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Purification of *Streptococcal nuclease* from *Streptococcus* sp.

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Abstract : *Streptococcal nuclease* was completely purified by stepwise CM-Sepharose column chromatography from crude extracts isolated from *Streptococcus* sp. The active enzyme fraction was eluted with the buffer containing 0.2 M NaCl. The purified enzyme showed a homogeneity on SDS PAGE and had a molecular weight of 35,000 daltons. The optimum pH and temperature for the enzyme were 9.0 and 50℃, respectively (Received August 25, 1994; accepted September 14, 1994).

Introduction

Streptococcal nuclease (SD) is an extracellular nuclease produced by group C *streptococci*.[1)] In combination with streptokinase, SD is used therapeutically, especially, for breaking down blood clots. While there have been many studies on streptokinase due to its commercial importance, there are very few reports available on SD.[2~6)]

The streptococcal extracelluar nuclease degrades both RNA and DNA endonucleolytically and the products are mostly oligonucleotides terminated in 5′-phosphate. The chain length of limit digest prepared from native calf thymus DNA was about 5.[7)] DNase activity is observed in the supernatant from cultures of streptococci group A, B, C, D, E.[7~9)] Also, it has been known that there are four classes of extracellular nuclease, A, B, C, D. Nuclease B, which is the largest component produced by streptococci group A, had a molecular weight of 23,000 daltons. In contrast to group A, group C appears to produce nuclease A. Recently, the isolation and nucleotide sequence of the SD gene from group C was reported.[10)] However, due to lack of information available in the literature, better understanding on molecular properties of SD is needed.

In this stuy, to further characterize SD, SD was completely purified by using a stepwise CM-Sepharose column chromatography, which is suitable for large scale purification of SD.

Materials and Methods

Materials

All the chemicals and reagents were of analytical grade.

Purification of streptodornase

After fermentation of *Streptococcus* sp. the broth was pretreated and applied to CM-Sepharose processing. Ammonium sulfate was added to the crude streptodornase (SD) solution, which was prepared by dissolving SD powder obtained after lyophilizing first CM-Sepharose processing, to a final concentration of 85% saturation. After centrifugation (7,500 rpm, 20 min), the precipitate was dissolved in 20 mM phosphate buffer (pH 7.5) and dialyzed against 5 mM phosphate buffer. The solution was applied to a column of CM-Sepharose (1.5×30 cm) equlibrated with the same buffer. The enzyme was eluted with stepwise gradient of 0 to 1 M NaCl in the same buffer. The enzyme fraction with the highest

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specific activity was pooled, dialyzed, and lyophilized.

Assay of streptodornase activity

Activity of SD was assayed as described by Kunitz.[11] To the reaction mixture with 0.8 ml calf-thymus DNA, the enzyme, 80 μmol glycine-KOH (pH 9.0), 0.8 μmol $MgSO_4$ and 0.8 μmol $CaCl_2$ was added and incubated at 37℃ for 10 min. The reaction was stopped by the addition of 80 μl of 5 N perchloric acid and was kept at −20℃ for 5 min. After centrifugation (12,000 rpm, 10 min), the amount of acid-solubilized fraction was determined by optical density of the supernatant at 260 nm. To examine the effect of pH on the enzyme activity, the following buffer systems were used; sodium acetate buffer (pH 4 and 5), hydroxylamine buffer (pH 6), tris buffer (pH 7 and 8), and glycine-KOH buffer (pH 9 and 10).

Protein assay

The protein concentration was measured by the method of Bradford with BSA as the standard.[12]

SDS PAGE

Molecular weight of purified SD was determined by SDS-PAGE using 4.5% stacking and 12.5% separating gel of 0.75 mm thickness as described by Laemmli.[13] The relative molecular weight of the purified SD was estimated by comparing its relative mobility with those of the following marker proteins: bovine serum albumin (66,000), egg albumin (45,000), phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), trypsin inhibitor (20,100), and alpha-lactalbumin (14,200). Gel was stained with 0.1% Coomassie Brilliant Blue R.

Results and Discussion

Purification of SD

Crude SD was further purified by using a stepwise CM-Sepharose column chromatography. For simplification of SD production processing, CM loading solution was loaded onto CM-Sepharose column and eluted by 0.25 M NaCl. As the result, purity of the crude SD was only 30%. Therefore, to obtain more than 95% pure SD, crude SD was reloaded onto CM-Sepharose column and eluted by using a stepwise NaCl gradient. CM elution profile (Fig. 1) indicates that 0.2 M elution peak has the highest enzyme activity. Also, SDS-PAGE pattern (Fig. 2) clearly indicates that 0.1 M elution peak contains 47 k Da protein and 30 k Da protein. Therefore, stepwise elution could eliminate impurities, producing pure SD at 0.2 M elution. This was also confirmed by specific activity of elution peak (Table 1). In contrast to crude SD, specific activity of 0.2 M elution peak had about 4 times higher than the crude SD solution. Therefore, stepwise CM-Sepharose column chromatography processing will be suitable for large scale preparation of SD.

Determination of molecular weight

The molecular weight of SD was determined by the SDS-PAGE. Its molecular weight was estimated to be about 35,000 (Fig. 2).

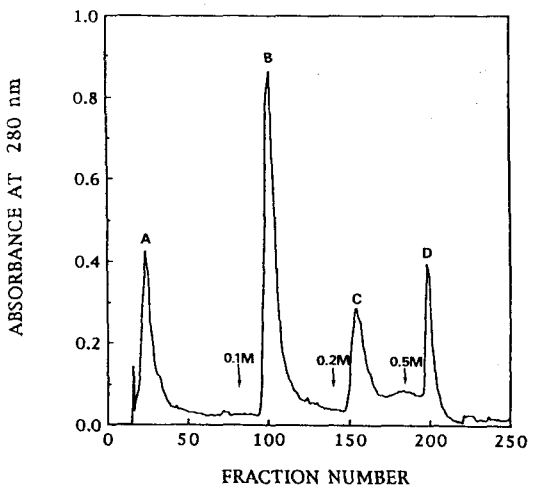

Fig. 1. Chromatography on CM-Sepharose. Crude streptodornase solution was applied on CM-Sepharose column (1.5×30 cm) equilibrated with 20 mM phosphate buffer, pH 7.5. The enzyme was eluted with stepwise salt gradient. The specific activity (×1000 units/mg) of elution peak was as follows. Peak A; 21, B; 37, C; 487, D; 58.

Ammonium sulfate fractionation

Large scale preparation of SD in pharmaceutical industry considers the ammonium sulfate (A/S) fractionation after taking out the broth from the culture tank. Compared to membrane processing, there are lots of advantage such as decrease of processing time and bulky broth in A/S precipitation processing step. To establish the optimum A/S fractionation range, at each addition of A/S, its remained activity of SD were determined. Fig. 3 shows that most SD was recovered between 40% and 60% cut.

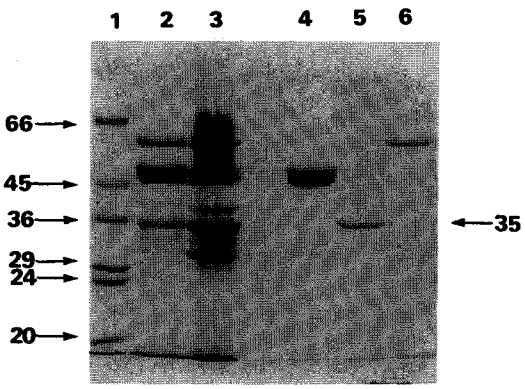

Fig. 2. SDS-PAGE of streptodornase isolated from *Streptococcus* sp. Lane 1; molecular weight marker, BSA (66 KDa), egg albumin (45 KDa), phosphate dehydrogenase (36 KDa), carbonic anhydrase (29 KDa), trypsinogen (24 KDa), trypsin inhibitor (20 KDa). Lane 2; crude extracts, 3; ammonium sulfate precipitate, 4; 0.1 M elution fraction, 5; 0.2 M elution fraction, 6; 0.5 M elution fraction.

Effect of temperature and pH on enzyme activity

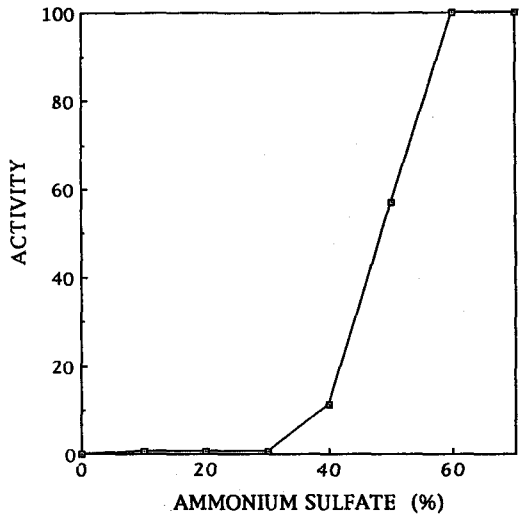

Fig. 3. Ammonium sulfate precipitation of the purified streptodornase. Enzyme activity of ammonium sulfate precipitate was detremined after addition of various amount of ammonium sulfate.

Table 1. Purification of streptodornase isolated from *streptococcus equsimilis*

Sample	Vol (ml)	Protein (mg)	Units	Specific activity (units/mg)	Purification folds	Yield (%)
A.*						
After U/F processing	200	427	1,654,000	3,900	1	100
After 1st CM	60	6.8	973,000	143,000	37	59
B.**						
Crude SD***	148	397	29,088,000	73,000	1	100
A/S precipitate	34.5	203	17,768,000	88,000	1.2	61
After 2nd CM	153.5	28	7,712,000	274,000	3.8	27

* Typical pattern of 1st CM-Sepharose processing.

** 2nd CM-Sepharose processing for optimization of SD production.

*** This was prepared from SD powder, which was obtained by lyophilization after 1st CM-Sepharose processing.

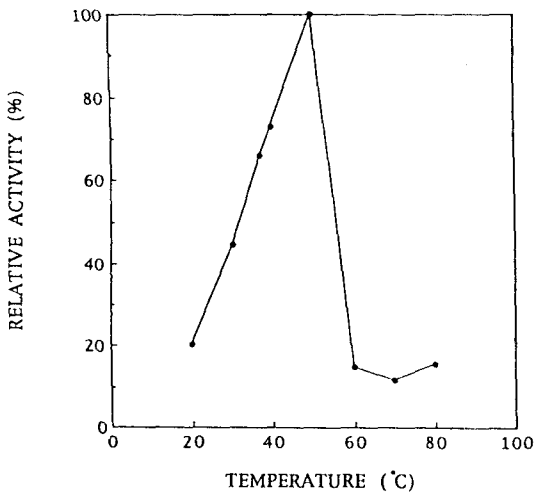

Fig. 4. Effect of temperature on the enzyme activity. The enzyme activity was measured at various temperature.

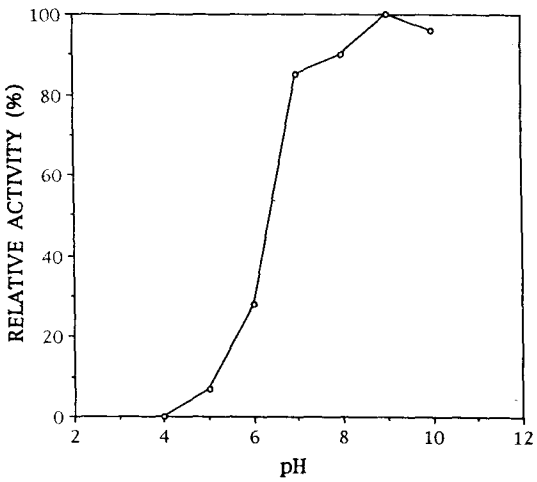

Fig. 5. Effect of pH on the enzyme activity. The enzyme activity was determined at various buffer systems mentioned in the text.

The effect of tempertaure on the enzyme activity is shown in Fig. 4. The SD activity was measured at various temperature. The optimum temperature was about 50℃.

Above 50℃, increase of temperature significantly decreased the activity. The effect of pH on the activity of the enzyme is shown in Fig. 5 The activity was measured at pH 4.0 to 10.0 using the various buffer systems. The optimum pH was around pH 9.0. And SD was quite active in the region of pH 7 through 10.

The molecular properties of the purified SD will be reported in the up-coming paper.

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Streptococcus sp.로부터 *Streptococcal nuclease*의 분리 정제

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초록 : *Streptococcal nuclease*가 *Streptococcus* sp. 발효액으로부터 stepwise CM-Sepharose 컬럼을 이용하여 완전히 분리 정제되었다. 역가 높은 효소 분획은 0.2 M 용리시 나왔고 분리 정제된 효소의 분자량은 35,000으로 전기영동상 밝혀졌으며 최적 pH와 온도는 각각 pH 9.0, 60℃ 로 밝혀졌다.