Purification and Properties of an Exo- α -1,3-glucanase from *Trichoderma viride*[†]

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An α -1,3-glucanase was partially purified from Meicelase, a commercial cellulase preparation from *T. viride*, and its properties were studied. Cellulase in the crude enzyme was removed by adsorption on cellulose powder, and α -1,3-glucanase was purified through salting-out with ammonium sulfate, chromatographies on DEAE-Sephadex, hydroxylapatite and Sephadex G-100. The α -1,3-glucanase gave 2 peaks on DEAE-Sephadex chromatography, and the enzyme of the second peak was further purified to about 300-fold purification. The enzyme was most active at pH 4.5, and 55°C, stable at 4°C at pH between 3 and 7, and stable up to 50°C on heat treatment at pH 4.5 for 30 min. The Michaelis constant for α -1,3-glucan was 7.1 mM (as anhydroglucose units). The molecular weight of the enzyme determined by gel filtration on Sephadex G-100 was 47,000. Only glucose was formed as reducing sugar liberated from α -1,3-glucan, showing that the enzyme was an exo- α -1,3-glucanase (EC 3.2.1.84).

Many strains of microbes have recently been found to degrade α -1,3-glucan,^{1~8)} and the enzyme α -1,3-glucanase produced by them was studied with some of the strains. All of the α -1,3-glucanases which have hitherto been reported seemed to act on α -1,3-glucan endo-wise except one,²⁾ although there were only few reports on the purification and characterization of the enzyme.

We have found the presence of α -1,3-glucanase in a commercial cellulase preparation, Meicelase, produced by Meiji Seika Co., Ltd., from *Trichoderma viride*. The enzyme was partially purified, its properties were studied, and it was revealed that the enzyme was an exo- α -1,3-glucanase (EC 3.2.1.84), which is different from an endo- α -1,3-glucanase (EC 3.2.1.59) reported by Hasegawa *et al.*¹⁾ with another strain of *T. viride*.

MATERIALS AND METHODS

Enzyme source. Meicelase powder, a commercial cellulase preparation from *T. viride*, supplied by Meiji Seika Co., Ltd., was used as a starting material of the enzyme purification.

Substrates. Pseudonigeran, an α -1,3-glucan, was prepared from the cell wall of *Aspergillus niger* QM 9270 (supplied by Dr. E. T. Reese, U. S. Army Natick Laboratories, Natick, Mass.) according to the method of Hasegawa *et al.*,¹⁾ and used as the substrate for α -1,3-glucanase.

Sodium carboxymethylcellulose (CMC) was obtained from Kokusan Chemical Works, Ltd., and cellulose powder, Avicel, was from Asahi Kasei Co., Ltd.

Chemicals. DEAE-Sephadex A-50 and Sephadex G-100 were the products from Pharmacia Fine Chemicals. Hydroxylapatite was prepared according to the method of Tiselius *et al.*⁹⁾ Proteins used as standards in the molecular weight determination were obtained from Sigma Chemicals.

Enzyme assays. 1) α -1,3-Glucanase activity was assayed by incubating 0.25 ml of enzyme solution with 0.25 ml of a 0.5% suspension of pseudonigeran in 50 mM acetate buffer, pH 4.5, at 40°C, and reducing sugar liberated in 30 min was determined by Nelson-Somogyi method.¹⁰⁾ One unit of α -1,3-glucanase was defined as the amount of enzyme which would liberate reducing sugar equivalent to 1 μ mole glucose per min

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under standard assay conditions.

2) Cellulase activities were estimated in the same way as above by using 0.5% CMC solution or 0.5% suspension of cellulose powder in place of pseudonigeran. They were referred to, respectively, as CMCase and CPase activities.

Protein concentration. The protein concentration of enzyme solutions was determined by measuring the absorbance at 280 nm in a Hitachi 101 spectrophotometer, and expressed in mg taking bovine serum albumin as the standard.

Paper chromatography. Reducing sugar produced in enzymatic digests was detected by means of paper chromatography on Toyo No. 50 filter paper with multiple ascending technique using the solvent system, *n*-butanol-pyridine-water (6:4:3). Spots were detected by the alkaline acetone-silver nitrate dipping procedure.¹¹⁾

RESULTS

1. Purification of the enzyme

All operations were performed in a cold room at 4°C. Meicelase powder (15 g) was dissolved in 600 ml of 50 mM acetate buffer, pH 4.5, the solution was stirred with 150 g of cellulose powder for several hr, and left overnight. The cellulose was filtered off, washed with 600 ml of buffer, and the filtrate and washing were combined. The enzyme was salted-out with 50% saturation of ammonium sulfate, dissolved in a small amount of water, dialyzed against 10-fold diluted McIlvaine buffer, pH 4.5, subjected to chromatography on DEAE-Sephadex column (3×50 cm), followed by linear gradient elution with 0 to 0.5 M sodium chloride at a flow rate 60 ml/hr. Elution pattern is shown in Fig. 1, in which 2 peaks of α -1,3-glucanase were observed. The enzyme of the second peak was further purified, because the specific activity of this peak was higher than that of the other. Thus, the chromatography was repeated with the second peak using a DEAE-Sephadex column (2×40 cm), and the enzyme was eluted as above. Active fractions were combined, dialyzed against 0.01 M phosphate buffer, pH 6.0, and applied to a hydroxylapatite column (5×5 cm), followed by elution with 10 to 300 mM

FIG. 1. The First DEAE-Sephadex A-50 Column Chromatography.

○, α -1,3-glucanase; ●, CMCase; ---, protein concentration (A_{280}); ↓, start of the gradient elution. One fraction 10 ml.

FIG. 2. Sephadex G-100 Gel Filtration.

○, α -1,3-glucanase; ●, CMCase; ---, protein concentration (A_{280}); one fraction 3 ml.

phosphate buffer, pH 6.0. Active fractions were concentrated in a Sartorius collodion bag under reduced pressure, subjected to gel filtration on Sephadex G-100, and eluted with 50 mM acetate buffer, pH 5.0. Elution pattern in the gel filtration is shown in Fig. 2.

A summary of the purification procedure is outlined in Table I. The overall purification was about 300-fold. The purified preparation showed no action on β -1,3-glucan (curdlan) and dextran, while trace hydrolytic activities were detected on CMC, maltose and starch. The purified enzyme was stored at 4°C, and used in experiments described below.

TABLE I. SUMMARY OF THE PURIFICATION OF THE α -1,3-GLUCANASE FROM *Trichoderma viride*

Fraction	α -1,3-Glucanase					CMCase	CPase
	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Purification (ratio)	Yield (%)	Total activity (units)	Total activity (units)
Crude enzyme	1000	25,000	0.04	1	100	17,000	1300
Cellulose powder treatment	950	16,000	0.06	1.4	95	14,000	60
Ammonium sulfate precipitation	520	1,900	0.27	6.8	52	4000	trace
1st DEAE-Sephadex	120	91	1.3	33	12	2000	0
2nd DEAE-Sephadex	90	45	2.0	50	9	1000	0
Hydroxylapatite	35	7.8	4.5	110	3.5	4	0
Sephadex G-100	25	2.2	11	290	2.5	0.4	0

2. Effects of pH and temperature on the enzyme

i) *pH-activity relationship.* Effects of pH on the enzyme activity were estimated in McIlvaine buffer. The optimum pH was found at pH 4.5 as shown in Fig. 3.

FIG. 3. Effect of pH on Enzyme Activity.

The enzyme activity was assayed as described under MATERIALS AND METHODS using 2-fold diluted McIlvaine buffer with varying pH values.

ii) *pH-stability.* The enzyme was stored in McIlvaine buffer of varying pH at 4°C or 30°C for 24 hr, and remaining activity was measured at pH 4.5. The enzyme was stable at pH between 3.0 and 7.0 at 4°C, and between 3.5 and 6.0 at 30°C for 24 hr.

iii) *Effect of temperature on enzyme activity.* The enzyme activity was measured at pH 4.5 at varying temperatures. The optimum temperature estimated was 55°C.

iv) *Thermal stability.* The enzyme ac-

tivity was measured after heating the enzyme in 0.05 M acetate buffer, pH 4.5, at 40~60°C for 30 min. Results showed that the enzyme was stable up to 50°C on these treatments.

3. The influence of substrate concentration on the initial velocity

The reaction velocity was measured at substrate concentrations between 2 and 20 mM (as anhydroglucose units), and the Michaelis constant, K_m , was calculated from Lineweaver-Burk plot shown in Fig. 4. The value of K_m 7.1 mM was obtained.

FIG. 4. Lineweaver-Burk Plot of α -1,3-Glucanase.

4. The estimation of molecular weight of the enzyme

The molecular weight of the enzyme was estimated by the method of Andrews¹¹⁾ using a Sephadex G-100 column (2×70 cm) equili-

FIG. 5. The Estimation of Molecular Weight of the Enzyme.

Column size, 2×70 cm; V_e , elution volume; V_o void volume; flow rate, 10 ml/hr; one fraction 3 ml. Molecular weight of standard proteins, bovine serum albumin 67,000, egg albumin 45,000, chymotrypsinogen 25,000 and myoglobin 17,800.

brated with 0.05 M acetate buffer, pH 5.0. One ml of each marker protein solutions (5 mg/ml) in the same buffer was applied to the column, and eluted at a flow rate 10 ml/hr. Void volume was determined with 1 ml of Blue dextran solution. A plot of the relative elution volume of marker proteins and α -1,3-glucanase *versus* the logarithm of molecular weight is given in Fig. 5. The molecular weight of α -1,3-glucanase determined is approximately 47,000.

5. The action pattern of the α -1,3-glucanase

In order to identify the products formed in the enzymatic digest, 0.5% pseudonigeran suspension 0.5 ml was incubated with 0.033 units of the enzyme at pH 4.5 and 40°C for 4 hr (up to 20% hydrolysis), and the digest was analyzed with paper chromatography at appropriate time intervals. Only glucose was detected as reducing sugar during the whole reaction period. Then the enzyme reaction was followed simultaneously by the following 3 methods: (1) reducing sugar determination with Nelson-Somogyi method,¹⁰⁾ (2) glucose determination with glucose oxidase method,¹²⁾ and (3) the determination of total solubilized carbohydrate with phenol-sulfuric acid meth-

FIG. 6. Production of Reducing Sugar and Glucose from α -1,3-Glucan by α -1,3-Glucanase.

R.S.: Reducing sugar determined with Nelson-Somogyi method, and expressed as glucose equivalent. Abscissa represents the amount of glucose determined with glucose oxidase method.

od¹³⁾ after removing insoluble substrate by centrifugation.

As can be seen in Fig. 6, a straight line with a slope of 1 was obtained between the amounts of reducing sugar and glucose formed. This indicates that glucose accounts for all of the reducing power and is the only product of the enzymatic reaction, which is confirmed by the results of paper chromatography on reaction products.

Then the ratio of total dissolved carbohydrate to reducing sugar was plotted against

FIG. 7. Action Pattern of the α -1,3-Glucanase.

P.S.: Total dissolved carbohydrate estimated with phenol-sulfuric acid method.

R.S.: Reducing sugar determined with Nelson-Somogyi method, and expressed as glucose equivalent.

the amount of glucose liberated during the enzyme reaction, according to Tung and Nordin.¹⁴⁾ As can be seen from Fig. 7, the results clearly indicates an exo-enzyme for the α -1,3-glucanase.

DISCUSSION

As described in the foregoing Section, an α -1,3-glucanase in a commercial enzyme preparation from *Trichoderma viride* was purified about 300-fold, and its properties were studied. Since the starting material contains large amounts of cellulase, the removal of cellulase was the main problem in enzyme purification. The adsorption on cellulose powder was highly effective in removing CPase, while CMCase was not removed by this treatment. On the other hand, CMCase could be reduced through chromatographies on DEAE-Sephadex, hydroxylapatite, and Sephadex G-100 to 1/40,000 of the crude enzyme.

α -1,3-Glucanase activity was separated into 2 fractions on DEAE-Sephadex chromatography, and the second fraction which showed higher specific activity was further purified in this study. Properties of α -1,3-glucanase in this fraction were studied, and it was proved that the enzyme is an exo-type α -1,3-glucanase which liberates only glucose from α -1,3-glucan.

The unequivocal evidence for the exo-wise attack of the enzyme was obtained from the ratio of total dissolved carbohydrate to reducing sugar, plotted against glucose production as shown in Fig. 7. If the attack would be of an endo-wise fashion, then the ratio would be high at early stages of the hydrolysis, and decrease rapidly thereafter.

Concerning to the first peak of enzyme activity separated on the first DEAE-Sephadex chromatography, it is not clear whether the peak represents an isozyme of exo- α -1,3-glucanase or another type of enzyme. But according to our preliminary experiments, the activity seems to be similar to that of the α -

1,3-glucanase purified here, because only glucose was detected on paper chromatography in the digest of α -1,3-glucan by the enzyme of the first peak.

The α -1,3-glucanase of the exo-type has been reported in *Aspergillus nidulans*.²⁾ But it is interesting enough that the enzyme of this type was also found in this study in *Trichoderma viride*, in which only endo-type α -1,3-glucanase has hitherto been reported.¹⁾

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