ANTIGENICITY OF CASEINOPHOSPHOPEPTIDES PREPARED FROM BUFFALO CASEIN

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ABSTRACT

In the present study, the antigenicity of caseinophosphopeptides (CPPs) enriched from the hydrolysis of buffalo casein with trypsin and neutrase was studied using a mouse model system. Relative levels of specific IgE and IgG made in respone to intraperitoneal sensitization to CPPs enriched samples were determined using indirect ELISA. It was found that the CPPs enriched samples prepared from both the enzymes, i.e. trypsin and neutrase, were less antigenic as compared to intact protein, i.e. sodium caseinate. Among the CPPs enriched samples, the antigencity of trypsin samples was less than that of neutrase samples. The results suggested that these CPPs enriched samples can be used as a food ingredient for food designed for hypoallergenic persons.

Keywords: buffaloes, *Bubalus bubalis*, caseinophosphopeptides, CPPs, antigenicity

INTRODUCTION

Food allergy is defined as an adverse clinical reaction due to an immune–mediated hypersensitivity response resulting from the ingestion of a food. Cow milk protein allergy represents the most frequent food allergy in infancy ranging between 2 and 7.5% of the infant population. Clinical challenges frequently reveal that patients who are allergic to milk react to the multiple protein fractions found in bovine milk (Savilahti and Kuitunen, 1992). Otani (1992) stated that minimum number of antigenic determinant sites for $\alpha s1$-CN, β-CN and κ-CN were 6, 6 and 4 respectively. The milk proteins mainly responsible for the allergy are α- and β-casein, followed by β-lactoglobulin and α-lactoalbumin to a lesser extent (Docena *et al*., 1996; Bernard *et al*., 1998; Jarvinen *et al*., 2001). Milk from other species, such as goat milk and sheep milk, has shown a high degree of cross reactivity (Spuergin *et al*., 1997; Bellioni-Businco *et al*., 1999). Milk from buffalo is a common drinking milk in many developing countries and is the main component of buffalo mozzarella eaten throughout the world. *In vitro* studies demonstrate a similar proteomic make-up and suggest antibody cross reactivity between cow milk and water buffalo milk proteins (Restani *et al*., 2002; D'Auria *et al*., 2005).Similarly, one human study showed a high degree of skin test positivity to water buffalo milk in patients with cow milk allergy (Katz *et al*., 2008). However, Sheehan and Phipatanakul (2004) described the first report of a child with cow milk allergy who is able to tolerate water buffalo milk.

Further the protein hydrolysates are widely used as milk substitutes for children with bovine

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milk allergy. Enzymatic hydrolysis is considered a desirable treatment for reducing the antigencity of milk caseins. The allergencity of casein hydrolysates that have been prepared using a single or a mixture of proteases that have specific cleavage sites have been reported (Boza *et al*., 1994; Nakamura *et al.,* 1993; Mahmoud *et al*., 1993, Otani *et al*., 1990 and Takase *et al*., 1979). Cordle (1994) reported that the allergencity declines with decreasing molecular weight, especially between 10000 and 2500 daltons. A number of biological active peptides have been identified in the casein hydrolysates. Among these the caseinophosphopeptides have been reported as mineral binding peptides which have a potential use as a food ingredient. Selective precipitation or enrichment of the phosphopeptides results in removal of potential antigens. Therefore, it is worth examining whether casein phosphopeptides are hypoallergenic. The main aim of the study was to compare the allergenicity of caseinophosphopeptides (CPPs) enriched products prepared from buffalo milk casein using membrane processing and selective precipitation method with two enzymes. The allergic potential of the proteins is generally evaluated as a function of serological responses i.e. IgG and IgE antibody production. The role of IgE in type I immediate a hypersensitivity allergic reaction is well understood in the scientific literature. It is also strongly agreed in the scientific literature that allergic reactions may occur independent of antigenic specific IgE. High affinity receptors for IgG, on human mast cells and basophils are activated in immediate hypersensitivity reactions. IgG-mediated immediate hypersensitivity, also known as IgG-mediated anaphylaxis, is not a new concept in allergy research. So in the present study, the allergenic activity as a function of the ability of proteins to provoke IgG and IgE antibody response in a mouse

model was studied by peritoneally injecting mice with sodium caseinate, phosphopeptides enriched ingredients and ova albumin with adjuvant (AlO_3). A sandwich ELISA was used to determine levels of IgG and IgE in the blood.

MATERIALS AND METHODS

Preparation of samples

Sodium caseinate prepared from buffalo milk was hydrolysed by two commercial enzymes, i.e. trypsin and neutrase, under optimized conditions (Saini, 2012). After hydrolysis the pH of the solution was adjusted to 4.6 and supernatant was collected after centrifugation. Then the pH of supernatant was adjusted to neutral and the solution was incubated at room temperature after adding calcium chloride 1.1%. After incubation, CPPs was enriched from the solution by using two methods: ethanol precipitation and ultrafiltration. A total of three samples were prepared; samples prepared with trypsin hydrolysates were named as trypsin CPPs i.e. TC (ethanol precipitation), trypsin retentate i.e. TU (ultrafiltration) and the sample from neutrase hydrolysed was named as neutrase retentate i.e. NU (ultrafiltration).

Intraperitoneal immunization for allergic response

Swiss albino male mice weighing 25-30 g each were taken from the Small Animal House, NDRI, Karnal, India. They were maintained on standard pellet diet and had access to tap water *ad libitum*. They were housed in groups of eight mice per cage and kept in polypropylene cages with stainless steel wire-bar lids, using a rice husk as a bedding material, under a 12 h light/dark cycle at room temperature of 22-24°C. Mice were then

acclimatized to the environment for one-week prior to the experiment. Animals were randomly assigned to one of six groups of eight mice each as given below:

Group 1: Control group injected with adjuvant in phosphate buffer saline (PBS) (pH 7.4)

Group 2: Group injected intraperitonially with ovalbumin + adjuvant in PBS (AlO$_3$)

Group 3: Group injected intraperitonially with TU + adjuvant in PBS (AlO$_3$)

Group 4: Group injected intraperitonially with TC+ adjuvant in PBS (AlO$_3$)

Group 5: Group injected intraperitonially with NU + adjuvant in PBS (AlO$_3$)

Group 6: Group injected intraperitonially with Na caseinate + adjuvant in PBS (AlO$_3$)

The mouse groups were immunized on the first day and a booster dose was given on the 15th day of the experiment. Seven days after the final injection, blood samples were collected aseptically by exsanguinations (cardiac puncture). Serum samples were prepared, diluted with PBS and used for antigen-specific IgE and IgG assay.

Immunoglobulin assay

The total IgE and IgG in serum was determined using an ELISA kit (Koma Biotech, Korea). The operating procedures were strictly followed as provided by the manufacturer. The principle of the ELISA kit was to employ the quantitative sandwich enzyme immunoassay technique. Polystyrene ELISA plates were coated overnight at 4°C with 100 µL of coating antibody (provided with kit) diluted in 50 mM carbonate-bicarbonate buffer, pH 9.6. After washing at 25°C with PBS containing 0.05% Tween 20, pH 7.4; the plates were post-coated with 200 µL bovine serum albumin (1%) in PBS, pH 7.4 for 1 h at 25°C. The plates were washed again with PBS containing 0.05% Tween 20, pH 7.4. Serum (100 µL) of optimal dilution (100-fold dilution for IgE, 2000-fold dilution for IgG) was added to each well, and plates were incubated for 1hr at 25°C. After washing the plates as above, 100 µL of optimally diluted detection antibody specific for IgE and IgG (provided with kit) were added to each well and the plates were incubated for 1 h at 25°C. After incubation, the wells were aspirated to remove liquid and the plate was washed as above. Finally, 100 µL of colour development solution was added to each well and incubated at room temperature for a proper colour development. (5-10 minutes). After sufficient colour development, 100 µL stop solution (2M H$_2$SO$_4$) was added to each well and the absorbance was measured at 450 nm using an ELISA plate recorder. Finally, 100 µL of 3, 3', 5, 5' tetramethyldiamine benzidine was added to each well and incubated at 25°C for a proper colour development. (5-20 minutes). After sufficient colour development, the reaction was stopped by addition of 100 µL of 2M H$_2$SO$_4$ to each well and the absorbance was measured at 450 nm using an ELISA plate recorder (TECAM InfiniteF200 Pro,).

RESULTS AND DISCUSSION

At 3 weeks postinjection, no significant difference (p≤0.05) was found in the level of serum IgE among the mouse groups injected with CPPs samples (TU, TC and NU) as compared to the control. Figure 1 shows the level of serum IgE towards peritoneally injected CPPs samples and ovalbumin. Whereas it is observed that serum IgE level in the group injected with Na caseinate (280.7±19.6 ng/ml) was significantly higher as compared to the control but significantly lower

than that of the ova+ve group (570.4±24.7 ng/ml). Among the CPPs samples the highest IgE level was observed in the NU sample (186.3±16.6 ng/ml) as compare to TU (170.6±12.9 ng/ml) and TC (178.5±11.8 ng/ml).

The level of serum IgG towards peritoneally injected CPPs samples and ovalbumin is given in Figure 2. At 3 weeks postinjection, IgG level in blood serum of mouse groups injected with CPPs samples did not differ significantly as compared to the control group (245.5±14.6 μg/ml). The serum IgG level of the group injected with Na caseinate (300.8±18.7 μg/ml) differed significantly from the control group and the ova+ve group (568.3±22.8 μg/ml). The highest value for IgG level was observed in the NU sample (266.5±15.6 μg/ml) as compared TU (255.6±10.8 μg/ml) and TC (247.2±12.7 μg/ml).

In this study, the levels of IgG in blood samples of mice was higher as compared to that of IgE induced by all the protein and peptides samples (Figures 1 and 2). There may be the possible explanation as reported by Wood and Wreghitt (1990) and Park and Allen (2000). The serum IgG may compete for antigen binding sites in the indirect ELISA assays or any IgE that was initially increased in the serum was suppressed by IgG, after circulating IgG secretion increased.

All the three caseinophosphopeptides enriched products i.e. TU, TC and NU showed significantly lower values for IgE and IgG as compared to that of Na caseinate and ovaalbumin. The levels of IgE and IgG in all these three products remained similar to that of the control. This clearly indicates that the fragments of casein (CPPs) are significantly less or non immunogenic than sodium caseinates. The reduction in antigenicity of the caseinophosphopeptides may be due to removal of antigenic peptides from these products by ultrafiltration (TU, NU) and selective precipitation (TC). Similar findings were reported by Park and Allen (2000), that the tryptic α_s-casein phosphopeptides induced significantly less IgG than the intact proteins or whole tryptic hydrolysates. They concluded that protein hydrolysis and subsequent purification of phosphopeptides using ultrafiltration might reduce the immune response.

Otani et al. (2000) observed that mice fed a caseinphosphopeptides added diet did not show enhanced IgG antibody level. Heddleson et al. (1997) also reported that specific antibody levels for the IgE in rats peritoneally injected with casein phosphopeptides rarely exceeded those of unimmunized rats, indicating that the allergenicity of casein phosphopeptides was inert when compared to native β-casein and skim milk proteins.

The present results suggest that the caseinophosphopeptides enriched products prepared from buffalo milk can be used in infant formulas and other functional foods to enhance mineral absorption and with reduced immunogenicity.

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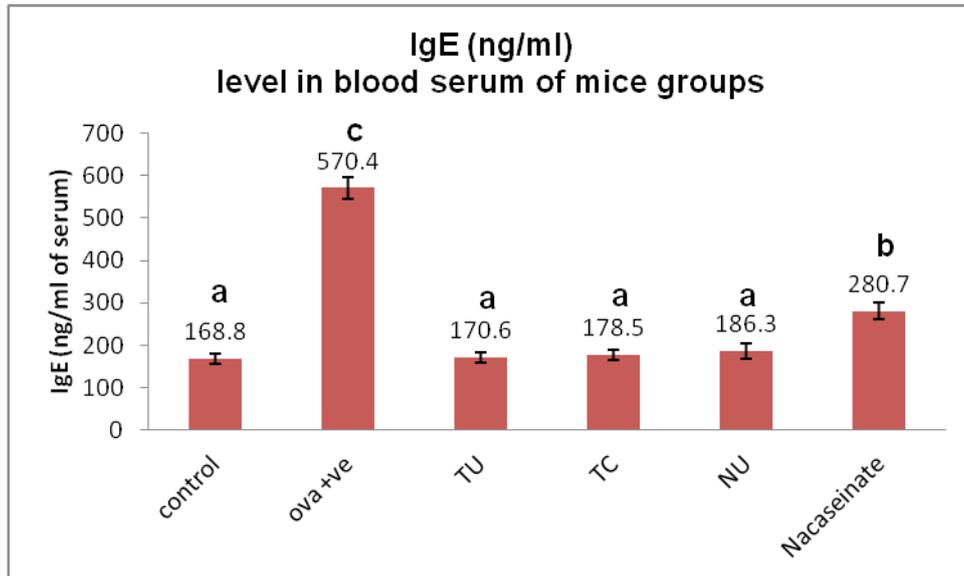
Figure 1. Graphs showing the IgE antibody level in blood serum of the control mouse group and groups injected with ovaalbumin, Na caseinate and different CPPs preparations (TU, TC and NU) (The values expressed as means ± SEM for eight mice per group. The values with different small letters superscripts differ significantly at 5% level of significance (P≤ 0.05), CD= 18.7).
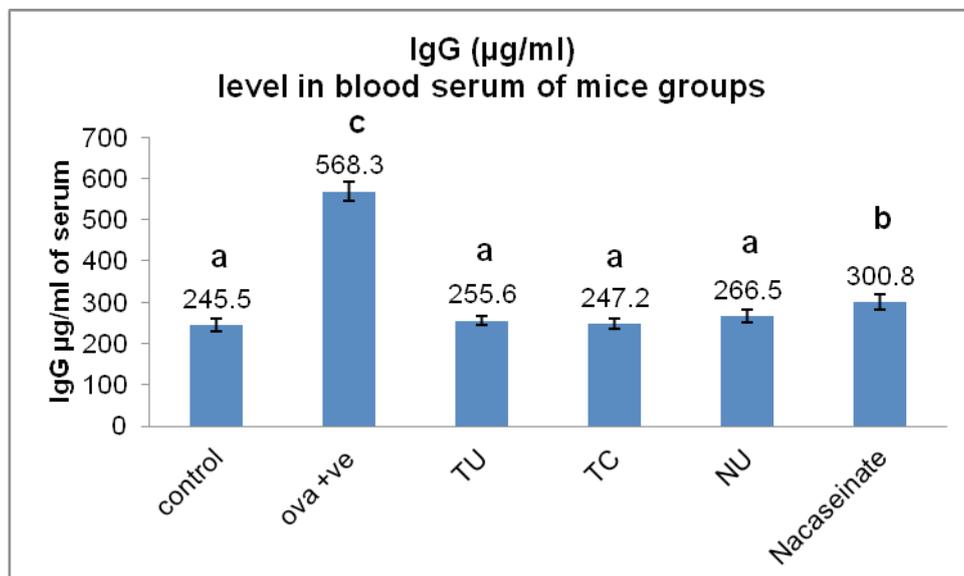

Figure 2. Graphs showing the IgE antibody level in blood serum of the control mouse group and groups injected with ovalbumin, Na caseinate and different CPPs preparations (TU, TC and NU) (The values expressed as means ± SEM for eight mice per group. The values with different small letters superscripts differ significantly at 5% level of significance (P≤ 0.05), CD= 23.34).

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