FEBS Letters 345 (1994) 135–138

FEBS 14032

Purification and properties of a novel enzyme from *Bacillus* spp. T-3040, which catalyzes the conversion of dextran to cyclic isomaltooligosaccharides

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Received 7 April 1994

Abstract

A novel enzyme, cycloisomaltooligosaccharide glucanotransferase (CITase), catalyzes the conversion of dextran to cyclic isomaltooligosaccharides by intramolecular transglucosylation (cyclization reaction). CITase was purified to homogeneity from the culture filtrate of *Bacillus* sp. T-3040 isolated from soil. The M_r of the enzyme was estimated to be 98,000 by SDS-PAGE. The enzyme catalyzed the cyclization reaction and gave three cyclic isomaltooligosaccharides (cycloisomalto-heptaose, -octaose, and -nonaose) at a total yield of about 20%. Coupling and disproportionation reactions were also observed. These results showed that this enzyme is a multi-functional enzyme which catalyzes intramolecular and intermolecular transglucosylation.

Key words: Cyclic isomaltooligosaccharide; Intramolecular transglucosylation; Dextran

1. Introduction

Cyclodextrin glucanotransferase (EC 2.4.1.19; CGTase) is known to catalyze the intramolecular transglucosylation of α-1,4 glucan [1]. No enzyme has been reported among α-1,6 glucan hydrolases that corresponds to CGTase. Recently, we isolated a bacterium which produced three novel cyclic oligosaccharides (cycloisomalto-heptaose, CI-7; -octaose, CI-8; and -nonaose, CI-9) in culture containing dextran, and found that these novel oligosaccharides were enzymatically produced from dextran [2]. This paper describes the purification and some properties of this novel enzyme, and we propose the common name cycloisomaltooligosaccharide glucanotransferase (CITase) for this enzyme.

2. Materials and methods

2.1. Reagents

Dextran 40 was purchased from Meito Sangyo Co., Japan. The series of isomaltooligosaccharides used as standards, from isomaltose to isomaltoheptaose, were purchased from Seikagaku Kogyo Co., Japan, and those from isomaltooctaose to isomaltodecaose were obtained from Funakoshi Co., Japan.

2.2. Cultures

Strain T-3040, isolated from soil and identified as *Bacillus* spp., was used for this study. It was cultured as described previously [2].

2.3. Enzyme assay

Enzyme activity was measured as follows. A reaction mixture containing 0.5 ml of 4% dextran 40, 0.4 ml of 100 mM acetate buffer (pH 5.5), and 0.1 ml of the enzyme solution was incubated at 40°C. for 1 h, and the reaction was stopped by boiling for 5 min. After removing denatured protein by centrifugation, the resulting cycloisomaltooligosaccharides (CIs; CI-7, CI-8, and CI-9) were measured by a high-performance liquid chromatography (HPLC) as described previously [2]. One unit of the enzyme was defined as the amount of enzyme that produced 1 μmol of CIs per min under the above condition.

2.4. Purification

Three-hundred liters of the culture broth was filtered using a hollow fiber (Microza; Asahi Kasei Kogyo Co.) for removing cells. The filtrate was concentrated to 6.3 l using a hollow fiber with a molecular weight cut-off of 6,000, and 900 ml of the concentrate was used for purification. The concentrate was dialyzed against 1 mM EDTA-10 mM phosphate buffer (pH 7.0) at 4°C for 16 h. After centrifugation, the supernatant was put on a DEAE-Sepharose CL-6B column equilibrated with 1 mM EDTA-10 mM phosphate buffer (pH 7.0). The enzyme was eluted with a linear gradient of NaCl (0–0.8 M). The active fractions were collected (total 320 ml), and then ammonium sulfate ($(NH_4)_2SO_4$) was added up to a final concentration of 1.0 M. The enzyme was subjected to an HPLC system (Tohso Co., Japan) using a preparative TSKgel Phenyl 5PW column (21.5 mm × 150 mm; Tohso Co.). Elution conditions were as follows: solvent, 10 mM EDTA–100 mM phosphate buffer (pH 7.0); elution, a linear gradient of $(NH_4)_2SO_4$ (1.0–0.0 M); flow rate, 4 ml/min; fraction volume, 8 ml/each fraction. The enzyme was eluted at around 0.1 M of $(NH_4)_2SO_4$. The active fractions were collected (total 150 ml) and then $(NH_4)_2SO_4$ was added again up to a final concentration of 1.0 M and subjected to rechromatography with the TSKgel Phenyl 5PW column. Elution conditions were almost the same except for linear gradient of $(NH_4)_2SO_4$ (0.2–0.0 M) and 4 ml/each fraction. The active fractions (total 88 ml) were concentrated to 1.0 ml by ultrafiltration using the PM 10 amicon membrane. The concentrate was diluted 20 times with 1 mM EDTA-10 mM phosphate buffer (pH 7.0) and applied to HPLC using a TSKgel DEAE 5PW column (7.5 mm × 75 mm, Tohso Co.) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of NaCl (0.0–0.4 M) and 1 ml/min flow rate. The active fractions (total 12 ml) were concentrated to 0.9 ml by ultrafiltration using the Centriprep (Amicon Co.). A portion (0.3 ml) of the concentrate was subjected to a molecular sieve HPLC using a TSKgel G3000 SW column (7.6 mm × 600 mm × 2, Tohso Co.) and eluted with 200 mM NaCl–10 mM EDTA–100 mM phosphate buffer (pH 7.0) and 1 ml/min flow rate. The enzyme was eluted as a single peak. The active fractions were combined (total 9 ml).

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SSDI 0014-5793(94)00418-U

2.5. General analytical methods

SDS-PAGE was done by the method of Laemmli [3]. Protein was determined by the method of Bradford [4]. Reducing power in the reaction mixture was measured by the method of Somogyi-Nelson [5].

3. Results and discussion

A novel enzyme, CITase, which catalyzes the conversion of dextran to CIs, was purified up to homogeneity with a 15.6% yield (Table 1). Specific activity of the purified enzyme was 2.18 U/mg protein. Although this value is far lower than those of hydrolases such as dextranase, the specific activity of our enzyme does not seem so low in comparison with that of cycloinulooligosaccharide fructanotransferase (CFTase; 10.8 U/mg) [6]. The enzyme was eluted as a symmetrical single peak, and its M_r was estimated at about 80,000 by gel filtration using a TSKgel G3000 SW column. It migrated as a single band on SDS-PAGE, and its M_r was estimated at about 98,000 (Fig. 1). These results suggested that the enzyme is a monomeric protein. The enzyme may have weak affinity for the TSKgel G3000 SW column, and hence may have been eluted with a delayed retention time.

The enzyme showed maximal activity at around pH 5.5, and was stable over the range of pH 4.5–8.5. When the thermostability of the enzyme was tested at 10–70°C in 0.1 M acetate buffer (pH 5.5) for 15 min, the enzyme was stable at temperatures below 40°C. The activity was almost completely inhibited by Cu^{2+} and Hg^{2+} ions.

Substrate specificity in the cyclization reaction was investigated with a reaction mixture of 0.5 ml of 4% substrate solution, 0.48 ml of 100 mM acetate buffer (pH 5.5), and 0.02 ml of the enzyme (2.71 U/ml). For the coupling reaction, a reaction mixture of 0.5 ml of 4% CI-8 solution, 0.1 ml of 1 M glucose solution, 0.38 ml of 100 mM acetate buffer (pH 5.5), and 0.02 ml of the enzyme (2.71 U/ml) was used, and for the disproportionation reaction, that of 100 μl of 2% isomaltopentaose solution, 96 μl of 100 mM acetate buffer (pH 5.5), and 4 μl of the enzyme (2.71 U/ml) was used. Each reaction mixture was incubated at 40°C for 1 h and stopped by boiling for 5 min. After centrifugation, the supernatant was analyzed by HPLC using a TSKgel Amide 80 column as described previously, and sugar peaks were identified with their retention times [2]. Isomaltohexaose and CI-7, which have the same retention times on HPLC analysis were distinguished by analyzing them after glucodextranase treatment. As shown in Fig. 2A, the enzyme produced three CIs (CI-7, CI-8, and CI-9) from dextran. The yields of CI-7, CI-8, and CI-9 were about 5%, 10%, and 3%, respectively (Fig. 3). The enzyme did not act on amylopectin and pullulan, which contained α-1,6 glucosidic linkage at the branch points. As shown in Fig. 2B and C, this novel enzyme produced linear isomaltononaose from CI-8 and glucose by the intermolecular transglucosylation reaction (coupling), and also produced several linear isomaltooligosaccharides from isomaltopentaose by the same reaction (disproportionation). These features closely resemble those of CGTase and CFTase. Consequently, we propose the common name of CI glucanotransferase (CITase) for the novel enzyme.

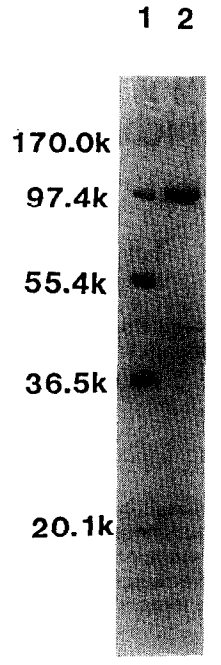

Fig. 1. SDS-PAGE of the purified CITase from *Bacillus* spp. T-3040. About 10 μg of protein was treated with 2.5% 2-mercaptoethanol at 100°C for 3 min and applied to each column. The gel was stained with Coomassie brilliant blue R250. Lane 1, marker proteins; lane 2, purified enzyme.

Table 1
Purification of cycloisomaltooligosaccharide glucanotransferase from *Bacillus* spp. T-3040

Procedure	Total volume (ml)	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Fold
Crude concentrate[a]	900	4936	154	0.03	100.0	1
DEAE-Sepharose CL6B	320	2620	78	0.03	50.6	1
TSKgel Phenyl 5PW 1st HPLC	150	147	70	0.48	45.5	16
TSKgel Phenyl 5PW 2nd HPLC	88	32	47	1.47	30.5	49
TSKgel DEAE 5PW HPLC	12	21	36	1.71	23.4	57
TSKgel G3000SW HPLC	9	11	24	2.18	15.6	73

[a]Corresponding to 43 liters of the culture broth.

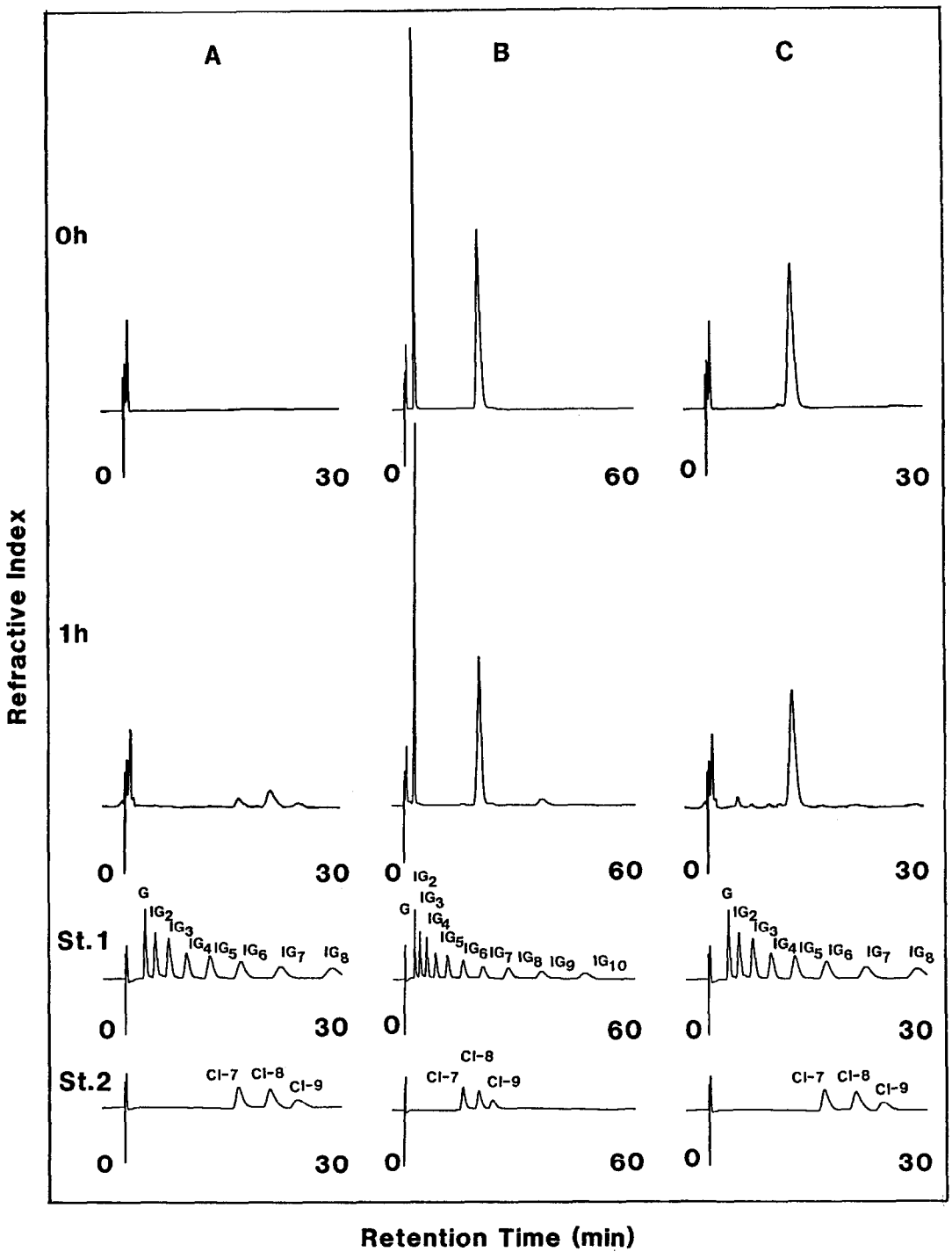

Fig. 2. Catalytic actions of CITase from *Bacillus* spp. T-3040. Details of the analytical conditions of HPLC and reaction conditions are described in the text. Panels A, B, and C show the HPLC profiles of the reaction mixture for cyclization, coupling, and disproportionation activity, respectively. St. 1 and St. 2 are standards of linear and cyclic isomaltooligosaccharides, respectively. Abbreviations: G, glucose; IG_2, isomaltose; IG_3, isomaltotriose; IG_4, isomaltotetraose; IG_5, isomaltopentaose; IG_6, isomaltohexaose; IG_7, isomaltoheptaose; IG_8, isomaltooctaose; IG_9, isomaltononaose; IG_{10}, isomaltodecaose; CI-7, cycloisomalto-heptaose; CI-8, cycloisomalto-octaose; CI-9, cycloisomalto-nonaose.

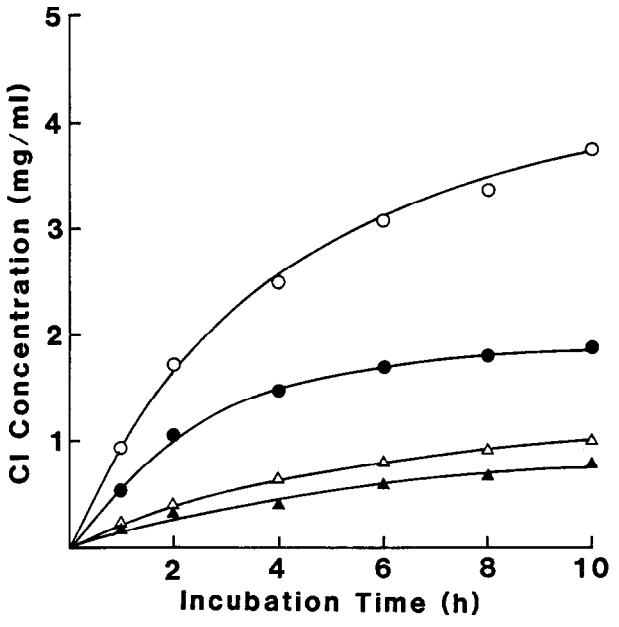

Fig. 3. Time course of cyclization reaction of CITase from *Bacillus* spp. T-3040. 1 ml of a 4.0% dextran 40 solution, 0.2 ml of 100 mM acetate buffer (pH 5.5), 0.78 ml of distilled water and 0.02 ml of the enzyme (2.71 U/ml) were mixed and incubated for 10 h at 40°C. 100 μl aliquots of the reaction mixture were withdrawn and boiled for 5 min. After centrifugation, the supernatant was analyzed by HPLC. CI-7 (●), CI-8 (○), and CI-9 (△).

Acknowledgements: The authors thank Drs. F. Yoshida, S. Sugiyama, S. Ishii, H. Sekine and T. Horiuchi for their useful advice. The authors also thank to Professors S. Chiba and S. Sagisaka of Hokkaido University for their encouragement.

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