

A novel catalytic material for hydrolyzing cow's milk allergenic proteins: Papain-Cu₃(PO₄)₂·3H₂O-magnetic nanoflowers

Nuan Feng^{a,b,1}, Haiyang Zhang^{b,1}, Yao Li^c, Yangkaixi Liu^c, Longquan Xu^a, Yi Wang^b, Xu Fei^{a,*}, Jing Tian^{b,*}

^a Instrumental Analysis Center, Dalian Polytechnic University, Dalian 116034, China

^b School of Biological Engineering, Dalian Polytechnic University, Dalian 116034, China

^c School of Light Industry and Chemical Engineering, Dalian Polytechnic University, Dalian 116034, China

ARTICLE INFO

Keywords:

Magnetic nanoflowers

Papain

Activity

β-lactoglobulin (β-Lg)

ABSTRACT

Milk allergenic proteins are the main reason of cow's milk allergy. The most common method for removing milk allergenic proteins is enzymatic hydrolysis. However, the direct application of protease to hydrolyze allergens will lead to the introduction of new allergenic proteins. An ideal strategy for removing milk allergenic proteins without introducing new allergenic proteins is immobilization of protease. Herein, we established a simple method to synthesize a novel papain-Cu₃(PO₄)₂·3H₂O-magnetic nanoflowers (PCMN). The PCMN demonstrated 1556% higher activity than the free alkaline papain. Moreover, the PCMN could hydrolyze most of allergenic proteins in cow's milk, generating low-sensitive milk. In cycle analysis, the PCMN also exhibited good reusability and were easily separated from the product.

1. Introduction

Cow's milk is a highly nutritious food and is the first food introduced into an infant's diet consumed in large quantities during lactation (Hochwallner, Schulmeister, Swoboda, Spitzauer, & Valenta, 2014). However, during ingestion of cow's milk, some infant's immune system will recognize some proteins in cow's milk as harmful substances, which will trigger cow's milk protein allergic reaction, leading to infant diarrhea, vomiting, respiratory asthma and skin allergies (Cao, Zhao, Yang, He, Ding, Wang, et al., 2011; Oliveira et al., 2019; Pessato, Carvalho, Tavano, Fernandes, Zollner, & Netto, 2016). Approximately 3% of infants develop the cow's milk protein allergic reaction in the first year after birth (Bu, Luo, Chen, Liu, & Zhu, 2013; Ji et al., 2017). Unfortunately, the prevalence of the cow's milk protein allergic reaction is increasing, because cow's milk feeding is more common than breastfeeding in recent years (Exl & Fritzsche, 2001). Based on clinical studies of large populations of allergic patients, β-lactoglobulin (β-Lg) and α-lactalbumin (α-La) are identified as the major allergen for the cow's milk protein allergic reaction (Ansari, Stoppacher, Rudolf, Schuhmacher, & Baumgartner, 2011; Chen, Lee, Yang, Lin, Wang, Yu, et al., 2014). Furthermore, it is noteworthy that β-Lg is not found in human milk, implying that β-Lg is difficult for infants to absorb

(Cheison, Lai, Leeb, & Kulozik, 2011). Currently, many methods, such as heat treatment, glycation reaction, high pressure, lactic acid fermentation, and enzymatic hydrolysis, are developed to reduce the cow's milk protein allergic reaction (Bu, Luo, Chen, Liu, & Zhu, 2013). Among these methods, enzymatic hydrolysis is the most effective to remove the cow's milk protein allergic reaction by breaking down allergenic proteins into small peptide molecules and amino acids (Li, Yu, Goktepe, & Ahmedna, 2016). Compared with other methods, the enzymatic hydrolysis of allergenic proteins is very appropriate to reduce the incidence of the cow's milk protein allergic reaction and increase the cow's milk absorption.

Enzyme is a highly excellent catalyst owing to its high catalytic efficiency and substrate specificity under mild conditions (Franssen, Steunenberg, Scott, Zuillhof, & Sanders, 2013; Garcia et al., 2011; Li et al., 2013). Papain is a thiol protease commonly present in plants and fungi and it can hydrolyze proteins including most of the proteins in cow's milk that cause allergies. (Homaei et al., 2014). Papain exhibits broad proteolytic activity and is used in various industrial applications, especially in food industry (Gu, Zhu, Li, & Xiong, 2018). For example, papain has been used to tenderize meat products, produce protein hydrolysate, clarify juice and beer, and produce cheese. However, the direct utilization of papain is limited by poor recovery and the

* Corresponding Author.

E-mail addresses: feixudlpu@163.com (X. Fei), tianjing@dlpu.edu.cn (J. Tian).

¹ These authors contributed equally to the work.

introduction of other non product proteins as contamination (Liu, Li, Dai, & Huang, 2017; Sheng, Xi, Zhang, Ye, & Zhao, 2018). The immobilization of free papain is an effective way to overcome these drawbacks and develop papain for further applications. The immobilized papain exhibits reusability and stability compared with free papain (He, Wu, Feng, Shao, & Cai, 2014).

Unfortunately, enzymes lose some of their activity in most immobilization methods, which is attributed to slight distortions in the enzyme structure by immobilization (Ding, Sun, Ren, & Qu, 2017; Rodrigues, Ortiz, Berenguer-Murcia, Torres, & Fernandez-Lafuente, 2013; Secundo, 2013). Recently, several nanotechnologies have been developed to improve the catalytic activity of immobilized enzymes (Ansari & Husain, 2012; Cipolatti et al., 2014; Sun et al., 2014; Wang et al., 2013). Among these nanotechnologies, hybrid enzyme-inorganic nanoflowers not only effectively improve the stability of free enzyme, but also increase enzyme activity. This peculiar result is mainly attributed to the high surface-to-volume ratio of hybrid enzyme-inorganic nanoflowers, which is beneficial for enhancement in the efficiency of surface reactions including efficient loading capability, strong adsorption capacity, and excellent catalytic ability (Ge, Lei, & Zare, 2012; Jing, Fei, Ren, Tian, Zhi, Xu, et al., 2017; Liang, Fei, Li, Tian, Xu, Wang, & Wang, 2015; Liu, Ji, & He, 2019). In contrast with conventional enzyme immobilization methods, the synthesis of hybrid enzyme-inorganic nanoflowers is simple, green, and consumes low amounts of energy (Ren et al., 2018). However, the nanoscale size of hybrid enzyme-inorganic nanoflower makes it difficult to separate from substrates, limiting their application. In continuous catalytic reactions, hybrid enzyme-inorganic nanoflowers need to be separated by centrifugation, which requires additional energy consumption (Li, Fei, Liang, Tian, Xu, Wang, Wang, 2016; Ren et al., 2019). As one of the most easily separated multifunctional materials, magnetic nanoparticles have been widely explored in lipase enzyme immobilizations (Sun, Ma, Liu, Su, Xia, & Yang, 2012). The immobilized enzymes with magnetic properties allow their easy separation from reaction solution by magnets (Du et al., 2018; Yang, Wang, Shi, Wang, Zhang, Han, & Jiang, 2016).

In this study, we report a novel and simple method to synthesize papain - $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ -magnetic nanoflowers (PCMN) with papain as organic component. This enzyme immobilization method not only improved the enzyme activity and thermal stability, but also simplified the separation process. The enzyme activity of PCMN was increased to 1556% of the free papain and the activity recovery was maintained at 597.6%. Moreover, PCMN have been successfully applied to hydrolyze β -Lg in cow's milk, generating low-sensitive milk. In cycle analysis, the PCMN also exhibited good reusability and reproducibility.

2. Materials and methods

2.1. Materials

Papain was obtained from Shanghai Yuanye Bio-tech Co. Ltd, and the activity was greater than 800 u/mg. Ethylene glycol (98%) and FeCl_3 (97%) were obtained from Guoyao Chemical Co. Ltd. Trisodium citrate (99%), urea (90%), *n*-hexane (99.5%) and cetyltrimethyl ammonium bromide (CTAB, 99%) were procured from Tianjin Damao Chemical Reagent Factory. Sodium acetate (99%), acetone (99.5%), *n*-butanol (99.5%) and tetraethyl orthosilicate (TEOS, 98%) were supplied by Tianjin Kemiou Chemical Reagent Co. Ltd. (3-aminopropyl) triethoxysilane (APTES, 98%) and *N*- α -benzoyl-L-arginine ethylester (BAEE, 98%) were purchased from Aladdin Reagent Co. Ltd. Dithiothreitol (DTT, 97%) was purchased from Beijing Solarbio Science and Technology Co. Ltd. Milk powder were procured from Shuangcheng Nestle Co. Ltd. All aqueous solutions were prepared by using deionized water.

2.2. Preparation of Fe_3O_4 nanoparticles with amino

Fe_3O_4 nanoparticles were synthesized as follow according to the method in the literature (Gao et al., 2017): FeCl_3 (2.60 g) and trisodium citrate (1.20 g) were dispersed in ethylene glycol (80 mL), and then sodium acetate (3.12 g) was added with stirring vigorously. After being stirred for 30 min, the mixture was sealed in teflon-lined stainless-steel autoclaves. The autoclave was kept at 200 °C for 20 h. Subsequently, the obtained black precipitates were collected by a magnet and washed for three times by deionized water and ethanol, respectively. The obtained Fe_3O_4 nanoparticles were dried by vacuum freeze-drier.

Chemical modification method was employed to achieve the amino functionalization of Fe_3O_4 nanoparticles. Amino-functionalized Fe_3O_4 nanoparticles had strong binding ability to enzymes. The specific steps of chemical modification method as follow: urea aqueous solution (0.4 M, 100 g) containing Fe_3O_4 nanoparticles (300 mg) was ultrasonicated at room temperature for 1 h. Then CTAB (1.25 g), *n*-butanol (1.25 g) and *n*-hexane (5 g) were added and the mixture was stirred for 30 min. With vigorous stirring, the solution of TEOS (0.875 g) and APTES (0.375 g) was added dropwise to the above mixture. After vigorous stirring for 30 min at room temperature, the reaction was continued at 70 °C for 24 h. The obtained brown precipitates were collected by a magnet and washed for three times by deionized water and ethanol, respectively. Subsequently, the precipitates were dispersed in 250 mL of acetone and refluxed at 80 °C for 24 h to remove the templates. Finally, Fe_3O_4 nanoparticles with amino were collected by a magnet and washed for three times by deionized water and ethanol, respectively. And the as-prepared Fe_3O_4 nanoparticles with amino were dried by vacuum freeze-drier.

2.3. Preparation of papain- $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ -magnetic nanoflowers (PCMN)

15 mL of PBS (10 mM, pH 7.4) containing Fe_3O_4 nanoparticles with amino (2.25 mg) was ultrasonicated at room temperature for 30 min. Then 5 mL of PBS (10 mM, pH 7.4) containing 22.5 mg of papain were added and the mixture was stirred for 2 h. Then the mixture was dispersed in 70 mL of PBS (10 mM, pH 7.4). 5.5 mL of aqueous CuCl_2 solution (120 mM) was added to the above mixture, and the mixture was incubated at room temperature for 3 days (Zhang et al., 2018). The obtained green precipitates were collected by a magnet and washed for three times by deionized water. Finally, the green precipitates were dried into the powders by using the technology of vacuum freeze-drying to obtain the papain- $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ -magnetic nanoflowers 1 (PCMN1). In order to determine the effect of Fe_3O_4 nanoparticles with amino on morphology and activity of PCMN, the PCMN (PCMN2, PCMN3, PCMN4 and PCMN5) containing different content of Fe_3O_4 nanoparticles with amino were synthesized as the same procedure (their feed ratios were provided in Supplementary material Table S1).

2.4. Weight percentage of papain in PCMN

In order to determine the content of papain, the thermogravimetric process of PCMN was investigated by TGA (Fig.S1). After removing the water molecules physically adsorbed on the PCMN (the water molecules disappear at 200 °C), the remaining weight loss was papain. The calculation of weight percentage of papain in PCMN was given as the following equation:

$$W = \frac{G_N - G_0}{G_N} \times 100\% \quad (1)$$

where *W* is the weight percentage of papain (%), *G_N* is the weight of PCMN, and *G₀* is the weight of inorganic component ($\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ and Fe_3O_4).

2.5. Determination of enzymatic activity

The enzymatic activity of papain and PCMN were measured at 25 °C and pH 7.4 with using N- α -benzoyl-L-arginine ethylester (BAEE) as substrate (Liang, Fei, Li, Tian, Xu, Wang, & Wang, 2015). Papain could catalyze the hydrolytic cleavage of the ester linkage in BAEE and produce N- α -benzoyl-L-arginine (BA), which can be detected at 253 nm by UV spectrometer. Briefly, 1 mL of DTT (30 mM) was added to 2 mL of PBS (10 mM, pH 7.4) containing 0.05 mg of papain (the amount of papain embedded in PCMN was equivalent to 0.05 mg free papain). After being incubated for 10 min at 25 °C, 3 mL of BAEE (2 mM) was added to above mixture and the reaction time was 5 min. The solution after the reaction was detected at 253 nm by UV-Vis. One activity unit of papain was defined as the amount of enzyme that produce a ΔA_{253} of 0.001 per minute with BAEE as substrate under experimental conditions. The calculation of enzyme activity for free papain and PCMN was shown in the following equation:

$$U = \frac{\Delta A_{253}}{0.001 \times G_E \times T} \quad (2)$$

where U is the enzyme activity of papain (U/mg), ΔA_{253} is the absorbance change at 253 nm, G_E is the amount of papain (free papain, or papain in PCMN, mg), T is the reaction time (min).

2.6. Encapsulation yield and activity recovery of PCMN

The encapsulation yield was defined as the ratio of the immobilized papain amount to the total amount of papain introduced to immobilization reaction. The immobilized papain amount could be given by the product weight of obtained PCMN and the weight percentage of papain in PCMN. The encapsulation yield was calculated by the following equation (Liang, Fei, Li, Tian, Xu, Wang, & Wang, 2015):

$$E = \frac{G_P \times W}{G_T} \times 100\% \quad (3)$$

where E is the encapsulation yield, G_P is the weight of obtained PCMN, W is the weight percentage of papain (%), and G_T is the total amount of papain added.

The activity recovery was defined as the percentage, which the activity of the immobilized papain in the total activity of free papain added. The activity recovery was calculated by the following equation (Bai & Zhou, 2004):

$$A = \frac{G_P \times W \times U_I}{G_T \times U_F} \times 100\% \quad (4)$$

where A is the activity recovery, G_P is the weight of obtained PCMN, W is the weight percentage of papain (%), U_I is the activity of the immobilized papain, G_T is the total amount of papain added, and U_F is the activity of free papain added (198 ± 56 U/mg).

2.7. The stability of free papain and PCMN3

The thermal inactivation of free papain and PCMN3 (the activity recovery of PCMN3 is highest) were investigated by incubating them in PBS (10 mM, pH 7.4) without substrate at 20, 30, 40, 50, 60 and 70 °C for 1 h, respectively. To examine the pH stability, the free papain and PCMN3 were incubated in PBS with pH of 6, 7, 8, 9 and 10 at 25 °C for 1 h, respectively. The residual enzyme activity was determined by the same procedure as described above.

2.8. Enzymatic hydrolysis of the cow's milk allergenic proteins and reusability of PCMN3

PCMN3 was used as a catalyst for enzymatic hydrolysis of cow's milk (milk powder aqueous solution). 50 mg of PCMN3 and 1 mL of 1% (w/w) milk powder aqueous solution were mixed and the hydrolysis

reaction was performed in a 10 mL beaker with a shaking speed of 200 rpm for 50 min at 25 °C (samples were taken every ten minutes for electrophoresis). The hydrolyzed milk was characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), according to the method of Laemmli (Laemmli, 1970).

The reusability of PCMN3 was investigated by enzymatic hydrolysis of milk powder solution for 4 times independently. 20 mg of PCMN3 and 0.3 mL of 1% (w/w) milk powder aqueous solution was added in a 10 mL beaker and enzymatic hydrolysis was carried out with a shaking speed of 200 rpm for 30 min. After the enzymatic hydrolysis, the PCMN3 was separated and collected. And then the PCMN3 was added into another 0.3 mL of 1% (w/w) milk powder aqueous solution.

2.9. SDS-PAGE analysis

SDS-PAGE was performed in polyacrylamide gel, which was composed of 5% w/v polyacrylamide stacking gel and 15% w/v polyacrylamide separation gel. The hydrolyzed milk (30 μ L) was dissolved in loading buffer (30 μ L) and the mixture was heated at 100 °C for 10 min. Then, 20 μ L of the mixture was loaded on the polyacrylamide gel. The hydrolyzed milk were separated in electrophoresis buffer and stained with staining solution (0.1% w/v coomassie brilliant blue R250, 0.1% w/v CuSO₄, 25% v/v isopropanol, 10% v/v acetic acid, 65% v/v water). The polyacrylamide gel was destained by using a methanol/acetic acid/water solution (V/V, 2:3:35). Blue Plus IV Protein Marker (10–180 kDa) was used as molecular mass standard.

2.10. Characterizations

The magnetization curves were examined by using a Physical Property Measurement System (PPMS, Quantum Design). IR spectra were conducted on a Spectrum Two Fourier transform infrared spectrometer (Perkin-Elmer). The X-ray diffraction (XRD) was performed on a Shimadzu XRD-7000S diffractometer with Cu K α radiation (50 kV, 200 mA, $\lambda = 0.154$ nm) and a scanning step of 0.02°. Scanning electron microscopy (SEM) was taken on a JEOL JSM 7800F electron microscope and energy dispersive X-ray spectroscopy (EDS) was performed with Oxford INCA. The UV-Vis absorption spectrum was measured on a Perkin-Elmer LAMBDA35. The XPS (X-ray photoelectron spectroscopy) was conducted on Thermo Scientific Escalab 250Xi. The element content was measured by a Perkin-Elmer Optima 8000 ICP spectrometer. SDS-PAGE was conducted on JM-250 electrophoresis apparatus.

3. Results and discussion

3.1. Structure of PCMN

PCMN as magnetic nanoimmobilized papain was synthesized, and its formation mechanism was illustrated in Fig. 1. Firstly, magnetic Fe₃O₄ nanoparticles with amino groups were synthesized through a series of chemical reactions. Secondly, papain was adsorbed to the surfaces of magnetic nanoparticles. Finally, CuCl₂ and magnetic nanoparticles with papain were placed into PBS to obtain PCMN with flower-like structures.

The structure and morphology of PCMN3 with the highest activity recovery were characterized through FT-IR, XRD, and EDS. IR spectra (Fig. S2) showed that PCMN3 had all of the characteristic absorptions of papain, Fe₃O₄ nanoparticles with amino groups, and Cu₃(PO₄)₂·3H₂O. However, the evidence cannot entirely prove that the PCMN3 contained Fe₃O₄ nanoparticles with amino and Cu₃(PO₄)₂·3H₂O because the characteristic peaks of Cu₃(PO₄)₂·3H₂O and Fe₃O₄ nanoparticles with amino overlapped. Furthermore, the XRD pattern of PCMN3 exhibited characteristic peaks attributable to Cu₃(PO₄)₂·3H₂O and Fe₃O₄ nanoparticles with amino groups (Fig. S3A). As shown in Fig. S3B, the EDS results of PCMN3 revealed the presence of copper (Cu), phosphorus (P), oxygen (O), carbon (C), silicon (Si), and iron (Fe) in the sample (their

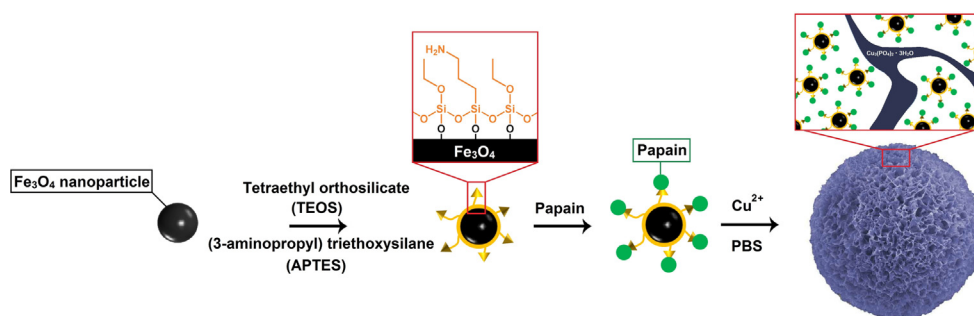


Fig. 1. Schematic diagram of the proposed growth mechanism of PCMN.

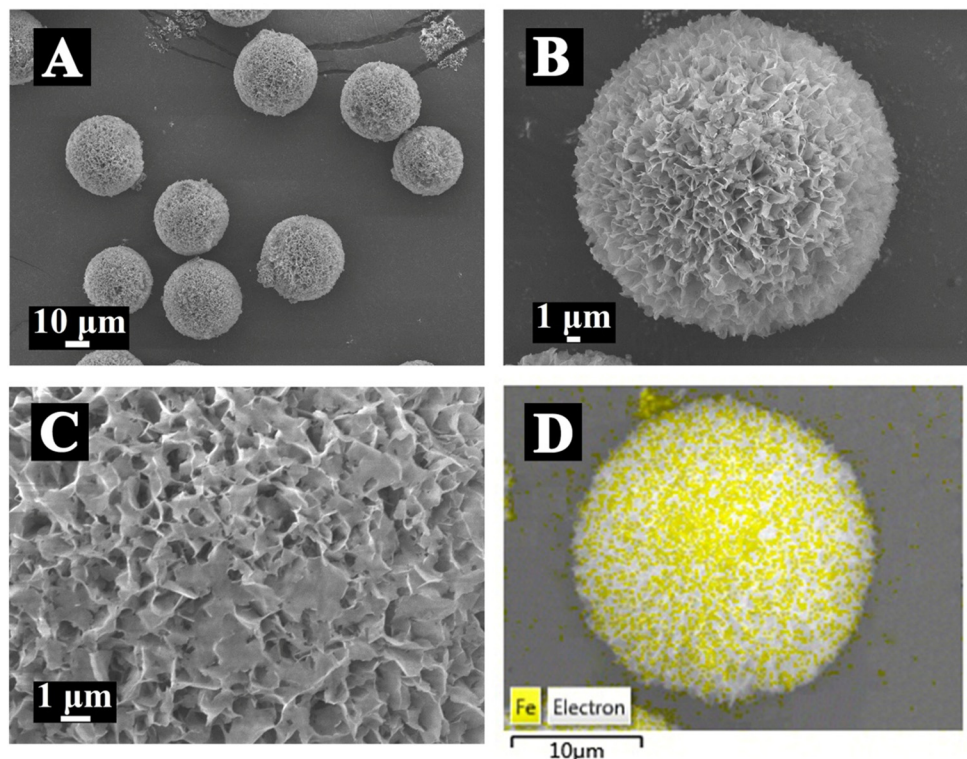


Fig. 2. SEM images (A–C) of PCMN and (D) EDS elemental mapping image of PCMN about Fe.

specific contents are provided in Table S2). These results clearly proved that PCMN3 was composed of crystallized $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ and Fe_3O_4 nanoparticles with amino groups.

The microstructure of freeze-dried PCMN3 was visualized through SEM. The SEM image of PCMN3 (Fig. 2A) showed the presence of flower-like structures with sizes of 30–40 μm . Clear flower-like structures of PCMN3 with increased SEM magnification are presented in Fig. 2B and 2C. Compared with the spherical structure without petals, the flower-like structure of PCMN exhibited significantly larger surface area, which was suitable for the contact between substrate and enzyme. Moreover, iron (Fe) was uniformly dispersed onto the surface of PCMN3, as shown in the EDS image (Fig. 2D). These results clearly proved that the Fe_3O_4 nanoparticles with amino have been successfully introduced into PCMN3.

3.2. Properties of PCMN

The magnetic property of PCMN3 was determined by physical property measurement system (PPMS) measurement. As shown in Fig. 3A, the saturation magnetization (M_s) value of the PCMN3 was 2 emu/g, which was lower than the Fe_3O_4 nanoparticles (51 emu/g)

and Fe_3O_4 nanoparticles with amino groups (40 emu/g). The main reason for the low M_s value was that only a few Fe_3O_4 nanoparticles with amino were added to PCMN3. Compared with the Fe_3O_4 nanoparticles and Fe_3O_4 nanoparticles with amino groups, M_s value of PCMN3 greatly decreased, but PCMN3 could still be separated from the reaction solution quickly by a magnet (Fig. 3B).

PCMNs (PCMN1–PCMN5) were synthesized by adding different proportions of Fe_3O_4 nanoparticles with amino groups. The weight percentage of papain in PCMNs should be obtained to appraise the enzyme activities of PCMNs. The weight percentage of the papain was determined by TGA and calculated using formula (1). The activities of the papain in PCMNs were obtained by using formula (2) and were presented in Table 1. The activities of PCMN1, PCMN2, PCMN3, PCMN4, and PCMN5 were 1556%, 1372%, 1323%, 1237%, and 1050% of free papain (the specific activity of free papain was 198 ± 56 U/mg), respectively. These results indicated that papain activity was increased during immobilization. The encapsulation yield of PCMNs was calculated by using formula (3). The encapsulation rate ranged from 35.8% to 46.8%, indicating that the immobilization degree of papain was quite high. Activity recovery was obtained by using formula (4) and was presented in Table 1. PCMN3 exhibited the highest activity

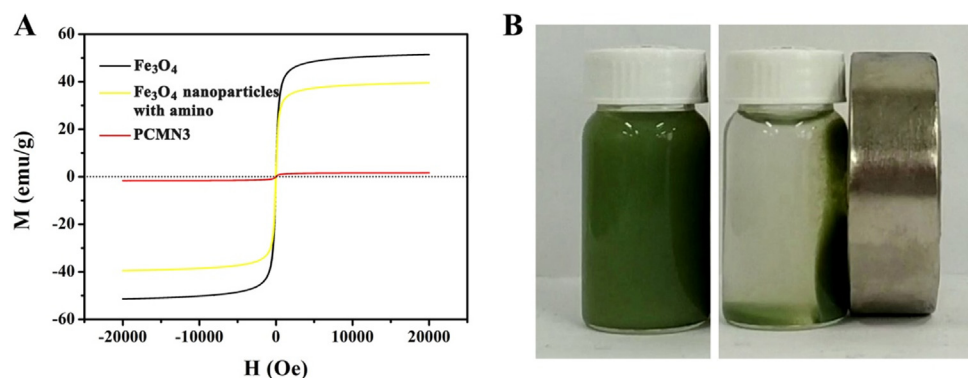


Fig. 3. The hysteresis loops (A) of the Fe₃O₄ nanoparticles, Fe₃O₄ nanoparticles with amino and PCMN3, and (B) the separation of PCMN3 from water.

Table 1

The specific activity, encapsulation yield and activity recovery of PCMN.

	PCMN (mg)	Weight percentage of papain (%)	Specific activity (U/mg)	Encapsulation yield (%)	Activity recovery (%)
PCMN1	60.5 ± 10.3	13.3	3082 ± 232	35.8	556.6
PCMN2	68.0 ± 9.1	13.4	2717 ± 459	40.5	555.7
PCMN3	76.4 ± 4.3	13.3	2620 ± 329	45.2	597.6
PCMN4	76.3 ± 2.3	13.0	2450 ± 397	44.1	545.5
PCMN5	78.5 ± 3.6	13.4	2080 ± 414	46.8	491.1

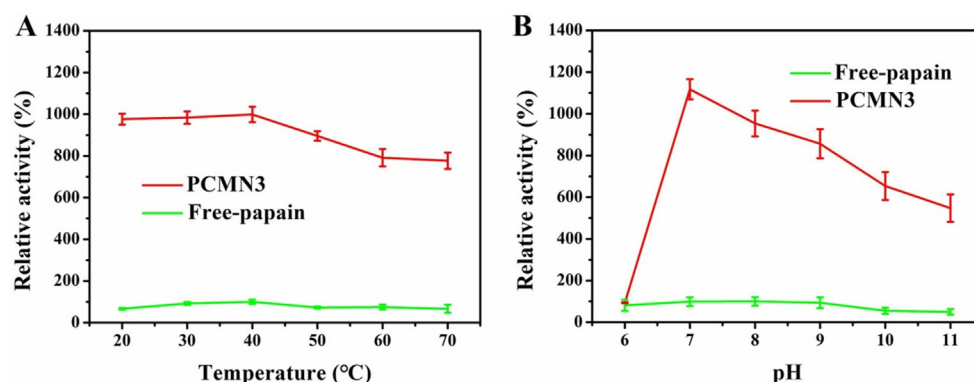


Fig. 4. Thermal stabilities (A) and pH stabilities (B) of free papain and PCMN3.

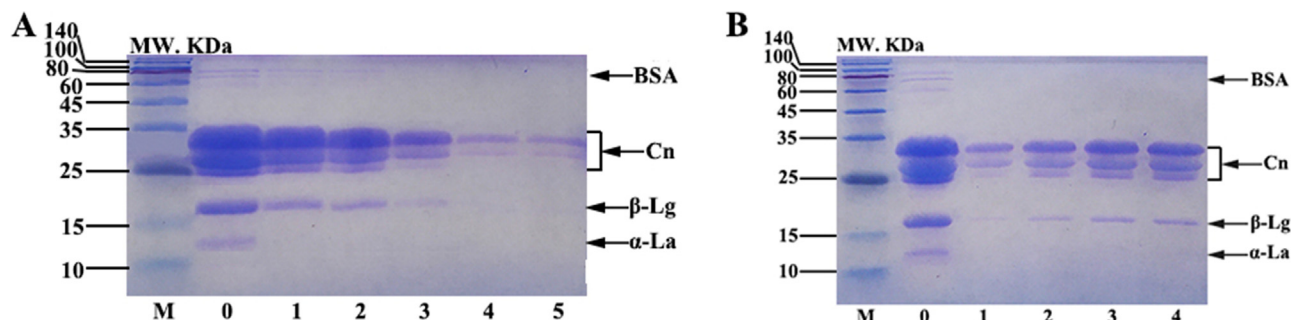


Fig. 5. SDS-PAGE patterns (A) of the hydrolysates, M: protein marker (140–10 KDa); 0:1% (w/w) milk powder aqueous solution; 1–5: the hydrolysates with different reaction times (10 min, 20 min, 30 min, 40 min and 50 min). SDS-PAGE patterns (B) of the hydrolysates from reuse experiment, M: protein marker (140–10 KDa); 0:1% (w/w) milk powder aqueous solution; 1–4: the hydrolysates with different reuse times (first, second, third and fourth time).

recovery, resulting in the most efficient catalytic conversion.

The enzyme activity is mainly dependent on its conformational integrity (Secundo, 2013). Sometimes, enzymes are exposed to extreme conditions or even to slight variations in temperature or pH that can induce abrupt conformational changes and subsequent activity loss. Therefore, the thermal and pH stabilities of free papain and PCMN3 were tested. For the thermal stability, the activities of free papain and

PCMN3 initially increased and then decreased with the increase of temperature. The activity of PCMN3 was generally higher than that of free papain (Fig. 4A). Specifically, PCMN3 still retained 77.8% of the highest activity at 70 °C for 1 h, whereas that of free papain was 66.4%. Fig. 4B showed that the specific activity of PCMN3 was also higher than that of free papain in the pH range of 7–11. This result indicated that the pH stability of PCMN3 was better than that of free papain. The

relative activity of PCMN3 was 1117% of free papain. Moreover, the activity of PCMN3 at pH 11 was much higher than that of free papain, although it was half of the highest activity. However, PCMN3 and free papain exhibited similar activity at pH 6. The reason for this phenomenon could be that the skeleton of PCMN3 composed of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ was unstable in an acidic environment, leading to the conformational change of papain. Therefore, PCMN3 was unsuitable for catalysis under acidic conditions. The results indicated that PCMN3 exhibited better thermal stability and pH stability than free papain. The reason may be that the immobilization enhanced the conformational stability of the active center of the enzyme molecule, reduced the influence from the external environment, and resisted the damage of temperature and pH.

3.3. Enzymatic hydrolysis of the cow's milk allergenic proteins and reusability of PCMN3

β -Lg and α -La can cause the abnormal immunologic reaction. The abnormal immunologic reaction to β -Lg has been found in 60%–80% of patients. Therefore, PCMN3 was used to hydrolyze β -Lg and α -La and the hydrolysis process was described in materials and methods 2.8. Hydrolysate of 1% (w/w) milk powder catalyzed by PCMN3 was determined by SDS-PAGE (Fig. 5A). Unhydrolyzed 1% (w/w) milk powder aqueous solution exhibited six major bands with apparent different molecular weights (MW). The first band with a MW of 66 kDa was characterized to be bovine serum albumin (BSA). Three bands with MW of between 25 and 35 kDa were casein (Cn). The two other bands (18 kDa and 14 kDa) corresponded to the β -Lg and α -La. As shown in Fig. 5A, the proteins in milk were hydrolyzed gradually as time increased. Notably, most of the allergenic proteins in milk (β -Lg and α -La) were hydrolyzed at 30 min.

To detect the reusability of PCMN3, a reuse experiment was performed, and the results were shown in Fig. 5B. During the first catalytic hydrolysis of milk, β -Lg, α -La and BSA were almost hydrolyzed, and even casein was largely hydrolyzed. After 4 times of catalysis, α -La and BSA were completely hydrolyzed, and most of β -Lg was hydrolyzed. With the increase of catalysis times, the hydrolysis efficiency of PCMN3 decreased slightly for β -Lg. This phenomenon was attributed to that some substances in milk were adsorbed on the surface of PCMN3 during the repeated hydrolysis process, which reduced the hydrolysis efficiency.

4. Conclusions

We developed an approach for the synthesis of a novel catalytic material for hydrolyzing allergenic proteins. The Fe_3O_4 nanoparticle and $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ nanoflowers were successfully combined to obtain PCMN that can be separated magnetically. PCMN exhibited high encapsulation yield of papain and activity recovery due to the binding force of papain, $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$, and Fe_3O_4 nanoparticles. The papain in PCMN showed significantly increased enzyme activity and exhibited good stability after being loaded on nanoflowers. Furthermore, PCMN3 was successfully used to hydrolyze allergenic proteins, such as β -Lg, α -La, and BSA. PCMN3 showed the advantages of low carrier cost, high catalytic activity, good stability, and fast separation speed, providing a new method for the production of hypoallergenic and easily digestible milk. Therefore, PCMN3 has great application potential as a biocatalyst to realize enzyme-based industrial processes.

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.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31771914, 31800498), Natural Science Foundation of Liaoning Province of China (20170540076, 20170540079), Research on science and technology of education department of liaoning province project (2017J078).

Author contributions

Nuan Feng and Haiyang Zhang performed the experiments and co-wrote the manuscript. Yao Li, Xu Fei and Jing Tian participated in the design of experiments and revised the manuscript. Yangkaixi Liu and Yi Wang assisted with FT-IR spectra.

Longquan Xu performed SEM imaging. All authors discussed the results and commented on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.foodchem.2019.125911>.

References

- Ansari, P., Stoppacher, N., Rudolf, J., Schuhmacher, R., & Baumgartner, S. (2011). Selection of possible marker peptides for the detection of major ruminant milk proteins in food by liquid chromatography-tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 399(3), 1105–1115.
- Ansari, S. A., & Husain, Q. (2012). Potential applications of enzymes immobilized on/in nano materials: A review. *Biotechnology Advances*, 30(3), 512–523.
- Bai, Z. W., & Zhou, Y. K. (2004). A novel enzyme support derived from aminated silica gel and polysuccinimide: Preparation and application for the immobilization of porcine pancreatic lipase. *Reactive and Functional Polymers*, 59(1), 93–98.
- Bu, G., Luo, Y., Chen, F., Liu, K., & Zhu, T. (2013). Milk processing as a tool to reduce cow's milk allergenicity: A mini-review. *Dairy Sci Technol*, 93(3), 211–223.
- Cao, Q., Zhao, H., Yang, Y., He, Y., Ding, N., Wang, J., ... Wang, G. (2011). Electrochemical immunosensor for casein based on gold nanoparticles and poly(L-Arginine)/multi-walled carbon nanotubes composite film functionalized interface. *Biosensors & Bioelectronics*, 26(8), 3469–3474.
- Cheison, S. C., Lai, M.-Y., Leeb, E., & Kulozik, U. (2011). Hydrolysis of β -lactoglobulin by trypsin under acidic pH and analysis of the hydrolysates with MALDI-TOF-MS/MS. *Food Chemistry*, 125(4), 1241–1248.
- Chen, F. M., Lee, J. H., Yang, Y. H., Lin, Y. T., Wang, L. C., Yu, H. H., & Chiang, B. L. (2014). Analysis of alpha-lactalbumin-, beta-lactoglobulin-, and casein-specific IgE among children with atopic diseases in a tertiary medical center in Northern Taiwan. *Journal of Microbiology, Immunology, and Infection*, 47(2), 130–136.
- Cipolatti, E. P., Silva, M. J. A., Klein, M., Feddern, V., Feltes, M. M. C., Oliveira, J. V., ... de Oliveira, D. (2014). Current status and trends in enzymatic nanoimmobilization. *Journal of Molecular Catalysis B: Enzymatic*, 99, 56–67.
- Ding, C., Sun, H., Ren, J., & Qu, X. (2017). Immobilization of enzyme on chiral polyelectrolyte surface. *Analytica Chimica Acta*, 952, 88–95.
- Du, Y., Gao, J., Kong, W., Zhou, L., Ma, L., He, Y., ... Jiang, Y. (2018). Enzymatic synthesis of glycerol carbonate using a lipase immobilized on magnetic organosilica nanoflowers as a catalyst. *ACS Omega*, 3(6), 6642–6650.
- Exl, B.-M., & Fritsche, R. (2001). Cow's milk protein allergy and its prevention. *Nutrition*, 17, 642–651.
- Franssen, M. C., Steunenbergh, P., Scott, E. L., Zuilhof, H., & Sanders, J. P. (2013). Immobilised enzymes in biorenewables production. *Chemical Society Reviews*, 42(15), 6491–6533.
- Gao, J., Kong, W., Zhou, L., He, Y., Ma, L., Wang, Y., ... Jiang, Y. (2017). Monodisperse core-shell magnetic organosilica nanoflowers with radial wrinkle for lipase immobilization. *Chemical Engineering Journal*, 309, 70–79.
- Garcia, J., Zhang, Y., Taylor, H., Cespedes, O., Webb, M. E., & Zhou, D. (2011). Multilayer enzyme-coupled magnetic nanoparticles as efficient, reusable biocatalysts and biosensors. *Nanoscale*, 3(9), 3721–3730.
- Ge, J., Lei, J., & Zare, R. N. (2012). Protein-inorganic hybrid nanoflowers. *Nature Nanotechnology*, 7(7), 428–432.
- Gu, Y. J., Zhu, M. L., Li, Y. L., & Xiong, C. H. (2018). Research of a new metal chelating carrier preparation and papain immobilization. *International Journal of Biological Macromolecules*, 112, 1175–1182.
- He, J., Wu, M., Feng, X., Shao, X., & Cai, W. (2014). Immobilization of papain on nanoporous silica. *RSC Advances*, 4(26), 13304–13312.
- 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.
- Homaei, A., Barkheh, H., Sariri, R., & Stevanato, R. (2014). Immobilized papain on gold nanorods as heterogeneous biocatalysts. *Amino Acids*, 46(7), 1649–1657.
- Ji, J., Zhu, P., Pi, F., Sun, C., Sun, J., Jia, M., ... Sun, X. (2017). Development of a liquid

- chromatography-tandem mass spectrometry method for simultaneous detection of the main milk allergens. *Food Control*, 74, 79–88.
- Jing, M., Fei, X., Ren, W., Tian, J., Zhi, H., Xu, L., ... Wang, Y. (2017). Self-assembled hybrid nanomaterials with alkaline protease and a variety of metal ions. *RSC Advances*, 7(76), 48360–48367.
- Li, X., Zhu, H., Feng, J., Zhang, J., Deng, X., Zhou, B., ... Peng, Y. (2013). One-pot polyol synthesis of graphene decorated with size- and density-tunable Fe₃O₄ nanoparticles for porcine pancreatic lipase immobilization. *Carbon*, 60, 488–497.
- Li, Y., Fei, X., Liang, L., Tian, J., Xu, L., Wang, X., & Wang, Y. (2016). The influence of synthesis conditions on enzymatic activity of enzyme-inorganic hybrid nanoflowers. *Journal of Molecular Catalysis B: Enzymatic*, 133, 92–97.
- 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.
- Liang, L., Fei, X., Li, Y., Tian, J., Xu, L., Wang, X., & Wang, Y. (2015). Hierarchical assembly of enzyme-inorganic composite materials with extremely high enzyme activity. *RSC Advances*, 5(117), 96997–97002.
- Liu, Y., Ji, X., & He, Z. (2019). Organic-inorganic nanoflowers: From design strategy to biomedical applications. *Nanoscale*, 11(37), 17179–17194.
- Liu, Z., Li, D., Dai, H., & Huang, H. (2017). Preparation and characterization of papain embedded in magnetic cellulose hydrogels prepared from tea residue. *Journal of Molecular Liquids*, 232, 449–456.
- 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.
- Pessato, T. B., Carvalho, N. C.d., Tavano, O. L., Fernandes, L. G. R., Zollner, R.d. L., & Netto, F. M. (2016). Whey protein isolate hydrolysates obtained with free and immobilized Alcalase: Characterization and detection of residual allergens. *Food Research International*, 83, 112–120.
- Ren, W., Fei, X., Tian, J., Li, Y., Jing, M., Fang, H., ... Wang, Y. (2018). Multiscale immobilized lipase for rapid separation and continuous catalysis. *New Journal of Chemistry*, 42(16), 13471–13478.
- Ren, W., Li, Y., Wang, J., Li, L., Xu, L., Wu, Y., ... Tian, J. (2019). Synthesis of magnetic nanoflower immobilized lipase and its continuous catalytic application. *New Journal of Chemistry*, 43(28), 11082–11090.
- Rodrigues, R. C., Ortiz, C., Berenguer-Murcia, A., Torres, R., & Fernandez-Lafuente, R. (2013). Modifying enzyme activity and selectivity by immobilization. *Chemical Society Reviews*, 42(15), 6290–6307.
- Secundo, F. (2013). Conformational changes of enzymes upon immobilisation. *Chemical Society Reviews*, 42(15), 6250–6261.
- Sheng, W., Xi, Y., Zhang, L., Ye, T., & Zhao, X. (2018). Enhanced activity and stability of papain by covalent immobilization on porous magnetic nanoparticles. *International Journal of Biological Macromolecules*, 114, 143–148.
- Sun, J., Ge, J., Liu, W., Lan, M., Zhang, H., Wang, P., ... Niu, Z. (2014). Multi-enzyme co-embedded organic-inorganic hybrid nanoflowers: Synthesis and application as a colorimetric sensor. *Nanoscale*, 6(1), 255–262.
- Sun, J., Ma, H., Liu, Y., Su, Y., Xia, W., & Yang, Y. (2012). Improved preparation of immobilized trypsin on superparamagnetic nanoparticles decorated with metal ions. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, 414, 190–197.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227, 680.
- Wang, L.-B., Wang, Y.-C., He, R., Zhuang, A., Wang, X., Zeng, J., & Hou, J. G. (2013). A new nanobiocatalytic system based on allosteric effect with dramatically enhanced enzymatic performance. *Journal of the American Chemical Society*, 135(4), 1272–1275.
- Yang, D., Wang, X., Shi, J., Wang, X., Zhang, S., Han, P., & Jiang, Z. (2016). In situ synthesized rGO-Fe₃O₄ nanocomposites as enzyme immobilization support for achieving high activity recovery and easy recycling. *Biochemical Engineering Journal*, 105, 273–280.
- Zhang, H., Fei, X., Tian, J., Li, Y., Zhi, H., Wang, K., ... Wang, Y. (2018). Synthesis and continuous catalytic application of alkaline protease nanoflowers-PVA composite hydrogel. *Catalysis Communications*, 116, 5–9.

Supporting Information

A novel catalytic material for hydrolyzing cow's milk allergenic proteins: papain-Cu₃(PO₄)₂•3H₂O-magnetic nanoflowers

Nuan Feng^{1, 2, #}, Haiyang Zhang^{2, #}, Yao Li³, Yangkaixi Liu³, Longquan Xu¹, Yi Wang²,
Xu Fei^{*1}, Jing Tian^{*2}

1. Instrumental Analysis Center, Dalian Polytechnic University, Dalian 116034, China

2. School of Biological Engineering, Dalian Polytechnic University, Dalian 116034, China

3. School of Light Industry and Chemical Engineering, Dalian Polytechnic University, Dalian 116034, China

* Correspondence to: Xu Fei, Instrumental Analysis Center, Dalian Polytechnic University, 1[#]Qinggongyuan Road, Dalian 116034, P. R. China

E-mail: feixudlpu@163.com

Tel: [+86-411-86323691-201](tel:+86-411-86323691-201)

Fax: [+86-411-86322038](tel:+86-411-86322038)

* Correspondence to: Jing Tian, School of Biological Engineering, Dalian Polytechnic University, 1[#]Qinggongyuan Road, Dalian 116034, P. R. China

E-mail: tianjing@dlpu.edu.cn

[#] These authors contributed equally to the work.

Tel: +86-411-86324485

Table S1. Feed ratios of the PCMN.

	Fe ₃ O ₄ nanoparticles with amino (mg)	Papain (mg)	Aqueous CuCl ₂ solution (120 mM, mL)	PBS (10mM, pH 7.4)	PCMN (mg)
PCMN1	2.25	22.5	5.5	90	60.5 ± 10.3
PCMN2	4.50	22.5	5.5	90	68.0 ± 9.1
PCMN3	6.75	22.5	5.5	90	76.4 ± 4.3
PCMN4	9.00	22.5	5.5	90	76.3 ± 2.3
PCMN5	11.25	22.5	5.5	90	78.5 ± 3.6

Table S2. Contents of several elements in PCMN5.

Element	Wt (%)	Element	Wt (%)
Cu	30.58	Na	3.42
O	29.71	Cl	2.43
C	20.30	Fe	0.59
P	9.43	Si	0.38

Determination of Cu²⁺ and Fe³⁺ concentration in hydrolyzed milk by Perkin-Elmer Optima 8000 ICP spectrometer

2 mL of 1% (w/w) hydrolyzed milk powder aqueous solution, 8 mL of nitric acid and 2 mL of perchloric acid were mixed, and the digestion reaction was performed in a 50 mL beaker at 150 °C. The obtained solution (5mL) was characterized by ICP spectrometer.

Table S3. ICP analytical results of deionized water, Milk, and hydrolyzed milk by PCMN3 .

sample	Solution concentration (g/L)	Fe ³⁺ concentration (mg/L)	Cu ²⁺ concentration (mg/L)
Deionized water	0	—	—
Milk	4	—	—
Hydrolyzed milk by PCMN3	4	—	—

—, Not detected

To detect the residue of particles in food, ICP (Inductively Coupled Plasma) analysis was carried out for the milk after catalytic hydrolysis. ICP results showed that there were no Cu²⁺ and Fe³⁺ in the milk after hydrolysis, which indicated that there were no residues of particles.

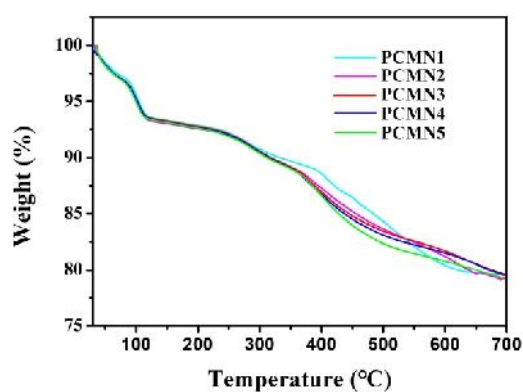


Fig.S1 TGA curves of PCMN.

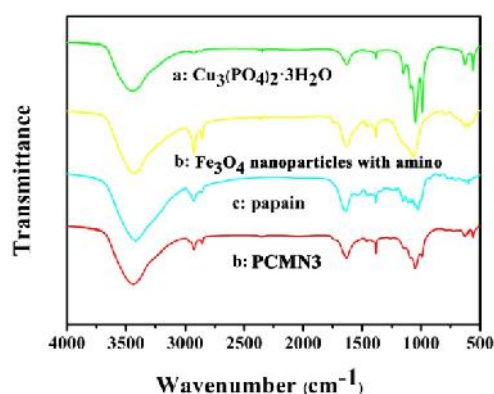


Fig.S2 FT-IR spectra of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$ (a), Fe_3O_4 nanoparticles with amino(b), Papain (c) and PCMN5(d).

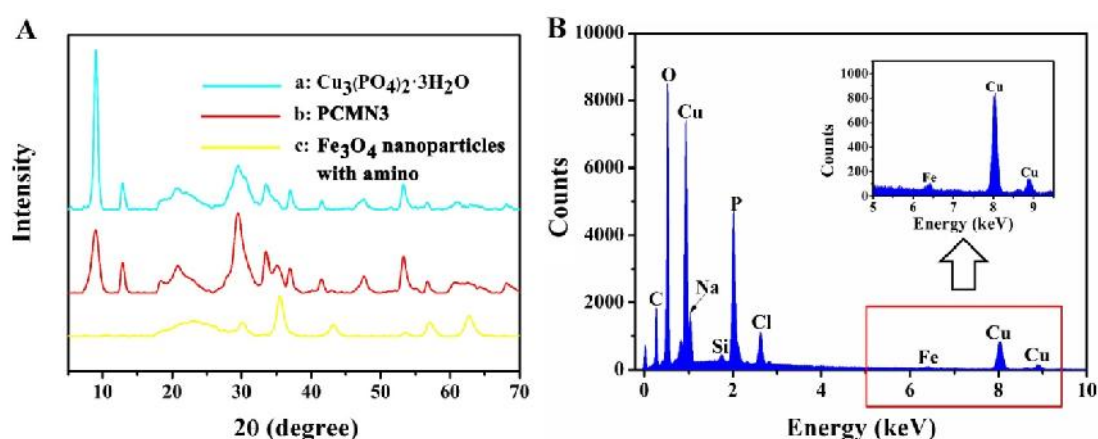


Fig.S3 (A) XRD patterns of $\text{Cu}_3(\text{PO}_4)_2 \cdot 3\text{H}_2\text{O}$, Fe_3O_4 nanoparticles with amino and PCMN3, and (B) EDS pattern of PCMN3

Enzymatic hydrolysis

Free papain was used as a catalyst for enzymatic hydrolysis allergenic proteins (milk powder aqueous solution). 6.65 mg free papain and 1 mL of 1% (w/w) milk powder aqueous solution were mixed and the hydrolysis reaction was performed in a beaker with a shaking speed of 200 rpm for 30 min (samples were taken every ten minutes for electrophoresis). The hydrolyzed milk was characterized by sodium

dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

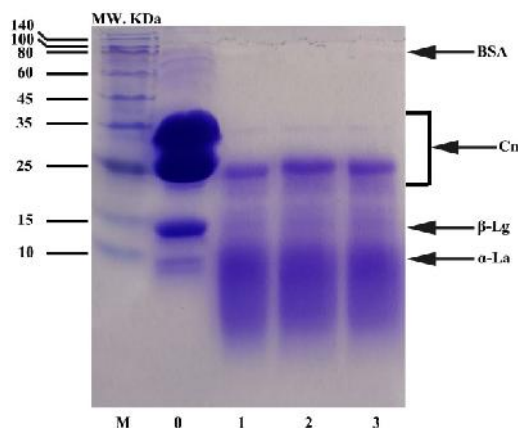


Fig.S4 SDS-PAGE patterns of M: protein marker (140-10KDa); 0:1% (w/w) milk powder aqueous solution; 1-3: the hydrolysates by free papain with different reaction times (10 min, 20min, 30min).

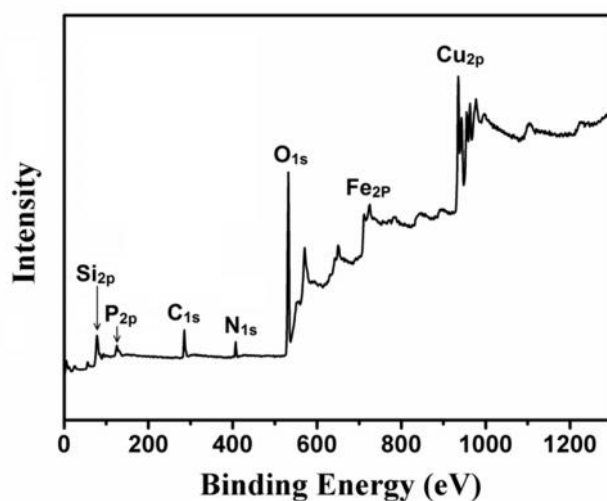


Fig.S5 XPS spectrum of PCMN3.

XPS analysis showed that the PCMN3 contained element cuprum, phosphorus, silicon, carbon, nitrogen, oxygen and iron on its surface. There were seven peaks in XPS spectrum at 964.88, 739.98, 544.88, 409.93, 297.88, 143.93, and 109.88 eV, which

were attributed to Cu2p, Fe2p, O1s, N1s, C1s, P2p, and Si2p, respectively.

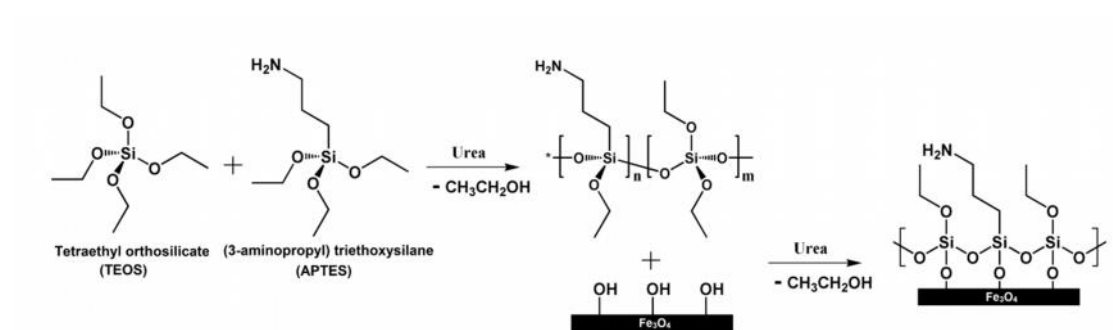


Fig. S6 Schematic diagram of the surface modification process of Fe_3O_4 particles.