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Purification and Characterization of an Intracellular Inulinase from *Bacillus sphaericus* 188-1

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Abstract

In order to obtain basal data for industrial application of inulinase from *Bacillus sphaeicus* 188-1, its intracellular inulinase was purified by ammonium sulfate fractionation and column chromatography on DEAE-Sephadex A-50 and Sephadex G-100. The enzyme was homogeneous as judged by SDS-polyacrylamide gel electrophoresis, with an apparent molecular weight of 29 kDa. Inulinase activity was optimal at pH 6.5 and 40°C. The enzyme activity was significantly inhibited by Cu^{2+}, Cd^{2+} and Hg^{2+}. The inulinase exhibited an apparent Km value of 0.014% for inulin.

Key words: *Bacillus sphaericus* 188-1, intracellular inulinase, characterization

INTRODUCTION

Inulin is a linear β-2,1 linked polyfructan terminated by a sucrose residue and is present as a reserve carbohydrate in *Jerusalem artichoke*, dahlia, chicory and other plants. Inulin has been used in the production of high-fructose syrups by enzymatic or acidic hydrolysis. However, the enzymatic hydrolysis of inulin is preferred because it produces less by-products than acid hydrolysis (1).

The inulin-decomposing enzymes are inulinase (E.C. 3.2.1.7), inulinase II (inulin fructotransferase; E.C. 2.4.1.93), exoinulinase (β-D-fructan fructohydrolase; E.C. 3.2.1.80) and cycloinulo-oligosaccharide fructanotransferase (CFTase) (2). Inulinase (β-fructosidase: β-fructan hydrolase: 2,1-D-fructan-fructanohydrolase; EC 3.2.1.7) splits β-2,1-fructanofuranosidic linkages of inulin to produce fructose (3). The classification of inulinase was based on inulinase activity on sucrose, inulin, hydrolysis patterns (endo-wise or exo-wise), types of reaction products (oligofructans or only fructose), and secretion patterns (intracellular or extracellular) etc. Inulinase from yeasts, in particular, are able to hydrolyze inulin and levan-type fructans exo-wise, and is an extracellular enzyme, partially associated with the cell wall and partially excreted into the culture media (4). Most inulinases are glycoproteins which can be induced exclusively by inulin (3).

Inulinase has been found in several yeasts and bacteria such as *Kluyveromyces* sp. (5-9), *Debaryomyces* sp. (10), *Saccharomyces* sp. (11,12), *Streptococcus* sp. (13,14), *Clostridium* sp. (15), *Arthrobacter* sp. (16-18), *Chrysosporium* sp. (19,20), *Pseudomonas* sp. (21,22) and in some filamentous fungi such as *Aspergillus* sp. (23-26), *Penicillium* sp. (27) and *Fusarium* sp. (28). However, only a few yeasts have been used as a source of inulinase in the food and pharmaceutical industries.

In a search for microorganism suitable for mass production of active inulinase, previous screening identified *B. sphaericus* 188-1 as a potent inulinase-producer, and investigated optimal production conditions of the inulinase (29). In this study, intracellular inulinase from *Bacillus sphaericus* 188-1 was purified by ammonium sulfate fractionation, ion-exchange chromatography and gel filtration, and its enzymatic characteristics were investigated in order to obtain data for industrial applications of the inulinase.

MATERIALS AND METHODS

Materials

Inulin (dahlia), Bacto-tryptone, Bacto-peptone, beef extract, yeast extract and Bacto-agar were purchased from Difco Lab. (Detroit, Michigan, USA) and inulin (*Jerusale artichoke*) was purchased from Merck (Darmstdt, Germany).

All chemicals were of analytical grade. Inulin (chicory), glucose, dinitrosalicylic acid, the electrophoresis reagents, standard markers of molecular weights were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). Sucrose, ampicillin, calcium chloride, sodium periodate and agarose were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan), and DEAE-Sephadex A-50 and Sephadex G-100 were purchased from Pharmacia Fine Chemicals

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(Uppsala, Sweden). Ammonium persulfate, blue dextran, Coomassie brilliant blue R-250, and the protein assay kit were purchased from Bio-Rad Lab. (California, USA).

Strain and culture condition

Bacillus sphaericus 188-1, isolated from soil, was used as the intracellular inulinase producer (29). It was grown in a batch culture in a 7 L fermentor (KFM-7, Korean Fermentor Co.) using inulin-peptone medium composed of 0.5% inulin, 0.4% peptone and 10 mM $MgSO_4 \cdot 7H_2O$ (pH 7.5). The agitation speed and aeration rate were maintained at 90 rpm and 1.0 vvm, respectively.

Assay of inulinase

Inulinase activity was measured by determining the amount of released reducing sugar from inulin (21,29). The reaction mixture containing 0.8 mL of 1% inulin in 100 mM sodium phosphate buffer (pH 7.0) and 0.2 mL enzyme solution was incubated at 37°C for 1 hr. Total reducing sugar was measured by the Somogyi-Nelson method (30). D-Glucose was assayed using the Glucose [HK] kit. D-fructose was determined as the difference between the amount of total reducing sugar and D-glucose. One unit of inulinase activity was defined as the amount of enzyme required to release of 1 μmole of reducing sugar per minute at 37°C and specific activity was expressed as unit per mg protein.

Purification of inulinase

The cell-free extract of *Bacillus sphaericus* 188-1 was adjusted to 30% saturation with ammonium sulfate. After removing the precipitate, the supernatant was brought to 70% saturation with ammonium sulfate. The precipitate was collected by centrifugation, and dissolved in a 100 mM sodium phosphate buffer (pH 7.0). After dialyzing against the same buffer, the enzyme solution was loaded onto a DEAE-Sephadex A-50 column equilibrated with the same buffer. The inulinase was eluted with a 0~0.5 M linear gradient of NaCl in 100 mM sodium phosphate buffer (pH 7.0). The elute fractions showing inulinase activity were pooled and dialyzed against 100 mM sodium phosphate buffer (pH 7.0). The sample was loaded onto a Sephadex G-100 column equilibrated with 100 mM sodium phosphate buffer (pH 7.0) and eluted with a 1 mL fraction at a flow rate of 6 mL/hr (22).

Determination of protein

Protein concentrations were determined by the method of Bradford (31) or Lowry (32) for the determination of specific with bovine serum albumin as a standard. The protein content of the elute during chromatography was estimated by reading the A_{280}.

Polyacrylamide gel electrophoresis

Molecular weight of enzyme subunits were determined by electophoresis on 10% SDS-PAGE slab gels using 25 mM Tris-glycine buffer (pH 8.3) (33). *E. coli* β-galactosidase (116 kDa), rabbit phosphorylase (97 kDa), bovine serum albumin (66 kDa), porcine fumarase (48.5 kDa) and bovine carbonic anhydrase (29 kDa) were used as molecular mass standard.

Electrophoresis for determining homogeneity of the purified enzyme was performed on a 8% native PAGE (33).

Measurement of K_m and V_{max}

Kinetic constants (K_m and V_{max}) were determined by Lineweaver-Burk polts. The velocity of hydrolysis for inulin was measured at 37°C in a series of tests with different concentrations of inulin.

RESULTS AND DISCUSSION

Purification of inulinase

Intracellular inulinase from *Bacillus sphaericus* 188-1 was purified by ammonium sulfate precipitation (30~70% saturation), followed by ion-exchange chromatography on DEAE- Sephadex A-50 (Fig. 1) and gel filtration chromatography on Sephadex G-100 (Fig. 2). One major activity peak was eluted at 0.3 M NaCl in ion-exchange chromatography. The results of the purification steps are shown in Table 1.

The specific activity of the purified inulinase was estimated to be 3700 Unit/mg, which is about 22 fold higher than that of the crude enzyme. However, the final yield was only 1.9% of the crude enzyme, which was lower than that of inulinase (P-I, P-II) from *Pseudomonas* No

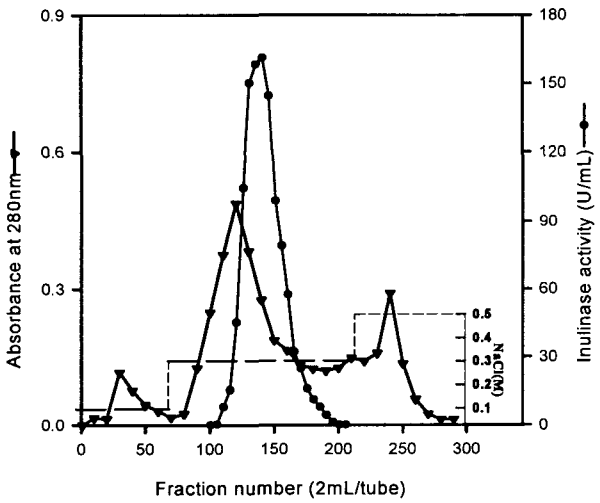
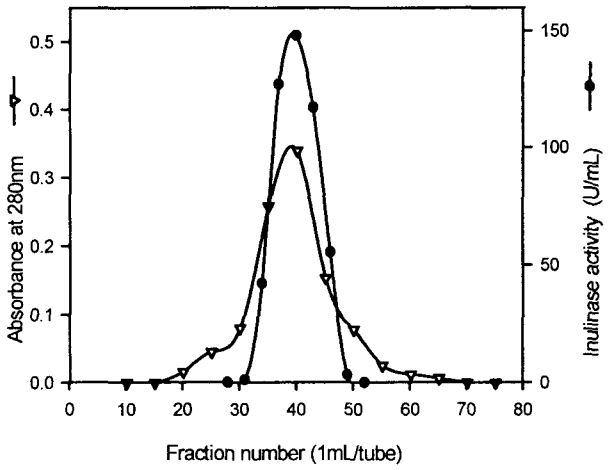


Fig. 1. DEAE-Sephadex A-50 column chromatography of inulinase from *B. sphaericus* 188-1.
The sample solution was applied onto a column (2.6×60 cm) of DEAE-Sephadex A-50 equilibrated with 100 mM sodium phosphate buffer (pH 7.0). The inulinase was eluted with a 0~0.5 M linear gradient of NaCl in 100 mM sodium phosphate buffer (pH 7.0) at a flow rate of 30 mL/hr.

able 1. Summary of the purification of inulinase from *Bacillus sphaericus* 188-1

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Cell free extract	527.4	87750	166.4	100.0	1.0
30~70% $(NH_4)_2SO_4$ precipitation	85.4	14548	170.3	16.6	1.0
DEAE-Sephadex A-50 chromatography	7.9	14453	1835.3	16.4	11.0
Sephadex G-100 chromatography	0.5	1658	3700.0	1.9	22.2



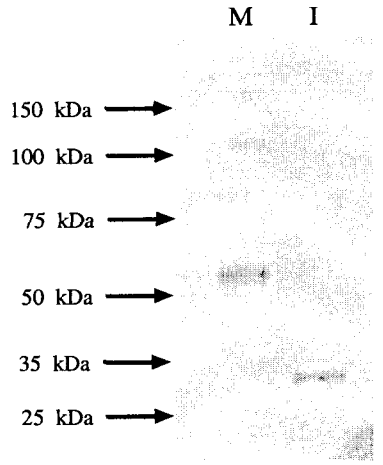
'ig. 2. Sephadex G-100 column chromatography of inulinase rom *B. sphaericus* 188-1.
'he elute fraction showing inulinase activity in DEAE-Sephadex \-50 column chromatography were applied on Sephadex G-100 olumn and then eluted with 50 mM sodium phosphate buffer pH 7.0) at flow rate of 6 mL/hr.

›5 (yield: P I-7.25%, P II-15.33%) (22).

Inulinase eluted from the Sephadex G-100 column was lectrophoretically homogeneous. Native PAGE of the pu-ified enzyme formed a single band (Fig. 3).

Properties of the inulinase

SDS-PAGE showed that the molecular mass of the pu-



'ig. 3. PAGE of the purified inulinase eluted from Sephadex 3-100 column.
.ane M is molecular weight marker. Line I is the purified nulinase.

rified enzyme was 29 kDa, suggesting that it forms a monomer (Fig. 4), as was also found with the enzyme prepared from *Pseudomonas* No 65 (22). It is known that the molecular masses of fungi inulinase subunits are larger with molecular weights of 74 kDa for *A. ficuum*, 81 kDa for *Aspergillus* sp., 85 kDa for *A. niger*, 70 and 84 kDa for *chrysosporium pannorum*, and 60 and 80 kDa for *Penicillum* sp.(8).

The optimum temperature for the enzyme activity was 37~40°C (Fig. 5) and the enzyme retained only 25% of the activity at 50°C for 10 min (Fig. 6). The optimum reaction temperature was similar to that of *Rhizopus* sp. TN-96 (40°C) (34), whereas it was lower than those of fungal, yeast and bacterial inulinase (50~60°C) (22). Vullo et al. (1) reported that inulinase activity from *B. subtilis* 430A decreased only 20% after 7 h at 45 or 50°C, but at 55°C the enzyme activity was rapidly lost.

The optimum pH for inulinase activity was determined by using different buffers ranging from pH 4.0~10.0 (Fig. 7). Maximum inulinase activity was at pH 6.5 and the enzyme was stable over a pH range of 6.0~8.0. This optimum pH was similar to those of inulinase from *Bacillus* sp., *Arthrobacter* sp. (16), *Pseudomonas* sp. (22), and

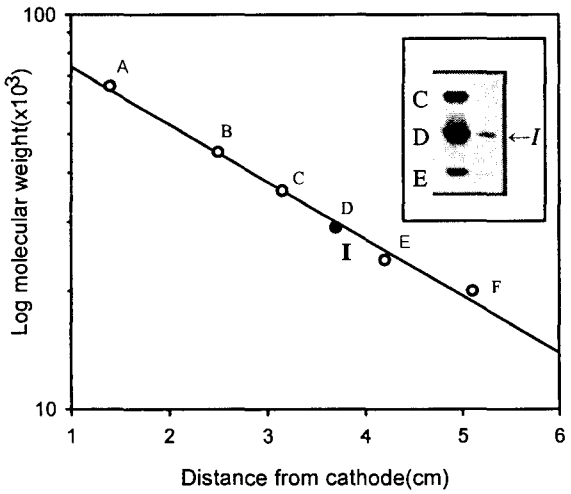


Fig. 4. SDS-PAGE and molecular mass of the inulinase.
A: BSA (66 kDa), B: Ovalbumin (45 kDa), C: Glyceraldehyde-3-phosphate dehydrogenase (36 kDa), D: Carbonic anhydrase (2 kDa), E: Trypsinogen (24 kDa), F: Trypsin inhibitor (20 kDa). ●, (I): Purified inulinase.
The inserted figure shows SDS-PAGE pattern of the purified enzyme.

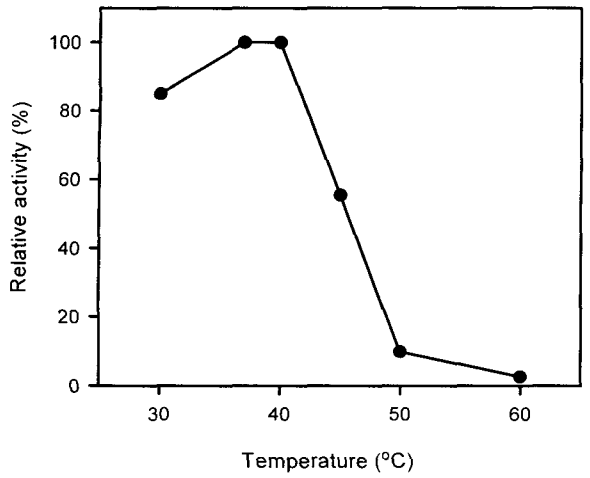


Fig. 5. Optimum temperature of purified inulinase from *Bacillus sphaericus* 188-1.
The reaction mixture containing 0.8 mL of 1% inulin in 100 mM sodium phosphate buffer (pH 7.0) and 0.2 mL of the enzyme was inculcated for 1 hr at various temperature (20~80°C).

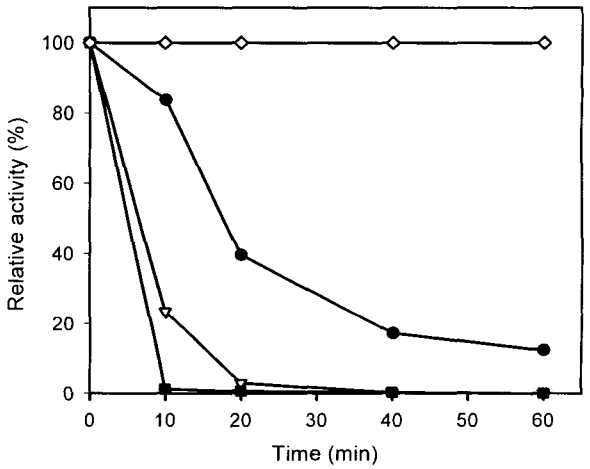


Fig. 6. Thermal stability of purified inulinase from *Bacillus sphaericus* 188-1.
The enzyme solution (0.1 mL) was mixed with 0.1 mL of 100 mM sodium phosphate buffer (pH 7.0), pre-incubated for the indicated time at various temperature (30~60°C), and then cooled to 0°C. The relative activity of the treated enzymes was assayed under the standard assay conditions.
◇: 30°C, ●: 40°C, ▽: 50°C, ■: 60°C.

Rhizopus sp. TN-96 (34), whereas it was different than those of fungal and yeast inulinase (pH 5.0) (25), probably due to difference between microbial species.

The substrate specificity of inulinase for poly- and oligosaccharide was examined by measuring the liberation of fructose (Table 2). The purified inulinase primarily hydrolyzed some kinds of inulin, stachyose, sucrose and raffinose; and a small extent levans were also hydrolyzed. However, melezitose, cellulobiose, lactose and maltose were not hydrolyzed. This result was different from the substrate specificity of *Pseudomonas* sp. inulinase (22)

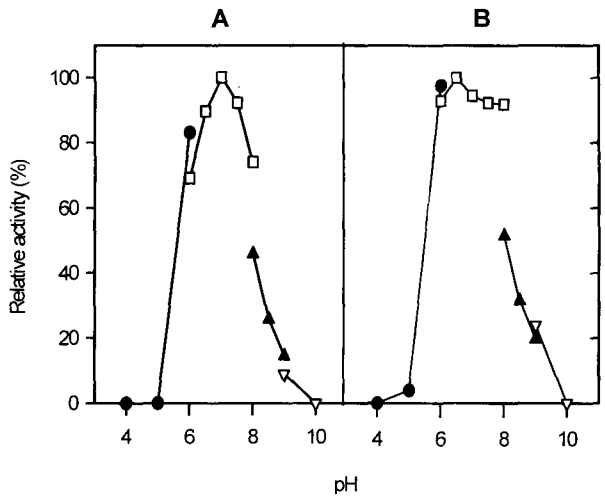


Fig. 7. Optimum pH (A) and pH stability (B) of the purified inulinase from *Bacillus sphaericus* 188-1.
The reaction mixture containing 0.8 mL of 1% inulin in 100 mM of various buffer (pH 4.0~10.0) and 0.2 mL of the enzyme was inculcated for 1 hr at 37°C (A). The enzyme reaction (0.1 mL) was mixed with 0.1 mL of 100 mM of various buffers (pH 4.0~10.0), preincubated for 1 hr. The relative activity of the treated enzymes was assayed under the standard assay conditions (B).
●: Citrate buffer (pH 4.0~6.0), □: phosphate buffer (pH 6.0~8.0), ▲: Tris buffer (pH 8.0~9.0), ▽: Borate buffer (pH 9.0~10.0).

Table 2. Substrate specificity of inulinase onto some kinds of sugar and inulin

Substrate[1]		Activity (Unit/mL)	Relative activity (%)
Levan		21.83	15.6
Melezitose		NH[2]	NH
Raffinose		84.17	60.1
Cellulobiose		NH	NH
Stachyose		121.33	86.7
Lactose		NH	NH
Maltose		NH	NH
Sucrose		145.67	104.1
Inulin			
Jerusalem artichoke	suspended	150.61	107.6
	dissolved	145.0	103.6
Chicory	suspended	127.39	90.9
	dissolved	140	100
Dahlia	suspended	111.67	89.8
	dissolved	135.06	96.5

[1]All substrates were dissolved or suspended in 50 mM sodium phosphate buffer, pH 6.5.
[2]NH: not hydrolyzed.

and *A. niger* P-III inulinase (26) which hydrolyzed only inulin (β-2,1-fructan).

Metal ions and EDTA were tested for their effects on activities of the purified inulinase. The enzyme activity was markedly inhibited by Cu^{2+}, Fe^{2+}, Cd^{2+} and Hg^{2+} at

inal concentrations of 1 mM, whereas Na^+, Li^+ and K^+ id not influence the enzyme activity (Table 3). Ohta et l. (34) reported that Mn^{2+} and Ca^{2+} appeared to stimulate he inulinase activity, increasing relative activities to 14% and 141% of controls, respectively, whereas an appreiable loss of activity was observed with ρ-chloromercurbenzoate (42.2%) and Fe^{3+} (43.4%).

The apparent K_m and V_{max} values of the purified inulinase vere estimated to be 0.014% and 10 M/min, respectively Fig. 8). Lee et al. (22) reported that the Km value for nulin (MW; 5400) of the purified inulinase (I) (II) from *Pseudomonas* sp. were 2×10^{-3} M and 5×10^{-3} M respecively. Up to now, it has been reported that microbial nulinase Km for inulin are widely varied, ranging from 1.2×10^{-2} mM for *Clostridium acetobutylicum* to 17 mM for *Candida salmenticensis* (1).

Table 3. Effects of metal ions and EDTA on the activity of urified inulinase from *Bacillus sphaericus* 188-1

Reagents[1)]	Concentration (mM)	Relative activity (%)
Control	-	100
K^+	25	101
Na^+	25	100
Li^+	1	98
Cu^{2+}	1	3
Ca^{2+}	1	89
Fe^{2+}	1	52
Mg^{2+}	1	88
Mn^{2+}	1	78
Cd^{2+}	1	25
Hg^{2+}	1	10
Al^{3+}	1	87
EDTA	1	95
	10	74

[1)]All of the cations were added as chlorides, except Mg^{2+}, Cu^{2+}, Fe^{2+} and Al^{3+} which were added as sulfates. The enzyme solutions containing 1 mM metal ions and EDTA except 25 mM of K^+ and Na^+ were preincubated at 37°C for 1 hr, respectively and the residual activity was measured.

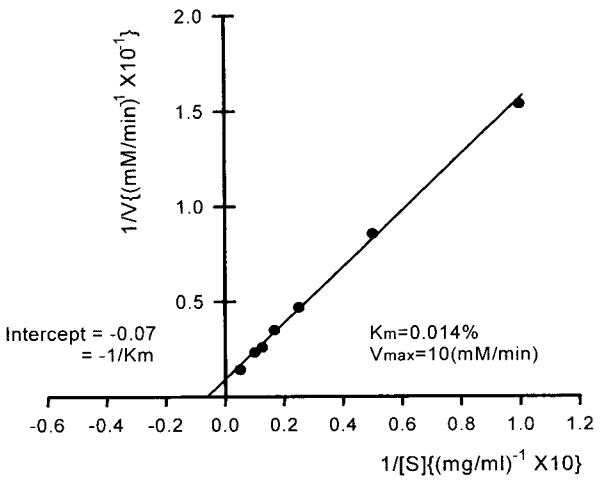


Fig. 8. Lineweaver-Burk plot for the determination of the Michaelis constant toward inulin.

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