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Ti^{4+}-phosphate functionalized cellulose for phosphopeptides enrichment and its application in rice phosphoproteome analysis

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ABSTRACT

In this study, a novel immobilized metal ion affinity chromatography (IMAC) material for phosphopeptide enrichment was prepared based on modified cellulose and was applied in rice phosphoproteome analysis. Firstly, cellulose was modified with phosphoric acid *via* esterification, and then Ti^{4+} was chelated onto the phosphorylated cellulose. The synthesized materials were ultrafine powders and had good dispersibility in acidic buffer, and as supporting matrix, phosphorylated cellulose exhibited good biocompatibility and chemical stability. Enrichment conditions were optimized and the optimum loading buffer was 40% acetonitrile (ACN) with 6% trifluoroacetic acid (TFA). Finally, the Ti^{4+}-phosphate functionalized cellulose was submitted to phosphopeptides enrichment prior to mass spectrometry (MS). For α-casein lysates, 14 phosphopeptides were detected with high intensities even though the sample concentration was as low as 2 pmol. Besides, 15 phosphopeptides were still identified by using the digest mixture of α-casein and bovine serum albumin with molar ratio of 1:100, which demonstrated high specificity and sensitivity for phosphopeptides enrichment. 19 phosphoproteins were identified from 200 μg of salt-free rice leaf protein lysates, while 30 phosphoproteins were identified from salt-stressed rice leaf protein lysates, and most of these proteins were related to the biological processes in response to abiotic stimulus.

1. Introduction

Protein phosphorylation is a pivotal post-translational modification (PTM) regulating many cellular functions, including cell growth, metabolism, apoptosis, and signal transduction [1–3]. It is estimated that approximately one-third of all proteins in eukaryotic cells are phosphorylated at any time [4] and protein phosphorylation is unanimously a major type of PTM observed under salinity stress in plants [5]. Because of the importance aforementioned, analytical methods for phosphoproteomics study have been concentrated on and developed rapidly. Conventional methods involved radioactive labeling followed by 2D gel electrophoresis, thin-layer chromatography, Edman degradation sequencing, and site-directed mutagenesis [6]. Recent years, mass spectrometry (MS) based methods, such as matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) [7–9] and electrospray ionization mass spectrometry (ESI-MS) [10,11], have emerged as extremely powerful tools in PTM study owning to its rapidness and high sensitivity. While MS based method is straightforward and simple, satisfying outcome are often hard to fulfil by direct analysis of protein digests. As lots of non-phosphopeptides suppress the ionization of phosphopeptides in the MS analysis and reduce the number of identified phosphopeptides in large-scale phosphoproteomic mapping [8]. To circumvent this obstacle, enrichment approaches of phosphopeptides before MS analysis are indispensable. Among the techniques, IMAC [12–16] has drawn lots of attentions and has been the most commonly used one.

IMAC was firstly introduced by Porath et al. at 1975 for the fractionation of human serum proteins [17]. In this technique, metal ions, such as Fe^{3+} [18], Ga^{3+} [19,20], Zr^{4+} [21], and Ti^{4+} [22], are immobilized on the supporting matrix *via* chelating ligands, such as IDA [16] and NTA [23]. The principle of phosphopeptides enrichment is based on electrostatic interactions between positively charged metal ions and negatively charged phosphate groups of phosphopeptides. Although this approach has been used successfully on phosphopeptides enrichment, it still suffers from insufficient specificity, as acidic peptides can also bound to the metal ions in the same way as phosphopeptides. Methylesterification of carboxylic acid has been suggested to improve the specificity of phosphopeptides enrichment [12,24], yet, it leads to unwanted side reactions and phosphopeptides loss during the tedious procedures [12,24,25].

Recently, Zhou et al. reported that using phosphate groups instead of traditional IDA or NTA as metal ions chelator was a

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new promising strategy for phosphopeptides enrichment [22]. As in the special MO_6 octahedra layer structure of metal phosphonate, each metal ion coordinates more than one phosphate group and *vice versa*, which provides superior metal chelating strength. Thus, the metal ions chelated on phosphate groups can still bind to the phosphopeptides with strong specific interaction. Based on this theory, a series of phosphate-functionalized materials have been synthesized with immobilization of Zr^{4+} or Ti^{4+} for enrichment of phosphopeptides [13,21,26–28] and the enrichment selectivity has been greatly improved. No matter what type of affinity systems, selecting a suitable supporting matrix is the pre-requisite and need to be emphasized primarily [29]. Up to now, most of the materials used for phosphate functionalization are nano- or polymeric materials, and they are either too expensive or complicated for phosphate modification. Therefore, development of a simple and low cost phosphate-modified substrate material is critical and essential for large-scale phosphopeptides analysis.

Cellulose is the most abundant natural polymer available and has been used in various fields due to its attractive properties, such as non-toxicity, biocompatibility, biodegradability, and easy to chemical modification [30]. Cellulose and its derivatives have been widely used as matrix in chromatographic column [31]. Feuerstein et al. successfully synthesized a new iron-IDA-cellulose IMAC material using cellulose as matrix, which exhibited high selectivity and recovery rates above 90% for phosphopeptides enrichment [32]. The hydroxyl group on the C-6 position of cellulose can be readily esterified by H_3PO_4, P_2O_5, or $POCl_3$, and the obtained phosphorylated cellulose has shown prominent ability in chelating metal ions [33]. Herein, we developed a novel IMAC material based on phosphorylated cellulose for selective enrichment of phosphopeptides. Briefly, cellulose was esterified by H_3PO_4 and then Ti^{4+} was chelated onto the phosphate-functionalized cellulose. Tryptic digests of casein were employed to evaluate its sensitivity and selectivity. To achieve better performance, optimization experiments were conducted by screening suitable loading buffer compositions. Finally, the Ti^{4+}-phosphate functionalized cellulose was employed in rice phosphoproteome to study the influences of salt stress on protein phosphorylation of rice leaf, and this investigation was aimed to examine its efficiency in real complex biological samples. Results revealed that the newly phosphorylated cellulose based Ti^{4+}-IMAC was indeed an efficient material, and the developed protocol could provide a potent and alternative approach for phosphoproteome study.

2. Experimental

2.1. Reagents and materials

Bovine serum albumin (BSA), α-casein (from bovine milk), β-casein (from bovine milk), 1-1-(tosylamido)-2-phenyl-ethyl chloromethylketone (TPCK)-treated trypsin (from bovine pancreas), and TFA were purchased from Sigma (St. Louis, USA). ACN and dimethyl formamide (DMF) were purchased from Beijing Lanyi Chemical Institute (Beijing, China). Dithiothreitol (DTT), iodoacetamide (IAA), β-mercaptoethanol, Tris, polyvinylpolypyrrolidone (PVPP), sodium fluoride (NaF), sodium orthovanadate (Na_3VO_4), phenylmethanesulfonyl fluoride (PMSF), sucrose, and Tris–phenol were obtained from Beijing Xin Jingke Biological Technology Co. Ltd. (Beijing, China). Microcrystalline cellulose, $Ti(SO_4)_2$, NH_4HCO_3, and H_3PO_4 were purchased from the Sinopharm Chemical Reagents Co. Ltd. (Shanghai, China). Cocktail tablets were obtained from Roche (Mannheim, Germany). Rice seeds (*Oryza sativa* L. cv. Nipponbare) were provided by Liping Zhan (Hua Zhong Agricultural University, Wuhan, China). ACN was HPLC grade and all other reagents were analytical grade. Water used in all experiment was purified by Milli-Q system (Millipore, Bedford, USA).

2.2. Synthesis of Ti^{4+}-phosphate functionalized cellulose

Reaction of cellulose with phosphorous acid was performed using a modified protocol according to the reported procedure [34]. Briefly, in a 250 mL, four-necked flask equipped with a condenser, a thermometer, a stirrer, and a nitrogen inlet, 0.5 g microcrystalline cellulose was soaked in 70 mL DMF along with 20 g urea. After the mixture was stirred at 100 °C for 1 h, 7 mL 98% H_3PO_4 was added dropwise. The reaction was allowed to proceed at 135 °C for 12 h. After cooling to room temperature, the mixture was centrifuged and washed with distilled water six times to remove the residual reagents. After lyophilized for 24 h, 0.1 g of the phosphorylated cellulose was immersed in 150 mM $Ti(SO_4)_2$ aqueous solution at ambient temperature overnight under gentle stirring. Finally, the Ti^{4+}-phosphate functionalized cellulose was filtered and rinsed with distilled water six times to remove the Ti^{4+} residue and lyophilized for usage.

2.3. Preparation of standard peptide samples

α-Casein (1 mg) and β-casein (1 mg) were dissolved in 1 mL of 50 mM ammonium bicarbonate buffer respectively and digested at 37 °C for 16 h with protein/trypsin ratio of 40/1 (w/w). BSA (6.6 mg) was dissolved in 1 mL of 50 mM ammonium bicarbonate buffer containing 8 M urea and incubated at 37 °C for 3 h. After adding 20 μL of 50 mM DTT, the mixture was incubated for 2 h. Then, 40 μL of 50 mM IAA was added and the mixture was incubated for another 30 min in the dark. The obtained solution was diluted 10 times with 50 mM ammonium bicarbonate and digested with trypsin at 37 °C for 16 h with protein/trypsin ratio of 40/1 (w/w). Semi-complex samples were mixtures originating from tryptic digestion of α-casein and BSA at molar ratio of 1:100 by keeping α-casein concentration at 10×10^{-12} mol. All the peptide samples were lyophilized for further usage.

2.4. Preparation of rice leaf peptide sample

Rice seeds germinated on wet filter paper at 30 °C for 3 days and grew in greenhouse at 25 °C in Hoagland solution according to the reported literature [35]. After cultivated for 15 days, roots were treated with 0 and 150 mM NaCl in Hoagland solution for 24 h and rice leaves were sampled. The plant tissues were frozen in liquid nitrogen immediately after harvest and stored at −80 °C before protein extraction.

Proteins were extracted with phenol method as follows: rice leaves were ground into fine powder in liquid nitrogen and 2 g of the powders were suspended in 6 mL of extraction buffer (0.7 M sucrose, 0.1 M KCl, 0.5 M Tris–HCl, 10 mM EDTA, 1 mM PMSF, 2% β-mercaptoethanol, 1% PVPP, 0.2 mM NaF, and 1 mM Na_3VO_4, pH 8.7). The mixtures were intensively homogenized for 10 min. Followed by adding an equal volume of saturated Tris–phenol, the mixtures were rehomogenized for another 10 min. The homogenate was centrifuged at 10,000 × *g* for 40 min at 4 °C, and then the upper phenol phase was mixed with five volumes of saturated ammonium acetate in methanol overnight at −20 °C. The precipitant was recovered finally by centrifugation at 10,000 × *g* for 40 min and washed with cold acetone three times. Protein pellet was then dried into powder with purging pure nitrogen gas and 80–100 mg of the obtained protein were redissolved in 1 mL rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 10 μL cocktail). After the mixture was incubated for 3 h at 37 °C, 5 μL of 1 M DTT was added and the solution was incubated for another 2 h. Then, 25 μL of 1 M IAA was added and the obtained solution was incubated for another 30 min

in the dark. The final protein concentration was quantified using protein assay kit according to the instructions of the manufacturer (GE Healthcare, NJ, USA). Finally, 200 μg of the protein was digested with trypsin for 16 h at 37 °C using an enzyme/protein ratio of 1:40 (w/w). The obtained digests were lyophilized before further usage.

2.5. Enrichment of phosphopeptides by Ti^{4+}-phosphate functionalized cellulose

For the enrichment of standard protein digests, peptide samples were dissolved in 50 μL loading buffer (40% ACN, 6% TFA), followed by addition of 5 μL Ti^{4+}-IMAC beads (10 mg/mL in 30% ACN and 0.1% TFA). After incubation for 30 min, mixtures were centrifuged at 13,200 × *g* for 5 min and the supernatant was removed. Then Ti^{4+}-IMAC beads were washed with 30 μL washing buffer 1 (50% ACN, 6% TFA, 200 mM NaCl) and 300 μL washing buffer 2 (30% ACN, 0.1% TFA), respectively. Finally, the bound phosphopeptides were eluted with 50 μL of elution buffer (10% NH_3 H_2O) under sonication for 10 min and the supernatant was collected and lyophilized after centrifugation for 15 min. For enrichment of phosphopeptides from rice leaf samples, protein digests were redissolved with an equal volume of loading buffer and 10 μL of Ti^{4+}-IMAC beads (10 mg/mL in 30% ACN and 0.1% TFA). The following procedure was the same as that of standard protein digests described above except that the elution buffer volume was 100 μL. All the phosphopeptides enriched by Ti^{4+}-IMAC were lyophilized prior to MS analysis. For the phosphopeptides enriched from standard protein digests, 2 μL of DHB solution (25 mg/mL in 70% ACN) containing 1% H_3PO_4 (v/v) was added and 0.5 μL of matrix solution was deposited on the MALDI target plate for MALDI-TOF MS analysis. The phosphopeptides enriched from rice leaf protein digests were subjected to ESI-Q-TOF MS analysis. To improve the selectivity of phosphopeptides enrichment, concentrations of ACN and TFA in the loading buffer were optimized. Three replicates were performed in all experiments.

2.6. MS and database searches

MALDI-TOF/TOF MS analysis was performed on a time-of-flight mass spectrometer (Bruker Daltonics GmbH) under the control of FlexControl™ 2.2 software. All the mass spectra were recorded in the positive ion reflector mode in a mass range from 900 to 4000 Da with accelerating potential at 25 kV. ±0.2 Da for MS-spectra fragment ions. A peptide mixture (Peptide calibration standard I, Bruker Daltonics Co.) was used for external calibration.

ESI-Q-TOF spectra were acquired on a Q-TOF Synapt High Definition Mass Spectrometry (Waters, Manchester, USA) utilizing automated data-dependent acquisition. The mass spectrometer was operated in positive ion mode with a source temperature of 90 °C. Rice leaf peptides were desalted on a column C_{18} (20 mm length, 180 μm inner diameter, and 20 mm outer diameter, Waters, Milford, USA) and eluted at 200 nL/min on an ACQUITY UPLC BEH 1.7 μm C_{18} analytical column (15 cm length, 75 μm inner diameter, and 250 mm outer diameter, Waters, Milford, USA) using a binary solvent system made up 0.1% formic acid in water (solvent A) and 0.1% formic acid in 100% ACN (solvent B). The peptides were eluted from the column with a linear gradient program that changed solvent A from 1% to 40% over 80 min and then to 80% at 10 min, keeping this concentration for 10 min and then reducing to 1% at 20 min. The two most abundant ions present in the survey spectrum were automatically mass-selected and fragmented in each cycle: 1-s MS *m*/*z* 350–1600 and maximum 4-s MS/MS *m*/*z* 50–2000, 30-s dynamic exclusion. After converting the MS/MS data to pkl file format, the information was searched against the NCBInr protein sequence database with *O. sativa* (134,567 sequences) as taxonomy using an online Mascot server (20110627). MASCOT scores >38 indicated that the results were reliable. Parameters of the protein sequence database were as follows: only tryptic peptides with up to one missed cleavage site were allowed; ±0.2 Da for MS/MS fragment ions; carbamidomethyl, oxidization, and phosphorylation (serine, threonine, and tyrosine) as variable modifications.

3. Results and discussion

3.1. Synthesis and characterization of Ti^{4+}-phosphate functionalized cellulose

Cellulose is a linear homopolymer composed of D-anhydroglucopyranose units (AGU), which are linked together by β-(1–4)-glycosidic bonds. Each of the AGU possesses hydroxy groups at C-2, C-3, and C-6, capable of undergoing different chemical modifications. Hydroxyl groups on the C-6 can be readily esterified by H_3PO_4 and phosphorylated cellulose with relatively high substitution can be obtained, which can chelate metal ions like Ti^{4+} with strong interactions. Once Ti^{4+} was chelated onto the phosphorylated cellulose, it will provide abundant binding sites for phosphopeptides, and this tight binding affinity between Ti^{4+} and anions in acid solution has been successfully employed in phosphopeptides isolation [22,30]. Fig. 1 illustrates the synthetic procedure of Ti^{4+}-phosphate functionalized cellulose. Phosphoric acid groups were introduced onto the surface of cellulose through esterification (Fig. 1a) and Ti^{4+} was chelated onto the modified cellulose by incubation of the beads in $Ti(SO_4)_2$ solution (Fig. 1b). Empty sites of Ti^{4+} ions were temporarily occupied by water molecules and were replaced afterwards by phosphorate groups of phosphopeptides during the enrichment of phosphopeptides. The

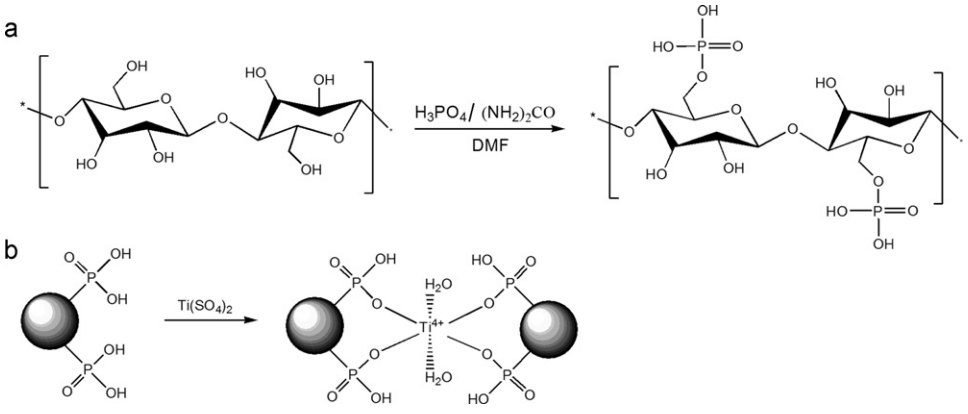

Fig. 1. Illustration of procedure for the preparation of (a) phosphorylated cellulose and (b) Ti^{4+}-phosphate functionalized cellulose.

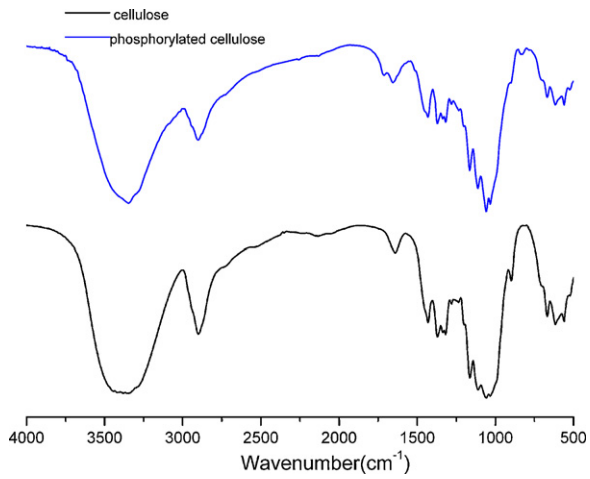

Fig. 2. FT-IR spectra of cellulose and phosphorylated cellulose.

whole procedure for preparation of Ti^{4+}-phosphate functionalized cellulose was extremely simple and safe.

FT-IR spectra were recorded with a 4 cm^{-1} resolution in the range of 500–4000 cm^{-1} on Nicolet NEXUS-470 FT-IR equipment (Madison, USA) from KBr pellets. As shown in Fig. 2, the following characteristic bands of cellulose are observed: a large band at 3400–3500 cm^{-1} corresponding to the vibration of OH groups; a band at 2800–2900 cm^{-1} attributed to CH_2 groups; characteristic bands at 1160 and 1120 cm^{-1} for C—O—C from the glucosidic units. In the IR spectrum of phosphorylated cellulose several new bands appeared: a band at 1210 cm^{-1} corresponding to the P=O bonds, a shoulder at 920–1000 cm^{-1} attributable to the P—OH group, and a band at 810 cm^{-1} corresponding to the P—O—C bonds. The results demonstrated the phosphorylated cellulose was successfully prepared.

Both the native cellulose and synthesized Ti^{4+}-phosphate functionalized cellulose are white ultrafine powders and SEM images are shown in Fig. 3. The surface morphology of phosphorylated cellulose was uniform and smooth. After immobilization of Ti^{4+} on the phosphorylated cellulose, plenty of particles appeared and distributed on the surface. The average diameter of the particles was estimated about 0.5 μm as shown in Fig. 3b. The pictures suggested that Ti^{4+}-phosphate functionalized cellulose was prepared successfully. Furthermore, we evaluated the robustness of this Ti^{4+}-IMAC by soaking into 0.1 M NaOH, TFA, and ACN respectively. The results revealed that the prepared material was very stable in strong alkali, acid, or organic solvents. Most importantly, the Ti^{4+}-phosphate functionalized cellulose was ultrafine powders which had large specific surface area, and had a good dispersion in acidic solvents as well. Thus, the obtained material with such characteristics was very suitable for phosphopeptides enrichment especially in complex buffer systems.

3.2. Optimization of loading conditions for phosphopeptide enrichment

Acidic loading/washing buffer has been widely adopted to ensure high selectivity through maintaining the carboxyl groups in protonated state while phosphate groups remaining in dissociated state. Up to now, TFA has been proved to be the most effective one compared with others, and the optimal concentrations of TFA in loading/washing buffer were quite different in the earlier reported literatures [26–28].

To investigate the influence of TFA concentrations and figure out the optimal condition regarding loading buffers during our selective enrichment, semi-complex sample consisting of α-casein/BSA with molar ratio of 1:100 was employed as the test sample. This relative complex sample was frequently used to mimic real biological sample and evaluate the enrichment efficiency. As shown in Fig. 4a, with 0.1% TFA in loading buffer, although 13 phosphopeptides (labeled by *) were detected with relatively high of intensity, non-phosphorylated peptides signals still dominated the mass spectra. With the increasing TFA concentration, intensities of non-phosphopeptides weakened dramatically (Fig. 4b) and the phosphopeptides dominated instead when the TFA concentration reached 6% (Fig. 4c). At this concentration, 14 phosphopeptides were detected and dominated the mass spectra with high intensity signals. However, further increasing of TFA concentration resulted in less identified phosphopeptides. As shown in Fig. 4d, only 10 phosphopeptides were detected with low intensity and abundant non-phosphorylated peptides signals were appeared. Experimental results described above can be explained that low concentration of TFA was insufficient for the protonation of carboxyl groups of acidic amino acid, yet high concentration suppressed the dissociation of phosphate groups. From the data presented in Fig. 4, adding 6% TFA in loading buffer yielded best phosphopeptide enrichment efficiency, and this result coincided with the conclusion of Zhou et al. [22].

Besides, appropriate concentrations of ACN in loading buffer not only can prevent non-specific binding between non-phosphopeptides and IMAC, but also enhance binding between metal ions and phosphate groups. To figure out the optimal loading buffer compositions, we also optimized the concentrations of ACN. Fig. 5 shows the effects of different ACN concentrations in loading buffers on the selectivity. Either low (20%, Fig. 5a) or high (80%, Fig. 5d) concentrations of ACN were relative ineffective, and the loading buffers with 40% (Fig. 5b) and 60% (Fig. 5c) ACN improved the selectivity of phosphopeptides enrichment where

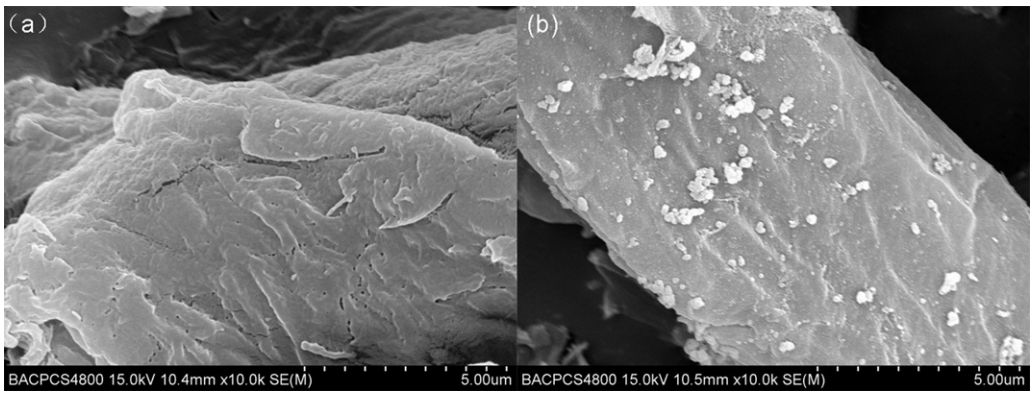

Fig. 3. SEM images of (a) cellulose and (b) Ti^{4+}-phosphate functionalized cellulose.

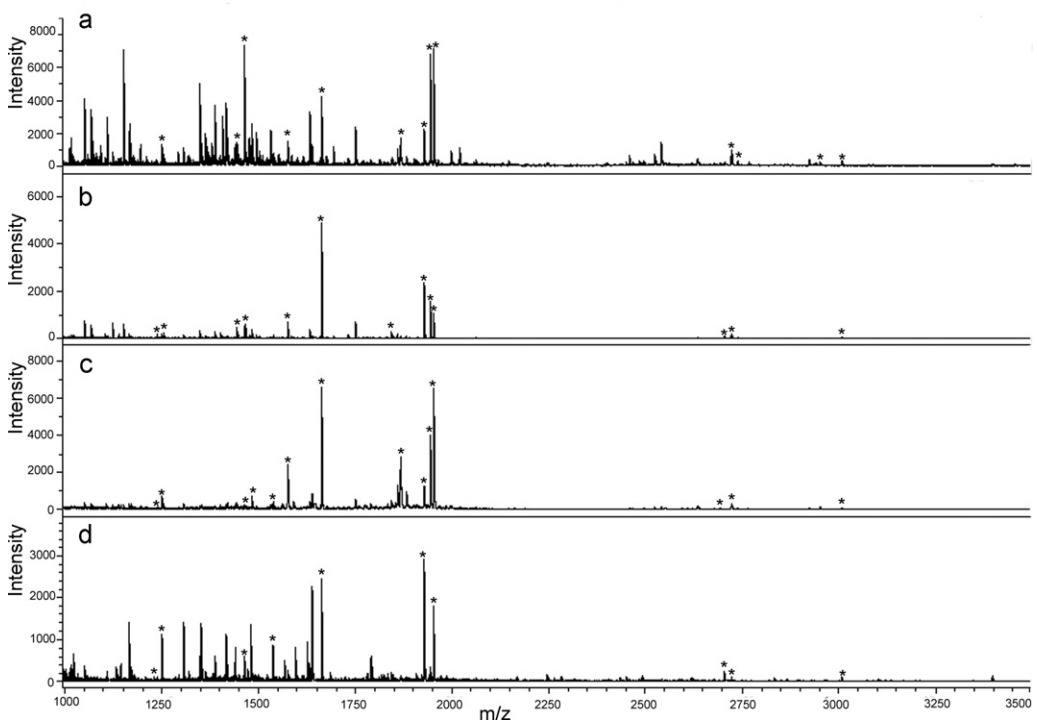

Fig. 4. MALDI mass spectra of phosphopeptides enriched by Ti^{4+}-IMAC with different TFA concentrations: (a) 0.1%, (b) 1.0%, (c) 6.0%, and (d) 8.0% in 80% ACN as loading buffer. The sample was mixture of tryptic digests of α-casein and BSA with ratio of 1:100. The peaks of phosphopeptides are marked with *.

as many as 15 phosphopeptides were detected. Compared to 60% ACN, 40% concentration was observed with higher intensity and fewer nonphosphopeptides. As an organic solvent, ACN with 40% concentration in loading buffer was the optimal one for the selective enrichment of phosphopeptides. Although better performance regarding both sensitivity and selectivity was achieved to some extent, the improvement was not so impressive and comparable to that of TFA.

Loading buffer is extraordinary important in phosphopeptides enrichment. Once non-phosphopeptides were bound to IMAC, it is very difficult to elute them [36]. Therefore, in the above section, we have focused on the optimization of the concentrations of ACN

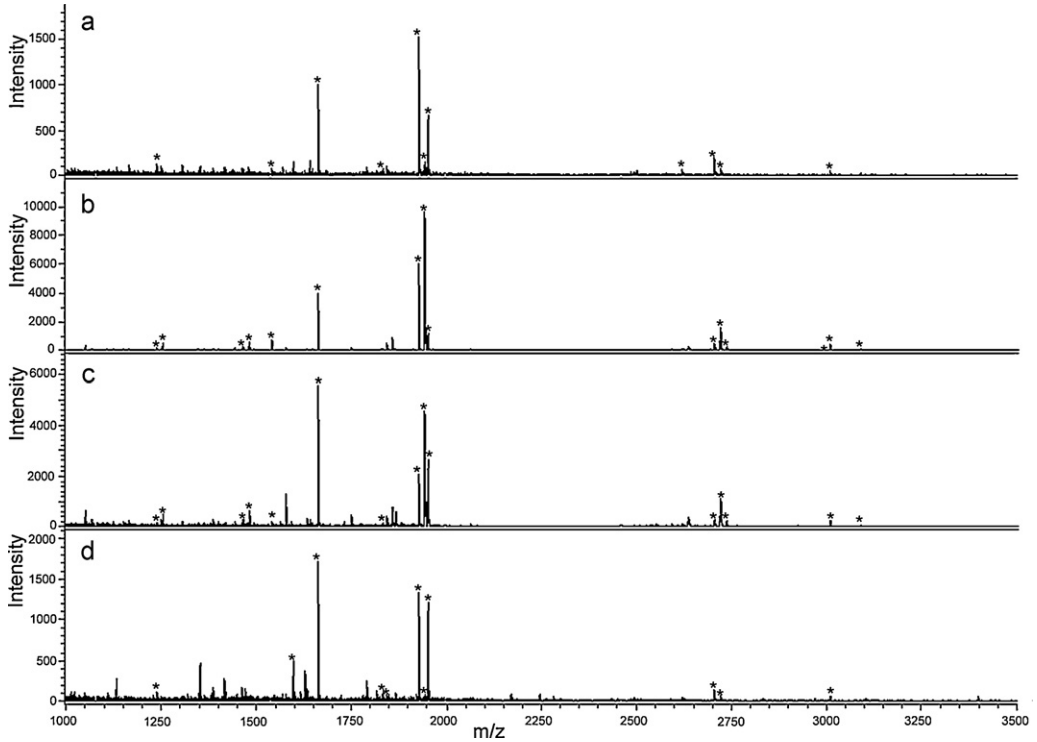

Fig. 5. MALDI mass spectra of phosphopeptides enriched by Ti^{4+}-IMAC with different ACN concentrations: (a) 20%, (b) 40%, (c) 60%, and (d) 80% in 6% TFA as loading buffer. The sample was mixture of tryptic digests of α-casein and BSA with ratio of 1:100. The peaks of phosphopeptides are marked with *.

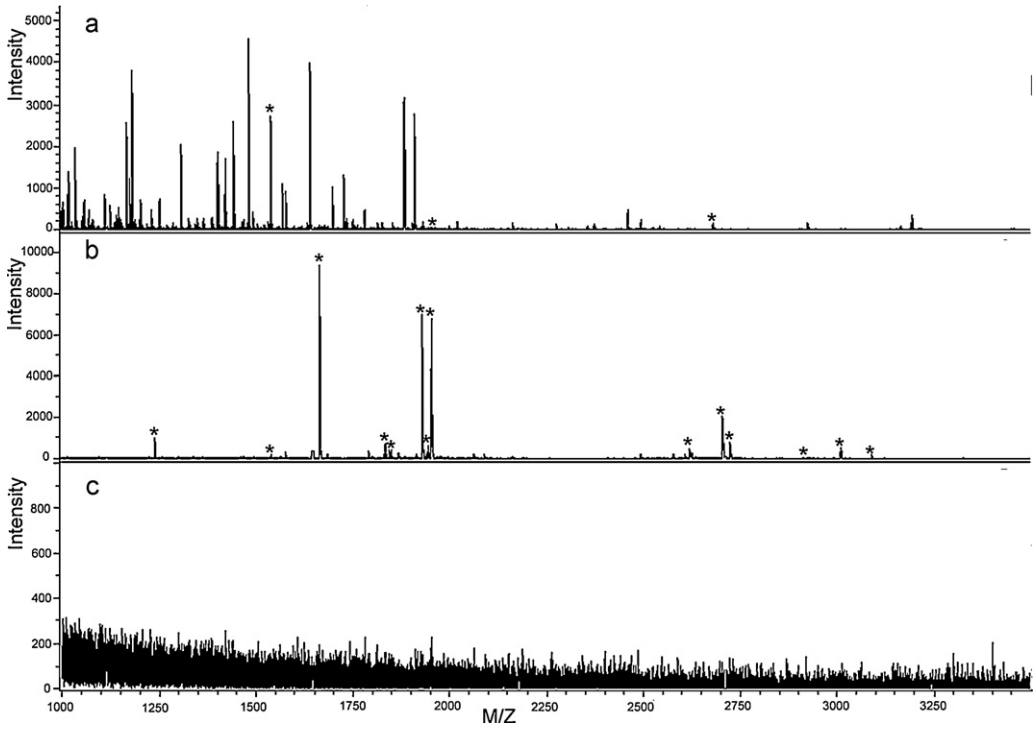

Fig. 6. MALDI mass spectra of the tryptic digest of α-casein (2×10^{-12} mol). (a) Direct analysis; analysis after phosphopeptides enrichment by (b) Ti^{4+}-phosphate functionalized cellulose and (c) phosphorylated cellulose. The peaks of phosphopeptides are marked with *.

and TFA in loading buffer, and other conditions including washing buffer and elution buffer were referred to the optimized protocol of Zou's group [22]. The final optimized enrichment protocol in our method was as follows: 40% ACN with 6% TFA as loading buffer; 50% ACN with 6% TFA containing 200 mM NaCl as washing buffer 1; 30% ACN with 0.1% TFA as washing buffer 2; and 10% NH_3 H_2O as elution buffer. If not stated otherwise, this enrichment protocol was used for phosphopeptides enrichment in all the subsequent experiments.

3.3. Specificity and sensitivity of standard phosphopeptide enrichment

Phosphoproteins are generally of low abundance in real samples. Therefore, investigating the specificity and sensitivity of Ti^{4+}-phosphate functionalized cellulose for phosphopeptides enrichment is necessary. Casein tryptic digests contain many phosphopeptides and have always been employed as standard sample to evaluate the efficiency of phosphopeptides enrichment of novel approaches. Fig. 6 shows the MALDI mass spectra of phosphopeptides enriched from 2 pmol α-casein tryptic digests. As shown in Fig. 6a, phosphopeptides detected were almost submerged by non-phosphopeptides and only 3 phosphopeptides could be observed without enrichment. However, after enrichment based on our Ti^{4+}-phosphate functionalized cellulose using optimized protocol described above, most non-phosphopeptides signals were eliminated and a total of 14 phosphopeptides signals dominated the MALDI mass spectra (Fig. 6b). The signal-to-noise ratio of phosphopeptides was enhanced dramatically as well. Among the detected 14 phosphopeptides, 6 were with a single phosphorylation site, 3 were with two phosphorylation sites, and 5 were with multiple phosphorylation sites (detailed information is listed in Electronic Supplementary Material Table S1). These results indicated that the Ti^{4+}-phosphate functionalized cellulose was able to selectively capture phosphopeptides not only with single but also multiple phosphorylation sites. Additionally, a large ratio of phosphopeptides was observed with multiple sites, suggesting the interactions between the ligands and phosphate groups were considerably strong. Moreover, the result analyzed above was achieved when the concentration of peptide sample was as low as 2 pmol. In comparison to the literature reported by Feng et al. [13], 12 phosphopeptides were detected from the same concentration of α-casein tryptic digests based on phosphonate-modified poly(glycidyl methacrylate-*co*-ethylene dimethacrylate), and only 10 phosphopeptides were observed with the commercial IMAC resin POROS 20 MC chelated with Fe^{3+}.

To further examine the biocompatibility of our matrix, control experiment based on the phosphorylated cellulose without Ti^{4+} binding was conducted. The enrichment procedure for 2 pmol α-casein tryptic digests was the same as Ti^{4+}-phosphate functionalized cellulose. As shown in Fig. 6c, no peptide peak was observed in the spectrum, which indicated no binding of peptides on phosphate functionalized cellulose. Therefore, the specific and sensitive enrichment of phosphopeptides with Ti^{4+}-phosphate functionalized cellulose mainly resulted from the strong interaction of phosphopeptides with Ti^{4+} that immobilized on the surface of phosphorylated cellulose.

As another standard phosphoprotein, β-casein was used to further evaluate the efficiency of phosphopeptide enrichment based on Ti^{4+}-phosphate functionalized cellulose, and results revealed high sensitivity and specificity of the material (detailed information are listed in Electronic Supplementary Material Fig. S1 and Table S1).

3.4. Comparative phosphoproteome analysis of rice leaves under salinity based on Ti^{4+}-phosphate functionalized cellulose

Salinity stress is a major abiotic stress that limits rice productivity and studies have revealed that protein phosphorylation plays a critical role in salt stress response in plants [37]. However, rice phosphoproteome analysis under salinity stress is still a challenge due to the generally low abundance and stoichiometry of

Table 1
Overview of the results for phosphoproteome analysis of rice leaves.

Treatments	No. of identified phosphopeptides	No. of identified phosphorylated sites and distributions				No. of identified phosphoproteins
		Total	Ser	Thr	Tyr	
Salt-free	21	27	17	9	1	19
Salt-stressed	33	48	36	12	0	30

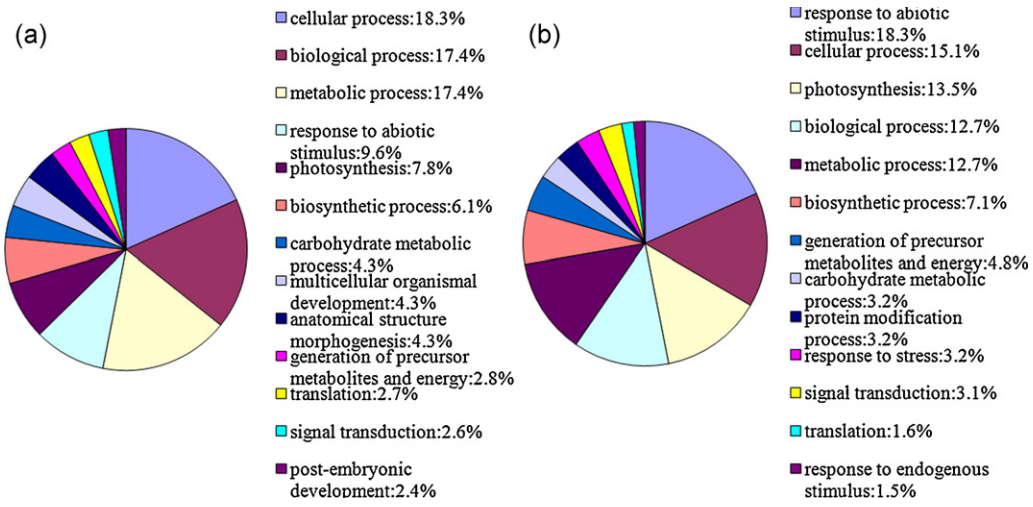

Fig. 7. Distribution of the salt-free (a) and salt-stressed (b) rice leaf phosphoproteins among GO categories based on biological processes. The pie charts were generated using the analysis results of the "GOSlimViewer" tool at AgBase.

phosphorylated protein forms. An efficient method for phosphopeptides enrichment with high sensitivity is demanded. We herein used Ti^{4+}-phosphate functionalized cellulose to selectively isolate rice leaf phosphopeptides prior to MS analysis.

The rice leaf protein was digested with trypsin, and the proteolytic digests were enriched by Ti^{4+}-phosphate functionalized cellulose. As shown in Table 1, 21 phosphopeptides and 19 phosphoproteins were identified when rice grow in normal condition. 33 phosphopeptides with 48 phosphorylation sites from 30 phosphoproteins were identified from salt-stressed (the peptide sequences and other detailed information about the phosphoproteins are listed in Electronic Supplementary Material Tables S2 and S3). It can be seen from the data that under salinity stress, both phosphoserine and phosphothreonine were detected. Among them, 75% of the identified phosphorylation sites were phosphorylated on serine residues, 25% of the identified sites on threonine residues, and tyrosine phosphorylation was not detected. To understand the biological processes of phosphoproteins identified from rice leaves based on Ti^{4+}-phosphate functionalized cellulose, gene ontology (GO) analyses was performed from the AgBase database by the GI number of NCBI. As shown in Fig. 7, the phosphoproteins identified from salt-free and salt-stressed rice leaves had very different distribution among the GO categories. The top two biological processes of the phosphoproteins identified from salt-free rice leaves were cellular process (18.3%) and biological process (17.4%), and for the phosphoproteins identified from salt-stressed rice leaves 18.3% were responding to abiotic stimulus and 15.1% were accounted for cellular process.

Rice leaf sample is extremely complex due to the existence of abundant RUBISCO and enrichment of phosphopeptides by IMAC soly may not give satisfying results. Our further work will focus on sample pretreatment based on our Ti^{4+}-phosphate functionalized cellulose by combining with other effective purifying technologies like PEG prefractionation and strong cation exchange.

4. Conclusions

Ti^{4+}-phosphate functionalized cellulose for phosphopeptides enrichment was prepared with simple method. The optimized loading condition was 40% ACN with 6% TFA. With the optimized buffer system, the material exhibited high sensitivity and specificity for phosphopeptides enrichment. As much as 15 phosphopeptides were detected in the digests of α-casein and BSA mixtures with molar ratio of 1:100. As α-casein concentration was as low as 2×10^{-12} mol, 14 phosphopeptides were observed with high signal-to-noise ratio. 19 phosphoproteins were identified from 200 μg of salt-free rice leaf protein lysates, and 30 phosphoproteins were identified from salt-stressed rice leaf protein lysates and most of these proteins were related to the biological processes in response to abiotic stimulus.

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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.2012.06.033.

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