Journal of Chromatography B, 893–894 (2012) 121–126

Contents lists available at SciVerse ScienceDirect

Journal of Chromatography B

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

Separation and purification of phosvitin phosphopeptides using immobilized metal affinity nanoparticles

Jing Zhang[a,b,1], Jun Sun[a,b,1], Yuntao Liu[a,b], Junhua Li[a,b], Yujie Su[a,b], Wenshui Xia[a,b], Yanjun Yang[a,b,*]

[a] State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, PR China
[b] School of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, PR China

ARTICLE INFO

Article history:
Received 1 December 2011
Accepted 28 February 2012
Available online 7 March 2012

Keywords:
Chondroitin sulfate (CS)
Phosvitin phosphopeptides (PPPs)
Immobilized metal affinity nanoparticles (IMANs)
Protein Purification
Metal ions

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

* Corresponding author at: State Key Laboratory of Food Science and Technology, Jiangnan University, Wuxi 214122, Jiangsu, PR China. Tel.: +86 0510 85329080; fax: +86 0510 85329080.
E-mail addresses: yangyanjun2009@hotmail.com, zhang315037418@163.com (Y. Yang).
[1] These authors contributed equally to this work.

1570-0232/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2012.02.046

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