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Fungal Glycosidases. V.[1] Purification and Properties of an Extracellular *exo*-(1→3)-β-D-Glucosidase from *Trichophyton mentagrophytes*

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An *exo*-(1→3)-β-D-glucosidase was isolated from the culture filtrate of the fungus *Trichophyton mentagrophytes* by ammonium sulfate fractionation, gel chromatography on Sephadex G-200 equilibrated with 0.1 M sodium phosphate buffer (pH 7.5), and ion exchange chromatography on DEAE-Sephadex A-50. The enzyme was purified 304 fold, with a recovery of 15%.

The *K*m value of the enzyme with *p*-nitrophenyl-β-D-glucopyranoside was 3.1 mM. The enzyme was strongly inhibited by Ag^{+}, Hg^{2+}, Cu^{2+} and Co^{2+} ions and also by N-bromosuccinimide and I_2. This enzyme hydrolyzed *p*-nitrophenyl-β-D-glucopyranoside but did not show any activity towards *p*-nitrophenyl-α-D-glucopyranoside or other *p*-nitrophenyl-glycosides. The enzymes also hydrolyzed (1→3)-β-linked glucosides, but not (1→4)-β- and (1→6)-β-linked glucosides.

When the enzyme hydrolyzed (1→3)-β-linked oligo and polyglucosides, the only product identified by paper chromatography was glucose monomer.

The molecular weight of the enzyme, as determined by sodium dodecyl sulfate disc gel electrophoresis, was 4.9×10^4 daltons.

Keywords——*Trichophyton mentagrophytes*; β-D-glucosidase; *exo*-(1→3)-β-D-glucosidase; fungal glycosidase; extracellular enzyme; hydrolysis of glucan

In our previous paper,[1] it was shown that α-D-mannosidase, N-acetyl-β-D-glucosaminidase and β-D-glucosidase are produced in the culture filtrate of *Trichophyton mentagrophytes*. In particular, the use of rat skin as a substrate made it possible to obtain large quantities of glycosidases. The properties of these enzymes obtained from the culture filtrate of *T. mentagrophytes* with and without rat skin as a substrate were the same.

This repot describes the purification and properties of a β-D-glucosidase obtained from the culture filtrate of the fungus using rat skin as a substrate.

Materials and Methods

Substrate——*p*-Nitrophenyl-β-D-glucopyranoside was purchased from Koch–Light Laboratories Ltd., England. Laminaran from brown marine algae, pachyman from *Poria cocus* and islandican from *Penicillium islandicum* IFO 4872 were prepared in our laboratory.[4,5] The following substrates were purchased: yeast glucan, O-carboxymethyl cellulose, nigeran, soluble starch, dextran, yeast mannan, esculin, maltose, melibiose, gentiobiose, turanose, *p*-nitrophenyl-α-D-glucopyranoside, lactose, cellobiose, sucrose, trehalose, methyl-β-D-glucoside, methyl-α-D-glucoside, salicin and phenyl-β-D-glucoside.

R-Laminaran was prepared by reduction of laminaran with 0.1% (w/v) $NaBH_4$. Oxidized laminaran was prepared by oxidation of laminaran with 0.017 M $NaIO_4$ for 24 hr at room temperature, followed by reduction with 0.1% $NaBH_4$.

Cytochrome c, chymotrypsinogen A and bovine serum albumin were purchased from Schwarz–Mann (U.S.A.).

Enzyme Assays——Liberation of *p*-Nitrophenol[6]: When *p*-nitrophenyl-β-D-glucopyranoside was used as a substrate, the incubation mixtures contained the following components (total volume, 1 ml): 0.5 ml of

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1 mM substrate in 0.05 M sodium citrate buffer, pH 4.5, and 0.5 ml of enzyme solution. Unless otherwise indicated, the mixtures were incubated for 30 min. The reaction was terminated by adding 2 ml of 0.4 M glycine–NaOH buffer (pH 10.5). The amount of *p*-nitrophenol released during incubation was measured by spectrophotometry at 400 nm.

Detection of Free Glucose: When laminaran, pachyman, yeast glucan, islandican, O-carboxymethyl cellulose, nigeran, soluble starch, dextran or yeast mannan was used as a substrate, the reaction mixture was the same as that for *p*-nitrophenyl-β-D-glucopyranoside, except that the substrate concentration was 0.1%. The reaction was stopped by the addition of 2 ml of Somogyi's alkaline copper reagent. The mixture was heated in a boiling water bath for 10 min then cooled. The amount of cuprous oxide formed was determined by adding Nelson's arsenomolybdate reagent, as described by Somogyi.[7]

Analytical Methods——Protein Assay: Protein was determined by the method of Lowry *et al.*[8] The protein concentration of the column effluent was estimated spectrophotometrically by the procedure of Kalcker.[9]

Paper Chromatography: A mixture of 2 mg of laminaran (or pachyman) in 0.5 ml of 0.1 M acetate buffer (pH 4.5) and 0.5 ml of the enzyme solution was incubated for 1 hr at 37°, deionized on Dowex 50-W resin, and then lyophilized. The resulting material was chromatographed on Toyo Roshi No. 50 filter paper. The solvent system was 1-propanol–ethylacetate–water (6 : 1 : 3). Reducing sugers were detected by spraying the paper with silver nitrate–sodium hydroxide.[10]

Polyacrylamide Gel Electrophoresis: The purity of the enzyme was examined by electrophoresis in 7.5% acrylamide gels using a modification of the Davis procedure.[11] Tris–glycine buffer (pH 8.3) was used and a current of 3 mA per tube was applied for 30 min. After electrophoresis, one gel was stained for protein with 0.04% Coomassie blue in 25% isopropylalcohol–10% acetic acid and 0.002% Coomassie blue in 10% isopropylalcohol–10% acetic acid, and then destained electrophoretically in 10% acetic acid. Another gel was cut into 1 mm sections with a razor blade. Each section was extracted with 0.5 ml of 0.1 M citrate buffer (pH 4.5), and the active fraction was detected by incubation of the extracts with 0.5 ml of 0.1 M citrate buffer (pH 4.5) containing 1 mM *p*-nitrophenyl-β-D-glucopyranoside at 37° for 30 min.

Purification of β-D-Glucosidase——Ammonium Sulfate Fractionation: Unless otherwise indicated, all operations were conducted between 0° and 4°. The filtrate of 25 day culture was used as a source of β-D-glucosidase. The filtrate was adjusted to 20% saturation with solid $(NH_4)_2SO_4$. The mixture was centrifuged to obtain a clear supernatant, which was then adjusted to 80% saturation with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in a minimum quantity of water, dialyzed and precipitated by adding acetone.[1]

Gel filtration: The crude enzyme fraction (3 ml, containing about 66 mg) was applied to a Sephadex G-200 column (2.0×97 cm) which had been equilibrated with 0.1 M sodium phosphate buffer (pH 7.5). The column was eluted with the same buffer, and 5 ml fractions were collected. The flow rate was adjusted to 5 ml/30 min.

Chromatography on DEAE-Sephadex A-50: A crude β-D-glucosidase preparation (15 mg of protein previously dialyzed against 0.04 M sodium phosphate buffer, pH 6.5) was applied to a DEAE-Sephadex A-50 column (1.9×45 cm) which had been equilibrated with 0.04 M sodium phosphate buffer, pH 6.5. The column was eluted with 300 ml of a linear salt gradient between 0.04 M sodium phosphate buffer, pH 7.5, and 1 M sodium chloride in the same buffer, and 3 ml fractions were collected.

Properties of β-D-Glucosidase——Effects of Various Metal Ions and Reagents: The effects of various metal ions and reagents on the enzyme activity were examined, using the following compounds: $AgNO_3$, $HgCl_2$, $CuSO_4$, $CoCl_2$, $ZnCl_2$, $MgCl_2$, $CaCl_2$, NaCl and ethylenediaminetetraacetic acid (EDTA), 2-mercaptoethanol, L-cysteine, N-bromosuccinimide and I_2. The relative activity was expressed as a percentage of the enzyme activity in the absence of metal ions and reagents.

Substrate Specificity: Measurements of substrate specificity were conducted under the standard assay conditions with the following substrates at a concentration of 0.1%: laminaran, pachyman, yeast glucan, islandican, O-carboxymethyl cellulose, nigeran, soluble starch, dextran and yeast mannan. The concentration of glucoside derivatives or disaccharides was 1 mM.

Molecular Weight Determination: Molecular weight (M.W.) determination in sodium dodecyl sulfate (SDS)–acrylamide gel was performed by a method similar to that described by Shapiro *et al.*[12] Mobility was calculated as the ratio of protein migration distance to gel length and plotted against the log of molecular

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weight. The sodium dodecyl sulfate–acrylamide gel was standardized with cytochrome c (1.24×10^4), chymotrypsinogen A (2.5×10^4) and bovine serum albumin (6.7×10^4).

*K*m Value: The effects of various substrate concentrations on the reaction rate at pH 4.5 (0.05 M citrate buffer) with *p*-nitrophenyl-β-D-glucopyranoside were determined. The simple Lineweaver–Burk plots[13] were linear.

Results

Purification of β-D-Glucosidase

The data on enzyme purification are summarized in Table I. Sequential ammonium sulfate fractionation resulted in 38 fold purification, with a recovery of 81%. By column chromatography on Sephadex G-200 (Fig. 1) and DEAE-Sephadex A-50 (Fig. 2), the β-D-glucosidase was purified 304 fold, with a recovery of 15%.

Table I. Purification of β-D-Glucosidase

Steps	Specific activity[a]	Yield	Purification factor
Culture filtrate	21.4	100	1
$(NH_4)_2SO_4$ precipitate 20 to 80% saturation	803.3	81	38
Sephadex G-200	2158.2	49	101
DEAE-Sephadex A-50	6508.0	15	304

Purification data are based on 1 l of culture filtrate.
a) mg of *p*-nitrophenol/mg protein.

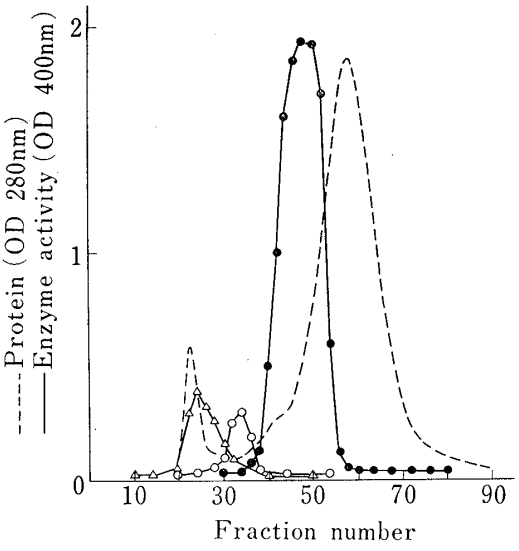

Fig. 1. Elution Pattern of the Crude Enzyme Fraction from a Sephadex G-200 Column (2.0×97 cm)

-------, absorption at 280 nm for protein; △—△, α-D-mannosidase activity; ○—○, N-acetyl-β-D-glucosaminidase activity; ●—●, β-D-glucosidase activity. The procedures for gel filtration and assaying the glycosidase activities are described in the text.

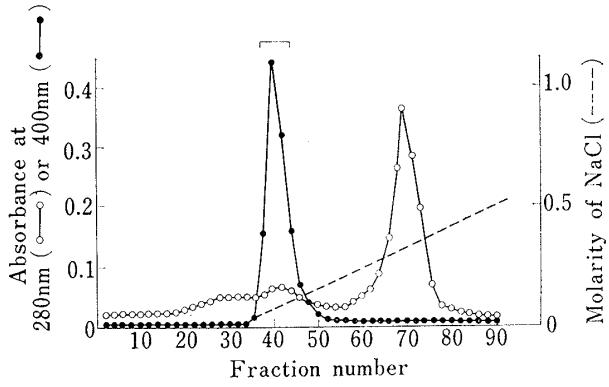

Fig. 2. Chromatography of Crude β-D-Glucosidase Fractions on a DEAE-Sephadex A-50 Column (1.9×45 cm)

Details are given in the text. ○—○, absorption at 280 nm for protein; ●—●, β-D-glucosidase activity expressed as optical density at 400 nm per 30 min per 0.05 ml of the fraction collected; -------, molarity of NaCl. Pooling was based on absorption peaks at 400 nm.

Properties of β-D-Glucosidase

Purity and Molecular Weight of β-D-Glucosidase——As shown in Fig. 2, the enzyme was eluted as a single peak which was free from protease and other glycosidase activities. Protease was assayed by using 1% casein solution in 0.028 M phosphate buffer, pH 7.8, as a substrate.

Purified β-D-glucosidase was found to be homogeneous by disc gel electrophoresis (Fig. 3). The activity peak coincided with the stained areas of the gel. The molecular weight of β-D-glucosidase was estimated by sodium dodecyl sulfate–gel electrophoresis. A single band was observed between chymotrypsinogen A and albumin, corresponding to a molecular weight of about 4.9×10^4 (Fig. 4).

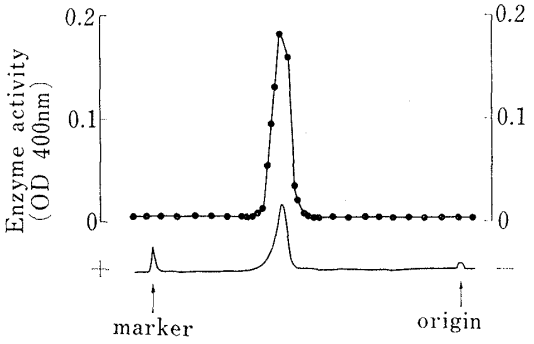

Fig. 3. Polyacrylamide Gel Electrophoresis of the Purified β-D-Glucosidase

Gels were run as described in the text and contained 30 ug of protein. One gel was stained with Coomassie blue. Protein of the stained gel was determined with a densitometer (lower curve). The other gel was cut into sections, which were extracted with 0.5 ml of 0.1 M citrate buffer (pH 4.5) and then assayed for β-D-glucosidase activity (upper curve).

TABLE II. Effects of Metal Ions and Various Inhibitors

Metal ions or inhibitors	Concentration mM	Relative activity %
None		100
$AgNO_3$	10	12
	1	5
$HgCl_2$	10	5
	1	3
$CuSO_4$	10	27
	5	84
	1	103
$CoCl_2$	10	21
	5	88
	1	128
$ZnCl_2$	10	68
$MgCl_2$	10	99
$CaCl_2$	10	102
NaCl	10	119
EDTA	10	105
2-Mercaptoethanol	10	107
L-cysteine	10	67
	5	69
	1	96
N-Bromosuccinimide	10	5
	1	3
I_2	1	8
	0.5	17
	0.1	87
ICH_2COOH	10	11

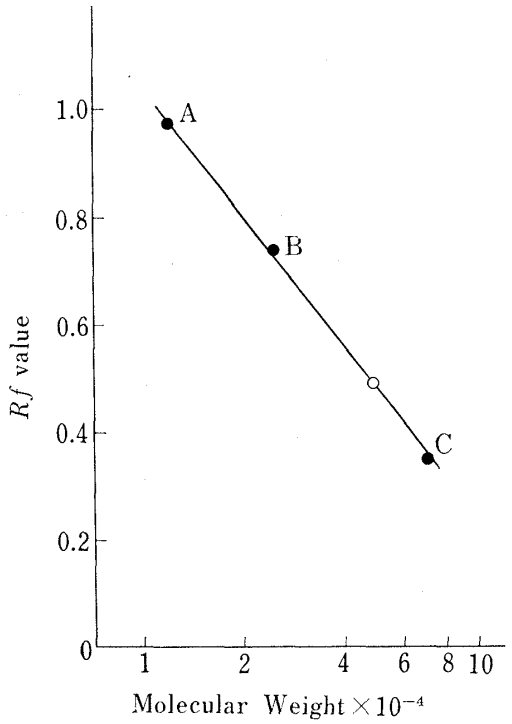

Fig. 4. Molecular Weight Determination of *T. mentagrophytes exo*-(1→3)-β-D-Glucosidase by Sodium Dodecyl Sulfate–acrylamide Gel Electrophoresis

The reference proteins are A, cytochrome c (1.24×10^4 M.W.); B, chymotrypsinogen A (2.5×10^4 M.W.); and C, bovine serum albumin (6.7×10^4 M.W.). The position of the enzyme is indicated by an open circle.

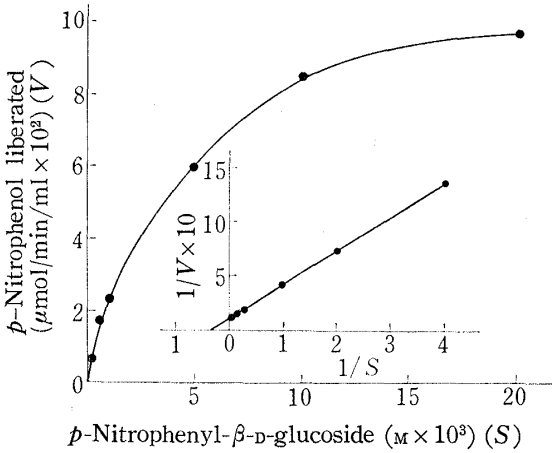

Fig. 5. Effect of *p*-Nitrophenyl-β-D-glucopyranoside Concentration on the Reaction Rate

For details concerning assay conditions, see the text.

Effects of Various Metal Ions and Reagents——Table II summarizes the effects of various metal ions and reagents on the enzyme activity. Among these metal ions, Ag^{+}, Hg^{+}, Cu^{2+} and Co^{2+} showed the highest levels of inhibition at a concentration of 10 mM. The enzyme was also inhibited by N-bromosuccinimide and I_2 at a concentration of 1 mM. Little or no inhibition was observed with EDTA, 2-mercaptoethanol and L-cysteine.

Effect of Substrate Concentration (*K*m Value)——The effect of substrate concentration on the reaction rate at pH 4.5 (0.05 M sodium citrate buffer) with *p*-nitrophenyl-β-D-glucopyranoside was studied. Fig. 5 shows the rate of hydrolysis as a function of *p*-nitrophenyl-β-D-glucopyranoside concentration. The *K*m value obtained from the plots was 3.1 mM.

Substrate Specificity——The purified β-D-glucosidase showed good activity towards *p*-nitrophenyl-β-D-glucopyranoside and esculin, but did not hydrolyze *p*-nitrophenyl-α-D-glucopyranoside, maltose, melibiose, gentiobiose, turanose, lactose, cellobiose, sucrose, trehalose or salicin (Table III-A). The enzyme was also tested on a number of polysaccharides such as laminaran, oxidized laminaran, pachyman, yeast glucan, islandican, O-carboxymethyl cellulose, nigeran, soluble starch, dextran and yeast mannan. The enzyme showed good activity towards laminaran and pachyman, but did not hydrolyze oxidized laminaran, islandican, O-carboxymethyl cellulose, nigeran, soluble starch, dextran or yeast mannan. When the enzymic hydro-

TABLE III. Substrate Specificity of the Purified Enzyme

A

Substrate	Relative activity (%)
p-Nitrophenyl-β-D-glucopyranoside	100
Esculin (6-(β-D-glucopyranosyloxy)-7-hydroxy-2H-1-benzopyran-2-one)	92
p-Nitrophenyl-α-D-glucopyranoside	0
Phenyl-β-D-glucoside	0
Methyl-β-D-glucoside	0
Methyl-α-D-glucoside	0
Maltose (4-O-α-glucopyranosyl-D-glucose)	0
Melibiose (6-O-α-D-galactopyranosyl-D-glucose)	0
Gentiobiose (6-O-β-D-glucopyranosyl-D-glucose)	0
Turanose (3-O-α-D-glucopyranosyl-D-fructose)	0
Lactose (4-O-β-D-galatopyranosyl-D-glucose)	0
Cellobiose (4-O-β-D-glucopyranosyl-D-glucose)	0
Sucrose (β-D-fructopyranosyl-α-D-glucopyranoside)	0
Trehalose (α-D-glucopyranosyl-α-D-glucopyranoside)	0
Salicin (2-(hydroxymethyl)phenyl-β-D-glucopyranoside)	0

B

Polysaccharide	Linkage	Relative activity (%)
R-Laminaran	(1→3)-β-D-, (1→6)-β-D-	100
Oxidized laminaran	(1→3)-β-D-, (1→6)-β-D-	0
Pachyman	(1→3)-β-D-	>100
Yeast glucan	(1→3)-β-D-, (1→6)-β-D-	10—30
Islandican	(1→6)-β-D-	0
O-(Carboxymethyl)cellulose	(1→4)-β-D-	0
Nigeran	(1→3)-α-D-, (1→4)-α-D-	0
Soluble Starch	(1→4)-α-D-, (1→6)-α-D-	0
Dextran	(1→6)-α-D-	0
Yeast mannan	(1→2)-α-D-, (1→3)-α-D-, (1→6)-α-D-	0

lysates of laminaran, pachyman and a mixture of pachyma-oligomers were examined at various times by paper chromatography, only glucose was found to be liberated (data not shown).

Discussion

T. mentagrophytes produces extracellular α-D-mannosidase, N-acetyl-β-D-glucosaminidase and β-D-glucosidase.[1] At present, the biological functions of these glycosidases in the fungus are not known. β-D-Glucosidase was produced to the greatest extent. With *p*-nitrophenyl-β-D-glucopyranoside as a substrate, the enzyme activity was maximum between pH 4.5 and 5.0.[1] The enzyme was stable at 50° for 5 min.[1] The molecular weight of the enzyme was 4.9×10^4 as determined by SDS disc gel electrophoresis (Fig. 4). This value was in agreement with the molecular weight of 5.0×10^4 calculated by Sephadex G-200 column chromatography.[1] The molecular weights of the enzyme from rat kidney, *Trichoderma viride* and *Saccharomyces cerevisiae* were $4.0—5.0\times10^4$,[14] 7.6×10^4,[15] and 3.0×10^5,[16] respectively. The molecular weight of the enzyme from *T. mentagrophytes* was different from all these values, but the pH optima of the enzymes were the same.

Preparations of β-D-glucosidase are often contaminated with other glycosidases. In the case of *Phaseolus vulgaris*,[17] β-D-glucosidase coexists with α-, β-galactosidases, β-N-acetyl-glucosaminidase and α-mannosidase. These enzyme preparations have been obtained almost free of cross-contamination with the exception of α-galactosidase and β-glucosidase, which were obtained as a mixture. However, the enzyme fraction from *T. mentagrophytes* obtained in this study was not contaminated with α-galactosidase. The method described in this paper for the preparation of β-D-glucosidase free from other glycosidases is relatively simple and reproducible. *R*-Laminaran, in which the reductive end group of laminaran has been reduced with $NaBH_4$, was hydrolyzed by β-D-glucosidase. However, the oxidized laminaran was not hydrolyzed by this enzyme. The enzyme hydrolyzes only the β-(Table III-A), and (1→3)-linkages (Table III-B), and is therefore an exo-(1→3)-β-glucosidase.

β-Glucosidic linkages are present in various glycoproteins, glycolipids and other carbohydrate complexes. The availability of β-D-glucosidase free from other glycosidase activities should therefore be useful for structural studies of many carbohydrate complexes containing β-glucosidic linkages.

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