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A Novel Enzyme of *Bacillus* sp. 217C-11 That Produces Inulin from Sucrose

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We found a bacterium that converts sucrose to a useful material, using about 6,000 samples of bacteria isolated from soil. This bacterium, *Bacillus* sp. 217C-11, was identified according to Bergey's manual, and produced a highly efficient enzyme that converted sucrose into inulin. So, the enzyme was purified to homogeneity through five chromatographic steps, to identify its enzymatic properties. The molecular mass of the enzyme was estimated to be 45,000, and this enzyme was a monomer protein (by SDS-PAGE). The optimum pH and temperature of this enzyme were 7–8 and 45–50°C, respectively. The enzyme reacted only with sucrose, but did not with other disaccharides, fructooligosaccharides and inulin. This paper will show that our enzyme is a novel one, which is different from the other well-known enzymes concerned in inulin production.

Key words: inulin; sucrose; fructosyltransferase; inulin-producing enzyme (IPE); *Bacillus* sp. 217-11

Inulin is a kind of polysaccharide, and is widely distributed throughout the plant kingdom. Among inulin-containing plants, the *Asteraceae* had been mainly used as raw materials for inulin production in Europe. For example, inulin exists as a reserve substance in the tubers or tuberous roots of the *Asteraceae* plant, such as dahlia, Jerusalem artichoke, and *Inula japonica*, *i.e.* British inula, and in the root of chicory.[1] In contrast with starch, inulin dissolves in warm water, and is a linear polymer having β-(2→1) linkages by which D-fructoses were polymerized. Plant-derived inulins are a group of compounds different in their molecular weights, and the average degree of fructose polymerization ranges from 32 to 34. Praznik and Beck[2] reported that the mean molecular weight is 2,282 to 17,000. Thus, the degree of fructose polymerization varies depending on plant species, but the molecular weights of inulin are limited within a certain range.

Inulin is a dietary fiber that is difficult to digest. Further, the promoting effect on the growth of *Bifidobacterium* is worthy of remark amid the recent boom in health-consciousness.

Inulin has not been commercialized in Japan, because commercial cultivation of these plants is difficult. To obtain inulin in Japan, there is no choice but to import. Such imported inulin is more expensive than some domestic substances having functions analogous to inulin.

In addition, the yield of plant-derived inulin depends on the harvest conditions, as the raw material of inulin is extracted from plant bodies. Also, the inulin content is rapidly reduced by autolysis, unless the inulin extraction is done just after the harvest. These facts must be fully considered in the industrial production of inulin.

Furthermore, the purification of plant-derived inulin is extremely difficult because of different fructose-chain lengths. Currently available plant-derived inulin is commercialized by spray-drying of a roughly fractionated crude solution containing inulin of different chain lengths.

Therefore, even though the purity of inulin may be high, a lack of uniformity in the chain lengths is unavoidable.

The above-mentioned higher plants from which inulin can be extracted naturally contain some enzymes for producing inulin. Luscher *et al.*,[3] and Edelman and Jefford[4] reported that inulin is produced from sucrose using two cooperative enzymes which were extracted from tubers of *Helianthus tuberosus*. Two enzymes were (1) sucrose:sucrose 1-fructosyltransferase (1-SST; EC2.4.1.99), which performs the transfer of fructosyl between sucroses, and (2) fructan:fructan 1-fructosyltransferase (1-FFT; EC2.4.1.100), which transfers fructose moieties between fructans having a degree of polymerization of 3 or more. But it is industrially impractical to use the above process requiring a large amount of enzymes from plant bodies, because it is time- and labor-consuming.

In addition to the plant-derived inulin, a method for producing analogues of inulin by the action of microbial enzymes has been reported. Kopeloff *et*

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Abbreviations: IPE, inulin-producing enzyme; 1-SST, sucrose: sucrose 1-fructosyltransferase; 1-FFT, fructan: fructan 1-fructosyltransferase; DP, degree of polymerization

al.[5] reported in 1920 that the conidiospores of *Aspergillus sydowi* have invertase activity and can produce a levan-type fructan from sucrose. After a fairly long time, Loewenberg and Reese[6] revealed in 1957 that the polysaccharide shows an inulin-typed structure having β-(2→1) linkages of fructose.

Later, Kawai *et al.*[7] reported in 1973 that both polyfructan and oligofructan are produced by conidiospores of *A. sydowi*, and that, like higher plant-derived inulin, the polyfructan consists of a linear chain which has β-(2→1) linkages lacking glucose at its end, and then that its molecular weight (about 20,000,000; DP ≒ 123,000) is much larger than that of higher plant-derived inulin.

In 1986, Nakakuki *et al.*[8] proposed a method for producing oligofructan and polyfructan by treating sucrose with cells of *A. sydowi*. The produced fructan was a linear polyfructan having fructose linked by β-(2→1) linkages with glucose at its end. This oligofructan showed the DP of 5 (828 in M.W.) or less, while the macromolecular fructan showed molecular weights ranging from 1.8×10^5 to 1.4×10^7.

Recently, Harada *et al.*[9] also proposed a method for producing polyfructan from sucrose using the conidiospores of *A. sydowi*, and reported that the molecular weight of the polyfructan is about 10,000,000 (DP ≒ 61,700).

On the other hand, Hidaka *et al.*[10] proposed a method for producing a linear fructan of β-(2→1) linkages from sucrose by using fructosyltransferases produced by *Aspergillus* or *Fusarium*. However, the fructan was an oligosaccharide in which 1 to 4 molecules of fructose are bound to sucrose. So, the fructan was defined as a substance different from inulin in molecular size.

Furthermore, *Rosell* and *Birkhed*[11] reported that *Streptococcus mutans*, which was considered as the pathogen of dental caries, induces an enzyme capable of producing an analogue of inulin. However, the inulin analogue is different from inulin, since it was a giant molecule having the molecular weight of 20,000,000 and it had β-(2→6) linkages in a linear chain of β-(2→1) linkages.

The above-described substances shall be referred to as inulin-type polyfructan, in order to distinguish them from higher-plant-derived inulin. Their properties are largely different from those of higher-plant inulin; for example, their molecular sizes are larger, and they have different structures. Concerning the inulin production by enzymes of microorganisms, no industrial technology has been established yet.

We continued the screening of microorganisms which change sucrose into valuable materials, and discovered a microorganism that can produce a highly efficient enzyme that converts sucrose into inulin. In this paper, we shall report the identification of the isolated microorganism, the purification of an induced enzyme, its enzymatic properties, and the identification and chemical compositions of the reaction products derived from sucrose.

Materials and Methods

Chemicals. Inulin was purchased from Orafti, Tienen, Belgium. Raftiline ST is unfractionated, spray-dried inulin isolated from the roots of *Cichorium intybus* L. This unfractionated inulin is polydisperse, and the average DP was 10. Raftiline HP is spray-dried inulin, in which low DP of inulin is removed by fractionation. The DP of Raftiline HP was 23. Levan obtained from *Serratia levanicum* was purchased from Wako Pure Chemicals. DEAE-Toyopearl 650M, Toyopearl HW55F, and phenyl-Toyopearl 650M were purchased from Tosoh Co. Other chemicals were purchased from Wako Pure Chemicals.

Screening method. The soil sample (1 gram) was suspended in sterilized water and spread on agar plates containing 2% sucrose as a carbon source, 1% peptone, 0.5% yeast extracts, 0.3% malt extract, 0.1% K_2HPO_4, and 0.05% $MgSO_4$ (pH 7.2), and incubated for several days at 30°C. Growing colonies were picked up, and were suspended in the same liquid medium. Twenty% (w/w) sucrose solution (final concentration) was added to the suspension, and it was incubated for 15 h at 30°C. Then, the resulting products were detected with thin-layer chromatography (TLC).

Microorganisms and their cultivation. One of the soil microorganisms screened was *Bacillus* sp. 217C-11. The strain was cultivated at 30°C for 15 h with agitation at 150 rpm in culture medium (pH 8.0) containing 0.5% sucrose, 1% peptone, 0.5% yeast extracts, and 0.2% K_2HPO_4. After cultivation, the bacterial cells were removed by centrifugation at 15,000 rpm for 15 min.

Thin-layer chromatography (TLC). TLC of sugars were done on a silica-gel 60 plate (Merck) developed with a solvent of acetone-water (9:1, v/v), and sugar spots were detected with 10% sulfuric acid by heating on a hot plate.

Quantitative analysis of sugars. High pressure liquid chromatography (HPLC) was done for quantitative analysis of sugars with the following equipment and detection conditions: column, Shinwa Chemical Industries ULTRON PS-80N (300 × 8.0 mm); mobile phase; distilled water; flow rate, 0.5 ml/min; temperature, 50°C; pump, Hitachi L-6000; and detector, Hitachi L-7490 RI detector.

Measurement of the average DP of inulin.

Enzyme hydrolysis. The average DP of inulin was

defined as the fructose/glucose ratio, after complete hydrolysis with Fructozyme inulinase (Novo Nordisk) for 15 h at 37°C at pH 7.0.[12,13] The amount of sugars was measured by HPLC.

HPLC. The average DP of inulin was measured by HPLC with a Tosoh TSK-Gel G3000PWXL (300 × 7.8 mm) gel permeation chromatography column under the following conditions: mobile phase, distilled water; flow rate, 0.5 ml/min; temperature, 50°C; pump, Hitachi L-6000; and detector, Hitachi L-7490 RI. Raftiline ST (DP = 10) and Raftiline HP (DP = 23) were used as standard materials.

Measurement of the polydispersity of inulin. The polydispersity in the chain length of inulin was evaluated qualitatively. The Dionex series DX-500 apparatus was equipped with an eluent degassing module, Dionex ED40, pulsed electrochemical detector, GP50 gradient pump and AS50 autosampler. The injected samples (50 μl, 3% solution) passed a CarboPac PA-1 column (4 × 250 mm). The bound material was eluted by a NaOH-NaOAc gradient.

^{1}H-and ^{13}C-NMR spectra. A sample was dissolved in D_2O, and the NMR spectrum was recorded at 500 MHz using a JOEL lambda-500FT-NMR spectrometer (Nippon Bunkoh).

Enzyme assay. The activity of the inulin-producing enzyme was assayed by measuring the amount of glucose released from sucrose. The reaction mixture (0.4 ml) containing 10 mM phosphate buffer (pH 7.0), 20% sucrose, and enzyme solution was incubated at 37°C for 30 min. The reaction was stopped by adding 1 N HCl. The glucose released was measured by the glucose oxidase-peroxidase method. One unit of enzyme activity was defined as the amount of enzyme that liberates glucose 1 μmol/min under these conditions.

Purification of inulin-producing enzyme (IPE).

The purification of IPE was done by the following five steps.

Ultrafiltration. The crude enzyme was concentrated 5 times by the ultrafilter (Nihon pole) for cutting off the molecular weight below 30,000.

Ammonium sulfate fraction. Powdered ammonium sulfate was added to the crude enzyme solution to obtain 70% saturation. After this was left standing for 2 h, the precipitation was collected by centrifugation and was dissolved in a minimum volume of 20 mM phosphate buffer, pH 7.0. Then, the enzyme solution was dialyzed against the same buffer.

DEAE Toyopearl 650M column chromatography. The dialyzed solution was put on a DEAE Toyopearl 650M anion exchange column (ϕ1.8 × 14 cm) equilibrated with 20 mM phosphate buffer, pH 7.0. The enzyme was eluted with a linear gradient of 0 to 500 mM KCl in the same buffer. The active fractions were pooled, concentrated by addition of ammonium sulfate to 70% saturation, and centrifuged. The precipitate was dissolved in a minimum volume of 20 mM phosphate buffer pH7.0. The enzyme solution was dialyzed against the same buffer.

Toyopearl HW55S gel filtration chromatography. The enzyme obtained after DEAE Toyopearl 650M treatment was put on a Toyopearl HW55S gel filtration column (ϕ1.8 × 137 cm) equilibrated with 20 mM phosphate, pH 7.0, containing 0.15 M KCl. The active fractions were pooled and dialyzed against 1.7 M ammonium sulfate in 20 mM phosphate, pH 7.0.

Phenyl Toyopearl 650M column chromatography. The dialyzed enzyme solution was put on a Phenyl Toyopearl 650M column (ϕ1.2 × 6 cm) equilibrated with 1.7 M ammonium sulfate in 20 mM phosphate, pH 7.0. The column was washed with this buffer, and the enzyme was eluted with a linear gradient of 1.7 M ~0 M ammonium sulfate in the same buffer. The active fractions were pooled and dialyzed.

Measurement of molecular weight of the enzyme.

Gel filtration method. The molecular weight of the enzyme was estimated by Fast protein liquid chromatography (FPLC) with a Sephacryl S300 gel filtration column (Pharmacia). Molecular weights of the marker proteins used were thioglobulin (670,000), gamma-globulin (158,000), ovalbumin (44,000), and myoglobin (17,000).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was done on a 2–15% polyacrylamide separating gel (Multy Gel 2/15, Daiichi Pure Chemicals Co., Ltd.) under reducing conditions. Protein was stained by Coomassie brilliant blue (CBB R-250). Molecular weights of calibration proteins used were myosin (200,000), β-galactosidase (116,248), bovine serum albumin (66,267), aldolase (42400), carbonic anhydrase (30,000), and myoglobin (17,000).

Protein assay. The protein concentrations were estimated with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, Calif.) for the Bradford dye-binding method, with bovine serum albumin as the protein standard.[14]

Analysis of N-terminal amino acid sequence of inulin-producing enzyme (IPE). After SDS-PAGE of the purified enzyme, the protein on the gel was transferred to PVDF membrane at 200 mM for 1 h. After the protein was stained as described above, the membrane corresponding to the protein band of IPE was cut off and analyzed with a HP G1005A Protein Sequencing system (Hewllett-Packard Co.).

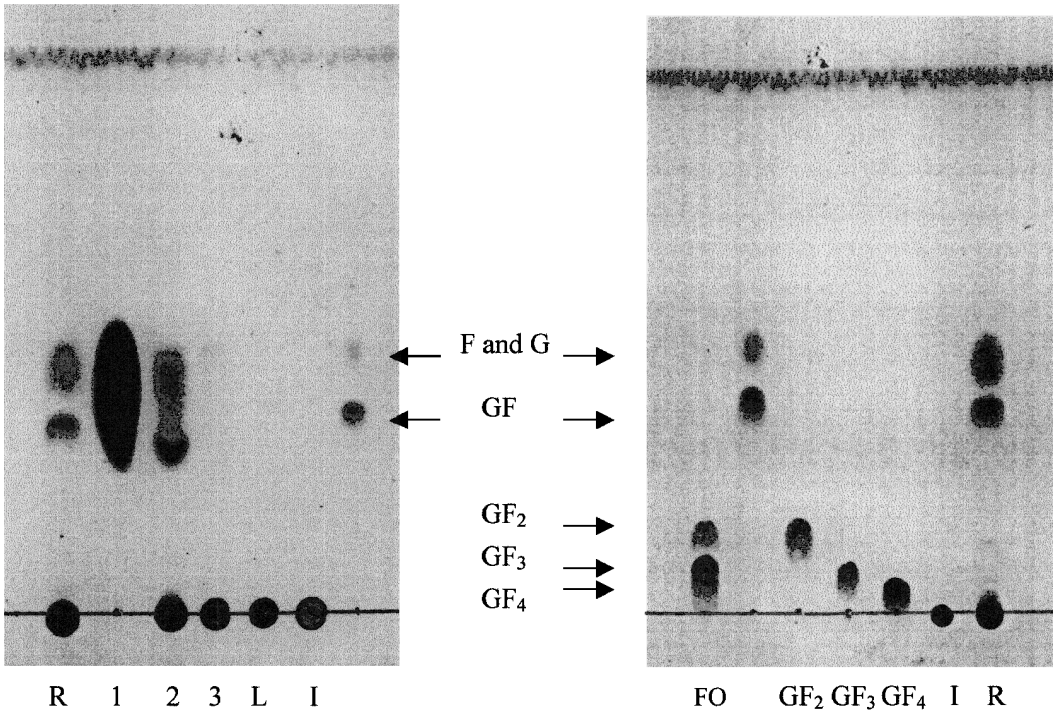

Fig. 1. TLC Analysis on Reaction Products from Sucrose.

The reaction mixture was composed of 20% (w/w) sucrose, 5 mM phosphate buffer (pH 7.0), and crude enzyme of IPE (Inulin-Producing Enzyme). The reaction was done at 37°C for 16 h. The reaction products were analyzed by TLC with acetone/distilled water (9:1, v/v) as a solvent.

Lane R, reaction mixture; Lane 1, R + inulinase; Lane 2, R + dextranase; Lane 3, levan + inulinase; Lane L, levan; Lane I, inulin; Standard materials were fructooligosaccharide (FO), fructose (F), glucose (G), sucrose (GF), 1-kestose (GF_2), nystose (GF_3), and fructosylnystose (GF_4).

Results and Discussion

Screening

During the screening, we found three types of bacterial strains producing inulin from sucrose. The first strain produced together the well-known fructooligosaccharides (1-kestose, nystose, and fructosylnystose) and inulin from sucrose. The second one showed very low invertase activity, and produced glucose, fructose, and also inulin from sucrose. The third one produced a relatively large amount of inulin from sucrose together with a slight amount of fructose. We took notice of the last strain, by which highly efficient inulin production will be obtained, and detailed investigation was done on the purification of related enzyme, reaction products, and so on. Taxonomical study of the isolate was done by NCIMB Japan Co., Ltd (Shizuoka). Taxonomic characteristics of the isolate, 217C-11, were Gram-variable, spore forming, motile, and rod-shaped ($1 \times 2 \sim 3\ \mu m$). The organism grew in both aerobic and anaerobic conditions. It was positive for both catalase and oxidase production, and negative for urease production and nitrate reduction. Thus, this strain was identified as a *Bacillus* sp.

TLC analysis on reaction products of sucrose

The enzyme was put in to a 20% sucrose solution and the reaction products were analyzed by TLC. As shown in Fig. 1, one of the products caused a strong spot. This product was not digested by dextranase (Sankyo Co., Japan), but was digested completely by inulinase. This inulinase could not digest levan, a kind of fructan, so it seems that this reaction product is inulin.

The lower molecular weight fructans indicated higher R_f-values in developing solvent systems of TLC analysis. Since reaction products were developed on lower positions than those of fructosylnystose, (pentasaccharide), it seems that their degrees of fructose polymerization are above hexasaccharide.

Time-course of inulin production by inulin-producing enzyme (IPE)

The time-course of changes of inulin production by IPE of *Bacillus* sp. 217C-11 were investigated. In the final concentration of 40% sucrose solution, the reaction was done at 37°C (Fig. 2). Inulin and glucose were gradually increased with the decrease in sucrose. When the reaction reached an the equilibrium, the composition of inulin and glucose had reached to 45%, respectively, and the release of fructose was hardly recognized. This will make the inulin purification easy.

Thus, the conversion rate of inulin from sucrose is 45%. When the concentration of the remaining sucrose solution reached to about 7%, the enzyme

reaction balanced. In the case of higher concentrations than about 7%, the reaction proceeded to the direction of inulin production. And, in lower than about 7% solution, inulin degradation occurred. So, this enzyme reaction seems to be a equilibrium reaction.

Identification of reaction product

After removal of low molecular weight sugars from reaction products by filtering through a reverse-osmosis membrane, the residue was refined three times with ethanol precipitation. The sample prepared to be uniform on HPLC was analyzed with NMR (Fig. 3). The same analyses were done for standard inulin and levan. The chart of the reaction product did not agree with that of levan, but did so with the standard inulin. These results and an additional analysis by mass spectroscopy indicated that the reaction product is inulin.

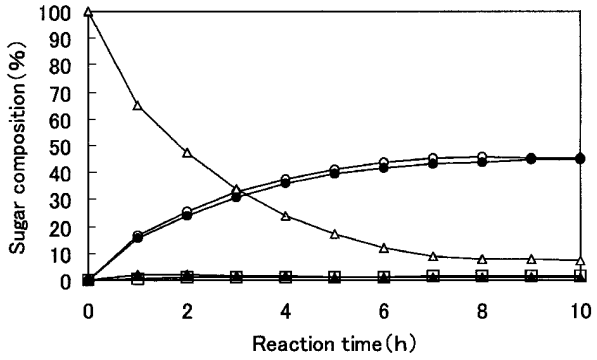

Fig. 2. Inulin Formation from Sucrose by IPE (inulin-producing enzyme).

The reaction mixture containing 40% (w/w) sucrose, 10 mM phosphate buffer (pH 7.0), and 1.5U of IPE was incubated at 37°C for 10 h. Samples were withdrawn at various intervals, and sugar composition in the reaction mixture was measured by HPLC. (△), sucrose; (○), inulin; (●), glucose; (□), fructose; (▲), 1-kestose.

DP of the reaction products

The result on the average DP of inulin was measured by the above-described method (Fig. 4). The DP obtained by this enzyme was 18. The glucose/fructose ratio of the sample digested completely with inulinase was 1:17. It was estimated that the DP is 18 (Table 1). Also, the polydispersity in chain length was investigated, and it was found that the DP of sucrose-origin inulin is in the range from 10 to 25, indicating a peak of about 16–17 (Fig. 5). On the other hand, the DP of plant-origin inulin was dispersed in a wider range from 10 to 60. Thus, each molecular weight of sucrose-origin inulin produced by this enzyme was relatively low, and these DPs concentrated in a narrower range from 10 to 30, compared with those of plant-origin inulin. Fortunately, we succeeded in producing a group of inulin molecules having approximately similar chain-length, using this enzyme. The inulin obtained by IPE was quite different from naturally occurring plant inulin. The significance of this difference is under consideration. At present, we are interested in several specific functions of the inulin produced by IPE. The details will be described in our next paper.

Purification of inulin-producing enzyme (IPE)

Table 2 shows a summary of the purification of IPE derived from *Bacillus* sp. 217C-11. The enzyme was purified about 7-fold with 17% yield from the culture. The specific activity of the purified enzyme

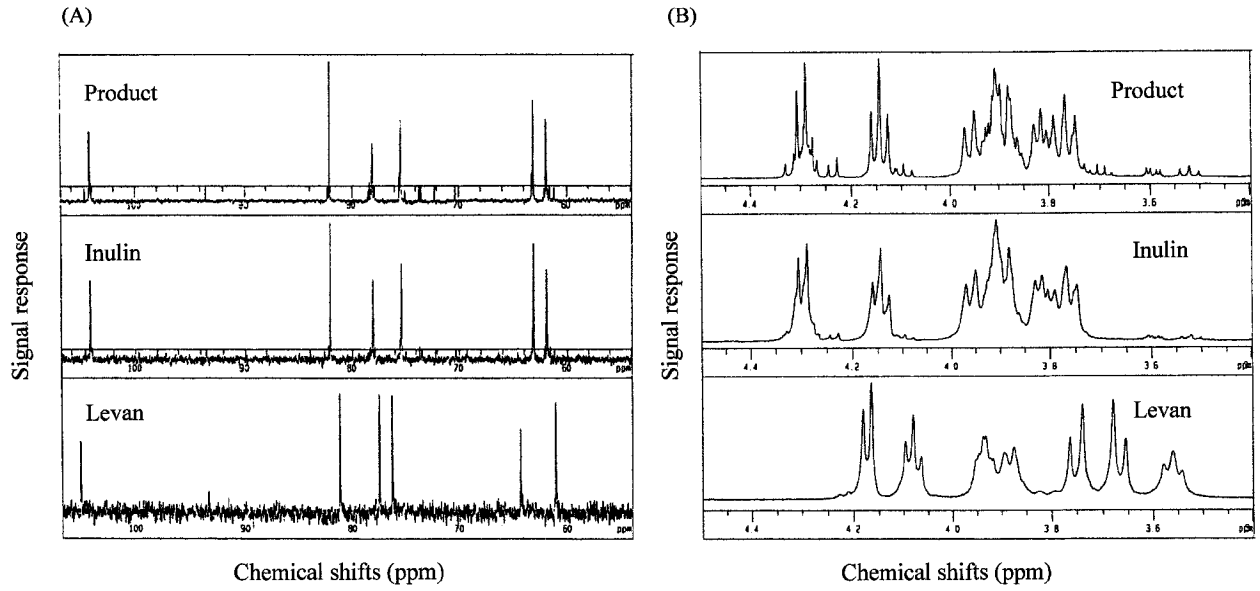

Fig. 3. NMR Spectrum of Reaction Product and Authentic Fructan.
(A) ^{13}C-NMR Spectrum, (B) ^{1}H-NMR Spectrum.

preparation obtained by a phenyl-Toyopearl 650M column chromatography was 53.3 U/mg protein. The purified enzyme preparation indicated a single band by SDS-PAGE. IPE purification was relatively easy.

On the gel filtration chromatography, the recovery of the enzyme was low. It seems that this slightly low recovery was caused by an unexpected large adsorption of the enzyme protein to gel materials.

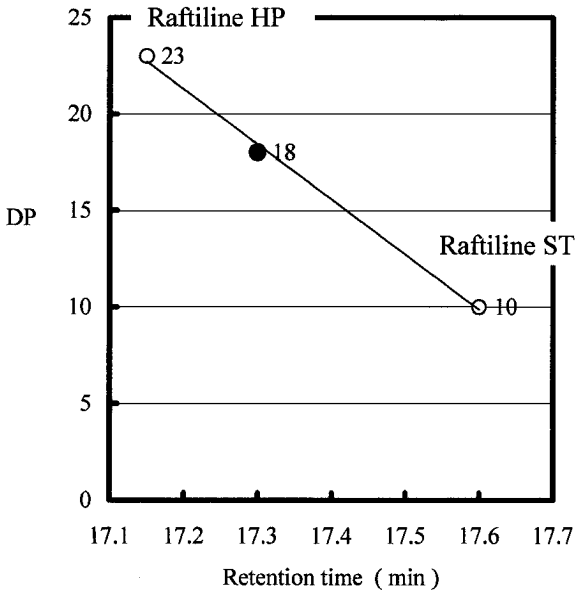

Fig. 4. Measurement of Average Degree of Polymerization of Reaction Product.
Symbols: (○), authentic inulin (Raftiline HP and Raftiline ST); (●), reaction product.

Some properties of IPE

The effects of pH and temperature on the IPE activity and stability are shown in Table 3. The optimum pH was 7 to 8, and the enzyme was stable from pH 6 to 9, and was unstable below pH 5. The optimum temperature was 45°C, and the enzyme was stable up to 40°C. The enzyme activity was completely inhibited by Cu^{2+} and Hg^{2+} (5mM), but was not inhibited by EDTA. So, heavy metal ions were not necessary to IPE activity. The molecular weight of the enzyme was estimated to be 45,000 by SDS-PAGE (Fig. 6), and 44,000 by gel filtration on a Sephacryl S-300 column. These results indicate that this enzyme is a monomer protein.

Substrate specificity

In order to investigate the substrate specificity of IPE, various disaccharides were examined as substrates. IPE acted specifically on sucrose to produce inulin with highly polymerized fructoses (DP>6), but was inactive on the other disaccharides such as maltose, lactose, trehalose, and cellobiose.

Furthermore, members of low DP fructooligosaccharide, that is, 1-kestose, 1-kestose and nystose, 1-kestose and fructosylnystose, did not act as substrates of high DP inulin production (Table 3). Also, in the enzyme reaction given sucrose as fructosyl

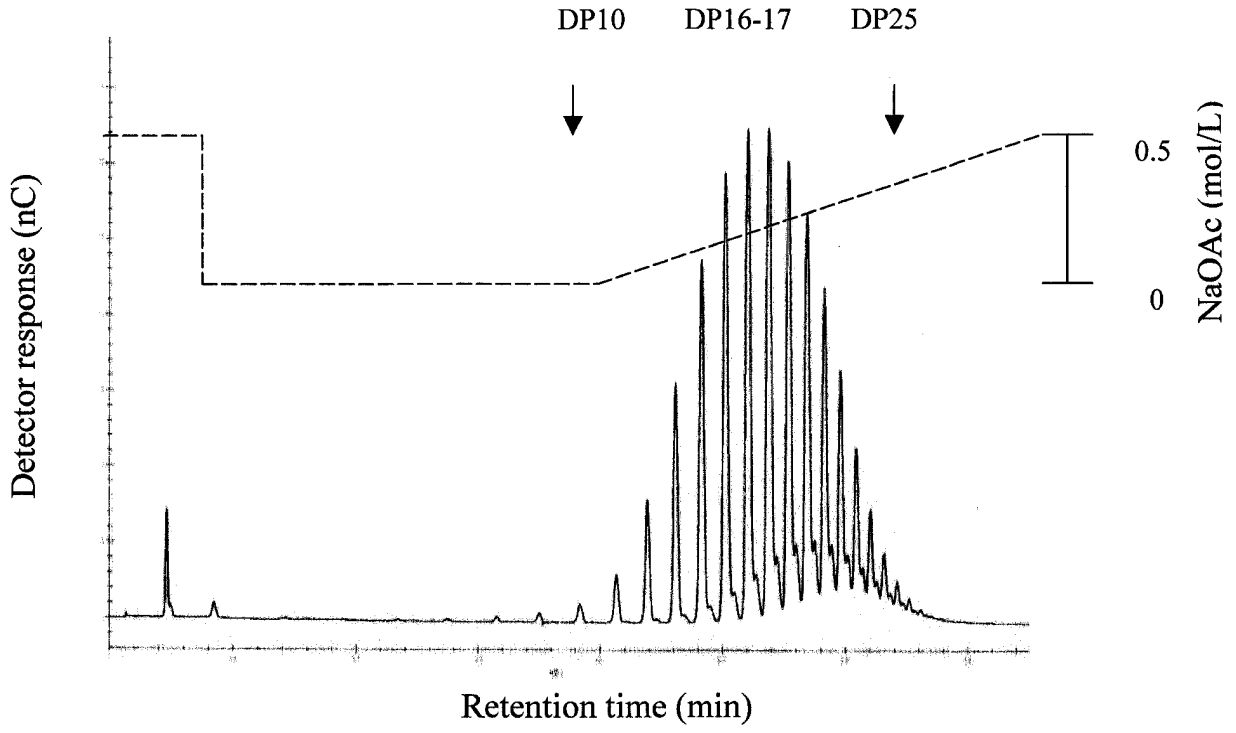

Fig. 5. HPAEC Analysis of Inulin on a CarboPack PA-1 Column.
Effluent, 150 mmol/l NaOH; Flow rate, 1 ml/min; Injection, 25 μl; Detector, pulsed amperometric detector.

Table 1. Sugar Composition of Inulin Treated with Inulinase

	Molar ratio to glucose	
	Glucose	Fructose
Raftiline ST	1	9
Product	1	17

Glucose and fructose were measured by HPLC.

Table 2. Summary of Purification Procedures of IPE* from *Bacillus* sp. 217C-11

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)
Culture filtrate	3720	466	8.0	100
Ultra filtration (30kDa)	3534	349	10.1	95
70% $(NH_4)_2SO_4$ ppt.	3225	375	9.2	87
DEAE-Toyopearl 650M	2780	97	28.7	75
Toyopearl HW55S	990	26	38.1	27
Phenyl-Toyopearl 650M	640	12	53.3	17

IPE*, Inulin- producing Enzyme.

Table 3. Properties of IPE* from *Bacillus* sp. 217C-11

Optimum pH	7–8	
pH stability	6–9	
Optimum temp.	45°C	
Theromostability	up to 40°C	
Molecular weight		
Gel filtration	44 kDa	
SDS-PAGE	45 kDa	
Inhibitor	Cu^{2+}, Hg^{2+} (5 mM)	
Substrates GF	→	high DP inulin
GF_2+GF_2	×→	high DP inulin
GF_2+GF_3	×→	high DP inulin
GF_2+GF_4	×→	high DP inulin

IPE*, Inulin-producing Enzyme.
GF, sucrose; GF_2, 1-kestose; GF_3, nystose; GF_4, fructosylnystose.

donor together with low DP fructooligosaccharides as the fructosyl acceptor, the production of high DP inulin was not recognized.

N-terminal amino acid sequence

The N-terminal amino acid sequence up to 30 amino acid residues of IPE was as follows: EEINSDYTSIWSRQQAEKVTP TDKTTAPKI. A comparison was made with the corresponding regions of other proteins using two data bases (FASTA and BLASTP), but no similarity to this sequence was found. A levansucrase derived from *Clostridium acetobutylicum* had a homologous arrangement (59%). This enzyme can produce levan (β-(2→6) fructan) from sucrose. This is a very interesting fact.

In conclusion, our results suggest that our enzyme, *i.e.*, the inulin-producing enzyme (IPE) of *Bacillus* sp. 217C-11, is a novel one unlike others found so far. The mechanism of inulin synthesis by this enzyme is being investigated.

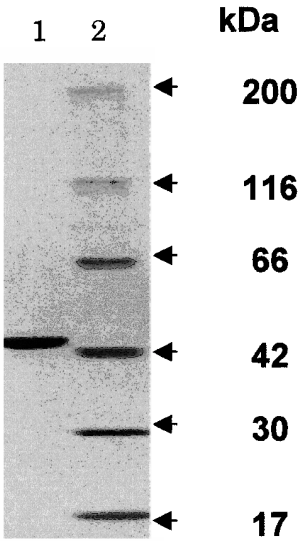

Fig. 6. Homogeneity of Purified IPE (inulin-producing enzyme) and the Molecular Weight Measurement by SDS-PAGE.
Lane 1, purified IPE; lane 2, standard protein mixture containing myosin (Mw 200,000), β-galactosidase (Mw 116,248), bovine serum albumin (Mw 66,267), aldolase (Mw 42,400), carbonic anhydrase (Mw 30,000), and myoglobin (Mw 17,000).

Acknowledgment

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