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Separation and purification of phosvitin phosphopeptides using immobilized metal affinity nanoparticles

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ABSTRACT

Monodispersed and functional immobilized metal affinity magnetic chondroitin sodium sulfate nanoparticles (short as IMAN @ Fe (III)) were prepared and employed in extracting of Phosvitin Phosphopeptides (short as PPPs) from egg yolk. It was found that the diameter of the magnetic CS nanoparticles was about 20 nm, and they could easily be aggregated by a magnet when suspending in the aqueous solution. The adsorption equilibrium of PPPs onto the obtained nanocarriers fitted well with the Langmuir model. The adsorption capacity of PPPs onto the superparamagnetic nanoparticles was influenced by pH and the initial concentration of the peptides solution. The final nitrogen/phosphorus molar ratios (short as N/P) of PPPs from crude egg yolk peptides and phosvitin peptides were low to 5.78 and 5.23, respectively. Compared with traditional methods, the need for preparation of phosvitin before purification is obviated and the higher purity of PPPs were obtained. In conclusion, this type of IMAN @ Fe (III) would bring advantages to the conventional separation techniques of PPPs from chicken egg yolk.

1. Introduction

Calcium (short as Ca) is an essential macronutrient for the body and the normal dietary intake of Ca, recommended for an adult, is 800–1200 mg/day. A deficiency of Ca will lead to metabolic bone diseases. Public concerns about osteoporosis among the aged population have been increasing the interest in dietary sources of Ca and the controversial use of Ca supplements for controlling aged-related bone loss [1]. Osteoporosis is now becoming one of the most serious adult diseases, and an approach needs to be found for increasing our calcium intake [2]. Early experiments have indicated that phosphorylated fragments of casein, casein phosphopeptides, increased the bioavailability in intestinal calcium and its retention by the body. Vitamin D was not required for phosphopeptide-induced changes in calcium metabolism. It has been confirmed that there is a key function to form a soluble complex with calcium in the phosphoserine moieties.

Hen egg yolk phosvitin is known to be richer in serine residues than casein and most of them are phosphorylated. Bo and Mine reported that phosvitin peptides, which were prepared by tryptic hydrolysis of phosvitin, enhanced Ca-binging property and inhibited the formation of insoluble Ca phosphate [2]. Choi and his partners found that the diets, fortified with phosvitin peptides, significantly enhanced Ca incorporation into bones [3]. Though the relationship between the molecular structure (the molecular size included) of phosphopeptides and their calcium-binging property has not yet been fully understood, many studies have indicated that the N/P, molecule size and calcium-binging property would be related roughly in such a way that: the lower the N/P, the higher the purity of PPPs, or the larger the density of a phosphoric group, the stronger the calcium-binging property. The N/P may reflect, on an objective and comprehensive basis, the peptide length and density of the phosphoric group, thus it can be used as a characteristic index of PPPs.

The three main traditional separation methods of PPPs, organic solvents precipitation, ion exchange chromatography and membrane separation, all have obvious disadvantages. The first one needs a large number of organic solvents and may cause pollution to the environment, Ion exchange chromatography requires acid and alkali regeneration, and the consumption of acid and alkali is large, the last one is relatively expensive and fails to get the purified PPPs. Today, among all available separation technologies for the purification of proteins, peptides, enzymes, nucleic acids, etc., those based on affinity interaction are most favored [4–7]. Using immobilized metal affinity nanoparticles (short as IMANs) as separation carriers is exactly a kind of such metal-affinity separations due to

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the following reasons: the preparation technology, product elution and metal ions regeneration may be achieved in a simple and cost-effective way and the separation may be conducted rapidly under the large surface-to-volume ratios [8–11]. Up to now, the most commonly used matrices material for IMANs were various polysaccharide or synthetic polymers coupled with organic ligands like iminodiacetic acid (IDA) and nitrilotriacetic acid (NTA) [12,13]. But these kinds of IMANs organic ligands, were relatively poisonous and expensive, would leak out of the matrix during purification steps that might limit their application in purification of products especially for food and pharmaceutical purposes [14–16]. At present, decorating non-toxic and metal chelating materials on magnetic nanoparticles would be a good choice and new development direction for IMANs. Natural polysaccharides have been investigated and becoming the hot research materials in recent years [17–19]. For this reason, natural chondroitin sodium sulfate was used to modify the magnetite nanoparticles and use IMAN @ Fe (III) nanoparticles to separate PPPs. PPPs were separated and purified effectively from both crude egg yolk hydrolysis polypeptides and phosvitin peptides using the specific magnetic nanoparticles and compared the N/P.

2. Experimental

2.1. Materials

The sources of the chemicals are as follows: trypsin (E.C.3.4.21.4, 3×10^6 IU/g), imidazole, chondroitin sulfate (CS), iron (II) sulfate heptahydrate ($FeSO_4{\cdot}7H_2O$), iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$), ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), 25% ammonia water ($NH_3{\cdot}H_2O$). Fresh chicken eggs were bought from local market. All the chemicals were of analytical reagent grade used without further purification and the water used in all experiments was prepared in a three-stage purification system and had an electrical resistivity of $M\Omega\,cm^{-1}$(highly pure water).

2.2. Preparation and characterization of magnetic chondroitin sulfate (CS) nanoparticles

Functionalized magnetic CS nanoparticles were prepared by adding Fe (II) irons and Fe (III) irons into 25% $NH_3{\cdot}H_2O$ solution, respectively, and then conjugating them with CS under hydrothermal conditions. The whole chemical reaction was made under a nitrogen atmosphere.

$FeSO_4{\cdot}7H_2O$ and $FeCl_3{\cdot}6H_2O$ were dissolved in the water–ethanol solution at the concentration of 0.03 M ions firstly (the molar ratio of $FeSO_4{\cdot}7H_2O$ to $FeCl_3{\cdot}6H_2O$ was 3:2 and the volume ratio of water to ethanol was 5:1), a certain amount of PEG6000 was added under continuous stirring. The chemical precipitation was achieved by adding 25% $NH_3{\cdot}H_2O$ into the fore-said solution at 60 °C for 10 min, and during the reaction process, the medium pH was at 10 approximately by the addition of aqueous solution of ammonia. Then 5% CS was added drop wise to the above reaction mixture over 10 min. After incubation for 60 min at 80 °C, the magnetite nanoparticles were precipitated with permanent magnet at room temperature, and rinsed with distilled water for more than six times to remove un-reacted chemicals thereof. Finally, a black precipitate (magnetite) was thereafter obtained by freeze-drying for about 48 h. And we prepared the naked Fe_3O_4 nanoparticles by the same method on the absence of CS as a comparison only.

The morphology of magnetic CS nanoparticles was observed employing a transmission electron microscope. Samples were prepared by placing two drops of nanoparticle suspension onto a carbon-coated copper grid, followed by drying at room temperature. The chemical functionalities present in a sample were determined by Fourier transform infrared spectroscopy (FTIR, Nicolte Nexus, Thermo Electrin Corporation). The freeze-dried samples and KBr powder (3:100) were then mixed together and made into pellets under high pressure. The sample was scanned from 4000 to 400 cm^{-1}. Three measurements for each sample were performed.

2.3. Preparation and characterization of immobilized metal affinity nanoparticles

10 mg of magnetic CS nanoparticles and 4 mL solution of Fe (III) ions (the concentration was 5 mg/mL) were mixed at pH 5 in a 5 mL centrifugal pipe and shaken in a thermostated shaker (200 rpm) at 37 °C until the adsorption had reached equilibrium. An atomic absorption spectrophotometer (AAS, Spectr AA 220/220Z, Varian, USA) was then used to measure the concentrations of metal ions in the obtained supernatant to determine the content of unimmobilized metal ions. The content of immobilized metal ions was determined according to the law of conservation of mass. Each experiment was performed in three times for the purpose of quality control and statistics. The synthesized IMANs were called IMAN @ Fe (III), FTIR was used to study the coupling mechanism between magnetic CS nanoparticles and Fe (III) ions. The chemical functionality groups present in a sample were detected by FTIR.

2.4. Preparation of egg yolk polypeptides from fresh chicken eggs

Chicken egg yolks were separated from fresh chicken eggs. And after drying at 60 °C, egg yolk powder was further purified with 95% ethanol at an powder-to-ethanol ratio of 1:6 for 1.5 h in three times. The mixture was then shaken in a thermostated shaker (37 °C, 200 rpm) and the mixed suspension was centrifuged at 10,000 rpm and 4 °C for 20 min, followed by drying at room temperature. The dried defatted egg yolk powder which suspended in 0.1 M NaOH solution was thereafter shaken in a thermostated shaker (200 rpm) at 37 °C for 3 h until the reaction had reached equilibrium. After the reaction, the solution was adjusted to the pH of 8.0 using 0.1 M hydrochloric acid and then filtered. The mixed suspension was ultra-filtered and the precipitate washed for 3–5 times with the highly pure water later to remove the free phosphate anion therein. The intercept fluid and washed precipitate were transferred to the enzyme bioreactor solution for reaction and the trypsin added to the sample solution at an enzyme-to-substrate ratio of 1:10 (w/w) and then incubated at 50 °C for 4 h. The pH of the solution was maintained at 8.0 with 0.1 M NaOH. The enzymatic reaction was stopped by maintaining the solution at 95 °C for 15 min, and then cooling it down to the room temperature before the adjustment of pH to 4.5. The tryptic digestion solution was centrifuged at $10{,}000 \times g$ under 4 °C for 20 min. The supernatant protein solution obtained was lyophilized and used as the source of PPPs. For comparison, the PPPs were also purified directly using phosvitin as the tryptic enzymolysis source. Chicken egg yolk phosvitin was separated according to the method of Losso and Nakai and partially dephosphorylated phosvitin and its phosphopeptides according to that of Bo and Mine [20,21].

2.5. Adsorption of phosphopeptides from aqueous solution

Easy to comprehended, the schematic of the metal affinity adsorption is shown in Fig. 1. As can be seen, the binding of PPPs to IMAN @ Fe (III) was mainly through the coordination between metal ions with electron-donating side chain of phosphoserine peptide residues. The adsorption methods are as follows.

40 mL crude egg yolk hydrolysis polypeptides were placed into Erlenmeyer flasks and 60 mg magnetite IMAN @ Fe (III) were added

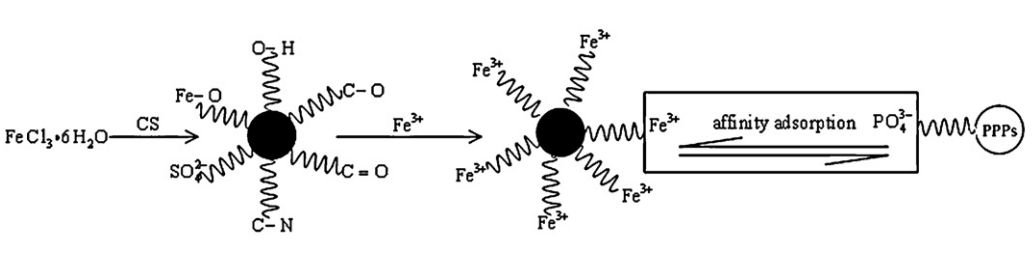

Fig. 1. The schematic of the metal affinity adsorption.

thereinto. The mixed suspension was shaken in a thermostated shaker (37 °C, 200 rpm) for a designated time (3 h). By changing the initial peptide concentration (1.25–20 mg/mL) and the pH value of the reaction (4–7), the effect of the pH was investigated. When equilibrium had been achieved for adsorption, permanent magnet was used to precipitate the magnetite nanoparticles. The obtained supernatant was then used to determine the contents of un-immobilized N and P, respectively. The content of nitrogen (N, %) was determined by Micro-Kjeldahl's method with 6.25 as the N-to-protein conversion factor, and the content of phosphorus (P, %) by the Molybdenum blue colorimetric method (GB-T 5537-2008). Each experiment was performed in three times for the purpose of quality control and statistics. The N/P was calculated with the following equation:

$$q = \frac{(Co_N - Ce_N)}{(Co_P - Ce_P)} \times \frac{31}{14} \quad (1)$$

where q is the N/P of the final products; Co_N and Ce_N are the quality content (mg/g) of the N in the initial solution and the supernatant phase after adsorption, respectively; Co_P and Ce_P are the quality content (mg/g) of P separately in the initial solution and the supernatant phase after adsorption. 31 is the relative molecular weight of P and 14 that of N.

2.6. Stability of the immobilized metal affinity magnetic nanoparticles in repeated use

The adsorption and desorption cycle was repeated five times using the same batch magnetite nanoparticles to determine the reusability of magnetic IMAN @ Fe (III). Desorption of PPPs from IMAN @ Fe (III) was investigated at 37 °C using 0.2 mol/L imidazole (pH 8.0) containing 0.2 mol/L NaCl as the desorbing agents. When equilibrium had been achieved for adsorption, the magnetic nanoparticles were recovered from the reaction mixture by external permanent magnet. The PPPs on the magnetic nanoparticles which washed with highly pure water for five to six times were mixed with the desorbing agents to desorb the PPPs at a constant shaking rate of 200 rpm. After 60 min of desorption, permanent magnet was used to precipitate the magnetite nanoparticles. The N/P was used as the evaluation index to assessing the stability of magnetic IMAN @ Fe (III).

The targeting nanocarriers can be recovered and reused by washing sequentially with EDTA (0.1 M) and Fe (III) metal ions solution.

3. Results and discussion

3.1. Properties of superparamagnetic nanoparticles

As the results of the study, the magnetic CS nanoparticles which suspended in the solution were aggregated quickly when a magnet was near the bottle, so it indicated that the nanoparticles could be easily and quickly separated from the solution by magnet. The biocompatible and biodegradable CS coating not only endowed the magnetic nanoparticles the water-soluble properties but also allowed the magnetic nanoparticles to be bio-conjugated with enzyme molecules by their functional group. In the paper, FTIR spectra of CS (a), naked Fe_3O_4 nanoparticles (b), and magnetic CS nanoparticles (c) were examined (Fig. 2) to confirm the binding of CS. As seen in the spectrum, three main characteristic peaks of the amino, C—N and carboxyl groups in CS at 1628 cm^{-1}, 1251 cm^{-1} and 1062 cm^{-1} were observed in the spectrum of magnetic IMAN. The FTIR spectrum also exhibited that: the presence of CS did shift IR vibrations of Fe_3O_4 but did not alter them very much (the band of Fe—O shifted from 562 cm $^{-1}$ to 568 cm $^{-1}$). Besides, no new bands were found in the spectra of magnetic CS nanoparticles. So we can conclude that CS was successfully coated on the surface of the Fe_3O_4.

The typical TEM micrographs of Fe_3O_4 after coating with CS are shown in Fig. 3. As can be seen, magnetic CS nanoparticles are spherical in shape with an average size of about 20 nm. The relationship between the particle diameter and adsorption carriers is that: smaller particles have larger surface-to-volume ratios and larger capacity to bind more substance on their surface [22]. So the nanoparticles have the good nanometer size and superparamagnetic properties.

3.2. The characteristics of the immobilized metal affinity nanoparticles

The advantages of IMANs separation are as following: firstly, separation rate is rapid for the large surface-to-volume ratios; secondly, it causes significant reduction in the operation cost because no expensive equipment was needed, and separation and recovery were only conducted through a magnet; and last but not the least,

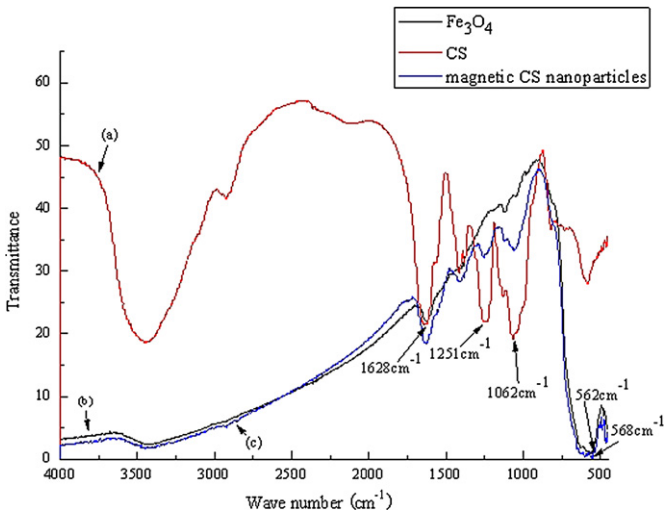

Fig. 2. FTIR spectra of CS (a), naked Fe_3O_4 nanoparticles (b), and magnetic CS nanoparticles (c).

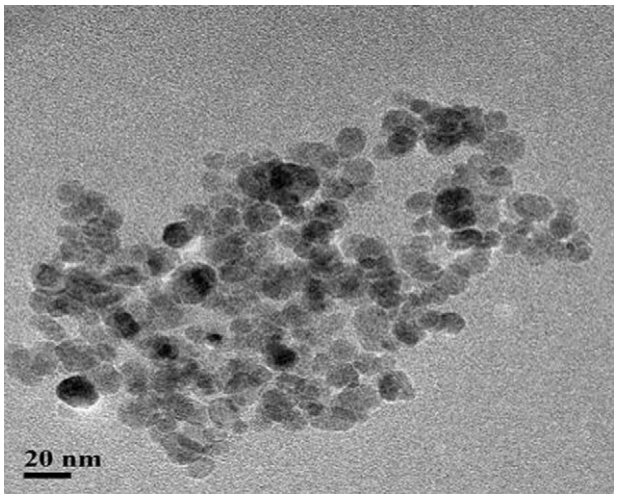

Fig. 3. The TEM micrographs of Fe_3O_4 after coating with CS.

it possesses high metal ions and peptide loading capacities. In this paper, monodispersed and superparamagnetic chondroitin sodium sulfate magnetic nanoparticles were prepared and then decorated with metal ions to adsorb the PPPs. Fe (III) ions were chosen, rather than Ni (II) which is often used in IMANs. It is because the leaking Ni (II) ions could cause human carcinogens even in trace amounts [23]. The biocompatible and biodegradable CS coating not only endowed the magnetic nanoparticles the water-soluble properties but also allowed the magnetic nanoparticles to be bio-conjugated with metal ions by their functional group.

FTIR technique was used to identify functional groups (like carboxyl, amino and hydroxyl, etc.) which are capable of adsorbing metal ions [24]. The FTIR spectra of IMAN @ Fe (III) (a) and magnetic CS nanoparticles (b) were exhibited in Fig. 4. As shown by the spectra of Fig. 4, the peak appeared at 1632 cm^{-1} corresponding to asymmetrical stretching vibration of carboxyl (C=O) which shifts to 1578 cm^{-1} after Fe (III) uptake, demonstrating that C=O group involve in Fe (III) complexation. The peak at 1062 cm^{-1} assigned to the stretching vibration of C—O which shifts to 1052 cm^{-1}, indicating that C—O also participate in Fe (III) adsorption, Furthermore, after Fe (III) adsorption, the band at 628 cm^{-1} which also assigned to SO_4^{2-} is found in complex's spectra, so SO_4^{2-} exists in the complex. From the FTIR spectra analysis, it can be concluded that Fe (III) was decorated on the surface of the magnetic CS nanoparticles

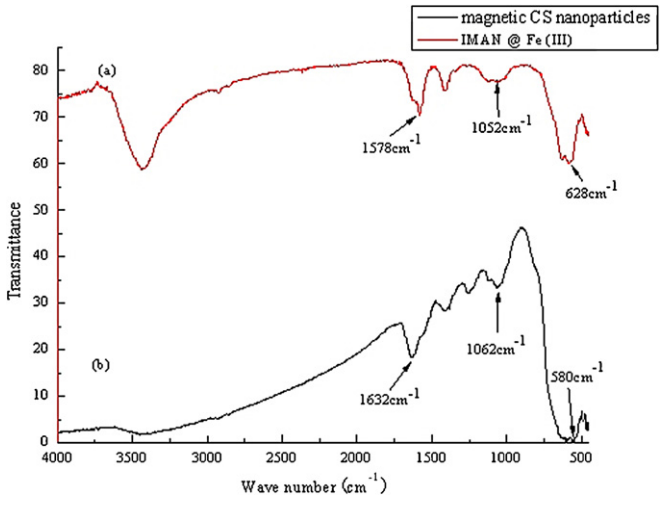

Fig. 4. FTIR spectra of IMAN @ Fe (III) (a) and magnetic CS nanoparticles (b).

Table 1
The relationship between the N/P and the pH.

	pH				
	4	4.5	5	6	7
N/P	9.755	5.78	7.378	8.02	8.76

successfully, and the C—O and C=O mainly participate in the formation of the metal complex. The charged Fe (III) amount in the magnetic CS nanoparticles was 152.12 mg/g.

3.3. Effect of the pH

The purity of a target peptide could be improved by controlling an adsorption process. The PPPs purity was also investigated at different adsorption medium pH that range between 4.0 and 7.0. It was found from Table 1 that the preferential medium pH for PPPs enrichment was at pH 4.5, the molar ratio of nitrogen to phosphorus (N/P), which reflects the sample purity was with a clear increase at lower and higher pH values. It could be drawn that PPPs enrichment was highly related to the pH and the enrichment efficiency increased when the pH was equal to 4.5, which is the isoelectric point of PPPs. Shamin studied the adsorption of lysozyme using nanomagnetic particles, the amount of lysozyme was adsorbed at pH 11, which is the isoelectric point of lysozyme. They concluded that maximum adsorption of a protein could be accomplished when it had a neutral charge that was at the isoelectric point [25].

The other reason may be that Fe (III) ions behave as Lewis acid and the pH of the reaction solution plays an important role in whether or not the Fe (III) ions can react as a strong Lewis acid. Fe (III) ions can selectively trap phosphopeptides in acidic solution because Fe (III) ions behave as a strong Lewis acid with positively charged. The Lewis acid property of Fe (III) ions and the selectivity of the IMANs for phosphopeptides diminishes with the pH value increasing, leading to high N/P and low purity of the products.

3.4. Adsorption model

Using 60 mg of IMAN @ (Fe (III)) and 40 mL of crude egg yolk hydrolysis polypeptides solution with different initial concentration of polypeptides (1.25–20 mg/mL), adsorption model was studied at pH 4.5. The relationship between the N/P and the original polypeptides solution concentration was studied. The results showed that the decrease in the N/P of the final product with increasing the original polypeptides solution concentration, but the decrease was slowed down when the original polypeptides solution concentration was higher than 5 mg/mL, and tend to the lowest N/P. So absorption of polypeptides on magnetite nanoparticles basically complies with Langmuir model which equation can be expressed as following:

$$\frac{C_e}{q_e} = \frac{C_e}{q_m} + \frac{1}{q_m k} \quad (2)$$

where C_e (mg/mL) and q_e are polypeptides concentration in the aqueous solution and that in the N/P of the purified product at equilibrium, respectively; q_m is the lowest N/P and k is the adsorption constant.

Experimental data are fitted to the Langmuir equation using nonlinear regression. In this work the equilibrium concentration and adsorption capacity have the expression as follows:

$$\frac{C_e}{q_e} = 0.1898Ce - 0.1674 \quad (3)$$

$q_m = 1/0.1898 = 5.29$, $R^2 = 0.997$, the relationship between the equilibrium concentration and adsorption capacity were clearly shown in Fig. 5. High R^2 values indicate that the model describes the

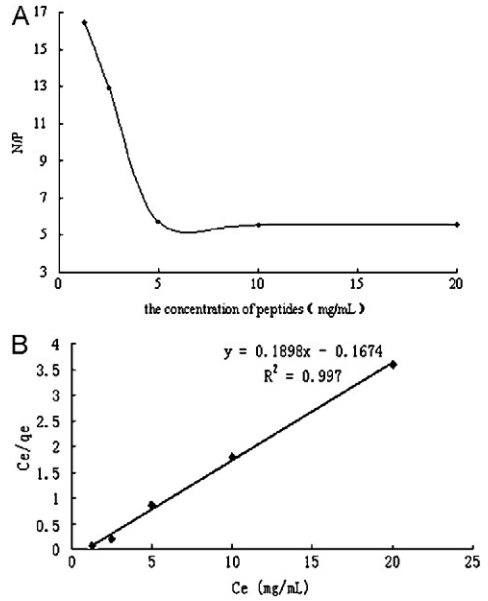

Fig. 5. The relationship between the equilibrium concentration and adsorption capacity.

adsorption behavior well. The lowest N/P of the final product at equilibrium for IMAN @ Fe (III) was calculated to be 5.29. This result may be particularly important in the design and optimization of processes using this metal affinity carriers.

As reported, metal affinity separation is based on the coordinating interaction of metal ions with proteins through their surface-exposed amino acid residues [8,9]. According to the principle of immobilized metal affinity separation, the binding of PPPs to IMAN @ Fe (III) was mainly through the coordination between metal ions with electron-donating side chain of phosphoserine peptide residues. Fe (III) iron was positively charged cationic while phosphorous group was negatively charged, and they would combine together through the electrostatic interaction. Previous studies have demonstrated that phosvitins were the principal phosphoproteins in the eggs and they had exceptionally high serine content, and most, or even all, of the serine residues were esterified to phosphate, the uninterrupted runs of as many as 28 phosphoserines [26,27]. The concentration of phosphate groups provides for numerous highly efficient metal-binding sites in clusters, so the exposed phosphoserine peptide residues should be the dominant affinity binding site in PPPs adsorption with immobilized Fe (III) ions.

3.5. Purification of phosphopeptides from polypeptides

Using IMAN @ Fe (III) to purify PPPs from both crude egg yolk hydrolysis polypeptides and phosvitin hydrolysis peptides was very simple and efficient. The purity of the PPPs was characterized by the N/P. Under the conditions with pH range of 4.5, temperature of 37 °C, as well as concentrations of magnetic nanoparticles and the peptides of 1.5 mg/mL and 5 mg/mL, respectively, the final product will be obtained within 3 h, with the N/P of 5.78 as to crude egg yolk hydrolysis polypeptides and 5.23 as to phosvitin hydrolysis peptides. The result shows that the N/P for PPPs separated from phosvitin peptides were slightly different from PPPs separated from crude egg yolk hydrolysis polypeptides under the same conditions. The products purified using organic solvents in their extraction, as reported, all had a N/P higher than 10 [2,27], no matter whether phosvitin is prepared before the purification or not. It is proved that using these magnetic nanoparticles, PPPs of higher purity would be obtained without the needs to prepare phosvitin before purification (the content of phosvitin is only 2% of the egg yolk dry matter, and the purification process needs vacuum freezing drying which consumes a lot of energy) and to use organic solvents during purification. Arica indicated that Fe (III) ions had a strong affinity for functional groups of peptides, so amino acid residues provided affinity-binding sites for Fe (III) ions through functional groups [28]. It may be therefore concluded that this type of superparamagnetic nanoparticles with high efficiency and cost-effectiveness would benefit the conventional separation techniques of PPPs from chicken egg yolk.

Table 2
The relationship between the adsorption and desorption times and the N/P.

	Times				
	1	2	3	4	5
N/P	5.73	10.78	28.76	34.41	36.68

3.6. Elution of adsorbed phosphopeptides

Imidazole, which can compete with the functional groups of protein for the immobilized Fe (III) ions, is usually used as an eluent in metal affinity separation. To evaluate the repeated use of the IMAN @ Fe (III) in this study, the adsorption–desorption operation cycles was repeated five times using the same batch IMAN @ Fe (III) for PPPs enrichment (as shown in Table 2), the N/P was clearly increasing after two times uses. It indicated that the stability of the IMAN @ Fe (III) was not so satisfied. It might be attributed to the existed strong interaction force between Fe (III) ions and imidazole molecules, this might result in the falling off the Fe (III) ions from the IMAN @ Fe (III). So the optimal eluent and elution conditions should be studied in future research work.

Jun studied the adsorption and desorption of lysozyme on immobilized metal affinity magnetic carboxymethyl chitosan nanoparticles (Fe_3O_4(PEG + CM-CTS) @ Fe (III)) and pointed out that the targeting materials could be recovered and reused by washing sequentially with EDTA (0.1 mol/L) and metal ions solution [16]. In the research, the IMAN @ Fe (III) has structural similarities with Fe_3O_4(PEG + CM-CTS) @ Fe (III), so we could surmise that IMAN @ Fe (III) could also be regenerated by washing sequentially with EDTA (0.1 mol/L) and metal ions solution. It further shows that during the whole process, the magnetic nanoparticles may be prepared and separated in a low-cost and reusable way, and it is very potential for superparamagnetic nanoparticles to be applied in the separation and purification of protein polypeptides.

4. Conclusions

High-efficiency and low-cost superparamagnetic IMAN @ Fe (III) have been developed using natural CS. The prepared IMAN @ Fe (III) possess high-density immobilized metal ions and then successfully applied to enrich PPPs from aqueous solution of egg yolk polypeptides, and the result showed that when the initial concentration of the peptides solution was 5 mg/mL, the pH was 4.5, we could obtain more purified PPPs with lower N/P. In addition, the peptides can be separated and purified in a very convenient and efficient way, without the demand on any expensive equipment, which makes feasible the routine production of PPPs.

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