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生物技术与方法

重组黄杆菌肝素酶 III 的纯化、表征及培养条件对酶生产的影响

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摘 要: 肝素酶 III 是一种特异性地裂解乙酰肝素的酶, 在大肠杆菌中表达时容易形成包涵体。为实现肝素酶 III 的可溶性表达, 利用谷胱甘肽-S-转移酶(GST)与肝素酶 III 融合性能, 通过构建相应的表达质粒 pGEX-heparinaseIII, 在大肠杆菌中实现了肝素酶 III 的可溶性表达。粗酶通过一步亲和纯化其纯度可达 95% 以上。通过对 LB 培养基摇瓶培养 *Escherichia coli* BL21 的诱导时机、诱导剂用量、诱导时间等培养条件的优化, 确定了该可溶性肝素酶 III 融合蛋白的最适生产条件。通过对纯酶的最适反应温度、pH、Ca^{2+} 浓度等一系列性质研究, 确定了该酶的最适反应条件。

关键词: 肝素酶 III, 谷胱甘肽-S-转移酶, 肝素黄杆菌, 可溶性表达, 表征

Purification and properties of recombinant GST-haparinase III and optimizationm of cultivation conditions

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Abstract: Heparinase III is an enzyme that specifically cleaves certain sequences of heparan sulfate. Previous reports showed that this enzyme expressed in *Escherichia coli* was highly prone to aggregation in inclusion bodies and lacks detectable biological activity. In this paper, we fused a glutathione-*S*-transferase (GST) tag to the N-terminus of heparinase III gene and expressed the fusion protein in *Escherichia coli* to develop an expression system of soluble heparinase III. As a result, approximately 80% of the fusion protein was soluble. The protein was then purified to near homogeneity *via* one-step affinity chromatography. A 199.4-fold purification was achieved and the purified enzyme had a specific activity of 101.7 IU/mg protein. This represented 32.3% recovery of the total activity of recombinant GST-heparinase III. The maximum enzyme production was achieved when bacteria were induced with 0.5 mmol/L isopropyl-β-D-thiogalactoside at 15°C for 12 h. The enzyme showed maximum activity at 30°C and pH 7.5. And the enzyme activity was stimulated by 1 mmol/L Ca^{2+} and 150 mmol/L NaCl.

Keywords: Heparinase III, glutathione-S-transferase (GST), *Flavobacterium heparinum*, soluble expression, characterization

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Introduction

Heparan sulfate proteoglycans (HSPGs) are a diverse group of molecules that comprise a protein core covalently linked to linear chains of heparan sulfate. HSPGs are an ubiqutious component of the cell membrane and the extracellular matrix and have important functions as structural molecules and cell surface receptors[1-4]. The heparan sulfate moieties of HSPG interact with a wide range of molecules, including extracellular matrix components[5-6], growth factors and cytokines[7-9], and enzymes[10]. By regulating the bioavailability and activity of these molecules, heparan sulfate plays a major role in a number of cellular processes, including cell adhesion, migration, differentiation, and proliferation[5-6,11]. The enzymatic cleavage of heparan sulfate by the heparanase is critical for the modulation ofthe biological function of heparan sulfate-binding proteins[12]. Heparinase has been used in various applications such as elucidation of the heparin structure[12-14], enzymatic assays for heparin[15-16], investigation of the anticoagulant mechanism[17], preparation of low molecular weight heparin anticoagulants[18] and anti-tumor agents[19], and the development of immobilized enzyme filters for blood de-heparinization[20]. *Flavobacterium heparinum* (*F. heparinum*) is a natural source of diverse polysaccharidases and sulfatases that degrade heparin and heparan sulfate[21]. However, the complexity of purification procedures and potential co-purification of multiple heparin lyases have generally hampered both analytical and commercial applications of heparinase and made them costly to produce.

To date, heparinase III gene from *F. heparinum* has been cloned and expressed in *E. coli* [22-23]. However, only very small amounts of soluble product could be recovered and the activity levels were too low to allow determining the specific activity of the enzyme. In addition, no detectable activity was obtained even after purification. In this report, we described the cloning, expression and recovery of biologically active recombinant heparinase III in *E. coli.* To our knowledge, this is the first report showing that soluble heparinase III with high specific activity could be obtained from *E. coli* under native conditions.

1 Materials and methods

1.1 Bacterial strains, plasmids and media

The strain *Pedobacter heparinus* (DSMZ 2366) which is synonymous with *F. heparinum* (ATCC 13125) was preserved in our laboratory. *E. coli* DH5α, *E. coli* BL21 (DE3) and pGEX-4T-2 were purchased from Novagen, USA. All restriction enzymes, T4 DNA ligase, RNase and DNA polymerase were from TaKaRa Biotechnology (Dalian, China). Glutathione Sepharose Fast Flow was purchased from Beijing Wsac Co Ltd. Other reagents were from major commercial suppliers.

F. heparinum was cultured as described [24]. Briefly, bacteria were cultured in a shaken flask at 23°C at 180 r/min for 24 h in medium containing the following components (*W*/*V*, %): yeast extract powder 0.5, tryptone 1.0, beef extract 0.5, NaCl 0.5. *E. coli* DH5α and BL21(DE3) were routinely grown in Luria Bertani (LB) medium supplemented with ampicillin (100 μg/mL).

1.2 Plasmid construction

F. heparinum genomic DNA was isolated using the Bacterial DNA kit (TianGen Biotech Co Ltd). To amplify the entire coding sequence without the putative signal sequence, two primers were designed based on the open reading frame of the heparinase III gene (GenBank Accession No. U27586). The 5′ primer (5′-TGAGGATCCCAAAGCTCTTCCATTAT-3′, *Bam*H I), 3′ primer (5′-TTACTCGAGCTAAGGAACCAACAC AAGCT-3′, *Xho* I) and purified *F. heparinum* genomic DNA was used as a template. The PCR product was ligated into pMD-18T vector to give pMD-heparinase III. The cloned heparinase III sequence was then released from pMD-18T by double restriction with *Bam*H I and *Xho* I and directionally inserted in-frame into corresponding sites of pGEX-4t-2.

1.3 Shaking flask expression

pGEX-heparinase III plasmids were transformed into *E. coli* BL21(DE3) for expression. A single colony was grown overnight and diluted 50 times in 200 mL of LB medium containing 50 μg/mL kanamycin. GST-heparinase III fusion protein expression was induced with 0.5 mmol/L isopropyl-β-D-thiogalactoside (IPTG) at 15°C for 12 h when OD_{600} reached 0.6. Cells were harvested by centrifugation at 4000 × g for 20 min.

1.4 Optimization of culturing condition

The methods adopted to study the optimization of culturing condition for enzyme production aimed to evaluate the effect of a single parameter at a time and later manifesting it as standardized condition before optimizing the next parameter. The experiments were conducted in triplicate and the results are average value of three independent experiments. To find out optimum induction opportunity, 0.5 mmol/L IPTG was

added to the broth medium at different culture time. The effect of IPTG concentration was studied by addition of different concentration of IPTG to the medium. To determine optimum induction time for enzyme production, the bacteria was induced at optimum induction opportunity with optimum IPTG concentration. The samples were withdrawn at different time intervals and the total enzyme activity and specific enzyme activity were assayed. To evaluate the effect of Ca^{2+} on enzyme production, steriled $CaCl_2$ was added to medium at the final concentration of 0, 1, 2, 5, 10 mmol/L, respectively. All the experiments were carried out in triplicate and the results are the average value of these three independent findings.

1.5 Enzyme purification

The cell pellet was resuspended in 10 mL cold binding buffer (50 mmol/L Tris·HCl, pH 8.0, 10% glycerol, 2 mmol/L $CaCl_2$, 2 mmol/L dithiothreitol) and the cells were collapsed by sonication for 15 min. Precipitation was discarded after centrifuged at 13 000×g for 20 min at 4°C. Soluble GST-heparinase III fusion protein in the supernatant was mixed with Glutathione Sepharose resin. The mixture was loaded on a column and washed with 10 column volumes of binding buffer at a flow rate of 1 mL/min. The bound protein was eluted with 5 column volumes of elution buffer (50 mmol/L Tris·HCl, pH 8.0, 10 mmol/L $CaCl_2$, 10% (*V/V*) glycerol, 10 mmol/L reduced glutathione (GSH)) at a flow rate of 0.5 mL/min.

1.6 Electrophoretic assays

The purity of the fusion protein was analyzed by a 10 % SDS-polyacrylamide gel. Gels were stained with Coomassie brilliant blue R-250 and destained by acetic acid-methanol-water solution (10:30:60, *V/V/V*). Protein concentration was determined by the Bradford assay[25] against a bovine serum albumin standard curve.

1.7 Enzymatic activity assay

GST-heparinase III fusion protein activity assay was performed essentially as described[26]. Briefly, the enzymatic activity was directly measured from an increase in absorbance at 232 nm as a function of time. 10 μL GST-heparinase III fusion protein was added to 990 μL reaction mixture which contained 50 mmol/L Tris·HCl buffer (pH 7.6), 1 mg/mL heparan sulfate (Shenzhen Hepalink Co. Ltd) and 2 mmol/L $CaCl_2$, thermally equilibrated at 35°C. The reaction mixture was then immediately returned to the spectrophotometer and the change in absorbance at 232 nm was measured at 30 s intervals over 3 min. Activity is expressed in units (μmol of product formed/min) using a molar absorption coefficient for the unsaturated C-4-C-5 bond of uronic acids of 3800 L/(mol·cm) [27].

1.8 Characterization of purified GST-heparinase III fusion protein

The optimum temperature for enzyme activity was determined in 50 mmol/L Tris·HCl buffer (pH 7.6) by 5°C increments from 25°C to 55°C. For temperature stability, enzyme solution was incubated at pH 7.6 at 25°C–55°C for 1 h. The optimum pH was assayed from pH 6.0–12.0. For pH stability, enzyme solution was incubated at pH 6.0–12.0 for 1 h. GST-heparinase III fusion protein activity was measured by the UV 232 nm assay.

To investigate the effect of Ca^{2+}, the assay was performed under the same conditions as described above except that 0, 0.5, 1, 2, 5, 8, 10 mmol/L calcium chloride were used. GST-heparinase III fusion protein activity was detected by the UV 232 nm assay.

Effect of salt(NaCl) concentration ranged from 0–400 mmol/L on heparinase III activity was also investigated. The assay buffers were prepared in 50 mmol/L Tris·HCl buffer pH 7.6, GST-heparinase III fusion protein activity was checked by the UV 232 nm assay.

1.9 GPC-HPLC assays

Heparan sulfate (20 mg/mL) was incubated with GST-heparinase III and native heparinase III (Shenzhen Hepalink Co. Ltd), respectively in 50 mmol/L Tris·HCl buffer (pH 7.6) containing 1 mmol/L $CaCl_2$ at room temperature for 12 h. The oligosaccharide products were analyzed by gel permeation chromatography (GPC)[28]. The reaction was terminated by boiling and subjected to TSK Gel G3000SW column (30 mm × 750 mm) connected with a UV detector. The flow rate of the mobile phase was set at 0.5 mL/min and the UV detection was done at 234 nm.

2 Results and discussion

2.1 Expression and purification of recombinant GST-heparinase III fusion protein

To facilitate expression and purification of recombinant GST-heparinase III fusion protein from *E. coli*, the coding sequence was amplified from *F. heparinum* genomic DNA and cloned to the downstream of sequences specifying a GST-tag in the expression vector pGEX-4T-2. The authenticity of the cloned sequence in the recombinant plasmids was confirmed by sequencing and restriction enzyme

profiling. GST-heparinase III fusion protein expression was induced with 0.5 mmol/L IPTG at the log phase and cultured at 15°C for 12 h. Cell lysis yielded a crude extract in which 80% of recombinant GST-heparinase III fusion protein was soluble. Soluble GST-heparinase III fusion protein was purified by Glutathione Sepharose Fast Flow chromatography to near homogeneity which appeared as a single band at the expected 101 kD position on an SDS-PAGE gel (Fig. 1).

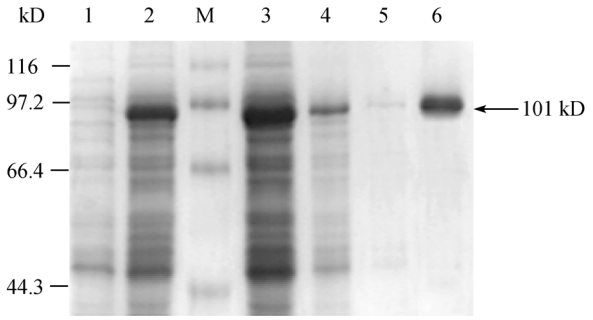

Fig. 1 Expression and purification of recombinant GST-heparinase III fusion protein. 1: un-induced total cell protein; 2: induced total cell protein; M: protein standards; 3: soluble fraction of crude cells lysate; 4: flow- through; 5: wash; 6: eluate with 10 mmol/L GSH. The arrow indicates the position of GST-heparinase III fusion protein (101 kD).

The results obtained using Glutathione Sepharose Fast Flow affinity chromatography for recombinant GST-heparinase III fusion proteins are summarized in Table 1. Recombinant GST-heparinase III fusion protein was purified 199.4-fold over the crude cell homogenate. The scheme provided an overall yield of 0.16% recovery from total expressed protein and 32.3% recovery of total activity. The specific activity of the purified enzyme was 101.7 IU/mg.

2.2 Optimization of the cultivation conditions

2.2.1 Effect of induction opportunity on enzyme production

All the optimization experiments were performed at 15°C. Fig. 2 shows the effect of induction opportunity on GST-heparinase III fusion protein production. With the increase of the initial inducing concentration of the culture, total enzyme activity and the specific enzyme activity are also enhanced. However, there is a slight decrease for the total enzyme activity and the specific enzyme activity when the OD_{600} of the culture was beyond 0.948 (Fig. 2). Because of IPTG can inhibit cell growth, the earlier of the inducing opportunity, the stronger of the inhibition on the cell growth. Thus the induction was selected at the late logarithmic phase (OD_{600} 0.8–0.9) in the following experiments.

2.2.2 Effect of IPTG concentration on enzyme production

The influence of the concentration of IPTG on the GST-heparinase III fusion protein production was studied by addition of different concentrations of IPTG to the medium. The enzyme activity and the specific enzyme activity were found to be maximal at 0.5 mmol/L IPTG (Fig. 3).

2.2.3 Effect of inducing time on enzyme production

In order to determine optimum induction time for GST-heparinase III fusion protein production, cells were induced with 0.5 mmol/L IPTG when OD_{600} reached of 0.8–0.9. As shown in Fig. 4, the enzyme activity was gradually increased with the induction time, whereas the specific enzyme activity became constant when the induction time was beyond 14 h.

2.2.4 Effect of Ca^{2+} concentration on enzyme production

The effect of additional Ca^{2+} on the GST-heparinase III fusion protein production was also studied. Fig. 5 showed that there was continuous raise in enzyme activity with increase in Ca^{2+} concentration. However, additional Ca^{2+} concentration above 0.5 mmol/L could not improve the enzyme productivity further, indicating that high concentration of calcium may inhibit cells growth and enzyme production. The highest specific activity was observed at 0.3 mmol/L Ca^{2+}.

2.3 Characterization of purified GST-heparinase III fusion protein

2.3.1 Effect of temperature on activity and stability of enzyme

The activity of purified GST-heparinase III fusion-protein was determined at different temperatures ranging from 25°C–55°C. The optimum temperature for activity was 30°C (Fig. 6) which was somewhat lower than that of the native heparinase III[27]. The slight differences may arise from difference assay conditions

Table 1 Purification summary of recombinant GST-heparinase III fusion protein

Step	Total protein (mg)	Total activity (IU)	Specific activity (IU/mg)	Activity recovery (%)	Purification fold
Crude extract	261.0	132.0	0.51	100	1.00
Glutathione Sepharose FF	0.42	42.7	101.7	32.3	199.4

The enzyme was purified from 1.0 L of culture broth.

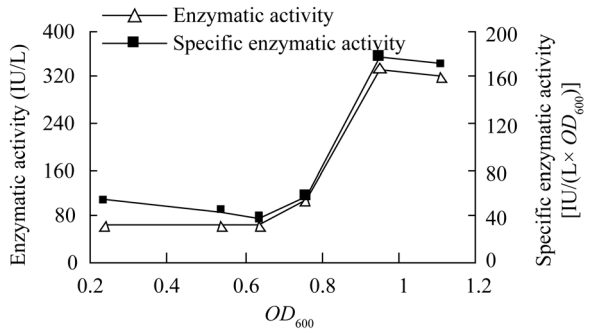

Fig. 2 Effect of induction opportunity on recombinant GST-heparinase III fusion protein production at 15°C.

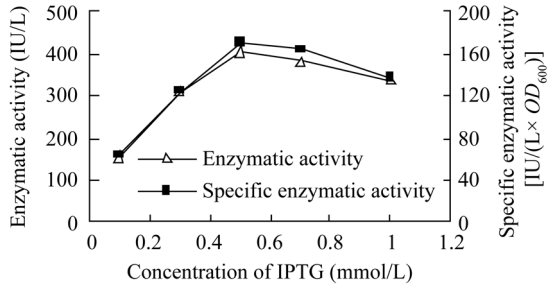

Fig. 3 Effect of IPTG concentration on recombinant GST-heparinase III fusion protein production at 15°C.

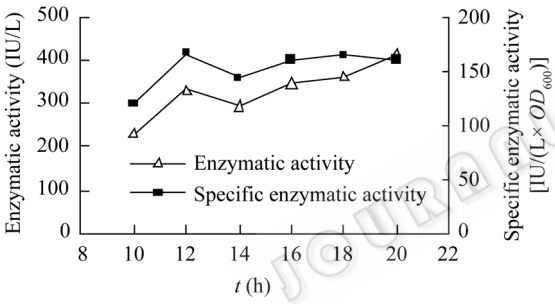

Fig. 4 Effect of induction time on recombinant GST-heparinase III fusion protein production at 15°C.

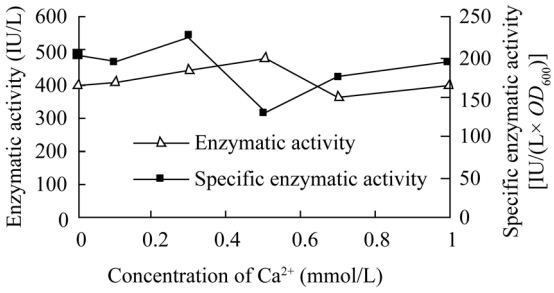

Fig. 5 Effect of Ca^{2+} concentration on recombinant GST-heparinase III fusion protein production at 15°C.

or differences in the purity of the enzyme studied. The studies on enzyme stability showed that GST-heparinase III fusion protein was stable (retained 90% of its activity) for 1 hour at 25°C–40°C. However, activity was significantly declined when temperature was beyond 40°C (Fig. 6).

2.3.2 Effect of pH on activity and stability of enzyme

The enzyme was active over a narrow pH range (pH 7.0−7.5) and showed optimum activity at pH 7.5(Fig. 7) which were similar to native heparinase III[27], indicating that the GST tag had no influence on pH dependence of this enzyme. The stability of the purified GST-heparinase III fusion protein was determined by the pre-incubation of the enzyme in various buffers with different pH for 1 h. The enzyme was relatively stable over a pH range of 7.0–11.0 retained 80% of the original activity.

2.3.3 Effect of Ca^{2+} on enzyme activity

The effects of Ca^{2+} on GST-heparinase III fusion protein activity was also examined (Fig. 8). As shown in Fig. 8, above the concentration of around 2 mmol/L, activation of GST-heparinase III fusion protein by Ca^{2+} became constant, but below this threshold, Ca^{2+} could be one of the main factors affecting the enzyme activity. A minimum concentration of 1 mmol/L Ca^{2+} is essential for enzyme activity. It is suggested that calcium acts as a possible co-factor for recombinant GST-heparinase III fusion protein activity.

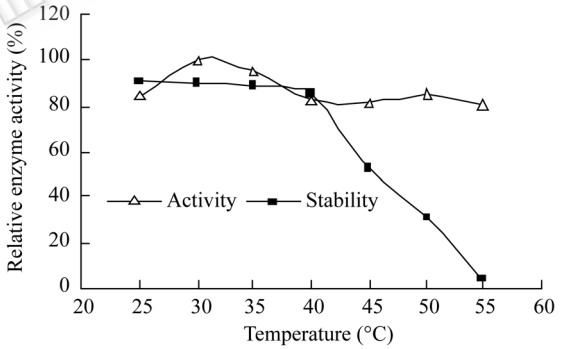

Fig. 6 Effects of temperature on activity (Δ) and stability (■) of recombinant GST-heparinase III fusion protein.

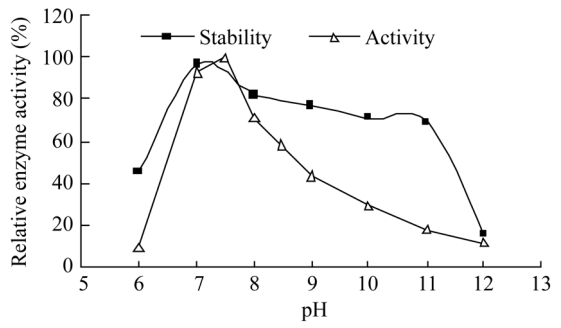

Fig. 7 Effects of pH on activity (Δ) and stability (■) of recombinant GST-heparinase III fusion protein.

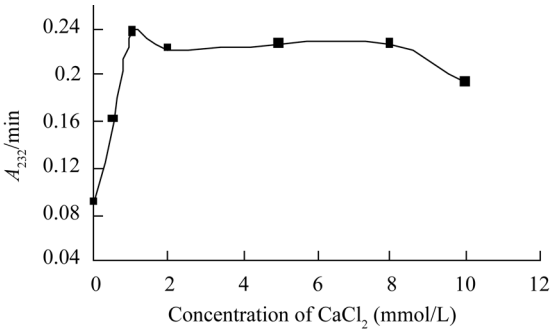

Fig. 8 Effect of Ca^{2+} concentration on recombinant GST-heparinase III fusion protein activity.

2.3.4 Effect of NaCl on enzyme activity

The effects of ion strength on GST-heparinase III fusion protein activity were shown in Fig. 9. The optimum ion strength for GST-heparinase III fusion protein activity was around 150 mmol/L NaCl, and it showed increasing activity with increasing NaCl concentrations in the range of 0–150 mmol/L and the activity decreased with adding more than 150 mmol/L NaCl (Fig. 9). The activity was almost completely lost in concentration of 300 mmol/L NaCl, reflecting a similar condition of native heparinase III as reported previously [27].

2.4 GPC-HPLC assay of heparin oligosaccharides

The oligosaccharides products degraded by GST-heparinase III fusion protein and native heparinase III were assayed using GPC-HPLC. The profile of recombinant enzyme was similar to that of native heparinase III, indicating that the GST tag had no influence on enzyme activity (Fig. 10).

3 Conclusion

In summary, the fusion of heparinase III to GST-tag significantly enhanced the soluble expression of heparinase III in *E. coli*. One-step purification by Glutathione Sepharose Fast Flow could effectively separate the target enzyme from the supernatant solution. This work provides a highly efficient method for large scale preparation of biologically active GST-heparinase III fusion protein which will facilitate the application in structure -function analyses of heparan sulfate and their derivatives as well as expand current understanding of the catalytic mechanism of heparinase III.

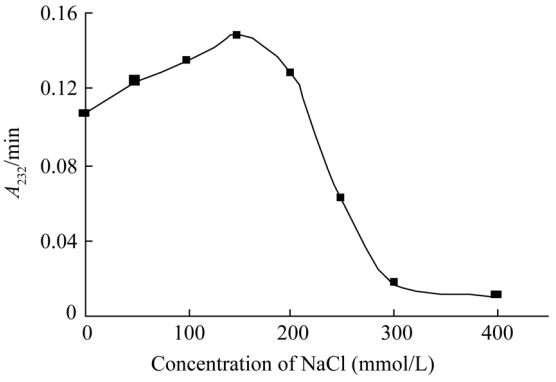

Fig. 9 Effect of NaCl concentration on recombinant GST-heparinase III fusion protein activity.

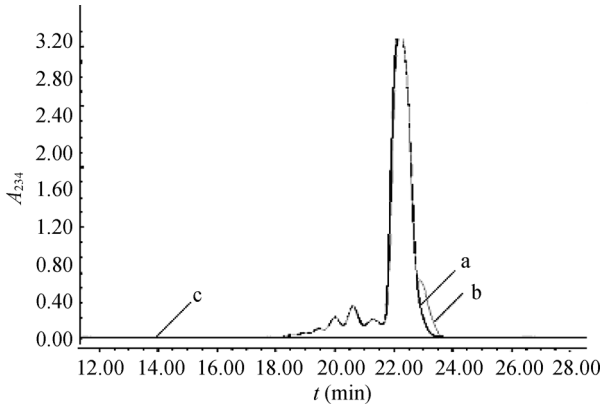

Fig. 10 GPC-HPLC analysis of oligosaccharides of heparan sulfate degraded by heparinase III. (a) The product profile of heparan sulfate degradation by GST-heparinase III fusion protein. (b) The product profile of heparan sulfate degradation by native heparinase III from *F. heparinum*. (c) The profile of heparan sulfate as a control.

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