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Purification and Properties of a Yeast Cell Lytic Enzyme from *Pellicularia sasaki*

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An endo β-1,3-glucanase (yeast cell lytic enzyme) has been purified from the culture medium of *Pellicularia sasaki* No. 89. The purification procedure involved ammonium sulfate fractionation, DEAE-Sephadex A-50 ion exchange column chromatography and Bio-Gel P-150 gel filtration. The final preparation was found to be homogeneous on the criteria of disc electrophoresis, ultracentrifugation analysis and substrate specificity test.

Several physicochemical and enzymatic properties of the enzyme were determined: molecular weight, 25,000; optimum pH, 6.0; optimum temperature, 40°C for living yeast cells distruption, and 60°C for yeast glucan hydrolysis; and stability, stable at pH 5~6. A complete amino acid analysis was presented.

The enzyme possessed considerable heat stability: the enzyme retained about 50 % of the original activity even after the heat-treatment at 100°C for 10 min.

KEY WORDS: Yeast cell lytic enzyme / Lytic enzyme, on yeast cell / β-1,3-glucanase (Endo) /

INTRODUCTION

Isolated preparation of β-1,3-glucanases is supposed to be a good toll for analyzing the structure and biochemical functions of yeast cell wall polysaccharides. The success in such application dependes upon the availability of the preparation as well as an accurate understanding of their substrate specificities and modes of hydrolytic action.

Previously, the authors had isolated and characterized endo β-1,3-glucanases capable of lysing viable yeast cells from *Rhizopus chinensis* R-69,[1~3] *Flavobacterium dormitator var. glucanolyticae*,[4] a fungi imperfecti[5] and a *Rhizoctonia* sp.[6,7] Also, it should be noted that endo β-1,3-glucanase have been isolated from *Arthrobacter luteus*[8] whose properties are significantly different from those of their glucanases.

This paper describes the purification of the endo β-1,3-glucanase from *Pellicularia sakaki* No. 89 and a determination of some of its physicochemical and enzymatic characteristics.

MATERIALS AND METHODS

Chemicals and substrates—All the reagents used were an analytical grade obtained

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commercially. Pachyman was prepared from air-dried Bukuryo (*Poria cocos*) following the method described by Whelan.[9] Yeast glucan was prepared from baker's yeast produced by Dainihon Seito KK., Japan, following the method described by Misaki *et al.*[10] Other substrates were supplied commericially.

Microorganism—*P. sasaki* No. 89 was kindly supplied by the Chemical Research Laboratory, Kumiai Chemical Industry Co., Ltd., and was grown as described previously.[6,7]

Analytical procedures—Analytical procedures and chromatographic procedures were the same as have been described in the previous papers.[1~7]

End groups analysis—NH_2 terminal residues were determined by the enzymatic method as described by Spackman *et al.*[11] For the determination of COOH terminal residues, carboxypeptidase Y digestion of the heat-denatured enzyme was carried out as described by Hayashi *et al.*[12]

RESULTS AND DISCUSSION

Purification

All the operations were carried out at 5°C unless otherwise specified.

Step 1: Extraction—Wheat bran koji, 1 Kg, cultured with *P. sasaki* No. 89 at 30°C for 10 days, was extracted with 4,000 ml of water for 24 hours.

Step 2: Ammonium sulfate fractionation—The extract was concentrated under reduced pressure at 30°C to approximately 1/10 of the initial volume. Ammonium sulfate was added to the concentrated solution to give 30 % saturation, the precipitate formed was discarded, and ammonium sulfate was further added to give 60 % saturation. The precipitate collected was dissolved in a minimum volume of 0.01M sodium acetate buffer, pH 5.8, and dialyzed against the same buffer for 50 hours.

Step 3: 1st DEAE-Sephadex A-50 column chromatography—The dialyzed enzyme solution was applied to a DEAE-Sephadex A-50 column which had been equilibrated with 0.01M sodium acetate buffer, pH 5.8. After washing the column with 0.1M sodium acetate buffer, pH 5.8, the enzyme was eluted from the column with 0.1M potassium phosphate buffer, pH 7.0. To the active fractions combined, ammonium sulfate was added to give 60 % saturation. The precipitate formed was collected, and dissolved in a minimum volume of 0.01M sodium acetate buffer, pH 5.8. After dialyzation of the enzyme against the same buffer, the solution was centrifuged at 24,000 g for 30 minutes.

Step 4: 2nd DEAE-Sephadex A-50 column chromatography—This step was performed by essentially the same procedure as used in the Step 3.

Step 5: 1st Bio-Gel P-150 gel filtration—Molecular sizing of the enzyme was performed on a column of Bio-Gel P-150 using 0.01M sodium acetate buffer, pH 5.8, as a working buffer solution. Fractions containing the enzyme were pooled and saturated with ammonium sulfate, and the precipitate formed was collected and dissolved in a minimum volume of 0.01M sodium acetate buffer, pH 5.8.

Step 6: 2nd Bio-Gel P-150 gel filtration—This step was performed by essentially the same procedure as used in Step 5. After the dissolution of the enzyme, the

Table I. Summary of Purification*

Step of purification	Total protein (mg)	Total activity (units)	Specific activity	Yield (%)
1. Extraction	238,750	31,750	0.13	(100)
2. Ammonium sulfate fractionation	35,671	20,716	0.58	65.2
3. 1st DEAE-Sephadex A-50 column chromatography	517	4,032	7.80	12.7
4. 2nd DEAE-Sephadex A-50 column chromatography	203	3,678	18.12	11.6
5. 1st Bio-Gel P-150 gel filtration	39	2,100	53.8	6.6
6. 2nd Bio-Gel P-150 gel filtration	28	1,800	64.3	5.6

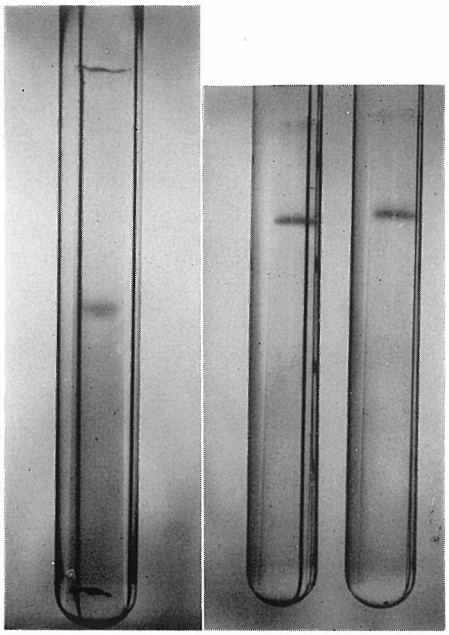
*: Activity was expressed as lytic activity.

enzyme solution was dialyzed against 0.01M sodium acetate buffer, pH 5.8 overnight.

The results of a typical purification are shown in Table I.

Physicochemical and Enzymatic Properties of the Enzyme

Purity—The enzyme was demonstrated to be pure by sedimentation in the ultracentrifuge, analytical disc electrophoresis on acrylamide gel (Fig. 1) and substrate specificity test using a wide variety of carbohydrates (see Table III).



A B C

Fig. 1. Polyacrylamide Disc Gel Electrophoresis of the Enzyme.
A. Electrophoresis at pH 9.4 in Davis gel.
B. Electrophoresis carried out at pH 7.4 in the presence of 1 % sodium dodecyl sulfate in Fairbanks' gel.
C. Electrophoresis carried out at pH 7.4 in the presence of 1 % sodium dodecyl sulfate and 1 % β-mercaptoethanol in Fairbanks' gel.

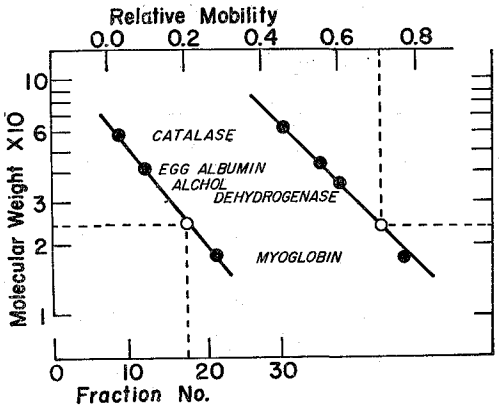


Fig. 2. Molecular Weight ot the Enzyme as Determined by Bio-Gel P-100 Gel Filtration and by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis.
A. Bio-Gel P-100 column (1×45 cm) was eluted with 10 mM sodium acetate buffer, pH 5.8, at a flow rate of 5 ml per hour. Fractions of 1 ml were collected.
B. The electrophoresis in the presence of 2 % sodium dodecyl sulfate by the method of Fairbanks was employed. The protein standards used in the construction of the standard curve are indicated in the graph.
For both graphs, the open circle marks the position of the enzyme.

Molecular weight—The molecular weight of the enzyme was determined by the methods of polyacrylamide gel electrophoresis and of gel filtration (Fig. 2). The observed electrophoretic mobility for the enzyme corresponded to a molecular weight of 25,000. Molecular weight measured by gel filtration through Bio-Gel P-100 was 25,000 in good agreement with that determined by electrophoresis.

Amino acid composition—The amino acid composition of the enzyme is shown in Table II. The minimum molecular weight calculated from the amino acid composition is in good agreement with that determined by disc electrophoresis and gel filtration. The amino acid composition of the enzyme appears to be similar to those reported for endo β-1,3-glucanases from *R. chinensis* R-69,[1,2] a fungi imperfecti[5] and *R. arrhizus* QM 1032.[13] Like these enzymes, it was high in acidic amino acids content and low in basic amino acids. In addition, it is notable that one residue of histidine and methionine was detected.

Since the enzyme was unreactive to 5,5′-dithio bis (2-nitrobenzoic acid), it is assumed that all cysteine residues (six residues as described in Table II) exist in the form of cystine and no free sulfhydryl group.

Amino acid sequence of amino and carboxyl terminal—When heat-denatured enzyme was digested with leucine aminopeptidase, serine was released at the fastest rate, in an amount corresponds to 0.9 residue per molecule of the enzyme. On the basis of the order of the release of amino acids, the amino acid sequence of the amino terminal of the enzyme seems to be -(Ala)-Gly-Ser-NH_2 (Fig. 3-B).

Table II. Composition of Amino Acids

Amino acid	Time of hydrolysis 20 hrs	40 hrs	60 hrs	No. of residues per molecule[a)]
	μmole amino acid/8.27 nmoles enzyme			
Aspartic acid	0.346	0.341	0.341	41
Threonine	0.139	0.132	0.122	18[b)]
Serine	0.145	0.135	0.118	19[b)]
Glutamic acid	0.063	0.064	0.061	8
Proline	0.176	0.172	0.168	21
Glycine	0.222	0.218	0.218	27
Alanine	0.153	0.148	0.148	18
Cystine/2	0.050	0.041	0.048	6
Valine	0.124	0.123	0.122	15
Methionine	0.008	0.009	0.009	1
Isoleucine	0.037	0.036	0.037	5
Leucine	0.105	0.103	0.103	13
Tyrosine	0.094	0.092	0.090	12
Phenylalanine	0.091	0.089	0.090	11
Lysine	0.035	0.034	0.034	4
Histidine	0.008	0.009	0.008	1
Arginine	0.059	0.062	0.061	7
Tryptophane				4[c)]
Total residue				231
Molecular weight				24,435

a) assuming a molecular weight of 25,000.
b) extrapolated to zero hour hydrolysis.
c) determined spectrophotometrically.

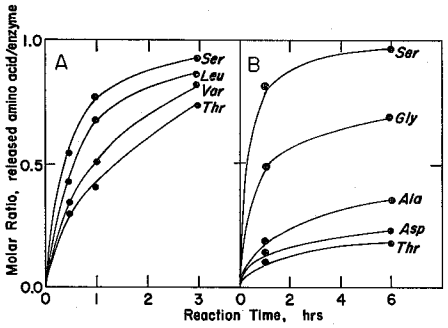


Fig. 3. Release of Terminal Amino Acids from the Enzyme by the Action of Leucine Aminopeptidase and Carboxypeptidase Y.
A. The heat-denatured enzyme, 1.608 mg, in 0.7 ml of 0.01 M sodium citrate buffer, pH 6.5, was digested with carboxypeptidase Y. 76.2 μg, at 30°C.
B. The heat-denatured enzyme, 1.595 mg, in 0.7 ml of 0.1 M Tris·HCl buffer, pH 8.0, was digested with leucine aminopeptidase, 235 μg, at 38°C.
The released amino acids were identified and quantitated by a Hitachi 034 amino acid analyzer.

The amino acid sequence of the carboxyl terminal of the enzyme seems to be -(Val)-Leu-Ser-COOH on the basis of the experimental results shown in Fig. 3-A. *Effect of pH and temperature*—Activities hydrolysing laminaran and yeast glucan and disrupting living yeast cells were assayed at various pH. The maximum activity was obtained at 6.0 for both substrates (Fig. 4-A). The enzyme showed maximum activity at 60°C for yeast glucan and at 40°C for living yeast cells (Fig. 4-B). This discrepancy may be resulted from the difference in the structure between these substrates.

When heated for 10 minutes at various temperatures, heat inactivation of the enzyme started at 70°C and reached approximately 50 % at 100°C. The enzyme

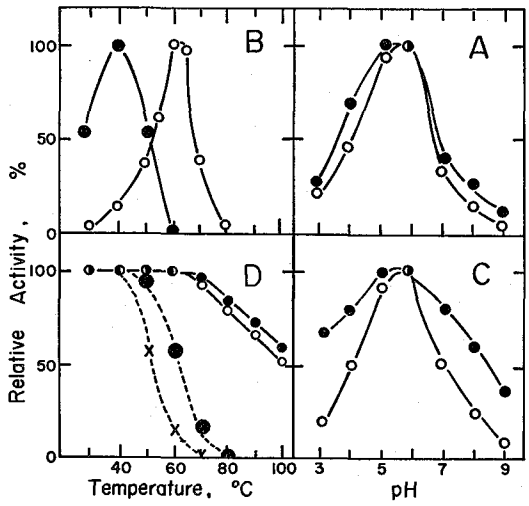


Fig. 4. Effect of pH and Temperature on Yeat Cell Lytic Activity and Yeast Glucan Hydrolytic Activity.

A. Activity-pH curve: Buffers were: pH 3~6, 100 mM sodium acetate; pH 6~8, 100 mM potassium phosphate; pH 8~9, 100 mM Tris·HCl. For the yeast cell lytic activity assay, protoplast forming buffer was adjusted with potassium hydroxide or hydrogen chloride to prescribed pH.

B. Activity-temperature curve: The activity was measured under the standard assay conditions at various temperature.

C. Stability-pH curve: The enzyme in the various buffer described in A, was heated at 80°C for 10 minutes and then the remaining activity was measured under the standard assay conditions.

D. Stability-temperature curve: The enzyme in 100 mM sodium acetate buffer, pH 5.8 was incubated for 10 minutes at prescribed temperature and then the remaining activity was measured under the standard assay conditions.

●: Yeast cell lytic activity

○: Yeast glucan hydrolytic activity

×: β-1, 3-glucanase activity used laminaran as substrate.

Straight line represents the activity of the purified enzyme and dotted line the activity of the crude enzyme.

Table III. Substrate Specificity of the Enzyme
The activity was measured by incubating the substrates at 0.5% concentration with 6.44 μg of enzyme in 50 mM sodium acetate buffer, pH 6.0. Reducing sugar produced was measured by the method of Somogyi-Nelson.

Substrate	Reducing sugar, μmole/ml	Substrate	Reducing sugar, μmole/ml
Pachyman	0.338	Amylopectin	0.000
Paramylon	0.234	Starch	0.000
Laminaran	0.104	Glycogen	0.000
Yeast glucan	0.143	Dextran (high MW)	0.000
Curdran	0.020	Dextran (low MW)	0.000
Screlotan	0.000	Yeast mannan	0.000
Lichenan	0.000	Dextrin	0.000
Cellulose	0.000	Xylan	0.000
Luteose	0.000	Pullulan	0.000
Amylose	0.000	Inulin	0.000

was found to be stable in the pH range of 5 to 6 when heated at 75°C for 10 minutes (Fig. 4-C). The inactivation curves are substantially identical between both activities, as shown in Fig. 4-D. Although the enzyme was heat stable in a purified form, but in crude form it behaved quite differently. The detailed study on the heat stability of the enzyme will be dscribed in the following paper.

Substrate spceificity—The activities of the enzyme on the broad range of carbohydrates with glycosidic linkages are given in Table III. Since the enzyme specifically hydrolyzed the glucans linked with β-1,3, the enzyme undoubtedly a kind of

Table IV. Effect of Various metals on Activity of the Enzyme
The enzyme, 50 μl (7.59 μg protein) was incubated at room temperature with 50 μl of 10 mM metal for 2 hours before the introduction of substrate. Metal ions were dissolved in 0.01 M sodium acetate buffer, pH 5.8.

Metal ion (5×10^{-3} M)	Relative activity	
	Yeast glucan depolymerizing activity	Living yeast disrupting activity
None	(100)	(100)
$ZnCl_2$	90	64
$CuSO_4$	84	52
$CaCl_2$	92	64
$CoCl_2$	98	80
$NiCl_2$	94	100
$MnCl_2$	104	72
$MgCl_2$	102	80
$AlCl_3$	80	76
$AgNO_2$	48	20
$HgCl_2$	18	32
$BaCl_2$	76	92
$Pb(NO_3)_2$	84	48

β-1,3-glucanase. The detailed study on the action pattern of the enzyme will be described in the following paper.

Effect of metal ion—As shown in Table IV, the enzyme was insensitive to the presence of various metal ions. However, silver and mercuric ions caused approximately 50 and 80 % inhibition, respectively. Fig. 5 shows the mercuric iondependent inhibition of the enzyme activity and the β-mercaptoethanol mediated reversion of the inhibition. These data led us to the presumption that the inhibition mechanism of mercuric ion is a formation of inactive dimer enzyme connected with mercuric ion. The presumption was supported by the experimental result that p-chloromercuribenzoate, as monovalent mercurous ion, did not affect on the enzyme activity at the same concentration (Fig. 5).

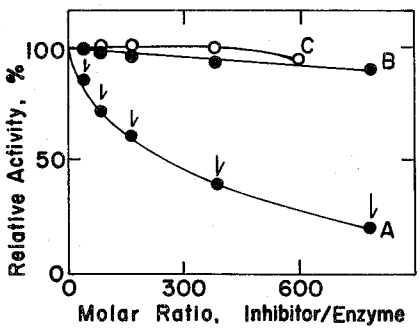


Fig. 5. Effect of Mercuric Ion and of Subsequent Treatment with β-Mercaptoethanol on the Activity of the Enzyme

The enzyme (15.9 μg=0.636 nmole) was incubated at room temperature at various concentrations of mercuric ions for 30 minutes before the determination of the activity. And then, β-mercaptoethanol was added to give 1.25 mM (indicated by arrow). After another 30 minutes, an aliquot was removed for the assay.

A: in the presence of $HgCl_2$.

B: after the addition of β-mercaptoethanol to A.

C: in the presence of p-chloromercurbienzoate.

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