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CARBOHYDRATE OXIDASE, A NOVEL ENZYME FROM *POLYPORUS OBTUSUS**

I. ISOLATION AND PURIFICATION

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1. Carbohydrate oxidase, an enzyme which catalyzes the oxidation of D-glucose, D-xylose, L-sorbose and δ-gluconolactone, thereby liberating H_2O_2, was isolated from the mycelium of the Basidiomycete *Polyporus obtusus*.

2. The enzyme was purified by fractional precipitation with polyethylene glycol and by acid precipitation.

3. Data are presented which indicate that the one enzyme acts upon all four of the carbohydrate substrates.

INTRODUCTION

Our laboratories have been engaged in the study of antitumor agents produced in laboratory cultures by the Basidiomycetes[1-3]. During the investigation of one of these agents for possible inhibitory action upon glucose uptake by ascites cells, an anomaly was noted. The results obtained by a reduction method for glucose were different from those obtained by the enzymic method using glucose oxidase (EC 1.1.3.4). The discrepancy was traced to the presence of H_2O_2, which was formed when the ascites cells were being incubated with glucose and crude fractions of the Basidiomycete. The source of the H_2O_2 was subsequently found to be a reaction catalyzed by a glucose-oxidizing enzyme present in the mycelial fractions. Since the specificity, reaction products, and electrophoretic properties of this enzyme were different from those of known glucose oxidases, we isolated it to study some of its properties. The enzyme was originally discovered in the mycelium of an alcohol oxidase-producing Basidiomycete[4,5]. Later, it was isolated from the mycelium of *Polyporus obtusus*, since this organism proved to be a better and more reliable producer. The present publication describes the production and purification of the enzyme. A following paper[6] will describe the specificity and nature of the oxidation reaction and the identification of the oxidation products. The enzyme will be called carbohydrate oxidase since it attacks several substrates belonging to different classes of carbohydrates.

* This paper is dedicated to the memory of RICHARD KUHN.

MATERIALS AND METHODS

Polyethylene glycol 6000. This material, molecular weight 6000–7500, was obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. It was pulverized in a ball mill before use.

Peroxidase. Horseradish peroxidase (EC 1.11.1.7), 400 units/mg, was purchased from Worthington Biochemical Corp., Freehold, N.J. A 0.1% stock solution in distilled water was prepared and stored at 4° under toluene until needed. The solution is stable for at least one month.

o-Dianisidine dihydrochloride (3,3-dimethoxybenzidine dihydrochloride). This material was purchased from Eastman Organic Chemical, Rochester, N.Y. A 1% stock solution was prepared in 0.25 M HCl. The solution was stored in the dark at 4°. It is stable for at least one month.

D-*Glucose.* A stock solution of 10 g of β-D-glucopyranose dissolved in 100 ml water was prepared. It was stored for at least 24 h before use (equilibrium mixture).

Peroxidase–dianisidine reagent. To 79 ml distilled water was added 10.0 ml of 0.5 M sodium phosphate (pH 7.0), 10.0 ml peroxidase stock solution and 1.0 ml of dianisidine stock solution. This reagent was prepared fresh daily. For some experiments a more concentrated reagent was needed. This was prepared in the same way, except that less water was added.

Glucose–peroxidase–dianisidine reagent. This reagent was freshly prepared as described for the peroxidase–dianisidine reagent, except that 10 ml of D-glucose stock solution replaced 10 ml of H_2O.

All other reagents were obtained in the highest purity available from commercial sources.

Culture for production of carbohydrate oxidase. The culture which produces the enzyme is a strain of *Polyporus obtusus* obtained from the Forest Research Institute, Dehradum, India. It was maintained on malt agar slants at 25°. Inoculum was obtained by a 7-day incubation of the cells from one slant in 100 ml of soy–peptone–glucose medium.

Medium for production of carbohydrate oxidase. 20 g Cerelose (Corn Products Co., Argo, Ill.), 20 g NZ Amine ET (Sheffield Chemical Co., Norwich, N.Y.) and 1 g KH_2PO_4 were added to 1 l tap water. The medium was adjusted to pH 6.5 and autoclaved 15 min at 121°.

Assay of carbohydrate oxidase activity. The enzyme was assayed by measuring the H_2O_2 produced in the following system: aliquots (1.0 ml) of appropriately diluted enzyme solution were dispensed in duplicate test tubes which were then placed in a 25° water bath. At zero time, 4.0 ml glucose–peroxidase–dianisidine reagent at 25° was added. The reaction was terminated after 5 min by addition of 0.2 ml of 4.0 M HCl. The color produced was read in a Klett–Summerson photoelectric colorimeter fitted with a No. 42 filter. The reading was corrected by subtracting the value for a blank containing the enzyme and reagent without the glucose. The amount of H_2O_2 produced was determined by comparison with standard H_2O_2 solutions treated in the same way. A unit was defined as the amount of enzyme that produces 1 μmole H_2O_2/min in this system. Specific activity was defined as the number of units/mg protein. The protein concentration of enzyme fractions was measured by the method of LOWRY *et al.*[7] except in the polyethylene glycol purification experiments where it was derived from

the nitrogen content by multiplying the latter by 6.25. This was necessary because polyethylene glycol interferes with the protein determination by the LOWRY method. Nitrogen was determined by the micro-Kjeldahl method of MINARI AND ZILVERSMIT[8]. In some experiments, fractions were assayed for activity upon D-xylose, L-sorbose and δ-gluconolactone, which are also substrates for the enzyme[6]. These assays were carried out as described for glucose, except that equivalent amounts of the desired substrate were substituted for glucose in the reagent.

Production of carbohydrate oxidase. The enzyme was produced in the mycelium of *Polyporus obtusus* during the growth of that organism. When optimum enzyme levels were obtained, the mycelium was collected by filtration on a coarse filter paper supported on a Büchner funnel by a fiberglass screen[9]. The mycelium was then washed with water and stored frozen until needed.

Extraction and purification. The frozen mycelium was homogenized in a Waring blendor with 0.05 M sodium phosphate (pH 7.0) (7.5 ml/g wet mycelium). The homogenate was maintained below room temperature by cooling in an ice bath between each of three 3-min homogenization periods. Centrifugation of the homogenate at 5100 $\times$ *g* for 20 min yielded a turbid supernatant which contained the enzyme. All subsequent operations were carried out at room temperature.

First polyethylene glycol precipitation. To 290 ml of supernatant, 65.2 g of polyethylene glycol was added slowly while mixing on a magnetic stirrer. The final polyethylene glycol concentration was 19% (w/v). The suspension was allowed to stand for 30 min and was then centrifuged at 7000 $\times$ *g* for 20 min. The sediment (Fraction 1) was dissolved in 30 ml of 0.2 M NaCl in 0.05 M sodium phosphate (pH 7.0). The insoluble material remaining after 30 min was removed by centrifugation at 33 000 $\times$ *g*.

Second polyethylene glycol precipitation. To 30.5 ml of yellow amber supernatant (Fraction 2), 3.36 g (11% w/v) polyethylene glycol was added while stirring. 30 min after the addition of polyethylene glycol the resulting suspension was centrifuged at 10 300 $\times$ *g* for 20 min. The yellow precipitate was dissolved in 25 ml of 0.05 M sodium phosphate (pH 7.0), resulting in an opalescent solution. Centrifugation of the solution at 33 000 $\times$ *g* yielded a clear yellow supernatant (Fraction 3).

Third polyethylene glycol precipitation. To 23 ml of Fraction 3, 2.53 g (11% w/v) of polyethylene glycol was added slowly while stirring, producing a copious yellow precipitate (Fraction 4). The precipitate was allowed to stand for 30 min, then was recovered by centrifugation at 10 300 $\times$ *g* for 20 min. Fraction 4 was dissolved in 5.0 ml of 0.05 sodium phosphate (pH 7.0) and stored at 4°.

Acid precipitation of carbohydrate oxidase. The mycelium was extracted as described for polyethylene glycol fractionation, except that 0.01 M sodium phosphate buffer (pH 8.0) was used. The homogenate was readjusted to pH 8.0 with 1.0 M NaOH before centrifugation. The extract was cooled in an ice bath and adjusted to pH 4.7 with 1.0 M acetic acid, resulting in the immediate formation of a flocculent precipitate. The suspension was kept in an ice bath for 30 min and then centrifuged at 18 400 $\times$ *g*. The precipitate (Fraction a) was suspended in 20 ml of 0.34 M NaCl in 0.1 M sodium phosphate (pH 8.0) and stored in an ice bath for 1 h. Centrifugation at 41 000 $\times$ *g* for 20 min yielded a heavy sediment (which was discarded) and a slightly turbid, light amber supernatant (Fraction b). The supernatant (19.0 ml) was dialyzed overnight at 5° against 1 l of 0.01 M sodium phosphate (pH 7.0) and centrifuged at 41 000 $\times$ *g*.

Adjustment of the clear supernatant to pH 4.7 with 1.0 M acetic acid resulted in the immediate formation of a flocculent yellow precipitate. The suspension was allowed to stand for 90 min at 0° and was then centrifuged at 20 000 × *g* for 20 min. The sediment (Fraction c) was dissolved in 10 ml of 0.01 M potassium phosphate (pH 7.0). The slightly turbid solution was dialyzed for 4 h against the same buffer. The bag contents were then centrifuged at 41 000 × *g* for 20 min, yielding a clear supernatant (Fraction d).

Electrophoresis on cellulose acetate. 2 µl aliquots of acid purified carbohydrate oxidase were applied to buffer-moistened Sepraphore III strips (1 inch × 6.75 inch, Gelman Instrument Co., Ann Arbor, Mich.). Electrophoresis was carried out at 250 V for 30 min. The strips were blotted lightly and placed on reagent agar to which the appropriate substrate and peroxidase–dianisidine had been added just before solidification.

RESULTS

Production of carbohydrate oxidase

The maximum enzyme production was about 0.8 unit/ml, and this level was achieved in 7 to 10 days. The results of a typical experiment are summarized in Fig. 1. The peak enzyme production coincided with a decrease in residual glucose and an increase in pH. Several days after maximum enzyme production was reached, lysis of mycelium occurred and the enzyme levels dropped sharply.

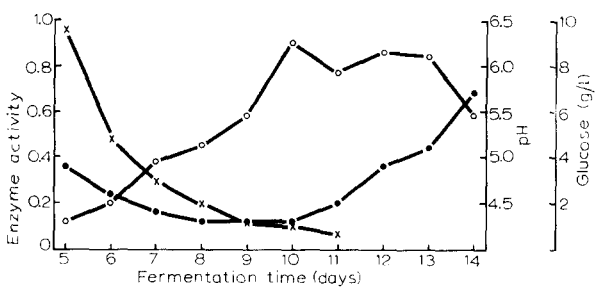

Fig. 1. Production of carbohydrate oxidase. For this purpose, 500 ml erlenmeyer flasks containing 150 ml of medium were inoculated with 5 ml of a 7-day culture. The flasks were then incubated at 25° on a reciprocating shaker at 90 strokes/min (4 inch/stroke). After the fourth day, a mycelial extract was prepared daily by homogenizing the mycelium from one flask in 150 ml of 0.05 M sodium phosphate (pH 6.8) for 5 min in a Waring blendor. The filtered mycelial extract was assayed for carbohydrate oxidase as described in the text, except that the reaction time was 30 min instead of 5. The residual reducing sugar of the filtered broth was monitored by the method of JOHNSON[10]. The pH of the broth was also determined daily. ○–○, carbohydrate oxidase activity; ●—●, pH; ×—×, glucose.

Polyethylene glycol purification of carbohydrate oxidase

Table I summarizes the polyethylene glycol purification of carbohydrate oxidase. The enzyme was purified about 17-fold with an overall recovery of 53%. Comparison of the activity of the fractions against D-glucose, D-xylose, L-sorbose and δ-gluconolactone shows that no fractionation into separate entities occurred during this purification.

TABLE I

PURIFICATION OF CARBOHYDRATE OXIDASE BY FRACTIONAL PRECIPITATION WITH POLYETHYLENE GLYCOL

Fractionation was carried out as described in the text, which also describes the methods for nitrogen determination and specific activity of the enzyme. The activity against the listed substrates was also determined as described in the text except that in the gluconolactone assay, the substrate was freshly prepared in distilled water and added to the reaction mixture at zero time. The glucose ratio represents the activity on the indicated substrate divided by the activity on glucose.

Enzyme fraction	*Volume (ml)*	*Total activity (units)*	*Total protein (6.25 × mg N)*	*Specific activity*	*Overall recovery (%)*	D-*Xylose*		L-*Sorbose*		*δ-Gluconolactone*	
						(μmoles H_2O_2 per min per mg protein)	*Glucose ratio*	*(μmoles H_2O_2 per min per mg protein)*	*Glucose ratio*	*(μmoles H_2O_2 per min per mg protein)*	*Glucose ratio*
Crude extract	290	1200	1600	0.75		0.27	0.36	0.57	0.76	0.50	0.67
Fraction 2 (clarified 19% polyethylene glycol ppt.)	32.5	1200	304	3.9	100						
Fraction 3 (clarified 11% polyethylene glycol ppt.)	25	1070	106	10.1	89	3.5	0.35	7.7	0.76	7.0	0.69
Fraction 4 (second 11% polyethylene glycol ppt.)	5	640	50	12.8	53	4.4	0.34	9.4	0.73	8.6	0.67

TABLE II

PURIFICATION OF CARBOHYDRATE OXIDASE BY ACID PRECIPITATION

Fractional precipitation with acetic acid at pH 4.7 is described in the text. The activity of the fractions was assayed against D-glucose, D-xylose, L-sorbose and δ-gluconolactone, also as described in the text. Protein was determined by the method of LOWRY *et al.*[7]. The glucose ratio is defined in Table I.

Enzyme fraction	*D-Glucose*	*D-Xylose*		*L-Sorbose*		*Gluconolactone*	
	(μmoles H_2O_2/mg protein)	*(μmoles H_2O_2/mg protein)*	*Glucose ratio*	*(μmoles H_2O_2/mg protein)*	*Glucose ratio*	*(μmoles H_2O_2/mg protein)*	*Glucose ratio*
Crude extract	0.88	0.34	0.39	0.57	0.64	0.17	0.20
Fraction b	5.25	2.04	0.39	3.6	0.68	0.73	0.14
Fraction d	8.64	3.4	0.40	6.03	0.70	1.01	0.12

Acid precipitation

Fractionation of carbohydrate oxidase by acid precipitation gave about a 10-fold increase in purity. Table II presents a comparison of the activity of various acid precipitated fractions against D-glucose, D-xylose, L-sorbose and δ-gluconolactone. Except for the gluconolactone oxidizing activity of the crude extract, the glucose ratios are reasonably constant throughout the fractionation. The glucose ratios for D-xylose and L-sorbose are approximately the same as those presented in Table I. However, for δ-gluconolactone the ratio is considerably lower than in Table I where a freshly prepared solution of δ-gluconolactone was used rather than the aged solutions in Table II. With aged solutions of δ-gluconolactone and polyethylene glycol purified carbohydrate oxidase a glucose ratio identical with that reported for acid purified carbohydrate oxidase was found (*cf.* Table I, ref. 6). The reason for this discrepancy is probably the rapid conversion of the δ-lactone to γ-lactone and gluconate in buffered solutions[11].

Carbohydrate oxidase has a broad pH optimum of 6.0 to 8.0. It is stable for at least 1 h at 25° over a pH range of 4.0–9.0. Electrophoresis of carbohydrate oxidase on cellulose acetate yielded diffuse zones when the strips were tested for enzyme activity against reagent agars containing one of the four substrates; D-glucose, D-xylose, L-sorbose and δ-gluconolactone. The active zone migrated about 1 cm toward the anode in Tris–barbital buffer (Gelman pH 8.8). The active zone was about 1.0 cm toward the cathode in strips which were run in 0.02 M sodium acetate buffer (pH 5.2). There was no evidence of separation of activities under either condition. Under the same conditions carbohydrate oxidase could be clearly distinguished from glucose oxidase.

DISCUSSION

Protein fractionation using polyethylene glycol was first described by POLSON *et al.*[12] who used this technique to prepare electrophoretically pure γ-globulin and fibrinogen from plasma. In a recent study[5] we used polyethylene glycol for the crystallization of alcohol oxidase, another enzyme from a Basidiomycete. Polyethylene glycol also proved to be a convenient and effective agent for the purification of carbohydrate oxidase.

A 17-fold increase in specific activity was achieved in only 3 steps. This compares with a 30-fold purification leading to a crystalline preparation in the case of alcohol oxidase[5]. Although the polyethylene glycol purified carbohydrate oxidase was not crystalline it was clear yellow in color and of sufficient purity to be used for the investigation of the oxidation products which is described in the following paper[6].

One of the objectives of the present study was to determine whether more than one enzyme is involved in the oxidation of D-glucose, D-xylose, L-sorbose and δ-gluconolactone. For this reason several attempts were made to fractionate carbohydrate oxidase into separate activities. Fractional precipitation with acetic acid, chromatography on DEAE-cellulose and hydroxyapatite as well as electrophoresis on cellulose acetate strips were studied in addition to the polyethylene glycol fractionation. No separation of activities was noted on either polyethylene glycol fractionation or acid precipitation: the glucose ratios remained practically constant over a 17-fold and a 10-fold purification, respectively. The glucose ratios are also almost identical in both purification schemes with the exception noted above. Carbohydrate oxidase was not adsorbed to DEAE-cellulose at pH 7.0 and 7.7. Carbohydrate oxidase was adsorbed on hydroxyapatite at pH 7.0 and eluted with a concentration gradient of phosphate buffers at the same pH. In all eluate fractions identical glucose ratios were found, however, little further purification of acid purified enzyme was achieved. Electrophoresis of carbohydrate oxidase on cellulose acetate strips showed no separation of activities. Thus the evidence suggests that the oxidation of D-glucose, D-xylose, L-sorbose and δ-gluconolactone is catalyzed by a single enzyme. While such a low degree of specificity appears unusual, a subsequent paper[6] will point out the close stereochemical relationship between all four of these substrates and that all four are oxidized at the same position. Additional evidence for the action of a single enzyme is the inhibition of gluconolactone oxidation by D-glucose, D-xylose and L-sorbose, also to be reported in the following paper.

While little is known about the distribution of the enzyme, it is found in at least two Basidiomycetes, *i.e.* in *Polyporus obtusus* and in an unnamed alcohol oxidase-producing species[4,5].

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