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Article

Isolation and Characterization of Endo-inulinases from *Arthrobacter* sp. S37

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The crude enzyme prepared from the culture supernantant of *Arthrobacter* sp. S37 was purified by Phenyl Toyopearl column chromatography. Six endo-inulinases were detected by activity staining on native PAGE and named Inu I to Inu VI. Endo-inulinase were further purified by DEAE cellulose column chromatography and band slicing. Inu II~VI produced mainly inulotriose (F3) and inulotetraose (F4) as well as a small amount of inulobiose (F2) and fructose in contrast to Inu I producing F3, F4 and F5 from inulin. The N-terminal amino acid sequence of native and six CNBr-cleaved fragment of Inu VI were determined. No homology was found in amino acid sequences between Inu VI and other fructan hydrolase including invertase reported.

Key words : *endo-inulinase, inulooligosaccharides,* Arthrobacter *sp. S37, N-terminal and internal sequence*

Inulin is a linear β-2,1 linked fructan with sucrose residue at the end of the chain. It occurs as a reserve carbohydrate mainly in the roots and tubers of Jerusalem artichoke and chicory. Endo-inulinase (2,1-β-D-fructan fructanohydrolase, EC 3.2.1.7) hydrolyzes inulin through endowise reaction producing fructo- and inulo-oligosaccharides, while exo-inulinase (EC 3.2.1.80) splits off the terminal fructose units from inulin. Although endo-inulinase has been purified from several microorganisms such as *Aspergillus niger,*[1] *Chrysosporium pannorum,*[2] *Penicillium purpurogenum,*[3] and *Aspergillus ficuum,*[4] which produce exo-inulinase as well. Inulo-oligosaccharides have been suggested to have bifidogenic effects.[5] For the industrial production of inulo-oligosaccharides, it is necessary to find a microorganism that produces only endo-inulinase. Previously, we isolated a novel endo-inulinase (Inu I) producing microorganism, *Arthrobacter* sp. S37 from soil,[6] purified the enzyme, and its enzymatic properties were investigated.[7] In this study, we report the purification and the mode of action of other five endo-inulinases from this organism, the N-terminal and internal amino acid sequence of one endo-inulinase.

Materials and Methods

Preparation of crude enzyme. The crude endo-inulinase was prepared as previously reported.[6] The super-natant of the culture broth was centrifuged (4°C, 25,000 g) after the addition of 80% ammonium sulfate, and the pre-cipitate was dissolved and dialyzed against 0.05 M Tris-HCl buffer (pH 7.5) at 4°C.

Hydrophobic interaction chromatography. The crude enzyme solution (30 m*l*, 92.1 mg of protein) was applied on HIC column, using a Phenyl-Toyopearl (1.7×18.5 cm, Tosoh Co., Ltd.). The column was pre-equilibrated with 2.0 M ammonium sulfate in 0.05 M Tris-HCl buffer (pH 7.5) and eluted with a decreasing linear gradient of ammonium sulfate from 2.0 to 0 M in the same buffer at a flow rate of 6 m*l*/10 min. The fractions showing inulinase activity (HIC A and B fractions) were pooled, concentrated by stirred cell (Amicon) after dialysis against distilled water, and subjected to DEAE-cellulose column chromatography.

DEAE-cellulose column chromatography. DEAE-cellulose column (1×11 cm) was pre-equilibrated with 0.05 M Tris-HCl buffer (pH 7.5). HIC A fraction was applied on the column and eluted with a linear gradient of NaCl from 0 to 0.2 M and stepwise gradient of 0.5 M NaCl in the same buffer at a flow rate of 6 m*l*/10 min. The fractions showing inulinase activity were analyzed by native PAGE. The fractions eluted at 0.5 M NaCl was dialyzed against 0.05 M Tris-HCl buffer, concentrated and subjected to DEAE-cellulose column chromatography. The elution was done with a linear gradient of NaCl from 0 to 0.5 M in the same buffer. The fraction showing inulinase activity was collected, dialyzed, and concentrated (HIC A fraction). The chromatography was performed as the same method in the rechromatography of HIC A fraction. The fraction showing inulinase activity was

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Abbreviations: DEAE, diethylaminoethyl; F, fructose; F2, inulobiose; F3, inulotriose; F4, inulotetraose; HPAEC, high pH anion exchange chromatography; HIC, hydrophobic interaction chromatography; PAD, pulsed amperometric detector.

collected, dialyzed and concentrated (HIC B fraction).

Gel slicing and electroelution. For gel slicing, the concentrated enzyme solutions were analyzed by native PAGE and protein staining. To identify inulinase bands, inulinase activity staining on polyacrylamide gel was done according to the method of Gabriel and Wang.[8] The protein bands coresponding to inulinase activity bands were sliced and electroeluted with Little Blue Tank (ISCO, USA).[9] The buffer of electroelution was 0.05 M Tris-HCl buffer (pH 8.0), and elution was done four times for one hour at 150 V. The eluents were dialyzed and concentrated.

Mode of enzyme action on inulin and sucrose. The mode of action on inulin and sucrose was investigated by using the reaction mixture containing 0.88 ml of 0.05 M Tris-HCl buffer (pH 7.5), 0.1 m*l* of 4% inulin (from dahlia tubers, Sigma) or 4% sucrose, and 0.02 m*l* of enzyme solution at 40°C for 44.5 hours. The reaction products were analyzed by TLC[10] and HPAEC equipped with a PAD (Dionex, Sunvayle, USA). CarboPac PA1 column (4×250 mm Dionex, Sunvayle, USA) was used, and elution conditions were as follows : an isocratic elution of 0.1 M NaOH followed by a linear gradient of sodium acetate from 0.001 to 0.6 M for 67 min.

Amino acid sequencing. For the N-terminal sequence, the slices of Inu VI were analyzed on 8% SDS PAGE, and the bands were electroblotted on PVDF membrane using modified Lauriere method.[11] For the analysis of internal amino acid sequencing *in situ* CNBr cleavage of Inu VI in gel was done with the modified method described by Pang and Mathieson.[12] The CNBr fragments of Inu VI were electroblotted on PVDF membrane after 16.5% SDS PAGE. The amino acid sequence were determined by automatic amino acid sequencer (Applied Biosystems Model 491A).

Inulinase activity. The inulinase activity was determined by the 3,5-dinitrosalicylic acid method.[13] The reaction mixture containing 2% inulin in 0.05 M Tris-HCl buffer (pH 7.5) and enzyme solution was incubated at 50°C for 15 min. The amount of reducing sugar liberated was measured using fructose as a standard. One unit of the enzyme activity was defined as the amount of the enzyme which produces 1 μmole of reducing sugar per min under these assay conditions.

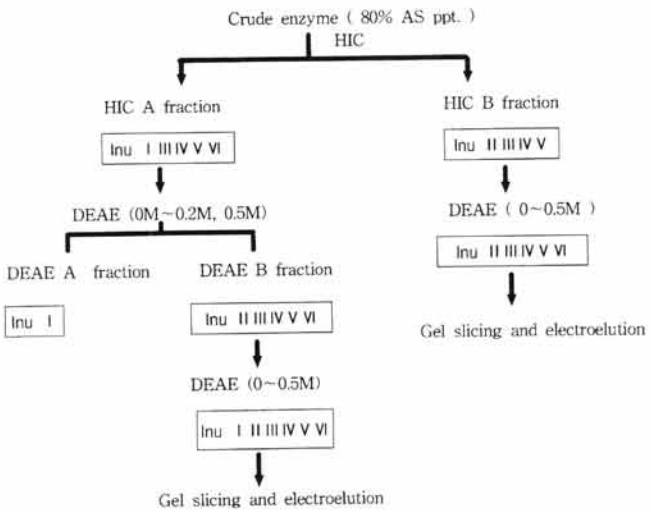

Fig. 1. The sheme of purification of endo-inulinase from *Arthrobacter* sp. S37. Inu I to Inu VI indicate endo-inulinases described on the text.

Results and Discussion

Purification of enzymes by HIC. The crude enzyme prepared from culture supernatant by ammonium sulfate precipitation (80%) was applied on phenyl Toyopearl column, and four protein peaks were detected (Fig. 2). Among these peaks, two peaks which were eluted at 1.3 M (HIC A) and 3 M (HIC B) $(NH_4)_2SO_4$ showed the inulinase activity (Fig. 2). Six bands showing inulinase activity were detected by activity staining (Fig. 3b), and six protein bands corresponding to inulinase activity bands were named sequentially Inu I~VI (Fig. 3). HIC A fraction contained five inulinases, Inu I, and, and HIC B fraction had four inulinases, Inu II~V (Fig 3). Inu I showed the same mobility and molecular weight to endo-inulinase already reported.[7] These results showed that *Arthrobacter* sp. S37 produces five additional inulinases other than endo-inulinase, Inu I.

DEAE-cellulose column chromatography. In the case of the first DEAE-cellulose column chromatography, two peaks eluted at 0.1 and 0.5 M NaCl showed an inulinase activity.

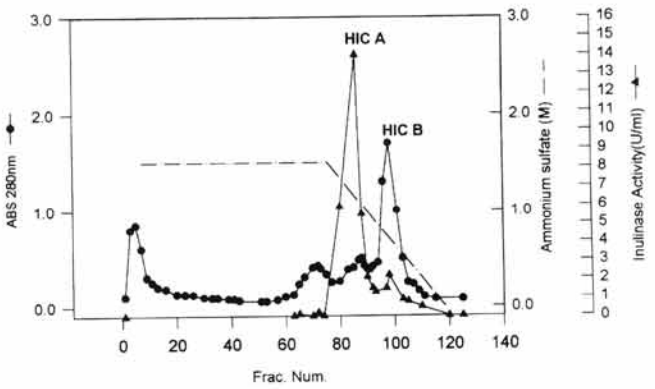

Fig. 2. Phenyl-Toyopearl column chromatography of crude endo-inulinase from *Arthrobacter* sp. S37 at a flow rate of 24 ml/h in 1.7×18.5 cm column.

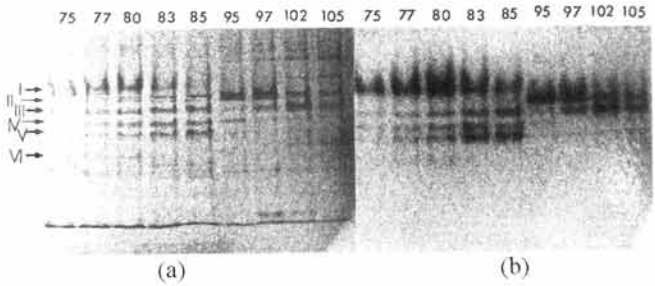

Fig. 3. 10% native PAGE of fraction on Phenyl-Totopearl column chromatography. (a) protein staining (b) activity staining the number on the top of the gel is fraction numger, 75~85; HIC A fraction, 95~105; HIC B fraction.

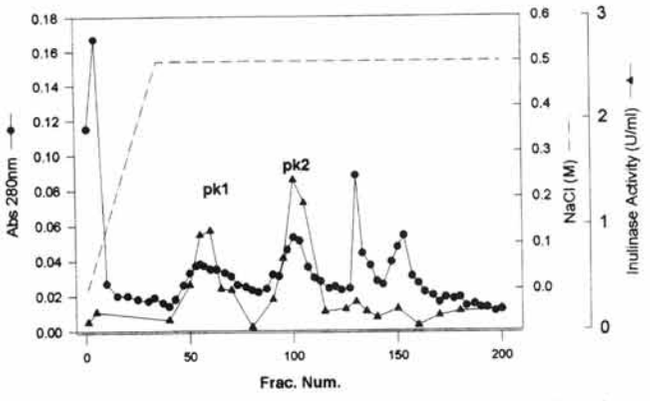

Fig. 4. DEAE cellulose column chromatography of HIC B fraction at a flow rate of 24 ml/h in 1×11 cm column. pk1; Inu I, pk2 ; Inu II, III and VI.

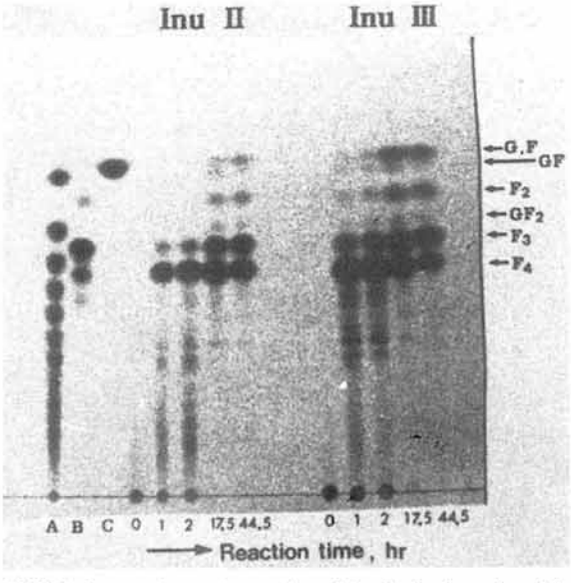

Fig. 5. Thin layer chromatography of inulin hydrosate with Inu II and Inu III. A, Jerusalen artichoke extract; B, inulooligosaccarides; C, G glucose; F, fructose; GE, sucrose; F2, inulobiose; GF2, 1-ketose; F3, inulotriose; F4, inulotetraose.

Activity at 0.1 M NaCl was caused by Inu I. In addition, the fraction of eluted at 0.5 M NaCl was performed rechromatography for further purification. Then the rechromatography showed two peaks eluted at 0.1 and 0.3 M NaCl fraction contained inulinase activity. The first was inferred by Inu I, the second by Inu III~VI, respectively (data not shown). Each band of Inu III~VI was sliced after native PAGE and electroeluted (HIC A fraction).

Five protein peaks were detected at fractions eluted by 0.5 M NaCl. The second and third peaks had inulinase activity (Fig 4). The first inulinase activity peak was mainly due to Inu V and the second due to Inu II, III and IV (data not shown). The inulinase bands were sliced after native PAGE and electroeluted (HIC B fraction).

Mode of enzyme action. The electroeluted enzyme solutions of Inu II~VI were reacted with sucrose and inulin. Sucrose

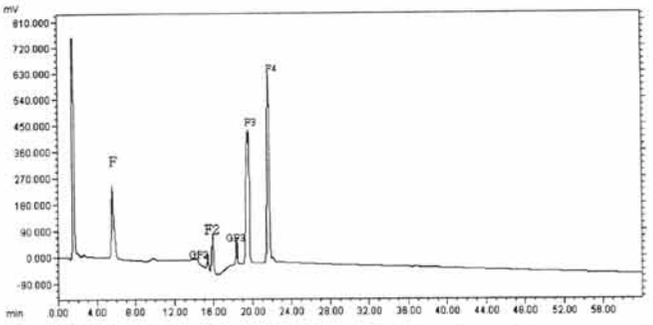

Fig. 6. High pH anion exchang column chromatography of inulin hydrosate for 44.5 hr with Inu II. F, fructose; GF2, 1-ketose; GF3, 1-nystose; F2, inulobiose; F3, inulotriose; F4, inulotetraose.

Table 1. N-terminal and internal amino acid sequence of Inu VI.

	Amino Acid Sequence
N-terminus	VNDPMHWWFDE
CN A	RKYKNGEQVATI
CN B	NEPHA
CN C	NEPHAPIWFDDKY
CN D	GLPVELTLTD
CN E	YRHGACGLELG
CN F	RLYLNGEQVATKDVGAK

was not hydrolyzed by the inulinase (data not shown), which indicates they had no invertase activity. The inulin hydrolysates with the enzymes in the course of reaction time were analyzed by TLC (Fig. 5, only the inulin hydrosates of Inu II and III are shown). In the case of Inu II, F4 and F3 were major products for 2 h without the detection of mono- and disaccharides. After 17.5 h, the reaction was completed to produce F3, F4 and a small amount of F2 and F. In the case of Inu III, though the major products were F4 and F3, F2 and F were also produced after 1 h incubation. After 44.5 h of reaction, more F2 and F were produced than in Inu II. HPAEC-PAD analysis of 44.5 h of reaction product of Inu II showed that F3 and F4 were major products, and fructooligosaccharides such as 1-ketose and 1-nystose were also produced (Fig 6). Inu I hydrolyzed inulin into F3, F4 and inulopentaose (F5) without the production of mono- and disaccharides.[7] From these results, it is suggested that Inu II~VI are endo-type inulinase producing manly F3 and F4 and they have affinity for F3 and F4 producing F2 and F.

Molecular weight of inulinase. The sliced band of Inu VI were analyzed on 7.5% SDS PAGE. The molecular weight of Inu II, III, IV, V and Inu VI were 181, 162, 139 131, and 97 respectively (data not shown). From the comparison of the molecular weights and the products of inulin hydrolysis with those of Inu I (molecular weight; 75 kDa), it is suggested that Inu II~VI are isozyme of Inu I.

N-terminal and internal amino acid sequence of Inu VI. The N-terminal and internal amino acid sequences of Inu VI were determined and are shown in Table 1. Eleven

amino acid residues of N-terminal were determined. The peptide fragments of CNBr cleavage were separated on 16.5% SDS PAGE. Six CNBr fragment (from CN A to CN F) were detected on the PVDF membrane (data not shown). All the amino acid sequences of CNBr fragments had no homology with Inu and other fructan hydrolases reported.

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References

1. Nakamuram, T., Kurokawa, T., Nakatsu, S. and Ueda, S. (1978) Crystallization and general properties of an extracellular inu1ase from *Aspergillus* sp. *Nippon Nogei Kagaku Kaishi* **52,** 159-166.
2. Xiao, R., Tanida, M. and Takao, S. (1989) Purification and some properties of endoinulinase from *Chrysosporiurm pannorum. J. Ferment. Bioeng.* **67,** 244-248.
3. Onoder, M., Murakami, T., Ito, H., Matsui, H., Honma, M., Chiba, S. and Shiomi, N. (1996) Molecular c1oning and nucleotide sequences of cDNA and encoding endo-inulinase from *Penicillium purpurogenum. Biosci. Biotech. Biochem.* **60,** 1780-1785.
4. Ettalibi, M. and Baratti, J. C. (1987) Purification, properties and comparison of invertase, exoinulase and endoinulases of *Aspergi11us ficuum. App1. Microbio1. Biotechno1.* **26,** 13-20.
5. Roberfroid, M. B., van Loo, J. A. E. and Gibson, G. R. (1998) The bifidogenic nature of chicoryinulin and its hydrolysis products. *J. Nutr.* **128,** 11-19.
6 .Kim, K. Y., Kang, S. I. and Kim, S. I. (1996) Product of a novel endo-inulinase from *Arthrobacter* sp. S37 *J Kor. Argic. Chem. Soc.* **39**, 99-103.
7. Kang, S. I., Chang, Y. J., Oh, S. J. and Kim, S. I. (1998) Purification and properties of an endo-inulinase from an *Arthrobacter* sp. *Biotech. Lett.* **20**, 983-986.
8. Gabriel, O. and Wang, S. F. (1969) Determination of enzymatic activity in polyacrylamide gels. *Anal. Biochem.* **27,** 545-554.
9. Harrington, M. G. (1990) Elution of protein from gels. *Methods Enzymol.* **182,** 488-495.
10. Ha, Y. J., Choi, E. H. and Kim, S. I. (1989) Production and characterization of endo-inulinase from *Streptomyces* sp. S 56. *Kor. J. Appl. Microbio1. Biotechnol.* **20**, 551-558.
11. Lauriere M. (1993) A semidry electroblotting system efficiently transfers both high- and low-molecular weight protein separated by SDS-PAGE. *Anal. Biochem.* **212**, 206-211.
12. Pang, A. S. D. and Mathieson, B. (1991) Peptide mapping of different *Bacillus tryringiensis* toxin gene products by CNBr cleavage in SDS-PAGE gels. *J. Inverteb. Pathol.* **57**, 82-93.
13. Melius, P. (1971) Isolation of yeast invertase by Sepadex gel chromatography. *J. Chem Educ.* **48**, 765-766.