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Purification and some properties of cycloinulo-oligosaccharide fructanotransferase from *Bacillus circulans* OKUMZ 31B †

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Abstract

Cycloinulo-oligosaccharide fructanotransferase was purified from the cultured medium of *Bacillus circulans* OKUMZ 31B, to electrophoretic homogeneity, by anion-exchange column chromatography on DEAE-Toyopearl 650M, hydrophobic column chromatography on Butyl-Toyopearl 650M, gel-filtration column chromatography on Sephacryl S-200HR and anion-exchenge column chromatography on SuperQ-Toyopearl 650M. The enzyme has a molecular weight of 132000 and a pI of 4.1. The enzyme was most active at pH 7.5 and 40°C, and was stable at pH 6.0–9.0 and below 40°C. The enzyme catalyses the conversion of inulin into cycloinulohexaose and cycloinuloheptaose in the ratio of ca. 4:1, and a small amount of cycloinulo-octaose. The enzyme has an isoform which may be a proteolyticaly modified species of the CFTase because of its reduced molecular weight, 126000.

1. Introduction

It is known that there are two types of enzymatic degradation of inulin; one is hydrolysis and the other is intramolecular transfructosylation. The enzyme which catalyses the latter reaction was first reported from *Arthrobacter ureafaciens* in 1972 [1]. The enzyme catalyses the conversion of inulin into di-D-fructofuranose 1,2′:2,3′-dianhydride through an intramolecular transfructosylation reaction, so that it was designated as inulin fructotransferase (depolymerising, EC 2.4.1.93) [2].

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Subsequently, several reports have been made relating to inulin fructotransferases [3]. Recently, we reported an enzyme which also catalyses the intramolecular transfructosylation reaction but converts inulin into the larger cyclic β-(2 → 1)-linked fructo-oligosaccharides. The enzyme produced from *Bacillus circulans* OKUMZ 31B [4] was arbitrarily designated as cycloinulo-oligosaccharide fructanotransferase (CFTase) and the products as cycloinulo-oligosaccharides. The main product of the enzymatic reaction was identified as cycloinulohexaose (CF6), together with two by-products, probably cycloinuloheptaose (CF7) and cycloinulooctaose (CF8) [4]. In this paper, the purification and some properties of the CFTase are described.

2. Materials and methods

Sugars.—Inulin from dahlia was purchased from Sigma. Cycloinulo-oligosaccharides as standards were purified from the mixture of the purified CFTase and inulin by the method described in ref. 4. CF7 and CF8 were separately eluted from a QAE-Toyopearl column following CF6. CF6 was crystallised,and CF7 and CF8 were rechromatographed on the QAE-Toyopearl column in order to enhance their purities up to 98% in HPLC [5].

Enzyme assay.—The activity of CFTase was assayed by the following method: enzyme solution (50 μL), 50 mM Tris–citrate buffer (pH 7.5; 50 μL), and 3% inulin solution (100 μL) were mixed and incubated at 30°C. The reaction was stopped by addition of MeCN (300 μL). The mixture was filtered through Ultrafree C3HV (Millipore, USA) and an aliquot (10 μL) was injected into the HPLC. One unit was defined as the amount of enzyme which produced one μmol of cycloinulohexaose from inulin per min. HPLC was carried out on a Shimadzu LC-6A instrument, employing a column of Capcell pak NH_2 (Shiseido, Japan, 4.6 × 250 mm), with elution by 65 : 35 MeCN–water at 1 mL/min, and differential refractomeric detection.

Cultivation of bacterium.—For enzyme purification, the bacterium was cultured at 30°C for 3 days in 10 Sakaguchi flasks containing medium (150 mL) with the following composition: 1.5% inulin, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, 0.2% KH_2PO_4, 0.1% corn steep liquor, and 0.5% yeast extract.

Purification of enzyme.—The cultured medium was centrifuged to remove the cells. The supernatant solution was dialysed against 20 mM Tris–citrate buffer (pH 7.5, TC buffer) and applied to a DEAE-Toyopearl 650M column (4.0 × 15 cm; Tosoh, Japan) equilibrated with TC buffer. After elution of nonadsorbed material, the enzyme was eluted with a linear gradient of sodium citrate (0.02 → 0.25 M) in TC buffer. The active fractions were collected, combined with the same volume of 40% saturated aq ammonium sulfate (the pH was adjusted to 7.5), and applied to a Butyl-Toyopearl 650M column (1.5 × 10 cm, Tosoh, Japan) equilibrated with TC buffer containing 20% satd aq ammonium sulfate. The column was washed with the equilibrated solution and the enzyme was eluted with a linear gradient of aq ammonium sulfate (concentration from 20 to 0% of saturation). The active

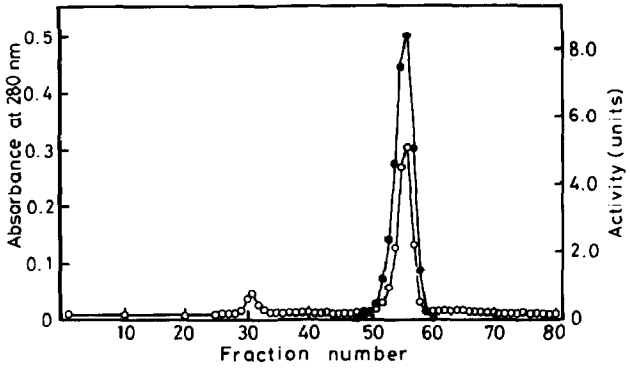

Fig. 1. Gel filtration on a Sephacryl S-200HR column: CFTase activity (•), absorbance at 280 nm (○).

fractions were collected and dialysed against TC buffer. After concentration with Poly(ethylene glycol) 20000, the sample was applied on a Sephacryl S-200HR (Pharmacia) column (2 × 60 cm) and eluted with TC buffer containing 0.2 M NaCl. The active fractions were pooled and concentrated with Molcut-II (Millipore, USA) to 1 mL. The sample was applied on a SuperQ-Toyopearl 650M column (1.5 × 45 cm; Tosoh, Japan) equilibrated with TC buffer containing 0.2 M NaCl, and eluted with the same solution. The active fractions were pooled for use in subsequent experiments.

Determination of protein.—Protein was assayed by the method of Bradford [6], using bovine γ-globulin as a standard.

Electrophoresis.—SDS polyacrylamide gel electrophoresis (SDS-PAGE) was effected according to the method of Laemmli [7]. Analytical isoelectric focusing was performed on a 4% polyacrylamide gel containing 2% of carrier ampholines (Pharmacia) in the pH range 3.5–10. The electrode solutions were 0.01 M H_3PO_4 and 0.4 M NaOH. Focusing was for 4 h at 200 V and 4°C.

3. Results

Purification of enzyme.—Although the activity peak coincided with the protein peak in the gel filtration step with Sephacryl S-200HR (Fig. 1), the sample was not purified to electrophoretic homogeneity (data not shown). Consequently, further purification using a SuperQ-Toyoperl 650M column was required. In this step, the enzyme activity was eluted with two peaks coinciding with protein (Fig. 2). Two fractions indicated by horizontal bars in Fig. 2 were collected and named FI and FII, respectively. The results of the purification are shown in Table 1. FI and FII were purified 50- and 46-fold over the crude solution, with 13.5 and 17.3% recovery, respectively. Their specific activities were 17.8 and 16.6 unit/mg of protein. As shown in Fig. 3A, FI and FII were purified to give a single protein band on SDS-PAGE.

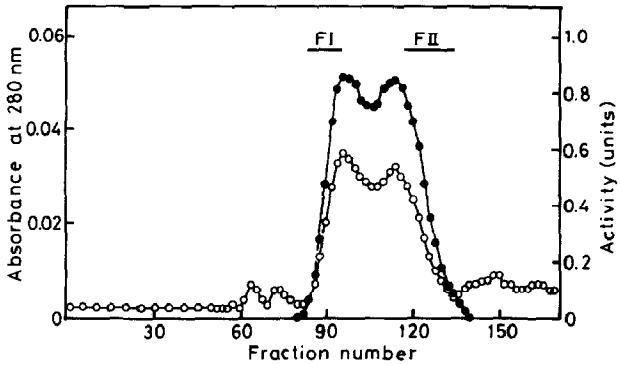

Fig. 2. SuperQ-Toyopearl column chromatography: CFTase activity (●), absorbance at 280 nm (○).f

Table 1
Purification of CFTase from cultured medium of *Bacillus circulans* OKUMZ 31B

Step		Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (-fold)
Crude enzyme		172	61.4	0.357	100	1
DEAE-Toyopearl		22.5	51.1	2.27	83.2	6
Butyl-TOYOPEARL		5.14	27.0	5.25	44.0	15
Sephacryl S-200HR		2.7	31.0	13.7	50.5	38
SuperQ-TOYOPEARL	FI	0.466	8.3	17.8	13.5	50
	FII	0.638	10.6	16.6	17.3	46

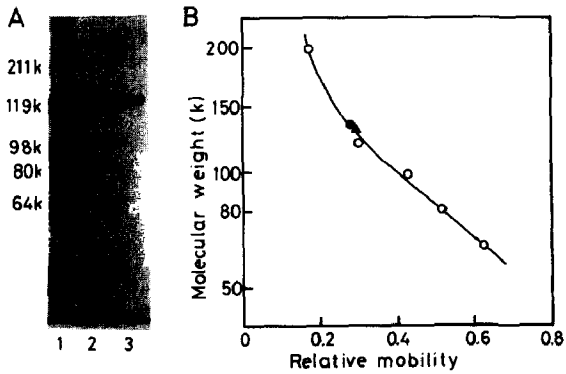

Fig. 3. SDS polyacrylamide gel electrophoresis of the purified enzymes and estimation of their molecular weights. A, Lane 1 is a prestained SDS molecular weight standard mixture (Sigma, Lot 61H9453); Lane 2, FI; and Lane 3, FII. B, The calibration curve: standard proteins (○), FI (●), and FII (▲).

Molecular weights and isoelectric points.—The molecular weights of FI and FII were estimated to be 132000 and 126000, respectively, by SDS-PAGE (Fig. 3B). Analytical isoelectric focusings gave the same pI value of 4.1 to FI and FII.

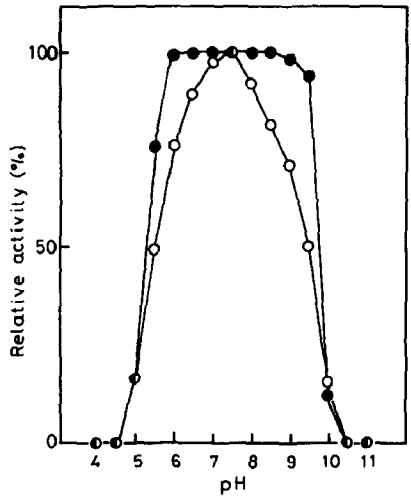

Fig. 4. Effects of pH on the enzyme activity and stability: relative activities at various pH values (○); remaining activities after incubation at various pH values for 30 min (●). A buffer consisting of 50 mM glycine, 50 mM Tris, and 50 mM sodium citrate was used.

Hereafter, we describe the enzymatic properties of FI; there was no significant difference in these between FI and FII.

General properties.—The enzyme had optimum activity at pH 7.5 and was stable in the pH range from 6.0 to 9.0 (Fig. 4). The enzyme was most active at 40°C and stable below 40°C (Fig. 5). The effects of several metal ions and chemicals on the enzyme activity were examined. As shown in Table 2, the activity was inhibited by

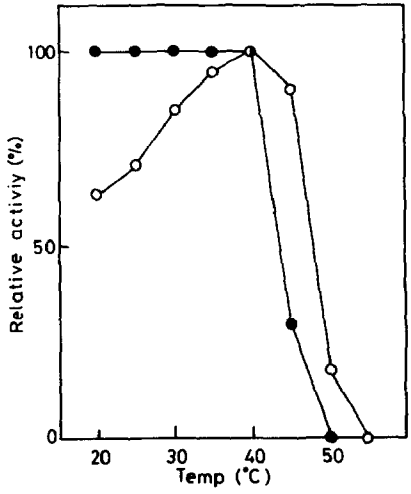

Fig. 5. Effects of temperature on the enzyme activity and stability: relative activities at various temperatures (○); remaining activities after incubation at various temperatures for 30 min (●).

Table 2
Effect of metal ions and some chemicals on CFTase activity

Chemical [a]	Concentration (mM)	Relative activity
None		100
$MnCl_2$	1	100
$MgCl_2$	1	100
$FeCl_3$	1	100
$CoCl_2$	1	104
$CaCl_2$	1	99
$ZnCl_2$	1	100
$CuCl_2$	1	97
$NiCl_2$	1	94
$HgCl_2$	1	2
$PbCl_2$	1	100
EDTA	1	87
PCMB	0.25	100
IAA	1	106

[a] PCMB, *p*-chloromercuribenzoic acid; IAA, iodoacetic acid.

$HgCl_2$ but not by *p*-chloromercuribenzoic acid and iodoacetic acid. This result suggested that cysteine residues were not essential for the catalytic activity.

Action of enzyme on inulin.—Fig. 6 shows a typical HPLC pattern for the enzyme reaction. The enzyme produced cycloinulohexaose and cycloinuloheptaose in the ratio of ca. 4:1, and a small amount of cycloinulo-octaose which was approximately 1% of the total products. After long incubation, up to 80% of the inulin was finally converted into cycloinulo-oligosaccharides. A K_m value of the

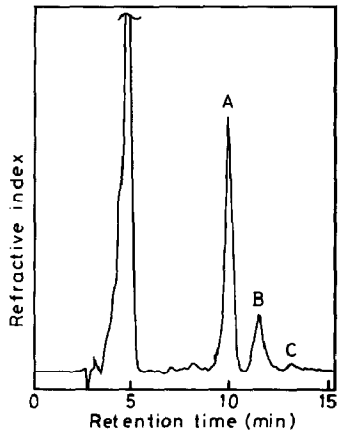

Fig. 6. A typical HPLC pattern of the enzyme products. The purified enzyme (0.02 unit) was incubated with inulin for 10 min, under the standard assay conditions in the text, and the mixture was applied to the HPLC system: A, cycloinulohexaose; B, cycloinuloheptaose; C, cycloinulo-octaose.

enzyme for inulin was estimated to be ca. 0.72 mM on the assumption that the molecular weight of inulin was 5000.

4. Discussion

The purification of CFTase from *B. circulans* OKUMZ 31B resulted in two enzyme fractions, FI and FII. They had similar enzymatic properties, but the molecular weight of FI was slightly larger than that of FII. FII may be an artifact formed from FI by limited proteolysis. The purified CFTase catalyses the conversion of inulin into cycloinulo-oligosaccharides. The reaction is similar to that catalysed by cyclomaltodextrin glucanotransferase (EC 2.4.1.19, CGTase) with starch as substrate [8]. CGTase produces α-cyclodextrin, β-cyclodextrin, and γ-cyclodextrin from starch just as CFTase produces CF6, CF7, and CF8 from inulin. Moreover, a recent report [9] has revealed that CFTase also catalyses intermolecular transglycosylation and hydrolysis in the same way as CGTase. The reactions catalysed by CFTase and CGTase seem to be analogous except for their substrates. It is of interest from the viewpoint of molecular evolution that these analogous enzymes exist for the categories of both glucan and fructan. Recently, a similar enzyme acting on dextran was reported, which produced cyclic (1 → 6)-α-D-glucans, namely cycloisomalto-oligosaccharides [10]. These reports suggest that this type of enzyme might commonly exist, corresponding to each natural polysaccharide.

The products of cycloinulo-oligosaccharides have crown rings constructed from β-(2 → 1) fructosidic linkages in the molecular centers [11]. Recently, a considerable interaction was observed between cycloinulohexaose and some metal ions [12]. Research into their applications as inclusion compounds is being undertaken.

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