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Studies on the Isolation, Purification and Characterization of a Cx Enzyme Produced by *Pyricularia oryzae*, C-7^{+t}

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도열병균에서 추출한 Cx효소의 순화 및 특성에 관한 연구

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Abstract: The $(NH_4)_2SO_4$ (70%) treated crude enzymes from the culture filtrates of theC-7^{+t} strain of *Pyricularia oryzae* which was grown on 2% CMC (carboxymethyl cellulose) for 8 days at 28°C, were chromatographied on Sephadex G-150 and DEAE-Sephadex A-25 columns. From the chromatography, three fractions of CMCase(C_x) was examined using Na-CMC as substrate. The C_x enzyme activity was optimal at pH 6.0 and 40°C, stable up to 40°C. The values of Km and Vmax of the enzyme were 2.8×10 mM and 5.9m moles/hour, respectively. The molecular weight determined by Sephadex G-150 column chromatography was around 80,000. Approximately sevenfold purified C_x enzyme gave a single protein band on the polyacrylamide gel electrophoresis.

Introduction

Cellulose is the major constituent of all vegetation, comprising from one-third to one-half of dry-plant material.

As such, it is the world's most plentiful renewable resource. Still, most vegetation is unused by man or animals and undergoes natural decay through the intervention of microorganisms capable of producing cellulases (Hainy and Reese, 1969). Earlier studies were concentrated on the prevention of decay of manufactured materials containing cellulose, but more recent work has aimed at the utilization of cellulose to produce sugar or SCP(single cell protein) via chemical or enzymic hydrolysis (Kim, 1977). It is now well established that cellulase is a multi-component enzyme system.

Using various chromatographic and electrophoretic techniques, the complex has been resolved into its component parts and it has become apparent that there are at least three different types of enzyme in the complex (Wood, 1975). C_1 component was believed to "activate" or "de-aggregate" the cellulose chains in preparation for attack by the hydrolytic enzymes of the cellulase complex.

C_x component can hydrolyse soluble derivatives of cellulose or swollen and partially degraded celluloses. Carboxymethyl (CM) cellulose is normally used for the assay of C_x activity. β-glucosidase hydrolyze cellobiose and short chain cellooligosaccharides to glucose, but have no effect on cellulose(Wood, 1975). *Pyricularia oryzae* is a casual organisms of rice blast which has long been known as one of the most important diseases of rice plant in this country. Many biological studies have been carried out on the classification of *P. oryzae*.

Sudo *et al.* (1973) had studied intensively on the cellulases secreted from the *P. oryzae*, separating the C_1 and C_x components.

Hirayama *et al.* (1978) first purified a β-glucosidase, which was induced strongly in the culture

medium of a phytopathogenic fungus *P. oryzae* grown in a cellulase medium. In the present studies, we isolated and chracterized some properties of C_x component induced from the *P. oryzae* $C-7^{+t}$ race grown on carboxymethyl cellulose(CMC) as the carbon source.

Materials and Methods

Cell growth: *Pyricularia oryzae* $C-7^{+t}$ was obtained from the Plant Pathology Department, Insitute of Agricultural Science and grown at 28°C for several days on a solid potato dextrose agar slant containing 20g of dextrose, 1,000ml infusion of 300g potato and 15 g of agar. The preculture was carried out by shaking culture at 28°C for 3 days in the medium of the following composition: Medium I

KNO_3 3.0g, KH_2PO_4 0.5g, $MgSO_4$ 0.5g, $CaCl_2$ trace, glucose 5.0g, D.W. 1,000 ml

A 10 ml amount of precultured mycelial suspension in the media was inoculated into a 500 ml culture flask containing 200 ml of the following medium: Medium II

carboxymethyl cellulose 20.0g, yeast extract 2.5g, $(NH_4)_2$ SO_4 1.0g, $MgSO_4 \cdot 7HO$ 0.5g, D.W. 1,000 ml

It was cultured for 8 days at 28°C.

Enzyme extraction and purification: The 8 day old culture was filtered and ammonium sulfate was added to the filtrate to make a 70% (W/V) saturation.

The precipitate was collected and dissolved in an acetate buffer (0.05M, pH 5) and dialyzed overnight against the same buffer by use of a dialysis sack. The dialyzate was chromatographed on Sephadex G-100 column equilibrated with the same buffer.

The gel filtration eluents were passed through DEAE-Sephadex A-25 column equilibrated with 0.05M, pH 8.3, Tris-HCl buffer.

Elution was carried out with an NaCl gradient using a two-chamber device, one contained 0.1M NaCl solution, the other 0.4 M.

Enzyme assay: (a) C_1 enzyme activity with 1% avicel(W/V) as the substrate was assayed by measuring the increase in the amount of reducing sugar according to the Somoigyi-Nelson method (Nelson, 1944; Somogyi, 1952). Glucose was used as a standard.

(b) C_x enzyme activity with 0.6% Na-CMC(W/V) as the substrate was assayed by the same method as used for C_1 enzyme activity.

(c) β-glucosidase activity with 0.1% salicin(W/V) as the substrate was assayed by the same method as used for C_1 enzyme activity. One unit of enzyme activity. was defined as the amount of enzme that released 1 mole of glucose from the standard assay condition. Specific activity of enzyme was expressed as units per mg of protsin present in the enzyme solution.

Determination of Protein: The relative amounts of protein in eluents of column chromatography were estimated at 280 nm with Beckmann Spectrophotometer DB-G. For the analysis of the specific activities of enzymes, the amount of protein was determined by the method of Lowry *et al.* (1951).

Estimation of molecular weight of C_x enzyme: The peak fractions in enzyme activity of the DEAE-Sephadex A-25 column eluents were concentrated by the Amicon PM-10 membrane and the molecular weight of the enzyme was determined by filtration of the concentrates through Sephadex G-150 column (2.3×65 cm) equilibrated with acetate buffer(0.05 M, pH 5.0).

Elution of the column was carried out at a flow rate of 12 ml per hour with the buffer, and 30 tubes of 4.5 ml fractions were collected in cold (4°C). After measurement of the peak enzyme activity in the fractions, the partition coefficient(K_{av}) was calculated. The molecular weight of C_x enzyme was estimated with its K_{av} values calculated on the standard curve of the marker proteins (Sigma).

Polyacrylamide gel electrophoresis: Disc gel electrophoresis was carried out on a 7.5% polyacrylamide gel with bromophenol blue as a tracking dye. A current of 3mA/gel was applied for approximately 5 hour at room temperature.

The gel was stained with 1% Amidoblack in the 7% acetic acid and destained by allowing to stand

in several changes of 7% acetic acid solution for 24 hours.

Results

Production of extracellular cellulases of *P. oryzae*: Fig. 1 illustrates the production of 3 extracellular cellulases during the 13 day culture of *P. oryzae* in the media at 28°C. The production of C_x enzyme and β-glucosidase appear quite closely parallel each other compared to that of C_1 enzyme. The C_x enzyme activity reached a maximum after 10 days, and C_1 enzyme and β-glucosidase activity reached a maximum after 9 and 11 days, respectively.

Effects of heat, temperature, pH and incubation time on cellulase activity: As shown in Fig. 2, the optimal heat stability of the 3 assayed cellulase activities were all at 40°C. The optimal temperature of C_x enzyme activity was at 40°C, while that of C_1 enzyme and β-glucosidase was at 50°C (Fig. 3). The optimal pH of C_x enzyme activity was at 6 and that of C_1 enzyme and β-glucosidase was at 5. (Fig. 4). Fig. 5 shows the optimal incubation time of C_1, C_x and β-glucosidase activities.

During 120 minute incubation the C_1 enzyme and β-glucosidase continued to be active with the former being more potent, whereas the C_x enzyme reached a maximum activity at 80 minute and the activity was tapering off thereafter.

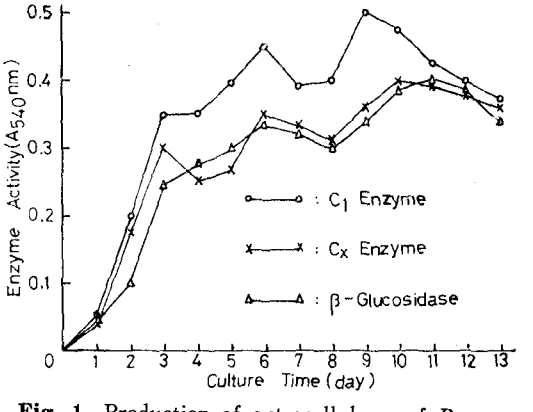

Fig. 1. Production of extracellulases of *P. oryzae*. The enzyme activities during 13 days were determined at pH 5.0 by measuring the amount of reducing sugar which was produced after incubation of the mixture of enzyme and subatrate for 1 hour at 50°C.

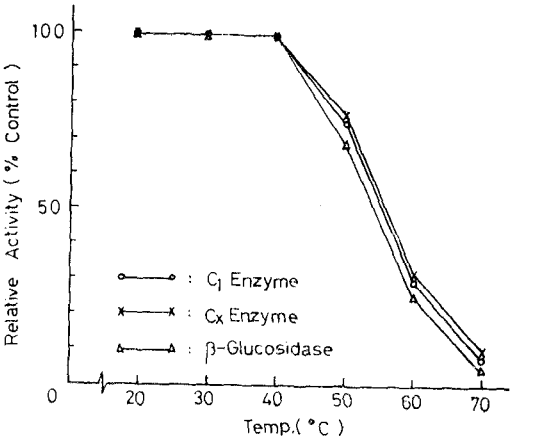

Fig. 2. Heat stability of cellulase activities of *P. oryzae*. The reaction mixtures consisting of 0.2ml of 0.1m acetate buffer, pH 5.0, and 1.0ml of enzyme solution were allowed to stand for 30min at each temperature, and enzyme activities were determined. The enzyme activities measured at 40°C were the control.

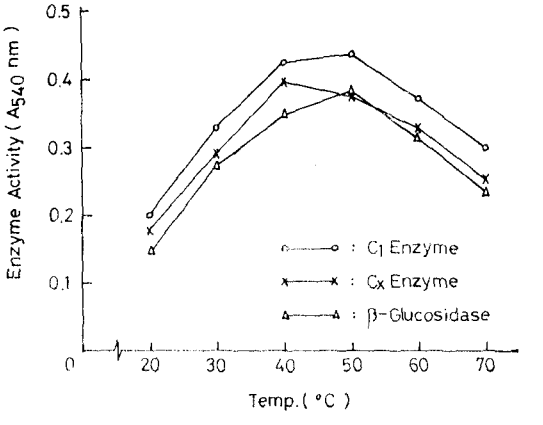

Fig. 3. Effect of temperature on cellulase activity of *P. oryzae*. The enzyme activity was determined at pH 5.0 for 1 hour incubation at various temperatures.

Purification of C_x enzyme: Step. 1-Salt precip itation. Fig. 6 is a flow diagram of the enzyme purification. After cultivation of *P. oryzae* on CMC for 8 days, the culture filtrate was treated with 70%

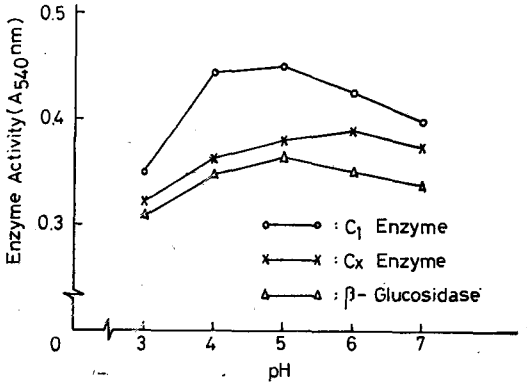

Fig. 4. Effect of pH on cellulase activity of *P. oryzae*. Buffers of 0.1M acetate(pH 3.0, 4.0, 5.0) and 0.1M phosphate(pH 6.0, 7.0) were used to measure the enzyme activity at 50°C after 1 hour incubation.

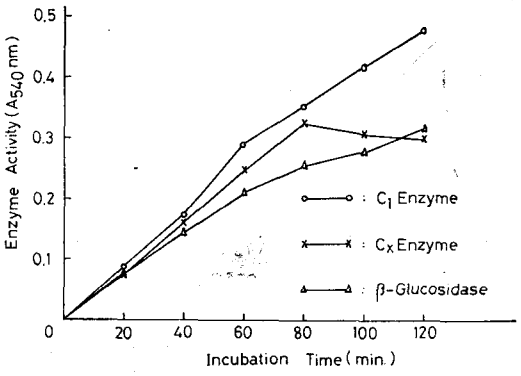

Fig. 5. Effect of incubation time on cellulase activity of *P. oryzae*. The enzyme activities were determined at 50°C, pH 5.0, with various incubation time intervals.

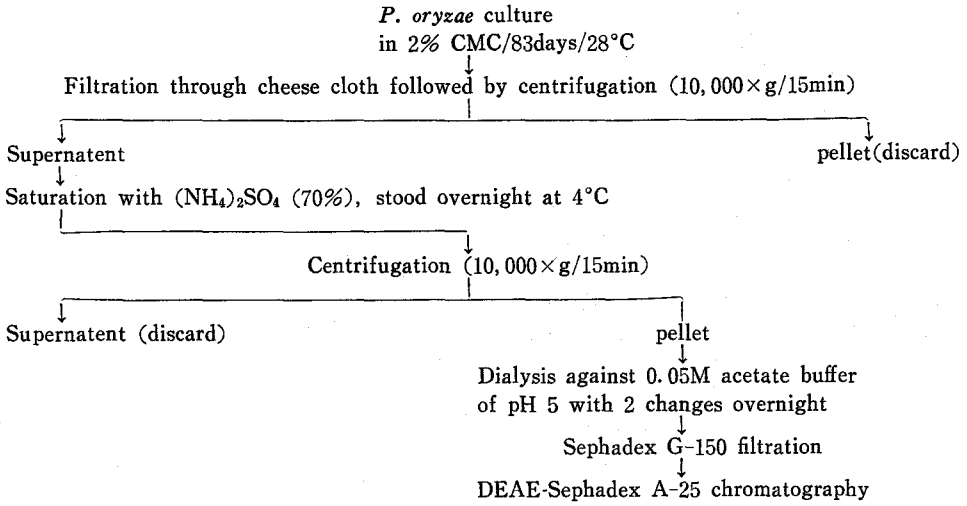

Fig. 6. A flow diagram of purification from C_x enzyme.

ammonium sulfate and stood overnight in cold. The precipitate was collected by centrifugation and dissolved with small amount of 0.05M, acetate buffer of pH 5.0, and then dialyzed against the same buffer overnight.

Step 2-Gel filtration. The column was packed with Sephadex G-150 and equilibrated overnight with 0.05M, acetate buffer of pH 5.0 before the dialyzate was applied on the column. As shown in Fig. 7, two protein peaks were appeared on the column chromatogram. Using different enzymatic subtsrates, several different enzymes could be separated; three CMCase(C_x)(F_1, F_2, F_3), an avicelase(C_1) and a β-glucosidase.

Step. 3-Ion exchange chromatography. The F 3 portion in Fig. 7 was pooled and concentrated by the Amicon PM-10 membrane.

The concentrates was used for further purification through DEAE-Sephadex A-25 column chromatography with NaCl gradient. Two protein peaks were appeared. Upon analysis of protein and enzyme activities, the first peak had only low specific activities indicating that this protein was escaped from the column unadsorbed, whereas the 2nd peak was the

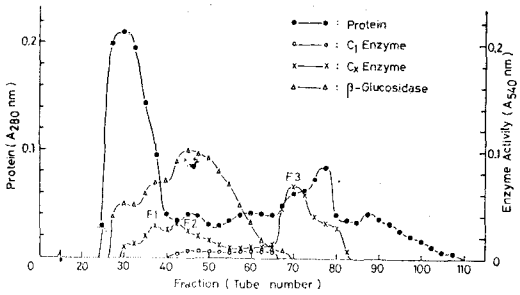

Fig. 7. Chromatography of crude enzyme on a Sephadex G-150 column.
Sample volume: 5ml
Flow rate: 12ml/hour
Column size: 2.3×65cm
Fraction volume: 3ml

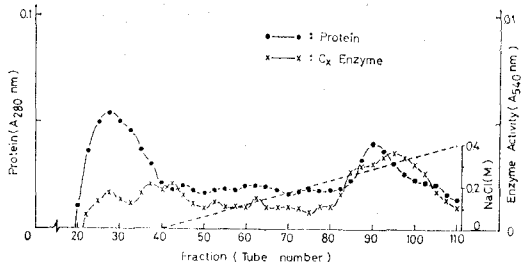

Fig. 8. Chromatography of F 3 on a DEAE-Sephadex A-25 column.
Sample volume: 15ml
Flow rate: 22.5 ml/hour
Column size: 2.3×25cm
Fraction volume: 4.5ml

Table I. Purification of C_x enzyme from *P. oryzae*, C-7.

Step	Total protein (mg)	Total activity (unit)	Specific activity (units/mg protein)	Yield of protein (%)	Recovery of activity (%)	Purification
Culture filtrate	3,000	20,833	6.9	100	100	1
$(NH_4)_2SO_4$(70%)	320	3,472	10.8	10.6	16.6	1.5
Sephadex G-150	80	2,416	30.0	2.6	11.5	4.3
DEAE-Sephadex-A-25	37	1,819	49.1	1.2	8.7	7.0

true enzyme protein which was active on CMC (Fig. 8).

The enzyme was purified about seven fold through the steps of purification as shown in Table 1.

Polyacrylamide gel electrophoresis: After each purification step, aliquots were analyzed by electrophoresis on polyacrylamide gel. Fig. 11 shows that the patterns obtained at different stages of the purification procedure. After first stage of purification the enzyme extract contained five components, and after second stage two components, and after the third stage, only one band.

Km, Vmax and molecular weight of C_x enzyme: The Michaelis constant was estimated with partially purified enzyme on a Lineweaver Burk Plot. The values of Km and Vmax of C_x enzyme were 2.8×

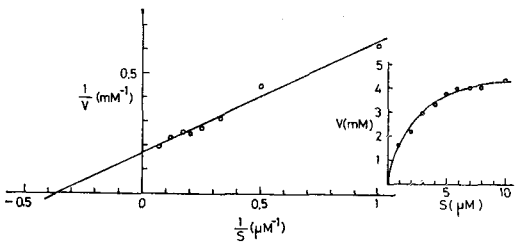

Fig. 9. A Lineweaver-Burk plot of C_x enzyme.
Calculated values of Km: 2.8×10mM,
and V_{max}: 5.9 mmoles/hour

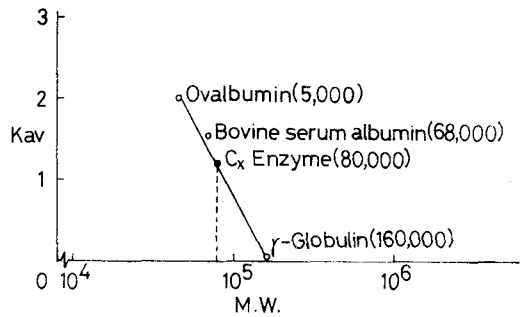

Fig. 10. Estimation of the molecular weight of C_x enzyme by the Sephadex G-150 column chromatography.
The molecular weight of the C_x enzyme determined was around 80,000.

10mM and 5.9m moles/hour on 0.6% Na-Cmc as substrate(Fig. 9). The molecular weight of the enzyme estimated by gel filtration method was about 80,000.

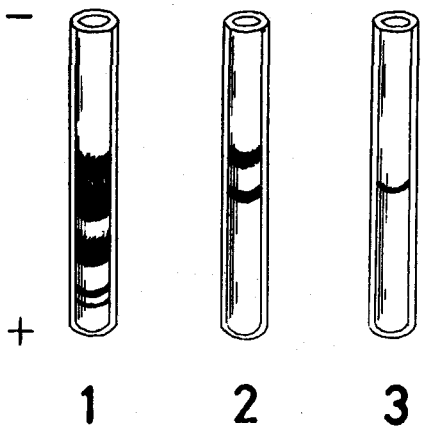

Fig. 11. Electrophoresis of C_x enzyme from *P. oryzae*. (24mA/75V)
Samples were taken
1. After$(NH_4)_2SO_4$(70%) precipitation,
2. After Sephadex G-150 column chromatography,
3. After DEAE-Sephadex A-25 column chromatography.

A single band of protein(enzyme) is seen in 3.

Discussion

The cellulase of *P. oryzae* was induced by the presence of carboxymethyl cellulose (CMC) in the medium and the exoenzyme in the culture filtrate was separated by Sephadex G-150 chromatography. From the gel filtration, three fractions of CMCases (C_x), a fraction of avicelase(C_1) and a fraction of β-glucosidase were identified. According to Sudo *et al.*(1973), 5-6 components of C_x enzyme were detected both in N-1 and C-3 strains grown on the rice plant powder as the carbon source.

Hirayama *et al.*(1976) reporten separation of 3 extracellular- and 2 intracellular C_x enzymes, while Cho and Kim(1981) reported separation of 2 CM Cases from T-2 strain cultured on CMC. In this connection, it may be concluded that cellulase is consisted of a multicomponent enzyme system.

In the present study, the optimum pH and temperature for the activity of C_x enzyme were at pH 6.0 and 40°C on CMC as substrate. Sudo *et al.*(1973) reported that cellulase activities of *P. oryzae* T-1, C-3, N-1 and 0 strains were optimal at pH 5.5 and 45°C, pH 5.2 and 48°C, pH 5.0 and 40°C and, pH 4.5 and 50°C, respectively. The data of optimal pH and temperature we obtained from enzymes of *P. oryzae* $C\text{-}7^{+t}$ were relatively high compared with data reported by Sudo *et al.* on the same enzyme substrates. It may be due to the fact that fungal strains used in two laboratories were different.

The Km value of C_x enzyme obtained in the present experiment was 2.8×10mM. Chun(1979) reported that the Km value of crude cellulase from *P. oryzae* N-2 strain was 1.05×10mM on Na-CMC as substrate. Recently, Cho(1981) reported that the Km value of crude CMCase from *P. oryzae* $T\text{-}2^{+t}$ was 1.1×10mM. The Km values appeared to be widely variable by the difference of enzyme sources and the substrates applied.

The Km value of the C_x enzyme we determined in the present paper was much higher than the values obtained by Chun(1979) and Cho(1981) in the previous reports, indicating higher enzyme-substrate affinity. This may be caused by the purer enzyme we prepared relative to the enzymes assayed by the others.

The molecular weight of C_x enzyme we determined was around 80,000 using Sephadex G-150 column chromatography.

Widely different data of molecular weight of C_x enzyme from different sources of microorganisms have been reported; The molecular weight of the C_x components of *Trichoderma viride*, *Trichoderma koningii*, *Fusarium solani*, and *Penicillium funiculosum* lie in the region 45,000~75,000, with the exception of the low molecular weight(13,000) C_x component which was removed from *T. koningii* cellulase on Sephadex G-75.

The smallest C_x yet found had a molecular weight of only 5,300. No report has been made on the molecular weight of C_x enzyme produced from *P. oryzae;* the higher value of the molecular weight of

C_x enzyme produced from other fungi was 75,000 which is significantly lower than the value we obtained in the experiment, 80,000.

The purified C_x enzyme was homogeneous on polyacrylamide gel electrophoresis and showed an approximately 7 fold increase of specific activity. Fungal as well as bacterial cellulases have often been reported to be present in multiple forms.

It is therefore of interest that at least in the culture filtrate of *P. oryzae* a single molecular species seems to be present after the present purification procedures were followed. This results demonstrate that a cellulolytic enzyme from the fungus *P. oryzae* can be purified by chromatographic and electrophoretic methods.

摘 要

2% CMC (Carboxymethyl cellulose)를 탄소원으로 하여 28°C에서 8일간 배양한 도열병균 (*Pyricularia oryzae*, C-7^{+t})의 배양액을$(NH_4)_2SO_4$ 염석, Sephadex G-150 및 DEAE-Sephadex A-25 column chromatography를 거쳐 순화하였다. 순화결과 F_1, F_2, F_3 3개의 CMCase (C_x), 1개의 Avicelase (C_1) 및 1개의 β-glucosidase를 얻었는데 그중 C_x 효소의 F_3만을 골라 실험을 계속했다. 이 효소의 활성도는 pH 6.0과 40°C에서 가장 높았으며 40°C까지 안정한 효소인 것을 알 수 있었다.

이 효소의 Km과 Vmax값은 각각 2.8×10^{-3}mM, 5.9 mmoles/hour이었고, 분자량은 Sephadex G-150 column chromatography에 의해 약 80,000으로 나타났다.

약 7배로 순화된 이 효소의 순화정도를 polyacrylamide gel electrophoresis에 의해 검증한 결과 1개의 band를 나타냈다.

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