Purification and Properties of a Maltotetraose- and Maltotriose-Producing Amylase from *Chloroflexus aurantiacus*

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A maltotetraose- and maltotriose-producing amylase which is stable at alkaline pHs and high temperatures was detected in the culture filtrate of a strain of *Chloroflexus aurantiacus* J-10-F1, a thermophilic, green, photosynthetic bacterium. The enzyme was purified to homogeneity, as demonstrated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, by means of ultrafiltration, ammonium sulfate fractionation, and DEAE-cellulose, hydroxyapatite, and high-performance liquid chromatographies. The molecular mass of the purified enzyme was estimated to be about 210,000 Da. The isoelectric point of the enzyme was estimated to be 6.24 by polyacrylamide gel electrofocusing. The amylase was stable up to 55°C and at alkaline pHs of up to 12.0. The optimum pH and temperature of the enzyme activity were 7.5 and 71°C, respectively. Metal ions such as Hg²⁺, Zn²⁺, Cu²⁺, Mn²⁺, and Ni²⁺ strongly inhibited the enzyme activity. The enzyme activity was reactivated specifically by Ca²⁺ after the enzyme was treated with 1 mM EDTA. This enzyme could digest various kinds of raw-starch granules from corn, cassava, and potato. Both maltotetraose and maltotriose were formed as the main enzymatic products from soluble starch.

Most amylases produce glucose and maltose from starch as their main products, but only a few amylases produce oligosaccharides specifically from starch. At present, the specific maltoligosaccharides have a range of potential uses in food, pharmaceutical, and fine-chemicals industries because of their unique nature and special properties. In the series of maltoligosaccharides from bacteria, such as *Pseudomonas stutzeri* (9–11), *Bacillus circulans* (14), *Bacillus subtilis* (15), and *Streptomyces griseus* (17). We have been interested in hydrolytic enzymes of various photosynthetic bacteria because there have been very few reports on hydrolytic enzymes such as amylase and protease from these organisms. In our laboratory, Banakari et al. purified an α-amylase from a nonsulfur, purple, photosynthetic bacterium and characterized its properties (1). This α-amylase could digest various kinds of raw starch as well as soluble starch, but it was thermostable. In the present report, purification and properties of a heat-stable amylase from a thermophilic, green, photosynthetic bacterium, *Chloroflexus aurantiacus* J-10-F1, are described. This amylase formed maltotetraose and maltotriose as the main products from soluble starch.

**MATERIALS AND METHODS**

**Microorganisms.** *C. aurantiacus* J-10-F1 was a kind gift from T. Nozawa, Faculty of Engineering, Tohoku University.

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**Protein determination.** The protein concentrations of the pooled samples were determined by the method of Lowry et al. (7), with bovine serum albumin as a standard. The protein content in the eluate from the column was measured by the A280.

**Ultrafiltration.** Crude enzyme solution was concentrated in a flat ultrafiltration plate (model Minitan Plates 4/PK 10,000 NM-HL; Millipore Corporation, Bedford, Mass.), and the enzyme samples from column chromatography were concentrated in a stirred cell (model 8050; Amicon Co., Danvers, Mass.) with YM-10 membranes.

**Thin-layer chromatography.** The enzymatic products were subjected to thin-layer chromatography with silica gel 60 (Merck art. no. 5553; 20 by 20 cm) with a solvent system of isopropanol–acetone–0.1 M lactic acid (4 ml:4 g:200 ml:30 ml). Glucose, maltose, and oligosaccharides produced from the reduced soluble starch by α-amylase from B. subtilis and maltose produced from the reduced starch by β-amylase from wheat were used as standards.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (6) after the samples were boiled in the presence of SDS and 2-mercaptoethanol. The stacking and the separating gels were 5% and 9% polyacrylamide, respectively. The proteins were stained with Coomassie brilliant blue R-250. The molecular weight standards used were myosin (200,000), β-galactosidase (116,250), phosphorylase b (97,400), bovine serum albumin (66,200), and ovalbumin (45,000).

**Isoelectric focusing.** Isoelectric focusing was performed at 4°C with ampholine (pH 3.5 to 10.0) according to the method of Garfin (5). The samples were run at 300 V for 17 h. The gel was stained with Coomassie brilliant blue R-250 to determine the location of the protein band. The isoelectric point of the enzyme was estimated by comparing the mobility of the enzyme with those of standard proteins. The pH standards used were phycocyanin (pH 4.65), β-lactoglobulin B (pH 5.10), bovine carbonic anhydrase (pH 6.00), human carbonic anhydrase (pH 6.5), equine myoglobin (pH 7.00), human hemoglobin A (pH 7.10), human hemoglobin C (pH 7.50), lentil lectin (three bands [pH 7.80, 8.00, and 8.20]), and cytochrome c (pH 9.60).

**Estimation of molecular weight.** The molecular weight of the enzyme was determined by two methods. (i) The purified enzyme was run on SDS-PAGE together with the standard proteins, and the molecular weight of the enzyme was estimated by comparing the mobility of the enzyme with those of the standard proteins. (ii) The purified enzyme was applied on a Toyopearl HW-65 gel filtration column (Tosoh Co. Ltd., Tokyo, Japan; 0.8 by 50 cm), and the elution volume of the protein peak was compared with those of the standard proteins.

**Hydrolysis of polysaccharides.** The reaction mixture containing 50 µl of the purified enzyme and 1% of each of the polysaccharides which were dissolved in 0.01 M Tris-HCl buffer (pH 7.5) was incubated at 60°C for 30 min, and the amount of reducing sugar produced was determined as described above. The polysaccharides used as substrate were glycogen (oyster), amylose (potato), pullulan, amylopectin (waxy maize), and dextran (Leuconostoc mesenteroides).

**Digestion of starch granules.** The digestibility of the raw starch was determined by incubating 0.025 ml of the enzyme solution with a 2-ml volume of 2% raw starch from corn, cassava, or potato that was suspended in a 10 mM Tris-HCl buffer (pH 7.5) containing 5 mM sodium azide. Digestion was carried out at 50°C with shaking. At time intervals, the reaction mixture was centrifuged to remove the remaining starch granules. The amount of total sugars in the resulting supernatant was determined by the phenolsulfuric acid method (3), with glucose as the standard.

**RESULTS**

**Growth of C. aurantiacus and amylase production.** At various time intervals during cultivation of C. aurantiacus in the medium containing 0.5% soluble starch as the main carbon source, the level of activity of amylase, the amount of reducing sugar produced in the culture broth, and cell growth were measured as shown in Fig. 1. The rapid growth of the bacterium and the production of extracellular amylase and reducing sugar proceeded concomitantly. After stationary-phase growth (after about 78 h of growth), the amount of cells decreased but production of reducing sugar continued to increase, and over 90% of the amylase activity at the stationary phase was still retained in the culture medium at the end of the culture. At the end of cultivation, the pH of the culture medium changed from the initial pH of 8.3 to 6.5 (data not shown).

**Purification of amylase from the culture medium.** A summary of the purification results is given in Table 1. The enzyme was purified about 810-fold, and the specific activity of the purified enzyme was 81 U/mg of protein.

Ten liters of the culture supernatant of C. aurantiacus was concentrated in a flat ultrafiltration plate with a flow rate of

<table>
<thead>
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<th>TABLE 1. Purification of amylase from C. aurantiacus</th>
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<td>Purification step</td>
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<tr>
<td>Culture supernatant</td>
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<td>Ultrafiltration</td>
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<td>30-60% (NH₄)₂SO₄</td>
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<td>DEAE-cellulose</td>
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<td>Hydroxypatite</td>
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<td>HPLC DEAE-5PW</td>
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FIG. 1. Time courses of growth and production of amylase and reducing sugar in the culture medium. O.D., optical density.
600 ml/min and pressure of 10 lb/in². The supernatant was concentrated about 50 times, with the recovery of 95% of the enzyme activity by this method.

Ammonium sulfate was added to the concentrated enzyme fraction to give 30% saturation. The precipitate formed was discarded by centrifugation. To the resultant supernatant was added ammonium sulfate to give 60% saturation. The precipitate was collected, dissolved in 0.01 M Tris-HCl buffer (pH 7.5), and dialyzed against 100 volumes of the same buffer overnight at 4°C.

The dialysate (14 ml) was applied on a DEAE-cellulose column (Wako Pure Chemical Industries Ltd., Osaka, Japan; 2.6 by 20 cm) equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). After washing with the same buffer, the elution was carried out with a linear gradient of KCl from 0.05 to 0.3 M in the same Tris-HCl buffer. Five-milliliter fractions were collected at a flow rate of 30 ml/h. The enzyme was eluted at a concentration of KCl of between 0.05 and 0.15 M. The enzyme fractions were collected, concentrated in a stirred cell, and dialyzed against 100 volumes of 0.01 M sodium phosphate buffer (pH 6.8).

The concentrated enzyme fraction from the DEAE-cellulose column (4 ml) was diluted 10 times with distilled water and applied to a hydroxyapatite column (Wako Pure Chemical Industries Co. Ltd.; 1.7 by 11.5 cm) that had been equilibrated with 1 mM sodium phosphate buffer (pH 6.8). The enzyme protein was eluted with a linear gradient of the buffer (pH 6.8) from 1 to 12.5 mM. Fractions (1.5 ml each) were collected. Two active peaks were separated in this purification step, and they were designated peak A and peak B, respectively. Peak A passed through the column, and peak B was eluted at a concentration of sodium phosphate of between 2.5 and 7.5 mM. The active fraction (a part of peak B) was subjected to high-performance liquid chromatography (HPLC) with a TSK gel DEAE-5PW column (Tohoh; 0.75 by 7.5 cm), which had been previously equilibrated with 0.01 M Tris-HCl buffer (pH 7.5). Elution was carried out with a linear gradient of KCl from 0 to 0.3 M in the same buffer. The flow rate was adjusted to 1 ml/min. The enzyme recovered from these steps showed one band of protein with SDS-PAGE (Fig. 2).

![FIG. 2. SDS-PAGE pattern of purified amylase (lane 1) and standard molecular mass markers (lane 2).](image)

**FIG. 3.** Thin-layer chromatogram of the enzymatic products from soluble starch by the action of amylase. Lanes: 1, maltose; 2, glucose; 3, glucose plus maltose; 4, reduced soluble starch; 5, hydrolysate of reduced soluble starch by α-amylase (B. subtilis); 6, hydrolysate of reduced soluble starch by β-amylase (wheat); 7 to 12, hydrolysate of reduced soluble starch by purified amylase (C. aurantiacus) after incubation of 10, 20, and 30 min and 1, 2, and 3 h, respectively. G1, glucose; G2, maltose; G3, maltotriose; G4, maltotetraose; G5, maltopentaose.

**Properties of the purified enzyme.** (i) **Molecular weight.** The molecular weight of the denatured enzyme was estimated by SDS-PAGE after the sample had been boiled in the presence of SDS and 2-mercaptoethanol, while that of native enzyme was estimated by the Toyopearl HW-65 gel filtration method. The molecular weights of denatured and native enzymes were both estimated to be about 210,000, indicating that the native enzyme has no subunit (data not shown).

(ii) **Isoelectric point.** The isolectric point of the enzyme was determined to be about 6.24 by isoelectric focusing (data not shown).

**Effects of pH and temperature on the enzyme activity.** The optimum pH for the enzyme activity was around 7.5. The enzyme was quite stable at alkaline pHs of up to 12.0, while it was rapidly inactivated at a pH of less than 7.5. The optimum temperature was about 71°C. The enzyme was stable until 55°C, and 72% and 20% of the original activity was retained after incubation at 63 and 67°C for 1 h, respectively.

**Effect of metal ions on the amylase activity.** Hg²⁺, Zn²⁺, Cu²⁺, Mn²⁺, and Ni²⁺ strongly inhibited the enzyme activity at their final concentrations of 1 mM. Co²⁺, Fe³⁺, Fe²⁺, Pb²⁺, Sn²⁺, Mg²⁺, and Ba²⁺ exhibited this inhibitory effect in decreasing order, while Ca²⁺ neither activated nor inhibited the enzyme. EDTA completely inhibited the enzyme activity at a final concentration of 1 mM. However, its activity could be partially recovered to 94%, 65%, 43%, and 23% of the original activity when 1 mM Ca²⁺, Ba²⁺, Mg²⁺, and Sn²⁺ was added to the EDTA-treated enzyme solution, respectively. These results suggest that this amylase requires a specific metal ion, Ca²⁺, for its full activity.

**Action of the enzyme on soluble starch.** Figure 3 shows an analysis of the enzymatic products formed from the reduced soluble starch by the purified enzyme in comparison with α-amylase from B. subtilis and β-amylase from wheat. G₂ (maltose) was formed only by β-amylase, and G₄ (maltotetraose) was formed mainly by the purified amylase of C. aurantiacus.
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Hydrolysis of polysaccharides by the purified amylase. The actions of the purified amylase on several polysaccharides were compared in terms of the relative initial rates of hydrolysis as shown in Table 2. Oyster glycogen, soluble starch, and amylase were hydrolyzed most rapidly, while pullulan was hydrolyzed at a rate of 17% of that of oyster glycogen.

Digestion of raw-starch granules by the purified amylase. The actions of the purified amylase on raw-starch granules of corn, cassava, and potato were examined, and the rates of digestion are shown in Fig. 4. The purified enzyme exhibited a high level of activity with raw corn and cassava starch, and a significant level of activity was observed with raw potato starch.

DISCUSSION

An amylase which produces maltotetrose and maltotriose from soluble starch as its main products was found in the culture filtrate of C.aurantiacus J-10-F1, which was isolated from the water of an alkaline hot spring in a canyon at Sokokura, Hakone district, Japan. The enzyme (peak B in hydroxylapatite column chromatography) was purified to a homogeneous protein on SDS-PAGE by means of ultrafiltration, ammonium sulfate fractionation, DEAE-cellulose and hydroxylapatite chromatographies, and HPLC (DEAE-5PW). Peak A which passed through the column was not purified in this experiment because peak A had less activity and more contaminated protein.

The estimated molecular weight (210,000) of the purified amylase (peak B) is higher than those of well-known maltotetraose- and maltotriose-forming amylases (approximately 12,500 to 57,000) (9-11, 14, 15, 17); therefore, this enzyme is considered one of the highest-molecular-weight forms of maltotetraose- and maltotriose-forming amylase. The purified amylase is quite stable at alkaline pHs, while it is rapidly inactivated at pHs of less than 7.5. The amylases of B. circulans (16) and nonsulfur, purple, photosynthetic bacterium (1) are also stable at pHs of up to 12. However, the purified amylase reported here is more thermostable than other maltotetraose- and maltotriose-forming amylases that have been reported previously (9-11, 14, 15, 17). There are some obscure data about the thermostability of the amylase from this bacterium; the enzyme was inactivated at 60°C, although the optimal temperature of this enzyme was 71°C. This difference may be caused by the difference in incubation conditions of the enzyme at various temperatures. In the enzyme stability experiment, the enzyme was preincubated at 60°C, while the enzyme was incubated with the substrate in the optimum-temperature experiment. Probably, the substrate protected the enzyme from heat inactivation under the latter experimental conditions.

It has been reported that Ca²⁺ is necessary for amylase activity and its stability. In the case of the purified amylase in this report, Ca²⁺ seems to have a similar role in activation, because the enzyme was reactivated by Ca²⁺ after it was inactivated by 1 mM EDTA, although some reactivation was also observed with Ba³⁺, Mg²⁺, and Sn²⁺.

Most of the product formed from soluble starch by purified amylase in the early stage of hydrolysis is maltotetraose, and it accumulated gradually. On the other hand, maltotriose, maltose, and maltopentaoose began to appear after 1 hr, although maltopentaoose and maltose were in trace amounts. This result suggests that this enzyme is a kind of α-amylase.

The purified amylase could digest raw starch from corn, cassava, and potato starch. Although a high level of activity was found with raw corn and cassava starch, digestion of potato starch was slow. The purified enzyme could hydrolyze oyster glycogen, amylase, and pullulan, as well as raw and soluble starch.

ACKNOWLEDGMENT

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REFERENCES