Purification and Properties of β-1, 3-Glucanase from *Pseudomonas stutzeri* KF13

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Pseudomonas-stutzeri KF13의 β-1, 3-Glucanase 精製 및 性質

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ABSTRACT: An extracellular β-1, 3-glucanase from *Pseudomonas stutzeri* KF 13 was purified about 390 fold with 26% recovery. The purified enzyme revealed a single band by polyacrylamide gel electrophoresis and SDS-polyacrylamide gel electrophoresis. The enzyme was stable in a pH 6.0 to 9.0, and relatively thermostable. The optimal pH and temperature on the enzyme activity were found to be 5.8 and 45°C, respectively. The activation energy was calculated to be 16,130 cal per mole. The *Km* value for laminarin was found to be 3 mg per m*l* and the molecular weight was determined to be 28,000 by gel filtration and 26,000 daltons by SDS-acrylamide gel electrophoresis. The enzyme was inhibited by 1.0 mM of Hg^{2+}, and strongly inhibited by 1.0 mM of *p*-chloromercuribenzoic acid.

KEY WORDS □ *Pseud. stutzeri* KF 13, β-1, 3-glucanase.

The β-1, 3-glucanase(β-1, 3-glucan glucanohydrolase, EC 3. 2. 1. 6) was originally reported by Reese and Mandels(1959) and has been found in many microorganisms including fungi(Clarke and Stone, 1965), yeast(Abd-E-lAl and Phaff, 1968, 1969; Notario *et al*, 1976) and bacteria(Horikoshi *et al*, 1963; Tanaka and Phaff, 1965). The β-1, 3-glucanase is a hydrolytic enzyme which hydrolyzes selectively the β-1, 3-glucosidic linkage of β-1, 3-glucan which distributed in algae, soybeans, germinating cereal grains, almond kernels and cell walls of yeast and fungi(Whitaker and Smart, 1953; Manners *et al*, 1973; Misaki *et al*, 1968; Marumi and Takemi, 1983). This enzyme is an inducible enzyme which is induced by addition of β-1, 3-glucanous materials such as cell walls of microorganisms, laminarin and pachyman(Tanaka *et al*, 1974; Kobayashi *et al*, 1974).

The β-1, 3-glucanase has particularly been employed in the formation of yeast spheroplasts and the lysis of yeast cell walls, and in the lysis of fungal cell walls by treating alone or with the chitinase(Mann *et al*, 1972; Tanaka and Phaff, 1965; Fleet and Phaff, 1974; Tanaka *et al*, 1974; Kobayashi *et al*, 1974; Skujins *et al*, 1965). But the constructions of fungal cell walls are slightly different from

those of yeast. And it is difficult to hydrolyze the intact cell of fungi. Therefore, there are still many problems in the lysis of fungal cell walls.

Hence, the purification system and some properties of the purified β-1, 3-glucanase from *Pseudomonas stutzeri* KF 13 which was described in the previous paper(Chung *et al*, 1986) are reported in this paper.

MATERIALS AND METHODS

Strains

Pseudomonas stutzeri KF 13 which was isolated and identified in the previous paper(Chung *et al*, 1986) was used for the production of the β-1, 3-glucanase. *Aspergillus fumigatus* IFO 5840 was used for the production of cell wall as an inducer.

Cultural Conditions and Preparations of Crude Enzyme

The medium for the production of β-1, 3-glucanase was consisted of 3.5% cell wall, 15% (v/v) yeast autolysate and 0.05% $MnSO_4$ in 0.1 M phosphate buffer at pH 7.5. These cells were grown with constant shaking(7cm, 120 strokes/min) on a reciprocal shaker at 30℃. After 24 hours of incubation, about 1 m*l* of the culture was transferred into a 500 m*l* shaking flask containing 100 m*l* of the same medium. The cells were then grown at 30℃ with constant shaking for 72 hours. After culture, the cells and other debris were removed by centrifuge at 10,000 rpm for 10 min(Hitachi 20 PR-52D, Japan). The supernatant was used as a crude enzyme solution.

Enzyme Assay

The β-1, 3-glucanase activity was based on the measurement of reducing sugar librated from laminarin as a substrate. The reducing sugars were assayed colorimetrically using dinitrosalicylic acid reagent(Sumners and Somers, 1949). The standard reaction mixture containing 0.1 M succinate buffer(pH 5.8) 1.0 m*l*, laminarin(6 mg/m*l*) 0.5 m*l* and enzyme solution 0.5 m*l* was incubated at 45℃ for 5 min. One unit of the enzyme was defined as the amount of the enzyme that released reducing sugar equivalent to μ mole of glucose per m*l* per min. Specific activity was expressed as the amount of enzyme per mg of protein.

Determination of Protein

Protein was determined according to the method of Lowry *et al*(1951) using crystalline bovine serum albumin as a standard.

Acrylamide Gel Electrophoresis

Analytical disc gel electrophoresis in 7.5% polyacrylamide gel at pH 8.3 using Tris-glycine buffer was performed according to the method of Davis(1964). About 60 μg of enzyme proteins were applied to each tube(0.5×10cm), and electrophoresis was carried out for 3 hours at 4℃ with a constant current of 2.5 mA per tube. After the run, the gels were stained with 0.25% Coomassie brilliant blue R-250 and destained in the solution containing 8% acetic acid and 25% methanol.

Activation Energy

The activation energy of β-1, 3-glucanase was calculated between 30 and 40℃ by the equation of Arrhenius(Segel, 1976).

Molecular Weight

The determination of molecular weight was carried out according to Andrews(Morris and Morris, 1976) by gel filtration on a Sephadex G-100 column(1.8×100cm) equilibrated with 0.02 M phosphate buffer(pH 7.2). The enzyme and maker proteins were eluted with the same buffer at a flow rate of 3 m*l* per hours.

SDS-polyacrylamide gel electrophoresis in the presence of 0.1% SDS was performed according to the method of Weber and Osborn (1969) on 10% acrylamide gels(0.5×10cm). Electrophoresis was performed at a constant current of 8 mA per gel for 5 hours. After the run, the gels were stained and destained with the above conditions, and stored in 7.5% acetic acid solution.

Chemicals

DEAE-cellulose(Brown, 0.91 meq/g) was obtained from Nakarai Chemical Co., Tokyo. Sephadex G-100(fine), laminarin, bovine

serum albumin(MW 67, 000), ovalbumin(MW 43, 000), trypsin(MW 23, 300) and lysozyme(MW 14, 300) were obtained from Sigma Chemical Co., St. Louis, Missouri. Phosphorylase b(MW 94, 000), carbonic anhydrase(MW 30, 000), trypsin inhibitor(MW 20, 100), α-lactalbumin(MW 14, 400) and blue dextran(MW 3, 000, 000) were obtained from Pharmacia Fine Chemical, Uppsala. The other chemicals employed through this work were products of the certified reagent grade such as extra pure reagent.

RESULTS AND DISCUSSION

Purification of β-1, 3-Glucanase

All the purification steps were carried out at below 5℃ unless otherwise stated.

Step 1: Ammonium sulfate fractionation; The crude enzyme solution was fractionated by salting out with ammonium sulfate. To 760 m*l* of the crude enzyme solution the solid ammonium sulfate was added to give a 0.3 saturation by magnetic stirring. After standing for 3 hours, the precipitate was discarded by centrifuge at 10, 000 rpm for 10 min, and to the supernatant the ammonium sulfate was added to give a 0.65 saturation. After standing for 5 hours, the precipitate was collected, dissolved in the 0.02 M phosphate buffer(pH 7.2), and dialyzed for 24 hours against the same buffer.

Step 2: DEAE-cellulose column chromatography; The dialyzed enzyme solution(66 m*l*) was applied to a DEAE-cellulose column(2.5×50 cm) equilibrated with 0.02 M phosphate buffer(pH 7.2). The column was washed with 200 m*l* of the same buffer at the flow rate of 100 m*l* per hour. The enzyme was linearly eluted with 800 m*l* of the buffer solution increasing the concentration of NaCl to 1.0 M. Seven m*l* of the fractions were collected. Active fractions, eluted at 0.2 M to 0.4 M NaCl, were collected and concentrated by the addition of solid ammonium sulfate to be 0.7 saturation. The precipitate, obtained by centrifuge, was dissolved in a minimal volume of the same buffer.

Step 3: Sephadex G-100 gel filtration; The concentrated enzyme solution(2 m*l*) was applied to a Sephadex G-100 column(1.8×100 cm) equilibrated with 0.02 M phosphate buffer(pH 7.2). The buffer was allowed to flow at a rate of 11 m*l* per hour and the fractions of 3 m*l* were collected.

As shown in Table 1, the β-1, 3-glucanase was purified about 390 fold with 26% recovery. The specific activity of the purified enzyme was 194.3 units per mg of protein.

The purification fold for the extracellular β-1, 3-glucanase from *Pseudomonas stutzeri* KF 13 was higher than 7 fold of *Basidiomycete* sp. QM 806 β-1, 3-glucanase(Huotari *et al*, 1968), 180 fold of *Bacillus circulans*(Horikoshi *et al*, 1963), 290 fold of *Penicillium italicum*(Sanchez *et al*, 1982), and 304 fold of *Rhizopus arrhizus* QM 1032(Clark *et al*, 1978), but the yield of recovery for that was lower than 70% recovery of *Rhizopus arrhizus* QM 1032 β-1, 3-glucanase.

Homogeneity of the Purified Enzyme

The purified enzyme preparation gave a single band on polyacrylamide electrophoretic gel as well as on SDS-polyacrylamide gel, as

Table 1. *Summary of the purification of β-1, 3-glucanase from Pseudomonas stutzeri KF 13.*

Procedure of purification	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification fold
Crude enzyme	7, 144	3, 729.7	0.5	100.0	-
Ammonium sulfate fractionation	636	2, 674.2	4.2	71.7	8.4
DEAE-cellulose chromatography	13	2, 000.0	153.8	53.6	307.6
Sephadex G-100 gel filtration	5	971.4	194.3	26.0	388.6

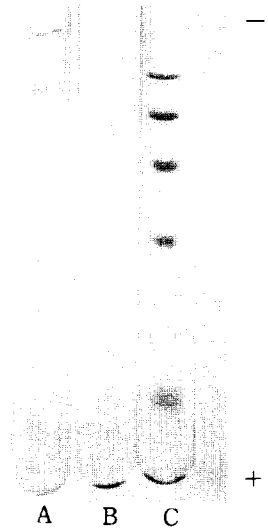

Fig.1. *Polyacrylamide gel electrophoresis of β-1, 3-glucanase in the absence(lane A) or presence(lane B and C) of sodium dodecyl sulfate.*

Lane A and B are the purified β-1, 3-glucanase. Lanc C is the standard proteins. The standard protins used and theii molecular weight are listed in Fig.6. Details are described in Materials and Methods.

shown in Fig.1. This results suggested that the enzyme was purified homogeneously.

Substrate Specificity

Several polymers were tested as possible substrates for the enzyme. The relative reaction rate of the enzymatic hydrolysis is presented in Table 2. The enzyme catalyzed highly the hydrolysis of laminarin, but slightly of starch and chitin. The enzyme showed a specificity for β-1, 3-glucosidic linkage.

Table 2. *Substrate specificity of the purified β-1, 3-glucanase.*

Substrate (6mg/ml)	Relative activity (%)
Laminarin	100.0
Corn starch	10.1
Chitin	6.4
Soluble starch	3.2
CMC	0.0
Cellulose	0.0

CMC; carboxymethyl cellulose.

Effect of pH and Temperature on Enzyme Stability

The effect of pH and temperature on enzyme stability were examined, and the results are shown in Fig.2. The enzyme was relatively stable in a pH 6.0 to 9.0 and up to 55℃, and then revealed the residual activity over 90%. The pH stability is similar to the results reported for *Flavobacterium dormitator* var. *glucanolyticae*(Nagasaki *et al*, 1976). The enzyme activity was remained only about 20% by heating at 65℃ for 10 min. But it showed slightly lower value than that of β-1, 3-glucanase from *Rhizopus* sp.(Yamamoto *et al*, 1974).

Effect of pH and Temperature on Enzyme Activity

As shown in Fig.3. the optimum pH and

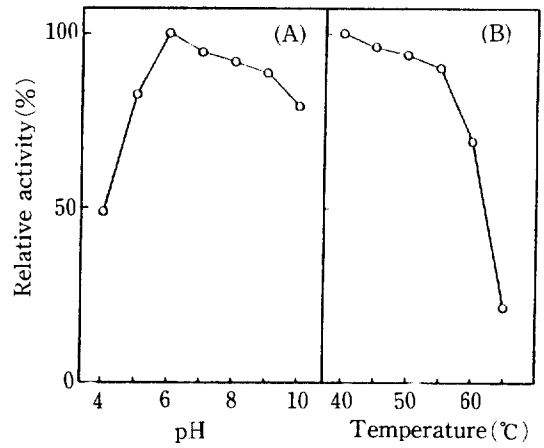

Fig.2. *The pH(A) and thermal(B) stability of β-1, 3-glucanase.*

(A); The enzyme solution was incubated in the indicated pH of buffers ranged from pH 4.0 to 10.0 at 35℃ for 10 min. After the pH was adjusted to pH 5.8 and then the residual activities were assayed under the standard conditions. Buffers used were McIlvaine buffer(pH 4.0 to 6.0), Sörensen buffer(pH 5.5 to 9.0) and 0.1 M Tris-HCl buffer(pH 9.0 to 10.0). (B); The enzyme solution in 0.1 M succinate buffer(pH 5.8) was incubated at the indicated temperatures ranged from 40 to 65℃ for 10 min. After being cooled, the residual activities were assayed under the standard conditions.

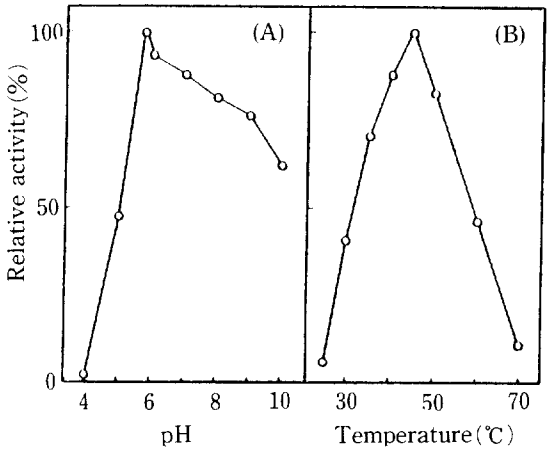

Fig.3. *Effect of pH(A) and temperature(B) on β-1,-3-glucanase activity.*

The enzyme activity was assayed under the standard conditions except that pH(A) was varied using a reaction mixture containing the indicated buffer from pH 4.0 to 10.0 and reaction temperature(B) was varied from 25 to 70℃. (A); Buffers used were McIlvaine buffer(pH 4.0 to 6.0), Sorensen buffer(pH 5.5 to 9.0) and 0.1 M Tris-HCl buffer(pH 9.0 to 10.0).

temperature on enzyme activity were found to be 5.8 and 45℃, respectively. The optimum pH on β-1, 3-glucanase from *Pseudomonas stutzeri* KF 13 is similar to those of other sources, ranged between pH 5.5 and pH 6.0 (Horikoshi *et al,* 1963; Yamamoto *et al,* 1974; Kobayashi *et al,* 1974; Nagasaki *et al,* 1976) except pH 4.5 from *Rhizopus arrhizus*(Reese and Mandels, 1959). The optimum temperature on β-1, 3-glucanase activity was generally showed in a 50℃ to 60℃(Reese and Mandels, 1959; Horikoshi *et al,* 1963; Huotari *et al,* 1968; Sanchez *et al,* 1982) except 40℃ from *Rhizopus* sp.(Yamamoto *et al,* 1974).

By the means of Arrhenius equation, the activation energy of hydrolytic reaction was calculated and found to be 16,130 cal per mole(Fig.4).

Molecular Weight

The molecular weight of β-1, 3-glucanase was determined by the gel filtration on Sephadex G-100 column and SDS-gel electro-

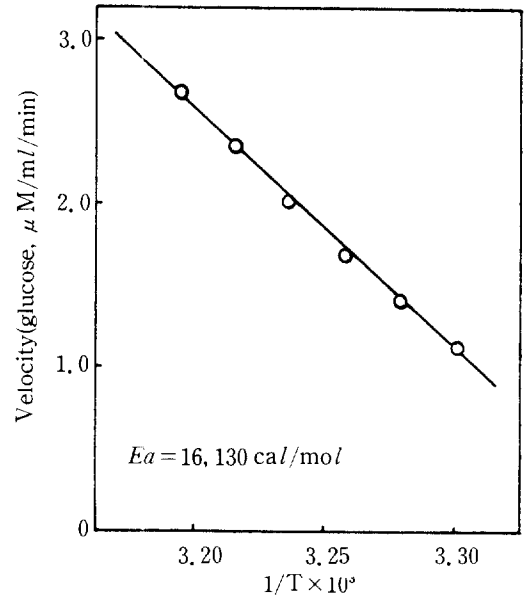

Fig.4. *Arrhenius plot of the effect of temperature on β-1, 3-glucanase.*

The data include the initial velocity determined at temperature between 30 and 40℃, plotted against the reciprocal temperatures. Velocity was expressed as μM of reducing sugar formed per ml of enzyme solution per min.

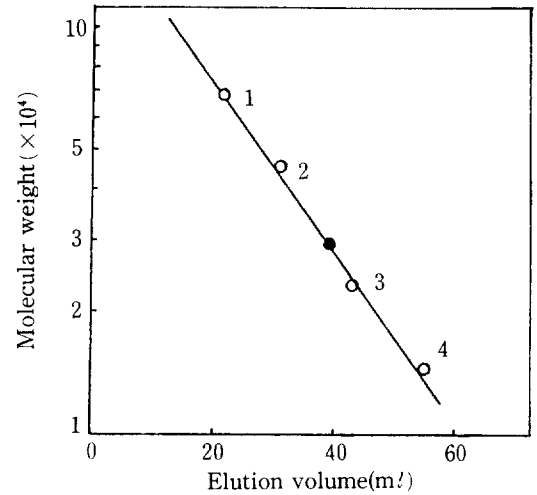

Fig.5. *Determination of the molecular weight of the β-1, 3-glucanase by Sephadex G-100 gel filtration.*

The standard proteins used and their molecular weight were: 1; bovine serum albumin(67,000), 2; ovalbumin(43,000), 3; trypsin(23,300), 4; lysozyme (14,300). ○; standard protein, ●; β-1, 3-glucanase.

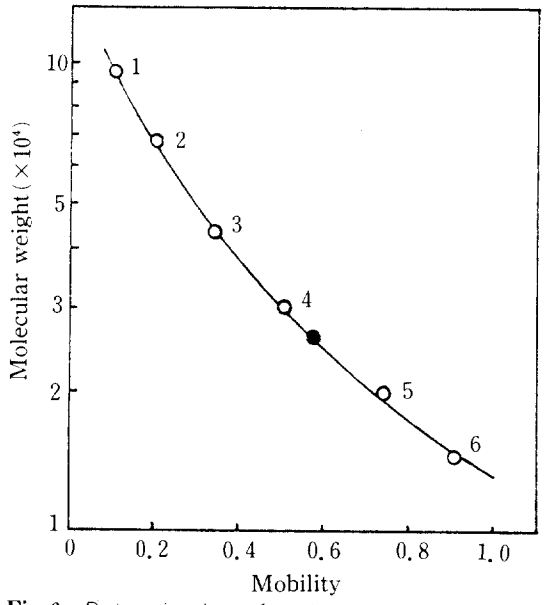

Fig.6. *Determination of molecular weight of the β-1, 3-glucanase by SDS-polyacrylamide gel electrophoresis.*

The standard proteins used and their molecular weight were: 1 ; phosphorylase b(94, 000), 2 ; bovine serum albumin(67, 000), 3 ; ovalbumin(43, 000), 4 ; carbonic anhydrase(30, 000), 5 ; trypsin inhibitor(20, 100), 6 ; α-lactalbumin(14, 400). ○ ; standard protein, ● ; β-1, 3-glucanase.

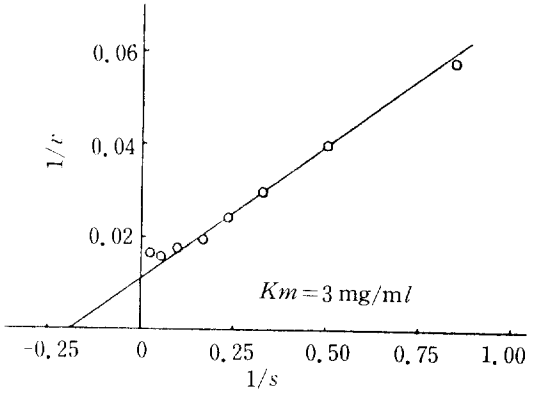

Fig.7. *Effect of substrate concentration on β-1, 3-glucanase activity.*

The reaction mixture contained 0.25 ml of the enzyme solution and the indicated amount of laminarin in the standard reaction mixture. Velocity(v) was expressed as μg reducing sugar per ml per min and substrate concentration(s) as mg per ml of laminarin.

Table 3. *Effect of metal ions on β-1, 3-glucanase activity.*

Metal ion	Relative activity (%)	
	1.0 mM	0.1 mM
Cu^{2+}	98.5	112.1
Mn^{2+}	115.0	136.0
Co^{2+}	119.1	–
Ca^{2+}	70.2	96.2
Zn^{2+}	76.2	–
Mg^{2+}	63.8	–
Hg^{2+}	14.9	21.8
Fe^{2+}	65.9	–
Na^{+}	74.5	89.5
K^{+}	87.2	98.4
None	100.0	100.0

The enzyme activity was assayed under the standard reaction conditions in the presence of metal ions at the indicated concentration and expressed as relative activity to that of control.

phoresis. From the results shown in Fig.5 and 6, the molecular weight was estimated as about 28,000 and 26,000 daltons, respectively. From these results, the β-1, 3-glucanase from *Pseudomonas stutzeri* KF 13 is seemed to be a monomer. The molecular weight of this enzyme was similar to that of *Rhizopus arrh-*

Table 4. *Effect of chemical reagents on β-1, 3-glucanase activity.*

Chemical reagent	Relative activity (%)
EDTA	122.6
p-CMB	0.0
Monoiodo acetate	98.7
o-Phenanthroline	117.9
Trichloro acetate	96.2
Sodium azide	96.8
Sodium cyanide	186.8
None	100.0

The reaction conditions were the same as those described in Table 3. Chemical reagents were added to the final concentration of 1.0 mM. EDTA; ethylenediamine tetraacetic acid, *p*-CMB; *p*-chloromercuribenzoic acid.

izus QM 1032 enzyme(Reese and Mandels, 1959; Clarke *et al*, 1978) as 29,000 except that of *Flavobacterium dormitator* var. *glucanolyticae* enzyme(Nagasaki *et al*, 1976) as 17,000-22,000 daltons.

Effect of Substrate Concentration on Enzyme Reaction

The Michaelis-Menten constant *Km* for laminarin as a substrate was determined by Lineweaver-Burk plot, as shown in Fig.7. The *Km* value was calculated to be 3mg per m*l* for laminarin. The enzyme reaction was weakly inhibited by a high concentration of laminarin.

Effect of Metal Ions and Chemical Reagents on Enzyme Activity

In order to investigate the effects of various metal ions and chemical reagents on β-1, 3-glucanase activity, the enzyme solution and substrate was incubated with 1.0mM or 0.1 mM metal ions and 1.0 mM chemical reagents as a final concentration at 45℃ for 5 min. The residual activity of the enzyme was determined and the results are shown in Table 3 and 4. The enzyme was slightly activated by the addition of 0.1mM Mn^{2+}, on the other hand inhibited by Hg^{2+} and Fe^{2+}. By the addition of the chemical reagents, the enzyme was activated by sodium cyanide, but strongly inhibited by *p*-CMB.

적 요

토양에서 분리한 *Pseudomonas stutzeri* KF 13의 배양여액으로부터 균체외 β-1, 3-glucanase를 수율 26%로서 비 활성도로 388.6배 정제하였고, disc 전기영동 및 SDS 전기영동에서 단일 단백질 band를 나타내어 순도를 확인하였다.

본 효소의 여러가지 성질을 검토한 결과 pH 6.0~9.0에서 및 55℃에서 10분간의 열 처리에도 비교적 안정하였고, pH 5.8 및 45℃에서 반응 최적조건을 나타내며 활성화 에너지 값(*Ea*)은 16,130 cal/mol 이었다.

또한 본 효소는 laminarin을 주 기질로 이용하며 laminarin에 대한 *Km* 값은 3mg/m*l* 이며, 분자량은 gel 여과법에 의하여 28,000, SDS-전기영동법에 의하여 26,000 dalton으로 측정되었으며, 본 효소는 monomer였다. β-1,3-glucanase의 효소활성은 1mM Hg^{2+}에 의하여 저해되었으며, 1mM *p*-chloromercuribenzoic acid에 의하여 강하게 저해되었다.

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