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Synthesis and Properties of an Insoluble Chitosan Resin Modified by Azamacrocycle Copper(II) Complex for Protein Hydrolysis

Xingyu Wang,[1] Dongfeng Wang,[1] Peng Liang,[1] Xingguo Liang[2]

[1]Department of Food Chemistry and Nutrition, College of Food Science and Technology, Ocean University of China, Qingdao 266003, People's Republic of China

[2]Department of Food Molecular Biology, College of Food Science and Technology, Ocean University of China, Qingdao 266003, People's Republic of China

Correspondence to: D. Wang (E-mail: wangdf@ouc.edu.cn)

ABSTRACT: We reported the synthesis process and the proteolytic ability of a novel crosslinked chitosan resin modified by azamacrocycle copper complex (CMCR). Characterization of CMCR showed that the Cu(II)Cyclen complex had successfully bound to the resin matrix covalently. Its proteolytic activity toward peptide bonds was evaluated by investigating the hydrolysis of bovine serum albumin in heterogeneous media. The maximum-observed pseudo-first-order rate constant (k_{obs}) was achieved under 60°C, pH 6.0, where protein was hydrolyzed into smaller peptides and amino acids confirmed by SDS–PAGE and ninhydrin reaction. The results indicated that the hydrolyzing efficiency delivered by CMCR was 4×10^5 times higher than that of spontaneous hydrolysis. CMCR also featured a desirable stability for chelating Cu^{2+} ions, which paved the way for its feasible recovery process and good reusability. © 2012 Wiley Periodicals, Inc. J. Appl. Polym. Sci 000: 000–000, 2012

KEYWORDS: proteins; resins; crosslinking; polysaccharides

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INTRODUCTION

Proteolysis, hydrolysis of protein into amino acids or small fragments, is widely applied to the utilization of agricultural yields, seafood, and meat byproducts as well as to the improvement of nutritional and functional properties of proteins. Some short peptide products generated by proteolysis show various kinds of bioactivity, which could be used in preparing functional food.[1] Amino acids and flavor peptides obtained from proteolysis have been used as novel additives in food in recent years, such as soy sauce, vinegar, and natto. Since their extensive applications, it has come to light that proteolysis protocols with higher efficiency and lower cost are required increasingly.

In industry, proteins are hydrolyzed conventionally by proteinases. Although their efficiency is high, they may be easily inactivated with slight alteration of condition and only selectively hydrolyze the substrates. Another shortcoming is that separating the enzyme from hydrolysate is not easy, and the recovery process is quite intricate. To solve the problem of subsequent recovery, immobilized enzymes have been developed.[2] However, their cost is high due to limited enzyme sources and long-term, complicated purification. Even more, immobilized enzymes are not capable of overcoming the instability of enzymatic catalysis, and so it leaves the problem standstill.

Many research teams have poured attention into the artificial enzymes constructed by metal ions and heterocyclic compounds due to their high stability. It has been proved that many metal complexes composed of Zn^{2+}, Co^{2+}, Ce^{4+}, La^{2+}, Pb^{2+}, and Pt^{2+} are capable of cleaving the peptide bonds and phosphodiester bonds under physiologic conditions.[3–6] The synthesis procedures of those complexes are feasible, and hydrolysis action occurs easily owes to their minor steric effect.[5] Subsequently, it was reported that Zr^{4+}, Cu^{2+}, and Pb^{2+} complexed with azacrown ether,[7] tridentate ligand,[8] and pyridine,[9] respectively, showed a gratifying prospect on designing synzymes. However, because most of the artificial metalloproteases are homogeneous, catalytic efficiency has been dramatically impaired by the strong polarity imposed by hydrones.[10] To solve the problem mentioned earlier, some groups used polymer materials such as polystyrene and polysilica particles[11] to construct heterogeneous carriers with a desirable hydrophobic microenvironment, which not only enhanced the hydrolytic activity but also administered to separate the catalyst from hydrolysate, because the separation procedure can be facilitated if the catalysts do not dissolve in

reaction system. Nonetheless, the synthesis protocols of these polymers are complicated, and some of the products are toxic. Moreover, these macromolecule reagents have to take millions of years to fully decompose spontaneously, which makes them no friend to environment.[12] As a natural alkaline polysaccharides with great abundance, chitosan has a distinct capability that can be modified effortlessly in addition to its biocompatibility, biodegradability, and bioactivity.[13] It also has been intensively investigated as a kind of biomaterial to prepare polymer functional material-like macroporous resin and edible film. Because nature has used polypeptides as the skeletons of enzymes, we are enlightened that polysaccharides may be exploited to build the scaffold of artificial metalloproteases likewise by the similar works reported earlier.[14] Studies demonstrated that chitosan-based polymers were allowed to be modified designedly to constitute regionally hydrophobic and hydrophilic microenvironment, which would redound to boost the activity of the artificial enzymes if the strategies could be applied to practical use.[15]

Given these premises, the aim of the present work is to create a novel modified chitosan copper(II) complex resin. To better mimic the features of natural enzymes, heterocyclic substance was introduced onto the chitosan backbone to constitute a hydrophobic microdomain, which according to the characterization mentioned earlier could promote hydrolyzing efficiency. Experimental results indicated that the novel chitosan-based complex indeed exhibited remarkable catalytic capability for peptide bonds hydrolysis and suggested that this chitosan complex could be further used to develop an artificial multinuclear protease.

EXPERIMENTAL

Materials and Reagents

Chitosan powder (MW: 50,000, deacetylation degree 92%, purchased from Li Zhong Co., Qingdao, China) was used without further purification. 1,4,7,10-Tetrazacyclododecane (Cyclen) was obtained from Kai Sai Chemical Co. (Shanghai, China). Bovine serum albumin (BSA; purity > 98%) was provided by Bio Basic (Beijing, China) and used without further purification. SDS–PAGE molecular weight markers (6.5–66 kDa) and other electrophoresis reagents were obtained from Sigma Chemical Co. (Beijing, China). All the reagents were analytical grade and purchased from local commercial sources.

Preparation of the Insoluble Catalyst

Chitosan-crosslinked resin (CCR) was prepared by the method of reverse phase suspension crosslinking. First of all, chitosan solution 5% (w/v) was prepared by dissolving powdery chitosan in 4% (v/v) acetic acid aqueous solution and added to 200-mL liquid paraffin-containing 2.5 mL Span-80, with stirring speed of 180 revolutions per minute (r/min) for 30 min. Second, 40 mL formaldehyde was continuously introduced and reacted for 30 min at 50°C. After this process, pH was adjusted to 9.0 using 0.1 *M* NaOH solution, followed by the addition of 20 mL epichlorohydrin and 3 h reaction under 60°C to form chitosan resin particles. Finally, the resin was washed by petroleum ether, absolute ethyl alcohol, and deionized water, respectively, and stored in DMF (*N, N*-dimethylformamide) for future use.

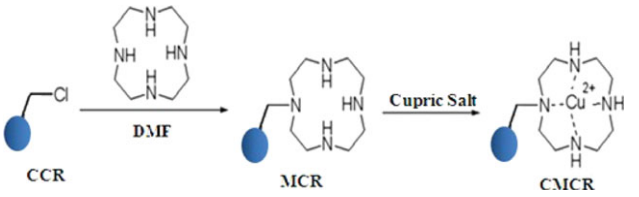

Figure 1. Preparation scheme of CMCR. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CCR was suspended in 200 mL, $5.0 \times 10^{-4} M$ cyclen solution, and the modification reaction was carried out under 40°C for 4 h. Then, certain amount of cupric salt was introduced to the system to make sure that the concentration of Cu^{2+} was 0.4 *M*. Similarly, the mixture was allowed to conduct the reaction under 40°C for 8 h. When it was finished, resin microspheres were collected by filtration and then flushed with distilled water and absolute ethyl alcohol repeatedly until no Cu^{2+} was detected. In the end, copper complex of modified chitosan resin (CMCR) was stored in deionized water. The preparation route of CMCR was shown in Figure 1.

Characterization of CMCR

Optical microscope and scanning electron microscope (SEM) were applied to obtain the surface detail of copper complex of modified chitosan resin (CMCR). Inductively coupled plasma atomic emission spectrometry (ICP–AES) was performed by using IRIS Intrepid IIXSP to determine the content of copper ions in the resin particles (CMCR). The parameters are as follows: RF power: 1150 W; nebulizer flow: 26.0 PSI; auxiliary gas: 1.0 LPM. The copper content of the unmodified chitosan resin copper complex prepared in our laboratory was 2.8% and that of CMCR was 3.4%, indicating a 21.4% improvement.

Resin samples were first pulverized with KBr. IR spectra were measured using a NEXUE 470 FTIR spectrometer (Nicolet Co., Waltham, MA) equipped with a deuterated triglycine sulfate detector (at room temperature) and OMNIC ESP V6 software. They were recorded from the accumulation of 32 scans in the 2000–500 cm^{-1} range at a resolution of 2 cm^{-1}. The spectral scan was repeated at least three times in order to correct the background noise. Data analysis was carried out using Origin 6.0 software (Microcal Software, Northampton, MA).

Incubation for CMCR Hydrolyzing Proteins

A certain amount of copper complex of modified chitosan resin (CMCR) was added in the reaction system that consisted of Tris–H^+ buffer solution (pH 7.0) and certain amount of BSA. The hydrolysis reaction was performed under a fixed temperature and shaking speed provided by isothermal water bathing shaking apparatus. Every 12 h, 0.2 mL samples were drawn from the reaction system and filtered with ultrafiltration membrane (aperture 22 μm), and then the clear liquid was stored at −18°C for further quantification. Incubation was carried out at 60°C enduring for 48 h.

Measurement of the Hydrolysate

After the incubation, hydrolysate was subjected to SDS–PAGE conducted according to the literature.[16] The intensity of protein bands in electrophoretic patterns, which represented the degradation of

substrate protein, was recorded by Tanon-4100 gel imaging system, and the data were processed to obtain initial rate plots. Ninhydrin reaction was used to confirm the hydrolyzing ability of CMCR. All runs were conducted in triplicate, from which standard deviations of typically 2–5% were obtained, as indicated by error bars in the data plots.

Catalytic Kinetics Study

For the limited solubility of CMCR in Tris–H^+ buffer, kinetic measurement was carried out under heterogeneous condition by adding the complex into a continuously shaking reaction system with fixed concentration of BSA. The concentration of the catalyst was calculated by assuming that all the CMCR used in the experiment has dissolved in the solution, where the copper content of the complex was calculated according to the results of ICP–AES. The effect of pH on catalytic efficiency was studied by altering the composite of buffer to create different pH at 60°C, and the measurement of relative parameters was carried out by varying the concentration of protein substrate and determining the degree hydrolysis of protein molecules at various time intervals. Also, the effect of temperature on catalytic efficiency was determined by altering the incubation temperature while the protein was being hydrolyzed. The temperature points were set to 40, 50, 60, and 70°C.

Recovery Procedure and Reusability

To evaluate the reusability of CMCR, the insoluble catalyst has been recycled from the reaction solution after the catalytic reaction enduring 48 h. The beads were recollected by filtration and shaken in Tris–H^+ buffer for 2 h to remove the peptide fragments and amino acid left. Then the beads were washed by water several times until the wash solution had no absorbance to be detected under 280 nm performed on spectrophotometer. The recovery procedure was carried out following each hydrolyzing reaction. The recycle conducted four times, and the catalytic efficiency was estimated by observed pseudo-first-order rate constant (k_{obs}).

RESULTS AND DISCUSSION

Structure of CMCR by Microscope

The surface detail of copper complex of modified chitosan resin (CMCR) particles was shown in Figure 2. The shape of the blue particles was spherical, and their surface was smooth; therefore, the surface friction between any beads was much weaker comparing to the insoluble catalyst with amorphism structure. The average diameter of these particles was 353 μm and more than 85% particles featured the diameter between 240 and 549 μm, which manifested a lower polydispersity. Generally, particles with smaller size own a high mobility and specific surface area in heterogeneous system. These two features signified the beads dispersed in distilled water more easily, which would be conducive to the intense interaction between substrates and active sites. As the matrix of CMCR, CCR has been studied as a novel functional material for a long time. Many groups have investigated the insoluble carrier intensely as a type of adsorbing agent for adsorbing heavy metal ions, industrial dyestuff, and toxic proteins. To better serve our purpose of designing artificial metallopeptidases, CCR microspheres were used as the support to provide polysaccharides backbone. Within the molecular domain, hydroxyls on the C-6 of glucosamines are crosslinked by epichlorohydrin. However, because epichlorohydrin has two active groups and higher mole concentration in the reaction, one of its active group (C—Cl) tends to suspend in space that will make the bonding of cyclen molecule possible.[17] CCR is also a good candidate for the backbone of immobilized mimic enzymes; thanks to its highly branched structure, availability of many reactive groups such as —NH_2 and —OH on its surface that are needed for the introduction of multiple catalytic elements in proximity on the polymer skeleton as well as large surface areas and high-mechanical strengths of its beads.

FTIR Analysis of CMCR

The IR spectra of chitosan resin (a), cyclen-modified chitosan resin (b), and copper complex of MCR (c) are shown in Figure 3. The Fit Multi-peaks function of Ominic software was applied to analyze the overlapping peaks.

The IR spectrum of chitosan resin showed in (a) 3420 cm^{-1} absorption peak represents the stretching vibration of ν(O—H) and ν(N—H), 2920 and 2867 cm^{-1}, $\nu(CH_3)$, and $\nu(CH_2)$, respectively. The 1469 cm^{-1} and 1395 cm^{-1} were assigned to the bending vibration of $\delta(CH_2)$ and $\delta(CH_3)$.[18] The absorption peak in the 1150 cm^{-1} was attributable to the stretching vibration of ν(C—O—C) and 715 cm^{-1} to the deformation vibration of the ring of glucosamine.[17] The vibration caused by bending δ(C—Cl) was spotted at 1296 cm^{-1}; however, in the IR spectrum of MCR showed in (b), the vibration intense of δ(C—Cl) has been weakened noticeably. This denotes that the cyclen molecule has been successfully coupled onto the —Cl of chitosan's backbone,[19] which paved the way for the construction of copper complex of modified chitosan resin (CMCR).

For CMCR complex, the most of adsorption peaks were mostly identical to cyclen-MCRs (b) except the wide adsorption band from 1544 cm^{-1} to 1369 cm^{-1}. The band revealed the existence of the Cu-cyclen skeleton referring to the description of the literature.[20] The adsorption peak of τ(N—H) at 1640 cm^{-1} also moved down to 1629 cm^{-1}, which indicated not only the azamacrocycles but also the amino hydroxyl, and groups were involved in complexation with copper ion.[21] All the analytical descriptions delivered earlier clearly point out that the metal-heterocycle has been successfully introduced on the backbone of chitosan resin giving rise to prospective hydrolyzation toward protein substrates.

Stability of Copper Ions on CMCR

Copper complex of modified chitosan resin (CMCR) is an artificial immobilized enzyme, which harnesses the copper ion to form the catalytic center. In this regard, the amount of copper ion chelated on CMCR is vital for its performance. To investigate the stability of the copper ions on CMCR, ICP–AES was used. The hydrolysate was separated from the dissociated copper ions by dialysis, and the sample was digested before test. The stability of Cu^{2+} was shown in Figure 4. According to the chart, after 48-h shaking, only 1.83% amount of ions dissociated off the matrix, which means that CMCR boasts an appreciable capacity of stabilizing Cu^{2+} ions.

Metal ions play versatile roles as Lewis acid catalysts in organic reactions. Normally, they are responsible for electron transfer and intermediate balance.[22] Here, copper ions mainly acted as strong

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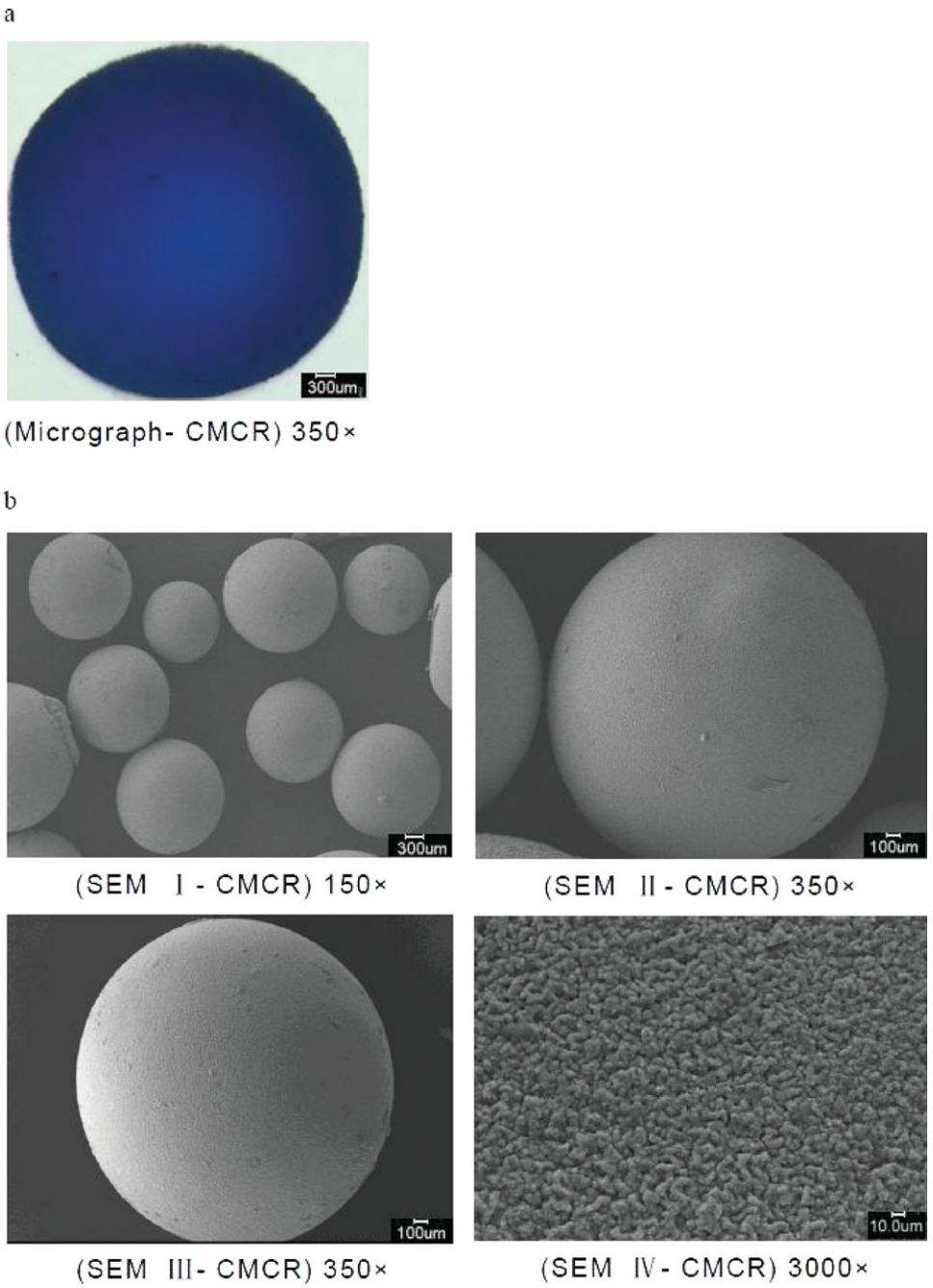

Figure 2. Micrographs (a) and SEM images (b) of CMCR particles. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Lewis acid for the activation of the peptide bonds and the generation of nucleophiles. Only by possessing a needed amount of metal ions, can mimic enzymes fully exhibit the hydrolyzing capability. Slight rate acceleration was also found when copper ions or copper-complexed unmodified chitosan resin was used alone.[23]

Catalytic Kinetic Studies

Apparent Rate Constants for BSA Hydrolysis. BSA was used as the model substance for evaluating hydrolyzing efficiency of CMCR. The half-life of peptide bond's spontaneous hydrolysis is nearly 1000 years at pH = 7.0, 25°C as reported.[24] It is logical that peptide bond should be much more stable than any other chemical bonds existing in human body, because a constant protein structure is vital for their functions. In this research, the buffer solution-containing protein substrate was shaken with the insoluble catalyst. The disappearance of the protein was demonstrated via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE), and the observed pseudo-first-order rate constant (k_{obs}) was measured by monitoring the decrease in intensity of the electrophoretic bands according to protein substrate.[25] The typical result of electrophoresis was illustrated in Figure 5(A). That the disappearance

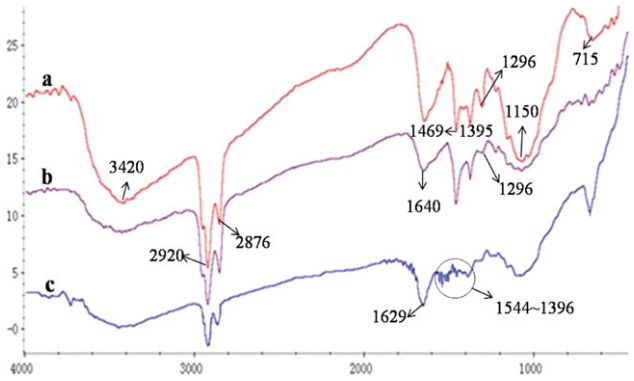

Figure 3. FTIR spectra of chitosan resins (a), cyclen-modified chitosan resins (b), and copper complex of modified chitosan resin (c). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

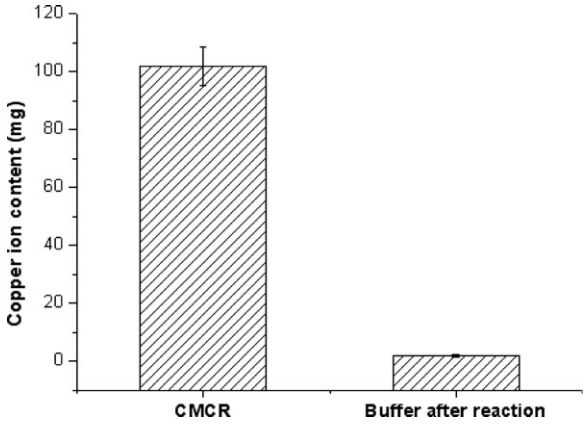

Figure 4. Stability of CMCR complexing Cu^{2+}. ICP–AES was used to test the stability of copper ions after 48-h hydrolysis reaction. The reaction was done in 20 mL, pH 7.0 Tris–H^+ buffer-containing 1.52×10^{-2} m*M* BSA. The amount of CMCR beads used in this part was 1.0 g.

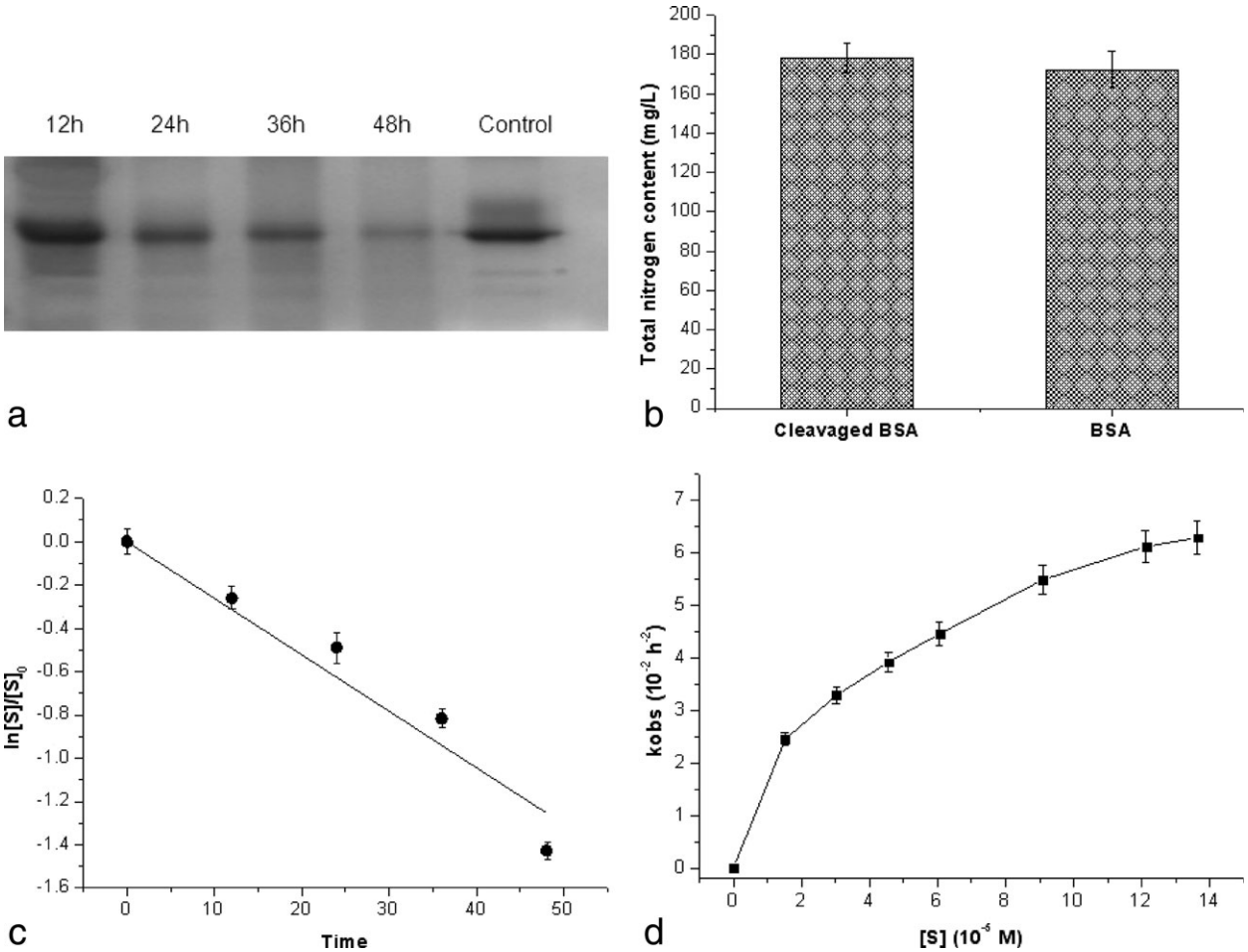

Figure 5. A: Results of SDS–PAGE were performed on BSA at 60°C and pH was 7.0. The catalyst amount was 1.0 g, and solution volume was 20 mL, pH 7.0 Tris–H^+ buffer-containing 1.52×10^{-2} m*M* BSA. B: Result of Kjeldahl determination toward different sources. The result of the *t*-text indicated that the significance level was $P > 0.05$. C: Plot of $\ln[S]/[S]_0$ against time for the electrophoretic bands presented in Figure 5(C). The relative concentration of substrate was measured by analyzing the density of the electrophoretic bands. The straight line corresponds to k_{obs} of 2.6×10^{-2} h^{-1}. D: Effect of initial BSA concentration on the initial rate for the hydrolysis of BSA by crosslinked chitosan resin modified by azamacrocycle copper complex at pH 7.0 and 60°C. The catalyst amount was 1.0 g, and solution volume was 20 mL.

of the electrophoretic band of each protein was not due to the adsorption onto the insoluble support was confirmed by measuring the total protein nitrogen content using Kjeldahl determination. The solution containing hydrolyzing product was obtained by collecting the supernatant after configuration. The control experiment was to measure the total protein nitrogen content of BSA in the same buffer yet without CMCR. The result [shown by Figure 5(B)] demonstrated that there was no significant difference between the control group and treatment group ($P > 0.05$), which means that the hydrolysis activity is mainly responsible for the decreasing electrophoretic band of BSA, because the protein nitrogen content would decrease if the adsorption effect existed.

The rate of protein cleavage at 60°C was measured by monitoring the decrease in the intensity of the electrophoretic bands corresponding to the substrate protein. The k_{obs} of CMCR hydrolyzing BSA was estimated from the plot of $\ln[S]/[S]_0$ ([S] represents protein concentration at the designated time point and $[S]_0$ stands for the initial concentration of protein) against time as shown as Figure 5(C). Electrophoresis of the reaction mixtures indicated that only the parent protein of was detected during the cleavage reaction catalyzed by CMCR. Under experimental conditions of electrophoresis, only proteins or peptides larger than several kilodaltons can be detected.[26] The number of peptide bonds of one BSA molecule interacting with the active sites of the insoluble catalyst is, however, not large, and the breakdown of the intermediate proteins is faster than the cleavage of parent molecule of BSA.[27] Therefore, it is reasonable that no proteins or peptides with sizes smaller than parent protein accumulate appreciably in the electrophoretogram. At the optimum pH and temperature, k_{obs} reached 3.21×10^{-2} h^{-1}, which is much faster than Zr^{4+} and Ce^{4+} complexes reported earlier.[28,29] Although the proteolytic potential of CMCR is weaker than trypsin hydrolysis at 32°C, pH = 7.0, the simpler structure and lower cost may entitle more investigation and application in prospect.

The effect of initial BSA concentration on the k_{obs} for the hydrolysis of BSA by CMCR at pH 7.0 and 60°C was shown in Figure 5(D). With a fixed catalyst concentration, the hydrolytic rates fitted the first-order reaction kinetic at lower BSA concentration but exhibited a saturation trend at higher substrate concentration, indicating that the catalytic hydrolysis of BSA might follow the Michaelis–Menten reaction mechanism. The equations elucidating this behavior are given below:

$$CMCR + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} CMCR{-}S \xrightarrow{k_{cat}} CMCR + P \quad (1)$$

$$v = \frac{k_{cat}[CMCR][S]}{K_M + [S]} = \frac{V_{max}[S]}{K_M + [S]} = k_{obs}[S] \quad (2)$$

$$\frac{1}{V} = \frac{1}{V_{max}} + \frac{K_M}{V_{max}} \times \frac{1}{[S]} \quad (3)$$

where k_1, k_{-1}, and k_{cat} are rate constants, V_{max} is the maximum reaction rate, and $K_M = (k_1 + k_{cat})/k_1$ is Michaelis constant. All the Michaelis–Menten parameters were calculated by Lineweaver–Burk double-reciprocal plot based on the data provided in Figure 5(D). According to eq. (3), the Michaelis–Menten parameters K_M were determined at $2.98 \times 10^{-5}M$, and the maximum reaction rate V_{max} was calculated at $7.01 \times 10^{-2}M$ h^{-1}. All the above-mentioned results indicated the validity of our proposed kinetics model. Additionally, the lower K_M value revealed the higher binding affinity between BSA and CMCR, indicating the competent catalytic activity for the hydrolysis of protein substrate. Although the proteolytic activity of CMCR was not as robust as natural metallopeptidase reported earlier,[30] it still boasts a desirable tolerance toward harsh reaction conditions and facilitates the subsequent separation.

Many research teams have reported the proteolytic activity of metal ions, which, generally speaking, act as Lewis acid catalysts in peptide bond hydrolysis. Under this situation, Cu^{2+} center can polarize the carbonyl group of the scissile peptide bond by binding at the carbonyl oxygen while facilitate Cu^{2+}-bound hydroxide ion, which acts as a potent nucleophile to promote hydrolyzing reaction.[31] However, the proteolytic activity of Cu(II)Cyclen complex itself is negligible. A possible explanation for this behavior is the dimerization of two Cu(II)Cyclen complexes and is formed without chitosan beads matrix.[32] Upon dimerization, the metal center would not be able to interact with peptide bonds of the substrate proteins. In fact, the hydrolyzing effect of resin beads can be related to the unique microenvironments created on the surface of the polysaccharides backbone. The microdomain is able to enhance the intrinsic interaction of the metal centers and protein substrates by screening the strong polarity of water molecules.[33] It confirmed a matter of fact that natural metallopeptidases often have created a hydrophobic pocket by folding peptide chains where the hydrolyzing reactions take place as well.[34] Because the bulky molecules like proteins tend to diffuse slowly to the reactive sites in resin beads,[35] shaking speed might have a great influence on BSA cleavage. As the speed we used in this study was only 160 r/min, the great catalytic enhancement could be achieved as long as the apparatus was able to provide much higher shaking pace, for the interaction that was conducive to hydrolysis would definitely be intensified at higher shaking speed.[32]

Effect of the Initial pH on Catalytic Hydrolysis of BSA. For natural enzymes, it is essential to exhibit their catalytic potential at the optimum pH. There are some research groups reported that artificial enzymes were much more stable than nature ones under harsh conditions.[36] As natural enzymes constructed by peptide chains, the molar concentration of H^+ in the reaction system truly affected the dissociation state of amino residues some of which are responsible for the catalytic efficiency. On the other hand, the mimic enzyme we prepared in this article showed a same behavior from pH 4.0–10.0. With the degradation of protein substrate, the pH of a specific system experiences a slight alteration caused by —COOH and —NH_2. So the initial pH was set as a parameter to be studied. The effect of initial pH on k_{obs} for the hydrolysis of BSA by CMCR was shown in Figure 6. It showed a bell-like curve with the maximum at pH = 6.0. CMCR speeds up the hydrolyzing reaction more than 4×10^5-fold comparing to spontaneous hydrolysis without relying on the extreme pH where the self-degradation will be noticeably intensified.

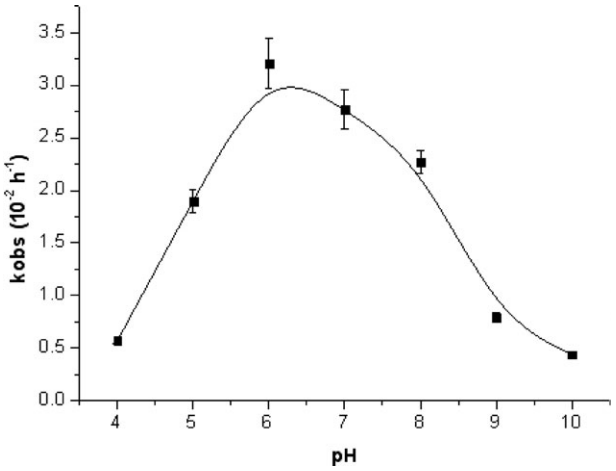

Figure 6. Optimum pH for CMCR hydrolyzing BSA. Buffers (50 m*M*) used for the optimum pH measurements were disodium-citric acid (pH 4.0 and 5.0), tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.0, 7.0, and 8.0), and borax-sodium hydroxide (pH 9.0 and 10.0). About 1.0 g CMCR was used, and the solution volume was 20 mL containing 1.52 $\times$ 10^{-2} m*M* BSA.

According to the chart given earlier, the optimum initial pH for degrading BSA by CMCR was around 6.0. Because the matrix we used to prepare CMCR was chitosan resin, it may be unstable under acidic conditions.[37] It is allowed to attribute the lower reactivity to the loose structure of CMCR induced by lower pH. On the other hand, when CMCR carriers were introduced into a system with higher pH, the excessive —OH groups were able to complex the associated Cu^{2+} ions from the active sites to form $Cu(OH)_2$ precipitation, which can be observed during experiment, depleting the Cu^{2+} ions in catalytic centers.[38] Therefore, the insoluble artificial enzyme failed to fully exhibit the hydrolysis potential.

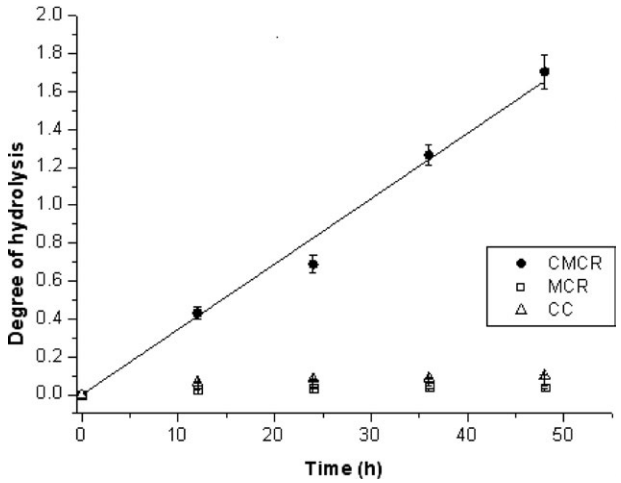

Figure 7. Catalytic activity of different kind of groups. All the reactions were performed in 20 mL, pH = 7.0 Tris–H^+ buffer with 1.52 $\times$ 10^{-2} m*M* BSA at 60°C for 48 h. Every 12 h, 20 μL supernatant was drawn and subjected to SDS-PAGE.

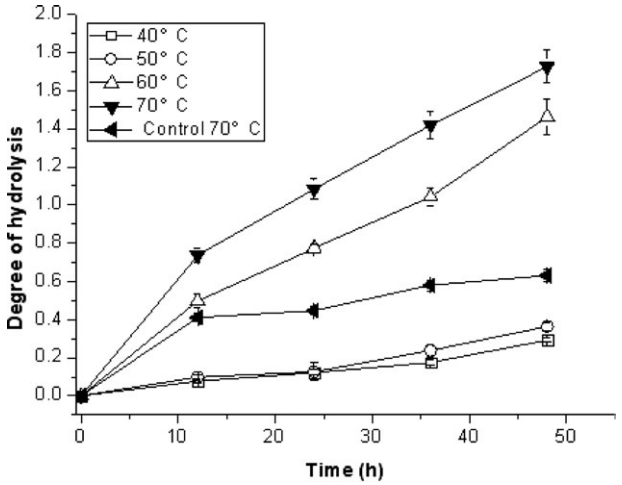

Figure 8. Effect of temperature on the hydrolyzing activity toward BSA. The hydrolysis reaction was performed in 20 mL Tris–H^+ buffer at pH 7.0 with 1.0 g insoluble catalyst, and the initial BSA concentration was 1.52 $\times$ 10^{-2} m*M*.

To confirm the hydrolyzing ability of CMCR on BSA, the control group was conducted by using MCR microspheres without chelating Cu^{2+} ions and cyclen complexed with Cu^{2+} ion (CC) on hydrolysis experiment. Degree of hydrolysis on BSA was estimated by the plot of $-\ln[S]/[S]_0$ against time. The result was shown in Figure 7. It was found that the cleavage rate delivered by MCR and CC was much lower than CMCR, pointing out the necessity of Cu^{2+} ions and cyclen moiety to exhibit hydrolyzing capacity. Some research teams discovered the hydrolysis potential of Cu^{2+} ions with complexes in homogenous systems.[39] However, the rate constants they provided were not appreciable owing to the absence of suitable microenvironment that only can be offered by the insoluble matrix. The resin particle not only enhanced the hydrolysis speed but also gave the facilities for separating the catalyst from the hydrolysate.

Effect of Temperature on CMCR Hydrolyzing Efficiency. A typical kind of enzyme fits a unique optimum temperature range. The proteolytic activity would be seriously impaired if the temperature is too low or too high. As an artificial metallopeptidase, copper complex of modified chitosan resin (CMCR) is supposed to follow that pattern as well. To evaluate the effect of temperature on the hydrolysis rate of BSA catalyzed by CMCR carrier, reactions were conducted from 40 to 70°C. Degree of hydrolysis on BSA was determined by the plot of $-\ln[S]/[S]_0$ against time. The result was illustrated in Figure 8. It manifested that the resin beads possessed an outstanding constancy under higher temperature. Within the range from 40 to 70°C, CMCR presented a remarkable catalytic activity. This artificial metallopeptidases released the fastest proteolytic activity at 70°C, where k_{obs} hits 3.10 $\times$ 10^{-2} h^{-1}. However, under this circumstance, BSA in control group experienced a serious self-degradation, which was even higher than the hydrolysis rate contributed by CMCR under the temperature of 40 and 50°C. Therefore, considering catalytic efficiency and energy costs of industrial applications, 60°C was adopted as the most suitable temperature for CMCR hydrolyzing BSA.

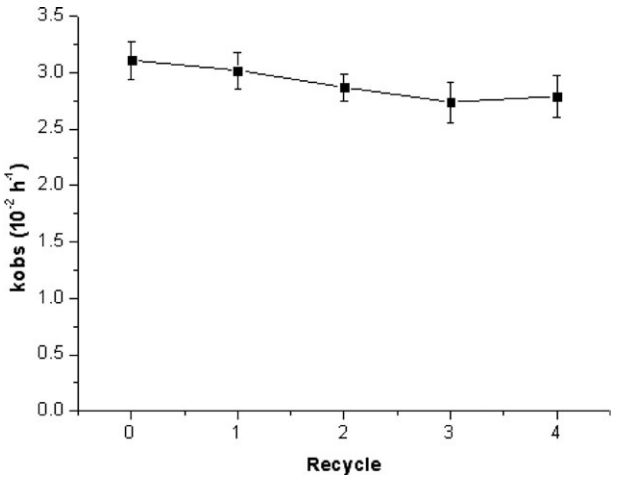

Figure 9. Reusable experiments of CMCR's hydrolyzing activity. For each cycle, the reaction was conducted at pH 7.0, 60°C, and the initial BSA concentration was 1.52 × 10^{-2} m*M*. The initial catalyst amount was 1.0 g, and the solution volume was fixed at 20 mL.

Reusability. As a new type of mimic enzyme, CMCR could also be taken as an immobilized metallopeptidase for the insolubility provided by chitosan resin matrix. Only by simple recycle routines can the resin beads be separated from the hydrolysate and used repeatedly, which gives rise to the most prominent advantages over natural enzymes. Moreover, the solid support also has the ability to improve CMCR's mechanical property and its tolerance under extreme pH and temperature environment.

The reusability of CMCR was demonstrated by Figure 9. Catalytic activity displayed minor decrease for each recycle, which might be mainly due to the flocculation of copper ions. After being reused for four times in 10 days, more than 85% of activity was maintained, suggesting that the deactivation or poison was not serious during the catalytic reaction and afterward recovery. It could be concluded that CMCR exhibited both excellent catalytic ability and stability. Thus, it is safe to hold a delightful prospect that this artificial metallopeptidase is appropriate for mass production in industrial application.

CONCLUSIONS

In this work, we successfully designed a new type of artificial metalloprotease-labeled CMCR. FTIR revealed that CMCR embodied the unique microdomain and special active moieties, which were able to accelerate the hydrolyzing reaction under mild conditions. Higher proteolytic activity was achieved under 60°C, pH 6.0, where the BSA substrate was hydrolyzed into smaller peptides and amino acids confirmed by SDS–PAGE. To the best of our knowledge, it is the first artificial proteolytic enzyme based on chitosan resin matrix. The immobilized artificial proteases can overcome thermal, chemical, and mechanical instabilities of natural proteases. By altering the composites of the active site of immobilized artificial enzyme properly, various types of chemical reactions can be catalyzed with higher rate acceleration. On top of that, the copper ions satisfactorily chelated on the chitosan carriers, which gave rise to the recovery and reuse of those beads with great stability.

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