

Combining experimental techniques with molecular dynamics to investigate the impact of different enzymatic hydrolysis of β -lactoglobulin on the antigenicity reduction

Xin-Xin Yu ^{a,b}, Wei-Yue Liang ^{a,b}, Jia-Yi Yin ^{a,b}, Qian Zhou ^{a,b}, Dong-Mei Chen ^{a,b}, Ying-Hua Zhang ^{a,b,*}

^a Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin 150030, PR China

^b Department of Food Science, Northeast Agricultural University, Harbin 150030, PR China

ARTICLE INFO

Keywords:

Molecular docking
 β -Lactoglobulin
Antigenicity
Active sites
Hydrolysis

ABSTRACT

β -Lactoglobulin (β -LG) is one of the major food allergens. Enzymatic hydrolysis is a promising strategy to reduce the antigenicity of β -LG in industrial production. The relationship between the cleavage sites of β -LG by protease and its antigenic active sites were explored in this study. Molecular docking and molecular dynamics (MD) were used to analyze the active sites and interaction force of β -LG and IgG antibody. Whey protein was hydrolyzed by four specific enzymes and the antigenicity of the hydrolysates were determined by ELISA. The results of MD showed that the amino acid residue Gln155 ($-4.48 \text{ kcal mol}^{-1}$) played the most important roles in the process of binding. Hydrolysates produced by AY-10, which was the only one with specificity towards cleavage sites next to a Gln, had the lowest antigenicity at the same hydrolysis degree. Antigenicity decrease was related to the energy contribution of the cleavage site in the active sites.

1. Introduction

Cow's milk protein (CMP) is widely used as a functional protein in the food industry with excellent functional properties and providing rich nutrients to the body (Hochwallner, Schulmeister, Swoboda, Spitzauer & Valenta, 2014). However, during the process of ingesting milk, the immune system of some infants will recognize some proteins in the milk as antigens, leading to cow's milk protein allergy (CMPA) symptoms such as vomiting, diarrhoea, respiratory asthma and skin allergies (Oliveira et al., 2019). CMPA is a common and symptomatic disease affecting approximately 2–3% infants, and over 21% patients still develop an allergic after 16 years old (Boyano-Martínez, García-Ara, Pedrosa, Díaz-Pena & Quirce, 2009). Because of the changes in living habits and social structure in recent years, milk feeding is more common than breast feeding. Considering that milk is one of the most exploited and consumed food products throughout the world, frequency and severity of milk allergies are medical issues of great importance.

Cow's milk proteins divided into two main compounds: casein and whey protein. Whey protein is commonly used as protein ingredients added to infant formulas, where β -lactoglobulin (β -LG) is one of the

major allergens in bovine whey protein because β -LG is not found in human milk (Eugenia Lucena, Alvarez, Menéndez, Riera & Alvarez, 2006). The β -LG monomer is a globular protein containing 162 amino acid residues with a molecular weight of about 18 kDa (Duan, Huo, Yang, Ren & Chen, 2012). It is predominantly a β -sheet protein with nine β -strands and one major α -helix, of which eight antiparallel β -strands to form a hydrophobic cavity. Each monomer contains two disulfide bonds, which were Cys⁶⁶-Cys¹⁶⁰ and Cys¹⁰⁶-Cys¹¹⁹, and Cys¹²¹ was the free thiol (Ma, Wang & Guo, 2018). The structure of β -LG makes it very stable at low pH and resistant to pepsin degradation. In addition, most of the hydrophobic amino acids (targets for pepsin) of β -LG point inward to form a β -barrel structure, which is not easily accessible to the enzymes (Chicón, López-Fandiño, Alonso & Belloque, 2008). At present, many methods have been developed to reduce milk protein allergic reactions, such as heat treatment, glycosylation, high pressure, lactic acid fermentation and enzymatic hydrolysis (Feng et al., 2020; Prioult, Pecquet & Fliss, 2005). Enzymatic hydrolysis can effectively reduce milk protein allergic reactions by decomposing antigen proteins into small peptide molecules and amino acids, and increasing the absorption rate of milk compared with other methods (Li, Yu, Goktepe & Ahmedna,

* Corresponding author at: Key Laboratory of Dairy Science, Ministry of Education, Northeast Agricultural University, Harbin 150030, PR China.

E-mail address: yinghuazhang@neau.edu.cn (Y.-H. Zhang).

2016). It has been studied that the structure of β -LG was destroyed by α -chymotrypsin, pepsin, trypsin, papain or alkaline protease hydrolysis treatment to reduce its antigenicity (Feng et al., 2020; Yu, Liu, Lu, Liu, Yin & Zhang, 2019). However, as protein hydrolysis carried on, the content of hydrophobic oligopeptides or amino acid residues increased, resulting in bitter taste (Kim, Li, & Eunice, 2006). The reduction of β -LG antigenicity depends on the specific cleavage site of enzymes and hydrolysis conditions. Under similar hydrolysis conditions, it has been clear that due to different cleavage sites, the reduction of β -LG antigenicity has great differences. The relationship between the sites destroyed by hydrolysis and the active sites where β -LG binds to antibody has not been reported.

Recently, molecular simulation technology has been widely used as a powerful means to analyze the interaction between receptor-ligand molecules (Tu, Cheng, Lu, & Du, 2018). Especially, the three-dimensional structures for many different allergens have been determined in the last few years, which have facilitated the development of computer virtual biomolecular interaction analysis. The purely virtual method of molecular modeling for antigen epitopes prediction is based on the assumptions derived from structural surface feature analysis found to affect antigenicity, such as increased solvent accessibility, backbone flexibility and hydrophilicity (Xu, Tack, Hughes, Ellington & Gray, 2014). The molecular docking methods or molecular simulation software such as RosettaDock, ZDOCK, RDOCK and Amber and Gromacs, have been successfully used to predict the active sites combined with experiments (Pandit et al., 2012). Many epitopes were predicted by molecular simulation methods such as anthrax protective antigen, β -LG and EGFR (Sivasubramanian et al., 2006; Cong & Li, 2012; Sivasubramanian, Maynard & Gray, 2008). RosettaDock has been increasingly used in protein docking and structure design in order to predict protein-protein interactions. It was originally introduced as a multi-scale Monte Carlo based docking algorithm that utilized a centroid-based coarse grain stage to quickly identify favorable docking poses and an all-atom refinement stage, which included simultaneously optimized rigid-body position and side-chain conformation (Chaudhury & Gray, 2009). In addition, RosettaDock has addressed the critical challenge of binding-induced backbone conformational changes in protein-protein docking. In this range, RosettaDock has been used in a wide range of applications from antibody-antigen docking (Sivasubramanian, Maynard & Gray, 2008), peptide docking (Chaudhury & Gray, 2009), enzyme inhibitor docking (Bocik et al., 2011) and symmetric docking (Andre et al., 2008).

Cow's milk protein allergy shows various clinical symptoms and involves different immunoglobulin antibodies. Although most of the identified epitopes and immunodominant epitopes overlapped with the informative epitopes identified in previous studies, there are some discrepancies between them because of the sensitivity of the assay and the selection of control. The immediate allergy reaction based on IgE antibodies is considered to be responsible for majority of the food-induced hypersensitivity. The atopic response to airborne pathogenesis-related group 10 protein molecules is characterized not only by an IgE response but also by an allergen-specific IgG response (Hofmaier et al., 2015). Some IgG binding regions identified colocalized with the IgE binding epitopes in milk-allergic patients, which means the IgE-binding epitopes also bind IgG (Jarvinen, Chatchatee, Bardina, Beyer & Sampson, 2001). Therefore, it is feasible that the interface structure and energy contribution of β -LG and IgG antibody were analyzed through molecular docking and molecular dynamics (MD). The antigenicity of β -LG can be reduced by different enzymatic hydrolysis. The comparative analysis of the predicted key epitopes and specific enzyme cleavage sites can explain the difference in the reduction of the antigenicity of hydrolyzed whey protein.

The major goal of our work was to use different specific enzymes to hydrolyze whey protein isolate (WPI), comparing the difference in the reduction of β -LG antigenicity in whey protein under the same degree of hydrolysis (DH). The molecular docking was used to combine β -LG with IgG antibody, and obtained the key epitopes through MD calculation in

this study. The reasons for the differences in the antigenicity of hydrolysates and predicted the antigenic active sites of β -LG were systematically analyzed and explained. It is the first time that molecular simulation method was used to explore the mechanism of enzymatic hydrolysis to reduce the antigenicity of β -LG. The results of studying the structural interaction of β -LG with IgG will provide basic data for more efficacious modification strategy in the future and promote the development of low-sensitivity ingredients in the food industry.

2. Material and methods

2.1. Sample preparation

The whey protein isolate (WPI) (87.95% protein content, Brewster Dairy, OH, USA) was hydrolyzed by four specific enzymes, including trypsin (250 units mg^{-1} , Solarbio, Beijing, China), α -chymotrypsin (1500 units mg^{-1} , Solarbio, Beijing, China), thermolysin (PC10-F) (90 units mg^{-1} , Amano Enzyme, Jiangsu, China) and Bacillus licheniformis protease (AY-10) (80 units mg^{-1} , Amano Enzyme, Jiangsu, China). The identified specific cleavage sites of these enzymes are Lys and Arg for trypsin; Phe, Trp and Tyr for α -chymotrypsin; Ile, Leu, Phe, Tyr, and Val for PC10-F; Gln, Lys and Leu for AY-10, respectively (Deng, van der Veer, Sforza, Gruppen & Wierenga, 2018; Dušeková, Garajová, Yavaşer, Varhač & Sedlák, 2018; Hernández-Ledesma, Ramos, Recio & Amigo, 2006; Vorob Ev, Butré, Sforza, Wierenga & Gruppen, 2016). Hydrolysis conditions of WPI (3%, w/v) were as follows: $2.5 \times 10^3 \text{ U g}^{-1}$ protein, pH 7.5, and temperature of 40°C for trypsin; $1.0 \times 10^4 \text{ U g}^{-1}$ protein, pH 8.0, and temperature of 40°C for α -chymotrypsin, 900 U g^{-1} protein, pH 8.0, and temperature of 60°C for PC10-F, 800 U g^{-1} protein, pH 8.0, and temperature of 55°C for AY-10, respectively. During the hydrolysis, the pH of the reaction mixture was stabilized by adding 0.5 mol L^{-1} NaOH. The hydrolysis reaction time of each enzyme was 4 h in this study. Within the first 30 min, 1 mL of hydrolysates were taken every 5 min, and then the hydrolysates were taken every 10 min. All samples were immediately inactivated in a boiling water bath for 5 min with 3% WPI as control. The free amino groups content was determined by OPA method. The absorbance of each diluted hydrolysate sample was measured by a UV-Vis spectrophotometer (UV-2401PC, Shimadzu, Japan) in triplicate. The hydrolysis curve was plotted with the DH as a function of the hydrolysis time. The hydrolysates of WPI with the same DH were prepared according to the hydrolysis curve.

2.2. Assays of a degree of hydrolysis

The degree of hydrolysis (DH) was assayed directly by quantification of the cleaved peptide bonds as assessed by the *ortho*-phthaldialdehyde (OPA) method with minor adjustments (Spellman et al., 2003). A standard curve was prepared using L-leucine so that the analysis system contained $-\text{NH}_2$ in a reasonable range. 3 mL diluted hydrolysates (1:500 with deionized water) and OPA reagent mixed well. After 5 min of reaction, the absorbance at 340 nm was measured by UV-Vis spectrophotometer (UV-2401PC, Shimadzu, Japan), and the $-\text{NH}_2$ groups were defined from the L-leucine standard curve. DH was calculated using the following equations:

$$Y = A \times \frac{N}{131.17 \times X} \quad (1)$$

where Y is $-\text{NH}_2$ content of sample (mmol g^{-1}), A is the content of L-leucine obtained from the standard curve ($\mu\text{g mL}^{-1}$), N is dilution times (500), X is protein content (%) of the sample.

$$\text{DH}(\%) = \frac{Y_1 - Y_0}{h_{\text{tot}}} \times 100 \quad (2)$$

where Y_0 is $-\text{NH}_2$ content in the sample before hydrolysis, Y_1 is $-\text{NH}_2$ content in hydrolysate, h_{tot} is the number of hydrolyzed bonds of β -LG as

7.8.

2.3. Elisa

The antigenicity of the samples was evaluated by indirect competitive ELISA (Yu, Liu, Lu, Liu, Yin & Zhang, 2019). The wells were coated with 100 μL 2 $\mu\text{g mL}^{-1}$ of standard β -LG (Sigma, Saint Louis, MO, USA) solution in 0.01 M phosphate buffer (PBS) pH 7.4, and incubated overnight at 4 °C. The plates (Corning, Cambridge, MA, USA) were washed three times with 250 μL of PBS containing 0.05% (v/v) Tween 20 (PBST). This washing system was used after each incubation step. To avoid nonspecific binding, the plates were blocked with 1% (w/v) gelatin in PBS solution. In parallel, the different concentrations of β -LG standard solutions and β -LG hydrolysates obtained with different enzymatic hydrolysis (diluted 1:500 with PBS, v/v) were incubated separately with the β -LG monoclonal antibodies (IgG) which prepared in our laboratory previously (diluted 1:1 $\times 10^5$, v/v) in a ratio 1:1. About 100 μL of these mixtures (β -LG hydrolysate samples and mAb) were added to the wells. Plates were incubated at 37 °C for 2 h and washed three times with PBST. After then, the well was filled with 100 μL of HRP-conjugated goat anti-mouse IgG (Shanghai Immune Biotech, Shanghai, China), diluted 1:2000 in PBS, and incubated for 1 h. Finally, the 50 μL 3,3',5,5'-tetramethyl-benzidine (TMB) (Sigma-Aldrich) was used as substrate for colour reaction at room temperature for 10 min, and the reaction was stopped with 50 μL H_2SO_4 (2 mol L^{-1}). The β -LG monoclonal antibody with a concentration of 1:1 $\times 10^5$ (v/v) as positive control, and 100 μL PBS were added as negative control to each plate, respectively. The absorbance of color products released in the titer wells was measured at 450 nm in a plate reader of the spectrophotometer (Bio-Rad Laboratories, NSW, Australia). The samples of 0.01–10 $\mu\text{g mL}^{-1}$ β -LG solution (dissolved in PBS) with gradient dilutions were used as competitors to measure the absorbance by the above method to obtain a standard curve of absorbance and β -LG concentration. The concentration of β -LG in the hydrolysate was calculated from the standard curves. The antigenicity was represented as a mean $\pm$ standard deviation (SD). Significant differences ($P < 0.05$) were analyzed using variance (ANOVA) by SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

2.4. Molecular docking

The three-dimensional crystal structure of the β -LG (PDB code: 1BSQ) as a ligand was obtained from the RCSB Protein Data Bank (<http://www.rcsb.org/pdb>) with a resolution of 2.22 Å. The immunoglobulin G antigen-binding fragment (IgG Fab) was selected by chain H and L in PDB code: 4LQF. Water molecules, other co-crystallized ligand and ions in protein complex were removed for further docking process and molecular dynamics (MD) simulations. As a receptor, the heavy (H) and light (L) chains of IgG antibody were modelled by the antibody modeling module of Discovery Studio 2.5 software (DS 2.5), and select the complementarity determining regions (CDRs) of the antibody template with the highest homology from the antibodies database by the BLAST search program to optimize the CDRs of IgG prior to docking (Biswas, & Bagchi, 2017).

The docking protocol was performed with RosettaDock software, which was implanted to explore possible binding interaction surfaces between β -LG and IgG antibody. The rigid body and protein backbone between the antibody and β -LG were optimized by the Monte-Carlo method (Chaudhury & Gray, 2009). Since only the complementarity-determining region (CDR) residues of an antibody were involved in binding to the antigen, all residues in the non-CDR (NCDR) region were not considered during the docking process (Pandit et al., 2012). The CDR domains of IgG antibodies serve as target libraries for binding site residues to filter output predictions (Xu, Tack, Hughes, Ellington & Gray, 2014), and at least 10,000 docking conformations were obtained for further analysis. A maximum number of 10 conformers were considered, and the conformation with the lowest binding energy was selected for

final MD simulations. Best complex structure model was selected then visually double-checked (to detect structural anomalies) using the molecular visualization PyMOL software (Wang, Chen, Huang, Diao, & Zhou, 2017).

2.5. Molecular dynamics simulations

The MD simulations were constructed using SANDER module of AMBER software (version 14), setting AMBER ff14SB force field for protein and the general AMBER force field (GAFF) as topology parameters for protein complexes (Daoud, Melkemi, Salah & Ghalem, 2018). After setting ionizable residues as their default protonation states at a neutral pH value, the complex was then solvated in a periodic box of explicit TIP3P water model which extended a minimum 10 Å distance from the box surface to any atom of the solute. Two steps of energy minimizations were carried out for the whole system, to eliminate possible bumps between the solute and the solvent.

After completion of the energy minimization, the whole system was heated gradually in the NVT ensemble from 0 to 300 K over 500 ps. Finally, a 20 ns MD simulation was performed under 1 atm with the NPT ensemble. The temperature was maintained at 300 K using Langevin dynamics with a collision frequency of 2 ps⁻¹ and the constant pressure was maintained employing isotropic position scaling algorithm with a relaxation time of 2 ps. All bond lengths were all constrained using the SHAKE algorithm and integration time step was set to 2 fs. H-bond and hydrophobic interaction analysis between the β -LG and IgG was performed using the PyMOL and LigPlot⁺ software, where potential H-bonds were accepted if they had a proton donor-acceptor distance of 3.5 Å (Suresh, Divya, Nidhi & Rajasekaran, 2018).

2.6. Binding free energy decomposition calculation

Five hundred snapshots were extracted from the last 1 ns trajectory for the final average structure of complex, which were applied for the calculation of the binding free energy (ΔG) and energy decomposition using molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) approach implemented as script (MMPBSA.py) in AMBER software to analyze the energy contribution calculation of each residue in the whole system (Evoli et al., 2014; Abro & Azam, 2016). The following Equations were applied:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - (G_{\text{receptor}} + G_{\text{ligand}}) \quad (3)$$

$$\Delta G_{\text{residue-pair}} = \Delta G_{\text{vdW}} + \Delta G_{\text{ele}} + \Delta G_{\text{sol}} = \Delta G_{\text{vdW}} + \Delta G_{\text{ele}} + \Delta G_{\text{pb}} + \Delta G_{\text{np}} \quad (4)$$

Where $\Delta G_{\text{complex}}$ is the total free energy of the β -LG—IgG complex, G_{receptor} represents the total free energies of the IgG antibody in solvent and G_{ligand} is total free energies of the β -LG in solvent, ΔE_{vdW} is van der Waals contribution, ΔE_{ele} is electrostatic contribution, and ΔG_{sol} represents solvation contribution in the free energy. The ΔG_{sol} includes two parts, the polar (ΔG_{pb}) and non-polar solvation (G_{np}) free energy.

3. Result and discussion

3.1. Docking and molecular dynamics simulation analysis

When docking locally, the RosettaDock software with the high accuracy predictions was used for the docking process of β -LG and IgG antibody (Chaudhury et al., 2011). In the present study, the determination of CDRs was based on the method developed by North et al. (2011). A total number of 10,000 complex conformations were taken out and ranked by docking energy value. The lower energy, the stronger binding ability. The energy of the complex structure was from −283.3 kcal mol⁻¹ to −232.4 kcal mol⁻¹. The complex with a lowest binding energy score (−283.3 kcal mol⁻¹) from precise docking process and its

structure was selected for 20 ns molecular dynamics simulation.

After molecular dynamics simulation, the root means square deviation (RMSD) of the backbone atoms of the complex system were calculated based on the starting snapshot, which was investigated to determine whether the complex has reached conformational stability during the equilibrium process. As shown in Fig. 1, we can observe that RMSD values of the whole system increase intensely within 2 ns and remains stable at 3 Å after 8 ns. The change in the RMSD value showed the motion state of the complex conformation within this solvent parameter, which had been stabilized at the end of the MD simulation and used for energy analysis.

A total of 20 ns simulation time was carried out, and MDs trajectory was applied for extracting the refined composite structure model (Fig. 2A). Obviously, a large number of intensive hydrogen bond interactions were formed at the interface between the protein β -LG and IgG antibody (Fig. 2B). Beside hydrogen bond interactions, hydrophobic interactions also play as a necessary role in complex stability (Abro & Azam, 2016). According to the hydrogen bonds and complementarity of hydrophobic/hydrophilic properties on the molecules surface of β -LG and IgG, 14 amino acids were determined as active sites of β -LG that drive interaction and stabilize the complex (Fig. 2C–E). The active site residues of β -LG were: Tyr20, Gln35, Arg40, Pro126, Glu127, Asp130, Phe151, Asn152, Pro153, Thr154, Gln155, Glu157, His161 and Ile162. Among them, LigPlot⁺ software indicated that Tyr20, Phe151, His161 and Ile162 did not form a hydrogen bonding with the IgG antibody, but the hydrophobic interaction promoted the binding, determined by the hydrophobic/hydrophilic nature of the amino acid itself, which could be further analyzed by energy decomposition. The IgG antibody interacted with active site residues of β -LG Tyr20, Gln35, Arg40, Phe151, Asn152, Pro153, Thr154, Gln155 and Glu157 by electrostatic interactions and a hydrogen bond. These results were agreed well with the conclusion obtained by Jarvinen, Chatchatee, Bardina, Beyer and Sampson (2001). The residues Tyr20, Glu157 and His161 contained on the outer surface of β -LG constitute the protein docking pocket, which was also identified by molecular docking for oleic acid (Zhong et al., 2018) and palmitic acid (Evoli et al., 2014).

3.2. Binding free energy decomposition

To investigate the binding free energy and molecular interactions between the β -LG–IgG complex conformation, MM-PB/SA module of the Amber software based on the MD trajectories was used for calculation. The predicted binding free energies of the complex obtained in Table 1, which discovered that the total binding free energies of complex system was $-58.90 \text{ kcal mol}^{-1}$, and the electrostatic energy ($-1090.31 \text{ kcal mol}^{-1}$) term was quite favourable and dominated the overall binding energy. Many research results indicated that electrostatic energy was the main driving force when two molecules were combined with each other (Wang, Chen, Huang, Diao, & Zhou, 2017). Furthermore, van der Waals interactions ($-48.83 \text{ kcal mol}^{-1}$) and the non-polar

part of the solvation free energy ($-37.13 \text{ kcal mol}^{-1}$) had similar values in the complex system and showed a favourable contribution to total binding energy. The extremely non-favourable contribution occurs due to the polar part of the solvation free energy ($1117.38 \text{ kcal mol}^{-1}$). Polar and non-polar components guide all the molecular interactions in a polar solvent, where non-polar energy contributions being more advantageous.

In order to further explore the interaction of β -LG and IgG at the atomic level, the binding free energy contribution of per residue of the ligand to the total binding energy of the complex was calculated by the MMPB/SA method and shown in Fig. 3. Residues contributing $\leq -1 \text{ kcal mol}^{-1}$ were considered to be key amino acids, indicating that they play an important role in stabilizing the complex Chu, Zheng, Wu, Zhang, and Zhang (2013). Obviously, Glu127 ($-2.87 \text{ kcal mol}^{-1}$), Phe151 ($-1.48 \text{ kcal mol}^{-1}$), Asn152 ($-3.39 \text{ kcal mol}^{-1}$), Thr154 ($-1.25 \text{ kcal mol}^{-1}$), Gln155 ($-4.48 \text{ kcal mol}^{-1}$), Glu157 ($-1.67 \text{ kcal mol}^{-1}$) and Glu158 ($-1.41 \text{ kcal mol}^{-1}$) were the residues that make high free energy contributions. Moreover, highly contributing residues were at the binding site of the complex except Glu158. It was speculated that AA Phe151–Glu157 were key active sites of β -LG. Gln155 illustrated an extremely low PB energy showing its great importance in the active sites. It has been reported that the above amino acid residues also exhibit strong antigenicity when subjected to peptide scanning or other methods for predicting β -LG epitopes (Cong & Li, 2012).

Then, we analyzed the electrostatic energy, van der Waals and solvation contribution decomposition to determine the detailed contribution of each major residue (Table 2). The energy contributions of the binding site residues indicated that the electrostatic energy dominates the total binding free energy, which demonstrated the results of previous total energy calculations. It was not observed in Fig. 2C–E that Tyr20, Phe151 and His 161 were involved in hydrogen bonding, however, exhibited favourable van der Waals forces and non-polar solvation energies due to the hydrophobic interaction between other residues. The non-favourable contribution of the total free energy of Asp130 ($0.92 \text{ kcal mol}^{-1}$) might be due to favourable contact with the polar solvent water, which indicated by the high polar solvent in Table 2.

3.3. Antigenicity of WPI hydrolysate with the same DH

The WPI hydrolysates with a degree of hydrolysis of 6% were prepared with different specific enzymes. At a lower DH, fewer hydrophobic amino acid residues were exposed, so the hydrolysates obtained an insignificant bitter taste, which had a benign impact on the quality of dairy products (Kim, Li, & Eunice, 2006). The antigenicity of the hydrolysate was determined by indirect competition ELISA. Since the different cleavage sites of four specific enzymes, the reduction in the antigenicity of β -LG was also different. As shown in Fig. 4, when the degree of hydrolysis was the same, the hydrolysis of AY-10 has the most significant impact on the antigenicity of β -LG, and the immunoreactivity of the hydrolysate drops to 10.23%, followed by α -chymotrypsin hydrolysate (20.17%) and thermolysin hydrolysate (40.26%). Moreover, the effect of trypsin hydrolysis to reduce the antigenicity of β -LG was not significant (50.36%), mainly because the key epitopes were not destroyed. It has been reported to hydrolyze β -LG by different enzymes to reduce antigenicity, and the commonly used enzymes are pepsin, α -chymotrypsin, thermolysin and trypsin. Chicon et al. treated β -LG with trypsin and α -chymotrypsin at atmosphere and found that the reduction effect of α -chymotrypsin was better than trypsin (Chicon, Belloque, Alonso, Martin-Alvarez & Lopez-Fandino, 2008), which result in the same conclusion as this study.

3.4. The relationship between energy of cleavage sites and antigenicity of hydrolysates

It was shown that the amino acid residues Tyr20, Arg40, Phe151, Gln155, and Ile162 located on the epitope of β -LG were also the cleavage

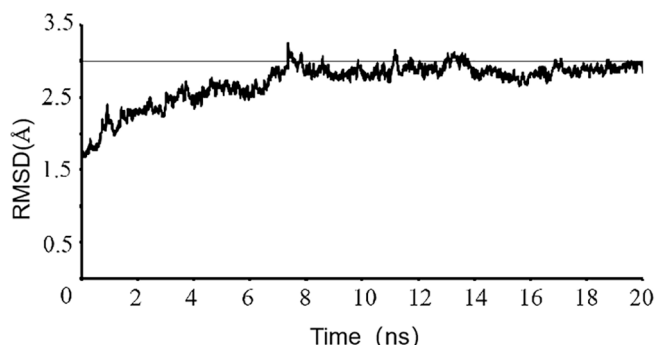


Fig. 1. RMSD value of the composite system over time during MD simulation.

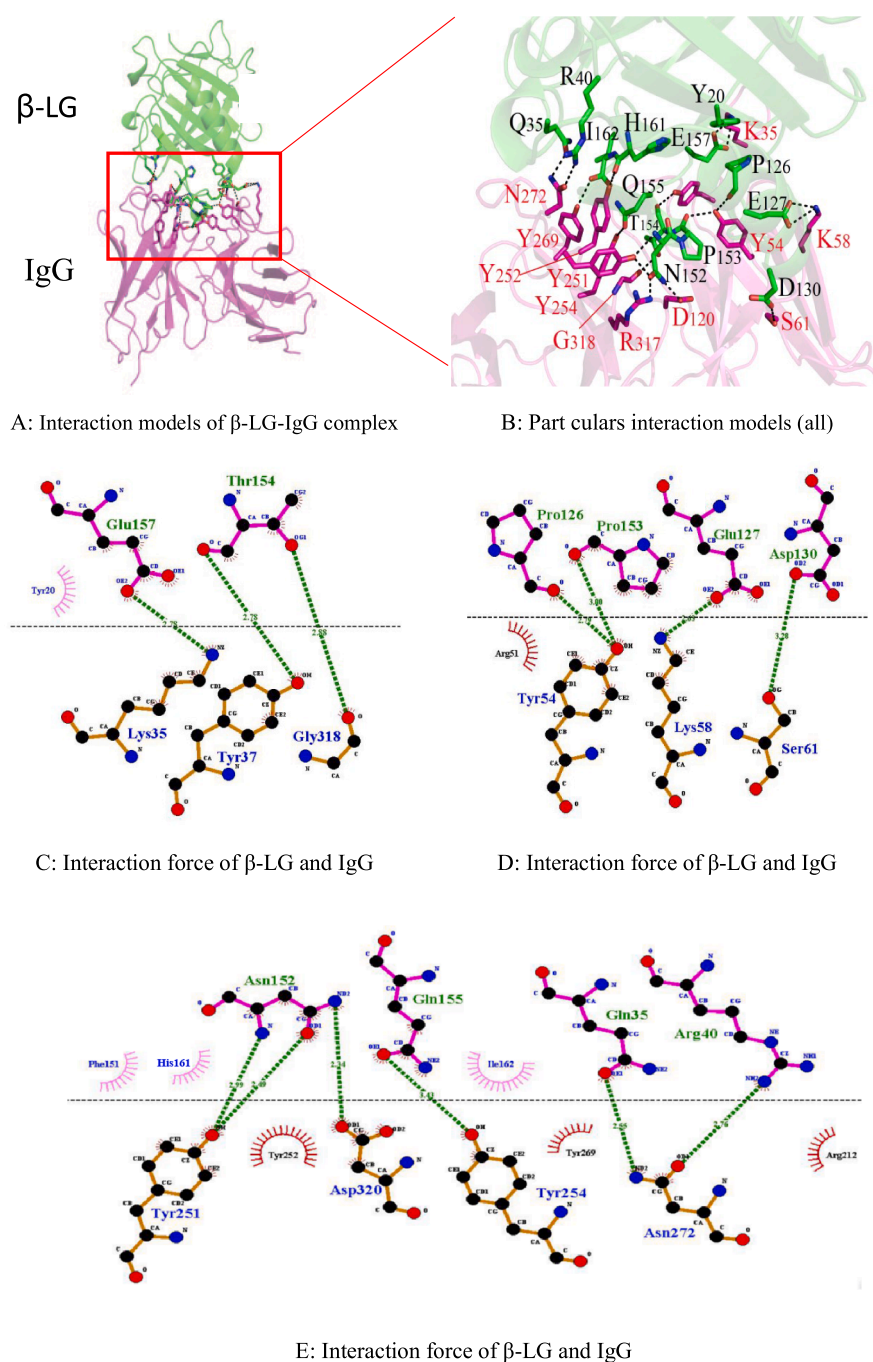


Fig. 2. Interaction models and interaction force between β -LG and IgG. Note: Picture A and B showed the active sites of β -LG and IgG binding, in which, β -LG and IgG antibody are colored in green and magenta, respectively; the key residue of β -LG and IgG antibody are marked by black and red, respectively. In the picture C, D and E showed interaction force of β -LG and IgG, in which, the β -LG and IgG molecules are represented by pink sticks and brown sticks, respectively; the black dashed lines indicate the molecular boundary, the green dashed lines indicate the hydrogen bond (H-bond), and the radiation arc indicates the hydrophobic interaction (β -LG are colored in pink and IgG are colored in red); The carbon atom (C) are colored in black, and all the oxygen atoms are colored in red and nitrogen atoms are colored in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Note: Picture A and B showed the active sites of β -LG and IgG binding, in which, β -LG and IgG antibody are coloured in green and magenta, respectively; the key residue of β -LG and IgG antibody are marked by black and red, respectively. In the picture C, D and E showed interaction force of β -LG and IgG, in which, the β -LG and IgG molecules are represented by pink sticks and brown sticks, respectively; the black dashed lines indicate the molecular boundary, the green dashed lines indicate the hydrogen bond (H-bond), and the radiation arc indicates the hydrophobic interaction (β -LG are colored in pink and IgG are colored in red); The carbon atom (C) are colored in black, and all the oxygen atoms are coloured in red and nitrogen atoms are coloured in blue.

sites of the enzyme from the molecular simulations results (Table 2 and Fig. 2), Fig. 4 and the hydrolysis sites of specific enzymes. The molecular basis of changes in antigenicity is the inactivation or destruction of epitope structures or increased inaccessibility to epitopes (Zhong et al., 2018). The reason for the decline in the antigenicity of β -LG was that

some epitopes were destroyed by hydrolysis. The cleavage sites of AY-10 (Gln35 and Gln155) played a crucial role in the binding of β -LG and IgG ($-5.66 \text{ kcal mol}^{-1}$). The cleavage sites of AY-10 (Gln35 and Gln155) played a crucial role in the binding of β -LG and IgG ($-5.66 \text{ kcal mol}^{-1}$). Although the hydrolysis sites of thermolysin were more than that of

Table 1The binding free energy components for β -LG—IgG.

Conformation	E_{ele} (kcal/mol)	E_{vdw} (kcal/mol)	G_{PB} (kcal/mol)	$G_{\text{non_polar}}$ (kcal/mol)	ΔG_{bind} (kcal/mol)
β -LG—IgG	-1090.31	-48.83	1117.38	-37.13	-58.90

Note: E_{ele} : electrostatic energy; E_{vdw} : van der Waals; G_{PB} : polar solvation energy; $G_{\text{non_polar}}$: non-polar solvation energy; ΔG_{bind} : binding free energy.

α -chymotrypsin, both of them hydrolyze only two sites at the antigenic active sites as Phe151 ($-1.48 \text{ kcal mol}^{-1}$) and Tyr20 ($-0.68 \text{ kcal mol}^{-1}$). The cleavage sites of trypsin at the active sites was only Arg40 ($-0.43 \text{ kcal mol}^{-1}$). Fig. 4 showed that the energy of the enzymatic hydrolysis sites was positively correlated with the antigenicity, and the lower energy, the lower antigenicity of the hydrolysate. AY-10 had the lowest energy at the cleavage sites and the lowest antigenicity of the hydrolysate (10.23%), indicating that AY-10 hydrolysis destroys the key epitope (Gln155) of β -LG. The hydrolysis energy of α -chymotrypsin and PC-10F was the same, but the antigenicity of PC-10F hydrolysate was higher than the former because the temperature of PC-10F hydrolysis conditions was significantly higher than the α -chymotrypsin. At this temperature, the structure of the β -LG was extended to expose the antigenic epitopes presenting inside originally (Rahaman, Vasiljevic & Ramchandran, 2017).

Under the same DH, the antigenicity of the hydrolysate of WPI hydrolyzed by different proteases was significantly different. The energy of the hydrolysis site of AY-10 was the lowest, and the antigenicity of its hydrolysates decreased most significantly, indicating that the hydrolysis of AY-10 undermined the key antigenic active site of β -LG. As the energy of the hydrolysis sites of the specific enzyme was different, the antigenicity of the hydrolysates was also different, and there was roughly a positive correlation.

4. Conclusion

In this paper, the molecular docking method was used to predict the antigen active sites of β -LG, and the interaction of β -LG and IgG antibody was analyzed by energy decomposition calculation method. The active sites of β -LG were Tyr20, Gln35, Arg40, Pro126, Glu127, Asp130, Phe151, Asn152, Pro153, Thr154, Gln155, Glu157, His161 and Ile162. Based on the results of energy decomposition calculations, Gln155 loaded the lowest energy contribution and played a major role in the

binding of β -LG and IgG. Under the same DH, the energy of the cleavage sites of specific enzymes was related to the antigenicity of the hydrolysate. Based on molecular docking and molecular dynamics, under the same degree of hydrolysis, the energy of the cleavage sites of a specific enzyme was related to the antigenicity of the hydrolysates. The lower the energy of the cleavage sites, the lower the antigenicity of the hydrolysates. AY-10 cleavage of Gln amino acid significantly reduced β -LG antigenicity. AY-10 was excellent in reducing antigenicity and might be used to develop a method that effectively reduces its antigenicity. The identification of epitopes has important practical value in guiding the development of hypoallergenic dairy products.

5. Ethics statement

No animals or humans were used in this study.

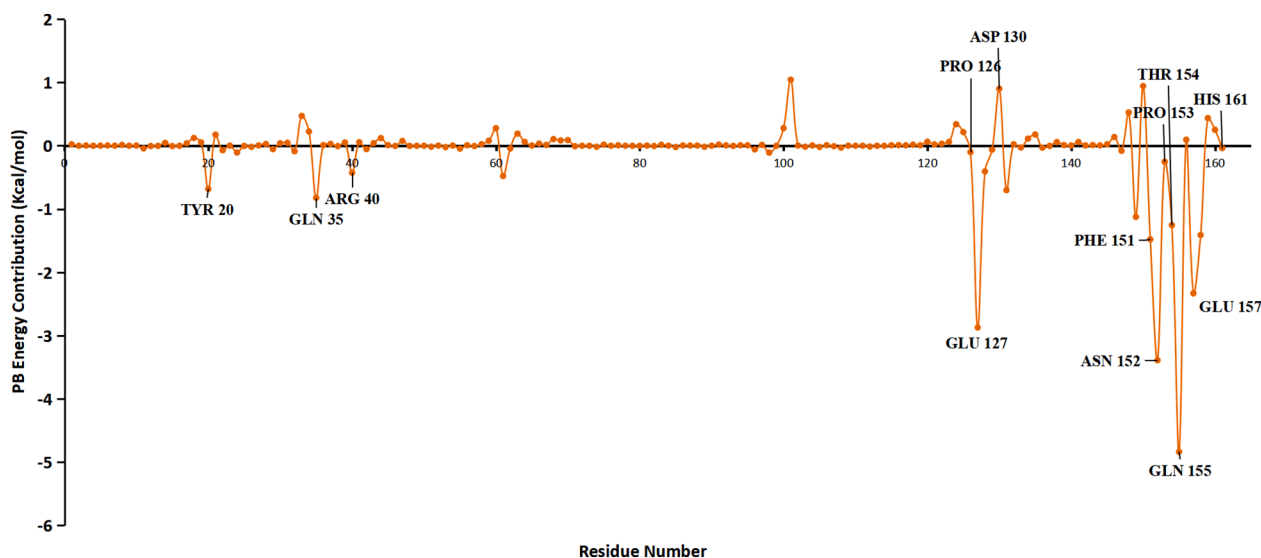
CRediT authorship contribution statement

Xin-Xin Yu: Data curation, Writing - original draft, Methodology. **Wei-Yue Liang:** Visualization, Investigation. **Jia-Yi Yin:** Supervision. **Qian Zhou:** Software, Validation. **Dong-Mei Chen:** Writing - review &

Table 2Free energy decomposition of β -LG residue at the interaction position.

Residue	ΔG_{vdw} (kcal/mol)	ΔG_{ele} (kcal/mol)	ΔG_{PB} (kcal/mol)	$\Delta G_{\text{non_polar}}$ (kcal/mol)	$\Delta G_{\text{residues}}$ (kcal/mol)
Tyr20	-0.22	-0.11	-0.32	-0.03	-0.68
Gln35	-1.26	5.00	-4.39	-0.18	-0.82
Arg40	1.19	-38.46	36.54	0.30	-0.43
Pro126	-0.54	-0.30	0.95	-0.10	-0.10
Glu127	2.06	-104.15	99.76	-0.54	-2.87
Asp130	-0.04	-43.19	44.15	-0.02	0.90
Phe151	-1.92	-1.23	1.88	-0.21	-1.48
Asn152	-3.80	-0.35	1.30	-0.54	-3.39
Pro153	-0.78	-0.92	1.50	-0.05	-0.25
Thr154	-2.74	-1.55	3.55	-0.51	-1.25
Gln155	-2.71	-3.96	2.20	-0.37	-4.84
Glu157	-0.64	-82.43	81.76	-0.36	-2.33
His161	-1.46	-2.60	4.11	-0.08	-0.04
Ile162	-	-	-	-	-

Note: E_{vdw} : van der Waals; E_{ele} : electrostatic energy; G_{PB} : polar solvation energy; $G_{\text{non_polar}}$: non-polar solvation energy; ΔG_{bind} : binding free energy. -: no calculating.

**Fig. 3.** Decomposition of MMPBSA free energy per residue in β -LG—IgG complex.

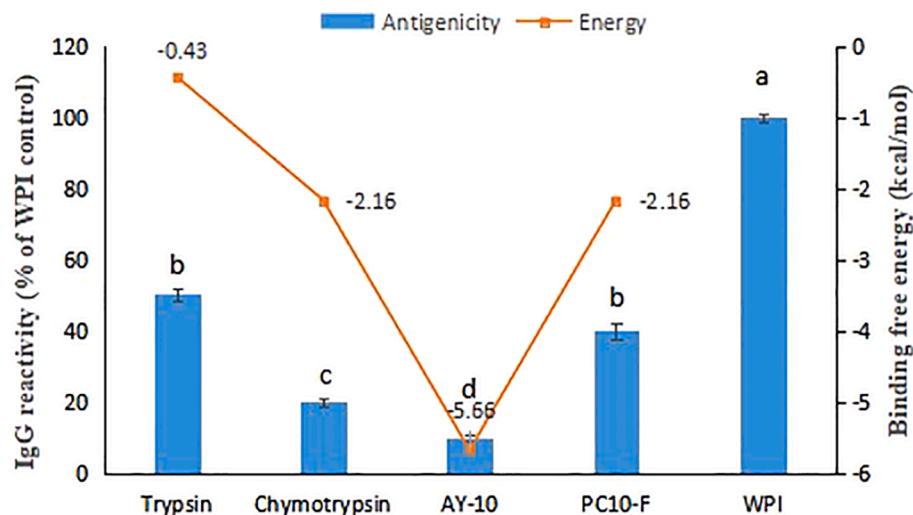


Fig. 4. Energy contribution of specific cleavage sites and antigenicity of enzyme-treated WPI hydrolysate with DH of 6%. Different letters (a–d) above columns indicate that the data were significantly different ($P < 0.05$).

editing. **Ying-Hua Zhang:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This work was funded by the National Natural Science Foundation of China (Grant No. 31571930).

References

- Abro, A., & Azam, S. S. (2016). Binding free energy based analysis of arsenic (+3 oxidation state) methyltransferase with S-adenosylmethionine. *Journal of Molecular Liquids*, 220, 375–382.
- Andre, I., Strauss, C. E., Kaplan, D. B., Bradley, P., & Baker, D. (2008). Emergence of symmetry in homooligomeric biological assemblies. *Proceedings of the National Academy of Sciences of the United States of America*, 105, 16148–16152.
- Biswas, R., & Bagchi, A. (2017). Inhibition of traf6-ubc13 interaction in nfkb inflammatory pathway by analyzing the hotspot amino acid residues and protein-protein interactions using molecular docking simulations. *Computational Biology & Chemistry*, 116.
- Bocik, W. E., Sircar, A., Gray, J. J., & Tolman, J. R. (2011). Mechanism of polyubiquitin chain recognition by the human ubiquitin conjugating enzyme Ubc2g2. *Journal of Biological Chemistry*, 286, 3981–3991.
- Boyano-Martínez, T., García-Ara, C., Pedrosa, M., Díaz-Pena, J. M., & Quirce, S. (2009). Accidental allergic reactions in children allergic to cow's milk proteins. *Journal of Allergy and Clinical Immunology*, 123(4), 883–888.
- Chaudhury, S., & Gray, J. J. (2009). Identification of structural mechanisms of HIV-1 protease specificity using computational peptide docking: Implications for drug resistance. *Structure*, 17, 1636–1648.
- Chaudhury, S., Berrondo, M., Weitzner, B. D., Muthu, P., Bergman, H., & Gray, J. J. (2011). Benchmarking and analysis of protein docking performance in rosetta v3.2. *PLoS One*, 6(8), e22477.
- Chicon, R., Belloque, J., Alonso, E., Martín-Alvarez, P. J., & Lopez-Fandino, R. (2008). Hydrolysis under high hydrostatic pressure as a means to reduce the binding of beta-lactoglobulin to immunoglobulin E from human sera. *Journal of Food Protection*, 71(7), 1453–1459.
- Chicón, R., López-Fandiño, R., Alonso, E., & Belloque, J. (2008). Proteolytic pattern, antigenicity, and serum immunoglobulin E binding of β -lactoglobulin hydrolysates obtained by pepsin and high-pressure treatments. *Journal of Dairy Science*, 91(3), 928–938.
- Chu, W. T., Zheng, Q. C., Wu, Y. J., Zhang, J. L., & Zhang, H. X. (2013). Molecular dynamics (md) simulations and binding free energy calculation studies between inhibitors and type ii dehydroquinase (dhq2). *Molecular Simulation*, 39(2), 137–144.
- Cong, Y. J., & Li, L. F. (2012). Identification of the critical amino acid residues of immunoglobulin E and immunoglobulin G epitopes in β -lactoglobulin by alanine scanning analysis. *Journal of Dairy Science*, 95(11), 6307–6312.
- Daoud, I., Melkemi, N., Salah, T., & Ghalem, S. (2018). Combined QSAR, molecular docking and molecular dynamics study on new acetylcholinesterase and butyrylcholinesterase inhibitors. *Computational Biology and Chemistry*, 74, 304–326.
- Deng, Y., van der Veer, F., Sforza, S., Gruppen, H., & Wierenga, P. A. (2018). Towards predicting protein hydrolysis by bovine trypsin. *Process Biochemistry*, 65, 81–92.
- Duan, C., Huo, G., Yang, L., Ren, D., & Chen, J. (2012). Comparison of sensitization between beta-lactoglobulin and its hydrolysates. *Asian Pacific Journal of Allergy and Immunology*, 30(1), 32–39.
- Dušeková, E., Garajová, K., Yavaşer, R., Varhač, R., & Sedláč, E. (2018). Hofmeister effect on catalytic properties of chymotrypsin is substrate-dependent. *Biophysical Chemistry*, 243, 8–16.
- Eugenia Lucena, M., Alvarez, S., Menéndez, C., Riera, F. A., & Alvarez, R. (2006). Beta-lactoglobulin removal from whey protein concentrates. *Separation and Purification Technology*, 52(2), 310–316.
- Evoli, S., Guzzi, R., & Rizzuti, B. (2014). Molecular simulations of beta-lactoglobulin complexed with fatty acids reveal the structural basis of ligand affinity to internal and possible external binding sites. *Proteins*, 82, 2609–2619.
- Feng, N., Zhang, H., Li, Y., Liu, Y., Xu, L., Wang, Y., ... Tian, J. (2020). A novel catalytic material for hydrolyzing cow's milk allergenic proteins: Papain-Cu₃(PO₄)₂·3H₂O-magnetic nanoflowers. *Food Chemistry*, 311, 125911.
- Hernández-Ledesma, B., Ramos, M., Recio, I., & Amigo, L. (2006). Effect of β -lactoglobulin hydrolysis with thermolysin under denaturing temperatures on the release of bioactive peptides. *Journal of Chromatography A*, 1116(1–2), 31–37.
- Hochwallner, H., Schulmeister, U., Swoboda, I., Spitzauer, S., & Valenta, R. (2014). Cow's milk allergy: From allergens to new forms of diagnosis, therapy and prevention. *Methods*, 66(1), 22–33.
- Hofmaier, S., Hatzler, L., Rohrbach, A., Panetta, V., Hakimeh, D., Bauer, C. P., ... Matricardi, P. M. (2015). "Default" versus "pre-atopic" IgG responses to foodborne and airborne pathogenesis-related group 10 protein molecules in birch-sensitized and nonatopic children. *Journal of Allergy and Clinical Immunology*, 135(5), 1367–1374.
- Jarvinen, K. M., Chatchatee, P., Bardina, L., Beyer, K., & Sampson, H. A. (2001). IgE and IgG binding epitopes on alpha-lactalbumin and beta-lactoglobulin in cow's milk allergy. *International Archives of Allergy and Immunology*, 126(2), 111–118.
- Kim, H. O., Li, C., & Eunice, C. Y. (2006). Quantitative structure-activity relationship study of bitter peptides. *Journal of Agricultural and Food Chemistry*, 54(26), 10102–10111.
- Li, Y., Yu, J., Goktepe, I., & Ahmedna, M. (2016). The potential of papain and alcalase enzymes and process optimizations to reduce allergenic gliadins in wheat flour. *Food Chemistry*, 196, 1338–1345.
- Ma, S., Wang, C., & Guo, M. (2018). Changes in structure and antioxidant activity of β -lactoglobulin by ultrasound and enzymatic treatment. *Ultrasonics Sonochemistry*, 43, 227–236.
- North, B., Lehmann, A., & Dunbrack, R. L. (2011). A new clustering of antibody CDR loop conformations. *Journal of Molecular Biology*, 406(2), 228–256.
- Oliveira, J. P. B., Candreva, A. M., Rizzo, G., Ramos, M. V., Oliveira, J. S., Oliveira, H. D., ... Freitas, C. D. T. (2019). Allergenicity reduction of cow's milk proteins using latex peptidases. *Food Chemistry*, 284, 245–253.
- Pandit, D., Tuske, S. J., Coales, S. J., Sook Yen, E., Liu, A., Lee, J. E., ... Hamuro, Y. (2012). Mapping of discontinuous conformational epitopes by amide hydrogen/deuterium exchange mass spectrometry and computational docking. *Journal of Molecular Recognition*, 25(3), 114–124.
- Prioult, G., Pecquet, S., & Fliss, I. (2005). Allergenicity of acidic peptides from bovine β -lactoglobulin is reduced by hydrolysis with *Bifidobacterium lactis* NCC362 enzymes. *International Dairy Journal*, 15(5), 439–448.

- Rahaman, T., Vasiljevic, T., & Ramchandran, L. (2017). Digestibility and antigenicity of β -lactoglobulin as affected by heat, pH and applied shear. *Food Chemistry*, 217, 517–523.
- Sivasubramanian, A., Chao, G., Pressler, H. M., Wittrup, K. D., & Gray, J. J. (2006). Structural model of the mAb 806-EGFR complex using computational docking followed by computational and experimental mutagenesis. *Structure*, 14(3), 401–414.
- Sivasubramanian, A., Maynard, J. A., & Gray, J. J. (2008). Modeling the structure of mAb 14B7 bound to the anthrax protective antigen. *Proteins*, 70, 218–230.
- Spellman, D., McEvoy, E., Cuinn, O. G., & FitzGerald, R. J. (2003). Proteinase and exopeptidase hydrolysis of whey protein: Comparison of the TNBS, OPA and pH stat methods for quantification of degree of hydrolysis. *International Dairy Journal*, 13(6), 447–453.
- Suresh, P. K., Divya, N., Nidhi, S., & Rajasekaran, R. (2018). Phenytoin-bovine serum albumin interactions – modeling plasma protein – drug binding: A multi-spectroscopy and in silico-based correlation. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 193, 523–527.
- Tu, M., Cheng, S., Lu, W., & Du, M. (2018). Advancement and prospects of bioinformatics analysis for studying bioactive peptides from food-derived protein: Sequence, structure, and functions. *TrAC Trends in Analytical Chemistry*, 105(8), 7–17.
- Vorob Ev, M. M., Butré, C. I., Sforza, S., Wierenga, P. A., & Gruppen, H. (2016). Demasking kinetics of peptide bond cleavage for whey protein isolate hydrolysed by *Bacillus licheniformis* protease. *Journal of Molecular Catalysis B: Enzymatic*, 133, S426–S431.
- Wang, H., Chen, Y., Huang, C., Diao, M., & Zhou, Y. (2017). Insight into the function of the key residues in the binding clefts of the substrate with CBM4-2 of xylanase Xyn10A by molecular modeling and free energy calculation. *Computational and Theoretical Chemistry*, 1111, 14–19.
- Xu, J., Tack, D., Hughes, R. A., Ellington, A. D., & Gray, J. J. (2014). Structure-based non-canonical amino acid design to covalently crosslink an antibody–antigen complex. *Journal of Structural Biology*, 185(2), 215–222.
- Yu, X., Liu, C., Lu, M., Liu, Y., Yin, J., & Zhang, Y. (2019). Impact of enzymatic hydrolysis followed by transglutaminase-induced cross-linking on decreasing antigenicity and reserving partial interfacial properties of whey protein isolate. *Food & Function*, 10(3), 1653–1660.
- Zhong, J., Fu, S., Yu, H., Zhou, L., Liu, W., Liu, C., & Prakash, S. (2018). Antigenicity of β -lactoglobulin reduced by combining with oleic acid during dynamic high-pressure microfluidization: Multi-spectroscopy and molecule dynamics simulation analysis. *Journal of Dairy Science*, 102(1), 145–154.