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# RAPID ENRICHMENT OF PEPTIDES WITH CALCIUM-CHELATING CAPACITY AND CHARACTERIZATION OF PHYSICAL CHEMICAL PROPERTIES

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Casein peptides with calcium-chelating capacity were rapidly enriched by using a novel ceramic matrix (CM)-based $Ti^{4+}$-IMAC adsorbent. The ability of calcium-chelating peptides (CCPs) to bind calcium and the physical properties of complexes formed between CCPs and calcium were investigated. Results demonstrated that the amount of calcium bound depended on the degree of hydrolysis (DH) of casein hydrolysates. The highest calcium binding capacity (683 mg $g^{-1}$) occurred when bovine casein was hydrolysed by pancreatin at a DH of 0.14%, meanwhile, the calcium content of CCPs-Ca complex exhibited the maximum level (134.96 mg $g^{-1}$). In addition, CCPs showed a higher radical scavenging capacity (50 μg $ml^{-1}$; 99% inhibition, or an equivalent activity of $9.91\times10^{-3}$ M Trolox) compared to casein digest. Moreover, Fourier-transform infrared spectroscopy and fluorescence spectroscopy were used to explore the interaction between CPPs and calcium, and the results demonstrated that phosphoserine residues as well as $COO^-$ groups of CCPs were involved in the formation of CCPs-Ca complex.

**Keywords:** casein hydrolysate, calcium chelate, enrichment, antioxidation

Calcium modulates several physiologic functions and its deficiency results in many diseases. As reported, calcium supplementation could increase physical activity in children, adolescents, and women. However, ionized calcium is prone to form calcium phosphate deposition in the intestinal environment, resulting in a low absorption. Calcium-chelating peptides (CCPs) have been proved to be effective in promoting Ca absorption, because peptide-Ca chelate could remain soluble, prevent dissociation and loss of Ca through the digestive system due to the bond between Ca and peptides.

Casein, accounting for approximately 80% of the total proteins in mammalian milk, is a family of four phosphorylated proteins, namely $\alpha_{s1}$-, $\alpha_{s2}$-, β-, and κ-casein. Currently, casein is widely used as a protein source in foods. A variety of reports show that casein peptides are both water soluble and have high nutritional values. Casein peptides are better absorbed than mixtures of free amino acids. Additionally, casein peptides, released either during food processing (by enzymatic hydrolysis or fermentation) or during gastrointestinal digestion, can be used as chelating agents with divalent metal ions (Liu et al., 2018), and also exhibit

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several physiological activities, such as antioxidant, angiotensin-converting enzyme (ACE)-inhibitory properties, antibacterial activity, partial cytoprotection (Wang et al., 2016), and so on. Thus, there is a great interest in developing casein peptides as nutraceutical for the formulation of functional foods.

Considering that ceramic matrix (CM) is a favourable carrier of immobilized enzyme with the properties of biocompatibility and abrasive resistance (Wu et al., 2014) and the high specificity of $Ti^{4+}$ to capture casein phosphopeptides (Zhou et al., 2013), a novel CM-based $Ti^{4+}$-immobilized metal ion affinity chromatography (IMAC) adsorbent was prepared and used for rapid enrichment of CCPs from casein hydrolysates in the present work. CM was easily prepared and mainly composed of some readily available metallic elements, such as calcium, silicon, aluminium, and magnesium, followed by modification with immobilized $Ti^{4+}$. CCPs were enriched rapidly from casein digests, and the calcium binding ability, the radical scavenging activity of CCPs, as well as the calcium content of CCPs-Ca complex were determined. FTIR and fluorescence spectroscopy were employed to reveal the interaction between CCPs and Ca.

## 1. Materials and methods

### *1.1. Materials and chemicals*

Bovine casein (Product Code C7078) of technical grade and porcine pancreatin (P7545) were purchased from Sigma Co. (USA). Acetonitrile (ACN) and trifluoroacetic acid (TFA) of HPLC grade were purchased from Merck (Darmstadt, Germany) and Dikma Co. (Beijing, China), respectively. Iminodiacetic acid (IDA) and 3-glycidoxypropyltrimethoxysilane (GLYMO) was purchased from Aladdin Chemical Reagent Co. Ltd. (Shanghai, China). Ultrapure water was obtained from an ELGA water purification unit (ELGA Ltd., Bucks, UK). All other reagents and solvents were of analytical grade and obtained from commercial source.

### *1.2. Preparation of casein digest*

Bovine casein was digested as previously described (Su et al., 2007). Briefly, the bovine casein (10 mg $ml^{-1}$) was hydrolyzed with pancreatin at an enzyme-to-protein ratio of 1/100 (w/w) in a batch reactor at 37 °C, and the pH was maintained at 8.0±0.1 by adding 0.1 M NaOH via potentiometric titration (Titro Line easy, Schott GmbH, Germany). During the reaction, aliquots of the hydrolysate were taken out at 10 min, 6 h, 12 h, and 24 h. Each aliquot was boiled at 100 °C for 10 min to stop the hydrolysis, and then lyophilized (FreeZone, LABCONCO, USA) after concentration in an R-2000 rotary evaporator (Shensheng Technology Co., Ltd., Shanghai).

### *1.3. Determination of the degree of hydrolysis*

The degree of hydrolysis (DH), defined as the percentage of cleaved peptide bonds, was estimated from the amount of base added to keep the pH constant during hydrolysis (Navarrete-Del-Toro & García-Carreño, 2002):

$$DH(\%)=\frac{BN_b}{\alpha M_p h_{tot}} \times 100$$

where $B$ is the volume (millilitres) of base used to maintain the pH; $N_b$ is the normality of the base; $1/\alpha$ is the average degree of dissociation of α-$NH_2$ related to the $pK$ of the $NH_2$ groups at particular pH and temperature; and $M_p$ (grams) is the mass of protein in the reaction mixture, $h_{tot}$ (meq/g) is the sum of millimoles of invidual amino acids per gram of protein associated with the source of protein used in the experiment. The total number of peptide bonds in the bovine casein protein substrate was assumed to be 8.2 meqv/g (Navarrete-Del-Toro & García-Carreño, 2002).

### *1.4. Enrichment of CPPs via $Ti^{4+}$-IDA-GLYMO@CM*

CM was prepared according to the procedure developed by Wu and co-workers (2013), then $Ti^{4+}$-IDA-GLYMO@CM was synthesized according to the method described by Zhou and co-workers (2013) as shown in Figure 1. The enrichment protocol for CCPs is shown in Figure 2A and described as follows: the synthesized $Ti^{4+}$-IDA-GLYMO@CM (100 mg) was mixed with 1 ml of casein digest solution (dissolved in the loading buffer of 80% (v/v) ACN and 6% (v/v) TFA, 10 mg $ml^{-1}$), and the mixture was stirred at 37 °C for 1 h; the $Ti^{4+}$-IDA-GLYMO@CM was isolated from the mixture by centrifugation (9000 r.p.m., 10 min), and then the peptide-loaded $Ti^{4+}$-IDA-GLYMO@CM was washed with solution 1 [50% (v/v) ACN and 0.5% (v/v) TFA containing 200 mM NaCl] and solution 2 [50% (v/v) ACN and 0.5% (v/v) TFA] three times; subsequently, the trapped CCPs were eluted by solution 3 [10% (v/v) $NH_3 \cdot H_2O$] and solution 4 [80% (v/v) ACN and 2% (v/v) TFA] three times; finally the eluate was collected and then lyophilized after removing organic solvents via a rotary evaporation.

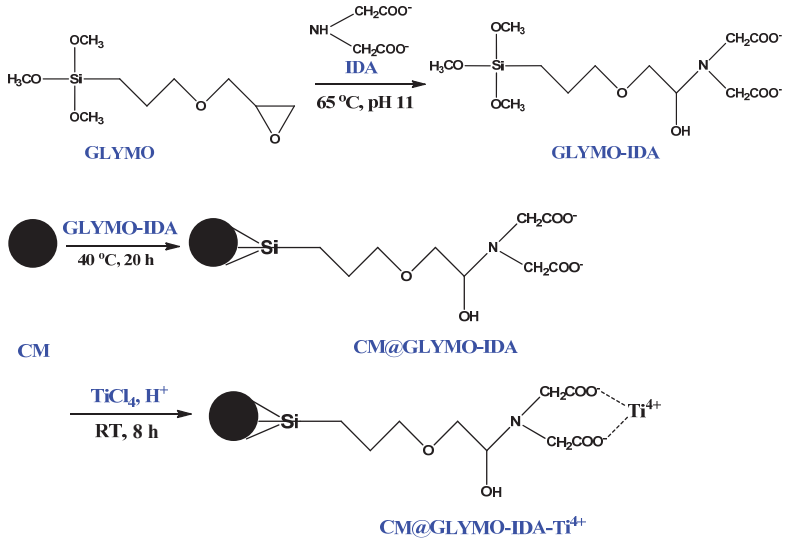


*Fig. 1.* Schematic illustration of the preparation of $Ti^{4+}$-IDA-GLYMO@CM

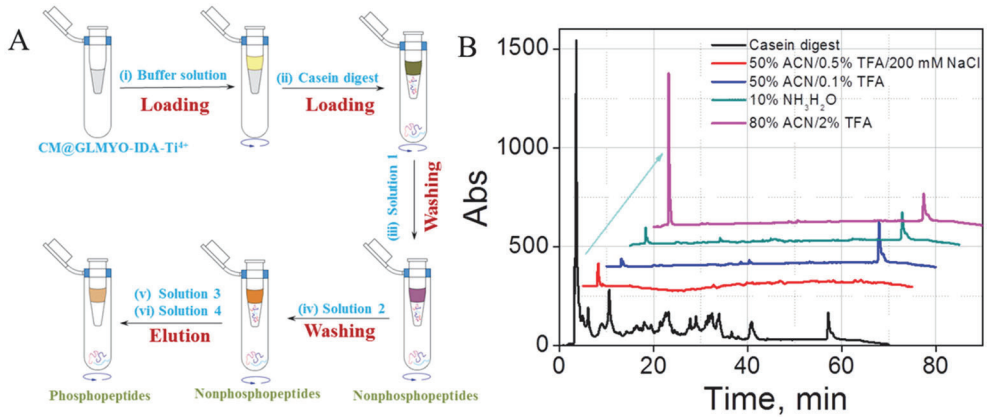


*Fig. 2.* (A) Flowchart indicating the enrichment of casein pancreatic hydrolysates by $Ti^{4+}$-IDA-GLYMO@CM, (B) RP-HPLC chromatograms of casein digest (24 h) as well as samples eluted by solution 1, 2, 3, and 4, respectively

### *1.5. Preparation of peptide-calcium complexes (CCPs-Ca)*

A 60-mg portion of each lyophilized hydrolysate was dissolved in 20 mM Tris-HCl (pH 7.4) buffer solution at a concentration of 10 mg $ml^{-1}$, followed by a 10-min incubation at 37 °C to ensure full dissolution. After adding of 3.75 ml of 0.8 M $CaCl_2$, the solution was maintained for 1 h at 37 °C. Then the mixture was placed in dialysis bags (MWCO 500, Spectrum, USA) and dialyzed in 400 ml of 20 mM Tris-HCl (pH 7.4) at 4 °C to remove free $Ca^{2+}$, with the dialysate changed every 4 h over a period of 48 h. Finally, the mixture solution was freeze-dried and the CCPs-Ca complex was prepared.

### *1.6. Measuring the inhibition of the formation of insoluble calcium phosphate*

The $Ca^{2+}$-binding abilities of different casein digest samples as well as CCPs were indirectly determined by measuring the inhibition of the formation of insoluble calcium phosphate according to the method reported previously (Berrocal et al., 1989).

### *1.7. Determination of calcium content*

Calcium binding ability was determined via titration with standard EDTA solution using calmagite as indicator according to the method described by Liu and co-workers (2013).

### *1.8. Measurement of ABTS radical scavenging activity*

ABTS radical scavenging activities of the hydrolyzed samples and CCPs were determined by the spectrophotometric method previously described by Re and co-workers (1999). Results were expressed as $10^{-3}$ mM Trolox.

### *1.9. Reversed-phase high-performance liquid chromatography*

The casein digest as well as samples eluted by solutions 1, 2, 3, and 4, respectively (Section 1.5), were analysed by reversed-phase high-performance liquid chromatography (RP-HPLC) according to the method described in our previous report (Wu et al., 2013).

### *1.10. Scanning electron microscopy*

The surface morphology analyses of $Ti^{4+}$-IDA-GLMYO@CM and CM were conducted on a scanning electron microscope (SEM) (FEI-Apreo). Each sample was previously fixed on a brass stub using double-sided adhesive carbon tape, which became electrically conductive by being coated with a thin layer of gold upon the surface for 30 s at 30 W in vacuum, and then was examined at an excitation voltage of 15 kV.

### *1.11. Fourier-transform infrared spectroscopy*

FTIR spectra were obtained in the transmission mode within the scanning range from 400 to 4000 $cm^{-1}$ using a Magna-IR560 instrument (Nicolet, USA). All operations were carried out in a dry atmosphere at room temperature (25±1 °C), and the results were recorded in transmittance units.

### *1.12. Fluorescence spectroscopy*

Fluorescence spectra were recorded on a spectrofluorimeter (Cary Eclipse, Agilent Technology Inc.) at 37 °C. The concentration of CCPs (10 min, 6 h, 12 h, and 24 h, respectively) was 4 mg $ml^{-1}$ (20 mM Tris-HCl, pH 7.4). A certain amount of $CaCl_2$ solution was added to the experimental groups to obtain a final concentration of $Ca^{2+}$ (0.160 mol $l^{-1}$). As for the control groups, the equal volume of $dH_2O$ was used. All groups were incubated for 30 min at 37 °C before measurement. An excitation wavelength of 220 nm was chosen, and emission spectra were recorded within the range of 230 to 420 nm.

## 2. Results and discussion

### *2.1. Preparation and characterization of $Ti^{4+}$-IDA-GLMYO@CM*

Due to biocompatibility and abrasive resistance of CM, CM-based $Ti^{4+}$-IMAC adsorbent was prepared (Fig. 1) and used to enrich CCPs. The images of surface structure of $Ti^{4+}$-IDA-GLMYO@CM and CM were visualized by SEM (Fig. 3). It is assumed that the superior macroporous structure would be beneficial for capturing CCPs efficiently.

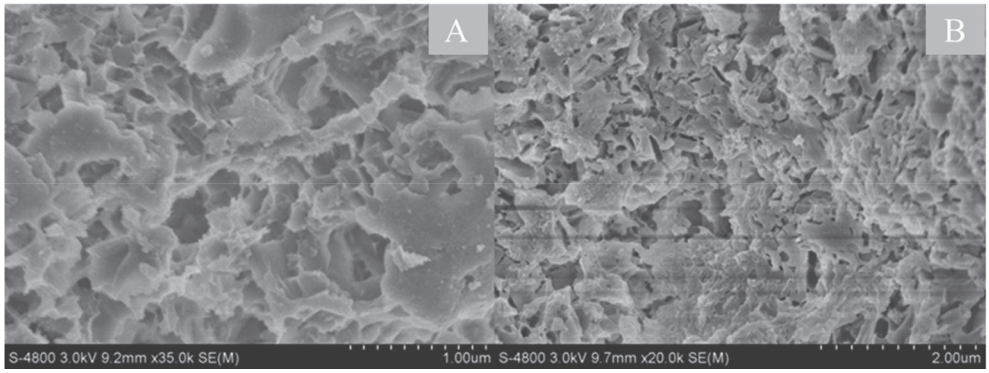


*Fig. 3.* SEM images of (A) CM and (B) $Ti^{4+}$-IDA-GLYMO@CM

### *2.2. Enrichment and evaluation of CCPs*

Peptides with calcium-chelating capacity in the casein digest were enriched via $Ti^{4+}$-IDA-GLYMO@CM according to the procedure shown in Figure 2A. As shown in Figure 2B, the RP-HPLC chromatogram of CCPs (eluted by 80% ACN/2% TFA) was much cleaner than that of casein digest, indicating that peptides with the ability of chelating metals were indeed purified.

The $Ca^{2+}$-binding abilities of different casein digest samples as well as CCPs were indirectly determined by measuring the inhibition of the formation of insoluble calcium phosphate. The results presented in Figure 4 show that the consumption of NaOH solution significantly decreased when CCPs were added, demonstrating that the formation of insoluble calcium phosphate could be inhibited by CCPs. It is worth noting that 10-min CCPs showed higher calcium-binding ability [683 mg($Ca^{2+}$)/g(CCPs)]. Similarly, the maximum value of the contents of calcium in CCPs-Ca complexes occurred in the case of 10 min-CCPs (Fig. 5A). These results might be explained by the fact that most of CCPs with the cluster sequence -SerP-SerP-SerP-Glu-Glu- were released within 10 min but hydrolyzed with the increase of DH (Su et al., 2007). Therefore, phosphoserine residues seem to play a pivotal role in calcium chelation.

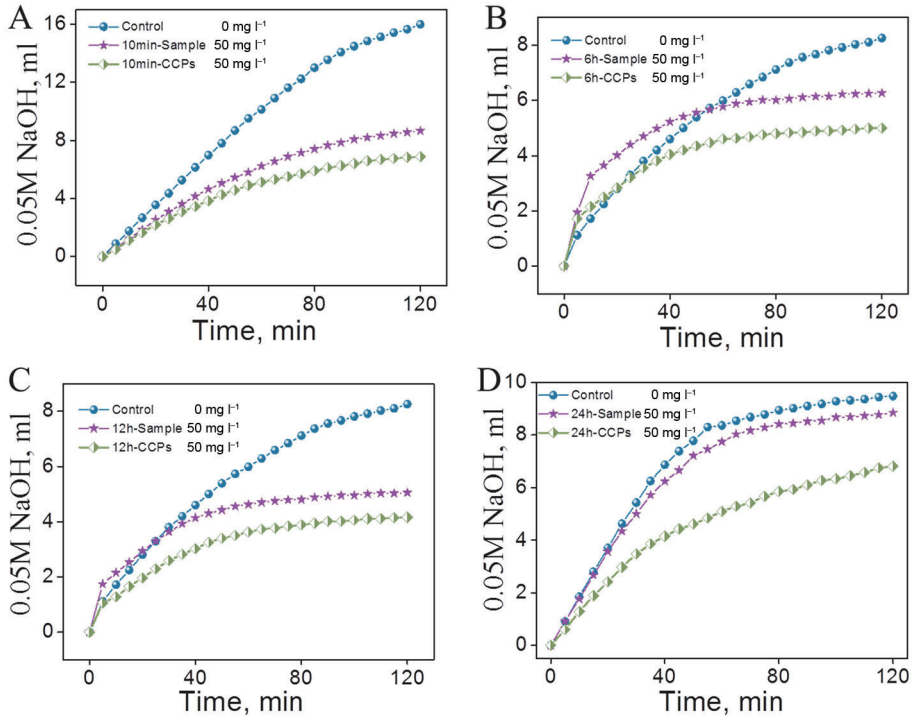


*Fig. 4.* Performance curves for the ability to inhibit the formation of insoluble calcium phosphate of casein digest samples as well as CCPs with different hydrolysis times (A) 10 min, (B) 6 h, (C) 12 h, (D) 24 h. Conditions: temperature 37 °C, pH 7.2, and the concentration of casein digest samples or CCPs 50 mg $ml^{-1}$

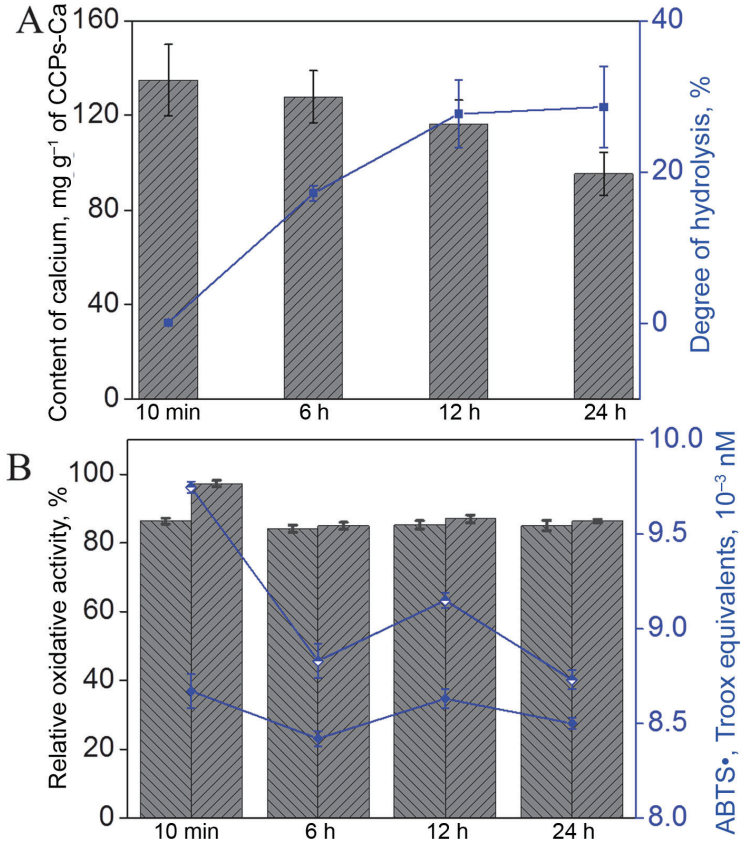


*Fig. 5.* (A) Degree of hydrolysis of casein hydrolysates with different reaction times and contents of calcium in CCPs-Ca complexes, (B) ABTS radical activity (inhibitory, %; Trolox equivalent, $10^{-3}$ mM) of casein digest (250 μg ml$^{-1}$) and CCPs (50 μg ml$^{-1}$) of different hydrolysis times (10 min, 6 h, 12 h, and 24 h)
A: ▨: Content of calcium; B: ▨: Casein digest; ▨: CCPs; —◆—: Casein digest; —◇—: CCPs

Additionally, the scavenging capacities of CCPs and the casein digest were estimated by the ABTS$^{\cdot+}$ decolourization assay and results, expressed as % inhibition of ABTS$^{\cdot+}$ as well as $10^{-3}$ M Trolox, are displayed in Figure 5B. Obviously, there was no significant difference among free radical scavenging activity of casein digest (250 μg ml$^{-1}$; 86.3%, 84.1%, 85.2%, and 85% for 10 min, 6 h, 12 h, and 24 h-casein digests, respectively). However, a maximum capacity (99% inhibition or an equivalent activity of $9.91\times10^{-3}$ M Trolox) was obtained for 10 min-CCPs at 50 μg ml$^{-1}$, although those of other CCPs were similar (90.1%, 91.2%, and 86.8% for 6 h, 12 h, and 24 h, respectively). Furthermore, at the hydrolysis time of 6 h, 12 h, as well as 24 h, the enriched CCPs at a lower concentration (50 μg ml$^{-1}$) provided similar scavenging activity as the casein digest at 250 μg ml$^{-1}$. All these results demonstrated that the CCPs enriched through $Ti^{4+}$-IDA-GLYMO@CM presented more effective primary antioxidant potential than casein digest.

Several amino acids have been proved to exhibit antioxidant activity. The radical scavenging activities of His and Tyr were thought to be due to the hydrogen-donating ability

of the imidazole group (Chan et al., 1994) and the phenolic hydroxyl group (Chen et al., 1996), respectively. Additionally, studies have indicated that the antioxidative activity of peptides depend on their constituent amino acids (Chen et al., 1996). As for CCPs, the potential functional domain (-SerP-SerP-SerP-Glu-Glu-) acting not only as metal sequestering, but also hydrogen/electron donation, was considered to provide more pronounced radical scavenging activity (Kitts, 2005).

### *2.3. Fourier-transform infrared spectroscopy*

The Fourier-transform infrared spectroscopy (FTIR) could provide information about amino acid residues in $Ca^{2+}$-binding peptides (Nara & Tanokura, 2008; Nara et al., 2013). FTIR spectra of CPPs and Ca-binding CCPs are shown in Figure 6. The results indicate that CCPs-Ca complexes have formed, since variations of FTIR spectra are obvious after addition of calcium. In the spectra of CCPs-Ca, red shifts in the region 1700–1600 $cm^{-1}$, assigned to the C=O stretch (mixed with the N-H bend and the C-N stretch), indicated the involvement of the carboxylic group in complex formation. Significant changes to the band around 1400 $cm^{-1}$ assigned to $COO^-$ (Nara & Tanokura, 2008) reflected the form of Ca-bound peptides. This suggested that the 'free' $COO^-$ groups of some amino acid residues, such as Asp and Glu, were also involved in Ca-peptide complex formation. Moreover, the absorption band near 1350–1100 $cm^{-1}$ attributed to the P=O stretch (Trindade et al., 1997) presented noticeable changes, suggesting that it was probably the calcium bound to CCPs through interactions with phosphoserine residues.

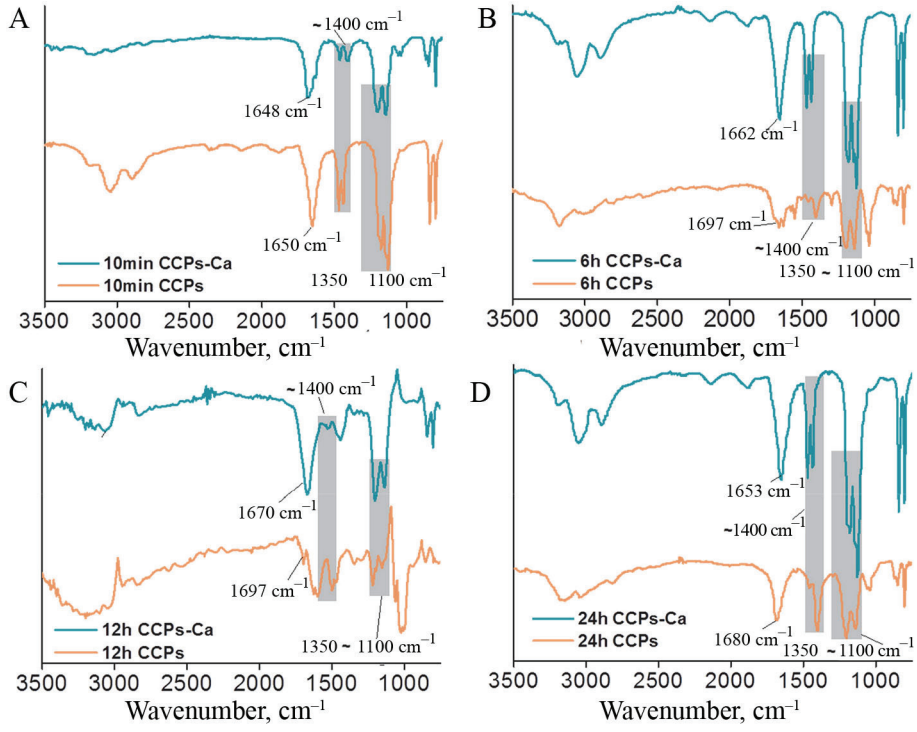


*Fig. 6.* FTIR spectra of CCPs and CCPs-Ca complexes at different hydrolysis times (A) 10 min, (B) 6 h, (C) 12 h, (D) 24 h

### *2.4. Fluorescence spectroscopy*

The fluorescence spectroscopy has provided valuable insights into the metal-binding properties of proteins (Kirberger et al., 2013; Tokunagao et al., 2013), since there are three aromatic amino-acid residues (phenylalanine, Phe; tyrosine, Tyr; tryptophan, Trp) that contribute to their ultraviolet fluorescence.

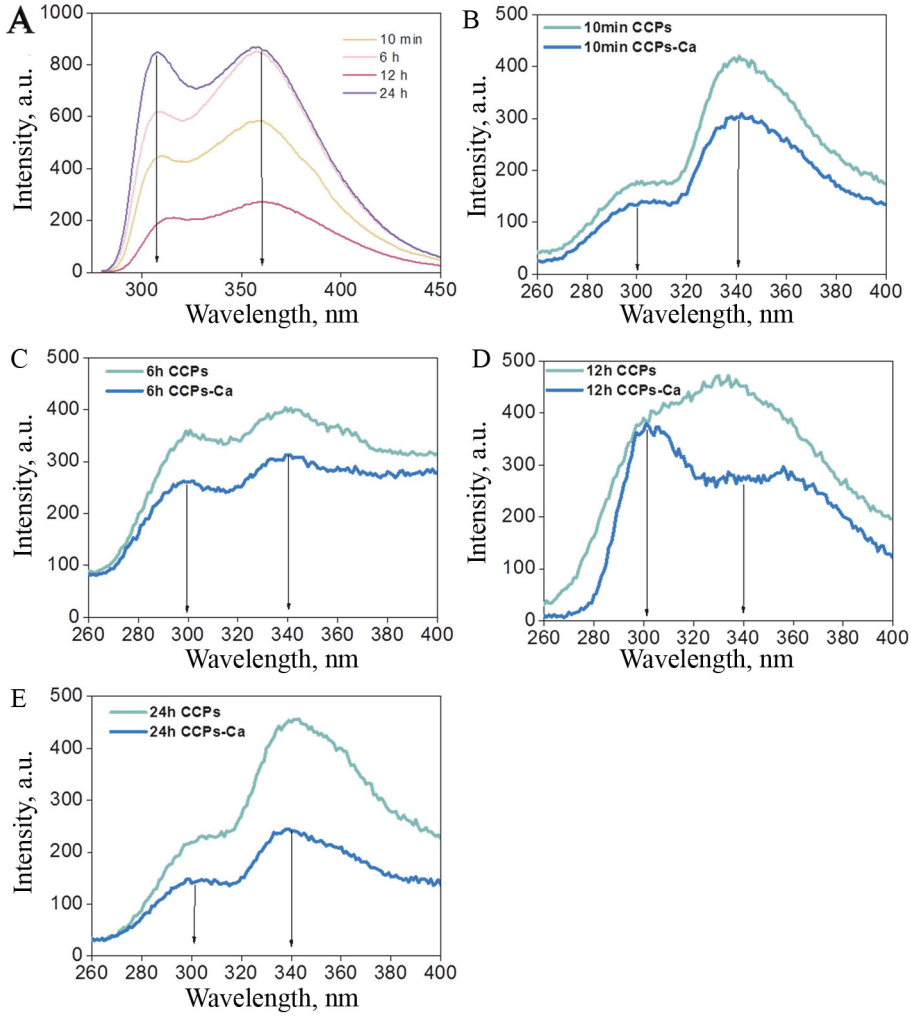


*Fig. 7.* (A) Fluorescence spectra of casein digest at various time points, conditions: concentration of samples, 5 mg $ml^{-1}$ (20 mM Tris-HCl, pH 7.4); excitation wavelength, 270 nm; emission spectra, 280–540 nm; temperature, 37 °C; (B-E) CCPs with or without calcium at different hydrolysis times (10 min, 6 h, 12 h, and 24 h); Conditions: $C_{CCPs}$=4 mg $ml^{-1}$; $C_{Ca}^{2+}$=0.160 mol $l^{-1}$; λex=220 nm; emission spectra, 230–420 nm; temperature, 37 °C

The emissions of Phe, Tyr, and Trp in water occur near 280 nm, 300 nm, and 350 nm, respectively. As shown in spectra of casein digest (Fig. 7A) and CPPs with or without calcium (Fig. 7B–E), Tyr and Trp emissions were observable, except that of Phe, which would be the

result of energy transfer (Lakowicz & Masters, 2008). Compared with the spectra of casein digest (Fig. 7A), blue-shifts happened to both Tyr and Trp emission in the spectra of CCPs (Fig. 7B–E). This phenomenon could be expected from the different degrees of solvent exposure of Tyr and Trp residues (Lakowicz & Masters, 2008), and again proved the fact that CCPs could be purified via $Ti^{4+}$-IDA-GLYMO@CM. Additionally, upon addition of calcium to CCPs, there was a decrease in intensity but no shifts of their Tyr and Trp emissions, showing that calcium-binding resulted in the quenching due to changes in the microenviroment around these two amino acid residues. Meanwhile, more significant change was observed in fluorescence intensity of Trp than that of Tyr, which might be due to the high sensitivity and the large effect of the local environment on molecular emission. Consequently, all above results proved the existence of interaction between calcium and CCPs.

## 3. Conclusions

In conclusion, CCPs with high calcium-chelating ability and radical scavenging capacity were successfully and rapidly enriched by CM based-$Ti^{4+}$-IMAC adsorbent. The present work has provided an alternative material for purifying not only CCPs but also proteins or even other macromolecules.

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