The Glucoamylase of *Coniophora cerebella*

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1. The major amylolytic enzyme in culture filtrates of *Coniophora cerebella* grown in starch-containing media has been purified and characterized as a glucoamylase (EC 3.2.1.3). 2. The activity/unit wt. of protein was increased 11-fold and the enzyme showed one major component on polyacrylamide-gel electrophoresis. 3. The glucoamylase had optimum pH 4.0–4.5. 4. Hg^{2+} completely inhibited the enzyme, but other ions tested had little effect on the activity at the concentration of ions used (5 mM). 5. The action of the enzyme on amylopectin, amylose and maltose was studied. Hydrolysis proceeded by the stepwise removal of glucose units from the non-reducing ends of the polymer chains, and the enzyme was able to bypass or to hydrolyse the α -(1 $\rightarrow$ 6)-glucosidic linkages at branch points in the amylopectin molecule. Glucose was the only product found in digests of these substrates. 6. At the same substrate concentration (0.1%, w/v) and enzyme concentration, the initial rates of glucose production from amylopectin, amylose and maltose were in the proportions 40:10:1. 7. K_m values at 40° and pH 4.0 were calculated for the enzyme acting on amylopectin, amylose and maltose.

The starch-degrading enzyme systems of the higher fungi have not been studied in the same detail as those of the commercially important moulds, yet many of these fungi are maintained on starch-containing media such as malt-agar or potato-dextrose-agar under conditions where amylolytic enzymes are probably the predominant extracellular carbohydrases.

Lyr (1959) claimed that the major amylolytic activity in the culture filtrates of a variety of wood-destroying Basidiomycetes, including *Coniophora cerebella*, was an α -amylase (EC 3.2.1.1). However, King (1966) presented evidence that a glucoamylase (α -1,4-glucan glucohydrolase, EC 3.2.1.3) was the predominant enzyme from *C. cerebella* degrading starch.

The present paper describes the purification of this enzyme and its study in more detail.

MATERIALS AND METHODS

Source of enzyme. *C. cerebella*, F.P.R.L. culture no. 11E, was grown on medium B as previously described (King, 1966) with 2% (w/v) soluble starch as carbon source and enzyme inducer. After growth for 10–14 days the crude enzyme solution was obtained by filtering the cultures through a no. 3 sintered-glass filter. The filtrate was immediately adjusted to pH 5.0 with 4N-NaOH, and NaN_3 added (0.31 g./l.) as preservative. This crude enzyme solution had α - and β -glucosidase (EC 3.2.1.20 and 3.2.1.21 respectively), laminarinase (EC 3.2.1.6) and glucoamylase (EC 3.2.1.3) activities.

Substrates. Soluble starch (A.R. grade) was obtained from British Drug Houses Ltd., Poole, Dorset. Waxy maize starch (amylopectin) was a gift from Professor D. J. Manners, and had an average chain length, as determined by periodate oxidation, of 24 glucose residues. Amylose was prepared from a commercial amylose (Koch-Light Laboratories Ltd., Colnbrook, Bucks.) by butan-1-ol fractionation. Cellobiose and maltose were chromatographically pure samples. Laminarin was purchased from Liverpool Borax Co. Ltd., Liverpool, 3.

Analytical methods. Total reducing sugar was determined by the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952), and glucose production by Dahlqvist's (1961) modification of the glucose oxidase method of Huggett & Nixon (1957). Glucose oxidase, peroxidase and o-dianisidine were obtained from British Drug Houses Ltd. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) after precipitation with 5% (w/v) trichloroacetic acid. The method was standardized with bovine serum albumin (crystalline; British Drug Houses Ltd.). Paper chromatography of the products of enzyme digestion was carried out in an ethyl acetate-pyridine-water (10:4:3, by vol.) solvent system. The separated sugars were detected by the aniline-phthalate reagent of Wilson (1959) or the alkaline AgNO_3 reagent of Trevelyan, Procter & Harrison (1950), with a final $\text{Na}_2\text{S}_2\text{O}_3$ wash solution to diminish the background colour.

Iodine staining. Starch solution or digest of starch solution (1 ml.; 2 mg./ml.) and iodine-iodide solution (10 ml.; 0.1 mg. of I_2 and 1.0 mg. of KI/ml.) were mixed and diluted to 50 ml. with water. The extinction of the complex was measured in 1 cm. cells in a spectrophotometer against a reagent blank containing no starch or digest of starch solution.

Electrophoresis. Polyacrylamide-gel electrophoresis was
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carried out according to Broome's (1963) modification of the disk electrophoresis technique (Davis, 1964). Cyanogum 41 (British Drug Houses Ltd.) gels (10%, w/v), prepared according to the manufacturer's instructions, were found to give better results than those prepared from mixtures of acrylamide and methylenebisacrylamide (Koch-Light Laboratories Ltd.), which proved difficult to polymerize and gave soft, easily damaged, gels.

Enzyme assay. Substrate (2mg.) in acetate buffer (2ml.; 0.1N-acetic acid adjusted to pH 4.0 with 4N-NaOH) and enzyme solution (0.1ml.) were incubated at 40° for 20 min. Under these conditions the rate of glucose release was linear throughout the assay period, as long as the total glucose produced did not exceed 150 μ g. (7% conversion into glucose with soluble starch as substrate). Enzyme solutions were diluted so that the activities fell within this limit. A unit of enzyme activity was defined as the amount of enzyme releasing 1 μ mole of glucose from the substrate/min. The standard assay procedure was used to measure 0–40 milliunits and a straight-line relationship existed between glucoamylase concentration and glucose produced in the standard assay over this range. Glucose produced was estimated either by the glucose oxidase method or, when possible inhibitors were present, by the Nelson-Somogyi method.

The enzyme activities recorded in Table 1 were derived from the amounts of glucose produced in the standard assay and the volumes of the various fractions, and are given in terms of the unit defined above.

Enzyme digests. For examination of the products of hydrolysis, digests consisted of substrate solution (5ml.; 2mg./ml.) and diluted enzyme solution (1ml.) in 0.1M-acetate buffer, pH 4.0, total volume 10ml. Incubation was carried out at 40° with toluene added as a preservative. Similar digests were used to measure the initial rates of hydrolysis of amylopectin, amylose and maltose, samples being removed at intervals (0–30 min.) and boiled, and the glucose produced was determined.

In experiments to determine the percentage conversion of amylose and amylopectin into glucose, the digests consisted of substrate (50mg.), undiluted, and purified glucoamylase solution (0.2ml.) in 0.1M-acetate buffer, pH 4.0, total volume 25ml. At intervals, a sample of the digest was removed and boiled, suitable portions were taken for the determinations of total reducing sugar and of glucose produced, and 1ml. was taken for the iodine-staining reaction.

Purification of the enzyme. The purification procedure described below is briefly summarized in Scheme I.

(1) Cold ethanol fractionation. Pooled culture filtrates were fractionated with ethanol at -10° . The precipitated fractions were dissolved in water and dialysed overnight against 50mm-phosphate buffer, pH 5.8 (46mm-KH₂PO₄–4mm-Na₂HPO₄). Any precipitate that formed was removed by centrifugation for 20 min. at 2000g, and the volume of solution was measured. The fractions were then assayed for enzyme activity and protein content and submitted to electrophoresis.

(2) Fractionation with (NH₄)₂SO₄. The fractions separated at 50–75% (v/v) ethanol were combined and fractionated with (NH₄)₂SO₄. The precipitated fractions were dissolved in water, dialysed against several changes of 50mm-phosphate buffer, pH 5.8, and centrifuged for 20 min. at 2000g to remove any residue, and the volume of

Scheme 1. Sequence of stages in the purification of the glucoamylase. See the text for further details.

the supernatant solution was measured. The fractions were assayed for enzyme activity and protein content and submitted to electrophoresis.

(3) Chromatography on hydroxyapatite. The fraction that separated at 55–100%-saturated (NH₄)₂SO₄ was dialysed against several changes of 5mm-phosphate buffer, pH 5.8 (4.6mm-KH₂PO₄–0.4mm-Na₂HPO₄), and the enzyme was adsorbed on a column (10cm. × 1cm.²) of hydroxyapatite prepared according to Tiselius, Hjerten & Levin (1956), and previously equilibrated with 5mm-phosphate buffer, pH 5.8, at 4°. The column was eluted with a series of phosphate buffers over the range 5–300mm, 15ml. of each buffer being used in turn. The eluting buffers were prepared from a stock buffer (460mm-KH₂PO₄–40mm-Na₂HPO₄), pH 5.8, by dilution. The enzyme was eluted in two successive fractions with 25mm- and 50mm-phosphate. The fractions were dialysed against 50mm-phosphate buffer, pH 5.8, assayed for enzyme activity and protein content, and examined by electrophoresis.

(4) Chromatography on DEAE-cellulose. The fractions eluted from the hydroxyapatite column with 25mm- and 50mm-phosphate buffers were combined and dialysed against 5mm-phosphate buffer, pH 5.8, and added to a column (5cm. × 0.8cm.²) of DEAE-cellulose that had been equilibrated with the same 5mm-phosphate buffer at 4°. The column was then eluted with 10ml. portions of a series of phosphate buffers, 5–50mm, pH 5.8. Very little enzyme was eluted in these fractions, but, when 50mm-phosphate buffer, pH 5.8, containing NaCl (50mm) was passed through the column, most of the enzyme was eluted in this fraction (10ml.). Subsequent elution of the column with 50mm-phosphate buffer, pH 5.8, containing higher concentrations of NaCl eluted only a little more enzyme. The fractions collected were dialysed against 50mm-phosphate buffer, pH 5.8, assayed for enzyme activity and protein content and examined by electrophoresis.

Table 1 summarizes the results obtained for the active fractions obtained during the fractionation procedure.

The fraction eluted from the DEAE-cellulose column with 50mm-phosphate buffer containing NaCl (50mm) gave essentially a single band on electrophoresis, and the subsequent characterization of the enzyme was carried out

Table 1. *Results of the fractionation procedures*

For details of the procedures and the enzyme assay, and for the definition of the enzyme activity units, see the text.

Fraction	Vol. of fraction (ml.)	Protein content of fraction (mg.)	Enzyme activity in fraction (units)	Recovery of enzyme activity (%)
Culture filtrate (starting material)	2000	108	134	100
Precipitated at 50–67% (v/v) ethanol	325	23.6	27.3	20
Precipitated at 67–75% (v/v) ethanol	355	39.1	60.3	45
Precipitated at 55–100% saturation with (NH ₄) ₂ SO ₄	465	24.2	39	29
Eluted from hydroxyapatite column with 25 mM-phosphate	15	1.14	5.2	3.9
Eluted from hydroxyapatite column with 50 mM-phosphate	15	1.46	11.0	8.2
Eluted from DEAE-cellulose column with 50 mM-phosphate containing 50 mM-NaCl	10	0.68	8.8	6.6

with this fraction. The final recovery of enzyme activity in this fraction was 6.6% and the activity/unit wt. of protein increased 11-fold over that of the starting material, while on the same basis the β -glucosidase and laminarinase activities fell to 0.25 and 0.3 of their original values respectively. The α -glucosidase activity/unit wt. of protein, with maltose as substrate, increased 12-fold during the purification. Transglucosylase activity was not detected by paper chromatography of digests containing up to 4% (w/v) of maltose.

The poorest recovery of protein and of enzyme activity occurred at the hydroxyapatite-chromatography stage, and considerable amounts of the enzyme activity were found to pass straight through the hydroxyapatite column. It was later found that this step could be omitted and the yield of enzyme increased if the active fraction after (NH₄)₂SO₄ fractionation was dialysed against 25 mM-phosphate buffer, pH 5.8, before passage through the DEAE-cellulose column, which was then eluted with a linear gradient of 0–0.3 M-NaCl in 50 mM-phosphate buffer, pH 5.8, applied over a volume of 80 ml. Fractions (3 ml.) were collected and assayed for glucoamylase activity. The enzyme eluted as a single peak that was essentially homogeneous on disk electrophoresis. Solutions of the enzyme prepared in this way were pale yellow, whereas the enzyme prepared by the original method gave colourless solutions.

RESULTS

The effect of pH on the enzyme activity was studied by varying the acetate buffer in the standard assay over the range pH 3–6. The enzyme exhibited a single maximum at pH 4.0–4.5.

The enzyme was completely inactivated by heating at 65° for 10 min. The purified enzyme was more heat-labile than the crude enzyme.

Various possible inhibitors and activators were added at a concentration of 5 mM in the standard assay, and the reducing sugar produced was deter-

Table 2. *Effect of possible inhibitors and activators on glucoamylase*

The possible inhibitors and activators were each at a concentration of 5 mM. For the standard assay procedure and other details, see the text. The results are given as percentages of the control activity.

Substance added	Enzyme activity (μ g. of glucose produced in standard assay)	Enzyme activity (%)
None (control)	46	100
Al ₂ (SO ₄) ₃	43	94
CaCl ₂	41	89
CuSO ₄	44	96
EDTA	50	109
HgCl ₂	0	0
KCN	40	87
KCl	46	100
MgSO ₄	46	100
NaF	46	100
Pentachlorophenol	52	113
Pb(NO ₃) ₂	50	109
ZnCl ₂	45	98

mined by the Nelson–Somogyi method. The results are shown in Table 2. None of the substances tested had much effect on the enzyme activity except for mercuric chloride, which caused complete inhibition.

A sample of the purified enzyme was submitted to gel filtration on a column (74 cm. $\times$ 2 cm.²) of Bio-Gel P-100 (Bio-Rad Laboratories, Richmond, Calif., U.S.A.). The column was pre-equilibrated with 0.1 M-phosphate buffer, pH 5.8 (92 mM-potassium dihydrogen phosphate and 8 mM-disodium

Fig. 1. Gel filtration of diluted, purified glucoamylase (2ml.) (●) and glucose (■) on Bio-Gel P-100. Fractions (2.2ml.) were collected and assayed for glucoamylase activity by the standard procedure described in the text.

hydrogen phosphate), and was calibrated for molecular-weight determinations by passing through it solutions of Blue Dextran 2000 [Pharmacia (G.B.) Ltd., London, W. 13], bovine serum albumin, cytochrome *c*, horseradish peroxidase, trypsin and glucose. The proteins were all purified commercial samples (British Drug Houses Ltd.). A diagram of molecular weight against elution volume was constructed, and from this and the elution volume of the enzyme its molecular weight was estimated to be about 48000. The elution pattern of the enzyme with glucose as a marker substance is shown in Fig. 1. Similar experiments with a calibrated column of Sephadex G-100 [Pharmacia (G.B.) Ltd.] gave variable elution volumes, and values for the molecular weight of the enzyme that were always less than that found with the Bio-Gel P-100 column. This may indicate an interaction of the enzyme with the dextran matrix of the Sephadex gel, similar to that found by Gelotte (1964) for pancreatic amylase.

The effect of amylolysis on the iodine-staining reaction of amylose and amylopectin is shown in Fig. 2. A progressive decrease in the extinction but little shift towards lower wavelengths were apparent. Even at very high values of percentage conversion into glucose, the iodine-staining reaction persisted, indicating that an endwise degradation was proceeding and that molecules with fairly long unbranched chains of α -(1 $\rightarrow$ 4)-linked glucose residues were still present at this advanced stage in the hydrolysis. At no time during the hydrolysis was any product other than glucose found on

Fig. 2. Effect of glucoamylase on the iodine-staining reaction. The reaction was carried out as described in the text. ●, Amylose; ○, amylopectin. The number beside each curve represents the percentage conversion of the substrate into glucose at the time when the sample was taken for the iodine-staining reaction.

chromatography of samples of the digests, and the very close parallel between the total reducing power of the digests, as determined by the Nelson-Somogyi reagent, and the true glucose formed, as measured by the glucose-oxidase reagent (Fig. 3), confirmed this. Amylopectin was initially degraded about four times as quickly as amylose at the same concentration (0.1%, w/v), indicating that the degradation proceeded from the non-reducing ends of the polymer chains. At the same concentration (0.1%, w/v), maltose was degraded at only about one-tenth of the rate of amylose, and it is therefore possible that the rate of hydrolysis increased with the increasing degree of polymerization of the substrate. The degradation of amylopectin proceeded beyond the β -limit dextrin, and hence the enzyme either was able to bypass the α -(1 $\rightarrow$ 6)-branch points or was associated with a debranching activity.

The K_m values for the purified enzyme acting on amylopectin, amylose and maltose were determined at 40° and pH 4 by varying the substrate concentration in the standard assay procedure. The results were treated according to the procedure of Lineweaver & Burk (1934) and are shown in Figs. 4 and 5. The values for K_m (amylopectin), K_m (amylose) and K_m (maltose) were 0.034%, 0.42% and 0.63% respectively. These are similar to the

Fig. 3. Percentage conversion of amylose and amylopectin into glucose. Amylose: $\blacktriangle$, Nelson-Somogyi method; $\bullet$, glucose oxidase method. Amylopectin: $\triangle$, Nelson-Somogyi method; $\circ$, glucose oxidase method.

Fig. 4. Lineweaver-Burk plot for the action of purified glucoamylase on amylopectin.

values determined for the glucoamylase from *Rhizopus delemar* of 0.062%, 0.58% and 0.23% for amylopectin, amylose and maltose respectively (Phillips & Caldwell, 1951b).

DISCUSSION

The purified enzyme appeared to be a glucoamylase of high specific activity. The results obtained previously with a crude enzyme preparation (King, 1966) could have been interpreted in terms of the combined action of a β -amylase, a debranching enzyme and a maltase, but such a

Fig. 5. Lineweaver-Burk plots for the action of purified glucoamylase on amylose ($\circ$), and maltose ($\bullet$). Two molecules of glucose are produced on hydrolysis of one molecule of maltose, and this has been taken into account when plotting the values of $1/v$ for maltose.

complex system seems to be unlikely for the purified enzyme. Although the purified glucoamylase had maltase activity and probably debranching activity, since glucose was the sole product of the hydrolysis of amylopectin even when a large proportion had been converted into glucose, such activities are nearly always associated with highly purified glucoamylase preparations (Pazur & Ando, 1960; Pazur & Kleppe, 1962; Abdullah, Fleming, Taylor & Whelan, 1963; Hiromi, Kawai & Ono, 1966) and are evidently due to the glucoamylase itself. Abdullah *et al.* (1963) showed that the hydrolysis of α -(1 $\rightarrow$ 6)-linkages in amylopectin was not likely to be a rate-limiting step in the hydrolysis of this polysaccharide by the glucoamylase of *Aspergillus niger*. It is therefore perhaps not surprising that branched oligosaccharides were not found in amylopectin digests with the *C. cerebella* enzyme.

Amylopectin was almost completely hydrolysed to glucose, values of over 90% conversion being obtained. The iodine-staining reaction persisted to the end of the experiment (summarized in Figs. 2 and 3) and, although it was much weakened, little shift in the wavelength of maximum extinction occurred. With amylose the conversion into glucose was less extensive than with amylopectin. Again

Table 3. *Summary of properties of glucoamylases from different organisms*

Organism	Optimum pH	Amylopectin conversion into glucose (%)	Amylose conversion into glucose (%)	Maltose hydrolysis	Hydrolysis of α -(1 $\rightarrow$ 6)-glucosidic bonds	Reference
<i>C. cerebella</i>	4.0-4.5	> 95	85-90	+	Probably +	Present paper
<i>A. niger</i>	4.8-5.0	80-90	About 60	+	+	Kerr <i>et al.</i> (1951)
<i>A. oryzae</i>	4.2-5.2	About 95	About 80	+	+	Okazaki (1954)
<i>Endomyces</i> sp.	4.8-5.0	95-100	70-80	+	+	Hattori & Takeuchi (1962)
<i>R. delemar</i>	4.5-5.1	84-90	76-90	+	+	Phillips & Caldwell (1951a,b)

the iodine-staining reaction persisted with little change in the position of the extinction maximum. Addition of fresh enzyme did not increase the extent of amylose hydrolysis. Since hydrolysis was incomplete and oligosaccharides were not found, and since the iodine-staining reaction persisted, it was concluded that a polymer molecule resistant to the action of the enzyme was present in the amylose digests. The nature of this resistance to amylolysis is not known, but it cannot be related to possible α -(1 $\rightarrow$ 6)-linkages in the amylose chains, as is the β -amylase limit dextrin of amylopectin, since amylopectin was degraded more completely than amylose by the *C. cerebella* enzyme. Amylose retrogrades readily under neutral or acid conditions, and the resistant polymer may represent a retrograded fraction of the amylose. Incomplete conversion of amylose into glucose has been found with other glucoamylases (Phillips & Caldwell, 1951a,b; Tsujisaka, Fukumoto & Yamamoto, 1958). Fukumoto, Tsujisaka & Ishimatsu (1961) showed that the incomplete hydrolysis of starch by the glucoamylase of *A. niger* was not due to the presence of phosphate groups on the starch molecules and suggested that minor unspecified stereochemical factors in the structure of the starch molecules were involved. However, their results did not preclude retrogradation as a major cause of the incomplete hydrolysis. Kerr, Cleveland & Katzbeck (1951), also working with an enzyme derived from *A. niger*, suggested that one polymer chain was completely hydrolysed before another was attacked. This mechanism would account for the persistence of the iodine-staining reaction with little change in the wavelength of maximum extinction, but not for the incomplete hydrolysis, unless some of the polymer molecules were in some unknown way resistant to attack by the enzyme. Barker, Bourne & Fleetwood (1957) favoured a theory of multi-chain attack by the *A. niger* enzyme, on evidence derived from the relative concentrations of the oligosaccharides produced during the hydrolysis of maltotetraose and maltopentaose. However, such small molecules might not be attacked in exactly the same way as polymeric substrates.

Table 3 summarizes the properties of the *C. cerebella* enzyme and of some of the mould glucoamylases. It is apparent that the *C. cerebella* glucoamylase is fairly typical of this group of amylolytic enzymes and it seems likely that this is a common starch-degrading enzyme in the fungi. In its action on amylose, it resembles the glucoamylases of *Aspergillus oryzae*, *Endomyces* sp. and *R. delemar* rather than that of *A. niger*. The *C. cerebella* glucoamylase has K_m values for amylopectin, amylose and maltose comparable with those determined for the enzyme from *R. delemar* (Phillips & Caldwell, 1951b).

Fungal glucoamylases often occur in association with enzymes of the α -amylase type, which can be preferentially denatured by prolonged exposure to low pH. Thus Phillips & Caldwell (1951a) recommended that solutions containing a mixture of glucoamylase and α -amylase from *R. delemar* be left at pH 2.4 at 5-10° for 9 days to destroy the α -amylase activity. The *C. cerebella* culture filtrates seemed to contain little α -amylase activity; however, this organism is known to produce large amounts of acid while being cultured (see King, 1966), and the culture medium often fell below pH 3 at an early stage in the growth of the fungus. It is therefore possible that, even if α -amylase was produced, it would not survive until the culture filtrate was harvested.

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