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Purification and Properties of an Extracellular Chitinase from *Streptomyces* sp.

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Streptomyces 속 균주로 부터 생산되는 Chitinase의 정제 및 그 성질

홍 용 기·서 정 훈

(1979년 6월 11일 수리)

Abstract

Streptomyces sp. 115-5 was selected as the most active microorganism of about 200 strains for the production of chitinase. The enzyme was purified by $(NH_4)_2SO_4$ treatment, lst-Sephadex G-100, DEAE-Cellulose, 2nd-Sephadex G-100 column chromatography, and evidence for homogenity was obtained from CM-Sephadex C-50 column chromatography and polyacylamide gel electrophoresis. The purified enzyme hydrolyzed chitin (N-acetyl glucosamine polymer) and chitosan (glucosamine polymer) but not cellulose. And with chitin as the substrate, a Km value of 3.6 mg of chitin per ml and a Vmax of 100 μmole per hr were found.

The activation of the chitinase was 3.66 kcal per mole. The molecular weight of the enzyme was estimated about 56,000 daltons by Sephadex G-100 chromatography and isoelectric point as pH 3.0.

Introduction

Chitin is found in the exoskeleton of arthropods, and also in fungi, about 3~5 % of chitin in the cellwall[1,2]. Thereə havebeen many studies on the enzyme responsible for the decomposition of chitin. Namely, chitinase was found in animals such as snals, custacea, *etc.* [1], and in microoranisms; *Serratia marcescens*[3], *Streptomyces sp.* [4,5], black-koii mold [6], *Phycomyces blakesleeanus*[7], *etc.*. A few studies on the chitosanase that was produced by *Myxobacter*[8], *Streptomyces*[9], *etc*, have, have been made. On cellulase there have been many reports up to now. In addition, there are some reports[10~15] on the lysis of fungal cell wall.

Our works were carried out on the chitinase produced by *Streptomyces* that is able to use in food and feed industry, and as an insecticidal aid.

In the previous papaer[16], the production and some properties of chitinase by *Streptomyces* sp. 115-5 was described. We present here the purification and the other properties of an extracellular

enzyme which has both chitinase and chitosanase activities.

Materiais and Methods

Microorganism.

About two hundreds of *Actimomycetes* strains from the culture collection of our laboratory were used for the screening test to produce chitinase. The greatest strain, *Streptomyces* sp. 115-5 was used for the following studies.

Cultural condition.

The culture medium for *Streptomyces* sp. 115-5 was 0.5% glucose, 0.2% chitin, 0.2% peptone, 0.1% K_2HPO_4, 0.05% $MgSO_4$ $7H_2O$, 0.05% NaCl. The initial pH was adjusted to 7.2. For the production of chitinase, cultivation was carried out in a 500 ml Sakaguchi flask containing 100 ml of the above medium on reciprocal shaker at 30°C for 48 hrs.

Preparation of Chitin and Chitosan.

Chitin was prepared by the treatment of conc-HCl as discribed previously[16]. Chitosan, a 97 % N-deacetylated procuct, was obtained from the above chitin by the aqueous sodium hydroxide procedure[17] described by Horton and Lineback.

Assay of Chitinase activity.

Chitinase activity was measured in a standard assay mixture which contained: 0.05M phosphate buffer (pH 7.0) 2ml, a suitably diluted enzyme solution 1 ml, 4 mg of chitin.

The mixture was incubated at 50°C for 1hr. One ml of sample was taken to assay for the reducing sugar that was released during incubation. The amount of reducing sugar resulting from the enzymatic hydrolysis was determined by the Somogyi-Nelson method[18], using glucose as standard. One unit of the chitinase is defined as that amount of enzyme which releases 1 μmole of reducing sugar equivalent, expressed as glucose per hr.

Determination of Protein.

Protein concentration of an enzyme preparation was estimated by measuring the absorbance at 280 nm using Hammarsten milk casein as a standard.

Preparation of crude enzyme.

Cellular material and remained substrate were removed from the culture fluid by centrifugation. Ammonium sulfate was added to the supernatant to the concentration of 0.6 saturation, precipitate was collected and dissolved in 0.01 M phosphate buffer (pH 7.0) solution, then dialyzed against the same buffer at 4°C for 1 day.

Materials used for Concentration and Purification of Chitinase.

The materials used for these processes were as follows: pressure ultrafiltration apparatus (Amicon design), Sephadex G-100 (Pharmacia Fine Chemicals), Diethylaminoethyl cellvlose (Nakarai Chemicals), GM-Sephadex C-50 (Pharmacia Fine Chemicals).

Determination of molecular weight.

Molecular weight of the purified enzyme was estimated by the method of Andrews[19]. with a column of Sephadex G-100 (2.5×65cm) about 6 mg amount of each protein was used and runned at a flow rate of 10 ml per hr. The reference proteins used were Human serum albumin (M. W. 68,500), Ovalbumin (M, W 45,000), Trypsin (M. W 24,000).

Electrophoresis on Polyacrylamide gel.

Polyacrylamide gel disc electrophoresis of the purified enzyme was carried out essentially by the method of Davis[20,21] with 7% polyacrylamide gel and 25 mM tris (hydroxymethyl) aminomethane-192 mM glycine buffer (pH 8.3).

Determination of isoelectric point.

Isoelectric point of the purified enzyme was determined by the paper electrophoresis, with variable buffer solution.

Toyo-filter paper No. 52 was used. Electropho-

resis was performed for 1 hr with a current of 3 mA per paper. Migrated protein spot was stained with 0.1 % ninhydrine.

Results

Purification of Chitinase.

All procedures were carried out below 4°C unless otherwise stated.

Ammonium sulfate precipitation.

To the supernatant removed cellular material and substrate, 0.6 saturation of ammonium sulfate was treated. The precipitate obtained by centrifugation (10,000 rpm, for 10 min) was dissolved and dialyzed against the 40-fold volume of 0.01 M phosphate buffer as described in methods.

lst-Gel filtration on Sephadex G-100.

The dialyzed enzyme was applied to gel filtration on a column of Sephadex G-100 (2.5×65 cm) which had been equilibrated with 0.01 M sodium phosphate buffer (pH 7.0). The column was eluted with the same buffer solution at a flow rate of 15 ml per hr and the effluent was fractionated into 5 ml portions. A typical chromatographic pattern is presented in Fig. 1 with the peak of enzymatic activity and the absorbance peak at 280nm. It appears as if two other chitinase peaks (fraction No. 36-48 and No. 49-69) existed, but fraction 49 to 65 were pooled and concentrated by ultrafiltration in an Amicon cell with a O1T mebrane.

Column chromatography on DEAE-cellulose.

The concentrated enzyme was applied to a column (1.8×18 cm) of DEAE-cellulose which had been washed previously with 0.1 N sodium hydroxide and equilibrated with 0.2 M tris (hydroxymethyl) aminomethane (Tris) buffer (pH 9.0).

The enzyme was eluted with the same equilibrating buffer. Fractions of 5 ml were collected, at a flow rate of 60 ml per hr. The absorbance at 280 nm and the enzyme activity were measured. A typical elution pattern is shown in Fig. 2. Fractions (No. 7 to 16) with high specific activity were combined and concentrated by ultrafiltration.

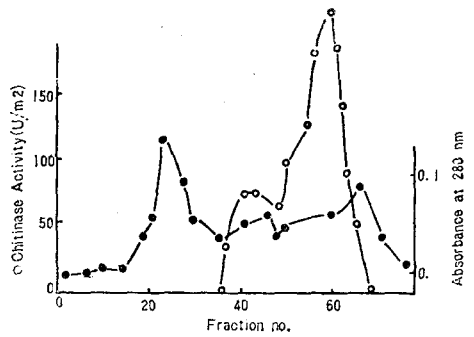

Fig. 1. Chromatography of Chitinase on Sedhadex G-100.
The column (2.5×65 cm) was equilibrated with the same buffer at a flow rate pf 15 ml per hr, and fraction ated 5 ml. Priotein was estimated by absorbance at 280 mn (•) and enyzme activity was estimated by the unit per ml (o).

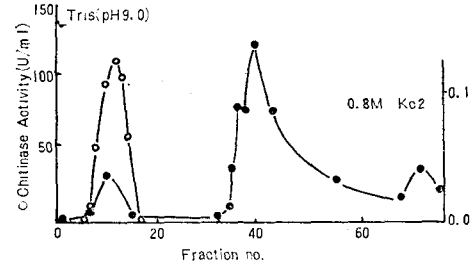

Fig. 2. Chromatograpy of Chitinase on DEAE-Cellulose.
The column (1.8×18 cm) was equilibrated with 0.2 M Tris buffer (pH 9.0). The enzyme was eluted with a stepwise addition of KCl. 5ml fractions were collected. Protein was estimated by absorbance at 280nm (•), enzyme activity was estimated by the unit per ml (o).

2nd-Gel filtration on Sephadex G-100.

The concentrated enzyme solution was applied to the column of Sephadex G-100 again and the enzyme was eluted with 0.01 M sodium phosphate buffer (pH 7.0) in the same manner as above. A typical elution is shown in Fig. 3, in which a symmetric protein peak was observed and the enzyme

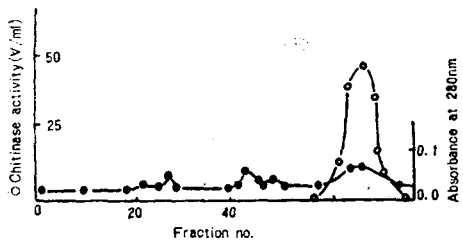

Fig. 3. 2nd Chromatography of Chitinase Sephadex G-100.

The column (2.5×65 cm) was equilibrated with 0.01 M-sodium phospate buffer (pH7.0). The enzyme was eluted with the equilibrating buffer at a flow rate of 10 ml per hr in the same manner as above. Protein was estimated by absorbance at 280nm (·) and enzyme-activity by the unit per ml (o).

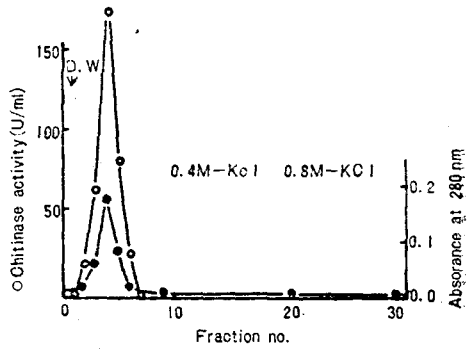

Fig. 4. Chromatograpy of Purified enzyme on CM-Sephadex C-50.

The column (1.9×11 cm) was. equilibrated with distilled water and eluted with a stepwise addition of KCl. Protein was estimated by absorbance at 280 mm (·), enzyme acti vity by the unit per ml (o).

activity agreed with the protein peak. The enzyme fractions were pooled, concentrated by ultrafiltration, and stored below 4°C.

Purification procedure and Efficiency

Chitinase activity measured by the amount of reducing sugar released from substrate chitin, and protein concentration were determined after each step. The values obtained are presented in Table 1.

The specific activity of the purified enzyme was 1328 units per mg of enzyme, and its purification fold was 422. The overall recovery was 36.4 %.

Purity of the Chitinase

The final enzyme preparation was dialyzed against distilled water and subjected to CM-Sephadex C-50 column chromatography and disc electrophoresis. A single protein band, indicating homogeneity,

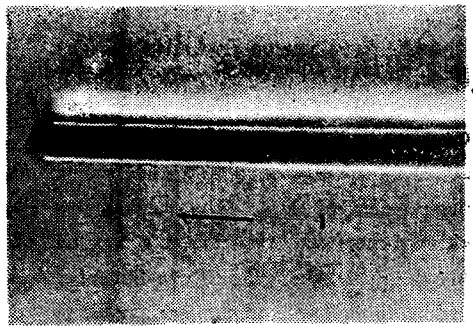

Fig. 5. Polyacrylamide Gel Electrophoresis of Purified Chitinase.

A 7% acrylamide gel was used in a Tris-glycine buffer (pH8.3). Samplecontaining about 240 μg of enzyme was mixed with concentrating gel solution and applied to the gel. Electrophoresis was performed for 3 hrs with a current of 3mA per tube. Gel was stained with amide black 10 B and destained with 7% acetic acid.

Table 1. Summary of the Purification of Chitinase

Purification steps	Vol (ml)	Total enzyme (u)	Total protein (mg)	Sp. Activity (u/mg protein)	Purification fold	Yield (%)
Culture sup't	480	6,000	1,908	3.14	1	100
60% $(NH_4)_2SO_4$	18.1	5,640	168	33.57	10.7	94
Sephadex G-100	24.3	4,129	21.4	193.07	61.4	67.8
DEAE-Cellulose	9.4	2,917	2.4	1214.36	386.2	48.6
Sephadex G-100	5.2	2,188	1.65	1328.33	422.2	36.4

was obtained on column chromatography and 7 % poly acrylamide gel electrophoresis, as shown in Fig. 4 and Fig. 5.

Michaelis-Menten Constant

With chitin as the substrate, the effect of substrate concentrations on the reaction rate of the enzyme was shown in Fig. 6. The Km value for hydrolysis of chitin 3.6 mg per ml, and Vmax was 100 μ-mole of reducing sugar per ml per hr.

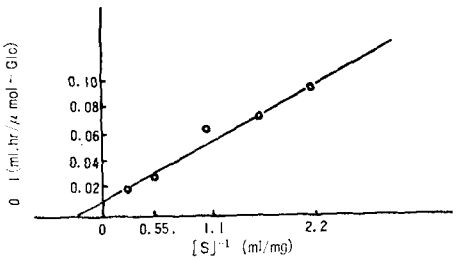

Fig. 6. Effect of Varying Chitin Concentrations on the Reaction Rate of Chitinase.
The 1 ml of purified chitinase (85 units perml) was incubated with the 2 ml of the indicated concentration of chitin, and the velocity was calculated as μmole of reducing sugar per ml per hr.

Activation Euergy for the Hydrolysis of Chitin Catalyzed by Chitinase

The temperature-enzyme activity relationship was examined at 25°C to 45°C. From Arrhenius equation: $k=A.e^{-Ea/RT}$, converting to logarithmic form, we have $\ln k=-\frac{Ea}{RT}+\ln A$. The plots of log k versus 1/T yield energy from the slope of the curve. The activation energy from the slope was calculated to be 3.66 kcal per mole, as shown in Fig. 7.

Molecular weight of the Chitinase

The molecular weight of chitinase was estimated by the gel filtration on Sephadex G-100. From the results shown in Fig. 8, the molecular weight was estimated as about 56,000.

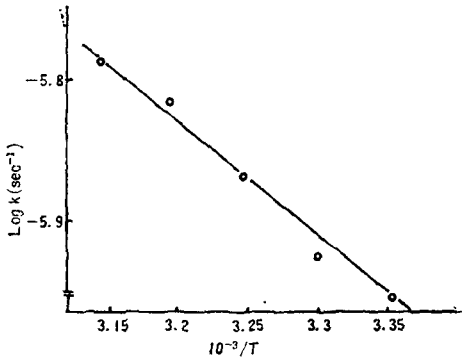

Fig. 7. Arrhenius plot of Chitinase Activity.
Amixture of the enzyme with chitin substrate was incubated at various temperatures at pH 7.0 for 1 hr, and the hydrolysis velocity was determined as μmole of reducing sugar per sec.

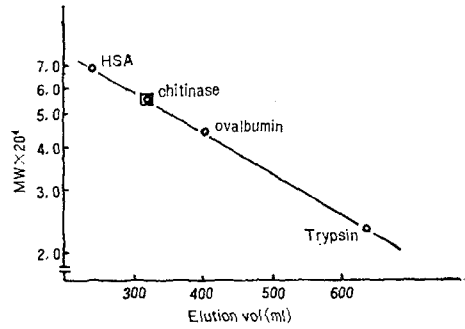

Fig. 8. Determination of the Molecular Weight by Sephadex G-100 Column Chromatography.
About 6 mg of each protein was applied to the column (2.5×65cm) and eluted with 0.01 M sodium phosphate buffer (pH 7.0) containing 0.2 M NaCl below 4°C. The position of chitinase is indicated by a square. The markers and their respective molecular weights were: A, Human serum albumin (68,500); B, Ovalbumin (45,000); C, Trypsin (24,000).

Isoelectric Point of the Chitnase

The isoeleciric point of the chitinase was determined by the paper electrophoresis method. The

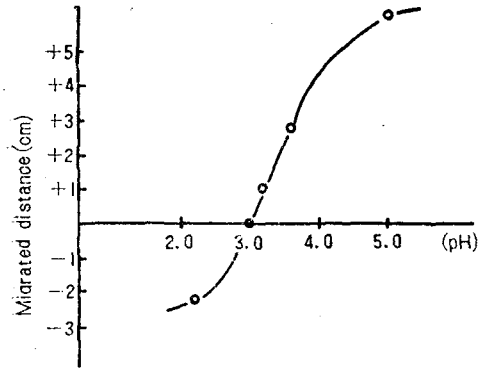

Fig. 9. Determination of the Isoelectric Point of Chitinase.

Paper electrophoresis was per formed for 1hr with a current of 3 mA per paper in various buffer: A, 0.05M Acetate buffer (pH 5.0); B-E, 0.05M McIlvaine buffer (pH 3.6, pH 3.2, pH 3.0, pH 2.2, respectively).

isoelectric point of the enzyme was estimated as 3.0 from no-migrating in the electric charge as shown in Fig. 9.

Substrate Specificity

Several polymers were tested as possible substrates for the enzyme. Only the chitin and chitosan were attacked, and cellulose powder, soluble starch, arabic gum were not attacked.

The enzyme showed a specificity for glucosamine and N-acetyl glucosamine polymers.

Table 2. Substrate Specificity of the Purified Chitinase

Substrate[a]	Relative rate[b]
Chitin	100
Chitosan	21.8
Cellulose powder	0
S-starch	0
Arabic gum	0

[a] The amount of each compounds were 4 mg, but chitosan was about 2 mg.

[b] The rate of hydrolysis was determined as relative to that of chitin.

Discussion

It permitted to purify to homogeneity by the above method described an enzyme which has an activity to the hydrolysis of chitin and chitosan, and has a reaction pattern of endotype hydrolysis.

Chitinase, chitosanase and cellulase are generally high specific enzymes. Of these enzymes, chitinase acts only on the β-1, 4-polymer of N-acetylglucosamine.

Removal of the acetyl group from chitin, chitosan and glycolchitosan, produce a nonsusceptible product (3, 5, 22). But the results presented here suggest that the purified chitinase is responsible for the hydrolytic activity on both N-acetylglucosamine polymer and glucosamine polymer. With the similarity between the chitin and chitosan, it is not unreasonable that one enzyme may attack both polymers.

In addition, Monreal and Reese[3] suggested that there is a prehydrolytic factor in the chitinolytic system (CH_1) as in the cellulolytic one (C_1) based on the susceptibility of crystalized versus swollen chitin. The purified chitinase in this work is assumed as the above prehydrolytic factor; as an enzyme which has endotype hydrolyzing pattern.

The magnitude of the activation energy which the *Streptomyces* sp. 115-5 chitinase-chitin system must surmount in order to pass from reactant to product was determined as 3.66 kcal per mole. In the Acid-chitin system, the activation energy for activated complex is known as 29.5 kcal per mole[1]. The chitinase-chitin system needs, thereforce, a unusually lower activation energy for the reaction with acid catalyst. The molecular weight of the chitinase was estimated as 56,000 daltons unlike the two chitinases, its molecular weights 25,000 and 33,000 respectively, described by Tominaga and Tsujisaka[5]. This finding raise interesting question whether *Streptomyces* sp. 115-5 chitinase is a dimer from one of the above two chitinases or a complex from chitinases or another chitinase with no relationship to the two chitinases. The purified chitinase was inhibited by product

(unpublished results). Addition of glucose, glucuronic acid, sorbitol and xylose inhibited the rate of chitinase action in the reaction mixture. Behaviour type of the product inhibition was the Mixed competitive-noncompetitive inhibiton in use of glucose as product inhibitor.

Acknowledgements

We are grateful to Dr. Inkoo Rhee for the experiments involving polyacrylamide gel electrophoresis and helpful discussions, and also to Dr. Bangho Song, and thank co-workers in our laboratory for technical assistance unceasingly during the couse of this research.

요 약

자연계에서 진균류와 절족동물의 외피를 이루는 주된 다당류인 chitin(N-acetyl glucosamine polymer)의 β-1, 4-lingkage를 가수분해하는 *Streptomyces* sp. 115-5 균주로부터 생성되는 chitinase를 정제하여 그 성질을 조사하였다.

48시간 진탕배양하여 생성된 chitinase를 ammonium sulfate처리, 1차 Sephadex G-100, DEAE-Cellulose, 2차 Sephadex G-100 column chromatography하여 정제하였으며 그 순도를 CM-Sephadex C-50 column chromatography 및 polyacrylamide gel electrophoresis로서 확인하였다.

이 chitinase는 chitin과 chitosan을 가수분해할수 있었으나 cellulose는 분해할수 없었고 chitin을 기질로서 사용하였을 경우 Km value가 3.6mg/ml이며 Vmax가 100 μmole/hr였다. Activation energy는 산 가수분해보다 훨씬 낮은 3.66 kcal/mole이었고 분자량은 Sephadex G-100을 사용한 column chromatography에서 56,000 daltons으로 나타났으며, 이 chitinase의 등전점은 pH 3.0에서 보여졌다.

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