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Purification and Characterization of Inulinase from *Penicillium* sp.

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Abstract

An extracellular inulinase from *Penicillium* sp. which isolated from soil sample was purified to a single protein through ammonium sulfate fractionation, DEAE-Sephacel ion exchange chromatography and Toyopearl HW 65 F gel filtration. The temperature and pH for the enzyme reaction were around 60℃ and 4.0, respectively. The enzyme was stable at 30℃-50℃ and in the pH range of 4 to 5. $CuCl_2$, $HgCl_2$ and EDTA inhibited the enzyme activity strongly. By contrast, MnCl2 and $CaCl_2$ activated the enzyme activity. The molecular weight of the purified enzyme was estimated to be 77,000 dalton by SDS-polyacrylamide gel electrophoresis. The Km values of the enzyme for inulin were calculated to be 2.2×10^{-3} M. TLC analysis suggested that purified enzyme is exo-type inulinase. The NH2-terminal amino acid sequences of the purified enzyme was determined to be NH2-X-Glu-Ser-Tyr-Thr-Glu-Lys/Leu-Tyr-Arg-Pro.

Key words : *Penicillium sp., inulinase, inulin*

Introduction

Inulin, a reserve carbohydrate of Jerusalem artichoke, dandelion, chicory, dahlias and Compositae family plant [2], is a linear β-2, 1 linked fructose polymer with a terminal glucose. Inulin has potential utilities for the production of high-fructose-syrups[27] and ethanol[24], and for medicinal use[23]. Chemical hydrolysis of inulin was used originally, but recently proved to be more costly and to give more undesirable degradations and by-product (such as difructose anhydride) than enzymatic hydrolysis[5,16]. Therefore, enzymatic hydrolysis has been intensively studied[26]. The enzyme used was inulinase [2, 1-β-D-fructanfructanohydrolase EC 3.2.1.7][5], which split β-2, 1 fructofuranosidic bonds of inulin. Most of the inulinases are exo-enzyme that split fructose units from the fructose end of the inulin molecule. A few endo-enzymes have been reported to liberate oligofructosides as primary products of hydrolysis[19]. The highest synergistic action of an exo- and an endo-inulinase was observed when an exo- and an endo-inulinase were mixed in the ratio of 1 to 13[19]. It was reported that the S/I ratio (activity on sucrose divided by activity on inulin) of invertase was 5,800-14,000 and that of inulinase was 3.5-26[4,20]. And immobilized enzyme technique was tried for the large scale inulin hydolysis and industrial use[14]. More receltly, inulin also can be source for the production of oligosacharides when endo-inulinase was acted on inulin, excluding exo-inulinase activity[18].

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Microbial inulinases have been mainly reported from *Kluyveromyces*[16,22], *Candida*[7], *Debaryomyces*[3], *Aspergillus*[28], and *Penicillium species*[13,17]. *Penicillium species*[1,25] which produced thermostable inulinase had been isolated from the soil sample in the rhizosphere of Jerusalem artichoke. Therefore, we have purified the inulinase from the isolated *Penicillium* sp. for better understanding of the enzymatic properties and investigated enzymatic properties of the inulinase.

MATERIALS AND METHODS

Isolation of *Penicillium* sp. Producing Inulinase

Penicillium sp. producing inulinase was isolated from the soil in the rhizosphere of Jerusalem artichoke. The isolated microorganism was considered as the *Penicillium* sp. on the basis of morphological characteristics.

Culture Conditions

The microorganism was precultivated at 30℃ for 5 days in a 500 mℓ Erlenmeyer flask containing 100 mℓ of medium B (1% Inulin, 0.8% $(NH_4)^2HPO_4$, 0.4% $NH_4H_2PO_4$, 0.05% KCl, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001 % $FeSO^4 \cdot 7H_2O$, 0.7% Yeast nitrogen base and 0.5% Casamino acid). The culture (5 mℓ) was inoculated into a 2 ℓ Erlenmeyer flask containing 500 mℓ of medium B followed by cultivation with shaking in reciprocal shaker at 30℃ for 6 days. After cultivation, the cells were removed by centrifugation. The resultant supernatant was used as the crude enzyme solution.

Enzyme Assay

The activities toward inulin or sucrose were measured by determining the released reducing sugar from inulin or sucrose by the DNS method[12]. After incubation of 50 μℓ of the enzyme solution in 75 μℓ of 0.2 M sodium acetate buffer (pH 4.7) and either 50 μℓ of 2% inulin (from Dahlia tubers) or 50 μℓ of 0.5 M sucrose at 40℃ for 10 min, the reaction was stopped by adding 500 μℓ of 3, 5 dinitrosalicylic acid reagent and heating at 100℃ for 5 min. One unit was defined as the amount of enzyme liberating one μmol of reducing sugar per min (inulin assay) or hydrolyzing one μmol of sucrose per min (sucrose assay) under the conditions described previously.

Protein Assay

The concentration of protein was determined according to the method of Lowry *et al.*[11] using bovine serum albumin as a standard protein.

SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis was performed on 10% polyacrylamide gel at pH 8.3 using Tris-glycine buffer, according to the method described by Laemmli[8]. Electrophoresis was carried out at room temperature for 3 hours with a eletric current of 30 mA. Protein was stained with Coomassie Brilliant Blue R-250.

The Estimation of Molecular Weight

The molecular weight of the purified enzyme was estimated by SDS-polyacrylamide gel electrophoresis. As standard proteins, Fructose-6-phosphate kinase (Mw 85,200), Albumin from bovine serum (Mw 68,000), Albumin from hen egg (Mw 45,000), Chymotrypsinogen A (Mw 25,000) were used.

Thin Layer Chromatography

Thin layer chromatography was done by a ascending technique on Kieselgel 60 F254 plate (silica gel) with a solvent system of n-buthanol : acetic acid : water (3 : 1 : 1, by volume) at room temperature. The spots of the reaction products were visualized with $AgNO_3$ 1.25 g+water 0.5 mℓ+aceton 10 mℓ.

The NH^2-Terminal Amino Acid Sequencing

After SDS-PAGE, gel was electroblotted onto PVDF (Polyvinylidene difluoride membrane filter) in transfer

buffer (10mM CAPS in 10% methanol) at 4℃ for 16 hours. Protein from the blotted PVDF was analyzed by Applied Biosystems, Inc. Model 476A.

Miscellaneous

DEAE-Sephacel column and Toyopearl HW 65 F column were obtained from Pharmacia Co. and Tosoh Co., respectively. Sodium dodecyl sulfate, polyacrylamide, inulin (from Dahlia tubers), coomassie brilliant blue R-250 and bovine serum albumin were purchased from Sigma Co.. Standard proteins were from Boerhinger-Mannheim. Silica gel was from Merck.. All other chemicals were obtained from commercial sources in reagent grade.

RESULTS

Purification Of Inulinase

The 2.2 ℓ of resultant supernatant was used as the crude enzyme solution and all steps were performed at 4℃

Step. 1. *Ammonium sulfate fractionation.* The collected crude enzyme solution was fractionated sequentially with ammonium sulfate precipitation. The precipitate that were revealed to be able to hydrolyze inulin was dissolved with 2 mM sodium acetate (pH 4.7), dialyzed against 2 mM sodium acetate (pH 4.7) at 4℃ overnight.

Step. 2. *Chromatography on* DEAE-*Sephacel column.* The dialyzate (30 mℓ) was applied on DEAE-Sephacel column equilibrated with 300 mℓ of 2 mM sodium acetate (pH 4.7). The enzyme was eluted with a linear gradient of 0 M to 1 M NaCl in the same buffer (Fig. 1). Each of fractions applied to the enzyme assay. The fractions (No 84, 85, 86) were pooled as an active inulinase and lyophilized after dialysis against 2 mM sodium acetate (pH 4.7) overnight.

Step. 3. *Chromatography on Toyopearl HW 65 F column.* The concentrated enzyme solution (2 mℓ) purified by step 2 was applied on Toyopearl HW 65 F column equilibrated with 300 mℓ of 2 mM sodium acetate (pH 4.7). The enzyme was eluted with the same buffer (Fig. 2). The fractions (No 27, 28) showing inulinase activity led to purify as a single band in SDS-PAGE (Fig. 3).

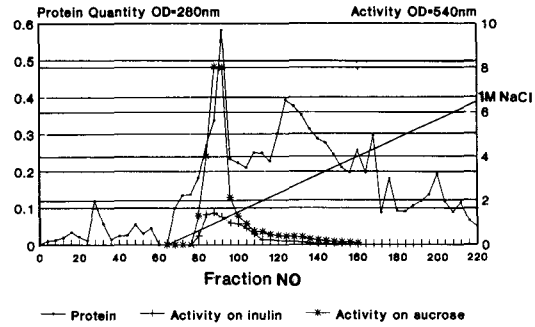

Fig. 1. DEAE-Sephacel column chromatography of inulinase from *Penicillium* sp.
The enzyme (30 mℓ) was applied on DEAE-Sephacel column (2.5×18 ㎝) equilibrated with 300 mℓ of 2 mM sodium acetate (pH 4.7). The elution was done with a linear gradient of 0 M to 1 M NaCl in the same buffer at a flow rate of 30 mℓ/hr.

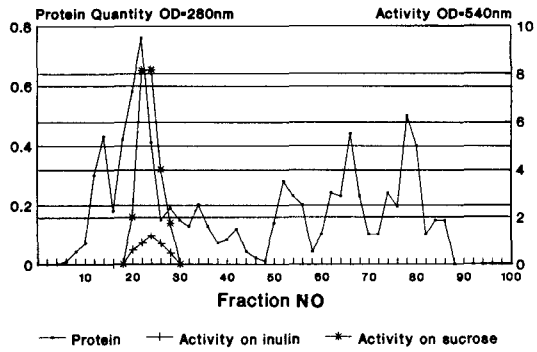

Fig. 2. Toyopearl HW 65 F column chromatography of inulinase.
The concentrated enzyme solution (2 mℓ) purified by step 2 was applied on Toyopearl HW 65 F column (1.4×60 ㎝) equilibrated with 300 mℓ of 2 mM sodium acetate (pH 4.7). The elution was done with the same buffer at a flow rate of 9 mℓ/hr.

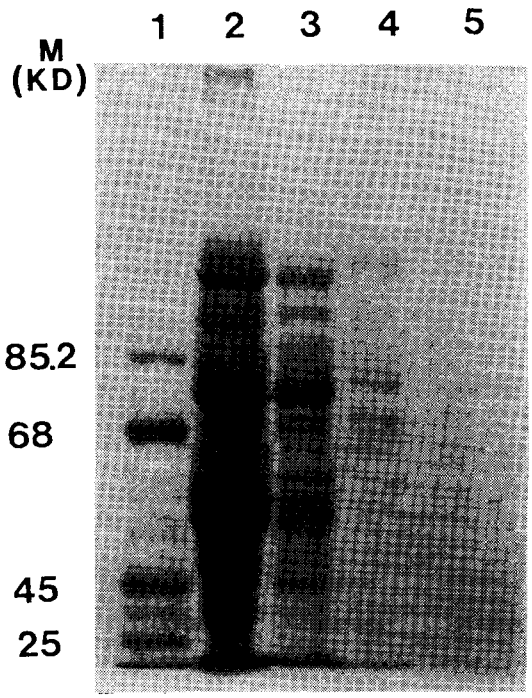

Fig. 3. SDS-polyacrylamide gel electrophoresis.
lane 1. Standard proteins : Fructose-6-phosphate kinase (Mw 85,200), Albumin from bovine pserum (Mw 68,000), Albumin from hen egg (Mw 45,000), Chymotrypsinogen A (Mw 25, 000), lane 2. Crude enzyme solution, lane 3. Proteins from salting out, lane 4. Proteins from DEAE-Sephacel column chromatography, lane 5. Proteins from Toyopearl HW 65 F column chromatography.

Table 1. Summary of purification procedures of inulinase from *Penicillium* sp.

Metal ions and Chemical	Relative Activity(%)
$MgCl_2$	100.7
$CaCl_2$	111.7
$MnCl_2$	172.7
$HgCl_2$	17.9
$CuCl_2$	52.3
$NiCl_2$	94.4
EDTA	90.5
NONE	100

We ascertained this protein band was active on inulin by gel slicing technique[9]. The purification procedures of inulinase are summarized in Table 1.

The Estimation of Molecular Weight

The molecular weight of the purified enzyme was estimated by SDS-PAGE. Plots of logarithmic molecular weight vs. protein mobility on 10% SDS-PAGE are shown in Fig. 4. The molecular weight of the purified emzyme was estimated to be 77,000 dalton.

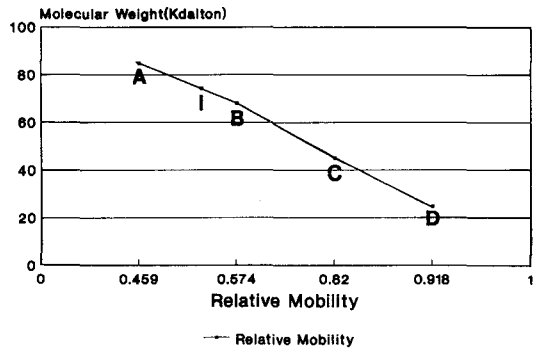

Fig. 4. The Estimation of the molecular weight of the purified enzyme by SDS-polyacrylamide gel electrophoresis.
Standard proteins : A : Fructose-6-phosphate kinase (Mw 85,200), B : Albumin from bovine serum (Mw 68,000), C : Albumin from hen egg (Mw 45,000), D : Chymotrypsinogen A (Mw 25,000), I : Purified enzyme.

Enzymatic Properties

Optimal temperature (Fig. 5) and pH (Fig. 7) for the enzyme reaction were around 60℃ and 4, respectively. The emzyme was stable at 30℃−50℃ (Fig. 6) and at pH 4−5 (Fig. 8). From the results of metal ions and chemical effect (Table 2.), the inulinase was considered to be activated by 1 mM of $MnCl_2$, $CaCl_2$. By contrast, it was inhibited by 1 mM of $CuCl_2$, $HgCl_2$ and EDTA. The products from inulin were analyzed by thin layer chromatography (Fig. 9). The major products were identified as monosaccharide. These results indicated the

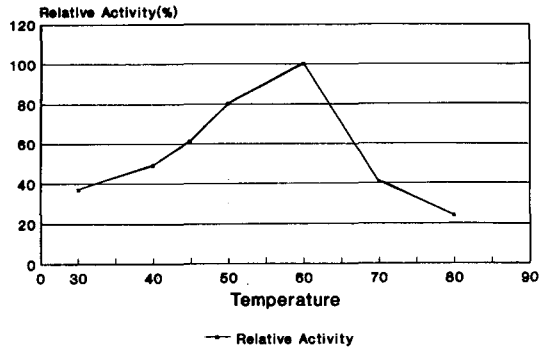

Fig. 5. Optimal temperature of inulinase.
The reaction mixture containing 50 μℓ of 2°F inulin, 75 μℓ of 0.2 M sodium acetate buffer (pH 4.7) and 40 μℓ of enzyme was incubated at various temperatures for 10 min.

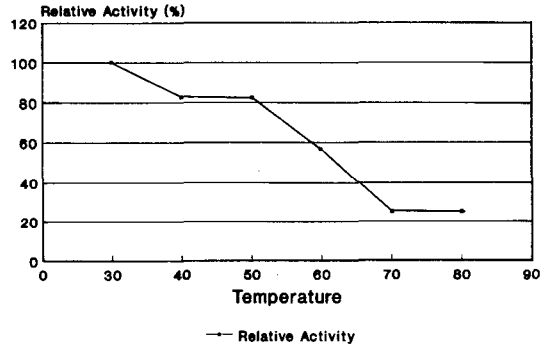

Fig. 6. Temperature stability of inulinase.
After the reaction mixture containing 40 μℓ of enzyme and 75 μℓ of 0.2 M sodium acetate buffer (pH 4.7) had been kept at various temperatures for 10 min, 50 μℓ of 2% inulin were added and then, the reaction mixture was incubated at 40℃ for 10 min.

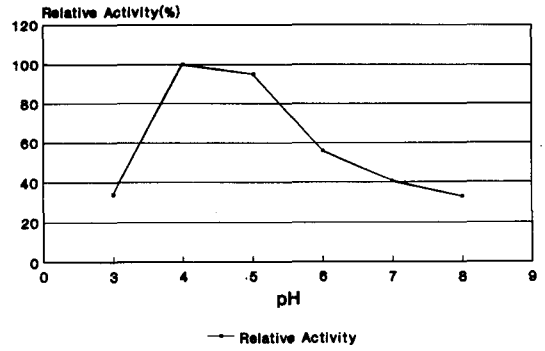

Fig. 7. Optimal pH of inulinase.
The reaction mixture containing 50 μℓ of 2% inulin, 75 8mℓ of various pH buffers and 40 8 mℓ of enzyme was incubated at 40℃ for 10 min.

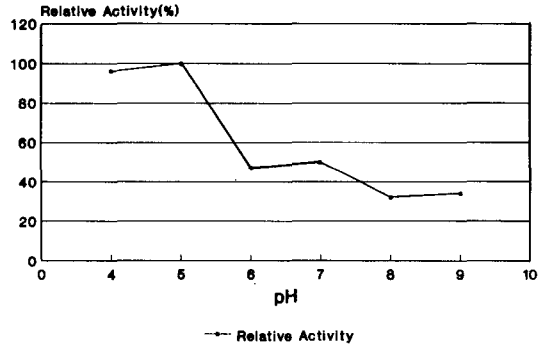

Fig. 8. pH stability of inulinase.
After the reaction mixture containing 40 8mℓ of enzyme and 75 8mℓ of various pH buffer had been kept at 4℃ for 25 hours, 50 8mℓ of 2% inulin were added and then, the reaction mixture was incubated at 40℃ for 10 min.

Table 2. Effects of metals (1 mM) and chemical on the inulinase activity

	Volume (mℓ)	Total protein (mg)	Total unit (U)	Specific activity (unit/mℓ)	Recovery (%)	Purification fold
Crude enzyme solution	2,200	2,600	1,960	0.75	100	1
50% − 80% $(NH_4)_2SO_4$	30	150	120	0.8	6.1	1.1
DEAE-Sephacel	9	4.8	56	11.7	2.9	15.6
Toyopearl HW 65 F	5	0.196	7.75	40	0.39	53.3

The enzyme reaction was performed in the reaction mixture containing 1 mMof each metal ion or chemical reagent at 4℃ and pH 4.7 for 10 min.

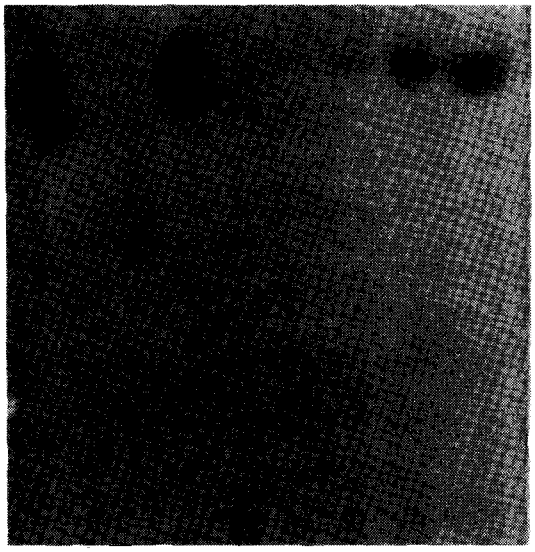

Fig. 9. Thin layer chromatography of inulinase reaction products after different incubation times : 30 sec, 5 min, 10 min, 30 min, 60 min.
References-G : Glucose, I : Inulin, S : Sucrose, F : FructoseThe reaction mixture containing 50 8 mℓ of 2% inulin, 75 8mℓ of 0.2 M sodium acetate (pH 4.7) and 50 8mℓ of enzyme was incubated at 40℃ for various times. After the indicated times, 20 8mℓ of the reaction mixture was pipetted out for TLC analysis. TLC analysis was done as described in Materials and Methods.

enzyme attacks inulin exo-wise. Also, it was demonstrated that KM value of the emzyme on inulin was 2.2×10^{-3} M (Fig. 10).

The NH_2-Terminal Amino Acid Sequencing

The NH_2-terminal amino acid sequence of the enzyme was determined to be X-Glu-Ser-Tyr-Thr-Glu- Lys/Leu-Tyr-Arg-Pro.

DISCUSSION

Inulin can be hydrolyzed by acid or enzymes to produce fructose syrups and oligofructosides. But, The enzyme hydrolysis is preferred because of a lower by-product formation than acid hydrolysis. Studies about hydrolysis of inulin enzymatically have been concentrated on industrial applications of microbial inulinase. An exo-type inulinase from *Penicillium* sp. was highly purified in our laboratory. The enzyme showed high activity toward inulin and sucrose. Nakamura and Nakatsu[15] reported that inulinase from *Penicillium sp.*. It could hydrolyze sucrose as well as inulin. The enzyme purified by us was similar to that inulinase for reaction mode for inulin. Both of them hydrolyzed inulin and sucrose rapidly, and only monosaccharides were produced from inulin. These enzymes might therefore be useful not only inulin hydrolysis but also for sucrose hydrolysis. The molecular weight of the purified enzyme was estimated to be 77,000 dalton by SDS-PAGE. It is a little larger than inulinase (Mw 74,000 dalton) from *Aspergillus ficcum*[6]. It was reported that the molecular masses of the exoinulinase were higher (74,000−76,000 dalton) than those of the endoinulinases (64,000−66,000 dalton)[6]. Inulinase was activated by 1 mM of $MnCl_2$,

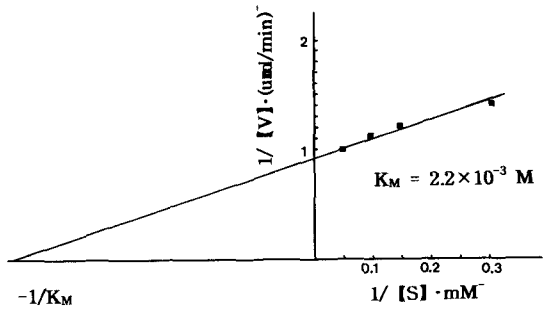

Fig. 10. Lineweaver-Burk plot of effects of various concentrations of inulin on reaction rate.
The reaction was carried out for 10 min with various concentrations of substrate in 0.2 M sodium acetate buffer (pH 4.7) at 40℃. Substrate concentrations were calculated by assuming the molecular weight of 5,400 for inulin (10).

$CaCl_2$. Especially, it was activated by 1 mM of $MnCl_2$ strongly. Also, Inulinases from *Pseudomonas* sp. and *Bacillus subtilis* were activated by $MnCl_2$. Therefore, it was suggested Mn^{2+} might be act as cofactor for inulinase. By contrast, $CuCl_2$ and $HgCl_2$ inhibited the enzyme activity strongly. The purified enzyme showed maximal activity around 60℃ and pH 4, respectively. A comparison of the inulinase purified by us with those from other microorganisms showed many similar properties.

Acknowledgments

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초록 : *Penicillium* sp. 유래 Inulinase의 정제 및 특성

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열안정성 inulinase를 분비하는 *Penicillium* sp.를 돼지 감자 서식지의 토양으로부터 분리하여 이 균주의 inulinase를 50%-80% 염석, DEAE-Sephacel column chromatography, Toyopearl HW 65 F column chromatography에 의해 정제하여 inulinase 활성을 가진 단일 band를 얻었다. 이 band의 단백질을 polyacrylamide gel electrophoresis 후 추출하여 inulinase 활성을 가지는 것으로 확인되었다. 이 단백질의 분자량은 SDS-PAGE에 의해 약 77,000 dalton으로 추정되었고, 최종 정제도는 53.3배이었다. 정제된 효소의 효소학적 성질을 조사한 결과, 최적온도는 약 60℃이었고, 열 안정성은 30-50℃에서 비교적 안정하였다. pH 4에서 가장 높은 활성을 나타냈으며, pH 4-5에서는 비교적 안정했고 염기성에서는 아주 낮은 활성을 나타내었다. 금속이온과 다른 화학물질의 영향을 조사한 결과 1mM의 $MnCl_2$와 $CaCl_2$에 의해 활성이 증가했으며 특히, $MnCl_2$는 1.7배까지 활성을 증가시켰다. 그러나, 1mM의 $CuCl_2$, $HgCl_2$와 1mM EDTA 시에는 활성이 저해되었다. TLC분석결과 모든 산물이 monosaccharide였으므로 이 효소는 exo-acting inulinase로 추정되었고, inulin에 대한 이 효소의 K_M치는 2.2×10^{-3}이었다. 이 효소의 결정된 N-terminal 아미노산 배열은 NH_2-X-Glu-Tyr-Thr-Glu-Lys/Leu-Tyr-Arg-Pro이다.