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Effect of $CaCl_2$ as activity stabilizer on purification of heparinase I from *Flavobacterium heparinum*

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Abstract

Heparinase I has been purified from *F. heparinum* by a novel scheme with 10 mM $CaCl_2$ added in crude extracts of cells. The enzyme was purified to apparent homogeneity through ammonium sulfate precipitation, Octyl-Sepharose chromatography, CM-52 chromatography, SP-650 chromatography, and Sephadex G-100 gel filtration chromatography. The specific activity of the purified enzyme was 90.33 U/mg protein with a purification fold of 185.1. The yield was 17.8%, which is higher than any previous scheme achieved. The molecular weight of the purified enzyme was 43 kDa with a p*I* of 8.5. It has an activity maximum at pH range of 6.4–7.0 and 41 °C. $CaCl_2$ is a good stabilizer of the purified enzyme in liquid form toward either storaging at 4 °C or freezing-thawing.

Keywords: $CaCl_2$; Activity stabilizer; Purification; *Flavobacterial heparinum*; Heparinase I

1. Introduction

Heparinases (or heparin lyases) are enzymes that can eliminatively cleave heparin or heparan sulfate into disaccharide and oligosaccharide products [1]. Heparinases have been used in numerous applications including structural determination of heparin [2], new bioassays for heparin [3], investigation of the anticoagulant mechanism [4], preparation of low molecular weight heparin anticoagulants [5] and anti-tumor agents [6,7], and the development of immobilized enzyme filters for blood deheparinization [8]. Heparinases of bacterial origin have been extracted or purified from various species [9–15].

Among bacterial heparinases, the enzymes of *F. heparinum* are the most widely used and best studied [1]. Linker and Hovingh [16] first separated these lyase activities, fractionating a crude lyase fraction into a heparinase and a heparitinase. Linhardt et al. [17] reported the purification of heparinase I to a single band on SDS–PAGE. Sufficient quantities of pure heparinase I for detailed characterization studies and amino acid analysis were first prepared by Yang et al. [18]. A scheme to simultaneously purify three kinds of heparinases from *F. heparinum* to apparent homogeneity is disclosed by Lohse and Linhardt [9].

However, none of the previous schemes could get high activity yield due to the instability of heparinases. Heparinase I retained only 50% of its activity when stored at 4 °C for a short time in liquid form [9]. Usually four to five steps were needed to obtain purified enzyme. The losing of activity was so significant that the yield by previous schemes could hardly attain 10%.

In the present study, several compounds were added to the crude enzyme extracts to test the ability on enhancing the activity storage. $CaCl_2$ was screened out as one of the best activity stabilizer of heparinase. And the protection effect of $CaCl_2$ was used in a novel scheme to obtain high yield of homogeneity heparinase I from *F. heparinum*.

2. Experimental procedures

2.1. Materials

The experiments were performed by using sodium salt of heparin from porcine intestinal mucosa (183 USP units/mg)

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(Hepalink Pharmaceutical Co., Shenzhen, China), Azure A, Coomassie brilliant blue, phenylmethylsulfonl fluoride (PMSF), 1,4-dithiothreitol (DTT) (Sigma, Chemical Co., MO, USA), electrophoresis molecular weight marker kits (MBI Fermentas Inc., Amherst, New England), Octyl Sepharose^{TM} fast flow, Carboxyl methylcellulose (CM-52), Sephadex G-100 (Amersham Biosciences, Sweden), Toyopearl SP-650 (TOSOH, Japan). These chemicals and all others were analytical grade.

Optical measurements were made with a spectrophotometer (UNIC UV-2100, Unico, Shanghai, China). For experimental work, incubator shaker (Xinrui Automatic Apparatus Co., Shanghai, China), electrophoresis (Tm-250, Jim-X Scientific Apparatus Co., China), and sonifer (Xinzhi Scientific Apparatus Co., Ningbo, China) were used.

2.2. Assays

2.2.1. Protein assays

Protein was measured by means of Lowry [19]. Assays were performed using a UNIC 2100 spectrophotometer.

2.2.2. Enzyme assays

Heparinase activity was measured according to the procedure of Lohse and Linhardt [9]. In this study, we observed that ammonium sulfate would interfere with the above assay. So heparinase activity was measured by Azure A assay [20] in ammonium sulfate precipitation and Octyl-Sepharose column chromatography because of the using of ammonium sulfate in these two steps.

2.3. Fermentation and enzyme recovery

The strain *Pedobacter heparinus* (DSMZ 2366), a subculture from *F. heparinum* (ATCC 13125), was maintained at 4 °C on slants contained (w/v) (%): yeast extract powder 0.5, tryptone 1.0, beef extract 0.5, NaCl 0.5, agar 2. Fermentation was conducted in 0.5-l shake flasks at 23 °C in all experiments. The bacterial cells on plate were transferred into a shake flask contained 100 ml medium which is same as slant except no agar added. The culture was incubated on a 2.5-cm stroke shaker at 150 rpm for 20–24 h as seed. Seeds were inoculated at the final concentration of 6% into 0.5-l flasks contained 200 ml fermentation medium {(w/v) (%): heparin 0.8, NH_4Cl 0.3, K_2HPO_4 0.25, NaH_2PO_4 0.25, $MgSO_4$ 0.05, L-histidine 0.05, L-methionine 0.05; trace salts ($NaMoO_3$, $CuCl_2$, $FeCl_2$, $CoCl_2$, $MnCl_2$, $CaCl_2$) 1×10^{-4} M}. The cultures were incubated on a rotary shaker (150 rpm) for 24 h, then harvested.

From 1 L of fermentation broth, about 4-g wet cell pellet was obtained by centrifugation for 15 min at 10,000 × *g* at 4 °C. This pellet was suspended in 100 ml of 10 mM Tris–HCl buffer at pH 7.0 and 4 °C. Cell suspension (20 ml at a time) was placed into a 30-ml glass cup and sonicated with cooling for 10 min at 150 W using a 50% pulsed mode. The disrupted cells were centrifuged at 15000 × *g* for 30 min at 4 °C and the pellet discarded. The 100 ml of supernatant, obtained by sonification and centrifugation, contained 1.5 mg/ml protein.

2.4. Screening of activity stabilizer for heparinase activity

Heparinase activity stabilities toward storage at 4 °C and freeze-thawing were investigated by using seven compounds, polyethylene glycol (PEG) 15,000 at 10% (w/v), glycerol at 20% (w/v), $CaCl_2$ at 10 mM, heparin at 20% (w/v), bovine serum albumin (BSA) at 2 mg/ml, PMSF at 0.1 μM, and β-mercaptoethanol at 10 mM. Crude extracts were mixed with each of the above compounds in 10 mM Tris–HCl buffer, respectively. These lyase solutions were then divided into two equal aliquots, and one of each was subjected to either freezing-thawing, or retained at 4 °C. Heparinase activity was determined at interval time of 1 day during storage at 4 °C or after freezing at −20 °C and thawing.

2.5. Purification of heparinase

The crude enzyme was brought to 45% saturation of ammonium sulfate by gradually adding solid $(NH_4)_2SO_4$ and was stirred for 1 h in ice-bath. The precipitate was removed by centrifugation at 15,000 × *g* for 15 min, supernatant was brought to 80% saturation of ammonium sulfate and stirred for 1 h. After centrifugation, the pellet was dissolved in 10 mM Tris–HCl buffer (pH 7.0, containing ammonium sulfate at 50% saturation).

Four steps of chromatography were performed for further purification of heparinase. The enzyme solution containing 50% saturation of ammonium sulfate, was loaded onto a Octyl-Sepharose (1.6 cm × 8 cm) column, which was pre-equilibrated with five column volumes of 10 mM Tris–HCl buffer (pH 7.0, containing ammonium sulfate at 50% saturation). The column was washed stepwisely by three column volumes of 10 mM Tris–HCl buffer (pH 7.0, containing ammonium sulfate at 50%, 40%, 30%, 20%, 10% saturation, respectively). Fractions with heparinase activity were collected and dialysed against 10 mM Tris–HCl buffer (pH 7.0, containing $CaCl_2$ at 10 mM) overnight with two changes.

Lyase purified by Octyl-Sepharose without concentration was subjected to a CM-52 column (1.6 cm × 4 cm) pre-equilibrated with 10 mM Tris–HCl buffer (pH 7.0, containing $CaCl_2$ at 10 mM). The column was eluted by a NaCl linear gradient of 0–300 mM in the same buffer at 0.5 ml/min flow rate. Fractions of every 3 ml elution were collected. The pooled fractions with heparinase activity were dialysed overnight against 10 mM Tris–HCl buffer (pH 7.0, containing $CaCl_2$ at 10 mM).

The lyase without concentration was then applied to a pre-equilibrated SP-650 column (1.6 cm × 4 cm), the buffer used to equilibrate and elution is the same as that used for CM-52 column, excepted a NaCl linear gradient of 0–500 mM was adopted. Those fractions with heparinase activity were pooled and condensed 10-folds by a Microncon ultrafiltration centrifuge tube (Millopore Co., USA).

The condensed enzyme was then applied to a Sephadex G-100 (1.5 cm × 60 cm) column which was previously equilibrated with the same buffer as above. Fractions of every 2 ml were collected at a flow rate of 8 ml/h. The fractions with heparinase activity were pooled and dialysed against 10 mM Tris–HCl

buffer overnight, then lyophilized or used straightly for properties investigation.

Absorbance at 280 nm, protein content and heparinase activity were monitored for each fraction and each purification step.

2.6. Characterization of the purified enzyme

The analysis of SDS–PAGE electrophoresis was performed by Laemmli method [21]. The gels were fixed with 12% (w/v) trichloroacetic acid, rinsed with distilled water and stained with a Rapid Coomassie Stain solution, and destained. The following proteins were used as SDS–PAGE electrophoresis molecular weight standards: rabbit phosphorylase *b* (97,400), bovine serum albumin (66,200), rabbit actin (43,000), bovine carbonic anhydrase (31,000), trypsin inhibitor (20,100), and hen egg white lysozyme (14,400).

Isoelectric point was determined by isoelectric focusing (IEF) on 5% polyacrylamide gels in the presence of 2% ampholines (pH 3–10). The purified enzyme (20 μg) was loaded onto the horizontal gel maintained at 2 °C. The voltage was increased stepwise: 100 V for 15 min, 200 V for 15 min, 450 V for 60 min by using Model 111 Mini IEF Cell (Bio-Rad, CA, USA). The gel was maintained at 2 °C during the run. The p*I* markers (Bio-Rad, CA, USA) ranging from 4.65 to 9.6 were co-electrophoresed to be estimated the p*I* of the protein. After IEF, the protein were stained with Coomassie brilliant blue R-250.

To analysis N-terminal, the purified enzyme (100 μg) was loaded on seven tracks of SDS–PAGE gel (15% polyacrylamide). After electrophoresis, the gel was electrophoretically transferred onto PVDF membrane cartridge. N-terminal amino acid sequence analysis was performed using an applied Biosystems sequencer (ABI491A, PE Co., Boston, USA).

The optimum pH of activity for the lyase was obtained by using Tris–HCl (pH 6.2–7.8). Heparinase assay solutions were made by diluting a 10 μl sample of the purified lyase (2–3 mg/ml protein concentration) with 90 μl of Tris–HCl buffer at 50 mM, pH 7.0, and placed on ice until required for assay. The activities of heparinase at different pH values were then determined.

The optimum temperature for activity was determined by using heparinase solution as above at pH 7.0 Tris–HCl buffer at 2 °C increments at temperatures between 25 °C and 45 °C. The temperature was adjusted in a temperature-regulated spectrophotometer and equilibrated for 10 min before the assay was started.

To study the effect of temperature and pH on the purified enzyme stability, enzyme assay stock solutions were prepared as above in buffer and placed in water baths at 10 °C increments at temperatures between 15 °C and 75 °C, samples were taken out 30 min later to measure remaining enzyme activity. To test the stability of the purified enzyme in $CaCl_2$ solution, enzyme solutions were dissolved in 10 mM Tris–HCl buffer, containing 1, 10, 100 mM $CaCl_2$, respectively. These solutions were then divided into two equal aliquots, and one of each was subjected to either retained in an ice bath or freezing-thawing. The activities were determined after brief storage at 4 °C or freezing at −20 °C and thawing.

3. Results

3.1. Screening of activity stabilizing additives

The stability curves of crude heparinase solutions containing different additives at 4 °C were shown in Fig. 1a. The enzyme aliquot containing $CaCl_2$ showed a good stability with only about 15% activity lost in the 5 days. Aliquots containing glycerol and heparin showed a good stability which could reserve about 45% activity in the 5 days. However, control enzyme lost 40% of its activity in the first day. Furthermore, 80% activity was lost after 5 days.

Enzyme solution containing β-mercaptoethanol shown fine stability in the first 3 days with 45% of its activity reserved, but from then on the activity decreased steeply. Enzyme solutions containing PMSF or BSA showed the same activity profile as control. Whereas enzyme solution containing PEG even shown a worse stability than the control.

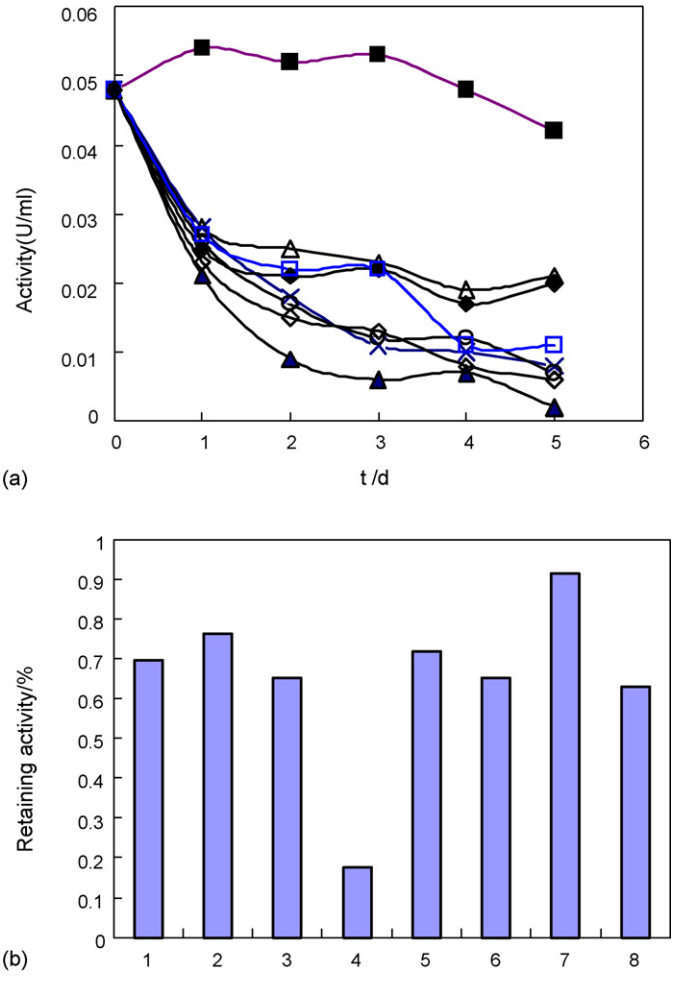

Fig. 1. Effect of additives on heparinase activity stability. (a) Time courses of activity of crude enzymes incubating in ice-bath with additives of PEG15000 at 10% (w/v) (▲), Glycerol at 20% (w/v) (△), $CaCl_2$ at 10 mM (■), Heparin at 20% (w/v) (♦), BSA at 2 mg/ml (◯), β-mercaptoethanol at 10 mM (□), PMSF at 0.1 μM (◊), and control (×). (b) Activities of crude enzymes after one time of freeze-thawing with different additives: (1) control; (2) BSA at 2 mg/ml; (3) PEG 15000 at 10% (w/v); (4) β-mercaptoethanol at 10 mM; (5) Glycerol at 20% (w/v); (6) PMSF at 0.1 μM; (7) $CaCl_2$ at 10 mM; (8) Heparin at 20% (w/v).

The effect of the additives on heparinase activity stability during freeze-thawing was shown in Fig. 1b. After one time of freeze-thawing, $CaCl_2$ showed fine protection effect on the enzyme activity with 90% activity preserved after freeze-thawing. Control enzyme solution reserved 70% of its activity, while β-mercaptoethanol shown a negative effect on activity protection with 85% activity lost. Others had little effect.

3.2. Purification step

The purified enzyme was obtained through five steps: $(NH_4)_2SO_4$ precipitation, Octyl-Sepharose chromatography, CM-52 chromatography, SP-650 chromatography, and gel filtration chromatography.

The highest recovered activity was obtained as 0.525 U/mg protein for 45–80% $(NH_4)_2SO_4$ in the bottom precipitated phase with 93% yield and with a 1.08-fold increase in specific activity.

Octyl-Sepharose column chromatography was employed to purify heparinase. The heparinase activity were observed in fractions eluted by $(NH_4)_2SO_4$ at 20% saturation (Fig. 2a). A 43.1% of heparinase activities was recovered in the pooled fraction with a 2.87-fold increase in specific activity.

CM-52 column chromatography was used to further purify heparinase. The heparinase activities were observed in fraction 21–30 (Fig. 2b). A 40.9% of heparinase activities was recovered in the pooled fraction with a 71.1%-fold increase in specific activity.

The active fractions were applied to a SP-650 column chromatography for further purification. Two peaks of heparinase activity were observed in the elution of fractions 31–42 and fractions 50–54 (Fig. 2c).

The two peaks of heparinase active fractions were applied to a sephadex G-100 column respectively for further purification. For peak I, enzyme was eluted from the column as a major single symmetrical peak resulting in a 185.1-fold increase in the specific activity with 17.8% yield (Fig. 2d). For peak II, enzyme activity cannot be detected in the elution.

A summary of the purification procedures presented in Table 1. SDS–PAGE analysis demonstrated the purification to homogeneity of the purified enzyme (Fig. 3).

3.3. Molecular properties of the purified enzyme

On SDS–PAGE, the purified enzyme gave a single band with a molecular weight of 43 kDa. This keep close agree with the data of heparinase I had been reported by literatures [9,18]. The p*I* value of the purified enzyme was determined as 8.5, which is also same as reported [18].

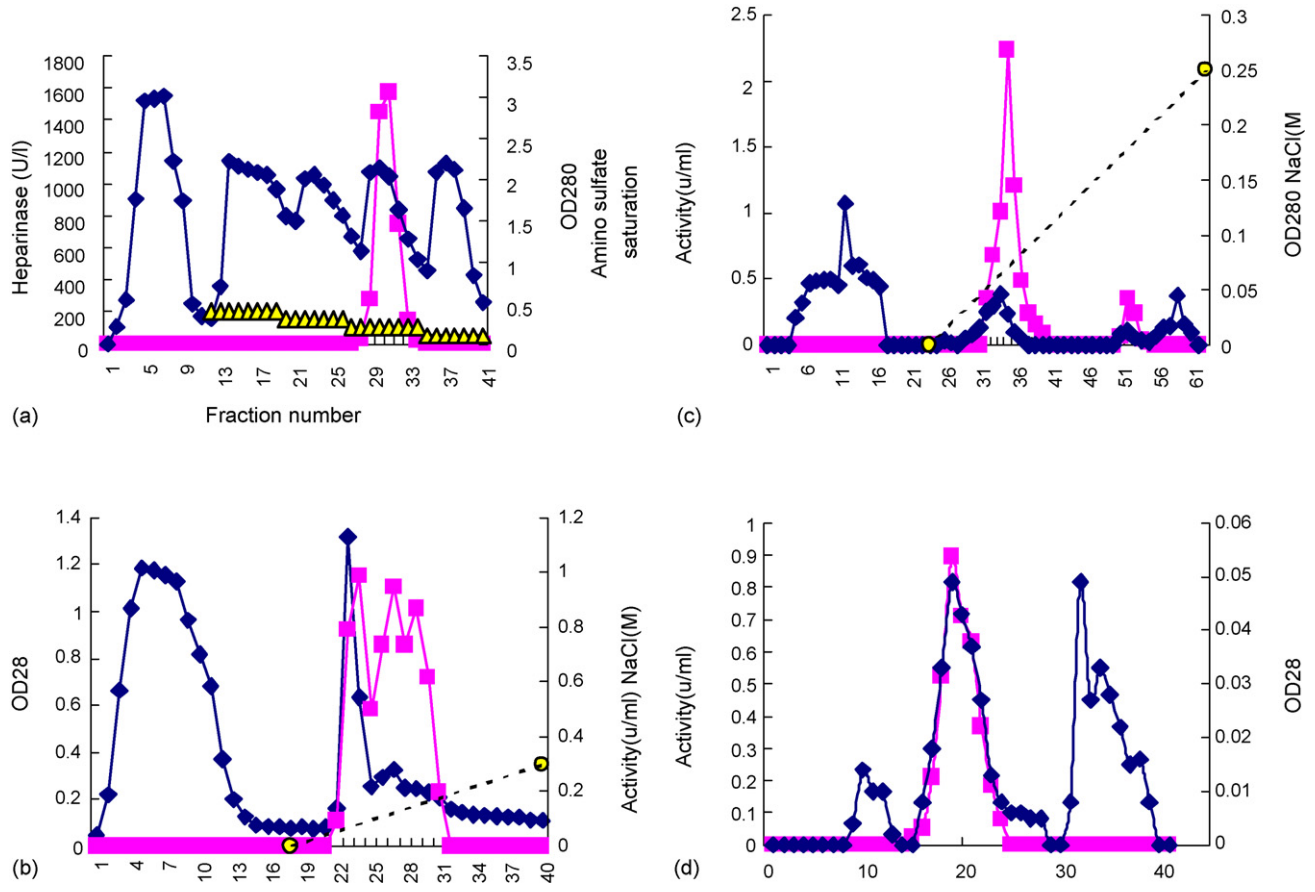

Fig. 2. Elution profile of heparinase from (a) Octyl-Sepharose chromatography, 129.7 mg of protein was placed on a column (1.6 cm × 8 cm): protein absorption (♦), heparinase activity (■) and Amino sulfate saturation (△); (b) CM-52 followed by (c) SP-650 chromatography, 22.5 mg and 0.846 mg of protein were subjected to two columns (1.6 cm × 4 cm) containing CM-52 and SP-650: protein absorption (♦); heparinase activity (■) and NaCl concentration (◊); and (d) Sephadex G-100 chromatography, 0.428 mg of protein was put on a column (1.5 cm × 60 cm): protein absorption (♦), heparinase activity (■).

Table 1
Summary of purification of heparinase

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	150	73.18	0.488	100	1
$(NH_4)_2SO_4$ precipitation	129.7	68.06	0.525	93.0	1.08
Octyl-Sepharose	22.5	31.5	1.4	43.1	2.87
CM-52	0.864	29.97	34.69	40.9	71.1
SP-650	0.428	20.09	46.51	27.4	95.3
Sephadex G-100	0.144	13.01	90.33	17.8	185.1

No N-terminal amino acid was detected in the purified heparinase. Neither Sasisekharan nor Daniel et al. could obtain an N-terminal amino acid of heparinase I [22]. Our attempts also failed presumably due to blocked N termini.

3.4. Variations of enzyme activity and stability with pH and temperature

Activity profiles of the purified enzyme was investigated at range of pH 6.2–7.8 buffer systems at 30 °C, or at temperature range of 25–45 °C at pH 7.0. Results shown the optimal pH of the enzyme was 7.0, the optimal temperature was 41 °C.

The thermo-stability variations were investigated by preincubating the purified enzyme in 10 mM Tris–HCl buffer, pH 7.0, at range of 15–75 °C for 0.5 h. The remaining activity values were determined under the standard assay conditions. Results indicated the enzyme was stable in 0.5 h under 15 °C, but was inactivated rapidly at 35 °C or above. No significant retained activity was found after incubation at temperature above 55 °C for 0.5 h. The purified enzyme remained comparatively stable in the region of pH 7.0–11.0 at 0 °C for 0.5 h, but was inactivated out of this range.

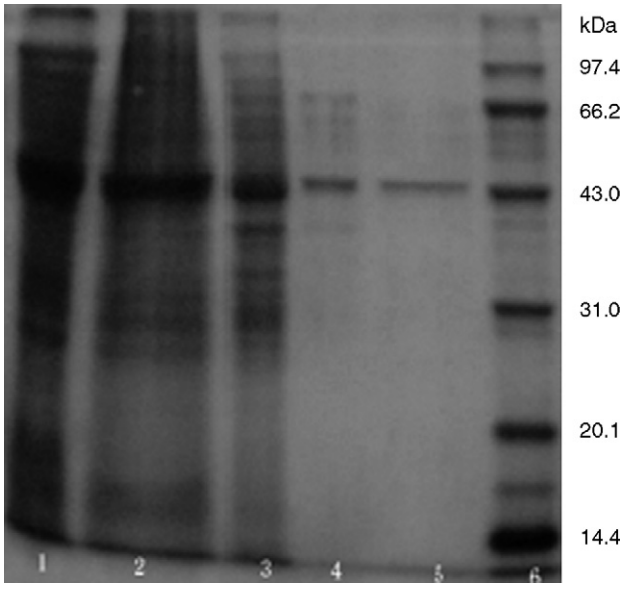

Fig. 3. Purification of heparinase as determined with SDS–PAGE. Electrophoresis was carried out using 12% polyacrylamide gel: (1) crude enzyme (40 μg protein); (2) enzyme solution after Octyl column (30 μg protein); (3) enzyme solution after CM-52 column (15 μg protein); (4) enzyme solution after SP-650 column (8 μg protein); (5) enzyme solution after Sephadex G-100 column (5 μg protein); (6) molecular mass markers (in kDa).

3.5. Effect of $CaCl_2$ on enzyme stability

The purified enzyme was incubated in 10 mM Tris–HCl buffer at pH 7.0, each containing $CaCl_2$ 0, 1, 10, 100 mM, respectively, for 5day at 4 °C. Samples were withdrawn every 24 h and assayed for retained activity (Fig. 4a).

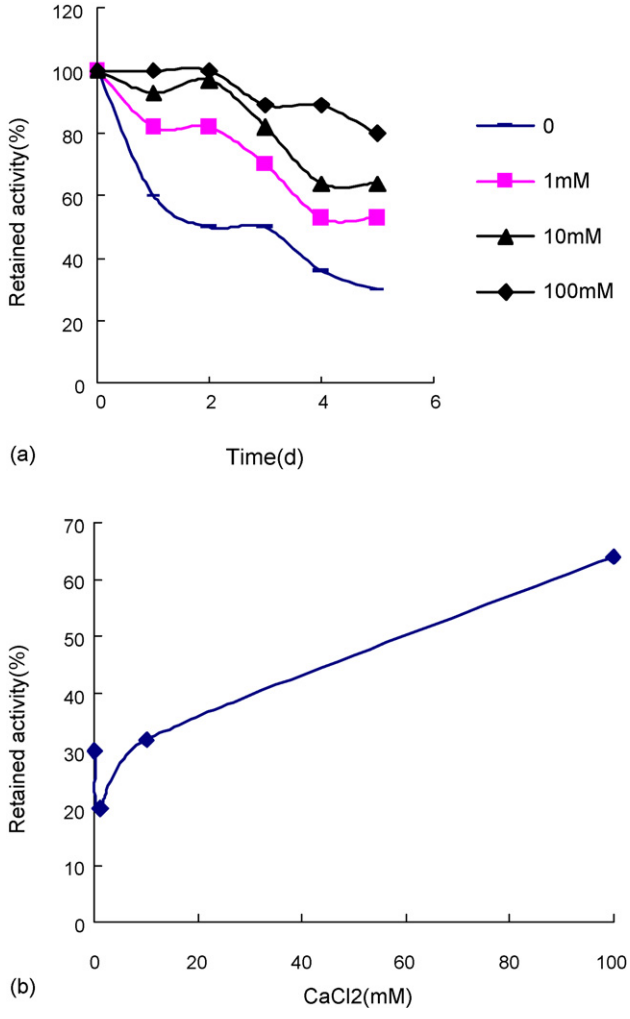

Fig. 4. Effect of $CaCl_2$ on heparinase I stability. (a) Time courses of retained activity percentages of the purified enzymes incubating in ice-bath with additives of 1 mM $CaCl_2$ (■), 10 mM $CaCl_2$ (▲), 100 mM $CaCl_2$ (♦), and control (▬); (b) retained activity percentages of the purified enzymes after freezing-thawing with additives of $CaCl_2$. Enzyme solutions were dissolved in 10 mM Tris–HCl buffer, containing 0, 1, 10, 100 mM $CaCl_2$, respectively. These solutions were then divided into two equal aliquots, and one of each was subjected to either retaining in an ice bath or freezing at −20 °C and thawing.

Table 2
Comparison of the efficiencies of purification haparinase I from *F. heparinum*

Reference	Culture volume (L)	Recovery of purified heparinase I (U/l)	Yield (%)	Specific activity (U/mg)
Yang et al. [18]	10	0.08	0.8	26.6
Zimmerman et al. [23]	100	9.8	11.8	75
Lohose and Linhardt [19]	5	62.4	10	130
This work	1	13	17.8	90.3

The control enzyme lost 40% of its activity after one day. Only 30% of the total activity retained after 5 days. The stability increased dramatically when $CaCl_2$ was introduced. At $CaCl_2$ concentration of 1 mM, the enzyme retained 80% of its activity after 2 days. 55% of the enzyme activity retained after 5 days. When the concentration of $CaCl_2$ increased, the enzyme could be even more stable. Near no activity lost in the enzyme solution containing 100 mM $CaCl_2$ after 2 days storage at 4 °C.

The effect of $CaCl_2$ on purified heparinase stability during freeze-thawing was also assayed. As shown in Fig. 4b, low concentrations ($\leq$10 mM) of $CaCl_2$ had little effect on the stability of enzyme during one time of freeze-thawing. But on a high concentration of 100 mM, the reserved activity increased from 30% to 65% compared to the control.

4. Discussion

Heparinase I has been purified by several schemes from *F. heparinum* since 1985. However none of the yields was high. The reasons may be bellow: (1) The co-production of other enzymes increased the difficulties of the purification so that four to five steps are usually needed; (2) the instability of heparinase [9,18] could leaded to activity losing in every purification step. It was reported that BSA and lactose [9], ammonium sulfate and calcium chloride [23] could give increased stabilization of the purified heparinase I. But nobody gave attention to the denaturation of the crude enzyme and low activity recovery leaded from it in purification procedures.

In this study, several compounds were used to assay the ability on enhancing the stability of crude heparinase extracts. PMSF was used to avoid the proteolysis because of its ability on inactivating proteases [24]. PEG [25] and glycerol [26] were added to increase the viscosity of the system and thus help prevent aggregation. β-Mercaptoethanol was used as reducing agent to prevent the oxidation reaction of thiol group of cysteine which is prone to denature the enzyme activity [27]. BSA was assayed since it was reported useful in preservation of protein [28]. Heparin might be a potent stabilizer of heparinase because it is the specific substrate of the enzyme. Calcium chloride was reported [23] as a stabilizer of purified heparinase I. These two were also assayed on increasing the activity stability of crude heparinase extracts.

The addition of PMSF and BSA shown little effect suggested that the loss of activity did not result from proteolysis. β-Mercaptoethanol shown good effect in 3 days on activity protection indicated oxidation reaction of the cysteine of the enzyme may contribute to the loss of heparinase activity. Glycerol shown good effect on heparinase stabilization indicated aggregation of protein should be another reason caused activity losing. Heparin shown good effect on heparinase stabilization because of its role as specific substrate. Among the additives being used, $CaCl_2$ exert a good stabilizing effect. This result indicated the denaturation of the enzyme is mainly because of the falling down of Ca^{2+} from the molecular of heparinase. When $CaCl_2$ was added, the high concentration of Ca^{2+} in the enzyme solution made it not easy to fall down from the enzyme molecular so that activity of the enzyme could be retained. It was reported that PEG could be a good stabilizer of invertase, or a denaturing agent of lysozyme [25]. Here our result kept agreement with the latter effect.

The purification was performed through following steps: $(NH_4)_2SO_4$ precipitation, hydrophobic interaction chromatography, anion-exchange chromatography, strong anion-exchange chromatography and gel filtration. The purified enzyme displayed one band on SDS–PAGE, with an activity of 90.33 U/mg protein. By using 10 mM $CaCl_2$ as stabilizer in purification steps, we obtained high yield of purified heparinase I with a total activity recovery of 17.8%, which is higher than the highest recovery of 11.8% ever approached by Zimmerman et al. [23]. The efficiency of purification method in this study is compared in Table 2 with the method of Lohse and Linhardt [9], the method of Zimmerman et al. [23], and the method of Yang et al. [18].

In this work, heparinase II and heparinase III could not been isolated. For heparinase III, we could not detect it because its specific substrate is heparan sulfate, not heparin which we used in enzyme assays. The reason we failed to obtain purified heparinase II may attribute to the Ca^{2+} used in the buffer system. Lohse and Linhardt [9] reported Ca^{2+} resulted in dramatically reduced heparinase II activity. We noticed the elution profile of SP650 chromatography shown two activity peaks, the first peak was purified as heparinase I, but the fractions of the second peak, which may be heparinase II, lost activity quickly.

Partial characterization of the purified enzyme was investigated. The enzyme had a molecular weight of 43 kDa and an isoelectric point of 8.5. The N-terminal amino acid sequence could not be determined. It has an activity maximum at pH range of 6.4–7.0 and 41 °C. The purified enzyme was stable in pH range of 7.0–11.0, but was very sensitive to thermal denaturation. All these are same or similar to the data of heparinase I which had been reported [9,18].

Zachary et al. discovered calcium could protect the enzyme from inactivation by modifying reagents [29]. Zimmerman et al. [23] mentioned calcium chloride could give increased stabilization of the purified heparinase I. But none of them shown detailed study on calcium chloride protecting heparinase I. In this study, the effect of calcium chloride on purified enzyme stability was

also assayed. Calcium at concentration of 1 mM exerted very fine effect on protection of enzyme in solution form during storage at 4 °C. And the effect was better when higher concentration of calcium was used. But in freeze-thawing, it seems high concentration as 100 mM of calcium was needed to protect the enzyme.

5. Conclusions

To solve the complex of low heparinase activity yield in purification procedures, $CaCl_2$ was selected out as an activity stabilizer of the crude extracts. And a novel scheme to obtain high heparinase I recovery was described in which the enzyme was protected by added $CaCl_2$. By the new scheme which mainly including four steps of chromatography, an activity yield of 17.8% was attained. The activity yield of this study is higher than any previous reported.

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