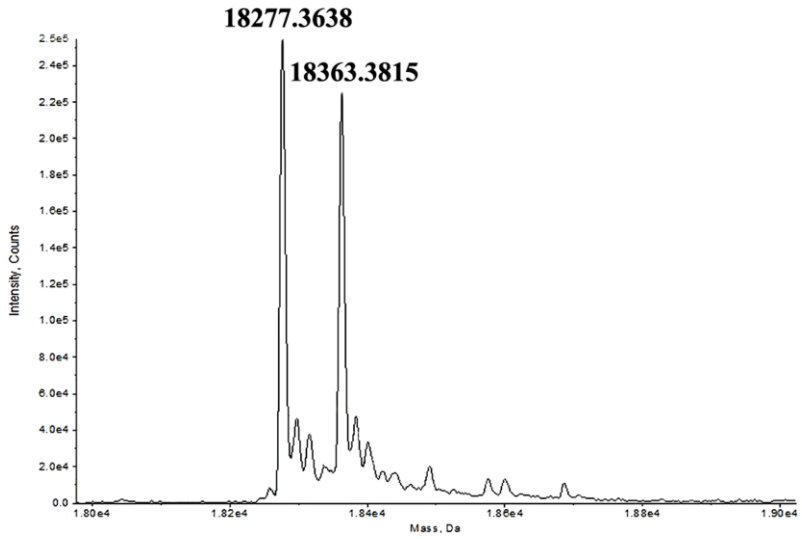
Intensity, Counts
Mass, Da
min

Figure 3.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18363.3815
Intensity, Counts
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2.2e5
2.0e5
1.8e5
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1.2e5
1.0e5
8.0e4
6.0e4
4.0e4
2.0e4
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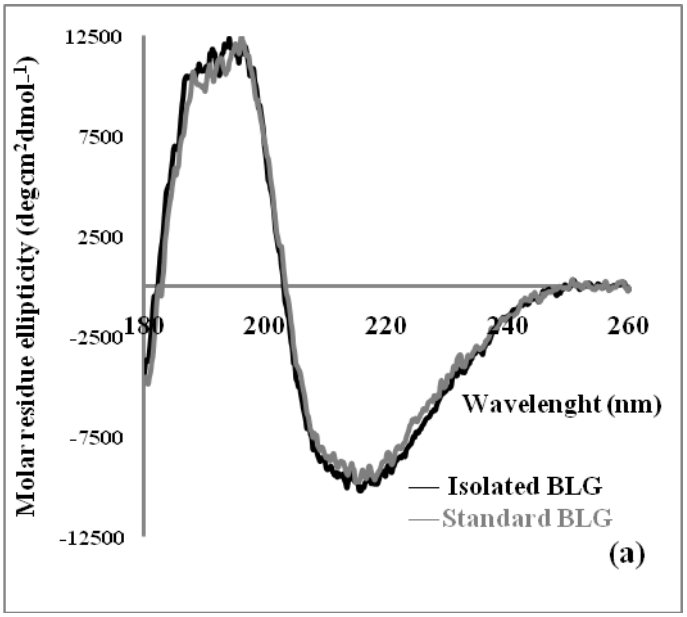
Mass, Da

Figure 4.



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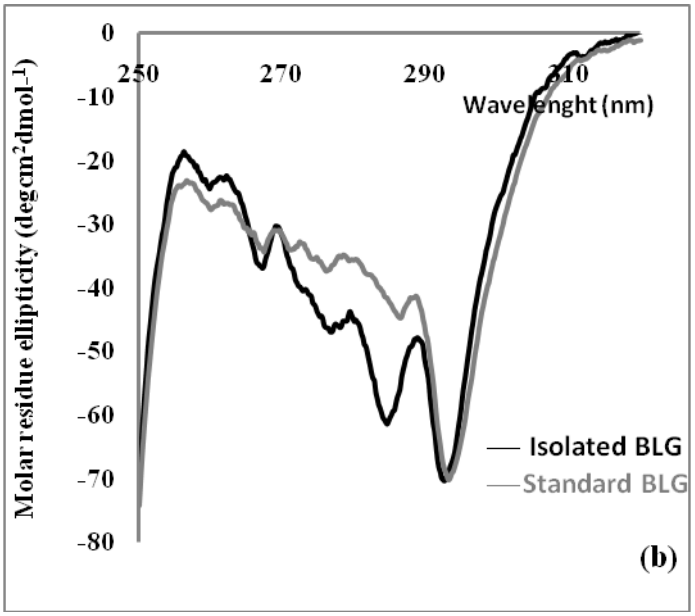

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591 **Figure 5.**

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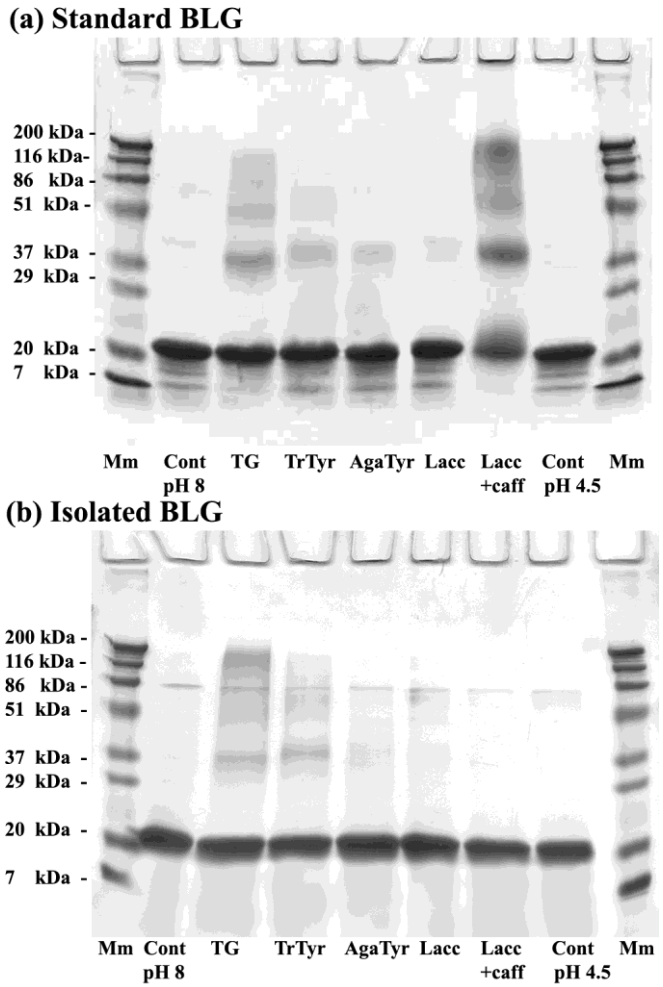
(a) Standard BLG
200 kDa -
116 kDa-
86 kDa -
51 kDa -
37 kDa -
29 kDa -
20 kDa -
7 kDa -
Mm
Cont pH 8
TG
TrTyr
AgaTyr
Lacc
Lacc +caff
Cont pH 4.5
Mm
(b) Isolated BLG
200 kDa -
116 kDa -
86 kDa -
51 kDa -
37 kDa -
29 kDa -
20 kDa -
7 kDa -
Mm
Cont pH 8
TG
TrTyr
AgaTyr
Lacc
Lacc +caff
Cont pH 4.5
Mm

Figure 6.

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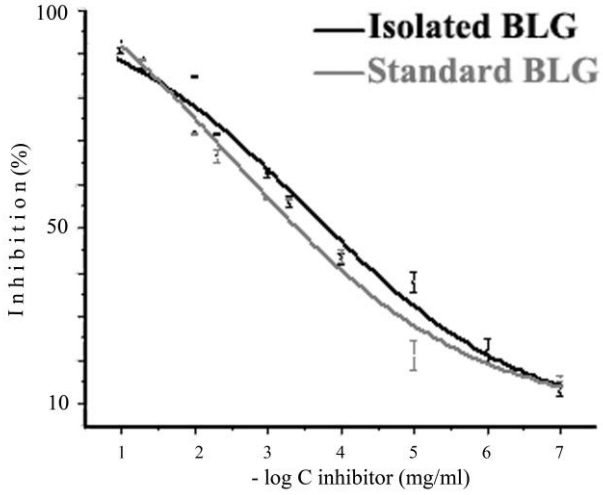

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