IMMUNOCHEMICAL CHARACTERIZATION OF PURIFIED BUFFALO BETA-LACTOGLOBULIN

Ranjit Aich[1], Subhasis Batabyal[2] and Siddhartha Narayan Joardar[2,*]

ABSTRACT

Beta-lactoglobulin (β-lg) is a major whey protein found in cow milk and other ruminants, deer, bison, and buffalo. β-lg is considered as one of the main allergenic components in bovine milk and therefore the modification of β-lg is considered as a promising treatment for milk allergy. Thus the present study was conducted to assess the antigenicity of purified β-lg that can be exploited for detection of buffalo milk-protein intolerance. β-lg was purified from milk of Murrah buffalo of Haringhata Farm, West Bengal (India) by combination of gel filtration (using Sephacryl S-200) and anion-exchange chromatography (using DEAE-Sepharose). The molecular weight was derived as 18.05 kDa in 15 percent one-dimensional SDS-PAGE. Antigenicity (seroreactivity) of purified β-lg was evaluated by double immuno diffusion (DID), western blotting, dipstick ELISA and indirect ELISA using hyperimmune sera raised in rabbit against purified buffalo β-lg. In indirect ELISA, titer of rabbit serum against buffalo β-lg was observed to be in between 200-400. Therefore, the study revealed that purified buffalo β-lg is highly antigenic and a seroreactive protein and may be used for detection of buffalo milk protein-intolerance using rapid, user-friendly, cost effective sero-diagnostic technique/tool.

Keywords: Beta-lactoglobulin, buffalo, western blotting, indirect ELISA, dipstick ELISA

INTRODUCTION

There are more than 20 allergic proteins in cow milk and one of the most important allergic proteins in milk is β-lactoglobulin to which about 82% of milk allergic patients are sensitive (Aoki *et al*., 2006; Kontopidis *et al*., 2004). 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; Wal *et al*., 2004). 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 biological functions of β-lactoglobulin are still not known; it could have a role in metabolism of phosphate in the mammary gland and the transport of retinol and fatty acids in the gut (Hill *et al.*, 1997). The complete amino acid sequence of β-lg has been reported and genetic variation in amino acid sequence has been identified (Creamer *et al*., 1983).

Eleven genetic variants have been discovered: A, B, C, D, E, F, G, H, I, J and W.

[1]College of Veterinary Science and Animal Husbandry, Mhow, Madhya Pradesh, India
[2]West Bengal University of Animal and Fishery Sciences, Belgachia, Kolkata, India,
*E-mail address: joardar69@gmail.com

Variant A and B are of the highest interest since they are associated with milk production performance, its quality and processing. β-lg is considered as one of the main allergenic components in bovine milk and therefore the modification of β-lg is considered as a promising treatment for milk allergy (Hst and Samuelsson, 1988). β-lg also has good emulsifying and foaming properties, and therefore offers a good model for elucidation of the adsorption characteristics of proteins at a surface. β-lg is also a model protein for studying the denaturation mechanism. These properties are important in the milk industry and bovine milk allergy. The present study was undertaken with the objectives to assess the antigenicity (seroreactivity) of purified β-lg that can be exploited for detection of buffalo milk-protein intolerance.

MATERIALS AND METHODS

Preparation of anti-sera

Buffalo β-lactoglobulin purified (Aich *et al*., 2014) by column chromatography (using Seopacryl S-200 and subsequently DEAE-Sepharose) was thoroughly mixed with equal volume of Freund's complete adjuvant (FCA) (1:1) and a total of 1 ml was injected intramuscularly at 3 sites into the thigh muscle and subcutaneously at 3 sites in the scapular region in two different New Zealand white rabbits with an average weight of 1.5 kg of each. Four booster doses of the same antigen emulsified with Freund's incomplete adjuvant (FIA) (1:1) were given with subsequent increase in amount after 14 days interval following the first injection (Williams *et al*., 1998). One week after the last injection, blood was collected from both and subsequently each rabbit's antiserum was separated by centrifugation at 5000 rpm for 10 minutes. Two serum samples were stored at -20ºC and antibody titers were determined by indirect ELISA.

Double immunodiffusion (DID)

DID test was performed according to Ouchterlony (1953) with some modifications. In brief, agarose solution (1.5%) was prepared with normal saline solution (NSS) and molten agarose was poured on a clean and grease free glass slide to give a thickness of 1.5 mm. Wells were then punched by gel puncher. Wells were filled with 20 µl of purified β-lactoglobulin from buffalo, 20 µl of hyper-immune serum raised against purified buffalo β-lactoglobulin as neat and 1:2 dilutions. The slide was placed in a humid chamber and incubated overnight at room temperature. On the following day the slide was washed in phosphate buffered saline (PBS) pH 7.2 and dried by blotting paper and visualized after coomassie brilliant blue staining.

Western blotting

The protein sample was separated by 15% SDS-PAGE (Laemmli, 1970) and then the resultant proteins were electroblotted to nitrocellulose filter paper (NCP) (Immobilon- NC) from gel according to the method of Towbin *et al*. (1979) and Svoboda *et al*. (1985).

Detection of immuno-dominant peptides by Western blotting

After electro-transfer, NCP was kept in blocking buffer (5% skimmed milk powder in PBS, pH 7.4) for two hr at 37ºC in incubator and then washed with washing buffer (0.05% Tween-20 in PBS, pH 7.4) for four times. Then the NCP was incubated with hyperimmune serum (1:40 dilutions in blocking buffer) for two hr and then washed for

four times. After that, the dried NCP was incubated with goat anti-rabbit horse radish peroxidase conjugate (1:500 dilutions in PBS, pH 7.4) for two hr and then washed for four times. The NCP was rinsed with substrate solution [Tris-HCl, H_2O_2 and Diaminobenzidine (DAB)]. Reactive protein band appearing after few min was observed. NCP was dipped into the distilled water to stop the enzyme substrate reaction. Lastly, it was dried up and preserved.

Dipstick Enzyme Linked Immunosorbent Assay (Dipstick ELISA)

Dipstick ELISA having similar methodology and principal as dot-ELISA was performed as per the method of Mialhe *et al*. (1992). Purified buffalo β-lactoglobulin (20 µg) in coating buffer (10 µl/ stick) was dispensed on to the membrane attached to the end of dipsticks and kept for overnight at 4ºC. Negative control was prepared by coating the stick only with coating buffer. Now these were dipped into blocking solution (5% skimmed milk powder in PBS, pH 7.4) for 2 h at 37ºC. Then washing was done for four times with PBS-T (0.05% Tween-20 in PBS). After washing, dipsticks were dipped into diluted (1:50) rabbit hyperimmune serum and incubated at 37ºC for 2 h. After washing with PBS-T for four times, the dipsticks were transferred to goat anti-rabbit horseradish peroxidase conjugate solution (1:1000 in PBS, pH 7.4) and kept for 2 h at 37ºC. After washing with PBS-T for four times, the sticks were dipped into substrate solution [Tris-HCl (50 mM, pH 7.5) 200 µl, PBS 10 ml, H_2O_2 10 µl and Diaminobenzidine (DAB) 5 mg]. After 3 minutes of incubation, the dipsticks were dipped into the distilled water to stop excess reaction and after drying the intensity of the colour development was observed.

Indirect Enzyme Linked Immunosorbent Assay (Indirect ELISA)

Hudson and Hay (1989) protocol with some modification was followed for this assay. Purified β-lactoglobulin from buffalo was used as coating antigens (5.0 µg purified protein in each well) after diluting with coating buffer (pH 9.6). The plate was kept at 4°C over night. The plate was blocked with blocking buffer (5% skimmed milk in PBS, pH 7.4) 200 µl per well. The plate was kept at 37ºC for two hr. Four times washing was done with washing buffer (0.05% Tween-20 in PBS). Serial two fold dilutions of hyper-immune serum was made and dispensed on the plate (100 µl/well). Goat anti-rabbit horseradish peroxidase conjugate (Genei, India) solution (100 µl/well) was added in the washed plate. Substrate buffer [3 µl H_2O_2, 0.025 gm of O-Phenylene diamine (OPD)] (100 µl/ well) was added and kept for 20 minutes in dark at room temperature. The absorbance was taken at 492 nm in an ELISA reader (ECI, India).

Determination of titre

Inverse of the dilution of hyperimmune sera in the well at which 50% of λ_{max} exist was considered as titre of the serum.

RESULTS AND DISCUSSION

Immunoreactivity of purified buffalo β-lactoglobulin was tested by DID, western blot, indirect-ELISA. Suitability of user-friendly tool dipstick ELISA was also assessed in the present study. In DID, after staining the slide with Coomassie brilliant blue R250, specific precipitation bands were observed in between purified buffalo β-lactoglobulin with its neat hyperimmune sera (Figure 1) only. In Western

blotting, β-lactoglobulin purified from buffalo milk showed the immunobtotting complexes with anti-buffalo β-lg rabbit serum (Figure 2). It indicated that the hyperimmune serum could recognize well its homologous antigen.

Dipstick ELISA was performed to show the intensity of the colour development due to immune complex formation. It was found that coloured dot was formed in case of β-lactoglobulin coated strips (Figure 3). However, no colour was developed in case of negative control. From this study, it may be stated that qualitative assessment of the presence of anti- β-lactoglobulin antibody in the serum may be done using dip-stick ELISA that has got immense diagnostic importance. Antigenicity of β-lactoglobulin purified from buffalo whey protein was evaluated by Indirect ELISA using hyperimmune serum against the buffalo β-lactoglobulin. The sero-reactivity is expressed in O.D. values at 492 nm and shown in Figure 4. The titer of rabbit hyperimmune serum was in between 200-400. Immunochemical protein specific identification was performed by Western blotting and result revealed that recognition procedure based on a well-known antigen-antibody interaction for clearly confirming the isolated β-lactoglobulin identity (Lozano *et al*., 2008). β-lactoglobulin purified by gel filtration using Sephadex G-50 or by DEAE-C anion-exchange chromatography from 50% ammonium sulfate precipitated globulins preserved their antigenicity to the same extent as standard proteins, demonstrated by Western blotting and the ELISA inhibition assay (Neyestani *et al*., 2003). Native and denatured β-lactoglobulin from ewes and goat milk was detected by Western blotting using commercially available polyclonal antibodies (Molina *et al*., 1996).

Altogether, these results proved that the purified buffalo β-lg is antigenically similar and its antigenicity retained well during purification process that corroborate the similar findings reported by the previous workers.

In short, the present study, after assessing antigenicity of β-lactoglobulin of buffalo origin, it

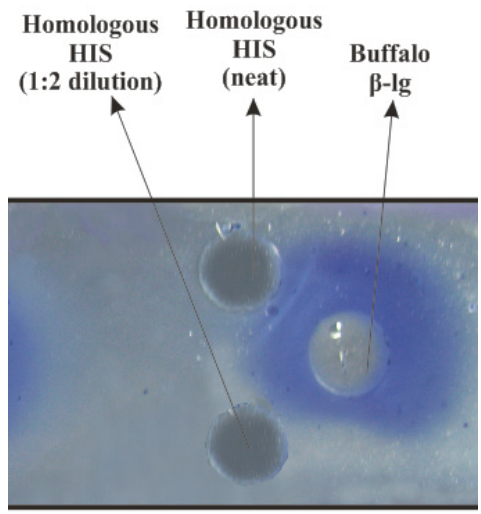


Figure 1. Double Immuno Diffusion test showing the precipitin line of purified buffalo β-lactoglobulin with its homologous antibody.

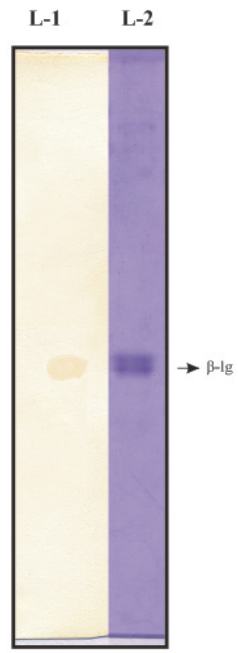


Figure 2. Western Blot analysis of purified buffalo β-lactoglobulin.
L 1: Immunoreactive buffalo β-lactoglobulin
L 2: Purified buffalo β-lactoglobulin in 15% SDS-PAGE

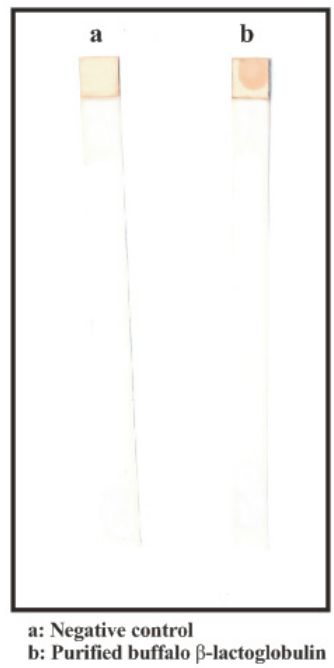
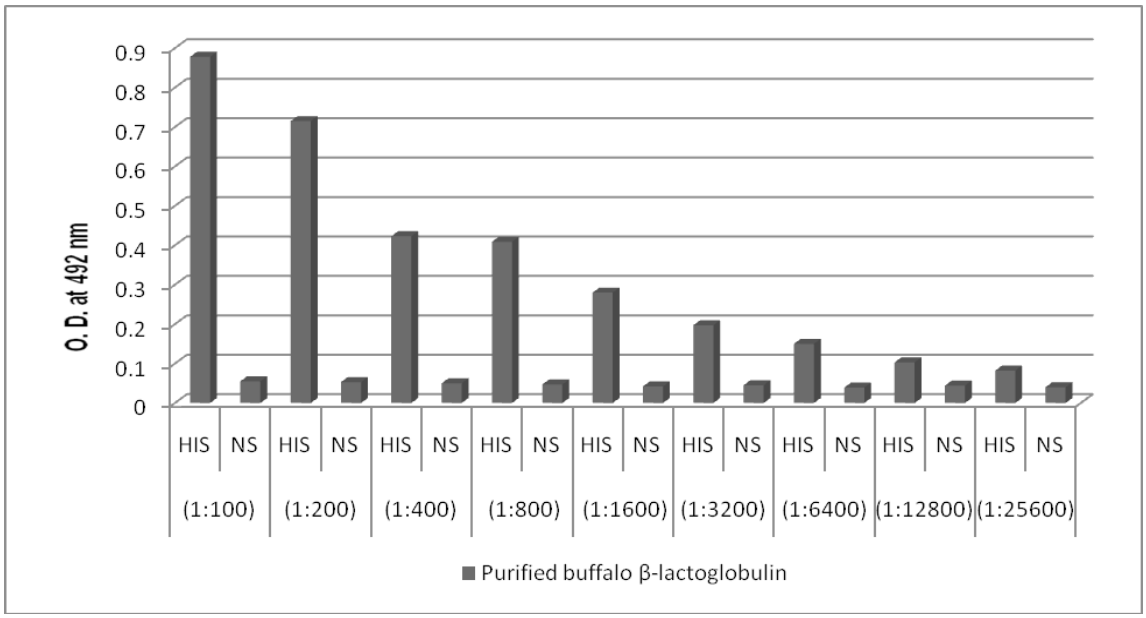


Figure 3. Assessment of antigenicity of purified buffalo β-lactoglobulin by dip-stick ELISA.



HIS = Hyperimmune sera NS = Normal sera

Figure 4. Assessment of seroreactivity of purified buffalo β-lactoglobulin by indirect ELISA.

has been observed that this protein may be used for detection of milk protein intolerance (CMPI) using rapid, user-friendly, cost effective sero-diagnostic technique like dip-stick ELISA. However, that needs validation using large number of samples.

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