Isolation and Some Properties of Glucoamylase from *Cephalosporium charticola* Lindau

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High glucoamylase (α-D-glucan glucohydrolase, EC 3.2.1.3.) activity was obtained in the cell-free culture fluid of *Cephalosporium charticola*. Glucoamylase seems to be the only amylolytic enzyme produced by *C. charticola*. The enzyme, purified on diethylaminoethyl-cellulose, was homogeneous by disc gel electrophoresis. The optimum pH on starch was 5.4, and optimum temperature was 60°C. Starch was degraded more rapidly than several other substrates; maltose was hydrolyzed about one-fifth as rapidly as starch. The molecular weight was 69,000, as determined by Sephadex G-100 filtration. The enzyme is a glycoprotein and contains about 6.6% sugars (mannose and glucosamine).

Glucoamylases from fungi are used to manufacture crystalline glucose and corn syrups. For this reason, the enzymes have received considerable attention. Glucoamylases have been described from *Aspergillus oryzae* (12), *Aspergillus awamori* (17), *Aspergillus phoenicis* (6), *Rhizopus delemer* (15), *Rhizopus javanicus* (18), *Endomycopsis capsularis* (3), and *Conioophora cerebella* (5). The glucoamylases from *Aspergillus niger* have been studied most thoroughly (7, 13).

Recently, a culture of *Cephalosporium charticola* Lindau was found in our laboratory to produce high amylolytic activity. The results of preliminary experiments suggested that an extracellular glucoamylase was present in the culture.

In this paper, the purification method and some properties of the glucoamylase will be presented.

**MATERIALS AND METHODS**

**Production of fungal glucoamylase.** A strain of *C. charticola* Lindau, obtained from the Botany Department of the University of Warsaw, was used as a source of glucoamylase. The fungus was cultivated on malt wort agar slants at 28°C for 96 h. Slants were rinsed with a minimal amount of water, and the resulting spore suspension was used to inoculate 100-ml portions of the following medium in Erlenmeyer flasks: NH₄H₂PO₄, 9.0 g; MgSO₄-7H₂O, 0.5 g; KH₂PO₄, 1.0 g; KCl, 0.5 g; FeSO₄-7H₂O, 0.001 g; starch, 20 g; vitamin B₆0.004 g; and tap water to 1,000 ml.

**Enzyme purification.** After 5 days of incubation at 28°C with shaking (140 rpm), the mycelium was removed by centrifugation. Two volumes of cold acetone (–10°C) was added to the culture fluid, and the precipitate was removed by centrifugation. The precipitate from 827 ml of culture fluid was dissolved in 150 ml of 0.02 M phosphate buffer, pH 7.0. This solution was applied to a diethylaminoethyl-cellulose column (2 by 45 cm) equilibrated with the above buffer, and the column was eluted with a linear gradient of NaCl, 0.0 to 0.5 M, with a flow rate of 0.7 ml/min; 5-ml fractions were collected.

**Enzyme assay.** For assay of the glucoamylase activity, 0.5 ml of enzyme solution and 0.5 ml of 1% starch in 0.02 M phosphate buffer, pH 7.0, were incubated for 30 min at 30°C. Reducing sugars were estimated according to Nelson's method (11). The amount of enzyme that produced reducing sugars equivalent to 1 μmol of glucose per min was adopted as one unit.

**Estimation of protein.** Protein was estimated according to the method of Lowry et al. (8).

**Paper chromatography of enzymatic products.** To characterize the products of hydrolysis of starch, enzyme reactions were stopped after 10, 30, and 60 min of incubation by inserting the tubes in a boiling water bath. The resulting hydrolysates (100 μl) were chromatographed on Whatman no. 1 filter paper at room temperature by descending method, the chromatograms being developed with a solvent consisting of butanol-acetic acid-water (4:1:5, vol/vol/vol), and the papers were sprayed with anisidine phthale.

**Hydrolysis of poly- and oligosaccharides.** Glucoamylase (2 ml, 0.28 mg of protein/ml) was incubated with 2 ml of 0.2% solutions (in 0.02 M phosphate buffer, pH 5.4) of starch, amylopectin, and amyllose for 60 min at 30°C. An identical procedure was performed with maltotetraose, maltotriose, and maltose using a 2-h incubation.

**Molecular weight determination.** The molecular weight of the enzyme was estimated by filtration through Sephadex G-100 (1). Pepsin, α-chymotrypsin, egg albumin, and bovine serum albumin served as the reference proteins. Elution was performed with 0.066 M phosphate buffer, pH 7.0; the flow rate was 3
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FIG. 2. Disc
gel electrophoresis of C. charticola glucoamylase. The separation was carried out in polyacrylamide gel (7.5%) at 5.0 mA/tube for 30 min. Tris(hydroxymethyl)aminomethane-glycine buffer, pH 8.4, was used.

ml/h, and 1-ml fractions were collected.

Carbohydrate analysis. The carbohydrates in the enzyme preparation were assayed by the phenol-sulfuric acid method according to Dubois (2). To establish the nature of the carbohydrate moiety of the enzyme molecule, the enzyme preparation was hydrolyzed with 2 N HCl at 100 C for 6 h, and liberated sugars were separated chromatographically as was described earlier. The Rf values of the sugars were compared with those of corresponding standards.

RESULTS AND DISCUSSION

Enzyme purification. The results of purification are summarized in Table 1. The specific activity of the enzyme after the diethylaminoethyl-cellulose purification step was equal to 23 U/mg of protein, the yield was 71%, and purification was 30-fold.

Only one peak of amyloolytic activity could be eluted from the diethylaminoethyl-cellulose column with about 0.2 M NaCl in phosphate buffer (Fig. 1). No further amyloolytic activity was eluted when a linear gradient of 0.5 to 1.0 M NaCl was applied. The obtained enzymatic fraction appeared to be homogenous when checked by polyacrylamide gel electrophoresis (Fig. 2). The only band visible was the protein moving toward the anode at pH 8.4 and displaying glucoamylase activity when eluted from the gel. Similarly, single forms of glucoamylases have been isolated from culture filtrates of Coniophora cerebella (5) and E. capsularis (3), whereas two or more forms have been isolated from A. niger (7), A. phoenicis (6), and A. oryzae (10).

Paper chromatography of enzymatic products. The products of enzymatic hydrolysis of soluble starch after various times of incubation were followed by means of paper chromatography. Glucose was the only liberated product in all samples tested after 10, 30, and 60 min of hydrolysis. Similar results were obtained, regardless of whether the enzyme was a purified preparation or the crude culture fluid (Fig. 3). Glucoamylase apparently is the only amyloolytic enzyme secreted by C. charticola under the conditions used.

Optimum pH and optimum temperature. The optimum pH was 5.4 (Fig. 4), and the optimum temperature was 60 C, when the incubation time was 30 min (Fig. 5).

Relative rates of poly- and oligosaccharide hydrolysis. When the velocities of hydrolysis of different carbohydrates were compared (Table

| Purification process | Vol (ml) | Total activity (U) | Total protein (mg) | Sp act (U/mg of protein) | Yield (%) | Purification (%) |
|----------------------|----------|--------------------|--------------------|--------------------------|-----------|------------------|
| Culture fluid        | 827      | 645.0              | 939.6              | 0.7                      | 100       |                  |
| After acetone (66%)  | 150      | 639.0              | 52.9               | 12.1                     | 99        | 16.7             |
| After diethylaminoethyl-cellulose | 50 | 331.5 | 14.0 | 23.7 | 71 | 33.6 |

Table 1. Purification of the glucoamylase from C. charticola
GLUCOAMYLASE FROM C. CHARTICOLA LINDAU

Fig. 4. Effect of pH on glucoamylase activity. The pH of the starch substrate was adjusted by using McIlvaine's buffer (mixture of 0.1 M citric acid and 0.2 M Na2HPO4·2H2O). Incubation time was 30 min at 30 C.

Fig. 5. Effect of temperature on glucoamylase activity. Reaction time was 30 min at pH 5.4.

C R B

Fig. 3. Paper chromatogram of the hydrolysis products of starch (1%) by glucoamylase present (B) in the culture fluid (0.4 U/ml) and (C) in the fraction after diethylaminoethyl-cellulose chromatography (0.4 U/ml), compared with a glucose standard (A). Incubation time was 30 min at 30 C.

2), starch was digested most rapidly and then, successively, amylopectin, amylose, maltotetraose, maltotriose, and maltose. Maltose was hydrolyzed one-fifth as rapidly as starch and two and three times less rapidly than amylose and maltotriose, respectively. Glucoamylase from C. charticola hydrolyzed amylose, maltotetraose, and maltose at relative rates similar to glucoamylase from E. capsularis (3). Two forms of glucoamylase of A. niger (7) were observed to act on maltose and maltotriose at about the same rate.

Molecular weight. The molecular weight of glucoamylase, estimated on Sephadex G-100, was 69,000 (Fig. 6); this is similar to the molecular weight of the glucoamylase from A. oryzae (10), but is less than the value of 97,000 daltons reported for the glucoamylase of A. niger (14). Some glucoamylases have smaller molecular weights; e.g., A. phoenicis (6) and Endomyces species (4) glucoamylases are reported to have molecular weights of 63,600 and 55,000, respectively.

Carbohydrate component. Our enzyme contains 6.6% sugars. They were identified by means of paper chromatography as mannose and glucosamine. In comparison, the glucoam-
Table 2. Hydrolysis of oligo- and polysaccharides by C. chartulae glucoamylase

| Substrate       | μmol of aldehyde groups/mg of protein/60 min | Relative rate of hydrolysis (%) |
|-----------------|---------------------------------------------|--------------------------------|
| Starch          | 766                                         | 100                            |
| *Amylopectin    | 642                                         | 84                             |
| Amylose         | 469                                         | 61                             |
| Maltotetraose   | 461                                         | 60                             |
| Maltotriose     | 360                                         | 47                             |
| Maltose         | 148                                         | 19                             |

![Graph](image)

**Fig. 6. Estimation of molecular weight by gel filtration.** Glucoamylase and marker proteins (2 μl) were applied to a Sephadex G-100 column (0.9 by 90 cm) and eluted with phosphate buffer, pH 7.0; 1-ml fractions were collected.

Glucoamylase from *A. phoenicis* contains 17% carbohydrates (6) and that from *A. oryzae* as much as 30% (9). Mannose is the sugar most commonly represented in the molecules of fungal glucoamylases (6, 16); the presence of a hexosamine was reported within the *A. oryzae* enzyme, but its exact chemical nature was not determined (9).

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