Note

Purification and Some Properties of a Glucoamylase from *Clostridium* sp. G0005

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Glucoamylase [1,4;1,6- α -D-glucanglucohydrazase, EC 3.2.1.3] catalyzes the release of β -D-glucose from the non-reducing ends of starch and related polysaccharides. Most of the reported glucoamylases are fungal or yeast enzymes.^{1–8)} Ishida *et al.* (Hitachi Research Laboratory, Hitachi, Ltd.) found a glucoamylase activity from an anaerobic thermophilic bacterium, *Clostridium* sp. G0005,⁹⁾ and we purified the enzyme to homogeneity. Glucoamylase activity was assayed by incubation in 125 mM sodium acetate buffer, pH 4.5, for 15 or 30 min at 60°C in the presence of 0.5% soluble potato starch (Sigma) as substrate. Glucose concentration of the incubated solution was measured by using a glucose analyzer (model 23, Yellow Springs Instrument Co., Inc. Yellow Springs, Ohio 45387). One unit of activity corresponds to the formation of 1 μ mol of glucose per minute. *Clostridium* sp. G0005 was cultured in a medium containing (per liter): dextrin, 15 g; peptone, 5 g; yeast extract, 5 g; KH_2PO_4 , 7 g; Na_2HPO_4 , 3.5 g; MgSO_4 , 0.1 g; sodium thioglycolate, 1 g; and resazurin, 0.2 g (as an oxidation-reduction indicator), under strictly anaerobic conditions with N_2 . Cultures were agitated at 200 to 250 rpm at 60°C. Glucoamylase activity was detected both in cells and in the medium. Both glucoamylase activities were increasing during log-phase in cell growth and reached a maximum at stationary phase. Glucoamylase activity of cells was 2.5–3-fold larger than the activity in the medium at stationary phase. Washed intact cells of *Clostridium* sp. G0005 showed glucoamylase activity and the cell-associated glucoamylase activity was solubilized by repeated sonication, the total activity being kept constant in the course of the operation (Fig. 1). The activity was also released from the cells by freezing and thawing of the cells. These results suggested that all of cell-associated glucoamylase (CGA) is located on the cell surface and its active site faces toward the outside. This characteristic localization of the enzyme is similar to that of glucoamylase and pullulanase activities in *Clostridium thermohydrosulfuricum*.^{10,11)}

CGA was purified from cells of *Clostridium* sp. G0005. Forty liters of full growth culture were centrifuged at $7,000 \times g$, separating the medium and cells. Harvested cells (50 g, wet weight) were frozen at -20°C , then thawed at room temperature and washed with 100 ml of 20 mM sodium phosphate buffer, pH 7.0, containing 0.1 M KCl, 1 mM PMSF, and 1 mM EDTA. Cells were removed by centrifugation at $10,000 \times g$ for 30 min and resuspended in the same buffer. Five hundred ml of the supernatant, collected by repeated extraction, was adjusted to 40% saturation of $(\text{NH}_4)_2\text{SO}_4$ and put on a column of Butyl-Toyopearl 650M (Tosoh Corp.). An active fraction was separated from the column with a decreasing linear gradient of 40 to 0% saturation of $(\text{NH}_4)_2\text{SO}_4$ in buffer A (25 mM sodium phosphate buffer, pH 7.0). After dialysis against buffer B (20 mM Tris-Cl buffer, pH 7.0), the enzyme fraction was further purified by a column of MonoQ HR 10/10 (Pharmacia) with a linear gradient of 0 to 0.5 M KCl in buffer B. Active fractions, eluted at 50–100 mM KCl, were put on a column of Butyl-Toyopearl 650S (Tosoh Corp.) and that of MonoQ HR 5/5 (Pharmacia) successively under the buffer conditions described above. This final purified enzyme was 1.8 mg, and gave a single band on SDS-polyacrylamide gel electrophoresis^{12,13)} and had a molecular weight of 77,000. Specific activity of CGA was 66 U/mg. Protein was measured by the method of Bradford¹⁴⁾ using bovine serum albumin as a standard.

Fig. 1. Solubilization of CGA by Sonication.

One ml and a half of a full growth culture of *Clostridium* sp. G0005 was centrifuged at $17,000 \times g$ for 5 min. The harvested cells were washed with 20 mM Tris-Cl buffer, pH 7.0 and suspended in 1.5 ml of the same buffer. The cell suspension was sonicated for 10 sec 6 times. After measurement of glucoamylase activity of the sonicated sample (circles), the suspension was centrifuged at $17,000 \times g$ for 5 min. The precipitant was resuspended in 1.5 ml of the same buffer. Glucoamylase activity of the supernatant fraction (triangles) and the suspension of the precipitant (squares) was measured. Solid symbols show the measurements of intact cells.

Fig. 2. Thermal stability of activity (A) and pH dependence of thermal stability (B) of CGA.

A, CGA (22.7 $\mu\text{g/ml}$) was incubated in 125 mM sodium acetate buffer (pH 4.5) at 60°C (solid circles), 65°C (triangles), 70°C (open circles) and 80°C (squares), for the indicated length of time. Then a sample of the enzyme was removed for assay. B, CGA (3.8 $\mu\text{g protein/ml}$) was incubated at 70°C for 1 hr at various pH in 125 mM glycine-HCl (squares), sodium acetate (triangles), MES-NaOH (circles), and 50 mM sodium phosphate (solid circles) buffers. Residual activities were measured.

The N-terminal amino acids of the CGA preparation were analyzed by a gas phase protein sequencer (Model 470A, Applied Biosystems Japan, Ltd.) and PTH-analyzer (Model 120A, Applied Biosystems Japan, Ltd.). No PTH amino acids were detected in significant amounts. The N-terminal of the protein was supposed to be modified. A glucoamylase found in the culture supernatant was also purified in the same manner and showed similar properties to the enzyme described above.

The temperature and pH dependence of CGA activity was investigated at various temperatures from 30 to 80°C in 125 mM sodium acetate buffer at pH 4.5, and at different pH, from 2 to 8 at 60°C using 125 mM glycine-HCl buffer (pH 2.0–4.0), 125 mM sodium acetate buffer (pH 3.5–5.5), 125 mM MES-NaOH buffer (pH 5.5–7.0), and 50 mM sodium phosphate buffer (pH 7.0–8.0). The CGA concentration in these assays were 0.3 $\mu\text{g protein/ml}$. The optimal temperature and pH for the enzyme were 65°C and pH 4.5. The thermal stability of CGA at pH 4.5 was measured (Fig. 2A). CGA was stable at 60°C but was inactivated above 65°C. To test the effects of pH on thermal stability, CGA was incubated at 70°C in various pH (Fig. 2B). The enzyme activity was lost abruptly at pH below 4 and above 7.

In this study, we purified a new bacterial glucoamylase, of which characteristics were similar to fungal and yeast glucoamylases.¹⁵⁾ Further analysis of the enzyme and comparison with other glucoamylases are expected to provide a lot of suggestions to elucidate the mechanism of the glucoamylase reaction.

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