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Purification and Characterization of Heparin Degrading Enzyme by isolated bacteria from brackish sediment

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Abstract

The heparinase-producing bacteria was isolated from brackish sediment and identified by morphological characteristic and 16S rRNA gene. The nucleotide sequence of 16S rRNA indicated highest similarity (97%-100%) with genus *Aeromonas*. The isolate was subsequently described as *Aeromonas* sp. RYA_En1. The crude intracellular enzyme produced by *Aeromonas* sp. RYA_En1 was partial purified by ammonium sulphate, ion exchange and gel filtration column chromatography, respectively. The active fraction obtained from gel filtration chromatography yielded enzyme production and enzyme specific activity of 410 U/L and 0.63 U/mg protein, respectively. Then, the purified fraction was determined for the optimal temperature and pH for the enzyme activities which were at 37°C and pH of 7.0, respectively. In addition, the molecular weight of the purified enzyme determined by the SDS-PAGE was approximately 90 kDa.
Keywords : *Heparinase, Heparin, Enzyme purification, Aeromonas* sp.

1. Introduction

Heparin and heparan sulfate (HS) are composed of linear chains of 20–100 disaccharide units of *N*-acetylated D-glucosamine ∝ (1-4) linked to glucuronic acid. Heparin and HS have been widely used for a variety of biological functions, anticoagulant activity, cell growth and proliferation, inhibition of angiogenesis and tumor growth and metastasis, as well as anti-viral activity (1, 2).

Heparinases are eliminase that cleave the major glycosidic linkages in heparin and HS (Figure 1) and create a C4-C5 double bond within the hexuronic acid located at the nonreducing end (3). These enzymes are designated as heparinase I (heparin lyase, EC 4.2.2.7), heparinase II (heparin lyase II, no EC number) and heparinase III (heparin lyase III, EC 4.2.2.8). The heparinase I primarily cleaves heparin and the heparinase II acts equally on both heparin and HS whereas the heparinase III specifically cleaves HS (4). Heparinase are commercially available from *Pedobacter heparinus* (formerly known as *Flavobacterium heparinum*). However, other microorganisms such as *Sphingobacterium* sp., *Bacillus circulans*, *Bacteroides stercoris* HJ-15,

Prevotella heparinolytica and *Aspergillus flavus* were also reported for the heparinase production (5-9). Finding the novel efficient heparinase producing bacteria may contribute the low cost of production.

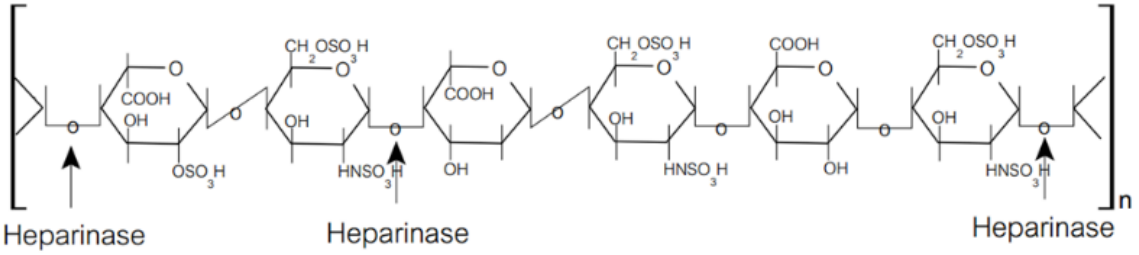

Figure 1. Structure of heparin and cleavage region by heparinase.

In this research, a bacterium was isolated from brackish sediment demonstratedpotential in heparinase producing. The crude enzyme obtained from the bacterium was purified and subsequently characterized some properties.

2. Materials and Methods

2.1 Materials

Sodium heparin salt from porcine intestinal mucosa and DEAE-sepharose Fast Flow were purchased from Sigma Chemical Co. Sephadex G-100 was purchased from GE Healthcare Life Science.

2.2 Microorganism and culture medium

Brackish sediment samples were collected from Krached canal in Rayong province of Thailand. Samples were collected from three different areas within location and stored at 4 °C in a refrigerator until use. The heparinase producing bacterial isolate RYA_En1 was selected for this research. The bacterium was identified by morphological characteristics and 16S rRNA gene sequence analysis. The 16s rRNA nucleotide sequence was amplified by universal primer 27F and 1492R.

2.3 Production and purification of the heparinase

The RYA_En1 isolate was inoculated in 1 L of optimized liquid medium containing 1% trypticase, 0.1% NaCl, 0.25% K_2PHO_4, 0.05% $MgSO_4$ and 1% maltose with initial pH of 7.0 (10). The culture was incubated on a rotary shaker adjusted shaking at 180 rpm, 30 °C for 24 h. Then, the culture was centrifuged at 6000 rpm at 4 °C for 20 mins. The resulting cell pellets were washed with sterilized saline containing 10 mM Tris-HCl buffer (pH 7.0) and then resuspended with the same buffer. The suspension was disrupted by sonication on ice for 30 mins at 150 W using a 50% pulse mode. Cells debris was removed by centrifugation at 12840×g for 20 mins at 4°C. The crude enzyme was obtained and purified by anion-exchange chromatography using DEAE Sepharose Fast Flow column which was pre-equilibrated with 10 mM Tris-HCl buffer pH 7.0. Proteins were eluted with a linear gradient of 0.1 – 0.4 M NaCl at a flow rate of 0.5 ml/min. The fractions with heparinase activity were further purified by gel filtration on Sephadex G-100 column, which was eluted with 10 mM Tris-HCl buffer pH 7.0 and finally collected. Purity and molecular mass

of the purified heparinase were determined with a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) system as described by Laemmli (11).

2.4 Analysis of protein and enzyme activity

Heparinase activity was determined by following the method of Lohse and Linhardt (12). Briefly, a reaction mixture contained 100 µl of 1.0 mg/ml of heparin in 0.1 M sodium acetate buffer at pH 7.0, 100 µl 10 mM calcium acetate and 20 µl of enzyme solution. The reaction mixture was incubated at 37°C for 10 min. Then, the reaction was stopped by incubation in water bath at 60°C for 10 min. The heparinase activity was calculated from the measured absorbance of reaction product at 232 nm. One unit of enzyme activity will form 0.1 µmole of unsaturated uronic acid per hour at pH 7.0 at 37°C. Total proteins were measured by Bradford assay (13).

2.5 Characterization of the purified enzyme

2.5.1 Effect of temperature on Heparinase activity

The enzyme activity was determined at different incubation temperature ranging from 20 to 45 °C at optimum pH obtained from the result of 2.5.2. Heparinase was incubated with 1 mg of heparin for 10 min, and enzyme activity was determined with the same manner with 2.4.

2.5.2 Effect of pH on Heparinase activity

The enzyme activity of the purified heparinase was examined at pH ranging from 6.0 to 9.0 (Tris-HCl buffer) at optimum temperature obtained from the result of 2.5.1. The enzyme activity was determined as described in 2.4.

Gram staining of bacterial isolate revealed that it was gram negative coccobacilli (Figure 2).

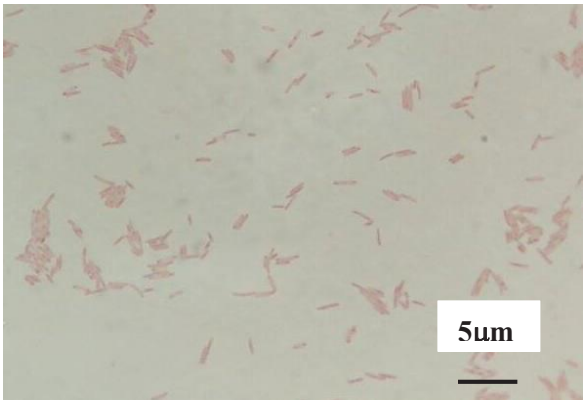

Figure 2. Gram-staining of the isolate RYA_En1 visualized under a microscope with 1000X resolution.

The bacterial isolate was identified by obtained 16S rRNA 1343 bp of the PCR product. The gene sequencing and homology of bacterial 16S rRNA gene revealed 95 – 100% homology to several Aeromonas strains. Subsequently, the isolate was described as *Aeromonas* sp. RYA_En1 (Figure 3).

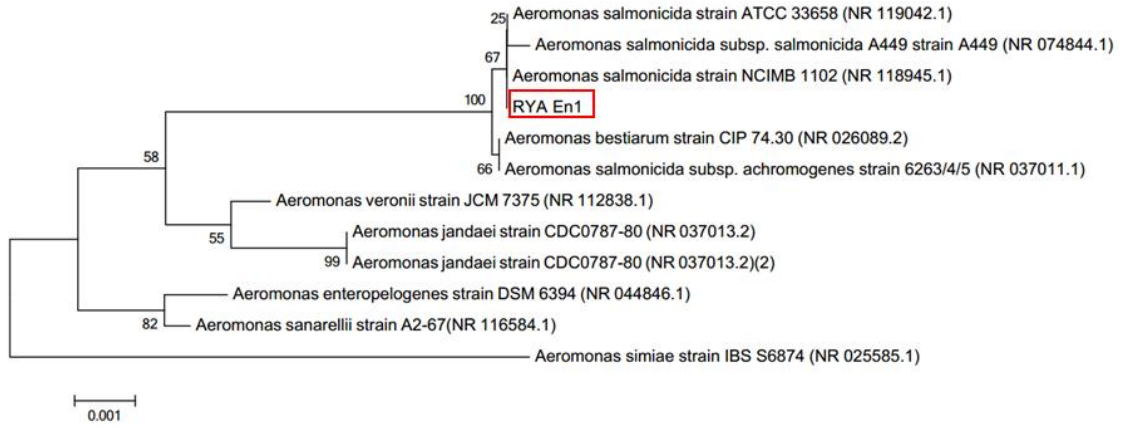

Figure 3. phylogenetic tree result of 16s rRNA sequencing of isolated RYA_En1.

3. Results and Discussion

3.1 Production and purification of the heparinase produced by *Aeromonas* sp. RYA_En1

After cultivation of *Aeromonas* sp. RYA_En1 in the optimized medium for 24 h, the intracellular heparinase the bacterium was released from the cell pellets by sonication, and the crude enzyme was precipitated by ammonium sulphate. The enzyme was sequentially purified by DEAE-Sepharose and Sephadex G-100 column chromatography, respectively, which resulted in a 2.42-fold purification and final yield of 0.83 % (Table 1). The proteins obtained from those sequential purifying steps were estimated for molecular weight as shown in Figure 4. Less protein bands were obtained for purified proteins using DEAE-Sepharose and Sephadex G-100 column chromatography.

Table 2. Enzyme activities of crude and purified heparinase from *Aeromonas* sp. RYA_En1.

Purification step	Total volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification Fold	Yield (%)
Culture extract	150	561.60	148.35	0.26	1.00	100.00
$(NH_4)_2SO_4$ precipitation	50	339.70	88.00	0.26	1.00	59.32
DEAE-Sepharose	10	7.11	2.47	0.35	1.35	1.67
Sephadex G-100	3	1.95	1.23	0.63	2.42	0.83

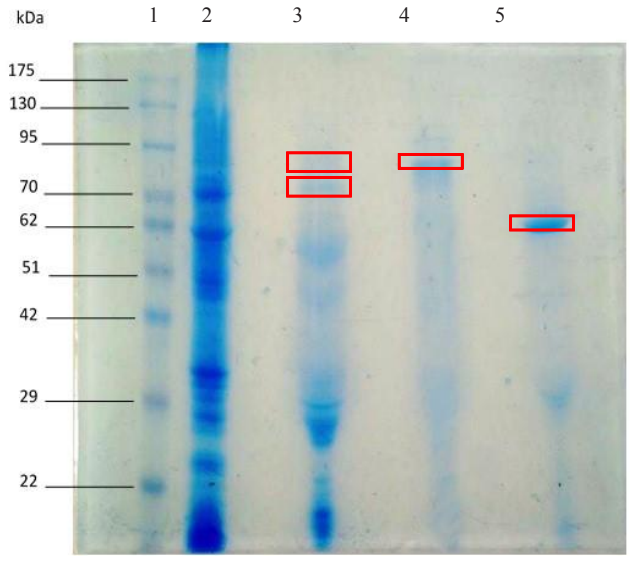

Figure 4. SDS-PAGE of the purification heparinase at various steps of purification Lane 1, marker; Lane 2, crude extract; Lane 3, Resuspension of proteins from precipitation by ammonium sulfate; Lane 4, purified proteins from DEAE Sepharose column chromatography; Lane 5, purified protein from Sephadex G-100 column chromatography.

The purification step using DEAE-Sepharose contributed four active fraction ranges of detectable enzyme activity as illustrated in Figure 5 and Table 3. Although the fraction of 2-24 contributed highest enzyme activity, the specific enzyme activity was only 0.37 U/ml which was slightly higher than the active fraction of 94-105 and 120-130.

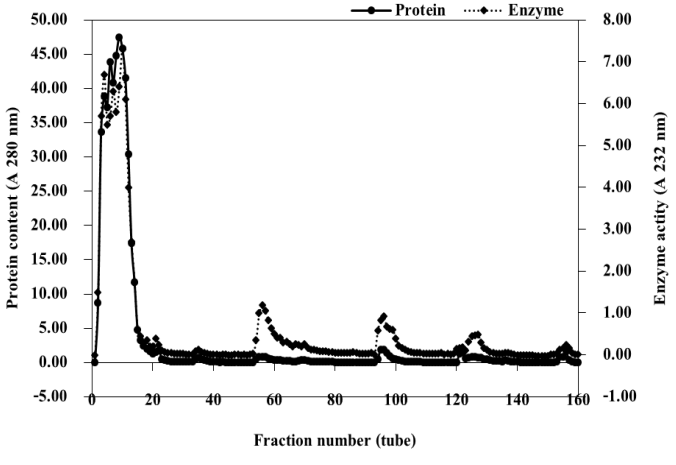

Figure 5. Elution profile of heparinase from DEAE Sepharose fast flow chromatography.

Table 3. Specific enzyme activity of active fractions of heparinase obtained DEAE Sepharose Fast Flow chromatography

Those four active fraction ranges were

Fraction.	Total Ac-tivity (U)	Total Proteins (mg)	Specific activity (U/mg)
Fraction 2-24	91.17±1.98	235.21± 5.26	0.37± 0.02
Fraction 53-78	1.18±0.02	7.00±0.06	0.17±0.01
Fraction 94-105	2.47±0.04	7.11±0.01	0.35±0.01
Fraction120-131	0.87±0.07	2.56±0.04	0.34±0.02

subsequently determined for molecular weight of proteins containing in the fractions by subjecting to SDS-PAGE (Figure 6). The fraction of 2-24 and 53-78 were found several protein bands which potentially indicated less purity compared to a sharp single protein band obtained from fraction of 94-105 and 120-131 at molecular weight of approximately 90 kDa. Due to the considerably high amount of enzyme unit and specific enzyme activity of protein from the fraction 94-105, Therefore the proteins from this fraction was selected for further purified using Sephadex G-100 column chromatography. The elution profile resulted from Sephadex G-100 chromatography contributed active fraction of 7-12 (Figure 7). The purified enzyme from this fraction yielded specific enzyme activity of 0.63 U/mg.

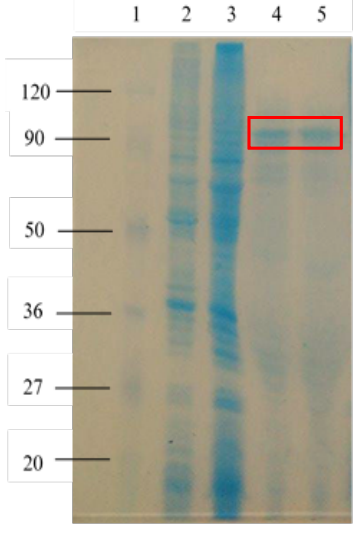

Figure 6. SDS-PAGE of elution profile of heparinase from DEAE Sepharose Fast Flow chromatography Lane 1, Marker; Lane 2, fraction of 2-24; Lane 3, fraction of 53-78; Lane 4, fraction of 94-105; Lane 5, fraction of 120-131.

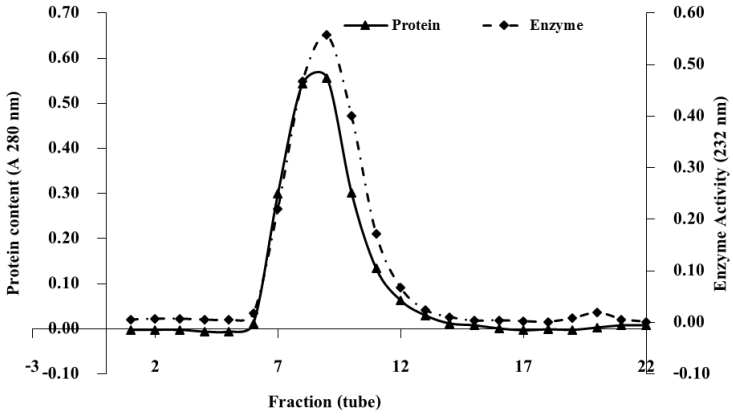

Figure 7. Elution profile of purified heparinase obtained from Sephadex G-100 chromatography.

3.2 Effect of pH and temperature on the purified heparinase produced by *Aeromonas* sp. RYA_En1

The influences of temperature and pH on enzyme activity of the purified enzyme were studied at temperature and pH range of 20 – 45 C and 6.0 – 9.0, respectively. The results showed that the optimal temperature and pH for enzyme activity of purified heparinase was 37°C and pH of 7.0, respectively (Figure 8 (A, B).

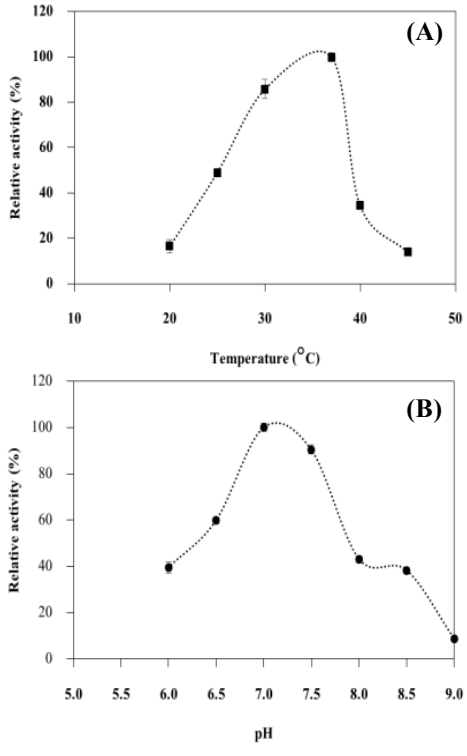

Figure 8. Influence of temperature (A) and pH (B) on enzyme activity of the purified heparinase.

4. Discussion

Heparinases has been purified from many microorganisms, such as *Flavabacterium heparinum* (14), *Sphingobacterium* sp. (5), *Bacteroides heparinolyticus* (15), and *Aspergillus flavas* (16). However, these heparinase were different in molecular weights, optimal pH and temperature.

In this study, the production of purified heparinase from *Aeromonas* sp. RYA_En1 reached 410 U/L. The apparent molecular weight of heparinase was approximately 90 kDa which was higher than the heparinase I from *Pedobacter heparinus* (85 kDa), and lower than the heparinase from *Bacillus circulans* (111 kDa) (6). In addition, the optimum temperature of the purified heparinase activity was 37°C. However, this optimum temperature was low compared to those of heparinases from Bacillus circulans (45°C) and Bacteroides stercoris HJ-15 (50°C). Nevertheless it was slightly higher that of Aspergillus flavus (35°C). The optimum pH of heperinase from RYA_En1 was 7.0-7.5 and it was similar to those of many reports (6-7, 16).

5. Conclusion

In the present work, heparinase-producing bacterial stain, Aeromonas sp. RYA_En1, was isolated from brackish sediment. The purified heparinase yielded 410 U/L and 0.63 U/g proteins for enzyme production and specific enzyme activity, respectively. The purified heparinase had apparent molecular weight of 62 kDa. The optimal temperature and pH for the enzyme activity were 37 C and 7.0, respectively.

6. Acknowledgments

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