

Purification and Characterization of Inulin Fructotransferase (Depolymerizing) from *Arthrobacter* sp. A-6

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Inulin fructotransferase (depolymerizing) (EC 2.4.1.93) was purified 34-fold from the culture broth of *Arthrobacter* sp. A-6 by using a combination of ammonium sulfate fractionation, DEAE-Sepharose CL-6B chromatography and Sephacryl S-200 gel filtration. The purified enzyme converts inulin into di-D-fructofuranose dianhydride III (DFA III) and small quantities of fructo-oligosaccharides. The temperature and pH optima of the enzyme were 70°C and 6.0, respectively. Molecular weight of the enzyme was determined to be 49 kDa by 12% SDS-polyacrylamide gel electrophoresis, and 145 kDa by Sephacryl S-200 gel filtration. This indicates that the functional inulin fructotransferase of *Arthrobacter* sp. A-6 has a homomeric trimer structure. The enzyme had an isoelectric point of pH 4.6. The N-terminal amino acid sequence of the purified enzyme subunit was Ala-Asp-Asn-Pro-Asp-Gly(?) -Ser-Asn-Met(or Glu)-Tyr-Asp-Val.

Inulin is a linear β -2,1-linked fructose polymer terminated with a sucrose residue. Large amounts of inulin are contained as an energy reserve in various plants, particularly in those of the composite family such as Jerusalem artichoke, chicory and dahlia. It has been known that inulin is converted to D-fructose, inulooligosaccharides, several kinds of di-D-fructofuranose dianhydride (DFA) and cyclofructans by respective enzymatic catalysis. Thus, inulin is of growing interest as a renewable carbohydrate raw material for various areas in biotechnology.

The inulin-decomposing enzymes are 2,1- β -D-fructan fructanohydrolase (E.C. 3.2.1.7) (inulinase) (19), inulin fructotransferase (depolymerizing) (EC 2.4.1.93) (inulinase II) (1-3, 11, 14, 21), inulin fructotransferase (DFA I-producing) (EC 2.4.1.200) (8, 9, 12, 18) and cycloinulooligosaccharide fructanotransferase (CFTase) (4, 6, 7).

As reported in a preceding paper (11), we isolated an inulinase II producer from a soil sample, and identified the bacterial strain as *Arthrobacter* sp. A-6. This enzyme converts inulin into di-D-fructofuranose 1,2':2,3' dianhydride III (DFA III).

DFA III is expected to be a low-calory sweetener which has half the sweetness of fructose. It is reported to be a stable compound due to its dioxane ring structure and also reported to have some physiological benefits such as *Bifidus* growth promotion, anti-caries effect and

the lowering of blood cholesterol level (5, 10, 13, 15, 16, 21).

In this study, we purified and characterized the inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. A-6.

MATERIALS AND METHODS

Bacterial Strain and Chemicals

A soil bacterium, identified as *Arthrobacter* sp. A-6 (11), was used to produce inulin fructotransferase (depolymerizing). Inulin (dahlia tuber), DEAE Sepharose CL-6B, Sephacryl S-200 were purchased from Sigma Co. (St. Louis, MO, U.S.A.). All other chemicals used were of analytical grade.

Enzyme Assay

Arthrobacter sp. A-6 was cultivated in the culture medium optimal for the production of the inulinase II (11) at 37°C, for 48 h. The crude enzyme solution was gained by centrifugation (10000 g, 15 min) of the culture broth. Inulin fructotransferase was assayed by mixing 10% inulin solution 0.5 ml, 0.1 M Sodium citrate buffer (pH 5.5) 0.45 ml and the enzyme solution 0.05 ml. Total 1.0 ml was used as a reaction mixture and incubated for 10 min at 60°C. The enzymic reaction product, DFA III, was analyzed by HPLC analysis. One unit of enzyme activity was defined as the amount of the enzyme that produced 1 μ mole of DFA III per min under assay conditions described above.

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Key words: inulin fructotransferase (depolymerizing), *Arthrobacter* sp. A-6, purification, characterization

HPLC Conditions

A Waters' carbohydrate column (4.6×250 mm) and RI detector were used. Other analysis conditions were as follows: column temperature, 70°C; mobile phase, acetonitrile : water (70 : 30); flow rate, 1.2 ml/min.

Purification Process

Crude enzyme solution was concentrated by ammonium sulfate precipitation (0-60% saturation) followed by dialysis at 4°C overnight against a 25 mM sodium phosphate buffer (pH 8.0). The dialyzed enzyme solution was loaded on a DEAE-Sepharose CL-6B column (2.6×20 cm). The column was preequilibrated with 50 mM sodium phosphate buffer (pH 8.0) and the enzyme proteins were eluted with the same buffer. The elution speed was 20 ml/h (3 ml/tube). The enzyme solution that passed through ion exchange chromatography without adsorption was gathered and loaded on Sephacryl S-200 column (1.6×75 cm) preequilibrated with 25 mM sodium phosphate buffer (pH 8.0). Then, the proteins were eluted with 50 mM sodium phosphate buffer (pH 8.0) at the elution speed of 20 ml/h (2 ml/tube).

Estimation of Purity and Molecular Weight

Purity of the purified enzyme was monitored by performing SDS-polyacrylamide gel electrophoresis (The separation gel contained 12% acrylamide). Molecular weight was estimated by 12% SDS slab gel electrophoresis and Sephacryl S-200 gel filtration.

Determination of Isoelectric Point

Isoelectric focusing (IEF) of the purified enzyme was carried out with 5% polyacrylamide gel containing a final concentration of 2.4% ampholytes of pH 3.0 to 10.0 (Bio-Rad Co.) Development was done for 90 min at 200 V and subsequently 90 min at 400 V. The developed gel was washed with 10% trichloroacetic acid (TCA) solution for 30 min, 1% TCA for 24 h, followed by Coomassie blue staining. IEF standard from Bio-Rad Co. was used as an internal standard for determining pI value.

Determination of N-Terminal Amino Acid Sequence

Determination of N-terminal amino acid sequence of the enzyme protein was committed to Korea Basic Science Center (Seoul Branch). The Edman degradation method was used with a Milligen 6600B protein sequencer.

RESULTS AND DISCUSSION

Purification of the Enzyme

The inulin fructotransferase (depolymerizing) was obtained as an extracellular enzyme solution (1.2 liter containing 20,278 units of the enzyme activity) from the culture broth of *Arthrobacter* sp. A-6. Table 1 summarizes results for the three-step purification procedure. In the final step of Sephacryl S-200 column gel filtration, the enzyme was effectively separated from the other proteins

Table 1. Summary of purification of inulin fructotransferase (depolymerizing) from *Arthrobacter* sp. A-6.

Step	Total protein (mg)	Total activity* (units)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude enzyme	578.0	20,278	35.1	100.0	1.0
Ammonium sulfate fractionation	50.9	11,968	235.1	59.0	6.7
DEAE-Sepharose CL-6B	42.2	11,299	267.8	55.7	7.6
Sephacryl S-200	2.0	2,390	1,195.0	11.8	34.0

*One unit of enzyme is defined as the amount of the enzyme liberating 1 μmole of DFA III per min.

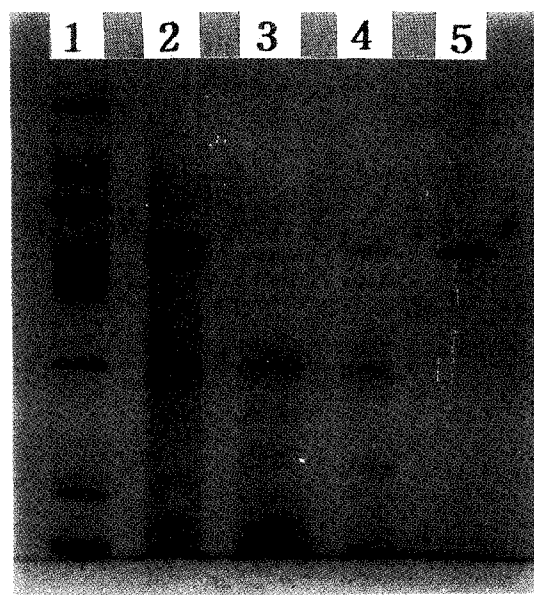


Fig. 1. 12% SDS-PAGE monitoring of the major steps of the enzyme purification.

Lane 1, protein size markers; lane 2, crude extract; lane 3, ammonium sulfate precipitate; lane 4, ion exchange column effluent; lane 5, Sephacryl S-200 gel filtrate. Arrow indicates the inulin fructotransferase.

in the enzyme solution gathered from the flow-through fractions of the DEAE-Sepharose CL-6B chromatography. The inulin fructotransferase in the active fractions of the third procedure was finally purified 34-fold with an 11.8% yield. This purified enzyme solution gave a single band on SDS-polyacrylamide gel electrophoresis as visualized by Coomassie brilliant blue R250 staining as seen in Fig. 1.

Characterization of the Purified Enzyme

Effect of pH and temperature on enzyme activity,

and thermal stability of the enzyme. The pH dependence of the inulin fructotransferase activity was examined in the pH range of 4.0-8.0, by using 0.05 M citrate/phosphate buffers. Fig. 2 indicates the effect of pH on enzyme activity, and maximal activity was observed at pH 6.0. The influence of temperature on enzyme activity is shown in Fig. 3. The optimum reaction temperature was found to be 70°C, which was the highest temperature noted as compared to the same enzymes from the other species. In addition, long-term heat treatment of the enzyme revealed that the inulinase II was highly stable at 70°C (Fig. 4). No significant loss of activity could be seen within 5 h at this temperature, whereas at 80°C the enzyme was inactivated rapidly and

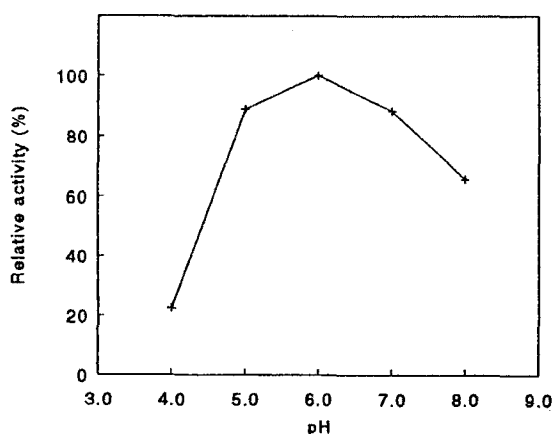


Fig. 2. Effect of pH on the enzyme activity. Buffers (0.05 M) used were as follows; citrate-phosphate buffer at pH 4.0, 5.0; phosphate buffer at pH 6.0-8.0. The enzyme reaction was carried out at 60°C for 10 min. Relative activity represents the relative value to the maximum enzyme activity at pH 6.0.

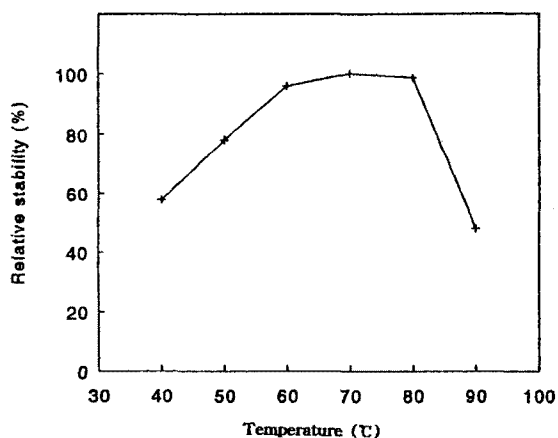


Fig. 3. Effect of temperature on the enzyme activity. The enzyme reaction was carried out for 10 min at various temperatures indicated in the figure. Relative activity was determined as the percentage of the enzyme activity at 70°C.

practically no activity was retained after 2 h treatment at this temperature. This high thermal stability of the enzyme is considered to be a property of high value for the enzyme for industrial applications.

Molecular weight estimation. Fig. 5 shows plots of logarithmic molecular weight versus protein mobility on 12% SDS-polyacrylamide gel. The molecular weight of the inulin fructotransferase was estimated to be 49 kDa (Fig. 5). While, by gel filtration with Sephacryl S-200, it was estimated to be approximately 145 kDa as shown in Fig. 6. From these results, the enzyme of *Arthrobacter* sp. A-6 was judged to have a trimeric structure. Molecular weight and other properties of the

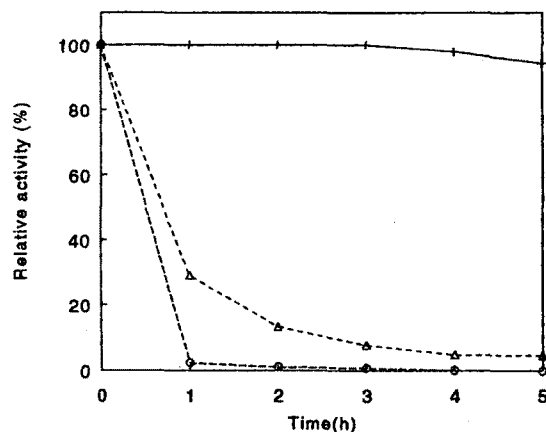


Fig. 4. Effect of temperature on the enzyme stability. The enzyme activity was measured at various temperatures for 10 min after allowing the enzyme solution stand at various temperatures for 1 to 5 h. Residual activity was determined as the percentage of the untreated control. —+, 70°C; ---△, 80°C; ···○, 90°C.

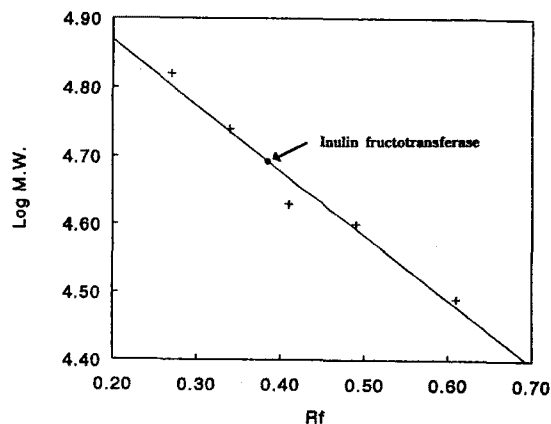


Fig. 5. Molecular weight estimation of the inulin fructotransferase (depolymerizing) by 12% SDS-PAGE (\log_{10} molecular weight vs. relative mobility). Size marker: bovine serum albumin, 97.4 kDa; glutamate dehydrogenase, 66.2 kDa; ovalbumin, 42.7 kDa; aldolase, 40.0 kDa; carbonic anhydrase, 31.0 kDa.

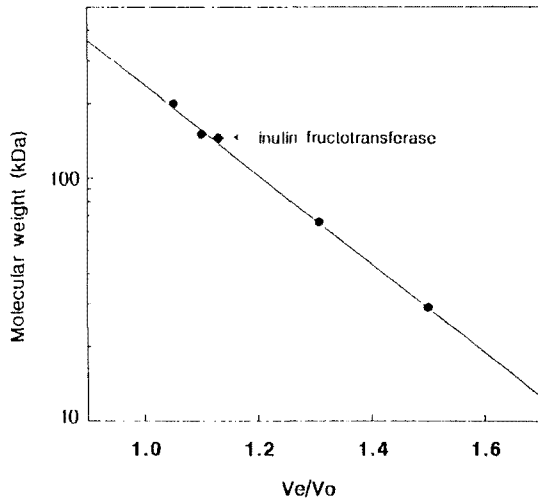


Fig. 6. Molecular weight estimation of the inulin fructotransferase (depolymerizing) by Sephacryl S-200.

Size marker: carbonic anhydrase, 29 kDa; albumin bovine serum, 66 kDa; alcohol dehydrogenase, 150 kDa; β -amylase, 200 kDa.

Table 2. Inulin fructotransferases from *Arthrobacter* sp.

Strains	M.W. (kDa)		Optimum		Ref.
	SDS-PAGE	Gel filtration	pH	Temp (°C)	
<i>A. ureafaciens</i>	–	80	6.0	50	17
<i>A. globiformis</i> C11-1	45	50	5.0	55	1
<i>A. ilicis</i> OKU17B	27	50	5.5	60	3
<i>Arthrobacter</i> sp. H65-7	49	100	5.5	60	20
<i>Arthrobacter</i> sp. A-6	49	145	6.0	70	This work

purified enzyme are shown in Table 2 together with those of other enzymes from *Arthrobacter* species reported on so far.

Analysis of the reaction products from inulin. An enzyme reaction was carried out at 60°C for 1 h, using 10 units of the purified enzyme in order to scan the products of the inulin lysate resulting from the catalysis. In searching by HPLC analysis, we could find one major peak of DFA III and two minor carbohydrate peaks identified as nystose (GF3) and 1- β -fructofuranosyl nystose (GF4) (Fig. 7).

Isoelectric point. The isoelectric point of the purified enzyme was estimated to be 4.6 by isoelectric focusing as described in detail in Materials and Methods (Fig. 8).

N-Terminal amino acid sequencing. The N-terminal sequence of the enzyme was analyzed as Ala-Asp-Asn-Pro-Asp-Gly (?) -Ser-Asn-Met (or Glu)-Tyr-Asp-Val. This result will be used for the determination of an open reading frame of the gene coding for the enzyme protein and also for post-translational modification analysis.

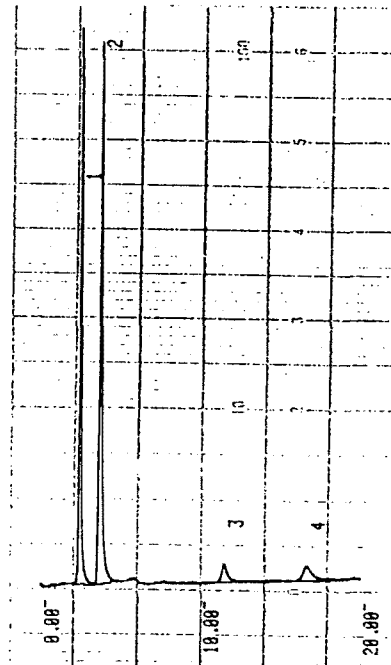


Fig. 7. Inulin lysates catalyzed by inulin fructotransferase (depolymerizing).

Exhaustive digestion of 5% inulin solution was carried out for 1 h, at 60°C. 1, water; 2, DFA III; 3, GF3 (nystose); 4, GF4 (1- β -fructofuranosyl nystose).



Fig. 8. pI determination of the purified enzyme by isoelectric focusing.

Focusing was carried out in 5% polyacrylamide gel containing Biolyte 3/10 under constant voltage condition at 200 V for 90 min and 400 V for 90 min. The standard proteins used were as the following: Bio-Rad IEF standards (from top to bottom) – cytochrome C, pI 9.6; lentil lectin (3 bands), pI 7.8, 8.0, 8.2; human hemoglobin C, pI 7.5; human hemoglobin A, pI 7.1; equine myoglobin, pI 7.0; human carbonic anhydrase, pI 6.5; bovine carbonic anhydrase, pI 5.1; phycocyanin, pI 4.65. Arrow indicates the inulin fructotransferase.

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