

Milk protein hydrolysates obtained with immobilized alcalase and neutrase on magnetite nanoparticles: Characterization and antigenicity study

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Abstract: Enzymatic hydrolysis is the most commonly used method to reduce the antigenicity of milk protein, but free protease is unstable and difficult to recycle after application. In this study, alcalase and neutrase were selected for immobilization on the modified magnetic Fe₃O₄ nanoparticles. The reusability of the immobilized enzyme was 68.23% of the total starting activity after 5 recycling batches. The optimal hydrolysis conditions were an enzyme to substrate ratio of 6000 U/g and reaction at 50°C and pH 8.5 for 3 h. Under these conditions, 22.76% hydrolysis of hydrolysate was achieved, and the antigenicity reduction rates of β -lactoglobulin and casein were 21.34% and 30.89%, respectively. In addition, 82.75% of the hydrolysate had a molecular weight less than 1 kDa, and free amino acids represented 13.65% of the sample. This result showed that the hydrolysis with immobilized enzyme was similar to that with free enzyme and the immobilized enzyme could be applied to produce hypoallergenic hydrolysate.

KEYWORDS

allergenicity, enzyme immobilization, hydrolysis, magnetic nanometer particles, milk protein

Practical Application: Reduces milk protein allergenicity.

1 | INTRODUCTION

Cow milk is a nutritional food and is the best substitute for breast milk, but its proteins, such as caseins (CAS), β -lactoglobulin (BLG), and α -lactalbumin (ALA), can produce allergic reactions (Bunyavanich & Mcba, 2019). The incidence of milk allergies is as high as 2–7.5% for infants and young children, which affects their growth and development and even threatens their lives (Solinas et al., 2010). Although food allergies are often overcome by elimination

from the diet, milk is an important source of nutrition for infants who cannot breastfeed. Thus, partially or extensively hydrolyzed milk formulas are used as an alternative for infants with milk allergies.

Enzymatic hydrolysis is one of the most commonly used methods to reduce protein allergenicity by destroying epitopes (Tavano et al., 2018). The specificity of the protease determines the hydrolysis site of the allergenic protein, thereby determining the allergenicity of the hydrolysate. Proteases, such as alcalase and neutrase, are referred to as

less specific proteases. These proteases are highly effective in reducing the allergenicity of proteins because they can cleave more epitopes than specific proteases. For example, alcalase exhibits an increased ability to reduce raw peanut allergenicity (the maximum reduction of Ara h 1, Ara h 2, and Ara h 6 were 100%, 100%, and 99.8%, respectively) (Yu & Mikiashvili, 2020). When different enzymes are used in combination, the synergy between them is anticipated to cleave more positions on the larger peptide to obtain hypoallergenic hydrolysates. The specific protease trypsin and less specific protease alcalase were used in combination to hydrolyze *Trachinus draco* protein, and the degree of hydrolysis was 78%, which was considerably greater than 44% reported for single enzyme hydrolysis (Kula et al., 2020).

In addition, enzymes in a free form need to be inactivated after hydrolysis, which is generally achieved by heating. Heat treatment can change the antigenicity of the hydrolysates by exposing or masking the unhydrolyzed epitope in the peptides (Rahaman et al., 2015; Taheri-Kafrani et al., 2009). The immobilized enzyme can eliminate the change in hydrolysate caused by heating inactivation, and the enzyme-free hydrolysate can be easily obtained. Magnetic nanoparticles are new organic-inorganic functional materials composed of polymers and magnetic materials. Among them, iron oxide (Fe_3O_4) nanoparticles are currently the most popular magnetic immobilization materials and have attracted attention in enzyme immobilization due to their high saturation magnetization, superparamagnetism, and large specific surface area (Cheng et al., 2016; Dong et al., 2015). Various functional groups, such as hydroxyl, amino, and aldehyde groups, can be attached through surface functionalization, which aims to promote the appearance of enzyme linkages and introduce chemical groups required for immobilization of enzymes. Magnetic nanoparticles can recover enzymes through the application of external magnetic field, and these enzymes can be reused in production, further expanding their application in the field of biotechnology (Lee et al., 2019; Rodrigues et al., 2019). Z. Chen et al. (2018) used chitosan and sodium tripolyphosphate-coated magnetic nanoparticles ($\text{Fe}_3\text{O}_4@CS\text{-}TPP$) as a carrier to develop a new and efficient method for immobilizing complex enzymes composed of alcalase and trypsin. They found that $\text{DE-Fe}_3\text{O}_4@CS\text{-}TPP$ nanoparticles exhibit better stability. After 35 days of storage, 86% of the activity remained. Moreover, after 10 rounds of repeated use, they retained greater than 60% of the initial activity.

The methods used to immobilize enzymes have been extensively studied, but studies on the complex hydrolysis of whole milk protein by immobilized enzymes to reduce allergenicity are limited. In this study, we immobilized alcalase and neutrase on amino functionalized Fe_3O_4

nanoparticles to prepare immobilized complex enzymes. The effectiveness of immobilized enzymes in reducing the allergens of CAS and BLG in milk proteins was evaluated.

2 | MATERIALS AND METHODS

2.1 | Materials

Milk protein concentrate (MPC 4766) containing 70.05 g/100 g protein, 1 g/100 g fat, 16 g/100 g lactose, 4.9 g/100 g moisture, and 6.9 g/100 ash was purchased from Beijing Milky Way Trade Corp (Beijing, China). Alcalase (200 U/mg powder) and neutrase (100 U/mg powder) were purchased from the Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). Nano Fe_3O_4 (99.5%, 20 nm spherical) was purchased from Tianjin Lian Electronic Technology Co., Ltd. (Tianjin, China). Bovine CAS ELISA kit and Bovine BLG ELISA kit were purchased from Genview Scientific, Inc. (Florida, USA). All other chemicals were of analytical grade.

2.2 | Complex enzyme immobilization

Amino-modified Fe_3O_4 nanoparticles were prepared using the method described by Akhond et al. (2016) with some modifications. Briefly, 0.145 g Fe_3O_4 was suspended in 40 ml anhydrous ethanol. Then 6 ml H_2O , 3 ml ammonia (25%, v/v), and 0.4 M tetraethyl orthosilicate were added at room temperature. This mixture was stirred at 650 r/min for 6 h to obtain the silica-coated Fe_3O_4 nanoparticles (MNPs). Then, (3-aminopropyl) triethoxysilane (APTEs) was reacted with MNPs at 50°C for 6 h under stirring to prepare amino-modified nanoparticles (AMNPs). The collected AMNPs were reacted with glutaraldehyde (5%, v/v) at room temperature for 6 h and separated with a permanent magnet for the immobilization. Complex enzyme composed of alcalase and neutrase (the mass ratio was 1:1) was mixed with 1 ml phosphate buffer (0.2 mol/L, pH 8.5) and 10 mg AMNPs were added to the solution. The mixture was incubated in a constant temperature shaker at 25°C for 6 h under gentle stirring. The immobilized enzyme (AMNPs-A+N) was collected with a permanent magnet, and washed thrice in phosphate buffer (0.2 mol/L, pH 8.5). The obtained AMNPs-A+N were freeze-dried and stored in a dryer preservation for the further study.

The chemical composition of the nanoparticles was analyzed using a Vector22 FTIR (Bruker, Karlsruhe, Germany) spectrometer with a distributed temperature gradient sensing (DTGS) detector. All spectra were recorded within the range of 4,000–500 cm^{-1} with a 4 cm^{-1} resolution and 16 scans. The morphology of the nanoparticles was observed using scanning electron microscopy (SEM)

(Hitachi SU1510, Japan). SEM samples were sputter coated with gold and captured at an acceleration voltage of 5 kV.

2.3 | Enzyme activity assay

Enzyme activity was determined in the free complex enzyme (Free-A+N) and immobilized complex enzyme (AMNPs-A+N) preparations using 2% (w/v) casein solution in 0.1 mol/L sodium phosphate buffer at pH 8.5. The reaction was incubated at 40°C for 10 min and stopped after adding 0.4 mol/L trichloroacetic acid. After centrifugation at $1300 \times g$ for 10 min at 4°C, the supernatant was collected and measured at 680 nm using a microplate reader (Synergy HTx, BioTek, Winsooki, VT, USA). One unit (U) of complex enzyme activity was defined as the amount of enzyme that hydrolyzed casein to form 1 μ g of tyrosine per minute under the described conditions (Shigenori Emi et al., 1976). The enzyme activity recovery was defined as the activity of the immobilized enzyme to that of the total activity of the free enzyme:

Activity recovery (%) =

(Immobilized enzyme activity/Total free enzymes activity)
 $\times 100$

2.4 | Reusability assay

The reusability of AMNPs-A+N was detected by the measurement of enzyme activity after immobilized enzyme hydrolyzed the MPC. In detail, AMNPs-A+N were added to the MPC 4766 solution (5%, w/v), and reacted at pH 8.5 and 50°C for 3 h. After each cycle, AMNPs-A+N were collected by a permanent magnet, and the residual activity was assayed as described earlier. The relative activity was expressed as a percentage of the initial enzyme activity.

2.5 | Optimization of hydrolysis conditions

MPC 4766 (5%, w/v) was dissolved in deionized water and agitated for 2 h in a 50°C water bath. Then, the MPC solution was cooled down to room temperature and hydrated at 4°C for 12 h. The reaction conditions for AMNPs-A+N hydrolysis of MPC were optimized. Identification of the optimal enzyme to substrate ratio (E:S), hydrolysis time, temperature, and pH was performed based on a one-variable-at-a-time approach. E:S (1000, 2000, 4000, 6000, and 8000 U/g), hydrolysis time (0.5, 1, 1.5, 2, 3 h), hydrolysis temperature (40, 45, 50, 55, and 60°C), and pH

(7.5, 8, 8.5, 9, and 9.5) were individually evaluated for their performances in hydrolysis MPC. The remaining reaction factors were fixed as an E:S of 6000 U/g, hydrolysis time of 3 h, hydrolysis temperature of 50°C, and pH 8.5. The pH of MPC solution (7.5, 8, 8.5, 9, and 9.5) was adjusted using 1 mol/L sodium hydroxide. After the reaction, AMNPs-A+N were separated by a permanent magnet, and the hydrolysates were freeze-dried and stored at -20°C.

2.6 | Degree of hydrolysis

The formaldehyde titration method was used to determine the degree of hydrolysis (DH) (H. Chen, 2003). One milliliter of hydrolysate solution was mixed with 60 ml distilled water, and the pH was adjusted to 8.2. After adding 10 ml formaldehyde, the pH was adjusted to 9.2 with 0.1 mol/L sodium hydroxide. A blank group with deionized water served as the control. The calculation method of DH as followed:

$$DH(\%) = \frac{c \times (V_1 - V_2)}{V_3 \times S \times W \times h_{\text{tot}}} \times 100$$

where c is the concentration of NaOH solution (0.1 mol/L); V_1 is the volume of standard NaOH solution consumed by sample during titration (mL); V_2 is the volume of standard NaOH solution consumed by blank (mL); V_3 is the volume of sample (1 ml); S is the substrate concentration (g/ml); W is the content of protein (%); and h_{tot} is the total number of peptide bonds in the protein (h_{tot} milk protein was 6.38 mmol/g).

2.7 | Antigenicity determination

The concentrations of residual BLG and CAS in the MPC hydrolysates obtained with Free-A+N and AMNPs-A+N were determined using ELISA kits according to the manufacturer's instructions. The antigenicity reduction rate was calculated using the following equation:

$$\text{Antigenicity reduction rate (\%)} = (M - N) / M \times 100$$

where M is BLG/CAS concentration in MPC (μ g/ml); N is BLG/CAS concentration in hydrolysates obtained with Free-A+N or AMNPs-A+N (μ g/ml).

2.8 | Electrophoresis

SDS-PAGE was performed using a 12% polyacrylamide separating gel and 5% stacking gel. The hydrolysate was

also elucidated by SDS-PAGE-Tricine according to the method of Pessato et al. (2016). The polyacrylamide concentrations of the separating gel, spacer gel, and stacking gels were 16.5%, 10%, and 4%, respectively. Samples were diluted with reducing buffer to a protein concentration of 25 mg/ml, and 10 μ l of each sample was loaded onto the gel. Electrophoresis was performed using a Mini-Protean II (Bio-Rad Laboratories Inc., Hercules, CA, USA), and the images were scanned with the Gel Doc XR system.

2.9 | Reversed-phase high performance liquid chromatography

Chromatographic analysis of hydrolysates was determined by an Agilent 1260 HPLC system (Agilent, Snoqualmie, WA, USA) equipped with a TSKgelG2000swxl column (300 mm $\times$ 7.8 mm, Waters Corporation, Milford, MA, USA) at 25°C. The mobile phase consisted of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B). The gradient was eluted with mobile phase B from 0 to 100% over 30 min at a flow rate of 0.5 ml/min. The absorbance at 220 nm was monitored using a UV detector. Prior to analysis, each sample (1 mg/ml) was filtered through a 0.45 μ m polytetrafluoroethylene membrane, and 20 μ l was injected. Cytochrome C (MW 12.5 kDa), aprotinin (MW 6.5 kDa), bacitracin (MW 1.45 kDa), acetamide-acetamide-arginine (MW 4.51 kDa), and acetamide-acetamide-acetamine (MW 0.189 kDa) (Bio-Rad, Hercules, CA, USA) were used as standards. The percentage relative area (corresponding to each peak) was analyzed using Star Chromatography Workstation software (Agilent 1200 series, USA).

2.10 | Statistical analysis

Statistical analysis was performed by one-way analysis variance (ANOVA) using SPSS Statistics 20.0 (IBM Corp., Armonk, NY, USA). All data were measured thrice and expressed as the mean $\pm$ standard deviation (SD). Significant differences ($p < 0.05$) were determined using Duncan's multiple range test.

3 | RESULTS AND DISCUSSION

3.1 | Complex enzyme immobilization

The modification of Fe₃O₄ nanoparticles and the binding of the complex enzyme onto AMNPs were analyzed by FT-IR (Figure 1). The wide absorption band at

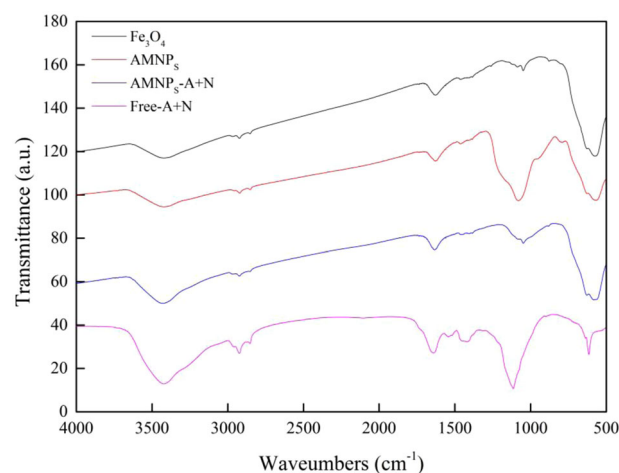
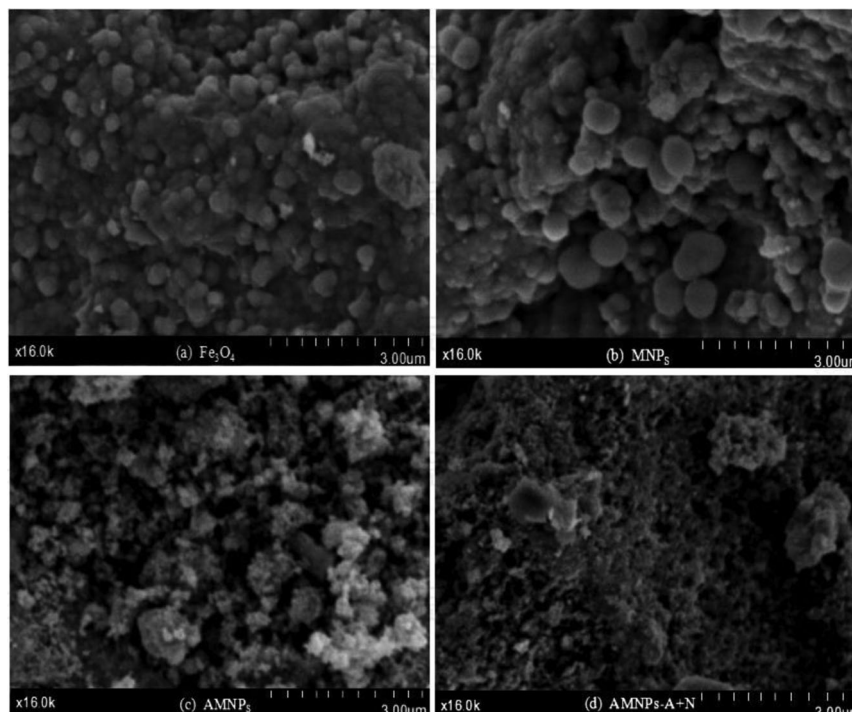


FIGURE 1 FT-IR spectrum of the sample: (a) Fe₃O₄, (b) AMNPs, (c) AMNPs-A+N and (d) Free-A+N. Fe₃O₄: Iron oxide nanoparticles; AMNPs: Amino-modified nanoparticles; AMNPs-A+N: Immobilized complex enzyme composed of alcalase and neutrase; Free-A+N: Free complex enzyme composed of alcalase and neutrase

approximately 594.5 cm⁻¹ in the FT-IR spectra corresponds to the stretching vibration of Fe–O, indicating the presence of Fe₃O₄ nanoparticles in AMNPs (Figure 1b) and AMNPs-A+N (Figure 1c) (Fang et al., 2016). The coating of silica and the amino modification of Fe₃O₄ nanoparticles were confirmed by the bands at 1080.7 cm⁻¹ and 1570 cm⁻¹, which correspond to the Si–O antisymmetric stretching vibration and N–H vibration, respectively (Poor Heravi et al., 2022). Compared with Figure 1b, the absorption peak of –OH near 3420 cm⁻¹ in Figure 1c has obvious changes, which can be attributed to the introduction of the CO–NH of the complex enzyme. Moreover, the characteristic absorption peaks of proteins at 1700 cm⁻¹–1500 cm⁻¹ (amide I and amide II) appeared in the spectra of AMNPs-A+N (M. Wang et al., 2018), indicating that the complex enzyme had been immobilized onto the surface of the AMNPs.

The micrographs captured using SEM showed the morphologies of the Fe₃O₄ nanoparticles (Figure 2a), MNPs (Figure 2b), AMNPs (Figure 2c), and AMNPs-A+N (Figure 2d). Fe₃O₄ nanoparticles were spherical or ellipsoidal with a diameter of 20 nm and clustered together according to the SEM observation (Figure 2a). As Fe₃O₄ nanoparticles magnetically self-aggregated into agglomerates, they were coated with SiO₂ (Figure 2b). The particle size increased after coating, but the uniformity was not good potentially due to uneven mechanical stirring during the coating process (Zhai et al., 2021). The surface of the aminated MNPs had depressions and holes, which were suitable as a carrier material for enzyme immobilization (Figure 2c) (N. Li et al., 2014). After

FIGURE 2 SEM images of (a) Fe_3O_4 , (b) MNPs, (c) AMNPs, and (d) AMNPs-A+N, the magnification was 16.0 k $\times$. Fe_3O_4 : iron oxide nanoparticles; MNPs: Silica-coated Fe_3O_4 nanoparticles; AMNPs: Amino-modified nanoparticles; AMNPs-A+N: Immobilized complex enzyme composed of alcalase



immobilization (Figure 2d), the depressions and holes were filled with enzymes, indicating that the enzymes were successfully immobilized. However, the particles seem to be aggregated potentially because the reaction occurred on the surface of the AMNPs nanoparticles (Aslani et al., 2018).

The activity of the immobilized complex enzyme was 84 U/mg protein, which was 66.6% of the activity of Free-A+N (126 U/mg protein). The low activity might be due to the limited binding sites (covalent amide bonds) on the surface of AMNP_s nanoparticles. In addition, the overload (saturation) of the enzyme led to the formation of intermolecular steric hindrance of lipoproteins on the surface of nanoparticles, inhibiting the diffusion of substrates and products and thereby affecting the catalytic activity and stability of the enzyme (K. Wang et al., 2019). Reusability is an important factor in the application of immobilized enzymes. AMNPs-A+N still retained 68.23% of its initial activity after 5 consecutive rounds of hydrolysis. The presence of APTes, a surfactant, could help stabilize the catalytic conformation of the enzyme and inhibit its inactivation (Santos et al., 2015). However, the substrate or product molecules were attached to the surface of the enzyme during the hydrolysis reaction, which masked its active sites and resulted in a gradual decrease in activity (L. Li et al., 2013). Moreover, enzyme shedding from the carrier and the loss of immobilized enzyme during recovery also led to a decrease in enzyme activity (Fang et al., 2016).

3.2 | Optimization of hydrolysis conditions

DH is usually used as a monitoring parameter for protein hydrolysis to evaluate the degree of hydrolysis of peptide bonds (Y. Zhang et al., 2018). Moreover, antigenicity testing of the hydrolysate is one of the significant components in the production of hydrolyzed milk formulas for infants and children diagnosed with cow's milk protein allergy (van Esch et al., 2020).

In this study, the factors of hydrolysis (E:S, hydrolysis time, temperature, and pH) were optimized. The DH and antigenicity reduction rate were chosen to evaluate the hydrolysis. As shown in Figure 3a, DH increased along with the increasing of E:S, but remained constant after E:S reached 6000 U/g. This finding might be due to the saturation of the substrate concentration relative to the enzyme. The antigenicity reduction rates of BLG and CAS were also increased and reached the maxima of 18.99 and 29.53% at 6000 U/g, respectively. Moreover, the allergen reduction rate of CAS was significantly greater than that of BLG. This finding was consistent with a previous study showing that whey protein had low susceptibility to enzyme hydrolysis (Dabrowska et al., 2020). The effect of hydrolysis time on the DH and antigenicity reduction rate were also studied, and the results are shown in Figure 3b. DH increased as the hydrolysis time increased from 0.5 to 2 h, and then started to increase slowly at 3 h, increasing by only 1.22%. The antigenicity reduction rate was increased within 3 h for CAS

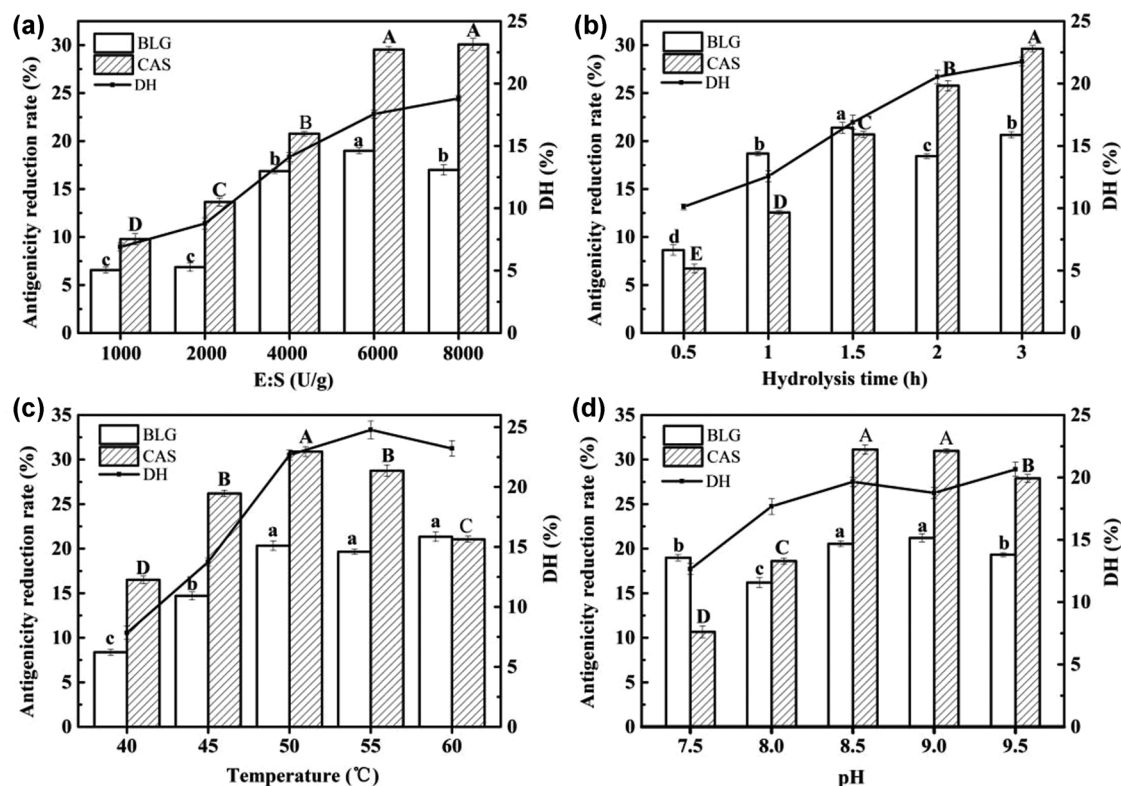


FIGURE 3 The effect of (a) E:S, (b) hydrolysis time, (c) hydrolysis temperature, and (d) pH on the antigenicity reduction rate and DH of hydrolysis milk protein. Data are given as means plus/minus standard deviation, $n = 3$. Different lower-case letters on the bars indicate significant differences in antigenicity reduction rate of BLG ($p < 0.05$); different capital letters on the bars indicate significant difference between antigenicity reduction rate of CAS values ($p < 0.05$). E:S: Enzyme to substrate mass ratio; DH: Degree of hydrolysis; BLG: β -lactoglobulin; CAS: Caseins

and 1.5 h for BLG. Dabrowska et al. (2020) found that the antigenicity of whey protein concentrate was reduced after 1 h hydrolysis, but a new reactive epitope would be exposed after hydrolysis for 5 h, thereby increasing the antigenicity. Considering the hydrolysis effect and actual production benefit, the comprehensive results showed that 6000 U/g and 3 h were the optimal E:S and hydrolysis time for MPC.

The DH and antigenicity reduction rate were assessed after hydrolysis of MPC by AMNPs-A+N under different temperatures and pH values. The reaction temperature ranged from 40 to 60°C. As shown in Figure 3c, increasing the temperature was able to improve the DH, but a steep decrease for DH was observed at 60°C. Regarding the antigenicity reduction rate of MPC, no significant difference was observed at 50, 55, and 60°C. This result indicated that the immobilized complex enzyme had a similar thermal stability to the free alcalase and neutrase (50–55°C). Figure 3d shows that both the DH and antigenicity reduction rate achieved maximum values at pH 8.5, 9.0, and 9.5. However, it should be noted that free alcalase (pH 9–10) and neutrase (pH 7–7.5) have different optimum pH ranges, whereas the immobilized complex enzyme exhibited better stability and activity over a wide pH range (8.0–9.5)

(Wan et al., 2022). Based on the earlier-mentioned experimental results, the optimal hydrolysis conditions were identified. The E:S was 6000 U/g, and the optimal hydrolysis time, temperature, and pH were 3 h, 50°C and 8.5, respectively.

3.3 | Characterization of milk protein hydrolysates

Under the mentioned optimal conditions, MPC was hydrolyzed by AMNPs-A+N, and Free-A+N was chosen as a control. The molecular weight (MW) distribution, DH, and antigenicity of the hydrolysate were evaluated.

3.3.1 | Electrophoresis analysis

The MW distribution of AMNPs-A+N hydrolysate was observed using SDS-PAGE and SDS-PAGE-Tricine (Figure 4). The MPC solution exhibited five major bands with apparent different MWs (Figure 4a, Lane 1). Three bands with MWs between 25 and 35 kDa were CAS.

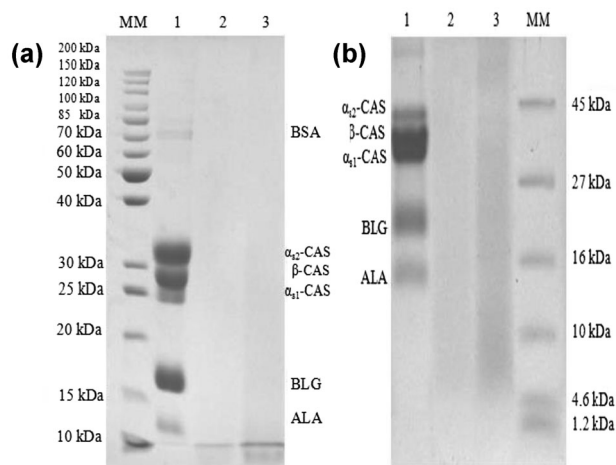


FIGURE 4 Electropherograms of MPC and hydrolysates with free and immobilized complex enzyme obtained under the conditions of E/S 6000 U/g, hydrolysis time 3 h, temperature 50°C, pH 8.5. (a) SDS-PAGE; (MM) Molecular mass marker (10–200 kDa); (1) MPC; (2) hydrolysate with Free-A+N; (3) hydrolysate with AMNPs-A+N. (b) SDS-PAGE/Tricine; (1) untreated MPC; (2) hydrolysate with Free-A+N; (3) hydrolysate with AMNPs-A+N; (MM) molecular mass marker (1.2–45 kDa). MPC: Milk protein concentrate 4766; Free-A+N: Free complex enzyme composed of alcalase and neutrase; AMNPs-A+N: Immobilized complex enzyme composed of alcalase and neutrase

The other two bands (18 and 14 kDa) corresponded to the BLG and ALA. The electrophoretic profiles of the hydrolysates obtained with Free-A+N and AMNPs-A+N showed almost complete disappearance of CAS, BLG, and ALA. Moreover, the MWs of the hydrolysates were mainly less than 10 kDa (Figure 4a, Lanes 2 and 3), indicating that the protein in the hydrolysate was mainly small molecular peptides. The SDS-PAGE-Tricine profile showed that the MW of the AMNPs-A+N hydrolysate was mainly concentrated in the 4.6–10 kDa fraction (Figure 4b, Lane 3), exhibiting a dispersed state without obvious protein bands. This result indicated that the majority of macromolecular proteins had been hydrolyzed to smaller peptides. However, no band was observed in the Free-A+N hydrolysate (Figure 4b, Lane 2), suggesting that the MW of peptides was too low to be stained. This finding indicated that the hydrolysis of the immobilized enzyme was weaker than that of the free enzyme. Z. Chen et al. (2018) evaluated the efficiency of protein hydrolysis by free enzyme and immobilized enzyme (DE-Fe₃O₄@CS-TPP MNPs) using rice bran, walnuts, adzuki beans, zein, and casein as substrates. The results showed that the DH of free enzyme on the earlier-mentioned proteins were slightly greater than that of DE-Fe₃O₄@CS-TPP MNPs, which was consistent with the earlier-given results.

3.3.2 | Chromatographic analysis

The chromatographic profiles (RP-HPLC) of the hydrolysates are shown in Figure 5. Before analyzing the MW distribution of hydrolysates, the relative MW must be calibrated with the standard. The relationship between the retention time (RT) and the logarithm of molecular weight (lgMW) is shown in Figure 5a. The calibration curve was $\lg MW = 7.454280.23007 \times RT$, $R^2 = 0.9914$. The MW distribution of hydrolysates was analyzed by the RT–lgMW relationship. The profiles of MPC exhibited three main proteins including CAS, BLG, and ALA, as shown in Figure 5b. The chromatograms of the two hydrolysates were similar, presenting more peak areas in the low MW region (< 1 kDa). This result indicated that the peptide chains in MPC were cleaved by less specific proteases, alcalase and neutrase. The MW distribution of the hydrolysates was further calculated using Star Chromatography Workstation software, and the results are shown in Figure 5c. In the hydrolysate obtained from AMNPs-A+N, MW > 20 kDa accounted for 2.36% of the sample, and MW < 1 kDa accounted for 82.75% of the sample. The result was similar to the MW distribution of Free-A+N hydrolysate (MW > 20 kDa was 1.89%, < 1 kDa was 85.31%). In general, MW < 180 Da was considered a free amino acid. The free amino acid contents in the Free-A+N and AMNPs-A+N hydrolysates were 13.65% and 15.92%, respectively, which met the requirements of free amino acid content in the extensively hydrolyzed formula (Alim et al., 2020; Vandenplas et al., 2014). Although no direct relationship existed between MW and antigenicity, many studies have showed that higher MW peptides (> 10 kDa) easily cause antigen reactions, whereas lower MW peptides (< 1 kDa) are less likely to cause milk allergies. ELISA analysis indicated that the ALA and BLG fragments with antigenicity after hydrolysis of whey protein mainly exist in the range of 1.5–6 kDa, whereas the peptides with a MW less than 4.8 kDa had low immunoreactivity and could be used as a peptide source for oral tolerance in infant formula (Wroblewska et al., 2004). Q. Zhang et al. (2020) found that the proportion of peptides with MW < 1.5 kDa in the hydrolysate of whey protein obtained by ultrasonic-ionic liquid pretreatment was obviously increased, and the antigenic decline rates of ALA and BLG upon alcalase or papain hydrolysis were greater than 80%.

3.3.3 | DH and antigenicity analysis

The DH of the hydrolysate from AMNPs-A+N was 22.76%, which was slightly lower than that of the Free-A+N

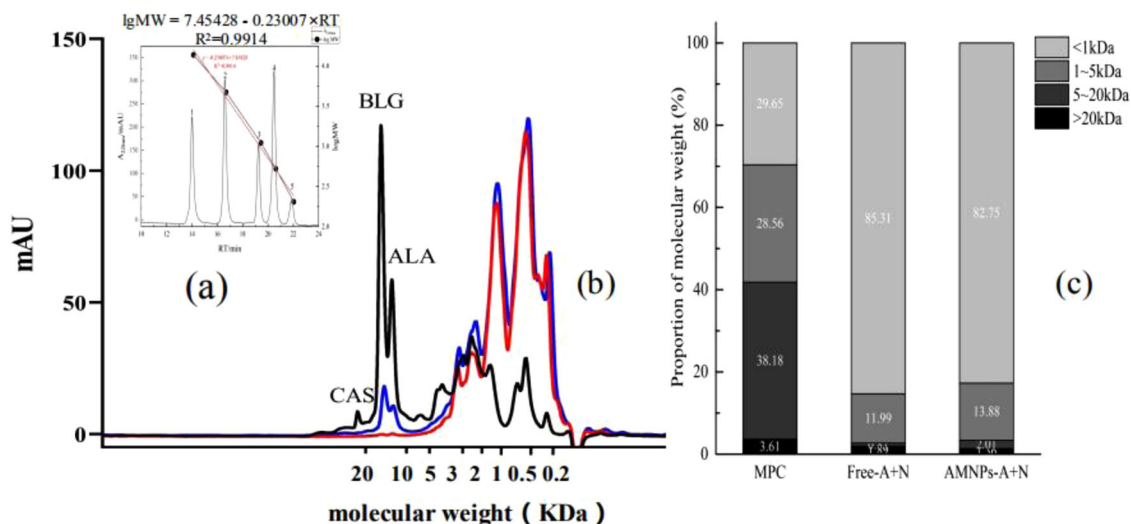


FIGURE 5 (a) High performance size exclusion chromatogram of standard proteins. (Cytochrome C; Aprotinin; Bacitracin; acetamide–acetamide–arginine and acetamide–acetamide–acetamine); (b) the molecular weight distribution content of hydrolysis milk protein. MPC (black line), Free-A+N hydrolysate (red line), AMNPs-A+N hydrolysate (blue line); and (c) proportion of molecular weight (%). MPC: Milk protein concentrate 4766; Free-A+N: Free complex enzyme composed of alcalase and neutrase; AMNPs-A+N: Immobilized complex enzyme composed of alcalase and neutrase

hydrolysate of 24.38%. This finding might be due to the proportion of complex enzymes changing during immobilization or the accessibility of the enzyme to the substrate or even the affinity of the enzyme toward a specific substrate changing due to the immobilization, resulting in a lower hydrolysis effect of the immobilized enzyme (Rocha et al., 2011).

The antigenicity reduction rates of BLG in hydrolysate of Free-A+N and AMNPs-A+N were 22.38% and 21.34%, respectively. No significant differences were noted between the two hydrolysates ($p > 0.05$). BLG existed in the natural conformation of the dimer, which was more difficult to hydrolyze than casein. Some antigenicity remained in the fragment, which was consistent with the results of Yao et al. (2013). For CAS, the antigenicity of the Free-A+N and AMNPs-A+N hydrolysates decreased by 33.76% and 30.89%, respectively. Although hydrolysis can reduce the antigenicity of BLG and CAS in milk protein, it did not completely eliminate their antigenicity. Aplebani et al. (1997) noted that in addition to specific extensively hydrolyzed casein formulas or amino acid-based formulas, residual antigenic activity could still be detected in most hydrolyzed products.

4 | CONCLUSION

In this paper, a complex enzyme composed of alcalase and neutrase was successfully immobilized onto amino-modified Fe_3O_4 nanoparticles via a glutaraldehyde cross-linking reaction. The immobilized enzyme was used to

hydrolyze milk protein, and the results showed that AMNPs-A+N had a similar effect as Free-A+N at 50°C in a pH 8.5 environment under a 3 h reaction using an E:S of 6000 U/g. Under the optimal hydrolysis conditions, the MW distribution, DH, and antigenicity reduction rate of milk protein hydrolysate met the requirements of extensively hydrolyzed formula. These findings indicate that the hydrolysate could be used for infants with milk allergies. Although the immobilized complex enzymes have been demonstrated to be a useful strategy for the production of hypoallergenic hydrolysate, further research is needed to improve the performance of immobilized enzymes.

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AUTHOR CONTRIBUTIONS

Hongbo Li: Project administration; writing—original draft; writing—review & editing. **Jingjing Yang:** Data curation; formal analysis; writing—original draft. **Airong Qin:** Resources; software. **Feifei Yang:** Methodology; resources. **Dingkuo Liu:** Investigation. **Hongjuan Li:** Project administration; supervision. **Jinghua Yu:** Project administration; supervision; writing—review & editing.

CONFLICT OF INTEREST

Authors declare no financial/commercial conflicts of interest.

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