Carbohydrate Research, 61 (1978) 229–238
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PURIFICATION AND SOME PROPERTIES OF *Bacillus macerans* CYCLOAMYLOSE (CYCLODEXTRIN) GLUCANOTRANSFERASE*

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(Received August 4th, 1977; accepted for publication in revised form, October 14th, 1977)

ABSTRACT

Bacillus macerans cycloamylose (cyclodextrin) glucanotransferase (EC 2.4.1.19) was purified by the technique of starch adsorption and DEAE-cellulose column chromatography, and then crystallized from an ammonium sulfate solution containing mM calcium chloride. The crystals of the enzyme were rod-shaped and showed a single band by disc-gel electrophoresis. The purified enzyme was dissociated into two subunits by sodium dodecyl sulfate-disc electrophoresis. The subunits had no enzyme activity. Details of each purification step and some properties of the enzyme are described in this paper.

INTRODUCTION

The cycloamylose glucanotransferase produced by *Bacillus macerans* has been known as "*Bacillus macerans* amylase" or simply "BMA" and produces cyclic oligosaccharides called "cycloamyloses", "cyclodextrins", or "Schardinger dextrins" from starch or other (1 → 4)-α-D-glucans. The enzyme has been studied by many workers[1–4], in order to elucidate the reaction mechanism for the production of cycloamyloses.

French and his co-workers[5] worked extensively on the mechanism of the enzyme to learn, e.g., the cosubstrate for the coupling reaction[6,7], the action pattern of disproportionation[2], and the conversion of cyclohexaamylose into cycloheptaamylose[8]. The action of this enzyme was considered to be complex, because the enzyme seems to catalyze at least three reactions, namely cyclization, coupling, and disproportionation. There have been very few reports on the action pattern and mechanism of the purified enzyme.

Hale and Rawlins employed a starch-adsorption technique for purification of the *Bacillus macerans* enzyme[9]. Purification of the enzyme was first studied by Schwimmer and Garibaldi, who used ethanol precipitation to obtain a single peak by ultracentrifugation[10,11].

DePinto and Campbell purified the enzyme by the combination of magnesium

*Dedicated to Professor Dexter French on the occasion of his 60th birthday.

chloride and ammonium sulfate precipitation, column chromatography, and electrophoresis; they obtained the purified enzyme displaying a single band by disc-gel electrophoresis[12].

Kitahata and Okada used the technique of starch adsorption and gel-filtration chromatography to obtain an enzyme giving a single band by disc-gel electrophoresis[13].

In these studies, large-scale preparation of the purified enzyme was difficult because it required many purification steps to obtain single-band enzyme. The other disadvantage was the use of a gel-filtration step, which limits the amount of protein that could be purified. For these reasons, *Bacillus macerans* cycloamylose glucanotransferase had not thus far been crystallized.

We have been working on the enzyme to elucidate the action pattern of cyclization, coupling, and disproportionation[14,15], and for preparation of several kinds of cycloamylose: hexa-, hepta-, octa-, and also branched cycloamyloses[16].

This paper summarizes our new purification procedure and a method for crystallizing the *B. macerans* cycloamylose glucanotransferase.

MATERIALS AND METHODS

Crude enzyme preparation. — *Bacillus macerans* (IAM 1243) was grown for 48 h at 40° in 15 l of culture medium consisting of 3% of wheat bran, 0.5% ammonium sulfate, and 0.5% calcium carbonate in tap water. The jar fermentor was agitated at 300 r.p.m. and aerated at the rate of 1 liter per liter of medium per min. The broth was filtered through cotton cloth and the filtrate used as a crude enzyme-preparation, which had 10 units of activity per ml.

Assays. — The enzyme activity was measured by the glucoamylase method[17]. One unit of enzyme activity is defined as the amount of enzyme that forms 1 μmol of cycloamylose per min at 50°. One unit determined by the glucoamylase method was equivalent to 1.7 unit by the Tilden–Hudson method[4], when the enzyme was produced in the wheat-bran medium. Protein was assayed by the Lowry method[18], with bovine serum albumin as a standard.

Starch adsorption. — Starch adsorption was performed at 3–5°. The heat–moisture treatment of commercial corn starch was effected for 20 min at 120°, and then the treated starch was used as adsorbent.

DEAE-Cellulose column chromatography. — DEAE-Cellulose was activated, equilibrated in 50mM acetate buffer (pH 6.0) containing mM calcium chloride, and then packed into a column (3 × 45 cm). Rechromatography was effected with a 2 × 35 cm column.

Analytical disc-gel electrophoresis and detection of starch hydrolytic activity. — Disc-gel electrophoresis was performed with 7.5% acrylamide gel (pH 8.9). Crude enzyme-preparation (100 μl), which had been dialyzed overnight in mM calcium chloride solution at 4°, was applied to each of two gels. One of the gels was stained by Amido Black, whereupon seven or more bands were observed in the gel. The other gel was incubated in 3% soluble-starch solution for 10 min at 50°, and then washed

with distilled water and stained by iodine in 0.2% iodine solution. No band other than the one of cycloamylose glucanotransferase was observed in the gel stained with iodine. Sodium dodecyl sulfate-disc electrophoresis was performed by the method of Weber and Osborn[20] with cytochrome C (horse heart, mol. wt. 12,500), albumin (hen egg, mol. wt. 45,000), and bovine serum albumin (mol. wt. 67,000) as standards.

RESULTS

Starch adsorption and desorption. — The heat–moisture treated starch was added to the crude enzyme preparation (pH 7.5–8.5) and the suspension was stirred for 15 h. The starch (5 g) adsorbed 90% of the activity from 5,000 U in the crude enzyme solution. After desorption, the starch was reused to sorb remaining enzyme. Residual activity (4%) was recovered by repeated starch adsorption, with 5 g of the starch. The starch that adsorbed enzyme on the first treatment was used for further purification.

The starch, containing adsorbed enzyme, was washed 4 times with 250-ml portions of cold 33% ethanol solution, to remove substances having no enzyme activity. Finally, the ethanol solution was filtered through a sintered-glass filter. Only 0.1–0.2% of the activity was lost during the washings, providing an extremely efficient purification of the enzyme. In order to desorb the enzyme, the washed starch was suspended in distilled water, stirring at 50°.

During the initial 15 min of stirring, 65% of the initially applied activity was desorbed, and the curve then leveled off. To recover more enzyme from the adsorbed starch, the desorption process was repeated by using fresh distilled water. Fig. 1 shows the relation between the desorption process and the recovery of the activity. After the second treatment, ~80% of the initial activity had been recovered. The

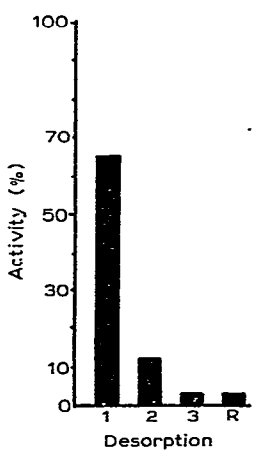


Fig. 1. Relation between the number of desorption treatments and recovered activity; 1–3: activity desorbed at each treatment; R: activity remaining in the starch cake. Activity is expressed as the percentage of the initially applied activity.

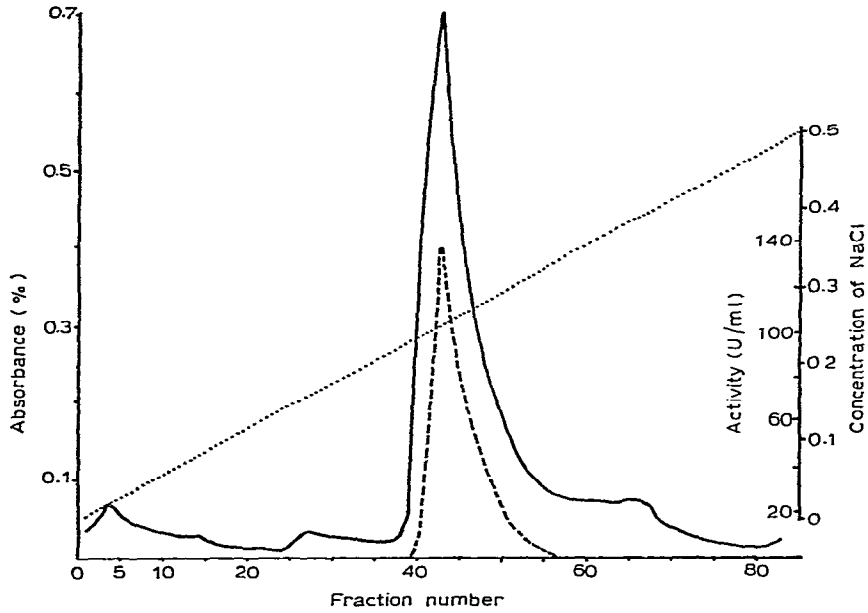


Fig. 2. Column chromatography on DEAE-cellulose of *B. macerans* cycloamylose glucanotransferase; ———, protein; - - - -, activity of the enzyme; . . . . . , gradient of NaCl. The column was eluted with a 0–0.5M gradient of sodium chloride at a flow rate of 50 ml/h, at 25°. Fractions (10 ml) were collected.

extract, in which the specific activity of the enzyme had increased 23-fold (as indicated in Table I), was almost colorless and clear.

Column chromatography on DEAE-cellulose. — Partially purified enzyme solution (200 ml, 172 mg of protein) obtained by starch adsorption, was applied to a DEAE-cellulose column, and the enzyme was eluted from the column with a gradient system of sodium chloride (0–0.5M) in 50 mM acetate buffer (pH 6.0) containing mM calcium chloride. The results of the chromatography are shown in Fig. 2. The active fractions were combined and dialyzed against 50mM acetate buffer containing mM calcium chloride (pH 6.0) and then rechromatographed.

Crystallization. — The active fractions were collected and concentrated to ~3–4% of protein in collodion bags by dialysis against mM calcium chloride solution under diminished pressure. Solid ammonium sulfate was slowly added to the concentrated enzyme solution to 10% saturation. The solution was kept at 3–5°. Within a week, the enzyme was observed to have crystallized. The crystals of the enzyme are rod-like, as shown in Fig. 3.

Purification at each step is summarized in Table I and a flow sheet of purification steps is shown in Fig. 4.

Properties of the crystalline enzyme. — The crystalline enzyme was washed with mM calcium chloride solution at room temperature, and then dissolved at 0° and dialyzed against the same solution. For analytical disc-electrophoresis, 40 μg of the

Fig. 3. Micrograph of crystals of *B. macerans* cycloamylose glucanotransferase.

TABLE I

SUMMARY OF PURIFICATION OF *Bacillus macerans* CYCLOAMYLOSE GLUCANOTRANSFERASE

	Potato–oatmeal[a]			*Wheat bran*[b]		
	Specific activity[c]	*Recovery* (%)	*Purification factor*	*Specific activity*[c]	*Recovery* (%)	*Purification factor*
Crude enzyme solution	1.4	100	1	7.0	100	1
Starch adsorption	98	75	70	163	80	23
DEAE-Cellulose column I	152	64	109	219	74	31
DEAE-Cellulose column II	220	37	157	245	68	35
Crystallization	—	—	—	245	50	35

[a]Potato–oatmeal: *B. macerans* was grown in a medium containing 5% of fresh potato, 0.5% of oatmeal, 0.5% of ammonium sulfate, and 0.5% of calcium carbonate. [b]Wheat bran: *B. macerans* was grown in a medium containing 3% of wheat bran, 0.5% of ammonium sulfate, and 0.5% of calcium carbonate. [c]Enzyme activity was measured by the glucoamylase method. Specific activity was defined as units/mg protein. The activity of one unit is the amount of the enzyme that forms 1 μmol of cycloamylose per min from soluble starch at 50° and pH 6.0.

Crude enzyme solution (5l, 7.0 units/mg protein

← Corn starch (50g)

Stir overnight at 5°
Decant

Starch cake

← 33% Cold ethanol (250 ml, 4 times)

Wash 4 times and decant

Starch cake

← Water (250 ml, twice)

Stir for 15 min at 50°
and filter (twice)

Filtrate (500 ml, 163 units/mg protein)

DEAE-Cellulose column chromatography
Dialyze and concentrate

← Solid ammonium sulfate (10% saturation)

Crystals of enzyme (units/mg protein)

Fig. 4. Flow sheet for purification of *B. macerans* cycloamylose glucanotransferase.

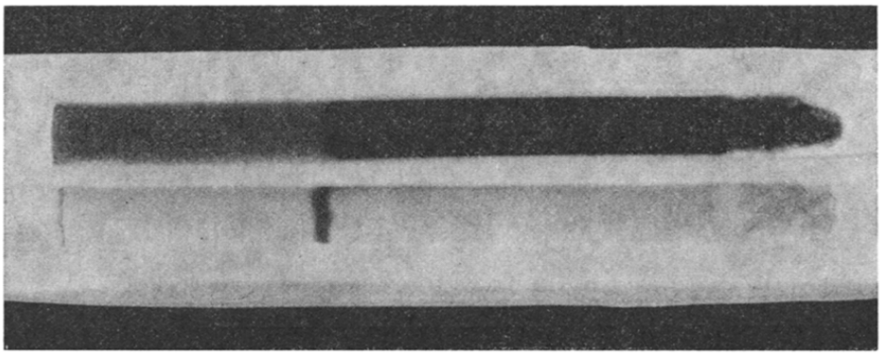


Fig. 5. Disc-gel electrophoresis pattern of crystalline *B. macerans* cycloamylose glucanotransferase. A 7.5% gel (pH 8.9) containing 0.2% of soluble starch was used. Conditions of electrophoresis are 4 mA/tube, for 1 h at room temperature. Upper gel: stained with 0.1% iodine solution; lower gel: stained with Amido Black.

purified enzyme was applied to each of two gels, which contained 0.2% soluble starch. One of the gels was stained by Amido Black, and only one protein band was observed, as shown in Fig. 5. The other gel was stained by iodine. We observed a yellow color where the enzyme moved, indicating that the enzyme acted on the starch during migration. The molecular weight of the enzyme was determined by acrylamide gel-filtration chromatography. Bio-Gel P-150(100–200 mesh, made by Bio-Rad Laboratories) was swollen and packed into a column (1.9 × 100 cm) that was calibrated by standard proteins. The enzyme protein was charged on the column and then eluted with 50mM Tris buffer containing mM calcium chloride (pH 7.50) at

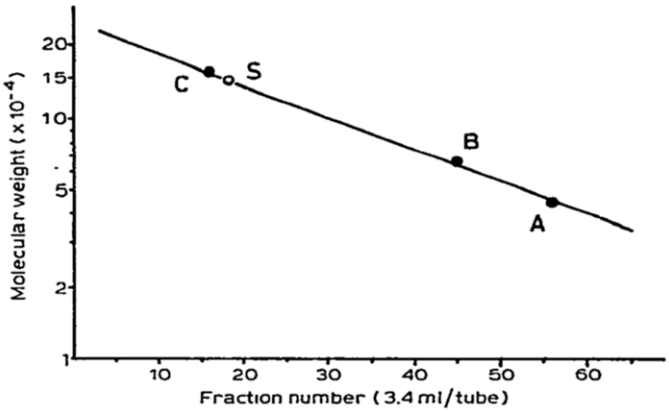


Fig. 6. The relation between fraction number and molecular weight: A, hen-egg albumin (mol. wt. 45,000); B, bovine-serum albumin (mol. wt. 67,000); C, rabbit-muscle aldolase (mol. wt. 158,000); and S, cycloamylose glucanotransferase. Experimental details are described in the text.

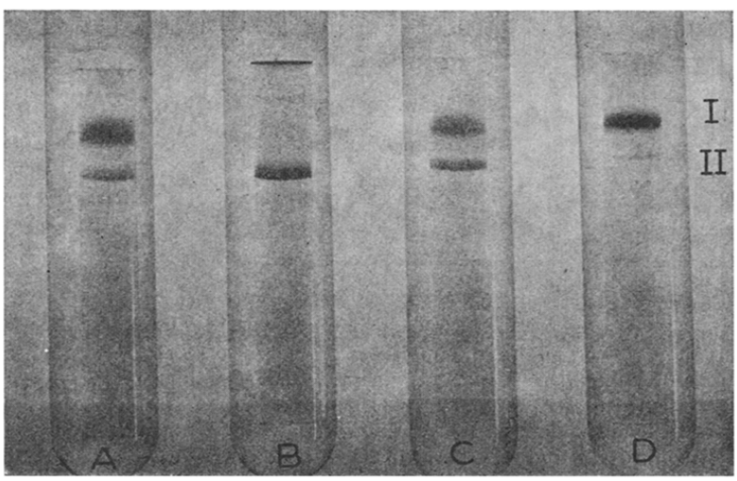


Fig. 7. Sodium dodecyl sulfate (SDS)-disc-gel electrophoresis pattern of crystalline *B. macerans* cycloamylose glucanotransferase; A: 1% of SDS; B: 1% of SDS and 20% of 2-mercaptoethanol; C: 1% of SDS and 5% of 2-mercaptoethanol; and D: reference (no addition). The reagents were added to the enzyme solution that had been buffered in 25% of glycerol–10mM phosphate buffer (pH 7.20). The mixtures were treated for 2h at 50°. Gels were stained with Amido Black.

3–5°, flow rate 8 ml/h. Fig. 6 shows the relation between molecular weight and elution volume. From these experiments, the molecular weight of the enzyme protein was calculated as 145,000. Also, the enzyme was treated under various conditions, in order to fractionate subunits, with sodium dodecyl sulfate and/or 2-mercaptoethanol, and then examined by sodium dodecyl sulfate-disc electrophoresis. The enzyme does not lose its activity in the presence of 1% sodium dodecyl sulfate and produces ~80% cycloamyloses from potato starch[21].

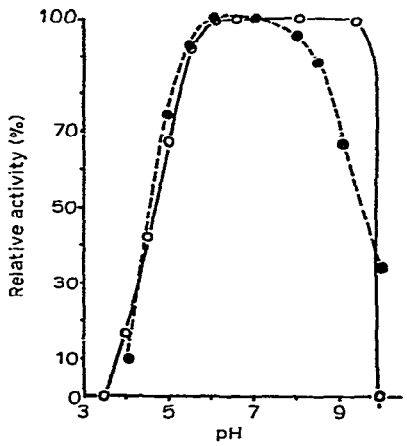
As shown in Fig. 7, the protein of the enzyme was dissociated and a new band

appeared by treating it with sodium dodecyl sulfate and 2-mercaptoethanol in 10mM phosphate buffer containing 25% glycerol (pH 7.20) for 2 h at 50°. This new band (II) corresponded to a molecular weight of 74,000. The band was cut out and the protein was extracted with 10mM acetate buffer containing mM calcium chloride. No enzyme activity was detected in the subunit having mol. wt. 74,000.

Effect of pH on activity. — Enzyme solution (100-μl aliquots, 0.18 units/0.75 μg protein) was mixed with 100 μl of various buffer solution containing mM calcium chloride (pH 3.5–6.0; 90mM acetate buffer, and pH 6.0–10.0; 45mM Tris buffer), and 100 μl of 3% soluble starch solution was added. The mixtures were incubated for 15 and 30 min at 15°. The amount of cycloamylose produced in the mixtures was determined by the glucoamylase method, and the enzyme activity was calculated. Fig. 8 shows the relative activity at various pH values. The enzyme shows high activity at pH 5.5–7.5, and the highest activity at pH 6.0 under the conditions tested.

Effect of pH on stability. — The enzyme (0.18 units/0.75 μg protein) was dissolved in 20 μl of various buffer solutions containing mM calcium chloride (pH 3.5–6.0; 9mM acetate buffer, and pH 6.0–10.0; 4.5mM Tris buffer) and the solutions were kept for 1 h at 25°. Soluble-starch solution (3%, 100 μl), 100 μl of 90mM acetate buffer containing mM calcium chloride (pH 6.0), and 80 μl of distilled water were added to the enzyme solutions, and the mixtures (pH 5.5–6.4) were incubated for 15 and 30 min at 50°. Fig. 8 shows the residual activity after the enzyme had been treated at pH 3.5–10.0 for 1 h at 25°. As Fig. 8 shows, the enzyme was stable over the pH range 5.5–9.5, and the activity was lost below pH 3.5 and above pH 10.0.

Effect of temperature on activity. — The mixtures were composed of 1% soluble starch, 30mM acetate buffer containing 0.3mM calcium chloride (pH 6.0), and 0.75 μg of the enzyme protein (0.18 units). After preincubation for 5 min, each component



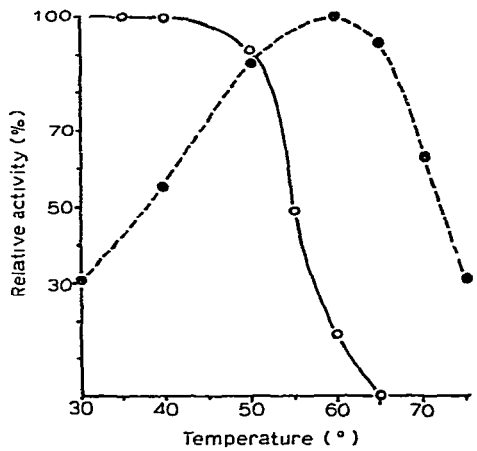


Fig. 8. Effect of pH on the enzyme activity and stability: ....●....: effect of pH on activity; — O —: effect of pH on stability. The experimental details are described in the text.

Fig. 9. Effect of temperature on the enzyme activity and stability; ....●....: effect of temperature on activity; — O —: effect of temperature on stability. Experimental details are described in the text.

was combined and the mixtures (300 μl) were incubated at various temperatures for 15 min. Fig. 9 shows the relative activity at 30–70°. The enzyme showed the highest activity at 60° under the conditions tested.

Effect of temperature on stability. — The enzyme (0.18 units/0.75 μg protein) was dissolved in 20 μl of 90mM acetate buffer containing mM calcium chloride (pH 6.0), and the solutions were kept for 15 min at various temperatures. After the treatment, an additional 100 μl of acetate buffer and 200 μl of soluble starch solution (1.5%) were added to the enzyme solutions. The mixtures were incubated for 15 min at 50° and the residual activity was measured.

As Fig. 9 shows, the enzyme was stable at temperatures below 50° and showed no significant loss of activity.

DISCUSSION

Cycloamylose glucanotransferase was adsorbed onto heat–moisture-treated corn starch in the absence of ethanol and ammonium sulfate. As other starch hydrolytic enzymes were not detected by disc-gel electrophoresis in the crude enzyme-preparation (as described in Materials and Methods), the enzyme had been effectively purified by starch adsorption.

Washing the enzyme-adsorbed starch was particularly effective for removing inactive protein and other substances. A large amount of the purified enzyme was obtained by column chromatography on DEAE-cellulose and it readily crystallized at a high concentration of the enzyme protein.

Although Schwimmer and Garibaldi have reported[10] that a small amount of hydrolytic activity was almost completely eliminated at the step of starch adsorption[9], the crystalline enzyme we obtained liquefied starch paste rapidly and formed reducing sugar gradually, as well as displaying the well-known three actions–cyclization, coupling, and disproportionation. We thus consider that the *B. macerans* glucanotransferase has hydrolytic activity. The difference between our result and that of Schwimmer may be attributed to the different culture of microorganism and a different medium for growing *B. macerans*.

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