Effects of enzymatic treatments on the hydrolysis and antigenicity reduction of natural cow milk

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Abstract

In this paper, the effects of different food-grade enzymes on the antigenicity of casein (CN), β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) in natural cow milk were studied. The degree of hydrolysis (DH), SDS-PAGE and molecular mas (MW) distribution of cow milk (CM) hydrolysates was assessed. Additionally, the residual antigenicity of CM hydrolysates was evaluated by using ELISA and western blotting with anti-CN, anti-β-LG and anti-α-LA rabbit polyclonal antibody. The results showed that Alcalase and Protamex hydrolysis could efficiently reduce the antigenicity of CN, β-LG, α-LA, showed a higher DH and the loss of density of CM proteins, as indicated by SDS-PAGE. The increasing of the low MW (<3 kDa) in CM hydrolysates was also presented. It was also found that Protamex, Alcalase could be more efficiently hydrolyzed major allergenic of CM than other enzymes for the development of

hypoallergenic cow milk. Our research will lay a theoretical foundation for the study of hypoallergenic cow milk.

Keywords: Cow milk; Enzymatic hydrolysis; Allergic protein; Antigenicity

1. Introduction

The incidence of food-induced allergies and related symptoms have been increasing around the world[1]. Epidemiological studies have found that cow milk allergy **(**CMA**)** is one of the eight most common food allergies[2]; the incidence of CMA is reportedly between 2%-7.5% in different countries[3]. CM is also one of the most common trigger foods causing food allergy within the first years of life, and 1%-2% of newborns exhibit allergenic responses to CM[4]. The immunological reaction can lead to respiratory, dermatological and gastrointestinal problems, including urticaria, atopic dermatitis, allergic rhinitis and angioedem[5]. These symptoms range from mild to anaphylactic reactions, which seriously impact the lives of allergic individuals and the growth of infants and children[6].

CM is one of the most important dietary components for humans in some parts of the world, and is considered a rich source of proteins, lipids, lactose and minerals[7,8]. Because of its high nutritional value, diverse mineral composition, proper calcium to phosphorus ratio and extensive sources, it is regarded as the best choice for infant formula and dairy products. However, allergy caused by CM is still a major concern in the dairy industry[9].

Previous research has revealed approximately 30 potentially allergenic proteins in CM that can induce immune responses in infants and young children[10]. Studies on large populations of allergic patients have suggested that the most abundant proteins in CM, CN (78%), β-lactoglobulin (β-LG, 9.7%) and α-lactalbumin (α-LA, 3.6%), are all major allergens[11]. Even proteins present in low amounts, such as bovine serum (BSA), lactoferrin (LF) and immunoglobulins (Igs) have shown the capacity to induce milk allergies[12]. Several studies have estimated the incidence of CM protein allergy as approximately 2%-6%[13]. Therefore, reducing the antigenicity of CM is considered to be a major challenge by milk manufacturers.

Researchers have developed a variety of methods to reduce sensitization to CN, β-LG and α-LA, or its certain components by optimizing and improving the processing conditions[14], including heat treatment, glycation and enzymatic hydrolysis, etc[15]. Among these, heat treatment is the most commonly used method to reduce pathogens, but it remains a controversial method for reducing the risk of allergies[16]. Glycation is one of the most frequent chemical modifications during industrial production and processing of milk, but due to the complexity of the product, its safety still needs to be evaluated [17]. However, enzymatic hydrolysis is the most effective method to modify proteins, it uses some digestive enzymes to alter the immunoreactivity of allergenic proteins[18]. During enzymatic hydrolysis, some peptide or disulfide bonds are damaged, which lead to the collapse of conformational or linear epitope. Thus, enzymatic hydrolysis could eliminate or reduce the allergenicity and antigenicity of milk proteins. Furthermore, it can yield a variety of new peptides, preserve the nutritional value of milk proteins, and also offer many physiological benefits for infants and young children[19].

Enzymatic hydrolysis is a well-known, safe and effective processing technologies to reduce the allergenicity of milk proteins. Recently, it has been widely used to produce a higher quality and hypoallergenic protein hydrolysates. Several studies have shown that whey (β-LG, α-LA) antigenicity could be decreased by hydrolysis with Alcalase[20]. Additionally, combined microwave and enzymatic hydrolysis of a whey protein concentrate (WPC) hydrolysate by Pronase, Chymotrypsin and other five different food-grade enzymes demonstrated effective proteolysis of whey proteins by either of the enzymes in combination with these treatments[21]. Some authors studied the antigenicity of whey proteins hydrolysates obtained with the combination of enzymatic hydrolysis and high pressure treatment, suggested that this method could decrease the immunoreactivity of whey protein hydrolysates[22]. However, previous studies have focused on the enzymatic hydrolysis of individual protein or the antigenicity of individual allergic protein in CM. Thus, in this work, natural cow milk was first subjected to enzymatic hydrolysis with different food-grade enzymes, the effects of different enzymes on the major allergenic proteins in natural cow milk were assessed. Briefly, DH, SDS-PAGE, MW distribution and residual antigenicity of CM hydrolysates were evaluated. The aim of this work was to lay a solid theoretical foundation for the production of hypoallergenic dairy products.

2. Material and methods

2.1 Materials

CN (purity >85%), α-LA (purity >85%), β-LG (purity >85%) alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies, Freund's complete and incomplete adjuvant, OPA, DTT, OPD, Papain and Pepsin were purchased from Sigma Chemical Company (St. Louis, MO, USA), Alcalase, Neutrase, Protamex and Flavourzyme were purchased from Novozymes (Bagsvaerd, Denmark). Three types of rabbit serum including polyclonal antibodies corresponding to CN, α-LA, β-LG were prepared in Shenyang Agricultural University.

2.2 Sample collection

Fresh CM was collected at the local farm (Shenyang, China), from 60 healthy cows (1-6 years of age) fed on grass. The main composition of CM was determined, including protein 3.05 g/100mL, fat 3.54 g/100mL, ash 1.12 g/100mL, moisture 87.80 g/100mL, lactose 4.98 g/100mL, dry matter 12.35 g/100mL, etc. Fat was removed from milk by high speed centrifugation at 15,000 rpm for 30 min at 4 ^{o}C. Skim milk was placed into bottles, transported to the laboratory (Shenyang Agricultural University) and stored at 4 ^{o}C.

2.3 Hydrolysis experiments

2.3.1 Cow milk hydrolysis

The enzyme solutions were prepared by dissolving the enzymes with distilled water (100 mg/mL) individually at room temperature, the CM and enzyme solutions were preheated separately with stirring (20 min) at suitable temperature (Alcalase 55±5 ^{o}C, Neutrase 50±5 ^{o}C, Flavourzyme 50±5 ^{o}C, Protamex 50±5 ^{o}C, Papain 20±5 ^{o}C and Pepsin 30±5 ^{o}C). Then, the enzyme solutions were added to the CM, the addition of enzyme according to enzyme activity-to-substrate ratio ranged from 2000 U/g to 10,000 U/g. The mixture was incubated for 120 min, and the enzymatic hydrolysis reaction was stopped by heating at 90 ^{o}C for 10 min, followed by immediate cooling. Later, the hydrolysates were centrifuged at 5,000 rpm for 10 min at 4 ^{o}C. Finally, hydrolysates were stored at -80 ^{o}C for further study. Untreated samples were used as control.

2.4 Determination of the degree of hydrolysis (DH)

The DH of the hydrolysates were evaluated using the ortho-phthalaldehyde (OPA) method, as previously described by Church and colleagues (1983)[23], with some modifications. The method quantified the amount of hydrolyzed peptide bonds using OPA. The OPA solutions were prepared by dissolving Na-dodecyl-sulfate (SDS), Na-tetraborate decahydrate, 97% OPA and 99% dithiothreitol (DTT). A solution of serine was used as a standard. 400 µL OPA reagent was added to 3 mL hydrolysates, swirled by inversion, incubated for 2 min in the dark, and the absorbance was measured at 340 nm. Each hydrolysate was analyzed in triplicate.

2.5 Determination of the molecular weight (MW) distribution

The MW distribution of the CM hydrolysates were assessed by gel permeation chromatography (GPC) using an Agilent PL aquagel-OH 10×300 mm column (LC1260, Agilent, USA) with UV detection at 214 nm under the following conditions: 100 μL injection volume, 30 min analysis time, 30°C column temperature, 0.1 mol/L sodium nitrate and 500 mg/L sodium azide aqueous solution mobile phase. The column was calibrated by using six types of protein as standards: BSA (MW 66.3 kDa), egg ovalbumin (MW 44.5 kDa), soybean trypsin inhibitor (MW 21.5kDa), cytochrome C (MW 12.3 kDa), pancreas aprotinin (MW 6.5kDa), Vitamin B_{12} (MW 1.3 kDa), which were purchased from Sigma Chemical Company (St. Louis, MO, USA).

2.6 SDS-PAGE and western blotting

SDS-PAGE was used to evaluate the hydrolysis of CN and whey (β-LG, α-LA) in CM hydrolysates, depending on the method described by Laemmli (1970)[24]. The separating, stacking gels were prepared by using 15%, 3% of acrylamide concentration, respectively. Prior to electrophoresis, the CM hydrolysates plus loading buffer were heated in the boiling water for 5 min. The content of protein transferred to every well was about 15 μg, the total protein in gels was stained with Coomassie Brilliant Blue G-250.

For western blotting, these separated proteins by using SDS-PAGE were submitted to 15% SDS-PAGE and then were transferred onto 0.45 μm nitrocellulose membrane (Bio-Rad, USA)

using miniVE blotter (Bio-Rad, USA) at 80 V for 120 min. Later, the membrane was incubated with 10 mL blocking buffer (1% BSA (w/v) in PBS (phosphate-buffered saline, pH 8.0) with 0.1% Tween $_{20}$ in PBS). Then, it was submitted to incubated with polyclonal antibodies (1:1000) for 1 h at 37^{o}C, the membrane was washed and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (Sigma, USA), diluted 1:5000 in blocking buffer for 1 h at room temperature. Image analysis of membranes was performed by gel scanner (Pharmacia Biotech, Sweden), densitometry of the protein bands using Gel-pro Analyzer software.

2.7 Determination of IgG-binding ability

The IgG-binding of CM hydrolysate were quantitatively analyzed by indirect competitive ELISA, depending to the method by Huang *et al.* (2019)[25], and some modifications were made. Microtiter plates (96-well) were coated with CN (or α-LA, or β-LG) diluted in 30 ug/mL (or 5 ug/mL for α-LA or 0.5 ug/mL for β-LG) of PBS and incubated overnight at 4^{o}C. The next day, microtiter plates were washed 3 times with 300 □L PBS including 0.05% Tween $_{20}$ (PBST). Then, the 0.5% gelatin in PBS were blocked for residual-free binding sites and maintained for 1 h at 37^{o}C;. Subsequently, the blocked solutions were removed, the wells were futher washed with PBST. The anti-proteins that reacted with the plate-bound antigens were incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG antibodies (100 μL,1:5000 in PBS) for 1 h at 37^{o}C. The wells were washed again, followed by the addition of 100 μL of O-phenylenediamine dihydrochloride (OPD) and incubated for 15 min at 37^{o}C in the darkness. After 15 min, the reaction was stopped by adding 50 μL of 2 mol/L H_2SO_4. The absorbance was measured at 490

nm by using an Eon microplate spectrophotometer (Biotek Instruments Inc., Winooski, VT, USA). The IgG reactivity inhibition (%) was calculated based on：

$$\text{IgG reactivity inhibition (\%)} = (1-\frac{B}{B_0})*100$$

Where B is the absorbance measured in the presence of CM hydrolysate, B_0 is the absorbance measured in the absence of CM.

2.8 Statistical analysis

These results are expressed as mean ± SD of three independent assays. Analysis of variance (ANOVA) tests for statistically significant effects of these treatments and between assay samples were analyzed by using SPSS Statistics for Windows software version 17.0 (SPSS, Inc., Chicago, IL, USA), following a one-way linear ANOVA model. Differences were considered significant at the $P < 0.05$.

3. Results and Discussion

3.1 The degree of hydrolysis

The DH of CM hydrolysates obtained with different enzymes was quantified using the OPA method, as shown in Fig .1. The DH of CM hydrolysates were observed ranging from 0.16% to 27.80%. This may be because different enzyme systems or activities are showed in the enzymatic

hydrolysis. It was obvious that the DH of CM hydrolysates obtained using Pepsin was 0.27%. However, it can be seen that CM subjected to Flavourzyme hydrolysis and exhibited a higher DH, which ranging from 18.06% to 27.80%. For hydrolytic efficiency, CM treated with Flavourzyme, Protamex and Alcalase hydrolysis was found to be more effective than others. In addition, Fig .1 suggested that with the increasing of enzyme activity-to-substrate ratio, the DH of CM hydrolyzed by Flavourzyme, Protamex, Alcalase increased gradually, and when enzyme activity-to-substrate ratio reached at 10,000 U/g, the DH all reached maximum hydrolysis (27.80%, 8.98%, 10.44%). It should be noted that Flavourzyme, Protamex and Alcalase played an important role in improving the DH of CM, it could be considered as being superior to other enzymes.

At present, enzymatic hydrolysis is the most efficient method to decrease allergenicity and antigenicity of CM proteins. The procedure of enzymatic hydrolysis could disrupt conformation or lineal epitopes, and prevent IgE-mediated allergic reactions. The enzymatic hydrolysis of milk proteins is a vital issue in the development of hypoallergenic milk for allergic infants and children children[26]. Previous studies have reported that the degree of milk proteins hydrolysis was influenced by enzyme specificity, types of enzymes, enzymatic hydrolysis conditions (pH, E/S, hydrolysis time and temperature) [27]. Ena *et al*. demonstrated that WPC hydrolysate obtained with Alcalase was characterised by a hydrolysis degree (DH) ranging from 14.5 % to 18%[28]. Spellman *et al*. also found a similar hydrolysis degree of WPC, *i. e*. 14% by using Alcalase 2.4 L[29].

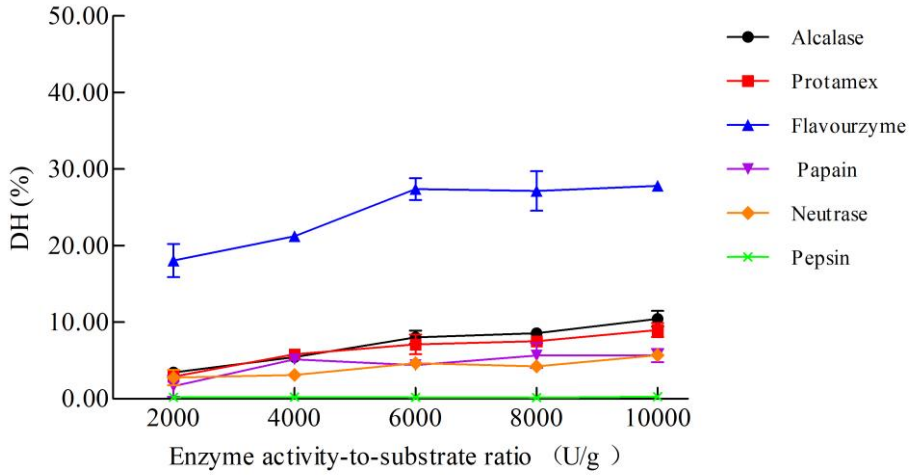


Fig. 1. The degree of hydrolysis (DH) of cow milk with different enzyme activity-to- substrate ratio (U/g). Each value represents the mean of three independent experiments ± SD.

3.2 SDS-PAGE

The antigenic proteins of CM are mainly made up of CN, β-LG, α-LA with MW of 19.0-25.2 kDa, 18.3 kDa, 14.2 kDa, respectively. The protein patterns of CM and hydrolysates were presented in Fig. 2. It indicated that after enzymatic hydrolysis, the electrophoregram of CM proteins were changed significantly. In comparison with CM, the amount of larger MW protein bands reduced, and lower MW proteins bands increased distinctly, the density of protein bands was also altered. As seen in Fig. 2, Alcalase, Protamex and Flavourzyme showed strong hydrolysis abilities for those major allergenic proteins CN, whey (β-LG, α-LA). Among these, Alcalase, Flavourzyme extensively hydrolyzed CN, whey (β-LG, α-LA), even at the lowest concentration assessed (enzyme activity-to-substrate ratio 2000 U/g), but with the increase of enzyme activity-to-substrate ratio, there was no significant change in protein bands. In addition, we observed concentration-dependent hydrolysis about Protamex, the density of protein bands was

reduced gradually with increasing enzyme activity-to-substrate ratio during enzymatic hydrolysis process. However, these results showed that Pepsin, Papain and Neutrase were unable to completely hydrolyze CN, whey (β-LG, α-LA). Therefore, among all the different enzymes used for this paper, Alcalase, Protamex, Flavourzyme were superior to other enzymes in degrading antigenic proteins of cow milk.

Enzymatic hydrolysis can break down the peptide bonds of milk protein, convert the whole protein into smaller peptide fragments. Due to differences in enzymes species, its degradation ability for proteins may be different. Smyth et al., hydrolyzed a WPC preparation applying Alcalase 0.6 L, after 30 min of enzymatic hydrolysis they observed that the presence of protein fraction of MW below 30 kDa[30]. Ena et al., studied conformation changes of protein in WPC preparation during hydrolysis with Pepsin and Corolase PP, after 30 min of hydrolysis, there were observed small fractions containing proteins with MW of over 16 kDa, suggesting different proteolytic enzyme specificities [28].

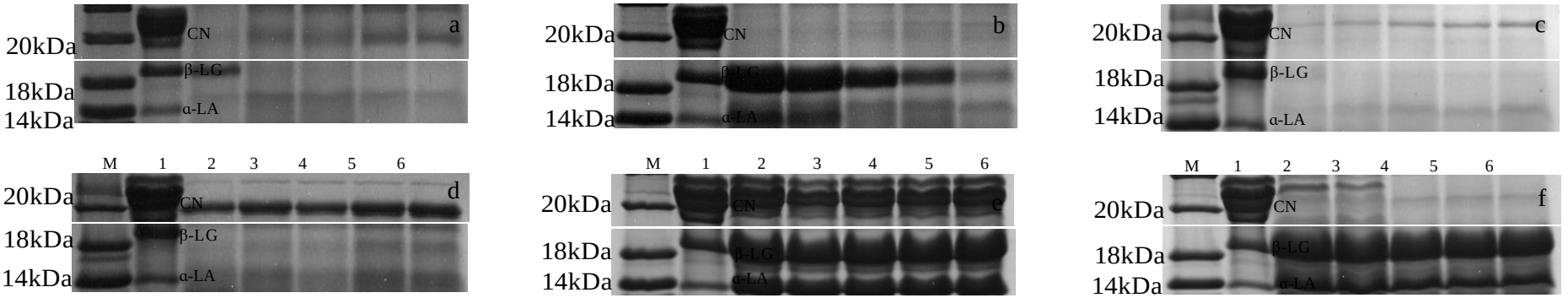


Fig. 2. SDS-PAGE analysis. a-f represent Flavourzyme, Protamex, Alcalase, Papain, Pepsin, Neutrase, respectively. M: marker; lane 1: cow milk; lane 2: 2000 :1; lane 3: 4000 :1; lane 4: 6000 :1; lane 5: 8000 :1; lane 6: 10,000 :1.

3.3 IgG reactivity reduction analysis

In order to identify the IgG reactivity reduction of samples obtained by these different enzymes, rabbit polyclonal antibody and indirect competitive ELISA were analyzed. As shown in Fig. 3, the IgG reactivity reduction of CM hydrolysates ranging from 4.02% to 81.27%. It can be observed that the IgG reactivity reduction of CM hydrolysates obtained with Pepsin was lowest (4.02%-12.02%). Regarding the Protamex, it was suggested that with the increasing of enzyme activity-to-substrate ratio, the IgG reactivity reduction rising gradually, until enzyme activity-to-substrate ratio reached at 8000 U/g, the IgG reactivity reduction reached maximum (72.25%). In addition, it could be seen that CM subjected to Flavourzyme, Papain, Alcalase hydrolysis, and exhibited a higher IgG reactivity reduction (69.00%-81.27%), which is in consistent with the results of Fig. 1. The IgG reactivity reduction indicated that the antigenic epitopes in CM were altered, although some effects of enzymatic hydrolysis seemed to vary slightly. Additionally, it also suggested that enzymatic hydrolysis can change the structure of the allergens in CM, interfere with the antigen-antibody complex, and thus reduce the IgG reactivity. Some studies have demonstrated that the residual antigenicity and IgE-binding ability of α-LA, β-LG, α-CN, β-CN in cow milk, and the reduction rate was about 15%-90%[31].

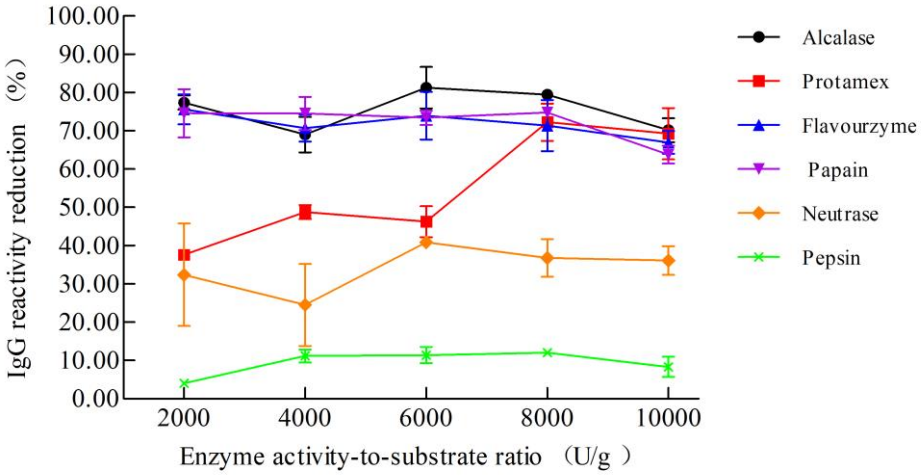


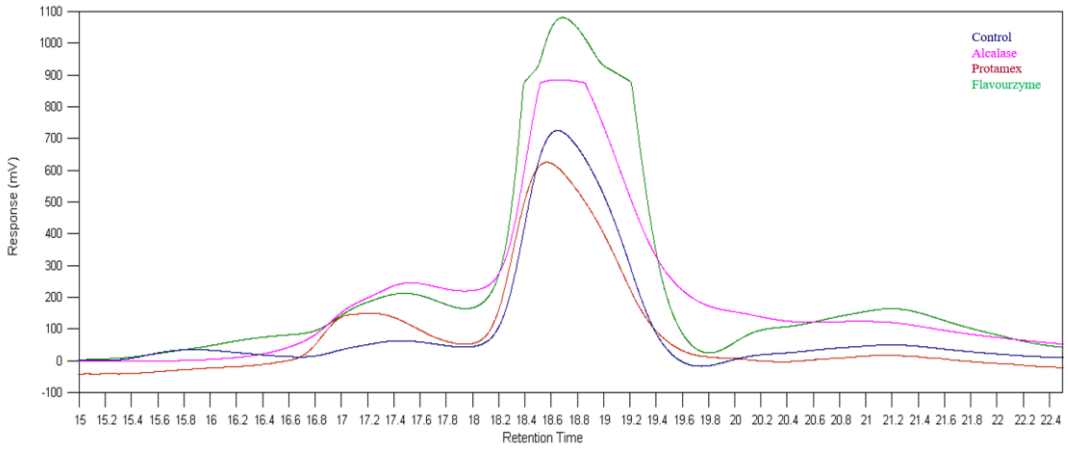
Fig. 3. IgG reactivity of cow milk hydrolyzed with different enzymes and enzyme activity-to- substrate ratio (U/g). Each value represents the mean of three independent experiments ± SD.

3.4 Molecular weight distribution

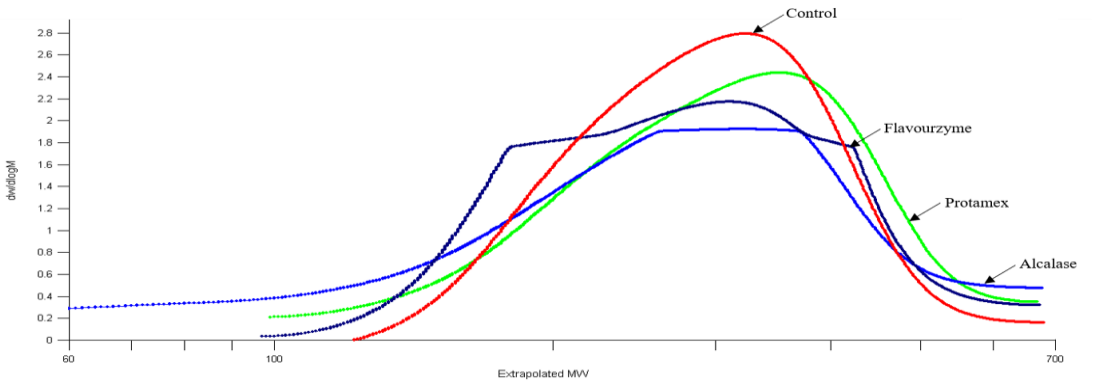
Enzymatic hydrolysis could break down milk proteins into short peptides and change the MW distribution, which can be evaluated by GPC. The MW distribution was calculated assuming an exponential relationship between MW and elution time. After enzymatic hydrolysis by different enzymes, the MW distribution of CM hydrolysates was significantly different, which reflected the differences of peptide chain lengths. The MW distribution of CM and CM hydrolysates are presented in Fig. 4. It was obvious that the MW of CM hydrolysates were mostly made up of small MW peptides <3 kDa. After enzymatic hydrolysis process, the percentage of milk proteins with MW distribution of 10-30 kDa, 5-10 kDa, 3-5 kDa significantly reduced ($P < 0.05$). The results demonstrated that during hydrolysis with different enzymes, because of the cleavage reaction and CM proteins were fragmented into low MW peptides. Furthermore, the MW distribution of CM

hydrolysates showed considerable differences. For CM hydrolysate obtained with Flavourzyme, the relative percentage of milk proteins with MW distribution of 3-5 kDa was about 2.05%, while CM hydrolysate treated with Alcalase was 0.76%. These results in combination with DH, SDS-PAGE suggesting that enzymatic hydrolysis with Alcalase and Protamex were much more effective in producing smaller peptides in CM. Several studies proved that the most effective mean to reduce the allergenicity of CM is by decreasing the MW of the principal CM allergens, namely CN, β-LG and α-LA, by enzymatic proteolysis[32]. Furthermore, some researches have shown that peptides with MW between 1,600 Da and 3,500 Da, prepared from either CN or whey, were unable to elicit an IgE-mediated allergic response[33]. Deeslie and Cheryan reported peptide MW was also one of the most key factors, governing the desired functional properties of CM hydrolysates used as a functional material[34].

(A)



(B)



(C)

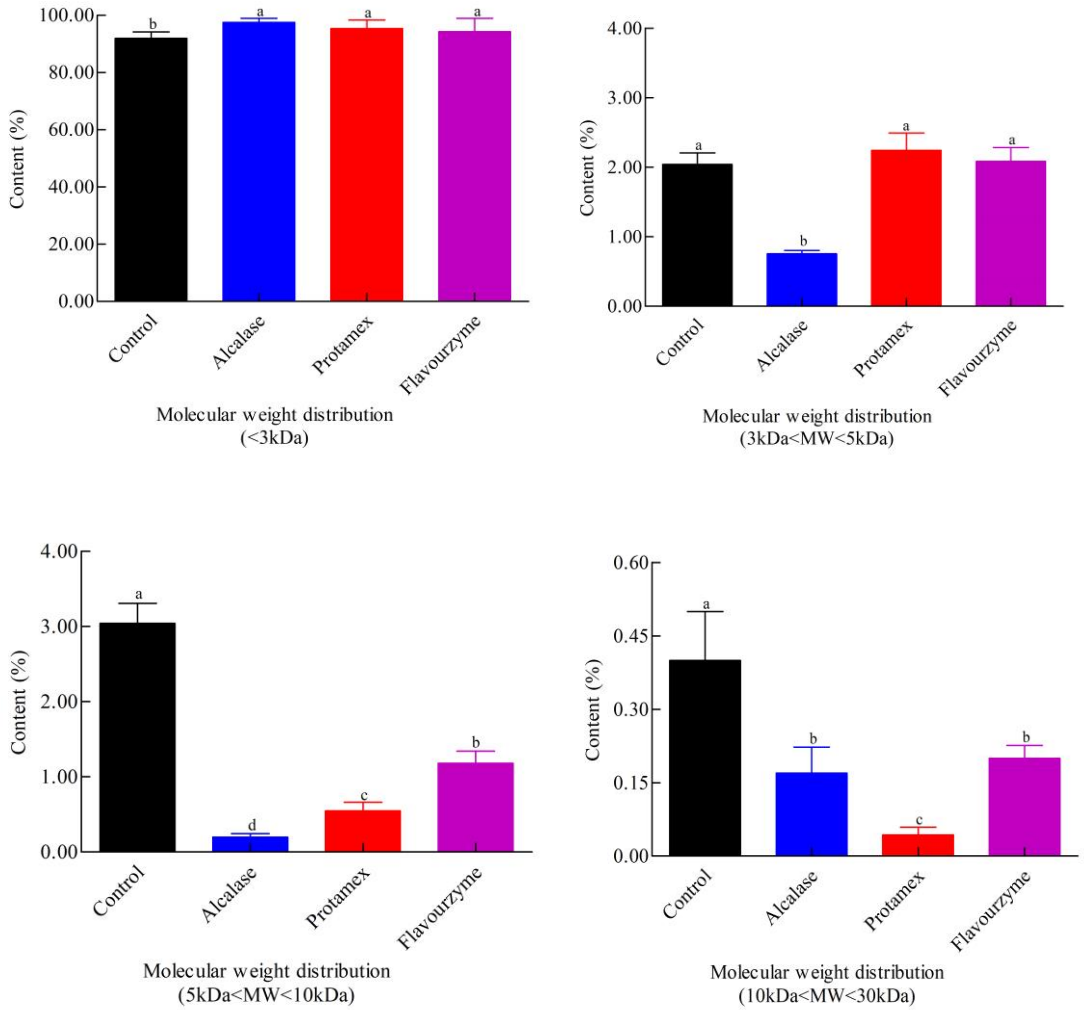


Fig. 4. Molecular weight distribution of cow milk hydrolysates determined by gel permeation chromatography. Different letters indicate significant differences among groups ($p < 0.05$).

3.5 Western blotting

In order to study the changes of allergenicity of the main allergenic proteins in the CM after enzymatic hydrolysis, we used anti-CN, anti-β-LG, anti-α-LA rabbit polyclonal antibodies and western blotting to qualitatively analyze the main allergenic proteins in CM. As seen from Fig. 5, the CM proteins before and after the enzymatic hydrolysis had an immune binding reaction with IgG antibody of the anti-CN, anti-β-LG and anti-α-LA rabbit serum, indicating that the CM proteins before and after the enzymatic hydrolysis had an immune reaction. Compared with CM, the IgG-binding capacity of antigen proteins (CN, β-LG, α-LA) in CM hydrolysate was reduced significantly, but the IgG-binding capacity of CN in CM hydrolysate was stronger than that β-LG and α-LA. Similar results proved that Alcalase was more effective at reducing antigenicity of milk proteins, decreasing in the IgG-binding capacity of α-LA, β-LG significantly[35]. Natália *et al* reported that Alcalase hydrolysis showed the similar inhibitory on the IgE or IgG-binding capacity of α-LA, β-LG in WPC[36].

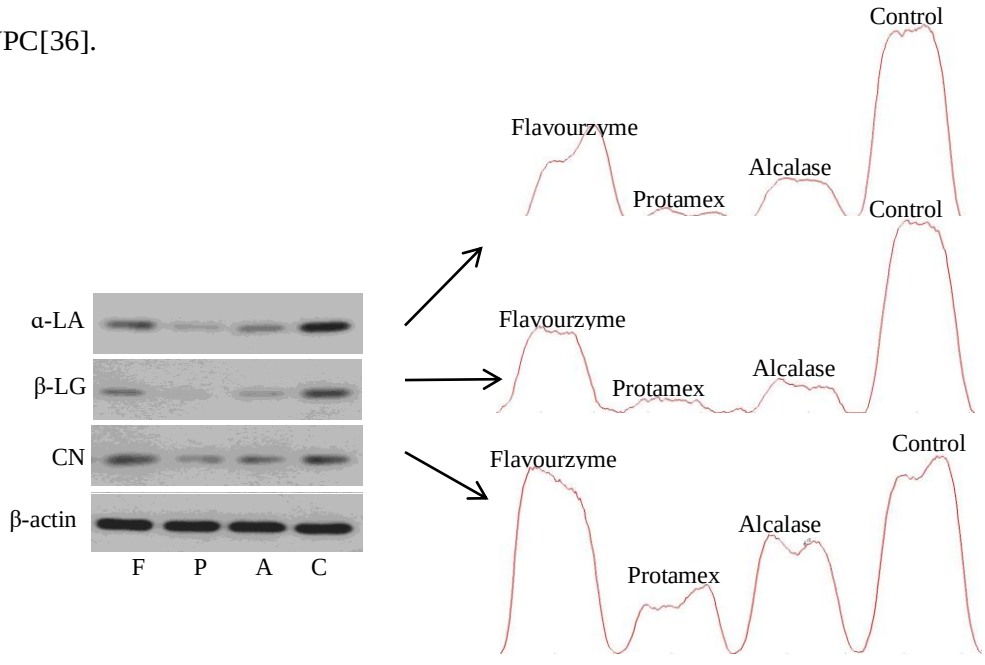


Fig. 5. Immunoblot of polypeptides obtained by hydrolysis of cow milk using three enzymes. C: Control (unhydrolyzed cow milk); A: Alcalase; P: Protamex; F: Flavourzyme.

3.6 IgG-binding ability

To determine the antigenicity of the CM hydrolysates, polyclonal antibodies were prepared, and competitive inhibition ELISA was used to evaluate the residual antigenicity of CN, β-LG, α-LA. As seen from that enzymatic hydrolysis could reduce the antigenicity of CM proteins, and the antigenicity reduction rate of β-LG, CN were much higher than that of α-LA ($p < 0.05$). Furthermore, Fig. 6 suggested that after enzymatic hydrolysis of CM by Alcalase and Protamex, the reduction rate in IgG-binding of α-LA were 11.28%, 6.10%, respectively, while that a higher residual antigenicity was obtained with Flavourzyme (-6.09%), which showed significant differences among these data ($p < 0.05$). Fig. 6 also demonstrated that the IgG-binding ability of β-LG during enzymatic hydrolysis with Flavourzyme and Protamex were reduced obviously, the reduction rate was59.09%, 79.37%, respectively, but enzymatic hydrolysis with Alcalase led to a dramatic reduction of 90.25%. These data also showed a significant difference ($p < 0.05$). Moreover, it can be seen that a distinct decrease in IgG-binding of CN during the enzymatic hydrolysis process, the significant reduction rate was 69.14%-91.21%. It is worth noting that Alcalase, Protamex played an important role in reducing the antigenicity of CN, β-LG, α-LA, therefore, it could be concluded as being superior to the other enzyme. Previous research are consistent with the present findings, Wróblewska *et al*. showed that the lowest immunoreactivity to anti-α-LA antibodies was found for whey protein hydrolysate

obtained using Alcalase and its double dose[37]. Quintieri *et al*. reported that the antigenicity of whey was slightly reduced when it was incubated with fungal proteinases and pancreatic extracts[38].

In this study, the amount of low MW peptides was significantly increased, and also the IgG-binding ability was considerably reduced in CM hydrolysates. The reason because of during the enzymatic hydrolysis, large MW peptides were hydrolyzed partially by enzymes, the reduction of antigenicity may be attributed to the splitting of epitope sequence. These results demonstrated that the change of IgG reactivity might be caused by the epitope change, which was consistent with the previous electrophoresis results. The allergic epitopes could be damaged or destroyed by the degradation of milk proteins, or conformational changes occurring, which resulted in reducing reactivity and IgG-binding ability. In addition, the enzyme specificity or types can also affect the degradation of allergic epitopes. Similar research has been found that the most comprehensive study carried out twelve different food-grade enzymes, these results proved that WPC incubated with Papain showed the lowest IgE-binding ability[39]. Villas-Boas *et al.* proved that enzymatic hydrolysis could reduce the amount of allergic epitopes, also the IgE or IgG-binding ability of β-LG was decreased significantly[40].

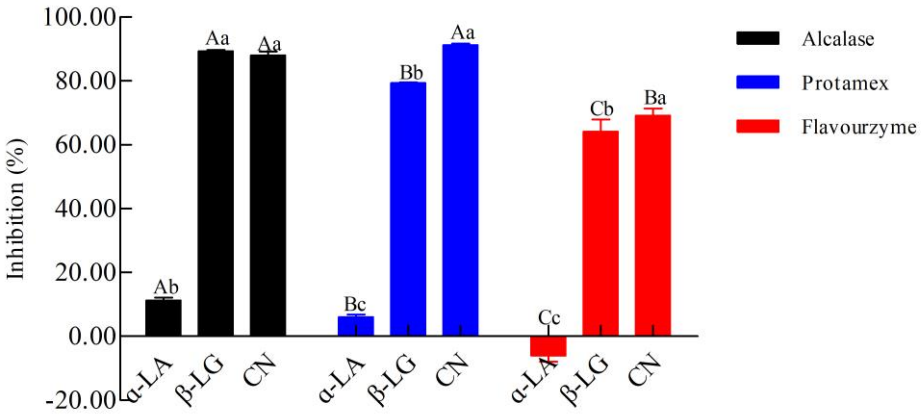


Fig. 6. Residual antigenicity of cow milk hydrolysates determined by ELISA using polyclonal antibodies against α-LA, β-LG and CN. Each value represents the mean of three independent experiments ± SD. Different letters indicate significant differences among groups ($p < 0.05$).

3. Conclusions

In this paper, natural cow milk was first subjected to enzymatic hydrolysis by different food-grade enzymes, and the effects of different enzymes on the major allergenic proteins in natural cow milk were assessed. Results showed that CM treated by Alcalase and Protamex hydrolysis could efficiently reduce the antigenicity of CN, β-LG, α-LA, especially for CN, β-LG, showed a higher DH and the loss of density of CM proteins, as indicated by SDS-PAGE. The increasing of the low MW (<3 kDa) in CM hydrolysates was also presented. It was also found that Protamex and Alcalase could be more efficiently hydrolyzed major allergenic of CM than other enzymes for the development of hypoallergenic cow milk. Furthermore, our work goes beyond previous studies, which have been limited to the enzymatic hydrolysis of individual protein or the antigenicity of

individual allergic protein in CM. This study will provide theoretical evidence for further research on hypoallergenic cow milk.

Conflicts of interest

The authors confirm that the contents of this article pose no conflicts of interest.

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