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Full Length Research Paper

Isolation and antigenicity evaluation of β-lactoglobulin from buffalo milk

X. Li[1,2], Z.L Luo[1,2], H.B Chen [1,2]* and Y.S. Cao[1,2]

[1]State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, P.R.China.
[2]Jiangxi-OAI Joint Research Institute, Nanchang University, Nanchang 330047, P.R.China.

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Buffalo β-lactoglobulin in phosphate buffer (0.02 M, pH6.8) was adsorbed on DEAE-Sepharose Fast Flow gel, and eluted with a linear gradient of NaCl (0-0.5 M) in 0.02 M phosphate buffer, pH 6.8. A further purification was performed on Sephadex G-75 gel by loading a concentrated and dialyzed fraction of samples containing buffalo β-lactoglobulin from ion-exchange chromatography, and seperating at a flow rate of 0.15 ml/min in 0.02 M phosphate buffer, pH 6.8. The purity of the isolated buffalo β-lactoglobulin was above 90% in comparison to the standard bovine β-lactoglobulin by SDS-PAGE and IEF-PAGE. The antigencity of the buffalo β-lactoglobulin was evualuted by indirect ELISA, Western-blotting and inhibition ELISA with anti-buffalo and anti-bovine β-lactoglobulin rabbit serum. The results showed that buffalo β-lactoglobulin could be seperated and purified by anion-exchange chromatography combined with gel filtration chromatography, and with a well-preserved antigenicity.

Key words: Buffalo milk, milk allergy, β-lactoglobulin, antigenicity.

INTRODUCTION

Like cross-reactivity between milk allergens from different mammalian species, the high cross-reactivity also exists between cow milk and buffalo milk because of their similar amino acid compositions (Restani et al., 2002; Carroccio et al., 1999; Docena et al., 2002). It is suggested that patients who suffer from cow milk allergy may be allergic to buffalo milk. Currently, an incidence of cow milk allergy ranges from 0.3 to 7.5% in population based on the studies in different countries, which is obviously linked to great consumption of cow milk, especially for infant and children (Bahna, 2002; Elsayed et al., 2004; Wal et al., 2004). Many studies have revealed there are more than 20 allergic proteins in cow milk and one of the most important allergic proteins in milk is β-lactoglobulin (β-lg) to which about 82% of milk allergic patients are sensitive (Aoki et al., 2006; Fritsché et al., 2005; Kontopidis et al., 2004; Zicarelli, 2006). On the other hand, due to superior nutritional and economic value, buffalo milk has gradually replaced cow milk in some areas of the world. The population of buffaloes has increased by 200% from 88 million to 174 million during the year 1961-2005, covering 42 countries around the world according to FAO (2004) (Cruz, 2006; Pang et al., 2006; Jiang et al., 2006). Up to the present, buffalo milk production is the second largest milk supply in the world after cow milk, representing more than 12% of total milk production (Ashmad et al., 2008). Therefore, much more attention has been paid to the allergy from buffalo β-lg for the increasing consumption of buffalo milk.

To better understand the mechanism of the allergy caused by buffalo β-lg, a purified buffalo β-lg and its antigenicity evaluation are quite necessary. Buffalo β-lg contains 162 amino acids (MW = 18.4 kDa) with only two different amino acids compared with the bovine β-lg variant B and its isoelectric point (pI) is 5.23 (Bolognesi et al., 1979; Ghosh et al., 1971). However, there is little information directly available on the purification of buffalo β-lg so far. Although some studies have demonstrated that highly purified bovine β-lg can be obtained by using high-performance liquid chromatography, fast protein liquid chromatography and isoelectric focusing, these methods are more complicated or expensive compared with gel filtration and ion-exchange chromatography (Nevestani et al., 2003; Godovac-Zimmermann et al., 1996; Edwin et al , 2004; Hahn et al., 1998).

*Corresponding author. E-mail: chbgjy@hotmail.com.

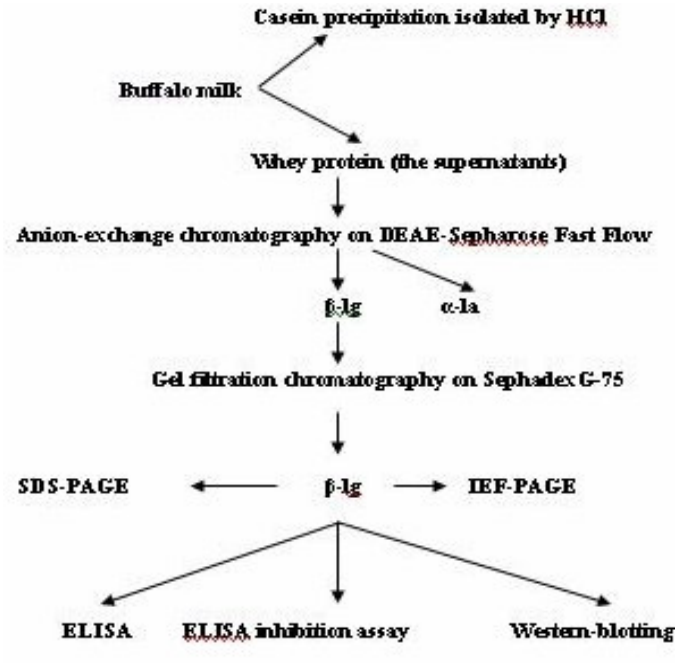

Figure 1. Flow chart for the purification and evaluation of the antigenicity of buffalo milk β-lg.

The purpose of this work is to introduce a protocol by combining ion-exchange chromatography and gel filtration chromatography in a tandem operation which renders purification of buffalo β-lg a simple and inexpensive task while preserving its antigenicity. The antigenicity of the purified buffalo β-lg was evaluated by indirect ELISA, Western-blotting and ELISA inhibition assay. Moreover, the method can be modified to purify buffalo β-lg for varying scales.

MATERIALS AND METHODS

Buffalo milk

Fresh raw milk samples were collected from the Murrah breed buffalo at the Cattle Farm of Guangxi Buffalo Research Institute, Nanning, China.

Chemicals

DEAE-Sepharose Fast Flow gels, Sephadex G-75 gel and pharmalyte™ 3-10 for IEF were from General Electric Company, USA. Ninty-six-well microplates were obtained from Labsystems, Finland. Standard bovine β-lg, gelatin from porcine skin, sheep anti-rabbit Ig/HRP, O-phenylene diaminc and methoxy-naphthol were from Sigma Company (USA). Polyvinyldifluoride (PVDF) membranes were obtained from Millipore (Bedford, MA, USA). All the other chemicals including Freund's complete adjuvant and Freund's incomplete adjuvant were purchased from Shanghai Sangon Biological Engineering Technology and Service Co., Ltd, China. All chemicals used were analytical reagent.

Preparation of buffalo whey protein

Buffalo milk was filtrated with quadrilayer gauze in order to remove some impurities. Then the milk was centrifuged at 3000 rpm for 30 min at 4°C and the top fat layer was removed by a spatula. The skimmed milk was acidified to pH 4.6 by adding 3 M HCl slowly (Hahn et al., 1998; Vyas et al., 2002). Furthermore, the solution was incubated for 30 min at 40°C and caseins were removed by centrifugation at 8000 rpm for 15 min at 4°C. The supernatant containing whey proteins was concentrated by hollow-fibre membrane with 10 kDa cut-off. The total concentration of buffalo whey proteins was 143.4 mg/ml determined by Lowry method (Lowry et al., 1951) and aliquots were stored at -80°C until used. The working flow chart is shown in Figure 1.

Anion-exchange chromatography

Anion-exchange chromatography was performed on DEAE-Sepharose Fast Flow gel packed in a column (30 cm length and 1.2 cm internal diameter). The loading buffer, equilibrating buffer and elution buffer were as follows: 0.02 M phosphate buffer, pH 6.8; 0.02 M phosphate buffer, pH 6.8; 0.02 M phosphate buffer containing 0 - 0.5 M NaCl, pH 6.8. The matrix was equilibrated with equilibrating buffer, and the column was washed with 200 ml equilibrating buffer after loading the sample diluted 1:8 with PBS (containing 71.7 mg whey protein, pH 6.8). The bound proteins were eluted at a linear gradient by using elution buffer with flow-rate and fraction volume being 1.5 ml/min and 30 ml, respectively. Finally, the target protein of the eluted sample was concentrated to 400 µl by centrifugation at 3500 rpm for 45 min at 4°C with Amicon Ultra-15 centrifugal Filter Unit (Ultracel-5 membrane, Millipore) (Lowry et al.,1951; Pourpark et al., 2004).

Gel filtration chromatography

Sephadex G-75 gel solution was degassed and packed into the column (1.2 × 75 cm). The matrix was equilibrated with 0.02 M phosphate buffer, pH 6.8 (Kinekawa and Kitabatake, 1996). About 300 µl protein sample collected by anion-exchange chromatography was mixed with 300 µl phosphate buffer (0.02 M, pH 6.8) and was loaded on the column. The elution was carried out by using 6 ml of fraction at a flow rate of 0.15 ml/min with 0.02 M phosphate buffer, pH 6.8. The absorbance of the fractions was monitored at 280 nm. The fraction sample was concentrated as the previous step, and the purity of protein preparation was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE).

Preparation of the anti-sera

Eight-week old Japanese white male rabbits were from Jiangxi Administration Committee of Laboratory Animal. Each of them was immunized by subcutaneous multi-sites injection with standard bovine and purified buffalo β-lg, respectively. 1 ml β-lg (1 mg/ml in PBS, pH 6.8) mixed with 1 ml of Freund's complete adjuvant was used for first injection. Then, each rabbit was injected for three times with booster dose 1 ml β-lg (1 mg/ ml) in suspension with 1 ml Freund's incomplete adjuvant on day 14, 28 and 42, respectively (Williams et al., 1998). One week after the last injection, rabbits were bled and each rabbit's antiserum was separated by centrifugation at 5000 rpm for 10 min. Serum samples from each two rabbits injected with the same antigen were pooled together. The two serum samples were stored at -20°C, and antibody titers were determined by indirect ELISA.

SDS-PAGE and IEF-PAGE

Fractions were analyzed by SDS-PAGE according to Laemmli (1970). Electrophoresis was run with a 5% stacking gel and a 15% separating gel, with a current of 15 and 35 mA, respectively. Loading samples were boiled at 100°C for 8 min prior to electrophoresis. The amount and volume of the purified protein solutions transferred to each well were about 10 µg and 20 µl, respectively. Protein staining was performed with Coomassie Brilliant Blue R250, 10% acetic acid and 25% methanol for at least 30 min or overnight. Distaining solution was carried out in 7.5% acetic acid and 5% methanol.

The gel for IEF-PAGE was prepared as follows: 29.1% acrylamide-0.9% bisacrylamide, 2.0 ml ; carrierampholytes, 0.5 ml; ultra-pure water, 5.5 ml; TEMED, 8 µl; 10% ammonium persulfate (AP), 50 µl. Preparation of samples and electrophoresis was performed according to the method described by Salaman and Williamson with minor modifications (Salaman and Williamson, 1971). 60 µg standard bovine β-lg sample as a marker was run concurrently with two 60 µg purified buffalo β-lg samples obtained from anion-exchange chromatography and gel filtration chromatography, respectively. IEF–PAGE was initially run at 60 V constant for 15 min, then at 8 mA constant until reaching 550 V. After removing the loading sample paper, it continued running with 580 V until the current become zero.

Western-blotting

Buffalo β-lg and standard bovine β-lg samples were electrophoresed as described above and electrotransferred to polyvinyldifluoride (PVDF) membranes with a current of 40 mA for 1 h. After PVDF membranes were blocked with 3% porcine skin gelatin in Tris-buffered saline (TBS, 20 mM Tris-500 mM NaCl, pH 7.5) for 1.5 h at 37°C.The same volume of antiserum from buffalo β-lg diluted 1:10,000 in TBS with 3% porcine skin gelatin was incubated for 1 h at 37°C. The membrane was washed three times for 10 min in TBS with 0.05% Tween 20 (TBS-T) and incubated for 1 h at 37°C with sheep anti-rabbit IgG /HRP diluted 1:5, 000 in TBS. Finally, the bands were developed on the membrane using 6 mg methoxy-naphthol dissolved in 3 ml methanol and 10 ml TBS with 6 µl of 30% H_2O_2 after the membrane was washed three times again in TBS-T.

Indirect ELISA for antibody titration and ELISA inhibition assay

Indirect ELISA was performed as follows. Labsystems 96-well microtiter plates were coated overnight at 4°C with 0.25 µg/ml β-lg (purified β-lg or standard bovine β-lg) in 0.05 M carbonate buffer, pH 9.6 (100 µl/well). Then, the plates were blocked for 1 h at 37°C with 1% porcine skin gelatin in 0.1 M PBS, pH 7.0. Furthermore, plates were washed three times with PBS containing 0.05% Tween 20 (PBST) and incubated with 100 µl of a 1:200 to 1:409,600 dilution of pooled rabbit sera for 1 h at 37°C. After washing the plates again for three times with PBST, 100 µl of sheep anti-rabbit IgG conjugate with horseradish peroxidase (1:10,000, Sigma) was added to each well for 1 h of incubation at 37°C. After further thorough washing of the plates for three times again, the reaction was developed with O-phenylene diaminc (OPD) in citrate buffer (0.1 M, pH4.6) in addition to 30% H_2O_2. The reaction was stopped with 50 µl of 2 M H_2SO_4 and the absorbance was measured at 492 nm in a plate reader (Bio-Rad, Model 600).

ELISA inhibition assay was done similarly to Indirect ELISA. Microtiter plates (96-well) were coated with 0.25 µg/well of purified buffalo β-lg in carbonate buffer (0.05 M, pH 9.6) for overnight at 4°C. Blocking buffer containing 1% porcine skin gelatin was added to each well and incubated for 1 h at 37°C. With regard to inhibition, pre-incubation of equal volume diluted anti-sera (1:60,000) and various concentrations of the standard bovine /buffalo β-lg were performed for 1 h at 37°C. A volume of 100 µl of each pre-incubated sample was transferred to the coated wells for competition with coated antigen for 1 h at 37°C. The other procedures were the same as described in indirect ELISA.

The percentage of inhibition was calculated as follows:

$$\text{Inhibition}(\%) = \frac{\text{total reactivity - remaining reactivity after inhibition}}{\text{total reactivity}} \times 100\%$$

Cross-reactivity was then calculated by using the following equation: $C_{St50}/C_{P50}\times100\%$, where C_{St50} and C_{P50} were the concentrations of the standard and the purified β-lg respectively, in which 50% inhibition had occurred.

RESULTS AND DISCUSSION

Anion-exchange chromatography

Anion-exchange chromatography profile of buffalo whey proteins on DEAE-Sepharose Fast Flow gel (Figure 2a) displayed two major fractions (b and d) and three minor fractions (a, c and e). Fraction b and d corresponded to α-lactalbumin and β-lg respectively, as shown by SDS-PAGE (Figure 2b). Fraction b and d appeared with 0.2 and 0.3 M NaCl in phosphate buffer (0.02 M, pH 6.8), respectively, and the total fraction volumes were 48 and 60 ml, respectively. The buffalo β-lg recovery rate was 50.26% of the total buffalo β-lg in the whey proteins (data not shown).

α-lactalbumin and β-lg are the major whey proteins. Separating the two kinds of proteins was the first step to isolate β-lg from whey proteins. The pI of buffalo β-lg was determined to be 5.23, whereas no related research on buffalo α-la was found in the literature. However, buffalo and bovine α-lactalbumin have a high homology in the composition of amino acids. It was suggested that the pI of buffalo α-la might be 4.8 similar to that of cow's. Therefore, it was easy to separate buffalo α-lactalbumin and β-lg in whey proteins by ion-exchange chromatography. The anion-exchange chromatography was run on DEAE-Sepharose Fast Flow gel with 0.02 M phosphate buffer, pH 6.8. This buffer solution can preserve a natural form of whey proteins well because the pH of fresh raw buffalo milk is 6.8 too. The results demonstrated that buffalo β-lg could be separated well from whey proteins while containing a little salt. Thus, the fraction of β-lg from anion-exchange chromatography is convenient to be performed on gel filtration for next purification.

Gel filtration chromatography

0.6 ml of concentrated buffalo β-lg solution (fraction d in Figure 2a) obtained by anion-exchange chromatography was submitted to Sephadex G-75 chromatography with 0.02 M phosphate buffer, pH 6.8. One major fraction (fraction B) appeared to be highly purified buffalo β-lg

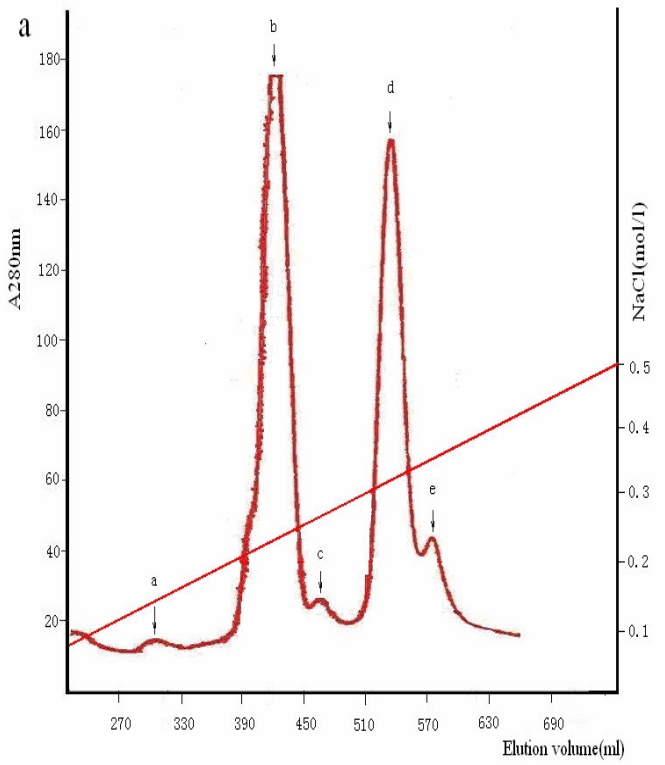

Figure 2a. Anion-exchange chromatography on DEAE–Sepharose Fast Flow of buffalo whey proteins. Chromatogram is only the eluted fractions without unbinding protein (0.02M phosphate buffer, pH6.8, with an 0-0.5 M NaCl linear gradient).

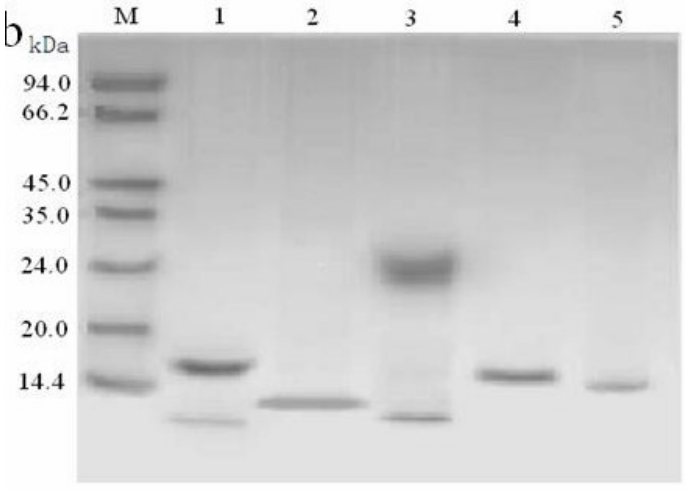

Figure 2b. Electrophoretic patterns of the fractions collected during ion-exchange chromatography. Lane M, Marker; Lane 1, fraction a; Lane 2, fraction b; Lane 3, fraction c; Lane 4, fraction d; Lane 5, fraction e.

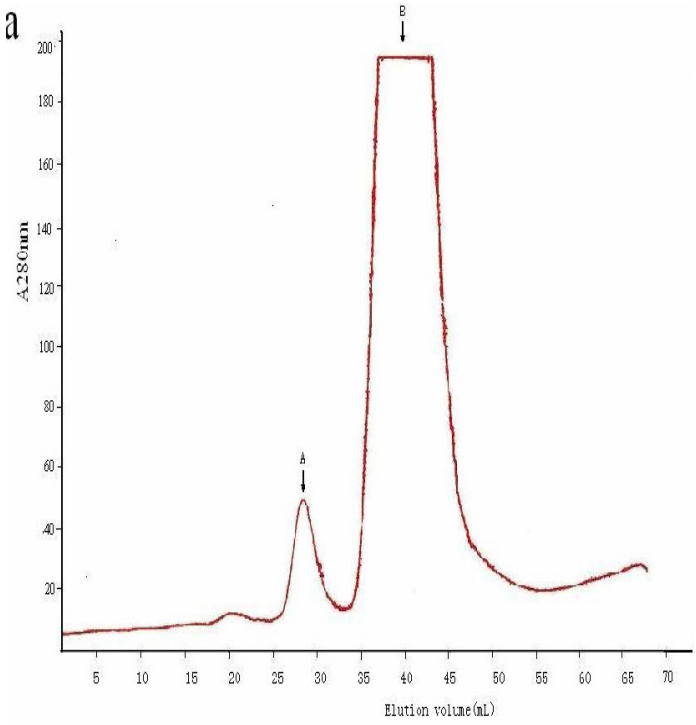

Figure 3a. Gel filtration chromatograpghy on Sephadex G-75 of fraction d issued from anion- exchange chromatography.

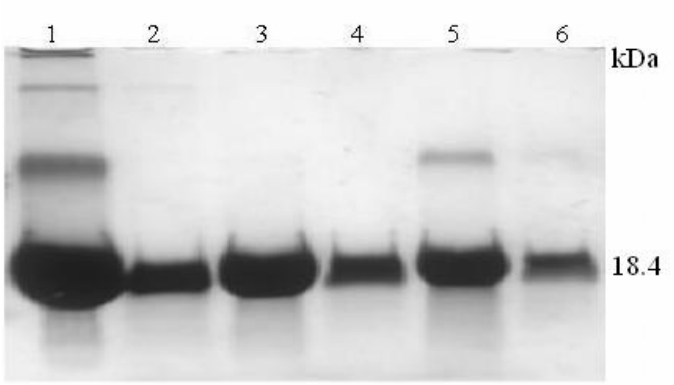

Figure 3b. SDS-PAGE pattern of standard bovine β-lg (Marker, Sigma, USA) and purified buffalo β-lg. Lane1 and 2 were concentrated fraction d (Figure 2a) issued from anion-exchange chromatography with 70 and 7 µg loading protein, respectively. Lanes 3 and 4 were concentrated fraction B (Figure 3a) obtained from gel-filtration chromatography with 50 and 5 µg loading protein, respectively. Lanes 5 and 6 were 40 and 4 µg standard bovine β-lg (Marker), respectively.

shown in Figures 3a, 3b and 3c. The purity of purified buffalo β-lg was higher than 90% determined on the basis of standard bovine β-lg by using SDS-PAGE and IEF-PAGE (Figures 3b and 3c). The total volume of fraction B was 14 ml with the recovery accounting for 91.2% of the total buffalo's β-lg in 0.6 ml of the loading protein samples (data not shown).

In gel filtration, proteins in solution are separated according to differences in their sizes as they pass through

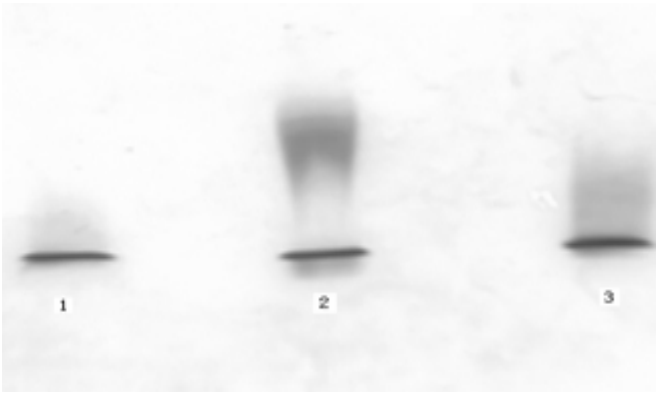

Figure 3c. IEF-PAGE pattern of buffalo β-lg(Fraction B in Figure 3a), buffalo β-lg (Fraction d in Figure 2a) and standard bovine β-lg on lanes 1, 2 and 3,respectively.The loading proteins were 60 µg.

a column packed with a chromatographic medium which is a gel. The G-type of Sephadex gels are commonly used medium in proteins separation. We chose Sephadex G-75 gel as the fraction medium because buffalo β-lg is a 18.4 kDa molecular existed in monomeric form in buffalo milk. Although DEAE-Sepharose Fast Flow anion-exchange chromatography was considerably fast and more efficient in the separation of buffalo β-lg from whey proteins, the fraction d (Figure 2a) still contained some contaminants as shown in Figures 3b and 3c. Thus, further purification was necessary. After fraction d was dialyzed in 0.02 M phosphate buffer and concentrated by Amicon ultra-15, the purification was finished on Sephadex G-75. Since the gel filtration was very gentle and the final purified buffalo β-lg was in a low saline solution, it would be very convenient to be used.

All these results demonstrated that the combination protocol of DEAE-Fast Flow anion-exchange chromatography and Sephadex G-75 gel filtration chromatography was valid for buffalo β-lg purification with high purity. This method may be an ideal tool for high buffalo β-lg recovery in laboratory or in medium size process, and relative low cost, simple procedure, it would be easy to perform.

Antigencicity

The antigenicity of purified buffalo β-lg was evaluated by indirect ELISA (Figure 4), Western-blotting (Figure 5) and inhibition ELISA (Figure 6). Figure 4 shows a cross-reactivity between buffalo and bovine β-lg by indirect ELISA. Both of rabbit sera of anti-buffalo β-lg and anti-bovine β-lg can react to each other of the two kinds of β-lg. The titer of rabbit antiserum from bovine β-lg determined by standard bovine and buffalo β-lg was the same (2^8), whereas that of rabbit antiserum from buffalo β-lg determined by bovine and buffalo β-lg were 2^9 and 2^{11} respectively. These results show that there is a strong cross-reactivity between buffalo β-lg and bovine β-lg, and suggest they share similar epitopes.

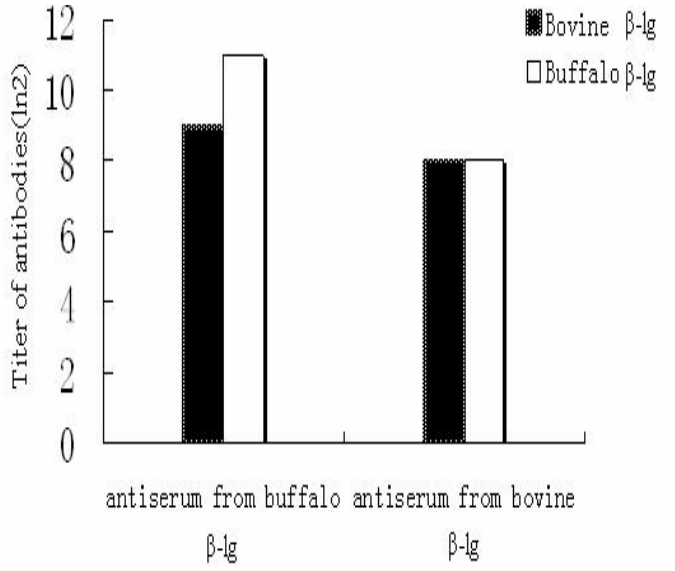

Figure 4. The cross-reactivity of bovine and buffalo β-lg by indirect ELISA.

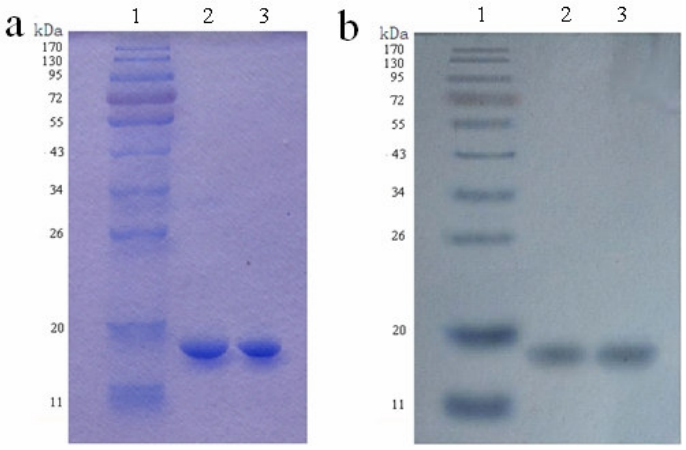

Figure 5(a). SDS-PAGE pattern of purified buffalo β-lg(2µg) and standard bovine β-lg (2 µg).(b) IgG-antigen complexes obtained in immunoblotting with anti-buffalo β-lg rabbit serum. Prestained Marker, purified buffalo β-lg and bovine β-lg on lanes 1-3, respectively.

Figure 5a shows the eletrophoretic pattern of purified buffalo β-lg and standard bovine β-lg, while the two bands in Figure 5b show the corresponding immunoblotting complexes of buffalo and bovine β-lg with anti-buffalo β-lg rabbit serum. It indicates anti-buffalo β-lg serum could recognize well both buffalo and bovine β-lg.

From Figure 6, a clear inhibition of the binding between rabbit anti-buffalo β-lg serum and buffalo β-lg (bovine β-lg) were observed when adding increasing concentration of the two kinds of β-lg. At the same antigen concentration, the percentage of inhibition was higher when the buffalo β-lg was used as inhibitor in comparison with bo-

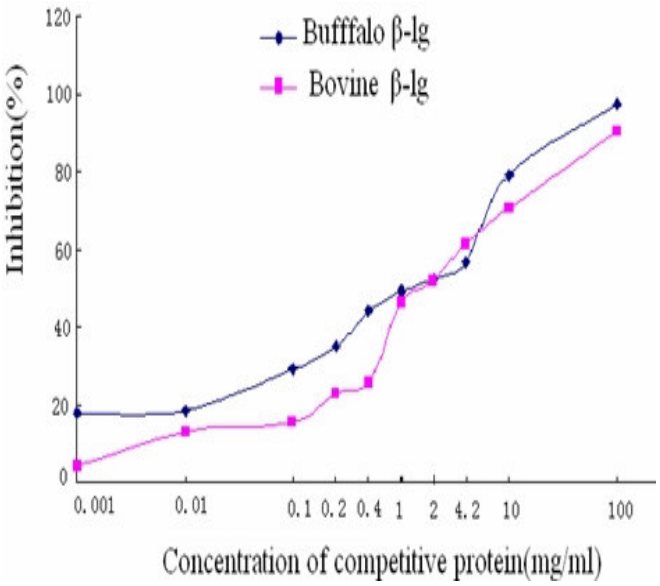

Figure 6. ELISA inhibition assays for buffalo β-lg and standard bovine β-lg.

vine β-lg except for the concentration of 4.2 mg/ml. When achieving 50% of ELISA inhibition (C_{P50} and C_{St50}), the antigen concentrations for buffalo β-lg and bovine β-lg were 1.79 ng/ml and 1.24 ng/ml, respectively. Thus, the cross-reactivity ratio between buffalo β-lg and bovine β-lg was 69.27%. It states clearly that the antigenicity of the purified buffalo β-lg was similar to that of standard bovine β-lg.

Altogether, these results proved not only that the purified buffalo β-lg is antigenic but also its antigenicity retained well during purification process.

Usually, antigenicity includes immunogenicity, the property of eliciting an immune response, and immunoreactivity, the reaction resulting from the recognition and binding of an antigen by its specific antibody or by a previously sensitized lymphocyte. The immunogenicity of the purified buffalo β-lg was evaluated by injecting it into the rabbits. The high titer of rabbit antiserum from buffalo β-lg indicated that the purified buffalo β-lg could stimulate a strong immune reaction. The similar binding capacity of anti-buffalo β-lg rabbit serum to both of the two kinds of β-lg also proved that buffalo β-lg had a good immunogenicity. The different titers of cross-reactivity between two kinds of anti β-lg rabbit serum might be due to the purity, for the buffalo β-lg was purer than that of standard bovine β-lg (Figures 3b and 3c).

In this study, all the results of indirect ELISA, Western blotting and inhibition ELISA have shown the immunoreactivity of buffalo β-lg. From the indirect ELISA experiment, we could find that buffalo β-lg reacted to both anti-buffalo β-lg rabbit serum and anti-bovine rabbit β-lg serum with a high binding capacity. It proved that purified buffalo β-lg had a good immunoreactivity. In immunoblotting, anti-buffalo β-lg recognized both buffalo and bovine β-lg and bound tightly to them with two obvious bands appeared even in a 20,000 dilution of anti-buffalo β-lg rabbit serum. It's a further evidence for indicating a good immunoreactivity of purified buffalo β-lg. After another investigation of inhibition ELISA, we found the binding to anti-buffalo β-lg rabbit serum affected by the concentration of buffalo β-lg. This result demonstrated the antigenicity of purified buffalo β-lg was preserved well once again.

Besides, we have quantified the cross-reactivity (69.7%) between buffalo and standard bovine β-lg for the first time. This data seem to show a contradition with homology of their amino acid sequences, in which only two amino acids are different between buffalo and bovine β-lg variant B. Actually, it is reasonable since the standard bovine β-lg used in this study was a mixture variant A and B of bovine β-lg . For another reason, the standard bovine β-lg was contaminated with a few other unknown proteins (Figures 3b and 3c). However, we think this bovine β-lg (Sigma) is still an alternative standard protein used in similar investigations before standard buffalo β-lg is available commercially.

Conclusion

Considering the increasing consumption of buffalo milk should be a new problem in milk allergy. It is important to isolate the allergens from buffalo milk for futher work. In the present study, we have established a method for isolating β-lg from buffalo milk by using anion-exchange chromatography combined with gel filtration chromatography. The method described here is efficient for preparing milligram of the purified buffalo β-lg with well-preserved antigenicity enough for laboratory research.

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REFERENCES

Aoki T, Iskandar S, Yoshida T, Takahashi K, Hattori M (2006). Reduced immunogenicity of β-lactoglubulin by conjugating with chitosan. Biosci. Biotechnol. Biochem. 70: 2349-2356.

Ashmad S, Gaucher I, Rousseau F, Beaucher E, Piot M, Grongnet JF, Gaucheron F (2008). Effect of acidification on physico-chemical characteristics of buffalo milk: A comparison with cow's milk. Food Chem. 106: 11-17.

Bahna SL (2002). Cow's milk allergy versus cow milk intolerance. Ann. Allergy Asthma Immunol. 89(6 Suppl 1): 56-60.

Bolognesi M, Liberatori J, Oberti R, Ungaretti L (1979). Preliminary crystallographic data on buffalo β-lactoglobulin.J. Mol. Biol. 131(2): 411-413

Carroccio A, Cavataio F, Iacono G (1999). Cross-reactivity between milk proteins of different animals.Clin. Exp. Allergy. 29(8): 1014-1016.

Cruz LC (2006). Buffalo development in the Philippines: current situation and future trends. Proceeding of the 5th Asia buffalo on so-

cial economic contribution of buffalo to rural areas, 28-36.
Docena G, Rozenfeld P , Fernándea R, Fassati CA (2002). Evaluation of the residual antigenicity and allergenicity of cow's milk substitutes by in vitro tests. Allergy, 57(2): 83-91.
Elsayed S, Hill DJ, Do TV (2004). Evaluation of the allergenicity and antigenicity of bovine-milk alphas1-casein using extensively purified synthetic peptides. Scand J. Immunol. 60(5): 486-493.
Edwin EGR, dos Reis Coimbra JS, Minim LA, Zuniga ADG, Saraiva SH, Rodrigues Minim VP (2004). Size-exclusion chromatography applied to purification of whey proteins from polymeric and saline phases of aqueous two-phase systems. Process Biochem. 39(11): 1751-1759.
Fritsché R, Adel-Patient K, Bernard H, Martin-Paschoud C, Schwarz C, Ah-Leung S, Wal JM (2005). IgE-mediated rat mast cell triggering with tryptic and synthetic peptides of bovine beta-lactoglobulin. Int. Arch. Allergy Immunol. 138(4): 291-297.
Ghosh SK, Chaudhuri S, Roy J, Sinha NK, Sen A (1971). Physico-chemical investigations on buffalo β–lactoglobulin: Studies on sedimentation, diffusion, and hydrogen ion titration. Arch. Biochem. Biophys. 144(1): 6-15.
Godovac-Zimmermann J, Krause I, Baranyi M, Fischer-Frühholz S, Juszczak J, Erhardt G, Buchberger J, Klostermeyer H (1996). Isolation and rapid sequence characterization of two novel bovine β-lactoglobulin I and J. J. Protein Chem. 15(8): 743-750.
Hahn R, Schulz PM, Schaupp C, Jungbauer A (1998). Bovine whey fractionation based on cation-exchange chromatography. J. Chromatogr. A. 795(2): 277-287.
Jiang HS, Wei YM, Jiang QY, Wei JW, Wei ZJ, Xie BK, Huang HP, Xiao H (2006). Pregnancies resulted from transfer of in vivo embryos derived from nonsurgical recovery in superovulated buffaloes. Proceeding of the 5th Asia buffalo on social economic contribution of buffalo to rural areas, pp. 53-58.
Kinekawa Y, Kitabatake N (1996). Purification of beta-lactoglobulin from whey protein concentrate by pepsin treatment. J. Dairy Sci. 79(3): 350-356.
Kontopidis G, Holt C, Sawyer L (2004). Invited review: β-lactoglubulin: binding properties, tructure and function. J. Dairy Sci. 87(4): 785-796.
Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227(5259): 680-685.
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein Measurement with the Folin phenol reagent. J. Biol. Chem. 193(1): 265-275.
Nevestani TR, Djalali M, Pezeshki M (2003). Isolation of alpha-lactalbumin, beta-lactoglobulin, and bovine serum albumin from cow's milk using gel filtration and anion-exchange chromatography including evaluation of their antigenicity. Protein Exp. Purif. 29(2): 202-208.
Pang K, Fan JB, Ren FZ, Yang YX (2006). Evolution and processing technology on China buffalo milk. Proceeding of the 5th Asia buffalo on social economic contribution of buffalo to rural areas: 299-305.
Pourpark Z, Mostafaie A, Hasan Z, Kardar GA, Mahmoudi M (2004). A laboratory method for purification of major cow's milk allergens. J. Immunol. Assay Immunochem. 25(4): 385-397 .
Restani P, Beretta B, Fiocchi A, Ballabio C, Galli CL (2002). Cross-reactivity between mammalian proteins. Ann. Allergy Asthma Immunol. 89(6 Suppl 1): 11-15.
Salaman MR, Williamson AR (1971). Isoelectric focusing of proteins in the native and denatured states. Anomalous behaviour of plasma albumin. Biochem. J. 122(1): 93-99.
Vyas HK, Izco JM, Jiménez-Flores R (2002). Scale-up of native beta-lactoglobulin affinity separation process. J. Dairy Sci. 85(1): 1639-1645.
Wal JM (2004). Bovine milk allergenicity. Ann Allergy Asthma Immunol. 93(5Suppl 3): s2 -s11.
Williams SC, Badley RA, Davis PJ, Puijk WC, Meloen RH (1998). Identification of epitopes within beta lactoglobulin recognised by polyclonal antibodies using phage display and PEPSCAN. J. Immunol. Methods. 213(1): 1-17.
Zicarelli L (2006). Buffalo development in Europe: current situation and future trends.Proceeding of the 5th Asia buffalo on social economic contribution of buffalo to rural areas, pp. 37-44.