Kor. J. Appl. Microbiol. Bioeng.
Vol. 11, No. 1, 47 – 52 (1983)

Studies on the Inulin Hydrolyzing Enzyme from *Aspergillu* sp. (C-58) (Ⅲ)

—Purification of inulase (P-I) from *Aspergillus* sp. (C-58)—

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(Received January 16, 1983)

Aspergillus sp. (C-58) 菌株가 生産하는 Inulin 分解酵素에 關한 硏究

— *Aspergillus* sp. C-58 菌株가 生産하는 inulase P-Ⅰ의 精製 —

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(1983年 1月 16日 접수)

The extracellular inulase produced by *Aspergillus* sp. C-58 was isolated by pH and charcoal treatment, precipitation with ammonium sulfate from the crude extract, and separated into 3 fractions (P-I, II, III) by DEAE-cellulose column chromatography in the ratio of 31.1:1.7:1 with respect to the activity. The ratio of inulase activity to sucrase activity of P-I, P-II and P-III fraction was 0.23, 0.24 and 1.1 respectively. The enzyme P-I fraction was purified 482 fold with a 22.8% yield by DEAE-Sephadex A-50, Sephadex G-75, Sephadex G-100 (1st and 2nd) column chromatography, and appeared homogeneous on polyacrylamide disc gel electrophoresis and ultracentrifugation.

Since Edelman *et al*[1] reported that the 60% ammonium sulfate precipitates of the extract of *Jerusalem artichoke* tuber has hydrolytic activity to fructose polymer, many inulases have been purified from various sources.

Edelman *et al*[2] investigated the mechanism of inulin hydrolysis with enzyme that was purified 7.3 fold by DEAE-cellulose from the crude extract of *Jerusalem artichoke*, and Edelman *et al*[3] also investigated the properties of ß-fructofuranosidase purified by DEAE-cellulose.

It was first purified yeast inulase that Kovaleva *et al*[4] reported the ß-fructosidase obtained from *Saccharomyces lactis*.

Thereafter, Negoro[5] compared *Candida kefyr* extracellular ß-fructofuranosidase purified by precipitation with 75% acetone, DEAE-Sephadex A-50 and SE-Sephadex C-50 column chromatography with the inulases obtained from *Saccharomyces cerevisiae* and *Candida utilis*. And the extracellular inulase of *Kluyveromyces fragilis* was purified 232 fold by Nahm *et al*[6] by DEAE-Sephadex A-50 column chromatography and Sepharose 6B gel filtration. Negoro[7] also purified intra-and extracellular inulase produced by *Kluyveromyces fragilis* with 203 and 119 fold purity respectively by precipitation with tannic acid, DEAE-Sephadex A-50 column chromatography. Nakamura *et al*[8] reported intracellular and extracellular inulases produced by *Penicillium* sp.-1. The extracellular enzyme was separated 3 different enzyme by DEAE-Sephadex column chromatography, and the intracellular enzyme[9] was purified DEAE-cellulose column chromatography and was separated into 4 kinds of inulase.

Aspergillus inulase was one of the well known microbial inulases.

Nakamura *et al*[10, 11] obtained extracellular inulase P-I, P-II, and P-III from *Aspergillus niger* K-12, and P-II and P-III were investigated in details.

Enzyme P-II was purified from culture filtrate by DEAE-cellulose column chromatography, Sephadex G-50, G-100, and G-200 gel filtration, and enzyme P-III was purified by DEAE-Sephadex A-50(1st and 2nd) and Sephadex G-100 column chromatography, and was obtained in crystalline form by ammonium sulfate precipitation.

In the present paper, to investigate the enzymatic properties of the inulase of *Aspergillus* sp. C-58, the enzyme was purified, so the result was reported.

Materials and Methods

Chemicals

Inulin and sucrose (Wako pure chemical industry, Ltd., Japan), DEAE-cellulose, DEAE-Sephadex A-50, Sephadex G-75 and G-100 were purchased from Pharmacia Fine Chemical company (Sweden). All other reagents were of analytical reagent grade.

Microorganism: The mold strain used throughout this work was *Aspergillus* sp.C-58 strain, which was isolated from garden soil as described previously[12].

Culture medium and method

The cultural medium and method used throught this work was followed as previous paper[13].

Enzyme assay: The enzyme activity was assayed as previous report[12].

I/S: I/S expresses the ratio of hydrolysis rate of inulin and sucrose.

Determination of protein: Protein was determined by the method of Lowry *et al*[14], and by measurement of absorbance at 280nm. Bovine serum albumin was used as a standard protein. Spectrophotometric measurement was carried out in Hitachi 200-10 spectrophotometer or a Shimadzu double beam spectrophotometer UV-200.

Acrylamide gel electrophoresis

Analytical disc gel electrophoresis in 7.5% polyacrylamide gel at pH 8.3 using Tris-glycine buffer was performed according to the method of Davis[15]. About 60ug, 80ug and 150ug of enzyme protein were applied to each tube (0.5 x 8cm), and electrophoresis was carried out for two hours at room temperature with a current of 3mA per tube. After the run, the gels were stained with 1% amido black 10-B and destained in 7% acetic acid.

Ultracentrifugal analysis

The sedimentation pattern was performed with Beckman model E analytical ultracentrifuge[16]. A multicell operation was employed for the sedimentation experiment. The enzyme was dissolved in 0.01M of sodium phosphate buffer, pH 7.0 to give protein concentration of 10.0mg per milliliter. The Rayleigh interference pattern was photographed at intervals of 38mins to ensure that equilibrium was established. The relation between the concentration of the enzyme and the fringe shift was determined with a synthetic boundary cell. To all calculations the partial specific volume of V = 0.7 *ml/g* was used. The sedimentation velocity was measured with a same ultracentrifuge as the sedimentation equilibrium method with 59,780 rev /min. The temperature of the ultracentrifugal experiments was maintained at 20°C.

Results and Discussion

Enzyme production

Optimal conditions for the enzyme production by *Aspergillus* sp. C-58 on wheat bran solid medium were established. These are cultivation method, media, addition of water volume, temperature and culture period. It was found that 50 to 55% of moisture content in wheat bran was suitable for the production of the enzyme by the strain[13]. Under these culture conditions, the enzyme production increased with the culture period, being maximum at 5 days, after which there was no or very little increase.

Purification of the inulase I

All the purification steps were carried out at 4-8°C unless otherwise stated. sodium phosphate buffer (0.01M) of pH 7.0 was used.

Preparation of crude enzyme solution: After extraction of wheat bran culture with addition of 8 volumes of cold distilled water, the filtrate was centrifuged at 8,000 x g for 20 min. The supernatant (1,382ml) was used as the crude enzyme solution

pH and heat treatments: To the crude enzyme solution (1,382ml), 14% ammonium hydroxide solution was gradually added with continous stirring to give pH of 8.2 and then treated at 45°C for 30min, followed by centrifuge at 8,000 x g for 20min, and adjusted pH to 5.0 with 4N HCL solution.

Charcoal treatment: To the resultant supernatant solution was added active charcoal (2%), and then the suspension was continuously stirred at5°C for 12 hrs. The suspension was filtrated under suction through a thick bet hyflosuper-cell over Toyo No.2 filter paper on Buchner funnel.

Ammonium sulfate fractionation: The charcoal-treated enzyme solution (1,333ml) was brought to 70% saturation by adding 635g of solid ammonium sulfate. After 3 hrs, the precipitate formed was removed by centrifugation at 8,000 x g for 30min. The supernatant was brought to 86% saturation by further addition of ammonium sulfate (200g). After standing overnight, the precipitate was collected by centrifugation and dissolved in a small amount of 0.01M buffer. The solution was dialyzed in cellulose tube for 10 hrs against 5 times change of 10 liters of distilled water.

DEAE-cellulose column chromatography: The dialyzed

enzyme solution (40ml) from the previous step was placed on a DEAE-cellulose column (2.5 x 54cm) which was previously equilibrated with 0.01M same buffer. The column was eluted by the same buffer with stepwisely increasing concentration of sodium chloride solution. The fractions having a volume of 15ml were collected at flow rate of 8.9ml per hour. A typical elution pattern of protein and enzyme activity is depicted in Fig. 1. Three protein fractions having inulase activity were designated as inulase I, II and III, respectively and the elution pattern was different from result of Nakamura and Nakatsu[8]. Total recovery of the enzyme activity was about 54.3% with the three fractions in the ratio of 31.1:1.7:1. The ratio was quite different from that of *Aspergillus niger*-12 which was cultivated by the submerged culture as reported by Nakamura *et al*[10]. Active fractions of inulase I (335ml) were combined, concentrated with a collodion bag (Sartorius Membrane Filter Co.) to 19ml, and then dialyzed against distilled water.

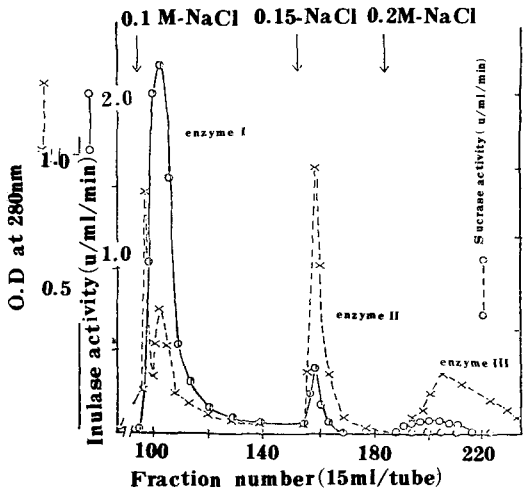

Fig. 1. Chromatography of the enzyme on DEAE -cellulose column.

Protein (826.6mg) was added to a column (2.5 x 54cm) equilibrated with 0.01M sodium phosphate buffer, pH 7.0 and eluted by the stepwisely increasing concentration of NaCl in equilibrating buffer. Fifteen milliliter fraction were collected at flow rate of 8.9/hr. o——o, activity toward inulin; •----•, activity toward sucrose; x----x, amount of protein.

DEAE-Sephadex A-50 column chromatography of the inulase I: The dialyzed preparation (19ml) from the previous step was applied to a DEAE-Sephadex A-50 column (2.2 x 30.5cm) equilibrated with 0.01M sodium phosphate buffer. After the column was washed with 1 liter of 0.01M sodium phosphate buffer containing 0.15M sodium chloride, the inulase I was eluted with 0.01M buffer containing 0.2M sodium chloride. The elution pattern is shown in Fig. 2. In the point of chromatographic pattern on the DEAE-Sephadex A-50 column, it was similar to the extracellular inulase reported by Nakamura and Nakatsu[8]. The active fraction (300ml) was concentrated with the collodion bag to 0.5ml, and then dialyzed in cellulose tube for 8 hrs against 4 times change of 2 liters of deionized water.

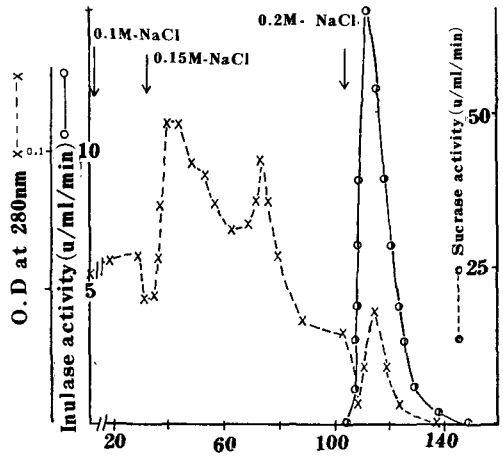

Fig. 2. DEAE-Sephadex A-50 column chromatography of inulase I.

The dialyzed enzyme I (139.9mg) solution was applied to a column (2.2 x 30.5cm) equilibrated with 0.01M sodium phosphate buffer, pH 7.0. Fifteen milliliter fractions were collected at flow rate of fifteen milliliter per hour. o——o, activity toward inulin; •----•, activity toward sucrose; x----x, amount of protein.

Sephadex G-75 column chromatography of the inulase I: The dialyzed inulase I which contained 13.0mg of protein was applied to a column of Sephadex G-75 (0.9 x 76cm) previously equilibrated with 0.01M sodium phosphate buffer, and eluted with same buffer. The fractions of each 4.0ml were collected at flow rate of 1ml per hr. Elution pattern is shown in Fig. 3. The active fractions (fraction No. 24 to 31) were concentrated in the collodion bag to about 4ml. First Sephadex G-100 column chromatography of the inulase I: The concentrated inulase I preparation from Sephadex G-75 column chromatography contained 10.7mg of protein was applied to a column of Sephadex G-100 (1.6 x 71.5cm) previously equilibrated with 0.01M same buffer. The fractions of each 0.95ml were collected at flow rate of

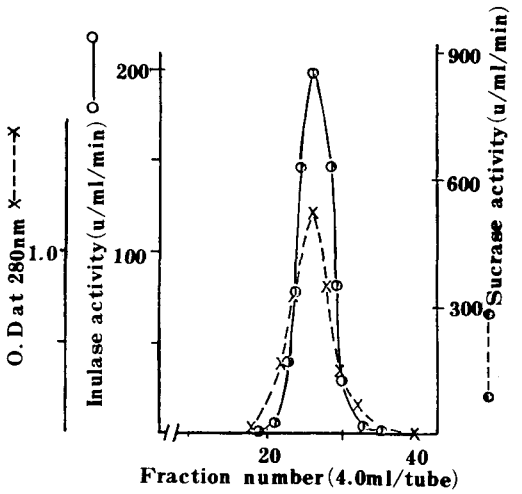

Fig. 3. Sephadex G-75 column chromatography of inulase I.

The dialyzed enzyme I (13.0mg) solution (obtained after chromatography on DEAE-sephadex A-50 column) was applied to a column (0.9 x 76cm) equilibrated with 0.01M sodium phosphate buffer, 7.0. The fractions of 4.0ml were collected at flow rate of 1.0ml/hrs. o——o, activity toward inulin; •----•, activity toward sucrose; x----x, amount of protein.

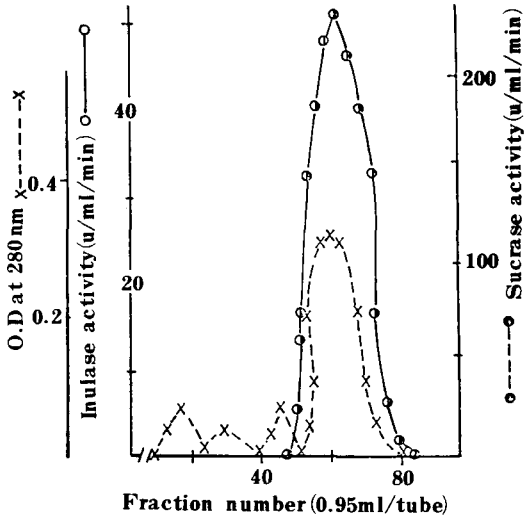

Fig. 4. First Sephadex G-100 column chromatography of the enzyme.

The dialyzed enzyme I (10.7mg) solution (obtained after Sephadex G-75 gel filtration) was applied to a column (1.6 x 71cm) equilibrated with 0.01M sodium phosphate buffer, 70. The fractions of 0.95ml were collected at flow rate of 1.35/hr. o——o, activity toward inulin; •----•, activity toward sucrose; x----x, amount of protein.

1.3ml per hour. The elution pattern is shown in Fig. 4 and it was resemble to P-II of *Aspergillus* inulase described by Nakamura *et al*[11]. The inulase I preparation (27ml) thus obtained had the specific activity of 465.4 fold against the crude enzyme extract. The active fractions (fraction No. 51 to 79) were concentrated in the collodion bag to about 3ml.

Second Sephadex G-100 column chromatography of the inulase I: The concentrated inulase I preparation from Sephadex G-100 first column chromatography contained 7.0mg of protein was applied to a column of Sephadex G-100 (1.6 x 71.5cm) previously equilibrated with 0.01M sodium phosphate buffer. The fractions of each 1.0ml were collected at flow rate of 1.3ml per hour. The elution pattern is shown in Fig.5. The enzyme preparation (26ml) thus obtained had the specific activity of 482.4 fold against the crude enzyme extract, and was designated as the purified enzyme. The yield of recovery for the extracellular inulase from *Aspergillus* sp. C-58 lie at the similar level as the enzyme reported by Nahm and Byun[6], and higher than those of *Penicillium* inulase of Nakamura and Nakatsu[8] and *Aspergillus* inulase described by Nakamura *et al*[10, 11]. The summary of the purification is shown in Table 1.

Homogeneity of the purified inulase I

Purity on the acrylamide gel electrophoresis: The purity

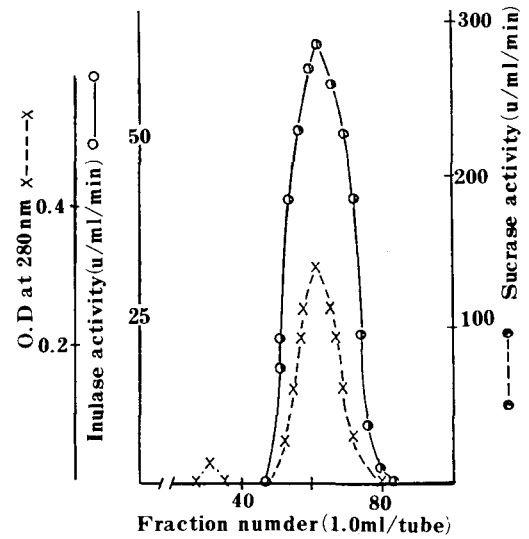

Fig. 5. Second Sephadex G-100 column chromatography of the enzyme.

The dialyzed enzyme I (7.0mg) solution (obtained after first Sephadex G-100 gel filtration) was applied to a column (1.6 x 71.5cm) equilibrated with 0.01M sodium phosphate buffer, 7.0 The fractions of 1.0ml were collected at flow rate of 1.3ml/hr. o——o, activity toward inulin; •----•, activity toward sucrose; x----x, amount of protein.

Table 1. Summary of enzyme purification

Purification Step	Protein* (mg)	Total Activity I	Total Activity S	Specific Activity I	Specific Activity S	Yield (%)	I/S	Purity (fold)
Crude extract	13,375	4,129	18,170	0.31	1.36	100	0.23	1.
pH treatment	11,147	3,980	17,990	0.36	1.61	96.4	0.22	1.15
Charcoal treatment	8,414	3,199	16,395	0.38	1.95	77.5	0.23	1.23
Ammonium sulfate 70-86%	826.6	2,240	9,333	2.71	11.3	54.3	0.24	8.74
DEAE-cellulose								
enzyme I	139.9	2,056	8,942	14.7	63.9	49.8	0.23	47.4
enzyme II	79.3	111.0	462.5	1.4	5.8	2.7	0.24	4.5
enzyme III	103.1	66.0	60.0	0.64	0.58	1.6	1.1	2.1
DEAE-Sephadex A-50								
enzyme I	13.0	1,645	7,151	126.7	550	39.8	0.23	408.7
Sephadex G-75	10.7	1,419	6,449	132	602	34.4	0.22	425.8
1st Sephadex G-100	7.0	1,010	4,591	144.3	655.9	24.5	0.22	465.4
2nd Sephadex G-100	6.3	945.5	4,298	149.5	682	22.8	0.22	482.4

I: activity toward inulin, S: activity toward sucrose.

* The amount of protein was estimated from absorbance at OD 280 nm, assuming $E^{1\%}_{cm} = 8.0$

of this preparation was examined by electrophoresis on polyacrylamide gel. The purified inulase I migrated towards the anode as a single band of protein (Fig. 6). The electrophoretogram was very sharp band, and the aspect was similar to *Penicillium* inulase[8] and *Aspergillus* inulase[11] reported by Nakamura *et al.*

The electrophoresis was performed in 7.5% polyacrylamide gel at pH 8.3 with a current of 3.0mA per tube. About 60ug, 80ug and 150ug of protein were applied. Gels were stained with Amido black 10-B and destained electrophoretically in 7% acetic acid at constant current of 5mA per tube.

Purity of the inulase I by ultracentrifugation: The sedimentation pattern (Fig. 7) obtained in a sedimentation velocity experiment indicated the presence of single component.

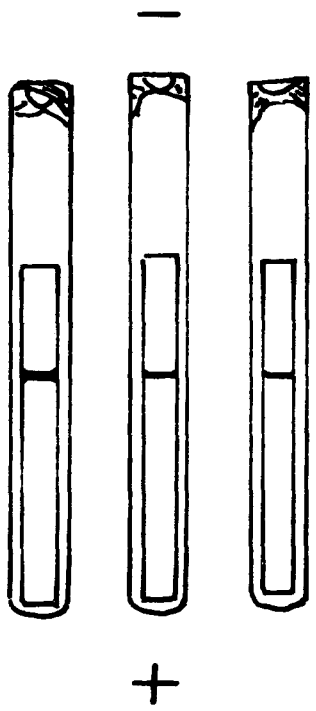

Fig. 6. Disc gel electrophoresis of the purified enzyme I.

Acknowledgements

The author wishes to thank Dr. H. Yamada and Dr. Y. Tani, Professor of Kyoto University, for their kind guidance and encouragement during the course of this work. The author wishes to express his sincere thanks to Drs. Y. Izumi, S. Shimizu and T. Nagasawa for their valuable advices. The author is highly thankful to Japanese Commis-

sion for UNESCO for awarding the fellowship and thus providing an opportunity to study applied Microbiology in Japan.

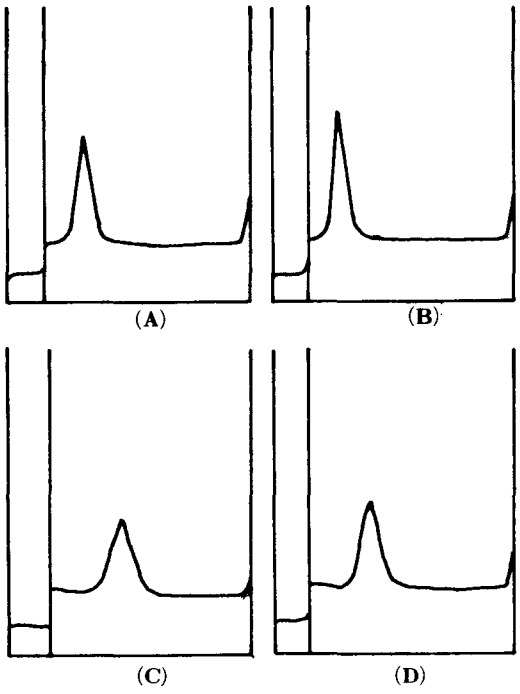

Fig. 7. Sedimentation patterns of the purified inulase I.

The purified enzyme I from Sephadex G-100 second column chromatography was used at a concentration of 1.0% in sodium phosphate buffer, pH 7.0. Photographs were taken 38(A), 62(B), 78(C), and 102(D) min after reaching 59,780 rev/min.
The sedimentation is left to right

要 約

Aspergillus sp.C-58 菌株가 生産하는 extracellular inulase에 대하여 pH, charcoal 처리 및 ammonium sulfate로 분별염석한 후 DEAE- cellulose를 利用한 column chromatography에 의하여 3個의 酵素蛋白質(Peak Ⅰ, Ⅱ, Ⅲ)로 분획되었으며 그 비율은 31.1 : 1.7 : 1이였다. P-Ⅰ, Ⅱ의 I/S는 그 비율이 0.23 및 0.24로 거의 同一하였으나 P-Ⅲ는 1.1로 P-Ⅰ 및 P-Ⅱ와 상이하였다.

Peak Ⅰ 효소에 대하여 DEAE-Sephadex A-50을 이용한 ion exchange chromatography에 의하여 추출효소에 비교하여 약 408배 정제되었으며 다시 Sephadex G-75 및 Sephadex G-100에 2회 gel filtration하여 약 482배 정제되었다. 이상과 같이 정제한 Peak Ⅰ의 효소액은 poly acrylamide를 이용한 disc gel electrophoresis 및 ultra centrifugation에 의하여 단일 단백질로 확인되었다.

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