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Comparison of a novel TiO_2/diatomite composite and pure TiO_2 for the purification of phosvitin phosphopeptides

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ABSTRACT

A novel TiO_2/diatomite composite (TD) was prepared and then characterized by scanning electron microscope (SEM) and Fourier Transform Infrared (FTIR). The results of SEM showed that after modification, the porous surface of diatomite was covered with TiO_2. Both diatomite and TD had clear disc-shaped structures with average grain diameters of around 25 μm. Then TD and pure TiO_2 were applied in the purification of phosvitin phosphopeptides (PPPs) from the digest of egg yolk protein, and a comparative study of adsorption properties of PPPs on TD and TiO_2 was performed. In the study of adsorption kinetics, the adsorption equilibrium of PPPs on TD and TiO_2 fitted well with the Langmuir model, and the time needed to reach adsorption equilibrium were both around 10 min. The maximum dynamic adsorption capacity of TD (8.15 mg/g) was higher than that of TiO_2 (4.96 mg/g). The results of repeated use showed that TD and TiO_2 were very stable after being subjected to ten repeated adsorption–elution cycles, and TD could easily be separated from aqueous solution by filtration. On the other hand, the present synthetic technology of TD was very simple, cost-effective, organic solvent-free and available for large-scale preparation. Thus, this separation method not only brings great advantages in the purification of PPPs from egg yolk protein but also provides a promising purification material for the enrichment of phosphopeptides in proteomic researches.

1. Introduction

Recently, many researchers have identified that PPPs had a great potential to improve the bioavailability of Ca^{2+} and thus increased the incorporation of Ca^{2+} into bones [1,2]. In traditional ways, PPPs are purified from phosvitin, which is the main phosphoprotein in egg yolk containing 10% phosphorus [3]. Although many studies have been carried out, the present purification methods still have some problems.

As the three main traditional separation methods of PPPs, organic solvent precipitation, ion exchange chromatography and membrane separation, all have their obvious disadvantages [4]. Our research team studied the application of immobilized metal affinity nanoparticles in PPPs purification. These methods were fast, high-loading and efficient, but the stability of these nanoparticles was not so satisfying. And the elution medium used was imidazole, which was toxic and not allowed to be used in food industry [4,5]. In addition, these methods are only used in research laboratories, the magnetic separation technology and equipment for large-scale industrial application are still immature. It is necessary to develop new separation technology for large-scale production of PPPs, which is more stable, specific and easier for expanded industrial application.

In recent years, TiO_2 is widely used as an affinity support for the enrichment of phosphopeptides, based on the strong selective bidentate binding of phosphorylated peptides onto the TiO_2 surface [6–10]. TiO_2 is an amphoteric oxide. In acid solutions,

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it acts as a Lewis acid with positively charged titanium atoms, displaying anion-exchange properties. While in base solutions, it displays cation-exchange properties. Meanwhile, TiO_2 has better corrosion resistance and biocompatibility than other organic or inorganic materials [11–15]. As TiO_2 is introduced for phosphopeptides enrichment only at the beginning of this decade, the current researches mainly concentrate in phosphoproteomic. Applying TiO_2 in the purification of PPPs has not yet been reported.

In our early studies, we discovered that PPPs could be specifically adsorbed onto the surface of TiO_2. However, due to the uneven distribution of grain diameters and surface hydrophilicity of TiO_2 powders, they could only be separated from aqueous solutions by high-speed centrifugal, which not only increased the cost of PPPs production but also limited the possibilities of large-scale application of TiO_2. Some researchers have reported several kinds of TiO_2 modification, composite materials such as TiO_2 microcolumns, Fe_3O_4@TiO_2 core–shell microspheres, and SiO_2/TiO_2 composite monolithic capillary column have been successfully synthesized and used for the selective enrichment of phosphopeptides [16–19]. But these methods are all laboratory studies, large-scale separation method for phosphopeptides still need further research.

In the modification researches of materials chemistry, TiO_2 has been loaded on various supporting materials such as glass plates, silica beads, zeolites, activated carbons, and diatomites [20–22]. As diatomite is cheap and widely used as a filter aid in food and pharmaceutical industries, and can easily be separated from aqueous solutions by filtration. What's more, its natural porous structure and stable physical and chemical properties (under acid and alkaline conditions) makes it a perfect support for materials modification.

In this work, a novel TiO_2/diatomite composite was prepared by chemical coprecipitation method and used for the purification of PPPs. The adsorption properties of PPPs on TD were studied and compared with that of pure TiO_2. The successful application of TD in PPPs purification may not only find a new purification method for PPPs production, but also provide a promising separation material for the enrichment of phosphopeptides from other complex biological samples in proteomic researches.

2. Materials and methods

2.1. Materials

Titanium dioxide (TiO_2, chemically pure), Hydrochloric acid (HCl), titanium tetrachloride ($TiCl_4$), 95% ethanol, acetone, trypsin (E.C.3.4.21.4.3 $\times$ 10^6 IU/g), sodium hydroxide (NaOH), ammonium molybdate, sodium sulfite, hydroquinone, sulfuric acid (H_2SO_4), perchloric acid ($HClO_4$), nitric acid (HNO_3), potassium sodium tartrate, copper sulfate pentahydrate ($CuSO_4{\cdot}5H_2O$) were purchased from Sinopharm Chemical Reagent Co., Ltd. Diatomite filter aid STD was purchased from Celite China (Beijing, PR China). The Fresh Egg Yolk powder was from Kang de Biologicals Co., Ltd. Nantong. All the other 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 18.2 MΩ cm^{-1} (highly pure water).

2.2. Preparation of crude yolk polypeptides from egg yolk powder

The fresh egg yolk powder was defatted with 95% (v/v) ethanol using Soxhlet's extraction method and the ethanol was removed by extraction filtration. The residue was dried at room temperature to volatilize the residual ethanol.

Firstly, the dried de-fat yolk powder was suspended in 0.1 M NaOH solution and shaken in a homothermal shaker for 3 h (37 °C, 200 rpm) to reach reaction equilibrium. After that, the mixture was adjusted to pH 8.0 with 0.1 M hydrochloric acid solution and centrifuged for 20 min (8000 $\times$ *g*, 4 °C). Then the suspension was ultra-filtered and the precipitate was washed with highly pure water for five times to remove the free phosphate anion therein. Secondly, the intercept fluid and washed precipitate were dried and transferred to a homothermal enzymatic reactor, and the trypsin was added to the solution above at an enzyme-to-substrate ratio of 1:20 (w/w). The mixture was incubated at 50 °C for 4 h for enzymatic hydrolysis, during which the pH value was maintained at 8.0 with 0.1 M NaOH. The enzymolysis was terminated by incubating the mixture at 95 °C for 15 min and cooling it down to room temperature before adjusting the pH value to 7.0. Then the tryptic digestion solution was centrifuged at 8000 $\times$ *g* (4 °C, 20 min). Finally, the resulting supernatant solution was lyophilized and used as crude egg yolk polypeptides.

2.3. Preparation and characterization of TD

For the preparation of TD, aqueous titanium tetrachloride ($TiCl_4$) and diatomite were used as the TiO_2 resource and support, respectively. Before synthesis, the diatomite was pretreated with 0.1 M HCl solution at a diatomite-to-liquid ratio of 1:20, followed by incubation for 30 min in a shaker at room temperature. The mixture was filtered after adjusting the pH to 7.0, and the precipitation was washed ten times by highly pure water and dried in a thermostatic drier. Through the pretreating process, the impurities in diatomite were removed and the refined diatomite was used for the preparation of TD.

Firstly, samples of dried diatomite (2.4 g) were immersed in 50 mL HCl (0.1 M) solution and stirred for 20 min to get a homogeneous suspension. Then 2.24 mL of aqueous $TiCl_4$ solutions were added dropwise to the above suspension under continuous stirring, and the mixture was incubated at 200 rpm for 30 min. As the hydrolytic action of $TiCl_4$ was very fast and the acid environment was helpful to slow down the process, the formation of TiO_2 was slow, which contributed to the well precipitation on the surface of diatomite. Secondly, the pH value of the mixture was adjusted to 7.0 gradually and continued stirring for 30 min for the reaction to reach equilibrium. Then mixture was the filtered, and the precipitation was rinsed with deionized water for six times to remove the unreacted chemicals. The initial composite was obtained by drying the precipitation above. Finally, the composite was calcined in a muffle furnace (600 °C, 3 h) for the combination of TiO_2 and diatomite to be more stable. After cooling down in a dryer, the TD was obtained.

The surface morphology of pure diatomite and prepared TD samples were examined by scanning electron microscope (SEM, S-4800, Hitachi Company). Fourier Transform Infrared (FTIR, Nicolte Nexus, Thermo Electrin Corporation) uses infrared radiation to determine the chemical functionalities present in the sample. The samples of diatomite, TiO_2 and TD were mixed with potassium bromide (KBr) powder (1:50) and the mixtures were made into pellets under high pressure. The infrared spectra of prepared samples between 400 and 4000 cm^{-1} were recorded.

2.4. Adsorption of PPPs from aqueous solution

Different initial concentrations of crude polypeptides solutions were prepared by dissolving freeze-dried crude egg yolk polypeptides in deionized water, stirring at 200 rpm for 30 min to get a homogeneous peptides solution.

2 g of TiO_2, 2 g of TD and 15 mL of crude polypeptide solutions (10–80 mg/mL, pH 1.0–6.0) were added into 150 mL triangular flasks, the mixed suspensions were shaken in a thermostated shaker (25 °C, 200 rpm) for a certain time, respectively. And during the incubation, the supernatants were withdrawn at suitable time