Purification and Properties of the Cyclodextrinase of *Bacillus macerans**

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ABSTRACT: A new enzyme, cyclodextrinase, from *Bacillus macerans* was purified 40-fold by means of ammonium sulfate precipitation, DEAE-cellulose, and recycling Sephadex chromatography. Sedimentation velocity experiments showed a major peak at 23 S and a minor peak at 13 S. The enzyme has a pH optimum of 6.2–6.4, and an energy of activation of 27,400 cal/mole over the temperature range 30–40°. The K_m for α-dextrin is $2.62 \pm 0.27 \times 10^{-3}$ M, whereas for β-dextrin it is $2.65 \pm 0.09 \times 10^{-3}$ M.

We have reported a new enzyme, cyclodextrinase, in *Bacillus macerans* that degrades Schardinger dextrins but cannot make these cyclic oligosaccharides (DePinto and Campbell, 1964). The enzyme which makes Schardinger dextrins from starch (the amylase of *B. macerans*) has been purified and some of its properties are reported in the accompanying paper (DePinto and Campbell, 1968a). This paper describes the partial purification and some properties of cyclodextrinase.

Materials and Methods

The organism used for enzyme production was *B. macerans* ATCC 8514. The media, conditions of growth, and preparation of cell-free extracts were described by DePinto and Campbell (1968a).

DEAE-cellulose was prepared and equilibrated with phosphate buffer as described by DePinto and Campbell (1968a). Sedimentation studies were performed in a Beckman–Spinco Model E analytical ultracentrifuge (DePinto and Campbell, 1968a).

Cyclodextrinase was assayed by the saccharogenic assay of Fischer and Stein (1961) as modified by DePinto and Campbell (1964). One saccharogenic unit is defined as that amount of protein which will release 1.0 mg of reducing groups, as maltose, in 5 min at 40° under the standard assay conditions. Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard. Schardinger dextrins were obtained from the Light Chemical Co. (England) and through the generosity of D. French and J. H. Pazur.

Results

Purification of Cyclodextrinase. Cells (150 g) were suspended in 0.01 M phosphate buffer (pH 6.2) to a volume of 400 ml. They were broken and centrifuged in the manner described by DePinto and Campbell (1968a). The supernatant fraction was adjusted to a protein concentration of 20 mg/ml and solid ammonium sulfate was added with stirring to 30% saturation. The inactive precipitate was removed by centrifugation and discarded. The supernatant liquid was then brought to 60% saturation with $(NH_4)_2SO_4$. The precipitate was collected by centrifugation, dissolved in 0.01 M phosphate buffer (pH 7.3), and dialyzed overnight (4°) against 10 l. of the same buffer.

Dry, equilibrated DEAE-cellulose was added to the enzyme preparation (10 mg of DEAE-cellulose/mg of protein) and the mixture was stirred for 1 hr at 4°. The DEAE-cellulose was removed by suction on a coarse sintered-glass filter and was washed with an equal volume of 0.01 M phosphate buffer (pH 7.3). The wash was combined with the original eluate.

To the above fraction, solid $(NH_4)_2SO_4$ was added to 50% saturation and the inactive precipitate was discarded. The precipitate formed between 50 and 65% saturation was collected by centrifugation, dissolved in a minimal volume of 0.01 M phosphate buffer (pH 6.2), and dialyzed overnight (4°) against 10 l. of the same buffer.

The sample was further purified by recycling column chromatography (Porath and Bennich, 1962) using a ReCyChrom recycling chromatography instrument equipped with a LKB Chopper bar recorder (LKB-producter, Stockholm, Sweden). Samples containing up to 1 g of protein were loaded onto a Sephadex G-200 column (3.5 × 75 cm). The column was maintained at 4° with a pump operated from a Buchler refrigerated fraction collector.The sample was eluted with 0.01 M phosphate buffer (pH 6.2) with a flow rate of 20 ml/hr. The first cycle was collected and assayed for cyclodextrinase activity. The peak fractions were pooled and concentrated to 15 ml with an LKB ultrafilter connected through a Dry-Ice trap to a vacuum pump. The con-

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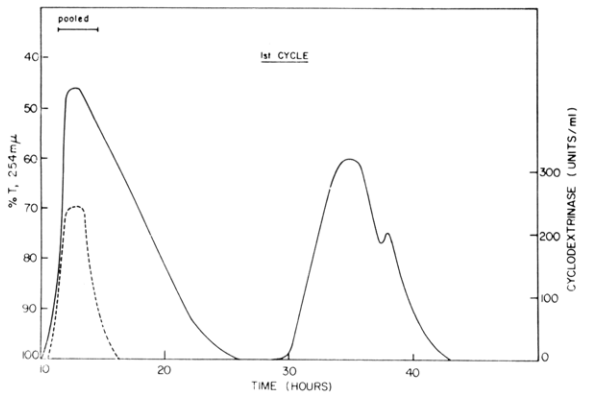

FIGURE 1: Sephadex G-200 chromatography of cyclodextrinase. Column size, 3.5 × 75 cm; flow rate, 20 ml/hr, upward flow. Solid line, protein; dashed line, cyclodextrinase activity.

centrated enzyme was recycled three times on a 50 × 3.5 cm column containing Sephadex G-200. The fourth cycle was assayed and the peak fractions were combined and concentrated by ultrafiltration. The results of a run starting with 400 mg of protein are shown in Figures 1 and 2. A typical purification summary is shown in Table I. Attempts to further purify the enzyme by preparative acrylamide disc electrophoresis resulted in complete inactivation of cyclodextrinase under the conditions employed (DePinto and Campbell, 1968a).

Properties of Cyclodextrinase. Figure 3 shows the sedimentation pattern of the enzyme. The $s_{20,w}$ was calculated to be 23 S. Although highly purified and free of amylase activity, the enzyme does not appear to be completely free from a small amount of contaminating material that has an $s_{20,w}$ of 13 S.

The effect of pH on cyclodextrinase activity is shown in Figure 4. The pH optimum is between 6.2 and 6.4.

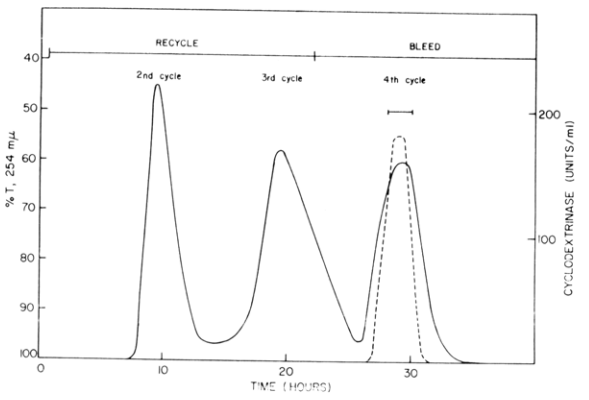

FIGURE 2: Recycling Sephadex G-200 chromatography of cyclodextrinase. Column size, 3.5 × 50 cm. Solid line, protein; dashed line, cyclodextrinase activity. The pooled fractions from the fourth cycle, as indicated, were used in the characterization studies.

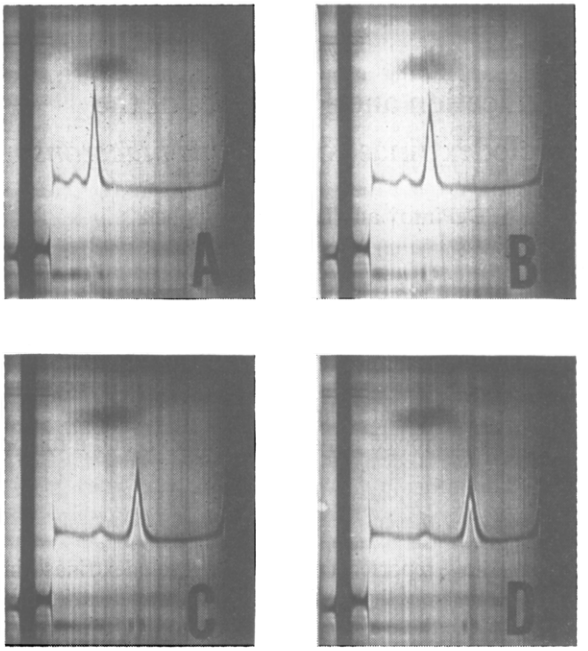

FIGURE 3: Sedimentation velocity pattern of cyclodextrinase at 59,780 rpm and 16°. The enzyme (8 mg/ml; specific activity, 203) was dissolved in 0.01 M phosphate buffer (pH 6.2). Direction of sedimentation is to the right. Time (minutes) after attaining speed: (A) 4, (B) 8, (C) 12, and (D) 18. The bar angle was 50°.

The effect of α- and β-dextrin concentration on enzyme activity is shown in Figures 5 and 6. The K_m for α-dextrin, calculated from duplicate experiments, was $2.62 \pm 0.27 \times 10^{-3}$ M. The K_m for β-dextrin was

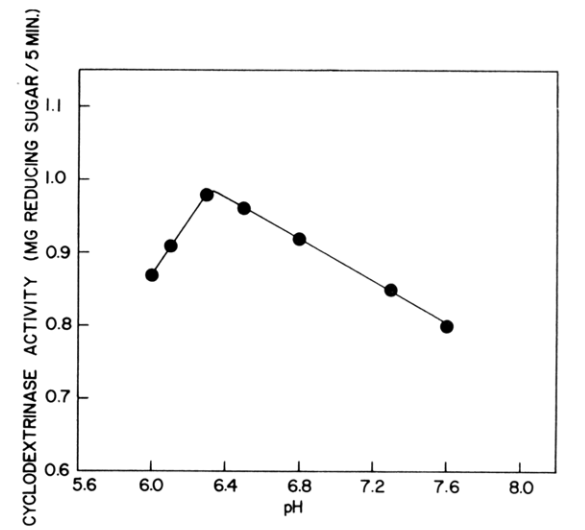

FIGURE 4: The effect of pH on cyclodextrinase activity. Approximately 1 unit of enzyme was incubated for 5 min at 40° with 1% α-dextrin, dissolved in 0.01 M phosphate buffer at the various pH values.

TABLE I: Purification Summary of Cyclodextrinase.

Step	Vol (ml)	Protein		Cyclodextrinase				
		mg/ml	Total (mg)	Units/ ml	Total Units	Sp Act.[a]	% Recov	Purifcn (-fold)
Crude extract	400	46.2	18,400	231	92,400	5.0	100	1.0
17,500-rpm supernatant fraction	220	51.2	11,264	402	88,440	7.9	95.7	1.6
$(NH_4)_2SO_4$, 30–60%	136	60.1	8,174	568	77,248	9.5	83.6	1.9
DEAE	775	5.1	3,953	87	67,425	17.1	73.0	3.4
$(NH_4)_2SO_4$, 50–65%	37	40.2	1,487	1,430	52,910	35.6	57.3	7.1
Sephadex G-200, four cycles	60	2.3	138	461	27,660	200.4	29.9	40.1
Ultrafiltration	14	9.7	136	1970	27,580	203.1	29.9	40.6

[a] Specific activity is expressed as units per milligrams of protein.

calculated to be 2.65 ± 0.09 × 10^{-3} M. Figure 7 is an Arrhenius plot of the effect of temperature on enzyme activity with α-dextrin as the substrate. The energy of activation calculated from the slope of the line between 30 and 40° was 27,400 cal/mole.

Discussion

Cyclodextrinase has a pH optimum nearly identical with that of the amylase of *B. macerans*. It differs, however, from the latter enzyme in its larger $s_{20,w}$ value (23 *vs.* 5.4) and in its action on starch and cyclic dextrins.

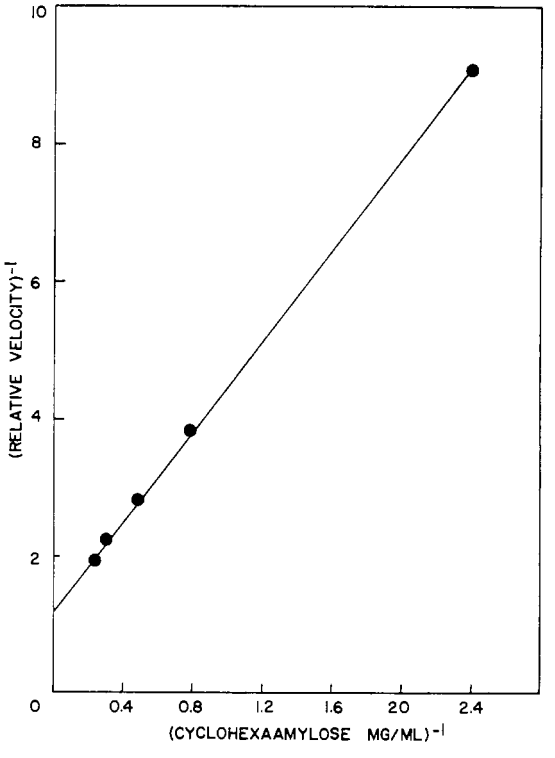

FIGURE 5: Lineweaver–Burk plot of cyclohexaamylose (α-dextrin) hydrolysis by cyclodextrinase.

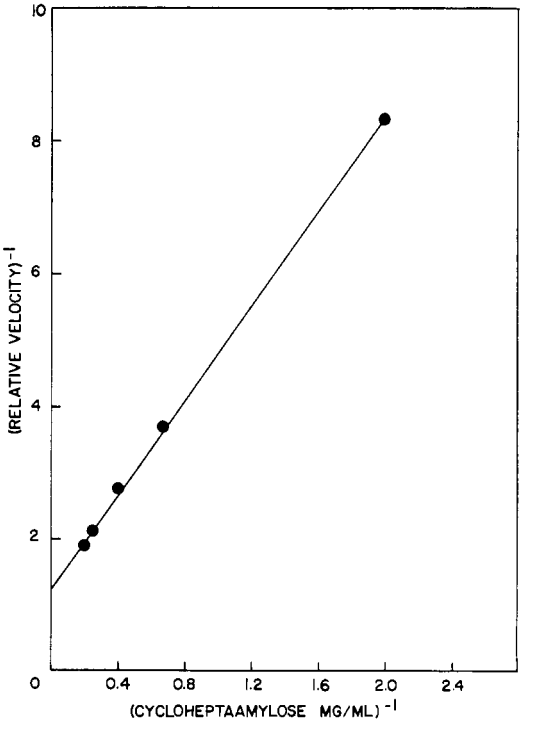

FIGURE 6: Lineweaver–Burk plot of cycloheptaamylose (β-dextrin) hydrolysis by cyclodextrinase.

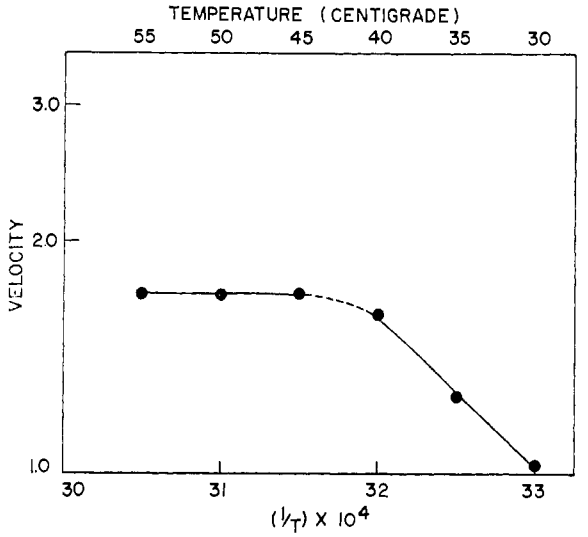

FIGURE 7: Effect of temperature on cyclodextrinase activity. Activity measurements were carried out at pH 6.2, over the temperature range 30–55° with 1% α-dextrin as substrate. Final concentration of the enzyme, which had a specific activity of 175, was 20 μg/ml. Incubation time was 2 min. The slope of the line from 30 to 40°, used to calculate the energy of activation, was -6.0×10^3.

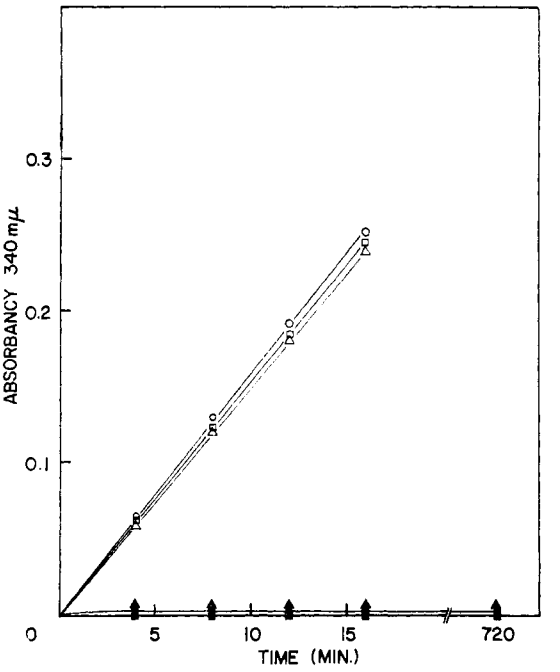

FIGURE 8: Effect of *E. coli* amylomaltase on Schardinger dextrins. The reaction was assayed by measuring the increase in absorbancy at 340 mμ as described by Wiesmeyer and Cohen (1960). The reaction mixture contained the following with final concentrations given as micromoles per milliliter: adenosine triphosphate, 0.5; triphosphopyridine nucleotide, 0.2; $MgCl_2$, 1.0; glucose 6-phosphate dehydrogenase, 150; phosphate buffer (pH 6.9), 0.4; hexokinase, 5 units; extract, 75 μg; and substrate as indicated. Symbols: (■) minus extract, (●) minus substrate, (▲) α-, β-, or γ-dextrin at 5 or 10 μmoles/ml, (△) 2 μmoles of maltose plus 5 μmoles of α- or γ-dextrin, (○) 2 μmoles of maltose plus 5 μmoles of β-dextrin, and (□) 2 μmoles of maltose, no cyclic dextrin.

The degradation of starch by cyclodextrinase is negligible and it does not form cyclic dextrins from starch or from linear maltooligosaccharides (DePinto and Campbell, 1964, 1968b). Cyclodextrinase initially opens the ring of the cyclic dextrins forming a linear molecule with the corresponding number of glucose units and then degrades the linear molecule into lower maltooligosaccharides (DePinto and Campbell, 1968b).

During the course of this investigation Dr. J. H. Pazur suggested that cyclodextrinase might be an amylomaltase. The enzyme does not incorporate [^{14}C]-glucose into linear maltooligosaccharides (DePinto and Campbell, 1968b) thereby ruling out that cyclodextrinase is an amylomaltase. The question arose however, as to whether the amylomaltase of *Escherichia coli* could catalyze the degradation of the Schardinger dextrins. We therefore prepared crude extracts of *E. coli* ML308 and measured amylomaltase activity as described by Wiesmeyer and Cohen (1960). Figure 8 shows that amylomaltase cannot form glucose from the Schardinger dextrins.

It should be noted that Ben-Gershom (1955) has presented evidence indicating that "cyclodextrinases" are present in takadiastase and that they are not identical with either takaamylase or takamaltase. The relationship of the "cyclodextrinase" activity of takadiastase to the cyclodextrinase of *B. macerans* is not clear. The saccharogenic activity of *B. macerans* extracts acting on starch had been previously observed by Schwimmer and Garibaldi (1952) and Schwimmer (1953). It is possible that these investigators were observing the resultant action of both the cyclodextrinase and *B. macerans* amylase.

Robyt and French (1964) have also reported that an amylase from *Bacillus polymyxa* degrades glycogen, the glycogen β-amylase limits dextrin, and cyclic dextrins. While the enzyme from *B. polymyxa* degrades β- and γ-dextrins, it degrades α-dextrin only very slowly ($^1/_{10,000}$ as fast as it does amylose). In contrast, cyclodextrinase has negligible activity on starch or glycogen and degrades α- and β-dextrins equally well.

Acknowledgment

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Purification and Partial Characterization of Glucose 6-Phosphate Dehydrogenase from Cow Adrenal Cortex*

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ABSTRACT: Bovine adrenal glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:$NADP^+$ oxidoreductase, EC 1.1.1.49) was purified by isoelectric precipitation, calcium phosphate gel adsorption, ammonium sulfate precipitation, and column chromatography on diethylaminoethyl Sephadex, carboxymethyl Sephadex, and Sephadex G-200. The enzyme was purified 2500-fold with a 20% yield. The average specific activity at pH 8.0 and 37° of the crystalline enzyme was 340 μmoles of oxidized nicotinamide–adenine dinucleotide phosphate ($NADP^+$) reduced/min per mg of protein. The preparation was demonstrated to be homogeneous by diethylaminoethylcellulose column chromatography, electrophoresis on cellulose acetate and polyacrylamide gel, velocity ultracentrifugation, and three consecutive crystallizations to a constant specific activity. The molecular weight was calculated to be 235,000, derived from a V_e/V_0 ratio of 1.58 on a calibrated Sephadex G-200 column and to be 238,700 by a sedimentation constant ($s_{20,w}$) of 9.8 S on the ultracentrifuge. The optimum pH in the absence of Mg^{2+} was 8.5; with Mg^{2+} it was the same but with a 20% greater activity. The K_m for $NADP^+$ at pH 8.0 and 25° in the presence of Mg^{2+} was 5.6×10^{-6} M as compared to a K_m of 4.7×10^{-2} M for NAD^+. The K_m for glucose 6-phosphate with $NADP^+$ at pH 8.0 and 25° in the presence of Mg^{2+} was 4.2×10^{-5} M compared to 1.9×10^{-4} M with NAD^+.

The activity of the pentose phosphate pathway is very high in endocrine tissues (Glock and McLean, 1954; McKerns, 1962a, 1965a,b). The first enzyme in this pathway is glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate:$NADP^+$ oxidoreductase,[1] EC 1.1.1.49) which catalyzes the reaction: glucose 6-phosphate + $NADP^+$ ⇌ 6-phosphate glucolactone + NADPH + H^+.

The specific activity and the amount of glucose 6-phosphate dehydrogenase can vary. Striking increases of this enzyme were observed in the mammary gland during lactation (Glock and McLean, 1954), in the ovary during estrus and after the administration of gonadotropin (McKerns, 1965b; McKerns and Nordstrand, 1965), in the liver upon refeeding a high carbohydrate diet following fasting (Tepperman and Tepperman, 1958), and in the adrenal upon administration of ACTH (McKerns, 1964b). Interest in glucose 6-phosphate dehydrogenase also derives from differences of the enzyme found in erythrocytes of people suffering from various congenital anemias (Kirkman *et al.*, 1964).

Stimulation of the activity of glucose 6-phosphate dehydrogenase would seem to be of major importance in the control of function and cell replication in many tissues regulated by hormones (McKerns, 1964b, 1967). There is evidence for unique species of the enzyme, each having binding sites for its activating hormone (McKerns, 1964b, 1966, 1967, 1968). Glucose 6-phosphate dehydrogenase controls the rate of entry of glu-

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[1] Abbreviations used: $NADP^+$ and NADPH, oxidized and reduced nicotinamide–adenine dinucleotide phosphates; ACTH, corticotropin; NAD^+ and NADH, oxidized and reduced nicotinamide–adenine dinucleotide.