

Purification and Characterization of an Inulin Fructotransferase from *Flavobacterium* sp. LC-413

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A bacterial strain LC-413, producing an extracellular inulin fructotransferase (depolymerizing) which converts inulin into di-D-fructofuranose dianhydride (DFAIII), was isolated from soil. Inulin fructotransferase from the isolate identified as a strain *Flavobacterium* sp. was purified from the culture broth by ammonium sulfate precipitation, followed by column chromatographies on DEAE-Toyopearl 650 M and phenyl-Toyopearl 650 M. The purified enzyme gave a single band on an electrophoretic disc-gel. The molecular weight of the enzyme was estimated to be 44,000 Da by SDS-polyacrylamide gel electrophoresis, and 45,000 Da by gel filtration, suggesting the monomeric state of the enzyme. The isoelectric point of the enzyme was about pH 4.5. The optimal pH and temperature for the enzyme reaction were 6.0 and 50°C, respectively. The purified enzyme digested inulin into di-D-fructofuranose-1,2':2,3'-dianhydride, confirming the enzyme was an inulin fructotransferase (inulinase II).

Inulin is a linear β -2,1 linked polyfructan terminated by a sucrose residue and contained as reserve carbohydrate in Jerusalem artichoke, dahlia, chicory and so on. Inulin-decomposing enzymes (inulinases, EC 3.2.1.7) which split β -2,1 fructofuranosidic linkages of inulin through either exo- or endo-type reaction have been studied in several microorganisms (9, 16, 17). Inulin fructotransferase (depolymerizing)(inulinase II, EC 2.4.1.93) is another type of inulin-decomposing enzyme. This enzyme was originally found in the culture broth of *Arthrobacter ureafaciens* and has been shown to convert inulin into di-D-fructofuranose-1,2':2,3'-dianhydride (DFAIII) and a small amount of inulo-oligosaccharides via an intramolecular transfructosylation reaction (14).

Interest in enzymatic formation of difructoanhydrides has been stimulated by the potential role of the oligosaccharides as food additives (5, 19). Thus, several reports on enzymatic formation of DFAI (6, 12), DFAV (8), and other isomeric difructoanhydrides of DFA produced by acid hydrolysis of inulin, as well as DFAIII (2), have been published.

Difructoanhydrides are expected to be used as a non-calorie or anti-tooth decaying sweetener, and also known to have a growth promoting activity for the enteric bacteria such as *Bifidobacterium*. It has been also studied

whether they have other peculiar physiological properties or not (3).

Recently, we isolated a new bacterial strain from soil, designated as *Flavobacterium* sp. LC-413, which produced extracellularly large amounts of inulin fructotransferase (inulinase II) and confirmed that the enzyme produced large amount of DFAIII from inulin (1). In this paper, we describe the purification and characterization of the enzyme produced by *Flavobacterium* sp. LC-413.

MATERIALS AND METHODS

Microorganism

Flavobacterium sp. LC-413 isolated from a soil sample was used throughout the present study as the inulinase II producer.

Cultivation

Flavobacterium sp. LC-413 was cultured in 500 ml conical flasks containing 50 ml of a medium comprising 1.5% inulin, 0.2% NaNO₃, 0.2% K₂HPO₄, 0.1% KH₂PO₄, 0.05% MgSO₄·7H₂O, 0.05% KCl, 0.3% malt extracts and trace amount of FeSO₄·7H₂O (pH 9.0). After incubation at 30°C for 24 h in a reciprocal shaker, 1 ml of the culture broth was used to inoculate 50 ml of the fresh medium (in 500 ml flask), which was then cultivated for 10 h. The culture supernatant obtained by centrifugation at 10,000×g for 20 min at 4°C was used as a crude enzyme solution.

Enzyme Assay

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Inulinase II activity was assayed by measuring the amount of the reaction product, after complete removal of residual inulin by acetone precipitation (13). The reaction mixture was composed of 0.2% inulin solution dissolved in 0.1 M phosphate buffer (pH 5.6) and 0.2 ml of the enzyme solution. Following incubation at 40°C for 30 min, the reaction was stopped by boiling at 100°C for 5 min. Immediately 0.4 ml of 0.8 M Clark & Lubs solution (mixture of KCl, H₃BO₄ and NaOH solution, pH 8.0), 0.02 ml of 3.2 M CaCl₂ and 3 ml of chilled acetone were added to the reaction mixture, and mixed well. After standing in an ice-bath for 10 min, the mixture was centrifuged at 10,000×g for 15 min at 0°C. One milliliter aliquot was taken from the supernatant and evaporated quickly to a thick syrup. It was dissolved in 1.0 ml of distilled water, and the amount of ketohexose was measured by the Resorcinol-HCl method (11) using fructose as a standard. One unit of enzyme activity was defined as the amount of the enzyme which produced 1 μmol of DFA per min under this assay condition. Protein concentration was determined by measuring the absorbance at 280 nm with a spectrophotometer.

Estimation of Molecular Weight

The molecular weight of the enzyme was estimated by SDS-polyacrylamide gel electrophoresis and gel filtration. Electrophoresis was performed on a 10% polyacrylamide gel in the presence of 0.1% SDS by the method of Laemmli (7). Gel filtration was carried out on a Sephadex G-100 (1×70 cm) column equilibrated with 5 mM Tris-HCl (pH 8.0). The standard proteins used for size calibration were β-galactosidase (116,000), phosphorylase B (97,000), bovine serum albumin (66,000), egg albumin (45,000), and carbonic anhydrase (29,000).

Measurement of Isoelectric Point

The isoelectric point of the enzyme was determined by 5% polyacrylamide gel isoelectric focusing using 0.2% Ampholine (pH 3.0-10.0). Two identical gels (0.8×8 cm) were prepared, one for the calibration of pH gradient and the other for detection of the enzyme. After electrofocusing at the constant voltage of 100 V per column for 3 h at 4°C, the inulinase II activity and pH of each slice were measured.

Analytical Methods

Thin layer chromatography (TLC) was carried out using a silica gel plate (Silica gel 60, Merck) with a solvent

system of butanol-ethanol-water (2:1:1, v/v/v). Spots on the plate were detected with 50% sulfuric acid. Bacterial growth was determined by measuring the optical density of the culture broth at 660 nm after appropriate dilutions.

Chemicals

Inulin from dahlia root was purchased from Difco Co. USA. Protein molecular weight standards for SDS-polyacrylamide electrophoresis and Sephadex G-100 were obtained from Sigma chemical co., St. Louis, Mo., U.S.A. DEAE-Toyopearl 650 M and Phenyl-Toyopearl 650 M were purchased from Tosoh, Tokyo. DFAIII and DFAI as standard oligosaccharides were donated by Kuniji Tanaka, a professor at Osaka Koiku university.

RESULTS

Purification of Enzyme

The extracellular inulinase II released into the culture broth of *Flavobacterium* sp. LC-413 was purified by the procedure summarized in Table 1. The supernatant obtained by centrifugation of culture broth was brought to 75% saturation with solid ammonium sulfate, and allowed to stand overnight at 4°C. After centrifugation at 10,000×g for 20 min at 4°C, the precipitate was dialyzed three times against 5 mM Tris-HCl (pH 8.0) at 4°C. The dialysate was applied on a column of DEAE-Toyopearl 650 M (2.2×12 cm) equilibrated with 5 mM Tris-HCl buffer (pH 8.0). After the column was washed with the same buffer, the enzyme was eluted with a linear gradient of 0 to 0.4 M NaCl in the same buffer (elution rate, 25 ml/h; fraction volume, 3 ml). The fractions showing enzyme activity (No. 31-55) were pooled and then saturated with solid ammonium sulfate to 30%. The enzyme solution was put on a column of phenyl-Toyopearl 650 M (1.8×7 cm) previously equilibrated with 5 mM Tris-HCl buffer (pH 8.0) containing 30% ammonium sulfate. After washing the column with the same buffer, the enzyme was eluted with a linear descending gradient from 30 to 0% of ammonium sulfate. However, the enzyme was not released from the column even by washing with the buffer containing no ammonium sulfate solution, and so was followed by stepwise elution with 1% ethylene glycol solution in Tris-HCl buffer (pH 8.0) to decrease the hydrophobicity of the enzyme. As shown in Fig. 1, the enzyme was eluted around fraction No. 35, showing a

Table 1. Purification steps of inulinase II from *Flavobacterium* sp. LC-413.

Purification steps	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Culture supernatant	970	1,601	81,209	51	100
Ammonium sulfate precipitaion	52	153	26,232	171	32
DEAE-Toyopearl 650 M	31	41	17,403	424	21
Phenyl-Toyopearl 650 M	15	1.4	8,019	5,728	10

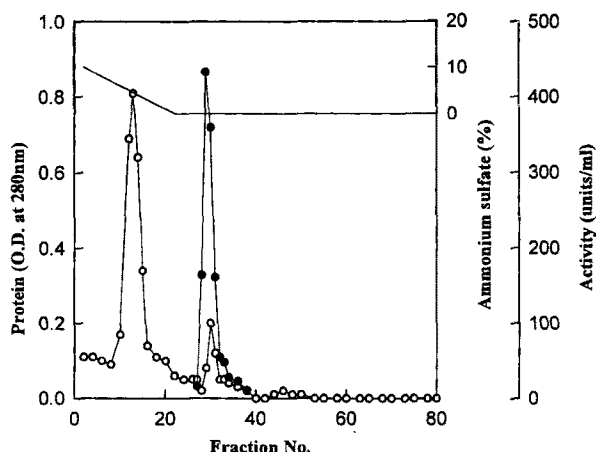


Fig. 1. Elution pattern of inulinase II from phenyl-Toyopearl 650 M column.

Enzyme solution was applied onto a column of phenyl-Toyopearl 650 M equilibrated with 5 mM Tris-HCl buffer (pH 8.0) containing 30% ammonium sulfate. After the column was washed with the same buffer, and eluted by descending gradient from 30% to 0% of ammonium sulfate, the enzyme was detached by 5 mM Tris-HCl buffer containing 1% ethylene glycol. Column size, 1.8×7 cm; flow rate, 25 ml/h; fraction volume, 3 ml; ○, absorbance at 280 nm; —●—, inulinase II activity.

single peak. The enzyme protein collected from the active fractions (No. 28-38) was almost homogenous, migrating as a single band on a disc gel (Fig. 2A). The specific activity of the purified enzyme was 5,728 units/mg protein, which was more than 112 times higher when compared to that of the culture broth.

Molecular Weight of Inulinase II

The molecular weight of the enzyme was estimated to be approximately 44,000 dalton by SDS-polyacrylamide gel electrophoresis as shown in Fig. 2B and Fig. 3. In case of gel filtration on a Sephadex G-100 column, the molecular weight of the enzyme was estimated to be about 45,000 (data not shown). These results suggest that the enzyme from *Flavobacterium* sp. LC-413 is monomeric.

Isoelectric Point

The isoelectric point of the enzyme was estimated to be about pH 4.5 (data not shown).

Effect of pH on Enzyme Activity

The effect of pH on enzyme activity was investigated by measuring the activity in the pH range of 3.0 to 10.0, using citrate, phosphate, and Tris-HCl buffers. As shown in Fig. 4, the maximal activity of the enzyme was obtained at pH 6.0.

To investigate the effect of pH on enzyme stability, enzyme was treated at various pHs for 24 h at 20°C, and then the remaining activities were measured. The enzyme was stable at the pH range of 5.0 to 8.0 (Fig. 4).

Effect of Temperature on Enzyme Activity

To examine the effect of temperature on enzyme activity,

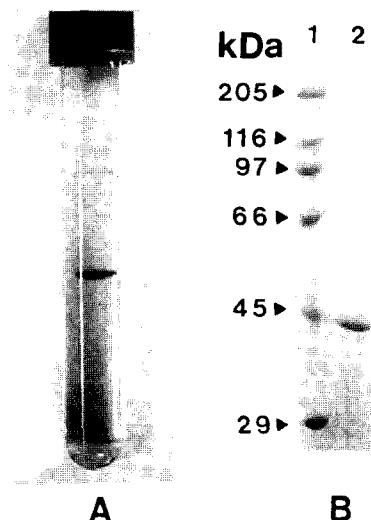


Fig. 2. Native-(A) and SDS-PAGE (B) of the purified enzyme. Native-PAGE of purified enzyme was carried out in 7% polyacrylamide gel using Tris-glycine buffer (pH 8.3). SDS-PAGE was performed as Materials and Methods. Lane 1, marker proteins; lane 2, purified enzyme.

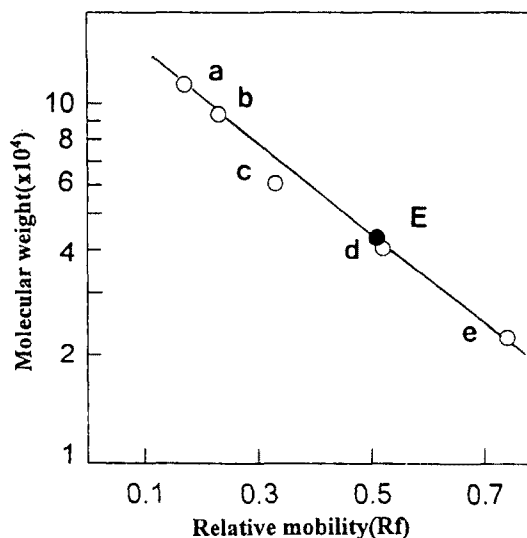


Fig. 3. Estimation of molecular weight of the enzyme by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis was performed on a 10% gel. Standard marker proteins: a, β-galactosidase (116,000); b, phosphorylase B (97,000); c, bovine serum albumin (66,000); d, egg albumin (4,5000); e, carbonic anhydrase (29,000); E, purified enzyme.

enzyme reaction was performed at various temperatures ranging from 30 to 90°C in 0.1 M phosphate buffer (pH 6.0). As a result, the maximal activity was observed at 50°C as shown in Fig. 5.

When the thermal stability of the enzyme was examined by assaying the residual enzyme activity after 30 min of

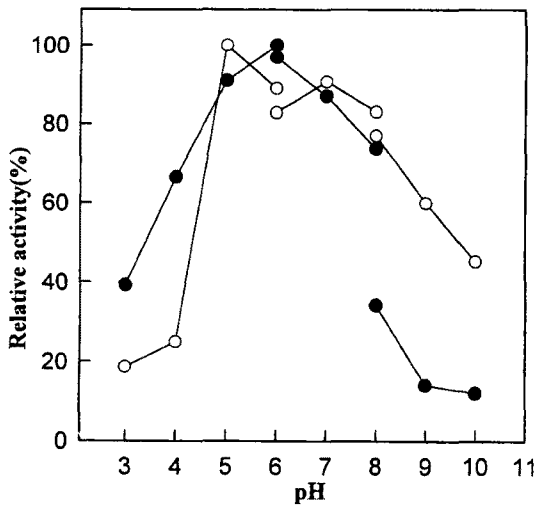


Fig. 4. Effect of pH on the enzyme activity. The enzyme activity was measured at pH 3.0 to 10.0 at 40°C to investigate optimum pH. To examine pH stability, the mixture of 0.1 ml of the purified enzyme and 0.9 ml of 100 mM buffer solution was incubated at 20°C for 24 h, and then was diluted 4-fold with 100 mM sodium acetate buffer (pH 5.6) and the residual activity was measured. —●—, enzyme activity; —○—, pH stability; pH 3.0–6.0, citrate buffer; pH 6.0–8.0, phosphate buffer; pH 8.0–10.0, Tris-HCl buffer.

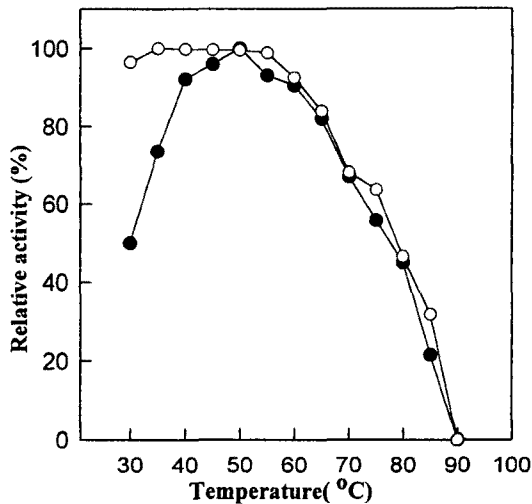


Fig. 5. Effect of temperature on the enzyme activity. The enzyme reaction was performed at various temperature ranging from 30 to 90°C in 0.1 M phosphate buffer (pH 6.0). To investigate thermal stability, the enzyme was incubated in 0.1 M phosphate buffer (pH 6.0) for 30 min, and then the residual activity was measured. —●—, enzyme activity; —○—, thermal stability.

preincubation of the enzyme at various temperatures, the enzyme remained relatively stable up to 65°C, but was rapidly inactivated at temperatures above that.

Substrate Specificity and K_m Value

The enzyme reaction was carried out with various

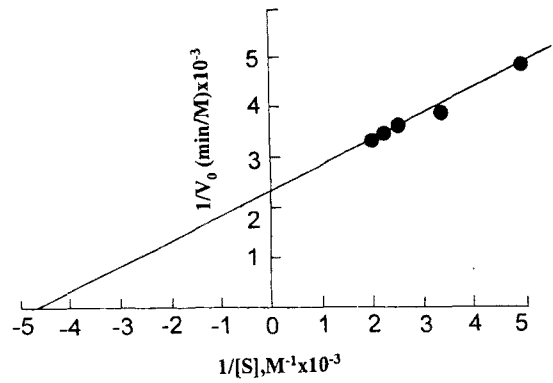


Fig. 6. Lineweaver-Burk plot of inulinase II reaction rate against inulin concentration. The enzyme reaction was carried out in 100 mM phosphate buffer (pH 6.0) at 50°C. The average molecular weight of inulin was assumed to be 5,000.

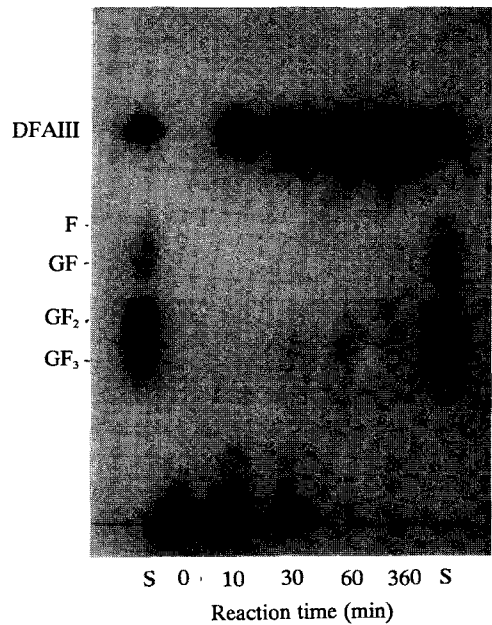


Fig. 7. TLC analysis of the digestion products of inulin by inulinase II. The enzyme reaction was carried out at 50°C for 6 h in a reaction mixture containing 20 ml of 5% inulin solution and 1.0 ml of the purified enzyme (535 units). As standards (S), DFAlII, fructose (F), sucrose (GF), GF₂ and GF₃ were used.

substrates to examine substrate specificity. That is, the purified enzyme was incubated at 65°C for 20 h with bacterial levans (from *Zymomonas and Serratia*), raffinose, stachyose, melibiose, sucrose, nystose (GF3), 1^F-fructofuranosyl nystose (GF4), starch, cellulose, or dextran as a substrate, and then the reaction products were analysed by TLC. As a result, none of these substrates listed above was degraded by the enzyme (data not shown),

suggesting that this enzyme had a very strict substrate specificity for inulin.

The affinity of the purified enzyme for inulin was examined with a Lineweaver-Burk plot as shown in Fig. 6. The enzyme exhibited Michaelis-Menten constant (K_m) of 2.1×10^{-4} M.

Reaction Mode of the Enzyme on Inulin

To investigate the reaction mode of enzyme on inulin, the purified enzyme was incubated with 20 ml of the substrate solution (5% inulin in 0.01 M phosphate buffer, pH 6.0). Small amounts of reaction mixture were withdrawn at suitable time intervals during the incubation, and the products were analyzed by TLC. As shown in Fig. 7, the amount of inulin was decreased as the reaction progressed, while the amount of DFAIII having Rf value of 0.64 increased rapidly. Rf value of DFAIII was greater than that of monosaccharides, such as glucose and fructose. After 6 h of reaction, inulin was degraded almost completely. Besides DFAIII, small amounts of 1-kestose (GF2) but not glucose or fructose were detected in the final reaction mixture. The identification of the reaction product by the enzyme was described in the previous paper (1).

DISCUSSION

We isolated useful microorganisms which converted inulin into inulooligosaccharides from soil, and identified as a strain of *Flavobacterium* sp. as reported in the previous paper (1). It was found that this strain produced an extracellular inulinase II which completely hydrolyzed inulin into DFAIII and small amounts of GF2 even in a short period of reaction time. Although this strain showed a high growth level in the presence of carbon sources such as glucose, fructose, soluble starch, and dextrin, no inulinase II activity was detected in the culture broth. However, the enzyme was markedly induced by the addition of inulin to the cultivation medium.

The inulinase II produced by the isolated *Flavobacterium* sp. was purified to electrophoretic homogeneity by comparatively simple procedures. Although the yield of the purified enzyme was relatively low, the specific activity of the finally purified enzyme was very high compared with those of other enzymes published previously (2, 4, 18), showing 5,728 units/mg protein and being 112 times higher than that of the culture broth. On purification, the enzyme was eluted through the column of Phenyl-Toyopearl 650 M, only when the column was washed with 5 mM Tris-HCl (pH 8.0) containing ethylene glycol, a surface tension-reducing agent that reduced hydrophobic interactions. Therefore, we assumed that this enzyme was more hydrophobic than a typical protein molecule of aquatic status. The K_m value of the purified enzyme for inulin was estimated to be 0.21 mM, in-

dicating that the enzyme has higher affinity to the substrate when compared with those of other previously reported enzymes (*Aspergillus niger* mutant 817; 0.48–0.5 mM, *Aspergillus* sp.; 1.25 mM) (10). The inulinase II was a monomeric enzyme with molecular weight of 44,000 Da, which was similar to that of enzyme from *Arthrobacter globiformis* C11-1 purified by Haraguchi *et al.* (2). Whereas, inulinases II from *Arthrobacter ilichis* OKU17B studied by Kawamura *et al.* (4) and from *Arthrobacter* sp. H-65-7 studied by Yokota *et al.* (18) were dimeric enzymes of 50,000 and 100,000 Da, respectively. The enzyme had maximum activity at pH 6.0 and at 50°C, and was stable at pH 5.0 to 8.0 and at temperature up to 65°C. These properties of the enzyme are not particularly different from those of other inulinases II reported previously (2, 4, 15, 18).

The enzyme could not decompose the saccharides having a similar linkage modes with inulin such as raffinose, starchyose, melibiose, sucrose, and levan and thus it had a strict substrate specificity for inulin only. The exhaustive digestion of inulin by the enzyme produced largely DFAIII and small amounts of GF2. Considering that most inulinases II reported previously (2, 4, 15, 18) produced small amounts of GF3 and GF4 as the final products in addition to DFAIII, we might conclude that the substrate specificity of the enzyme has a different characteristic from that of other inulinases II and that this enzyme is therefore more suitable for the production of DFAIII from inulin.

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