Carbohydrate Research, 49 (1976) 383—388

SOME PROPERTIES OF A FUNGAL β-D-GLUCANASE PREPARATION*†

DAVID J. MANNERS, J. ROGER STARK, GLYNN WILSON††, AND JAMES Y. BRODIE
Department of Brewing and Biological Sciences, Heriot-Watt University, Edinburgh EH1 1HX (Great Britain)
(Received December 2nd, 1975; accepted for publication, January 2nd, 1976)

ABSTRACT

A commercial enzyme preparation, of fungal origin, contained a mixture of β-D-glucanases which were fractionated by ion-exchange chromatography to give a mixture of an endo-(1→4)- and an exo-(1→3)-β-D-glucanase. These two enzymes were then separated by molecular-sieve chromatography on Sephadex G-150. The purified exo-(1→3)-β-D-glucanase has a relatively high specificity for (1→3)-β-D-glucosidic linkages, and has no action on lichenin.

INTRODUCTION

Enzymes capable of hydrolysing β-D-glucosidic linkages are widely distributed in fungi[2–6]. These enzymes exhibit a variety of substrate specificities and action patterns[7], and these properties have been used to advantage in the structural analysis of β-D-glucans. An exo-(1→3)-β-D-glucanase[8] from *Basidiomycete* species QM806 and an endo-(1→3)-β-D-glucanase from *Rhizopus arrhizus*[2,9] have been widely used for this purpose. We now describe initial studies on the characterization of the β-D-glucanases from a commercially available, fungal-enzyme preparation, which is marketed as "Glucanase GV" (A/S Grindstedvaerket, Brabrand, Denmark) for use in the Brewing Industry[10].

EXPERIMENTAL

The enzyme preparation was supplied as a liquid concentrate which contained a high proportion of reducing sugar, as detected by the modified Nelson[11] and phenol–sulphuric acid reagents[12]. Protein was therefore precipitated from a 1-litre sample of enzyme by the addition of solid ammonium sulphate to 80% saturation (yield, 5.3 g).

The preparation of substrates and the procedures for the assay of β-D-glucanase activities have been described in detail previously[1,13].

---

*Dedicated to the memory of Professor Edward J. Bourne.
†Studies on β-Glucanases: Part IV. For Part III, see Ref. 1.
††Present address: The Rockefeller University, New York, N.Y. 10021, U. S. A.

RESULTS

The 0–80% ammonium sulphate precipitate showed activity towards laminarin, lichenin, and CM-cellulose (reductometric assays), and barley-β-D-glucan and CM-cellulose (viscometric assays). Fractionation of this material on DEAE-cellulose (Fig. 1) showed several peaks of β-D-glucanase activity. A single peak of (1→3)-β-D-glucanase activity was eluted at the beginning of the salt gradient and was partially separated from a peak having activity towards lichenin. Several peaks of (1→4)-β-D-glucanase were eluted at higher concentrations of salt. These peaks also showed activity towards lichenin. It should be noted that, in Fig. 1, the size of the samples used for assay against CM-cellulose were one-tenth of those used for the laminarinase and lichenase assays, so that the preparation has a relatively high endo-(1→4)-β-D-glucanase activity.

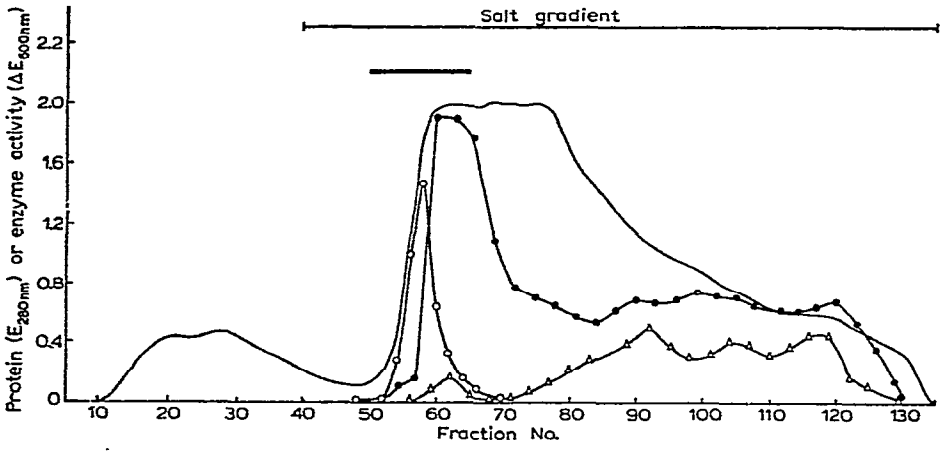

Fig. 1. Fractionation of "Glucanase GV" on DEAE-cellulose. The 0–80% ammonium sulphate fraction (500 mg) in 20mM citrate–phosphate buffer (pH 8.0) was applied to a column (20 × 2.5 cm) of Whatman DE-52, equilibrated with the same buffer. Protein (————) was eluted by buffer followed by a salt gradient (0→1.0M NaCl in 20mM citrate phosphate, pH 8.0). Fractions (8 ml) were collected and aliquots (0.1 ml) were assayed against laminarin (○——○) and lichenin (●——●). Aliquots of 0.01 ml were assayed against CM-cellulose (△——△) by a reductometric method[11]. Fractions under the heavy bar were combined, and concentrated by ultrafiltration.

The (1→3)-β-D-glucanase was completely separated from lichenase activity and further purified by molecular-sieve chromatography on Sephadex G-150 (Fig. 2 and Table I). Although the overall degree of purification was only 11·6-fold, this level is similar to that reported for other fungal exo-(1→3)-β-D-glucanases, *e.g.*, the enzymes from a *Basidiomycete*[14] QM806 and from *Sclerotinia libertiana*[15] were obtained in a homogeneous or crystalline state after only a 7- or 8-fold increase in specific activity. These observations suggest that a high proportion of the protein secreted into the medium by these organisms is exo-(1→3)-β-D-glucanase.

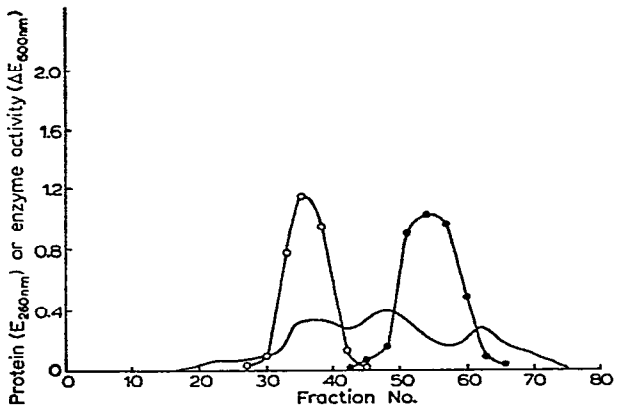

Fig. 2. Fractionation of (1→3)-β-D-glucanase on Sephadex G-150. Combined fractions from DEAE-cellulose chromatography were applied to a column (60×2.5 cm) of Sephadex G-150 in 0.1M acetate buffer (pH 5.0). Fractions (4 ml) were collected, and assayed for protein (————), and against laminarin (O——O) and lichenin (●——●).

TABLE I

PURIFICATION OF THE (1→3)-β-D-GLUCANASE

Stage	*Total protein (mg)*	*Total units*[b]	*Specific activity (units/mg of protein)*	*Purification (fold)*	*Yield (%)*
Ammonium sulphate[a]					
0–80%	315	175	0.55	—	100
DEAE-cellulose	109	133	1.20	2.2	76
Sephadex G-150	12.6	81	6.40	11.6	46

[a]The amount of starting material was 500 mg. [b]1 unit is defined as the amount of enzyme liberating 1 μmol of D-glucose per min. in a digest containing 0.5 ml of 1% laminarin, 0.48 ml of 0.1M sodium acetate buffer (pH 4.8), and 0.02 ml of appropriately diluted enzyme solution at 37°.

In 0.05M sodium citrate–phosphate buffer, the enzyme showed optimum activity towards laminarin between pH 4.0 and 5.5, with a maximum at pH 4.8. It had a molecular weight of ~63,000 based on molecular-sieve chromatography using a column of Sephadex G-100, calibrated with bovine serum albumin, ovalbumin, pepsin, α-chymotrypsinogen, and ribonuclease.

The enzyme was characterized as an exo-(1→3)-β-D-glucanase by the following observations: (*a*) it degraded both the soluble and insoluble forms of laminarin, liberating D-glucose as the only major product throughout the incubation; (*b*) it did not diminish the viscosity of CM-pachyman solutions; (*c*) with insoluble laminarin, the rate of release of D-glucose determined by reducing-power measurements was the same as that from D-glucose-oxidase assays (Table II); (*d*) it did not attack periodate-oxidised laminarin (prepared by Dr. G. Fleet) at a significant rate, although Smith-degraded laminarin was readily hydrolysed (Fig. 3). In the latter substrate, the

non-reducing D-glucose residues in the original laminarin, which are oxidised by periodate and then subsequently reduced, are removed during the mild hydrolysis with acid.

TABLE II

RATE OF HYDROLYSIS OF LAMINARINS BY EXO-(1→3)-β-D-GLUCANASE[a]

Substrate	*Insoluble laminarin*[b]	*Insoluble laminarin*[c]	*Soluble laminarin*[b]
Time (min)			
5	12	9	6
10	23	20	18
15	—	—	31
30	68	64	62
60	124	120	116

[a]The digests consisted of 7.5 ml of 0.1% laminarin, 6.0 ml of sodium acetate buffer (0.1M, pH 4.8), and 1.5 ml of a 1:250 dilution of purified enzyme in buffer, and were incubated at 37°. Samples (1 ml) were analysed at intervals, the enzyme being inactivated by heating (100°, 5 min). [b]μg of D-glucose liberated (Nelson–Somogyi assay). [c]μg of D-glucose liberated (D-glucose-oxidase assay).

The action of the purified enzyme on various substrates was investigated by incubating substrate (5 mg) with acetate buffer (0.1 ml; 0.02M, pH 5.0) and enzyme solution (0.1 ml, 1:250 dilution) for 24 h at 37°. The digests were analysed by paper chromatography after 6 and 24 h. Compared to the rapid attack on laminarin, laminaritriose was only slowly attacked, and the rate of hydrolysis of paramylon[16],

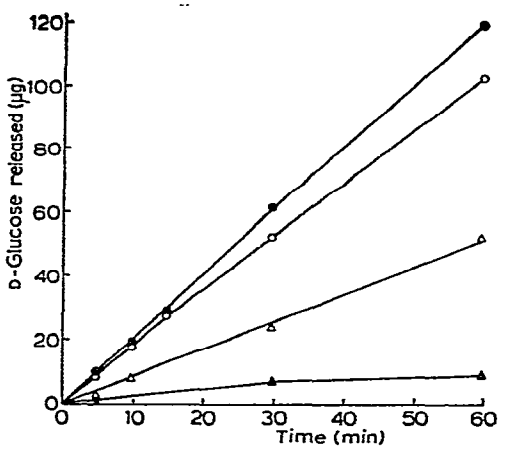

Fig. 3. Rate of hydrolysis of laminarin derivatives by purified exo-(1→3)-β-D-glucanase. Digests contained 6.0 ml of 0.1% substrate, 4.8 ml of sodium acetate buffer (0.1M, pH 4.8), and 1.2 ml of enzyme solution (1:250 dilution of purified enzyme, containing 0.108 unit/ml), and were incubated at 37°. Samples (1 ml) were withdrawn at intervals for analysis[11]. Key: ●——●, Smith-degraded laminarin; O——O, borohydride-reduced, soluble laminarin; △——△, CM-laminarin (average degree of substitution, 0.31 per D-glucose residue); ▲——▲, periodate oxidised laminarin. For the last substrate, the enzyme concentration was increased 5-fold.

laminaribiose, and laminaribiosyl-mannitol was even lower. The slow attack on paramylon is most probably due to the low solubility of the substrate. The following substrates were not attacked: laminarin polyalcohol, luteose, xylan, cellodextrin, cellobiose, cellobiitol, glucosyl-mannitol, laminaribiitol, gentiobiose, sophorose, and β,β-trehalose. The enzyme therefore shows a high degree of specificity for substrates containing linear sequences of three or more (1→3)-linked β-D-glucose residues which are terminated by a non-reducing residue. However, a moderate degree of substitution of the D-glucose residues may be tolerated, since CM-laminarin is slowly hydrolysed (Fig. 3). Borohydride-reduced, soluble laminarin, as expected, was readily hydrolysed by the enzyme. Sclerotan, from *Sclerotinia sclerotiorum*[5], was hydrolysed to give glucose and gentiobiose.

The enzymic activity towards lichenin (Fig. 2) decreased the viscosity of solutions of barley β-D-glucan and also released reducing sugars from CM-cellulose. With lichenin, the major product was a trisaccharide, tentatively identified as 4-*O*-β-laminaribiosyl-D-glucose from its paper-chromatographic mobility; other products were cellobiose and traces of a higher oligosaccharide. Endo-(1→4)-β-D-glucanases with a similar ability to degrade mixed-linkage β-D-glucans have been identified from *Aspergillus niger*[17] and *Trichoderma viride*[18].

DISCUSSION

The present studies have demonstrated the presence of several β-D-glucanase components in a commercially available enzyme preparation of fungal origin. Since two of these components, an exo-(1→3)-β-D-glucanase and an endo-(1→4)-β-D-glucanase capable of hydrolysing both lichenin and CM-cellulose, can be readily separated and purified, they should provide useful tools for the structural analysis of β-D-glucans containing (1→3)-linkages, either alone, or together with either (1→4)- or (1→6)-linkages. For example, laminarin has been fractionated by chromatography on DEAE-Sephadex (molybdate form) to give the D-glucose-terminated molecules (G-chains) and mannitol-terminated molecular (M-chains)[19]. Incubation of the G-chains with the exo-(1→3)-β-D-glucanase gave D-glucose and gentiobiose as the only products. Similar digestion of the M-chains gave D-glucose, gentiobiose, and D-glucosyl-mannitol. These results provide clear evidence that the small proportion of (1→6)-β-D-glucosidic linkages[20] in laminarin are present in both the G- and the M-chains. The results also show that the enzyme can by-pass resistant linkages, and hence, complete the hydrolysis of laminarin. Details of these and related experiments on the structure of yeast cell-wall β-D-glucans will be reported elsewhere.

The recent literature contains several references to exo-(1→3)-β-D-glucanases, including enzymes isolated from *Helix pomatia*[21], sea-urchin eggs[22], three species of yeast (*Fabospora fragilis*[23], *Hansenula anomala*[23], and *Saccharomyces cerevisiae*[23,24]) and from other fungi, *e.g.*, *Phytophthora palmivora*[25], *Sclerotinia libertiana*[15], *Coniothyrium minitans*[5], and *Trichoderma viride*[5]. As a group, these enzymes show various degrees of specificity. The enzyme from *Helix pomatia*[21] can slowly hydrolyse

some (1→4)- and (1→6)-β-D-glucosidic linkages, in addition to the (1→3)-type, whilst certain yeast exo-β-D-glucanases act on both laminarin and pustulan[23,24]. The purified Grindsted enzyme resembles that from the other fungi[5,15,25] in its relatively high specificity for (1→3)-β-D-glucosidic linkages, and its ability to by-pass (1→6)-inter-chain linkages in branched substrates such as laminarin or sclerotan, and hence complete the degradation of the substrate. This latter property contrasts with the hydrolysis of starch by certain exo-amylases whose activity is arrested by inter-chain linkages.

ACKNOWLEDGMENTS

This work was supported by a grant from the Science Research Council. We are indebted to Grindsted Products Ltd for generous supplies of "Glucanase GV", and to Dr. J. S. D. Bacon for the sample of sclerotan.

REFERENCES

1 D. J. MANNERS AND G. WILSON, *Carbohyd. Res.*, 48 (1976) 255–264.
2 E. T. REESE AND M. MANDELS, *Can. J. Microbiol.*, 5 (1959) 173–185.
3 C. G. C. CHESTERS AND A. T. BULL, *Biochem. J.*, 86 (1963) 31–38.
4 A. E. CLARKE AND B. A. STONE, *Biochem. J.*, 96 (1965) 793–801.
5 D. JONES, A. H. GORDON, AND J. S. D. BACON, *Biochem. J.*, 140 (1974) 47–55.
6 G. H. FLEET AND H. J. PHAFF, *J. Biol. Chem.*, 249 (1974) 1717–1729.
7 D. R. BARRAS, A. E. MOORE, AND B. A. STONE, *Advan. Chem. Series*, 95 (1969) 105–138.
8 T. E. NELSON, J. JOHNSON, E. JANTZEN, AND S. KIRKWOOD, *J. Biol. Chem.*, 244 (1969) 5972–5980.
9 F. W. PARRISH, A. S. PERLIN, AND E. T. REESE, *Can. J. Chem.*, 38 (1960) 2094–2104.
10 J. ENKELUND, *Process Biochem.*, August 1972, pp. 2–4.
11 J. F. ROBYT AND W. J. WHELAN, in J. A. RADLEY (Ed.), *Starch and its Derivatives*, Chapman and Hall, London, 4th edition, 1968, p. 432.
12 M. DUBOIS, K. A. GILLES, J. K. HAMILTON, P. A. REBERS, AND F. SMITH, *Anal. Chem.*, 28 (1956) 350–356.
13 D. J. MANNERS AND G. WILSON, *Carbohyd. Res.*, 37 (1974) 9–22.
14 F. I. HUOTARI, T. E. NELSON, F. SMITH, AND S. KIRKWOOD, *J. Biol. Chem.*, 243 (1968) 952–956.
15 J. EBATA AND Y. SATOMURA, *Agr. Biol. Chem.*, 27 (1963) 478–483.
16 D. J. MANNERS, J. F. RYLEY, AND J. R. STARK, *Biochem. J.*, 101 (1966) 323–327.
17 A. E. CLARKE AND B. A. STONE, *Biochem. J.*, 99 (1966) 582–588.
18 O. IGARASHI, M. FUJIMAKI, AND Y. SAKURAI, *Hakko Kogaku Zasshi*, 47 (1969) 456–462.
19 J. R. STARK, *Carbohyd. Res.*, 47 (1976) 176–178.
20 W. D. ANNAN, E. L. HIRST, AND D. J. MANNERS, *J. Chem. Soc.*, (1965) 885–891.
21 J. J. MARSHALL AND R. J. A. GRAND, *Arch. Biochem. Biophys.*, 167 (1975) 165–175.
22 A. V. MUCHMORE, D. EPEL, A. M. WEAVER, AND R. T. SCHIMKE, *Biochim. Biophys. Acta*, 178 (1969) 551–560.
23 A. T. H. ABD-EL-AL AND H. J. PHAFF, *Biochem. J.*, 109 (1968) 347–360.
24 V. FARKAS, P. BIELY, AND S. BAUER, *Biochim. Biophys. Acta*, 321 (1973) 246–255.
25 V. Z. HOLTEN AND S. BARTNICKI-GARCIA, *Biochim. Biophys. Acta*, 276 (1972) 221–227.