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Enzyme–polysaccharide interaction and its influence on enzyme activity and stability

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ABSTRACT

An attempt was made to probe and elucidate the influence of three kinds of polysaccharides including the negatively charged sodium carboxymethyl cellulose (CMC), the uncharged methyl cellulose (MC) and the positively charged sodium carboxymethyl chitosan (CMCS), on the catalytic activity and stability of the model enzyme, β-D-glucuronidase (GUS). DSC analysis showed that the denaturing temperature of GUS was increased by 7 °C in the presence of CMC, but decreased in the presence of MC or CMCS by 5 and 3 °C, respectively. This variation was in good accordance with changes in the enzyme's catalytic activity. Circular dichroism was employed to characterize the conformational changes of GUS before and after the addition of the polysaccharide. It suggested that charged polysaccharides, CMC and CMCS, were favorable for improving the pH stability and the storage stability of GUS, whereas uncharged MC did not show such a stabilizing effect. At an elevated temperature up to 70 °C, GUS in CMC solution remained 78% activity and displayed the highest thermal stability among the three enzyme-polysaccharide pairs. The electrostatic interaction between enzyme and polysaccharides was closely relevant to the enzyme conformation, activity and stability.

1. Introduction

Recently, much attention has been given to maintenance of biocatalytic functions of enzyme including its activity and stability. Enzymes in nature are present in the cytoplasm or organelle of living cells wherein the cytosol is actually a complex biocolloid which is rich in proteins, nucleic acids, lipids and mono/polysaccharides (Miyoshi & Sugimoto, 2008). The interactions between the enzyme and these ambient biomacromolecules play crucial roles in stabilizing the structure and function of enzymes. For example, β-D-glucuronidase (GUS), a hydrolase which catalyzes the cleavage of terminal glucuronic acid, exists in the lysosome. Lysosome is a membrane-bounded vesicle providing an acidic (pH 4–5) colloid microenvironment with a high density of biomacromolecules (Bechet, Tassa, Taillandier, Comaret, & Attaix, 2005). Enzyme molecules have nonspecific interactions with neighboring biomacromolecules in the extracellular liquid microenvironment, exerting a notable influence on enzyme activity (Chen, Xu, & Wang, 2007; Cui, Du, Zhang, & Chen, 2008). It has been demonstrated that polysaccharides are very important biomacromolecule components for enhancing the stability of free enzyme against pressure and temperature fluctuation (Allison et al., 2000; Atheš & Combes, 1998; Zhang, Foegeding, & Hardin, 2004). In our recent work, polysaccharides were chosen as the liquid core to prepare enzyme-containing microcapsule with a cell-like or organelle-like structure (Zhang et al., 2008; Zhang et al., 2009). The compatibility and tolerance against harsh condition of these capsules were both improved. Thorough understanding and elucidation of enzyme-additive interaction will enable the control and optimization of the enzyme microenvironment and an enhancement in the enzyme activity as well as stability.

It is believed that there are at least two factors pertaining to the effect of polysaccharides on enzyme thermal stability. First, the molecular crowding or molecular confinement effect caused by addition of polysaccharides not only maintains the native structure of enzyme but also inhibits enzyme aggregation (Allison et al., 2000; Eggers & Valentine, 2001). Second, the presence of hydrophilic polysaccharides enhances the preferential hydration of enzyme to build up a more stable hydrate water layer around the enzyme surface and the hydrophobic interactions among the non-polar amino acid residues on the enzyme are simultaneously strengthened (Atheš & Combes, 1998; Back, Oakenfull, & Smith, 1979; Cui et al., 2008; Sathish, Kumar, & Prakash, 2007). However, little research on the electrostatic interaction between enzyme and polysaccharides has been reported.

Herein, three polysaccharides with similar molecular structure but different charges were chosen to alter the microenvironment of the model enzyme GUS. The enzyme GUS possesses an isoelectric

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point (p*I*) of 4.8–5.0 (Witcher et al., 1998) and is thus negatively charged under a neutral pH condition. Different electrostatic interactions would be generated between GUS and the negatively charged sodium carboxymethyl cellulose (CMC), uncharged methyl cellulose (MC) and positively charged sodium carboxymethyl chitosan (CMCS). Conformational changes were monitored by CD to tentatively analyze how the polysaccharide-enzyme electrostatic interactions affected the enzyme activity and stability.

2. Experimental

2.1. Materials

β-Glucuronidase (GUS) (EC 3.2.1.31) from *Escherichia coli* (type IX-A, lyophilized powder, 1,000,000–5,000,000 units/g protein) was purchased from Sigma Chemical Co. Methyl cellulose (MC), sodium carboxymethyl chitosan (CMCS) and sodium carboxymethyl cellulose (CMC) were obtained from Tianjin Reagent Chemicals Co. Ltd. Baicalin and baicalein standards for analysis were obtained from the National Institute for the Control of Pharmaceutical and Biological Products of China. Baicalin (purity >98%), used as the substrate, was purchased from Sichuan Xieli Pharmaceutical Co. Ltd. All the other chemicals were of analytical reagent grade.

2.2. Characterizations

The thermodynamic property of GUS in the presence or absence of various polysaccharides was determined using a PerkinElmer Pyris Diamond differential scanning calorimeter (DSC). GUS and polysaccharide were dissolved in 30 mM sodium phosphate buffer (pH 7.0) to get a final concentration of 23 mg/mL for GUS and 0.5% (w/v) for polysaccharide. 40 mL of the above solution was sampled, hermetically sealed in a stainless steel pan and weighed. A sealed empty pan was used as a reference. Three replicates of each sample suspension were scanned at a heating rate of 10 °C/min in the temperature range of 20–150 °C.

Changes in the secondary structure of GUS in the presence or absence of polysaccharides at room temperature were determined by circular dichroism spectroscopy (CD) on a JASCO J715 spectropolarimeter (JASCO, Japan). The enzyme and the polysaccharide were dissolved in 5 mM Tris–HCl buffer (pH 7.0). The final enzyme concentration was 62.5 μg/mL and the polysaccharide concentrations were, respectively, 0.025% (w/v) for CMC, 0.125% (w/v) for MC and 0.125% (w/v) for CMCS. Six scans were conducted and averaged for each sample from 200 nm to 260 nm at a 0.5 nm interval with a rate of 50 nm/min and a response time of 8 s. The optical path was 1 cm.

2.3. Enzyme activity

Bioconversion of bailcalin to baicalein catalyzed by GUS in the presence or absence of polysaccharide was carried out. The enzyme activity was determined by measuring the amounts of baicalein produced with time. All the chemicals used were dissolved in 30 mM Tris–HCl buffer (pH7.0). A certain amount of baicalin and Na_2SO_3 were added into the polysaccharide solution followed by preheating at 37 °C for 15 min. Na_2SO_3 was used as an antioxidant. Then, the GUS solution (37 °C) was added into the above mixture to induce the reaction. The total volume of reacting solution was 20 mL containing baicalin (0.4 mg/mL), GUS (0.01 mg/mL) and Na_2SO_3 (0.2%, w/v). To get an equal viscosity for the three reacting systems, the concentrations of the three polysaccharides were kept at 0.1% (w/v) for CMC, 0.5% (w/v) for MC and 0.5% (w/v) for CMCS, respectively. The difference in diffusion resistance caused by the different viscosity property of various polysaccharides could be ignored. 100 μL of the reacting solution was sampled every 15 min, diluted with methanol and analyzed by HPLC (HP1100, Agilent, equipped with Agilent ZORBAX SB-C18 column) to determine the amount of baicalein produced. A mixture of methanol/H_2O/H_3PO_4 (60/40/0.2) was used as the mobile phase at a flow rate of 1 mL/min. The detection wavelength was set at 274 nm. The relative enzyme activity was calculated by comparing the activity in the presence of polysaccharides with that without any polysaccharides.

2.4. Thermal stability

GUS and the polysaccharides were dissolved separately in 30 mM Tris–HCl buffer (pH 7.0) to get a GUS solution with a concentration of 0.5 mg/mL, a CMC solution with a concentration of 0.2% (w/v), a MC solution with a concentration of 1.0% (w/v) and a CMCS solution with a concentration of 1.0% (w/v). 0.4 mL of GUS solution was mixed with 10 mL polysaccharide solution and the mixture was incubated at different temperatures (30–70 °C) for 1 h and then heated up or cooled down to 37 °C. The residual activity of GUS after incubation was measured at 37 °C, pH 7.0. The relative activity of GUS after incubation was determined by comparing the activity with that without thermal incubation.

2.5. pH stability

The GUS and the polysaccharides were dissolved in 30 mM Tris–HCl buffer at different pH 4.0–8.0. The concentrations of GUS and polysaccharides of each sample were the same as those described in the above Section 2.4. The 0.4 mL GUS/10 mL polysaccharide mixture was incubated at different pH conditions (pH4.0–8.0) for 1 h. Then the residual enzyme activity was measured at 37 °C, pH 7.0. The relative activities of GUS after incubation under different pH conditions were determined by comparing the activity with that without incubation.

2.6. Storage stability

The GUS/polysaccharide mixture was prepared according to the above procedure at room temperature and pH 7.0 and stored at 4 °C. The residual activity of GUS was monitored with time. The initial GUS activity was regarded as a standard to calculate the remaining activity.

3. Results and discussion

3.1. Effect of polysaccharides on the enzyme activity

The influence of the three polysaccharides on the bioconversion reaction could be intuitively observed by the color change of the solutions (Fig. 1). The initial substrate solutions were bright yellow. As the reaction proceeded, the solutions containing CMCS and MC turned brown and yellow-brown, respectively. In contrast, little change in color was observed for the CMC-containing solution. The notable color change for CMCS system was due to the reduced stability of baicalin in the presence of CMCS. This unfavorable effect on the substrate usually caused a decrease in production of baicalein.

The enzyme activities for various polysaccharide-containing systems were presented in Table 1. The addition of negatively charged CMC and uncharged MC led to an increase in the enzyme activity by 12% and 5%, respectively, compared with the blank one without any polysaccharides. A loss of 4% in enzyme activity was found in the presence of positively charged CMCS. The influence of polysaccharides on the enzyme activity was likely attributed to the following two aspects. On one hand, the interaction between polysaccharide and enzyme result in some conformational changes of the enzyme molecules. This aspect could be further studied by

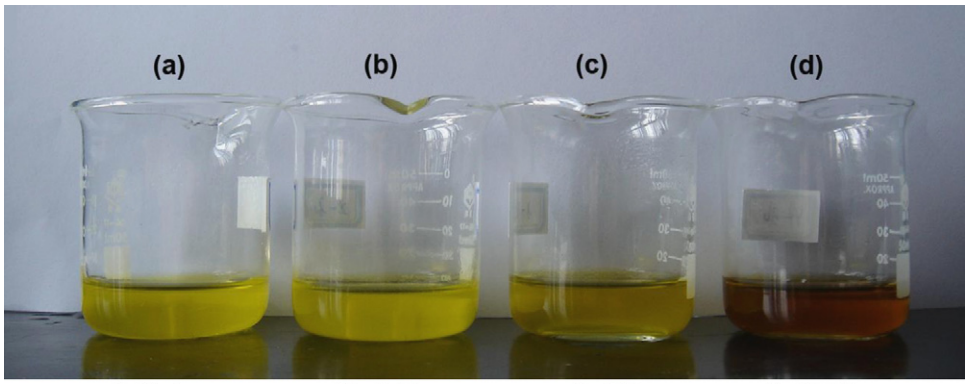

Fig. 1. Effect of polysaccharides on the color of the reaction liquid.

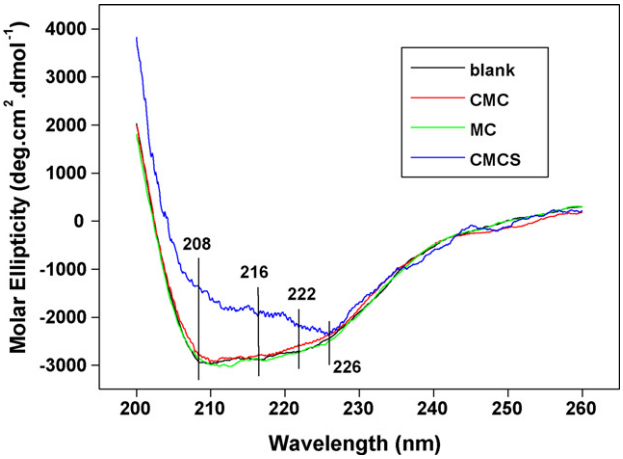

Fig. 2. Effect of polysaccharides on the CD spectra of GUS.

the analysis of CD spectra. GUS molecule is composed of four subunits and its secondary structure is mainly in α-helix (Jain et al., 1996). As shown in Fig. 2, the GUS in blank Tris–HCl buffer presented three negative peaks at 208, 216 and 222 nm in the CD spectrum. The introduction of CMC and MC exerted little effect on the CD spectrum of GUS, indicating that the original secondary conformation of GUS was well retained. In contrast, the CD spectrum of GUS changed remarkably after mixing with CMCS. The negative peaks at 208 and 216 nm disappeared, the mole ellipticity decreased and the negative peak at 222 nm was red-shifted to 226 nm. This change in secondary conformation was likely due to the electrostatic attraction between the negatively charged GUS and the positively charged CMCS that destroyed the secondary bonds in GUS molecule.

Table 1
Effect of polysaccharides on the relative activity, denaturation temperature and storage stability of GUS.

	Blank	CMC	MC	CMCS
Enzyme activity[a] (relative activity, %)	100	112	105	96
Denaturation temperature[b] (°C)	103	110	98	100
Storage stability[c] (remaining activity, %)				
0 day	100	100	100	100
10 days	74	115	57	97
26 days	4	41	7	94

[a] The enzyme activity was expressed in terms of relative activity calculated by setting the activity in buffer without polysaccharide as 100%.
[b] Denaturation temperature was determined by DSC.
[c] Storage stability was shown and compared by setting the fresh (0 day) activity as 100% for each polysaccharide.

On the other hand, the electrostatic interaction between polysaccharide and substrate was another reason that influenced the enzyme activity by affecting the combination of enzyme and substrate. Since baicalin molecules with dissociable carboxyl groups are negatively charged under neutral pH condition, the electrostatic repulsion between baicalin and CMC (also negatively charged) enhanced the molecular movement of baicalin. As a result, the enzymatic reaction rate was accelerated because the probability of collision between substrate and enzyme increased. The electrostatic attraction between CMCS and baicalin, on the contrary, inhibited the molecular movement of baicalin. In addition, the active center of GUS might be shielded by CMCS due to the electrostatic attraction between CMCS and GUS. The above two factors weakened the effective combination between the substrate and enzyme, resulting in a decrease in enzyme activity.

3.2. Thermal stability

DSC analysis was made to test the thermal stability in terms of denaturation temperatures (data listed in Table 1). The denaturation temperature of native GUS was 103 °C, while in the presence of CMC, MC and CMCS, the denaturation temperatures were, respectively, increased by 7 °C, decreased by 5 and 3 °C. The denaturation temperature indicates the destruction of secondary structure, but the destruction of secondary structure is not the only reason for enzyme deactivation. Hydrate layer destroying, enzyme aggregation and some other unfavorable factors could also lead to a notable decrease and even a total loss of enzyme activity even when temperature was much lower than its denaturation temperature. Therefore, the effect of polysaccharides on the enzyme deactivity was further investigated by examing the activity after incubation at temperatures range from 30 to 70 °C (Fig. 3). For native GUS, no loss in activity was found after incubation at 30 °C for 1 h and only 3% loss after incubation at 40 °C. However, when the incubation temperature increased to 50 °C or higher, the enzyme lost activity remarkably, 43% loss at 50 °C and nearly 100% loss at ≥60 °C. In the presence of the negatively charged CMC, 78% of original activity could be preserved after incubation at 50 °C. A negative effect was found for the uncharged MC and positively charged CMCS, the relative activity being even lower than native enzyme. This variation in deactivity temperatures regarding the three polysaccharides was in accordance with that found in the denaturation temperature.

The secondary conformation of GUS after thermal incubation in the presence of polysaccharides was studied by CD. Comparing Fig. 4a with Fig. 2, little change in CD spectra for GUS blank sample was found before and after 30 °C incubation. In contrast, after incubation at 70 °C, the peaks attributed to α-helix at 208 and 222 nm completely disappeared and transformed into a random-coil struc-

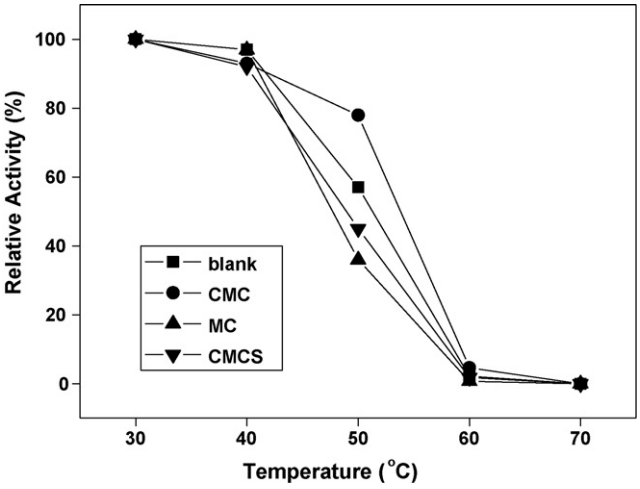

Fig. 3. Effect of polysaccharides on the thermal stability of GUS.

ture. The enzyme lost all its activity after 70 °C incubation although part of its secondary structure retained. In the presence of CMC, the CD spectra of GUS did not change after thermal incubation, only the mole ellipticity decreased slightly (Fig. 4b). This suggested a stabilizing effect of CMC on the conformation of GUS, which resulted in an enhancement in enzyme thermal stability. Because of the heat-sensitive sol–gel transformation of MC at 40–50 °C, the secondary structure of GUS with addition of MC underwent complex changes with increase of temperature, while the results was depicted in Fig. 4c. The molar ellipticity began to decrease at 40 °C and the α-helix peak showed a red shift when the temperature increased to 50 °C. Correspondingly, the activity of GUS decreased rapidly. Regarding the positively charged CMCS, the original ordered structure of GUS was destroyed during thermal incubation (Fig. 4d), resulting in a significant reduction in activity.

With the increase of temperature the hydration layer would be gradually destroyed and thus resulting in enzyme aggregation, conformational change and deactivation. In this study, only the negatively charged CMC was found to be able to improve the thermal stability of GUS. This result implied that the electrostatic

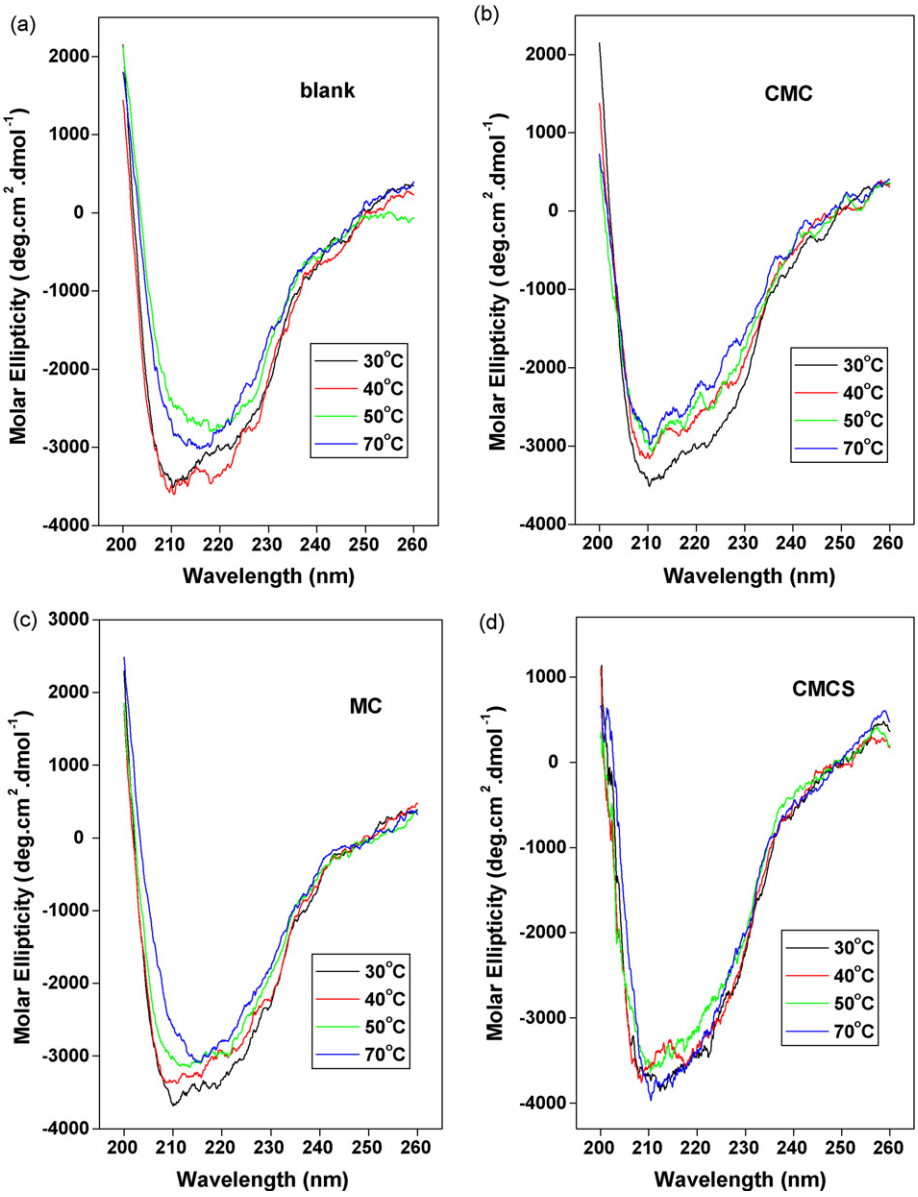

Fig. 4. Effect of polysaccharides on the CD spectra of GUS after thermal incubation.

interaction was a very important factor for enzyme stability. For GUS/CMCS mixture, the net charge of GUS decreased because of the electrostatic attraction between the positively charged CMCS and negatively charged GUS, the hydration layer on enzyme surface was easier to destroy hence, weakening the thermal stability. In regard to gelation of MC via physical cross-linking at 40–50 °C (Desbrieres, Hirrien, & Ross-Murphy, 2000; Lin, 2002), the original hydration layer on the enzyme surface was destroyed by the interaction with hydroxyl groups of MC through hydrogen bonds, resulting in a decrease in thermal activity. The addition of negatively charged CMC could inhibit conformational changes as proved by CD study. Under neutral pH conditions, the electrostatic repulsion between CMC and GUS prevented the polysaccharide molecules from approaching the enzyme, thus retaining the hydration layer on the enzyme surface. As this protective effect on the hydration layer inhibited the aggregation of enzyme molecules, the steric confinement effect of the polysaccharides further restricted enzyme unfolding. Coincidentally, the native microenvironment for GUS provided by lysosome is also a negatively charged one created by negatively charged neighboring macromolecules and the inner lysosome membrane surface. It could be concluded that charge property is crucial in generating a suitable microenvironment mimicking their natural existence for enzymes.

3.3. pH stability

Enzymes will generally undergo reversible or irreversible conformational changes under extreme pH conditions. The conformational changes of GUS under different pH conditions with or without polysaccharides were characterized by CD (Fig. 5). The conformation stability in α-helixes and β-sheets of enzyme mainly depends on the hydrogen bonds formed between the hydrogen atoms and the oxygen atoms on neighboring amino acid residues (Federici, Masulli, Gianni, Ilio, & Allocati, 2009). These hydrogen bonds as well as the net charge of the enzyme surface would be greatly influenced by pH variation (Boscolo, Leal, Salgueiro, Ghibaudi, & Gomes, 2009). A significant change in GUS conformation under acidic or alkaline conditions was observed compared

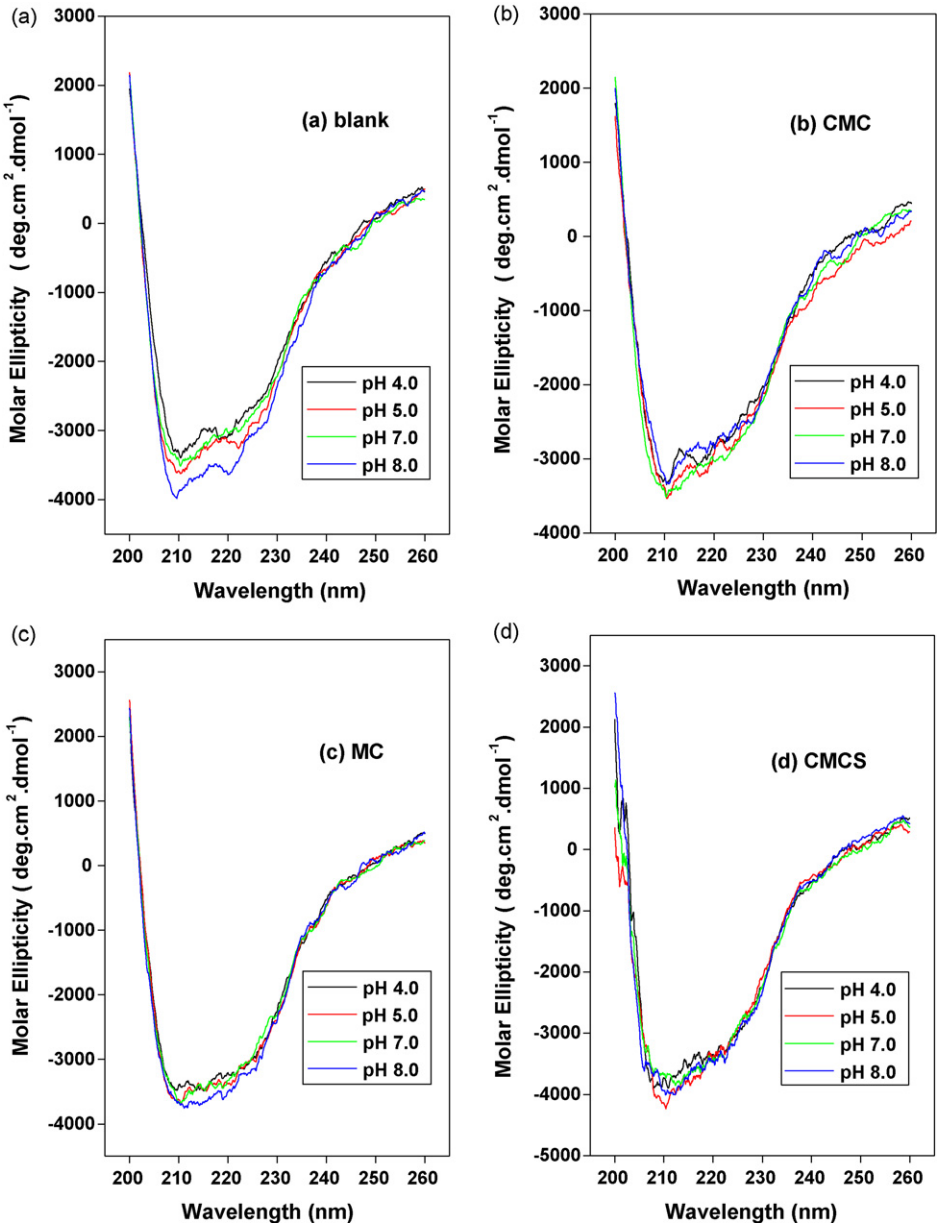

Fig. 5. Effect of polysaccharides on the CD spectra of GUS under different pH conditions.

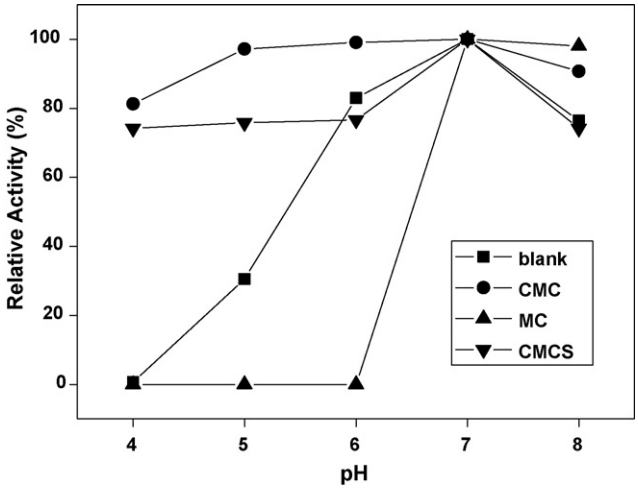

Fig. 6. Effect of polysaccharides on the pH stability of GUS.

with that at neutral pH. Correspondingly, as shown in Fig. 6, a sharp decrease was found after blank GUS was incubated under either acidic or alkaline condition and complete deactivation appeared at pH 4.0.

In native microenvironment of enzyme, the pH change was regulated through the buffering function of some weak acids and their conjugate bases, such as –COOH/–COO^-, –NH_3^+/–NH_2, $H_2PO_4^-$/HPO_4^{2-} and H_2CO_3/HCO_3^-. The buffering function of the polysaccharides used herein was tested by measuring the change of pH value before and after addition of polysaccharides (Table 2). MC with no dissociable groups showed no buffering ability. Both the anionic CMC and amphoteric CMCS showed buffering capacity, being able to buffer the solution of pH 4.0–8.0 to around neutral pH (6.5–7.5). The –COOH/–COO^- and –NH_3^+/–NH_2 pairs on CMC and CMCS consume H^+ under acidic conditions or release H^+ under alkaline conditions, thus regulating the pH condition in microenvironment.

Table 2
Effect of polysaccharides on the pH value of 30 mmol/L Tris–HCl buffer solution.

	pH value				
Tris–HCl blank	4.0	5.0	6.0	7.0	8.0
CMC	6.5	6.7	6.7	7.0	7.5
MC	4.0	5.0	6.0	7.0	8.0
CMCS	7.2	7.5	7.5	7.5	7.5

The enzyme activity of GUS after incubation under different pH (4.0–8.0) conditions was measured to test the influence of various polysaccharides on the pH stability of GUS. After mixing with MC solution, a remarkable reduction in the pH stability of GUS was found with a total lost of activity in acidic environments (pH ≤ 6.0). This could be explained by the incapability of the uncharged MC to prevent the hydrogen bonds inside GUS from being destroyed under the pH fluctuation. In contrast, the presence of polysaccharides with buffering function (CMC and CMCS) remarkably improved pH stability of GUS due to the buffering effect, especially under acidic conditions. At pH 4.0, GUS retained nearly 80% activity in presence of CMC or CMCS. It could be deduced from the above results that electrostatic interactions played an important role in maintaining pH stability of GUS.

3.4. Storage stability

The storage stabilities of GUS with and without polysaccharides were compared in Table 1. The activity of GUS in blank solution decreased sharply by 26% after 10-day storage and continued to decrease thereafter. On the 26th day of storage, only 4% of its initial activity was found. Storage stability of GUS could be significantly improved by adding CMC. No loss but even a slight increase in activity was found during the first 10 days, and 41% of activity could be retained in 26 days. GUS with CMCS pre-

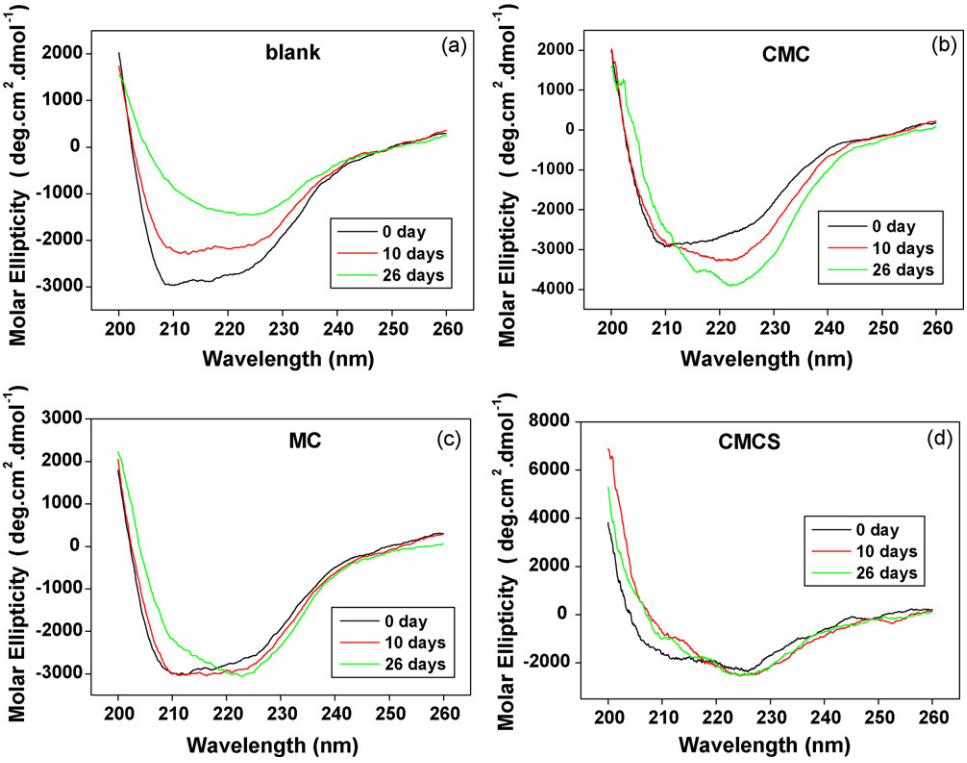

Fig. 7. Effect of polysaccharides on the CD spectra of GUS during storage.

sented the highest storage stability and 94% of activity was retained after 26 days. However, MC showed little effect on the storage stability of GUS. The above results suggested that charged polysaccharides (CMC, CMCS) had a more favorable effect on the storage stability of the model enzyme GUS than the uncharged polysaccharide (MC). The electrostatic interaction (attractive or repulsive) between polysaccharide and enzyme was supposed to be a crucial factor determining enzyme storage stability. As for the different influence extent of CMC and CMCS on storage stability, it may be tentatively explained as follows. The model enzyme GUS (p*I* 4.8) was negatively charged in the neutral storage environment and it seemed that the attractive electrostatic interaction between CMCS and GUS exerted a more favorable effect on storage stability than the repulsive electrostatic interaction between CMC and GUS.

The CD spectra clearly displayed the conformational change of enzyme during storage. For GUS storaged in blank Tris–HCl buffer system, a remarkable decrease in molar ellipticity from 205 to 230 nm and an approx. 4 nm red-shift of CD bands were observed after 26-day storage (Fig. 7a), indicating substantial denaturation which led to a sharp decrease in activity. GUS in the presence of CMC underwent an interesting change in its secondary structure. Both the content of α-helix (222 nm) and β-sheet (216 nm) increased with increase of storage time (Fig. 7b). This increase was supposed to be due to the electrostatic repulsion and hydrogen bonding between CMC and GUS. These unusual changes in conformation made a positive contribution to the storage stability of GUS. A remarkable change of conformation was found upon addition of MC, i.e., the negative peak at 208 nm which was assigned to α-helix totally disappeared after 26-day storage (Fig. 7c). In the presence of CMCS, the conformation of GUS remained stable with a constant mole ellipticity (Fig. 7d). In summary, both the negatively charged CMC and the positively charged CMCS were able to create a charged microenvironment and caused conformational changes which were favorable for GUS storage.

4. Conclusions

The presence of polysaccharides significantly influenced the enzyme activity and stability by the electrostatic interaction between polysaccharide and enzyme. Since the enzyme GUS was negatively charged at neutral pH, the positively charged CMCS reduced catalytic activity of GUS, while the negatively charged CMC and uncharged MC enhanced catalytic activity of GUS. The electrostatic repulsion between CMC and GUS reinforced the hydration layer on the enzyme surface, thus improving the enzyme thermal stability. The buffering effect of charged polysaccharides CMC and CMCS enhanced the pH stability and storage stability of GUS. Considering all the influences on enzyme activity and stability in the presence of the three variously charged polysaccharides under study, the negatively charged CMC was the most favored one. This result was in accordance with the natural existence microenvironment of GUS.

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