Carbohydrate Research, 48 (1976) 209–216

AN ENDO-(1→6)-β-D-GLUCANASE FROM *Mucor hiemalis**

TOSHIO MIYAZAKI AND NAOKO OIKAWA
Department of Microbial Chemistry, Tokyo College of Pharmacy, Hachioji, Tokyo 192-03 (Japan)
(Received October 2nd, 1975, accepted for publication, December 30th, 1975)

ABSTRACT

An endo-(1→6)-β-D-glucanase (EC 3 2 1), isolated from the culture filtrate of *Mucor hiemalis*, was purified by ammonium sulphate fractionation and gel filtration The homogeneity of the enzyme was confirmed by disc electrophoresis The enzyme had a wide range of temperature and pH stability, high substrate specificity, and an action pattern of the endo-type

INTRODUCTION

(1→6)-β-D-Glucans have been isolated from lichens[2–4], yeast[5], and Penicillium sp [6–9], and (1→6)-β-D-glucanases are also known (1→6)-β-D-Glucanase activity has been detected in *Sclerotinia libertiana*[10], Rhizopus sp [10 11], *Penicillum brefeldianum*[12], Actinomycetes[13], *Bacillus circulans*[14], Streptomycetes[15], *Shizophillum commune*[16], and *Aspergillus usami*[17] The enzymes isolated from *Gibberella fujikuroi* by Shibata *et al* [18] and from *Rhizopus chinensis* by Nagasaki *et al.*[19] have been studied in detail. We have studied various glycosidases secreted by fungi[1], and we now report on the purification and some properties of a (1→6)-β-D-glucanase isolated from *Mucor hiemalis*

MATERIALS AND METHODS

Micro-organism — After screening tests using a medium containing (1→6)-β-D-glucan as the sole carbon source, *Mucor hiemalis* was selected as the source of the (1→6)-β-D-glucanase

Enzyme assays — 1 Unit is defined as the amount of enzyme which yields 1 μmol of reducing sugar when a mixture of 0 5 ml of (1→6)-β-D-glucan (0 2%), 0.5 ml of enzyme solution, and 1 ml of 0 1M acetate buffer (pH 4 5) is incubated for 1 h at 55° 0 2–0 4 U of enzyme was a convenient amount to use experimentally For the detection of (1→3)-β-D-glucanase activity, 0 2% of laminaran was used. Reducing sugar was measured by the Somogyi–Nelson method[21,22]

*Fungal Glycosidases Part II For Part I, see Ref 1

Protein assays — Protein content was determined by the absorbance at 280 nm or by the method of Lowry *et al* [23].

Substrates — (1→6)-β-D-Glucan (islandican) was purified from a culture filtrate of *Penicillium islandicum* IFO 4872 according to the method of Nakamura *et al* [8,9] Pachyman[24], laminaran[25], yeast glucan[26], yeast mannan[27], and nigeran[28] were purified by the literature procedures The following substrates were purchased commercially· dextran, carboxymethylcellulose (Na salt), soluble starch, phenyl β-D-glucopyranoside, methyl α-D-glucopyranoside, trehalose, turanose, cellobiose, maltose, sucrose, melibiose, gentiobiose, isomaltose, melezitose, and raffinose Oligosaccharides of the (1→6)-β-D-linked series were prepared by partial, acid hydrolysis (0 3M H_2SO_4, 1 5 h) of the (1→6)-β-D-glucan extracted from *Gyrophora esculanta*[3]. After hydrolysis, the oligosaccharides were isolated by elution from charcoal–Celite, using a 0→30% ethanol gradient, followed by p c [Whatman 3MM paper, multiple development, 1-propanol–ethyl acetate–water, 6 1 3 (Solvent *A*)][29]

Purification of the enzyme — A culture of *M hiemalis* (1 9 l) was incubated in a reciprocal shaker (115 times/min) at 25° The culture medium contained 0 2% of islandican, 0 2% of $(NH_4)_2SO_4$, 0 2% of KH_2PO_4, 0 1% of $MgSO_4$ $7H_2O$, and 0 01% of yeast extract (Wako Pure Chemical Ind Ltd.) After 3 days (see Table I), the culture was filtered through a nylon cloth, dialyzed against distilled water for 24 h at low temperature, and then lyophilized to give a white powder (*L-1*) (2 498 g) A solution of *L-1* in distilled water (50 ml) was centrifuged, ammonium sulphate was added to the supernatant to 0 7% saturation, and the mixture was stored in a refrigerator overnight The precipitate was collected after centrifugation at 10,000 r p m for 10 min, and dissolved in distilled water (5 ml), and the solution was dialyzed against distilled water at 5° overnight and then lyophilized to give *L-2* (0 044 g) *L-2* was eluted from a column (1 5×90 cm) of Sephadex G-100, using 0 01M phosphate buffer (pH 7 2) containing 0.5M KCl A portion (0 1 ml) of each fraction (3 ml) was assayed for enzyme activity

TABLE I

PRODUCTION OF (1→6)-β-D-GLUCANASE DURING GROWTH OF *Mucor hiemalis*

Time of growth (days)	1	2	3	4	6	8
pH	5 1	4 1	3 7	3 6	3 6	3 6
(1→6)-β-D-Glucanase activity (unit/0 5 ml)	0 004	0 390	0 419	0 386	0 382	0 297

General properties of the enzyme. — (*a*) *Effect of pH.* The pH stability was examined by treatment of the enzyme with the appropriate buffers for 20 h at 25°, and the pH optimum was determined by using the enzyme assays described above

(*b*) *Effect of temperature* The heat stability of the enzyme was determined by assaying enzyme activity after incubation of the enzyme with acetate buffer (pH 4.5) for 10 min at each temperature.

(*c*) *Effect of metal ions and bovine serum albumin.* The activity of the enzyme

was determined in the presence of each of the following salts $MgCl_2$ $6H_2O$, $CaCl_2 \cdot 2H_2O$, $ZnCl_2$, $CdSO_4$, $CuSO_4 \cdot 5H_2O$, $HgCl_2$, $FeCl_3 \cdot 6H_2O$, and also in the presence of bovine serum albumin (Wako Chemical Ind. Ltd.).

(*d*) K_m *value* The K_m value was obtained by incubating the enzyme with various concentrations of islandican for 10 min

(*e*) *Substrate specificity.* 0 2% of each substrate was used

(*f*) *Mode of action of the enzyme* After the enzyme had acted on islandican for 0 5, 1, 2, and 3 h, each solution was concentrated, and subjected to chromatography on Toyo Roshi No 50 paper (solvent *A*). Likewise, the products from each (1→6)-β-D-oligosaccharide were investigated Detection was effected with alkaline silver nitrate[30], and molar ratios were determined, after paper chromatography, by the phenol–sulphuric acid method[31]

(*g*) *Disc electrophoresis* Disc electrophoresis of the enzyme was carried out in glycine buffer (pH 8 3) for 2 h and β-alanine buffer (pH 4 5) for 1 8 h with a current of 2 mamp per Biogel column After electrophoresis, the column was cut into 20 2 5-mm slices Each slice was extracted with 0 5 ml of 0 1M acetate buffer (pH 4 5), and each extract was incubated with the substrate at 55° for 3 h

RESULTS AND DISCUSSION

Although (1→6)-β-D-glucanases have been detected in various fungi, the production of a (1→6)-β-D-glucanase by the Mucor genus has not been documented hitherto.

The specific activity of the culture filtrate was increased 2-fold by means of ammonium sulphate fractionation (Table II), and 14 4-fold after gel filtration on Sephadex G-100 The recovery of the activity was 92 4% and 13 6% after ammonium sulphate fractionation and gel filtration, respectively Generally, the culture filtrate contained, in addition to the (1→6)-β-D-glucanase, other, interfering enzymes *Penicillium brefeldianum*[12] also yielded substantial amounts of a (1→3)-β-D-glucanase and a β-D-glucosidase which were isolated by zone electrophoresis

TABLE II

PURIFICATION OF (1→6)-β-D-GLUCANASE

Purification step	*Total activity (units)*	*Total protein (mg)*	*Specific activity (units/mg)*	*Yield (%)*
Culture filtrate	1782 2	82 5	21 6	100
Ammonium sulphate fractionation	1646 9	39 9	41 2	92 4
Sephadex G-100 gel-filtration	243 0	0 782	310 7	13 6

By elution of the crude glucanase (prepared from *M. hiemalis*) from Sephadex G-100, using 0.01M phosphate buffer (pH 7.2) containing 0 5M KCl, the (1→6)-β-D-glucanase could be separated from the (1→3)-β-D-glucanase (Fig 1). Elution with

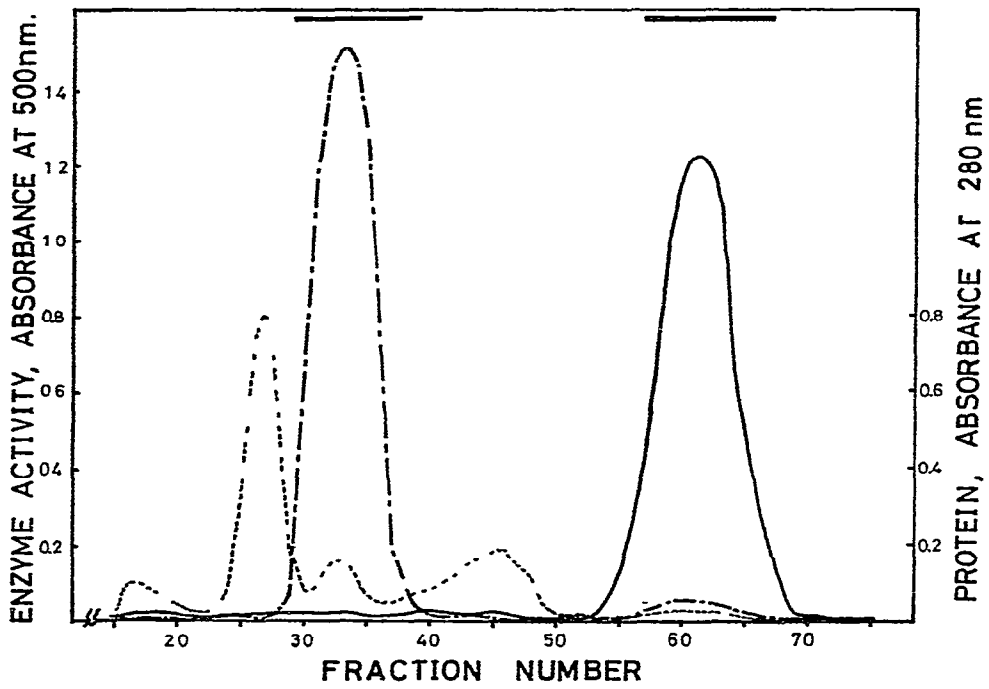

Fig 1 Gel filtration of crude enzyme on Sephadex G-100 – – – –, protein, ————, (1→6)-β-D-glucanase activity, — · — —, (1→3)-β-D-glucanase activity

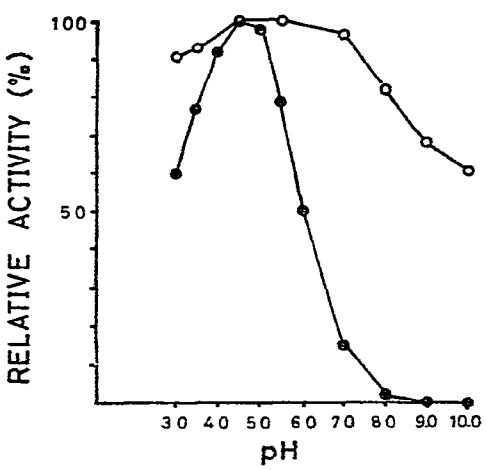

Fig 2 Effect of pH on the enzyme activity: O——O, pH stability, ●——●, pH optimum.

the KCl-free buffer gave the (1→3)-β-D-glucanase. Since Sephadex G-100 is prepared from a (1→6)-α-D-glucan, the adsorption of (1→6)-β-D-glucanase may be promoted and this effect could contribute to the high purity of the enzyme isolated

The pH optimum of the enzyme was 4 5, and the enzyme was stable in the pH range 3–7 (Fig 2) It is of interest that the optimum pH of the enzyme should be on the acid side, as *M hiemalis* was cultured in an acidic medium. The pH optima of the other (1→6)-β-D-glucanases[18,19] are 5–6

In studies of the endo-(1→6)-β-D-glucanase from *Rhizopus chinensis*, Nagasaki *et al*[19] stated that potassium phosphate inhibits enzyme action. The (1→6)-β-D-glucanase from *M hiemalis* was not inhibited in this way.

The optimum temperature of the enzyme was 55° and it was stable in the range 30–60°. Even at 70°, ~56% of the activity remained (Fig 3)

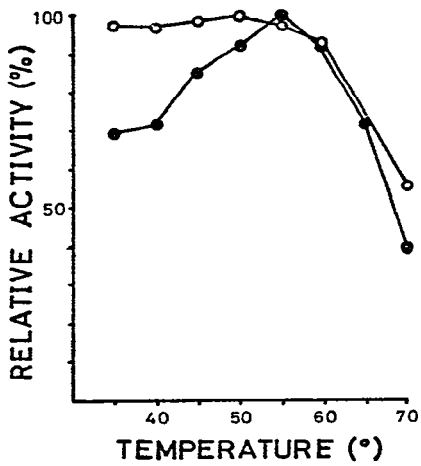

Fig 3 Effect of temperature on the enzyme activity O——O, heat stability, ●——●, temperature optimum

TABLE III

EFFECT OF METAL IONS ON THE ENZYME ACTIVITY

	Relative activity (%)		
	10^{-2}M	10^{-3}M	10^{-4}M
None		100	
Mg^{2+}	104 1	102 5	98 9
Ca^{2+}	96.2	93 4	100 7
Zn^{2+}	25 9	62 8	98 0
Cd^{2+}	46 8	70.2	101.1
Cu^{2+}	0 4	25 1	69 4
Hg^{2+}	0	18 7	85 8
Fe^{3+}	0	53 3	62 3

The enzyme was not affected by Mg^{2+} and Ca^{2+}, but was moderately inhibited by Zn^{2+} and Cd^{2+}, and strongly by Cu^{2+}, Hg^{2+}, and Fe^{3+} (Table III)

The activity of the enzyme was protected and enhanced (119% and 124%, respectively) by the addition of 100 or 200 μg/ml of bovine serum albumin

The K_m value of the enzyme, obtained by a Hofstee plot, was 0 173% (Fig 4)

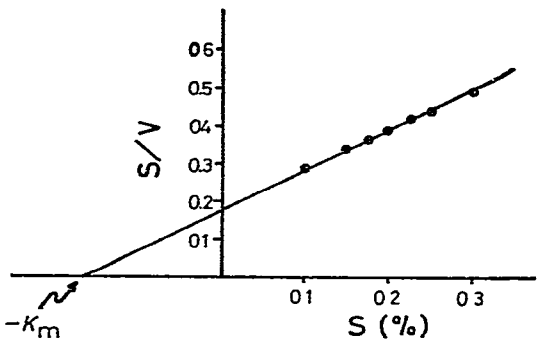

Fig. 4 Hofstee plot of the (1→6)-β-D-glucanase

Taking the activity of the (1→6)-β-D-glucanase against islandican as 100%, the enzyme was slightly active against laminaran, but not against the other substrates tested (Table IV), laminaran contains some (1→6)-β-D-linkages

TABLE IV

SUBSTRATE SPECIFICITY OF PURIFIED ENZYME[a]

Polysaccharide	*Linkage*	*Activity* (%)
Islandican	(1→6)-β-D-	100
Laminaran	(1→3)-β-D-, (1→6)-β-D-	2 7
Yeast glucan	(1→3)-β-D-, (1→6)-β-D-	1 0
Pachyman	(1→3)-β-D-	0
Carboxymethylcellulose (Na salt)	(1→4)-β-D-	0 9
Dextran	(1→6)-α-D-	0 4
Nigeran	(1→3)-α-D-, (1→4)-α-D-	0
Soluble starch	(1→4)-α-D-, (1→6)-α-D-	0
Yeast mannan	(1→6)-α-D-, (1→3)-α-D-, (1→2)-α-D-	0

[a]No activity was observed against phenyl β-D-glucopyranoside, methyl α-D-glucopyranoside, trehalose, turanose, cellobiose, maltose, gentiobiose, isomaltose sucrose, melibiose, melezitose, and raffinose

The enzyme appeared to be of the endo-type and had no exo-action For example, it did not attack phenyl β-D-glucopyranoside and, when acting on islandican, produced the series (di- to octa-saccharide) of (1→6)-β-D-oligosaccharides, but D-glucose was not formed Similarly, the enzyme did not attack gentiobiose or

gentiotriose, but the following conversions were observed tetramer→dimer; pentamer→dimer and trimer; hexamer→dimer, trimer, and tetramer The enzyme acting on the hexamer gave, initially the tetramer, trimer, and dimer, but, on prolonged action, the dimer increased at the expense of the tetramer. The final molar ratio of the trimer and dimer produced from the hexamer was 0 34 1 0 Thus, the enzyme cleaved the inner linkage of the hexamer.

It is of interest to note that the endo-(1→6)-β-D-glucanase produced by *Bacillus circulans* gave gentiobiose and D-glucose from pustulan[14]

After disc electrophoresis in glycine buffer (pH 8 3) and β-alanine buffer (pH 4 5), the active component was present in a single slice A protein band was not detected because of the low concentration of the enzyme protein

Although the yield is not high, the enzyme has high specificity and stability, and is likely to be useful for structural analysis of glycans

(1→6)-β-D-Glucanase activity was not detected in the culture fluid when D-glucose was used instead of islandican as the carbon source, therefore, the enzyme is induced On the other hand, (1→3)-β-D-glucanase was detected, even in the substrate-free culture

Application of the enzyme in the structural investigation of glycans will be described elsewhere

ACKNOWLEDGMENTS

We express our gratitude to Mr. K. Ohshima, Mr N. Matsumoto, and Mr N Ito for technical assistance, to the village headman of Ueno village of Gumma prefecture for the present of *G. esculenta*, and Dr A Ito of this college for the gift of isomaltose.

REFERENCES

1 S YAMATO, T YADOMAE, AND T MIYAZAKI, *J. Ferment Technol* 52 (1974) 706–712
2 B LINDBERG AND J MCPHERSON, *Acta Chem Scand*, 8 (1954) 985–988
3 S SHIBATA, Y. NISHIZAWA, T TAKEDA, AND M TANAKA, *Chem. Pharm Bull*, 16 (1968) 2362–2369
4 A H HECKENDORF AND Y SHIMIZU, *Phytochemistry*, 13 (1974) 2181–2183
5 D J MANNERS, A J MASSON, AND J C PATTERSON, *Biochem J*, 135 (1973) 31–36
6 C G ANDERSON, W N HAWORTH, H RAISTRICK, AND M STACEY, *Biochem J*, 33 (1939) 272–279
7 J BADDILEY, J G BUCHANAN, AND E M THAIN, *J Chem, Soc*, (1953) 1944–1946
8 N NAKAMURA, J OOYAMA, AND O TANABE, *Nippon Nogei Kagaku Kaishi*, 35 (1961) 949–953
9 E EBERT AND M H ZENK, *Arch Mikrobiol*, 54 (1966) 276–296
10 Y SATOMURA, M ONO, AND J FUKUMOTO, *Bull Agr Chem Soc Japan*, 24 (1960) 317–321
11 A KURODA, S OHWADA, AND Y TOKUMARU, *J Ferment Technol*, 46 (1968) 930–937
12 E T. REESE, F W. PARRISH AND M MANDELS, *Can J Microbiol*, 8 (1962) 327–334
13 N NAKAMURA AND O TANABE, *Agr Biol Chem*, 27 (1963) 80–87
14 H TANAKA AND H J PHAFF, *J. Bacteriol*, 89 (1965) 1570–1580
15 H SUGIMOTO, *Agr. Biol Chem*, 31 (1967) 1111–1123
16 J G. H WESSELS, *Biochim Biophys Acta*, 178 (1969) 191–193
17 Y SHIBATA AND T FUKIMBARA, *J Ferment Technol*, 50 (1972) 388–396

18 Y. SHIBATA AND T FUKIMBARA, *J Ferment. Technol*, 51 (1973) 216–226
19 S YAMAMOTO, R. KOBAYASHI, AND S NAGASAKI, *Agr Biol Chem*, 38 (1974) 1493–1500
20 S YAMATO, T. YADOMAE, AND T MIYAZAKI, *J Ferment Technol*, 52 (1974) 706–712
21 M. SOMOGYI, *J Biol Chem*, 195 (1952) 19–23
22 N NELSON, *J Biol Chem*, 153 (1944) 375–380
23 O H LOWRY, N J ROSEBROUGH, A L FARR, AND R. J RANDALL, *J Biol Chem*, 193 (1951) 265–275.
24 S A WARSI AND W J WHELAN, *Chem Ind (London)*, (1957) 1573
25 W A. P BLACK, *Methods Carbohyd Chem*, 5 (1965) 159–161
26 D J MANNERS, A J MASSON, AND J C. PATTERSON, *Biochem J*, 135 (1973) 19–30
27 T. E EDWARDS, *Methods Carbohyd Chem*, 5 (1965) 176–179
28 S A BARKER, *Methods Carbohyd Chem*, 5 (1965) 165–167
29 D R. BARRAS AND B A STONE, *Biochim Biophys Acta*, 191 (1969) 329–341
30 W E TREVELYAN, D P PROCTER, AND J S HARRISON, *Nature (London)*, 166 (1950) 444–445
31 M DUBOIS, K A GILLES, J K. HAMILTON, P. A REBERS, AND F SMITH, *Anal Chem*, 28 (1956) 350–356