Journal of Chromatography B, 915–916 (2013) 33–38

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Highly efficient enrichment of phosvitin phosphopeptides by novel magnetic carboxymethyl chitosan nanoparticles decorated with Fe (III) ions

Jun Sun[a], Yuntao Liu[b], Yujie Su[b], Wenshui Xia[b], Yanjun Yang[b,*]

[a] School of Food and Biological Engineering, Jiangsu University, Zhenjiang, Jiangsu 212013, PR China
[b] School of Food Science, Jiangnan University, Wuxi, Jiangsu 214122, PR China

ARTICLE INFO

Article history:
Received 6 March 2012
Accepted 16 December 2012
Available online 25 December 2012

Keywords:
Magnetic nanoparticles
Bioactive peptides
Phosvitin
Phosphopeptides

ABSTRACT

Functional immobilized metal affinity magnetic carboxymethyl chitosan nanoparticles (abbreviated as Fe_3O_4 (PEG + CM-CTS) @ Fe (III)) were conveniently applied for phosvitin phosphopeptides (PPPs) enrichment for the first time. The morphology of magnetic nanoparticles was observed by transmission electron microscope (TEM). It was found that the diameter of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) was about 20 nm, and could easily aggregate by a magnet when suspending in the aqueous solution. In the PPPs enrichment study, the results obtained emphasized the role of pH, temperature and the initial concentration of the peptides solution in governing the efficiency and mechanism of affinity interactions. Due to the large specific surface area, the enrichment of PPPs onto the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles was promising. The adsorption equilibrium of PPPs onto the obtained magnetic nanoparticles fitted well with the Langmuir model, and the nitrogen/phosphorus molar ratio (N/P) which at the maximum enrichment capacity for PPPs was 4.83. Due to the small diameter, the decrease of the N/P is particularly rapid in the early enrichment stages (0–30 min) to reach a plateau after 60 min. Compared with traditional methods, the need for preparation of phosvitin before purification is obviated and PPPs of higher purity were obtained. Since the preparation, surface modification and affinity separation processes of the magnetic nanoparticles are cost-effective, convenient and efficient, this type of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles would bring advantages compared to conventional separation techniques of PPPs from chicken egg yolk, as well as for phosphopeptides enrichment in proteomics research.

© 2012 Elsevier B.V. All rights reserved.

1. Introduction

Bioactive peptides have shown the most promising potentials as health promoting agents. There are many kinds of bioactive peptides with various biological properties, such as antioxidant, antimicrobial, anticancer, metal binding, immunomodulatory, antihypertensive and so on [1–3], among these peptidic molecules, phosphopeptides are most interesting since it can effectively bind calcium and inhibit the formation of insoluble calcium phosphates, thus resulting in an increased calcium-binding capacity [4]. A great number of natural phosphopeptides have been derived from casein; however, a subunit of casein has only 1–13 phosphoserine residues to stabilize amorphous calcium phosphate, whereas a molecule of phosvitin has approximately 120 phosphoserine residues [5]. The phosvitin phosphopeptides (PPPs), extracted from chicken egg yolk phosvitin have been shown to bind and solubilize calcium more efficiently than commercial casein phosphopeptides [6,7]. Furthermore, it was reported that PPPs exhibit free radical scavenging and antioxidant properties against lipid peroxidation and that they are also effective against oxidative stress in vitro [8,9]. Moreover, Samaraweera et al. showed that PPPs could be used as naturally derived antioxidant and antiaging metal-binding peptides in the cosmetics industry [10]. Thus, we consider that PPPs could be integrated into several different applications in the food, medical and cosmetic industries in the near future.

Presently, specific enrichment of phosphopeptides is possible using several analytical strategies, such as high-performance liquid chromatography (HPLC), the barium ethoxide precipitation method, and immobilized metal ion affinity chromatography (IMAC) [6,9,11]. Of all the available separation technologies for the enrichment of phosphopeptides, those based on the metal ion affinity interactions are the most favoured [12,13]. Immobilized metal affinity magnetic nanoparticles (IMANs) which are based on the separation principle of IMAC have been proved to be useful in the enrichment of phosphopeptides as they encompass the following advantages: firstly, their adsorption rate is rapid since they have large surface-to-volume ratios; secondly, they significantly reduce operation costs because the equipment used is not expensive since the immobilization and the recovery of PPPs specifically through

* Corresponding author. Tel.: +86 0510 85329080; fax: +86 0510 85329080.
E-mail addresses: sunjunwin@126.com (J. Sun), yangyj2005@hotmail.com (Y. Yang).

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.jchromb.2012.12.013

magnetic-based processes and last but not the least, they possesses high metal ions and target molecules loading capacities [14–16]. PPPs enrichment from chicken egg yolk proteins using IMANs has never been reported.

In our previous study, we have successfully applied chemical coprecipitating and metal ions conjugation reaction to large-scale synthesis of IMANs (Fe_3O_4 (PEG + CM-CTS) @ Fe (III)) [17,18]. In this paper, we used Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles as a new magnetic nanocarrier for PPPs enrichment. Considering that the magnetic nanoparticles possess the advantages of high efficiency, cost-effectiveness and easier separation from the reaction system, the present work provides quantitative information on the enrichment of PPPs when using Fe_3O_4 (PEG + CM-CTS) Fe (III) nanoparticles. Our final objective was to develop potential applications of such magnetic nanoparticles in IMAC technology as chromatographic resin agents for the separation of PPPs from chicken egg yolk, as well as for phosphopeptides enrichment in proteomics research.

2. Materials and methods

2.1. Materials

The sources of the chemicals are as follows: Trypsin (E.C.3.4.21.4, 3×10^6 IU/g), imidazole, ethylenediaminetetraacetic acid disodium salt (EDTA-2Na), iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$). Fresh chicken eggs were bought from local market. All the chemicals were of analytical reagent grade used without further purification. The water used in all experiments was prepared in a three-stage purification system and had an electrical resistivity of 18.2 MΩ cm^{-1} (highly pure water).

2.2. Preparation of immobilized metal affinity magnetic nanoparticles (IMANs)

In order to investigate the enrichment of PPPs on IMANs, firstly Fe_3O_4 (PEG + CM-CTS) nanoparticles which were prepared by chemical coprecipitating iron (II) and iron (III) in 25% (w/v) $NH_3 \cdot H_2O$ solution and subsequently conjugated with PEG 6000 and CM-CTS under hydrothermal conditions have been synthesized. Iron (II) and iron (III) were dissolved in water–ethanol solutions, a certain amount of PEG6000 were added under continuous stirring. Chemical precipitation was achieved by adding $NH_3 \cdot H_2O$ solution (25%) into the above solution at 60 °C for 10 min, then, 5% CM-CTS was added dropwise 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 six times to remove unreacted chemicals. The synthetic procedures of Fe_3O_4 (PEG + CM-CTS) nanoparticles have been presented in detail in our previous paper [17]. Secondly, Fe (III) ions were immobilized onto the Fe_3O_4 (PEG + CM-CTS) nanoparticles. The reaction procedures of the IMANs which abbreviated as Fe_3O_4 (PEG + CM-CTS) @ Fe (III) have been previously reported in detail by Sun et al. [18]. The concentrations of Fe (III) were measured using an Atomic Absorption Spectrophotometer (AAS, Spectr AA 220/220Z, Varian, USA). Shape and morphology analysis of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) were examined by transmission electron microscope (TEM, JEOL JEM-2100 (HR)). Each experiment was performed in duplicate for quality control and statistical purposes.

2.3. Preparation of egg yolk polypeptides from fresh chicken eggs

Chicken egg yolks which were isolated from fresh chicken eggs were further defatted with 95% (v/v) ethanol using Soxhlet's extraction method then dried in oven at 60 °C. The dried defatted egg yolk powders were suspended in a 0.1 M NaOH solution and shaken in a thermostated shaker (200 rpm) at 37 °C for 3 h. Then, the pH of the solution was adjusted to 8.0 using a 0.1 M hydrochloric acid solution and the mixture was then diluted 3–5 times using highly pure water. The free phosphate anions were removed by Ultrafiltration (Mw = 3000 Da). The trapped fluid was transferred to the enzyme solution reactor, and the pH of the solution was maintained at 8.0 with 0.1 M NaOH. Trypsin was added to the above-mentioned solution at an enzyme-to-substrate ratio of 1:10 (w/w). Then, the mixture was incubated at 50 °C for 4 h. 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 adjusting the pH to 4.5. The tryptic digestion solution was centrifuged at 10,000 rpm at 4 °C for 20 min. The resulting supernatant protein solution was lyophilized and used as the source of PPPs.

For comparison purposes, enrichment of the PPPs was also performed according to conventional a procedure using phosvitin as the tryptic enzymolysis source. Chicken egg yolk phosvitin was isolated according to the method of Losso and Nakai [19]. Partially dephosphorylated phosvitin and its phosphopeptides were prepared according to the method of Jiang and Mine [6].

2.4. Phosvitin phosphopeptides enrichment

Magnetic Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles were used in each experiment. Typically, 60 mg of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles was mixed with 40 mL of a crude egg yolk hydrolysis polypeptides solution (1–20 mg/mL) which containing PPPs (pH 4.0–7.0). The mixture was added into a 100 mL of triangular flask and the mixed suspension was shaken in a thermostated shaker (200 rpm) at (10–37 °C) for 15–180 min until the adsorption had reached equilibrium. Aliquots were withdrawn at suitable time intervals. Changing the initial concentration of crude egg yolk hydrolysis polypeptides solutions, adsorption model was studied. After the adsorption reaction, the obtained supernatant was then used to determine the contents of unimmobilized nitrogen (N) and phosphorus (P), respectively. Each experiment was performed in duplicate. The content of N was determined by Micro-Kjeldahl's method; and the molybdenum blue colorimetric method (GB/T 5537–2008) [20] was used for P content determination. The nitrogen/phosphorus molar ratio (N/P) was calculated with following equation:

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

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.

2.5. Stability of the IMANs in repeated use

To determine the reusability of the magnetic Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles, the adsorption and desorption cycles were repeated five times by using the same batch of magnetite nanoparticles. Desorption of PPPs from Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles was investigated at 37 °C using 0.2 M imidazole (pH 8.0) containing 0.2 M NaCl as the desorbing agents. When equilibrium had been achieved for adsorption, the magnetic nanoparticles were recovered from the reaction mixture using an external permanent magnet. The PPPs bound on the magnetic nanoparticles were washed with highly pure water for five to six times were mixed at a constant shaking rate of 200 rpm in the presence of the desorbing agents so as to desorb the PPPs from the

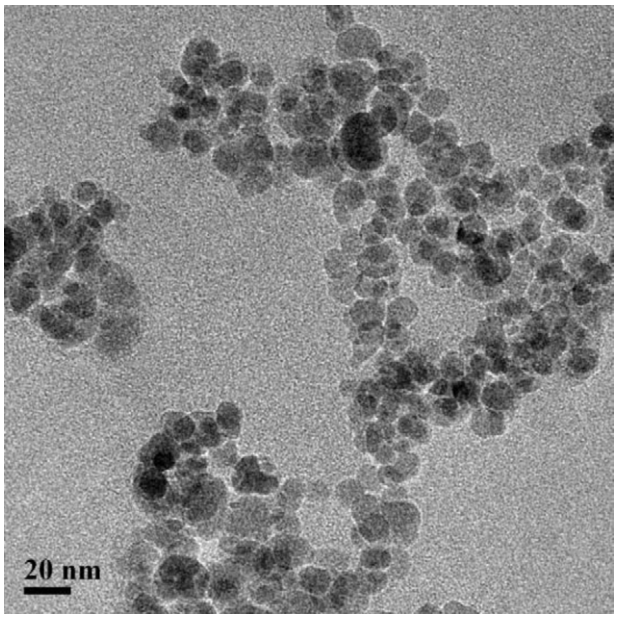

Fig. 1. TEM image of the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles.

nanoparticles. After 60 min of desorption, a permanent magnet was used to precipitate the magnetic nanoparticles. The N/P ratio was used as the evaluation index for assessing the stability of magnetic Fe_3O_4 (PEG + CS) @ Fe (III) nanoparticles.

The targeted 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. The characteristics of the IMANs

As shown in our previous study [17], magnetic Fe_3O_4 (PEG + CM-CTS) nanoparticles which are not decorated with Fe (III) ions were essentially spherical, monodisperse, and with a mean diameter of about 15 nm. After conjugation with Fe (III) ions, the resulting IMANs remained spherical and monodispersed (Fig. 1), indicating that the process of Fe (III) conjugation did not significantly lead to a change in size and dispersibility of the nanoparticles. This can be attributed to the fact that the Fe (III) conjugation reaction occurred only on the surface of the magnetic Fe_3O_4 (PEG + CM-CTS) nanoparticles.

The IMAC method is the most widely used technology for the selectively enrichment of phosphopeptides from a complex mixture system since it is a convenient, highly efficient, and economic procedure. In this paper, we chose Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles as the magnetic nanocarriers for PPPs enrichment. The chosen Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles possess the following advantages: firstly, the presence of hydrophilic group (—COOH—OH and $—NH_2$) on their surface allows for higher dispersibility in water-based solutions, a characteristic that improves the interaction between PPPs and the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles. Secondly, the larger surface/volume ratio of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles endowed them a higher binding rate for conjugation with PPPs. Interestingly, the synthetic process of the magnetic Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles is very easy to perform, and suitable for large-scale synthesis.

The IMAC technology which applied for phosphopeptides enrichment is based on the specific interaction between Fe (III) and the phosphate groups of phosphopeptides. Based on this concept, the more Fe (III) immobilized on the surface of the IMANs, the more phosphopeptides enrichment will be achieved. AAS was used to determine Fe (III) content in magnetic Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles. For two repeated determinations an average content of 153.8 mg/g of Fe_3O_4 (PEG + CM-CTS) nanoparticles for Fe (III) was obtained. However, the amount of immobilized metal ions only represents the binding capacity of the support and does not provide any information about the absorption capacity of the corresponding IMANs. Therefore, enrichment of PPPs from chicken egg yolks was studied.

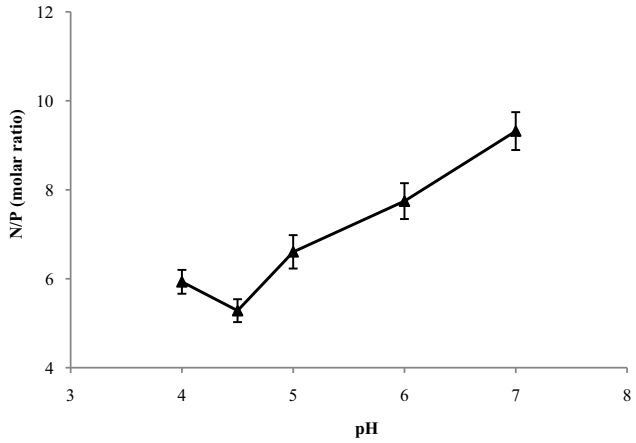

Fig. 2. Effects of pH on PPPs enrichment on the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles (initial concentration of peptides solution: 5.0 mg/mL; temperature: 37 °C; time: 120 min).

3.2. Phosvitin phosphopeptides enrichment

The final degree of hydrolysis (DH) of chicken egg yolk protein was 13.02%. The low DH value can be explained by the high specificity of trypsin which cleaves at basic amino acids, Lys and Arg, thus a limited number of peptides might be generated. Furthermore, the phosphoserine residues are arranged in a core section, which forms blocks that can carry up to 15 consecutive residues alternated with the basic amino acids [21], so the trypsin cleavage sites are presumably hindered and less accessible.

Theoretically, all the PPPs with a phosphate groups should be able to be captured and enriched by such an IMAC technology. In the present work, the resulting magnetic Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles were used for direct enrichment and purification of PPPs in tryptic hydrolysates of crude chicken egg yolk proteins. Different enrichment parameters were studied on PPPs enrichment.

3.2.1. Effect of the pH

The purity of a target peptide could be improved by controlling the adsorption process and the pH of the medium which is a very important parameter that determines the adsorption capacity of the IMANs. The pH of the adsorption medium affects the net charge of the immobilized metal ions as well as the protein molecule. The optimal pH values for enrichment of PPPs on Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles were investigated in the pH range of 4.0–7.0 (Fig. 2). The preferential medium pH for PPPs enrichment was at pH 4.5. At higher pHs, the molar ratio of nitrogen to phosphorus (N/P) which reflects the sample purity [22] was clearly increased. Therefore, it could be postulated that PPPs enrichment was highly related with the pH of the solution given that the optimal medium pH for PPPs enrichment was observed at pH 4.5. This phenomenon may result from the fact 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

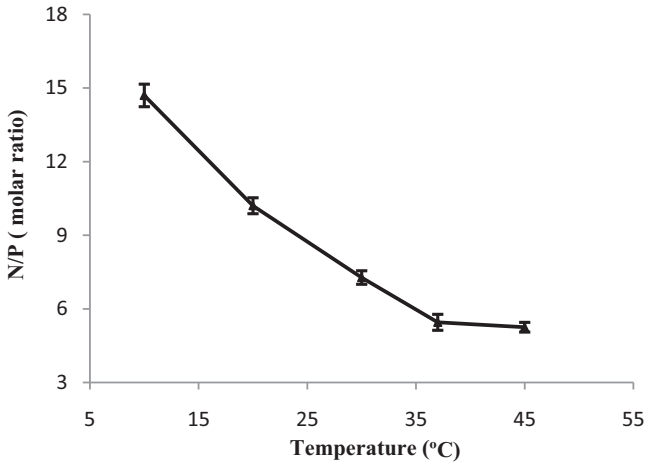

Fig. 3. Effects of temperature on PPPs enrichment on the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles (initial concentration of peptides solution: 5.0 mg/mL; pH: 4.5; time: 120 min).

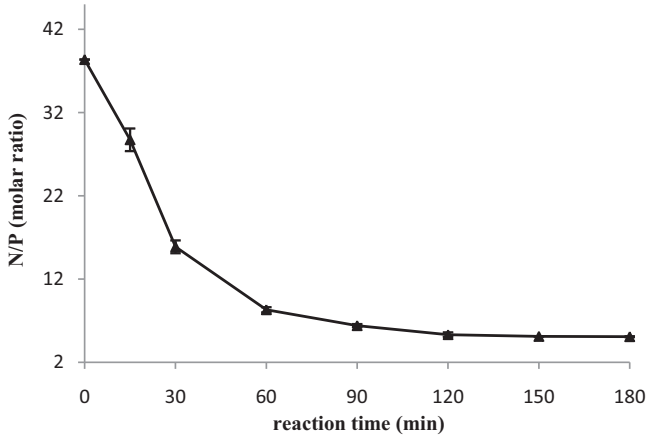

Fig. 4. Effect of reaction time on PPPs enrichment on the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles (initial concentration of peptides solution: 5.0 mg/mL; pH: 4.5; temperature: 37 °C).

selectively trap PPPs in acidic solution because Fe (III) ions behave as a strong positively charged Lewis acid. The Lewis acid property of Fe (III) ions and the selectivity of the IMANs for PPPs diminishes with increasing the pH value, leading to high N/P and low purity of the products (Fig. 2).

3.2.2. Effect of temperature

The medium temperature is another important parameter which can affect the absorption of bioactive molecules. At present, a growing number of studies on the effect of temperature on bioactive molecules absorption have been reported [23–26]. Some researchers had drawn the conclusion that bioactive molecules absorption is an exothermic process, in which the absorption rate decreased gradually as temperature increase [25]. Other authors inferred that the process is endothermic [26]. In this paper, the effect of temperature on PPPs enrichment from the mixed protein solution shown in Fig. 3 shows that the adsorption of PPPs onto the functional Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles significantly increased when increasing temperature from 10 to 45 °C. The N/P (molar ratio) of the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles for PPPs enrichment decreased from 14.7 to 5.25. This indicated that the increase in temperature was favourable to PPPs enrichment onto the magnetic nanoparticles. This result is consistent with the fact that a rise of temperature induces a rise in kinetic energy of the nanoparticles which increases molecular movements and collision frequency between the sorbent and adsorbates.

3.2.3. Kinetic properties

With regards to further studying the adsorption characteristics of PPPs onto the surface of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles, the effect of contact time (15–180 min) on PPPs enrichment was examined using 60 mg of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles with 40 mL of crude egg yolk polypeptides hydrolyzates at the initial concentration of 5.0 mg/mL, and results are shown in Fig. 4. Since the nanoparticles have a very small diameter and large specific surface area, the decrease of the N/P (molar ratio) is particularly rapid in the early enrichment stages (0–30 min) to reach a plateau after 60 min. This data indicates that efficient enrichment of PPPs onto the magnetic Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles occurs fairly rapidly and that IMANs nanoparticles are a promising approach to PPPs purification.

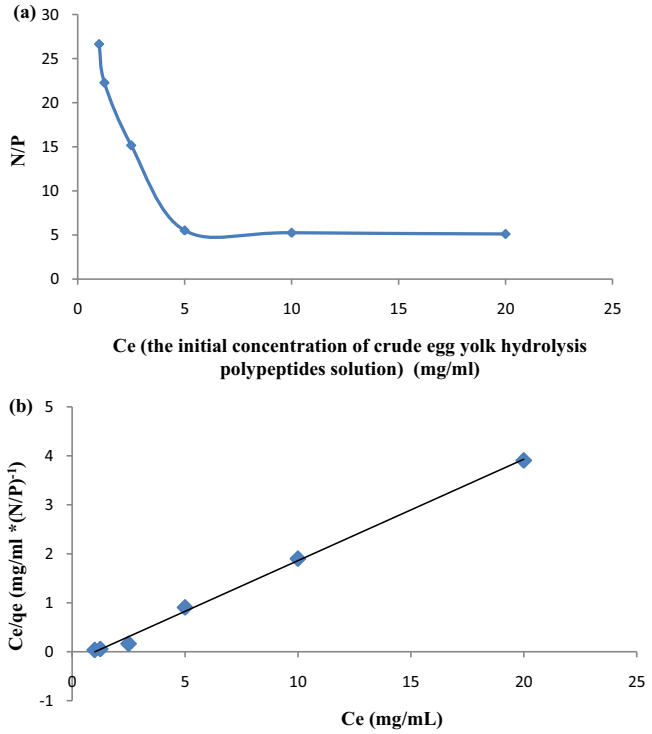

Fig. 5. (a) Effect of the initial concentration of peptides solution of the PPPs enrichment on the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles. (b) Curve fitting of Langmuir model.

3.2.4. Adsorption model

Using 60 mg of magnetic Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles and 40 mL of crude egg yolk polypeptides hydrolyzates solution with different initial concentration, the adsorption process was studied. The relationship between the enrichment capacity and the initial crude egg yolk polypeptides hydrolyzates concentration is presented in Fig. 5a. The results show a correlation between the decrease of the N/P (molar ratio) and the increasing initial concentration of the crude egg yolk polypeptides hydrolyzates solution. However, the decrease of the N/P molar ratio was slowed down when the original crude egg yolk polypeptides hydrolyzates solution concentration was 5 mg/mL and remains unchanged at concentrations values higher than 5 mg/mL, it may be due to adsorption sites saturation of the

magnetic nanoparticles. Also, a good linear relationship is observed between the concentration of the crude egg yolk polypeptides hydrolyzates solution and N/P molar ratio (Fig. 5b) indicating that the enrichment of PPPs on the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles basically complies with Langmuir model which can be used to describe the absorption process. The equation can be expressed as follows:

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

where C_e (mg/mL) and q_e are the concentration of crude egg yolk polypeptides hydrolyzates solution and the N/P at time t (min), respectively. q_m is the minimum N/P which at the maximum enrichment capacity for PPPs and k is the adsorption constant.

In this work the equilibrium concentration and the N/P molar ratio at the maximum enrichment capacity can be expressed as follows: $C_e/q_e = 0.207C_e - 0.2097$, $R^2 = 0.9973$; $q_m = 1/0.207 = 4.83$.

The related coefficient (R^2) is 0.9973; high R^2 values indicate that the Langmuir model provides a good description of the behaviour of the adsorption process. This suggests that the enrichment of PPPs by Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles is monolayer-type, and the PPPs might form a layer on the surface of the magnetic nanoparticles.

According to the principle of immobilized metal affinity separation, the binding of PPPs onto Fe_3O_4 (PEG + CS) @ Fe (III) is mainly occurring through coordination between metal ions with the electron-donating side chain of phosphoserine peptide residues. However, Fe (III) iron is cationic while phosphorous group is negatively charged, and they would bind together through an electrostatic interaction. Furthermore, the electron-donating side chain of phosphoserine peptide residues provides numerous highly efficient metal-binding sites, so the exposed phosphoserine peptide residues should be the dominant affinity binding sites in PPPs adsorption with immobilized Fe (III) ions.

3.3. Purification of phosphopeptides from polypeptides

PPPs were also enriched from the tryptic solution using the phosvitin as hydrolysis substrate. The enrichment efficiency of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles with PPPs obtained from the phosvitin hydrolysis solutions was examined using 60 mg of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles with 40 mL of phosvitin hydrolysis solution at the initial concentration of 5.0 mg/mL. The mixed suspension was shaken in a thermostated shaker (200 rpm) at 37 °C for 120 min when the adsorption had reached equilibrium. Although the N/P (molar ratio) was 4.97 which was comparable with that obtained with the above method, it did not improve appreciably. However, the preparation of the phosvitin hydrolysis solutions was more complicated (the content of phosvitin is only 2% (w/w) of the egg yolk dry matter, and the purification process needs vacuum freezing drying which consumes a lot of energy) than the procedure which uses the crudes chicken egg yolk protein as the hydrolysis substrate. Thus this type of superparamagnetic Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles can result in potential application in the enrichment of PPPs from the tryptic hydrolysis solutions of crude chicken egg yolk proteins.

3.4. Stability of magnetic nanoparticles in repeated use

The PPPs-loaded Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles were placed within the elution medium containing 0.2 M NaCl at 0.2 M imidazole (pH 8.0), imidazole, which can compete with the functional groups of phosphate ions which covalent coupling with Fe (III) ions, is usually used as an eluent in metal affinity absorption.

To evaluate the repeated use of the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles, the adsorption–desorption operation cycles was repeated five times by using the same batch of Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles for PPPs enrichment. The N/P molar ratio clearly increased after three repeated absorption–desorption cycles. This result indicated that the stability of the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles was low probably due to the strong interactions between Fe (III) ions and imidazole molecules, which most likely removed some of the Fe (III) ions from the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles. The selection of the optimal eluent and elution conditions will be studied in future research work. It should be noted that, the Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticle could be regenerated by washing sequentially with EDTA (0.1 M) and Fe (III) ions solutions.

4. Conclusion

Functional immobilized metal affinity magnetic carboxymethyl chitosan nanoparticles (abbreviated as Fe_3O_4 (PEG + CM-CTS) @ Fe (III)) were conveniently applied for PPPs enrichment for the first time. The adsorption equilibrium of PPPs onto the obtained nanocarriers fitted well with the Langmuir model, and the nitrogen/phosphorus molar ratio (N/P) which at the maximum enrichment capacity for PPPs was 4.83. Compared with the enrichment of PPPs from phosvitin hydrolyzates, Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles would bring potential application in PPPs enrichment from egg yolk protein hydrolyzates with the simpler procedures and higher enrichment efficiency. The purification process avoiding expensive equipment makes routine PPPs enrichment feasible. However, the nanoparticles were not very stable when subjected to three repeated adsorption-elution cycles, so the selection of the optimal eluent and elution conditions will be studied in future research work. It is anticipated that such Fe_3O_4 (PEG + CM-CTS) @ Fe (III) nanoparticles may have great potential to be used in large-scale purification of PPPs from chicken egg yolk, as well as for phosphopeptides enrichment in proteomics research.

Acknowledgements

Authors express sincere thanks to the senior talent scientific research founds of Jiangsu University(12JDG077), the Nature Science Foundation of China (NSFC, SKLF-MB-200805), Doctoral Research Funds of Jiangnan University (JUDCF11018) and Graduate Education Innovation Project in Jiangsu Province (CXZZ, 110489) for supporting this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.12.013.

References

[1] E. Haque, R. Chand, Eur. Food Res. Technol. 227 (2008) 7.
[2] N.P. Möller, K.E. Scholz-Ahrens, N. Roos, J. Schrezenmeir, Eur. J. Nutr. 47 (2008) 171.
[3] I. Choi, C. Jung, H. Choi, C. Kim, H. Ha, Food Chem. 93 (2005) 577.
[4] B. Jiang, Y. Mine, Biosci. Biotechnol. Biochem. 65 (2001) 1187.
[5] O. Castellani, C. Guérin-Dubiard, E. David-Briand, M. Anton, Food Chem. 85 (2004) 569.
[6] B. Jiang, Y. Mine, J. Agric. Food Chem. 48 (2000) 990.
[7] I. Choi, C. Jung, H. Seog, H. Choi, Food Sci Biotechnol. 13 (2004) 434.
[8] D. Young, F. Nau, M. Pasco, Y. Mine, J. Agric. Food Chem. 59 (2011) 9207.
[9] S. Katayama, X.M. Xu, M.Z. Fan, Y. Mine, J. Agric. Food Chem. 54 (2006) 773.

[10] H. Samaraweera, W.G. Zhang, E.J. Lee, D.U. Ahn, J. Food Sci. 76 (2011) R143.
[11] F. Tan, Y.J. Zhang, W. Mi, J.L. Wang, J.Y. Wei, Y. Cai, X.H. Qian, J. Proteome Res. 7 (2008) 1078.
[12] S.B. Ficarro, G. Adelmant, M.N. Tomar, Y. Zhang, V.J. Cheng, J.A. Marto, Anal. Chem. 81 (2009) 4566.
[13] J.Y. Wei, Y.J. Zhang, J.L. Wang, F. Tan, J.F. Liu, Y. Cai, X.H. Qian, Rapid Commun. Mass Spectrom. 22 (2008) 1069.
[14] A.H. Lu, E.L. Salabas, F. Sch, Angew. Chem. Int. Ed. 46 (2007) 1222.
[15] P.R. Levison, S.E. Badger, J. Dennis, P. Hathi, M.J. Davis, I.J. Brue, D. Schimkat, J. Chromatogr. A 816 (1998) 107.
[16] K.B. Lee, S. Park, C.A. Mirkin, Angew. Chem. Int. Ed. 43 (2004) 3048.
[17] J. Sun, Y.J. Su, S.Q. Rao, Y.J. Yang, J. Chromatogr. B 879 (2011) 2194.
[18] J. Sun, S.Q. Rao, Y.J. Su, R.R. Xu, Y.J. Yang, Colloids Surf. A 389 (2011) 97.
[19] J.N. Losso, S.A. Nakai, Simple procedure for the isolation of phosvitin from chicken egg yolk, in: Egg Uses and Processing Technologies, New Developments, Oxon, UK, 1994, p. 150.
[20] GB/T 5537-2008, PR China.
[21] B.M. Byrne, A.D. van het Schip, J.A.M. van de Klundert, A.C. Arnberg, M. Gruber, A.B. Geert, Biochemistry 23 (1984) 4275.
[22] O. Castellani, V. Martinet, E. David-Briand, C. Guerin-Dubiard, M. Anton, J. Chromatogr. 79 (2003) 1273.
[23] G. Bayramoglu, Y. Tunali, M. Yakup Arica, Catal. Commun. 8 (2007) 1094.
[24] X. Chen, J.H. Liu, Z.C. Feng, Z.Z. Shao, J. Appl. Polym. Sci. 96 (2005) 1267.
[25] K.Z. Elwakeel, Desalination 250 (2010) 105.
[26] Y.S. Ho, G. McKay, Process Biochem. 38 (2003) 1047.