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PURIFICATION AND PROPERTIES OF *ARTHROBACTER UREAFACIENS* INULASE II

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SUMMARY

1. *Arthrobacter ureafaciens* inulase II, which converts inulin to di-D-fructofuranose 1,2′:2,3′ dianhydride (difructose anhydride III) and a small amount of oligosaccharides, was purified about 150-fold in the 43% yield from the cultured liquid of the bacteria by means of ammonium sulfate fractionation, followed by acetone fractionation and Sephadex G-100 gel filtration. A polarimetric measurement was carried out for the estimation of enzymatic activities.

2. The purified enzyme was found to be homogeneous by polyacrylamide disc gel electrophoretic studies in support of an intramolecular transfructosidation reaction of the enzymatic reaction. The enzyme was optimally reactive at pH 6.0 and at 50 °C, and was stable within a broad pH range (pH 4 to 11) and below 50 °C. The activity was strongly inhibited by $HgCl_2$.

INTRODUCTION

In the previous study[1], a strain of *Arthrobacter ureafaciens* isolated from soil was reported to produce an extracellular enzyme which efficiently converts inulin to difructose anhydride III, leaving a small amount of oligosaccharides. On the basis of the fact that the main reaction product was essentially different from that of the common inulase which splits off the terminal fructose units from the inulin molecule and is known to be β-2,1-fructan fructanohydrolase (EC 3.2.1.7), the common inulase and the present enzyme were arbitrarily designated as inulase I and inulase II, respectively, and the action of the latter enzyme in the formation of difructose anhydride III was assumed to be an intramolecular transfructosidation reaction. However, the enzyme used in the study was not of a satisfactorily pure form.

In our second paper on this enzyme, we report on its purification and properties of the purified enzyme. In these studies, the enzymatic activity was estimated polarimetrically, utilizing the property of high specific rotation of difructose anhydride III.

MATERIALS AND METHODS

Preparation and purification of enzyme

Cultivation of the strain of *A. ureafaciens* for the enzyme preparation of the cultured liquid (culture supernatant), and the lyophilized preparation of the crude enzyme ($(NH_4)_2SO_4$ precipitate) were performed according to the method described in the previous paper[1]. This lyophilized material, 2 g, was dissolved in 100 ml distilled water, and the solution was centrifuged to remove insoluble material. To the supernatant solution, chilled in ice, 1.5 vol. of cold acetone (−10 °C) was slowly added at 0–5 °C under stirring. The precipitate formed was collected by centrifugation at 0 °C for 15 min, 10 000 × *g*, and washed with ice-cold acetone, ice-cold ether, and dried *in vacuo* over $CaCl_2$ (acetone precipitate). The yield was 25 to 75 mg. In the Sephadex G-100 gel filtration, a 50-mg portion of the acetone precipitate was dissolved in 2.0 ml of 0.05 M acetate buffer (pH 6.0), and subjected to gel filtration using a 3.2 cm × 60 cm column of Sephadex G-100 (Pharmacia, Uppsala, Sweden; particle size, 40–120 μm) preequilibrated with 0.05 M acetate buffer (pH 6.0). The column was eluted with the same buffer, and 3-ml fractions were collected at a flow rate of 10 ml/h. The fractions showing high enzymatic activity were pooled and stored in a refrigerator until use (Sephadex G-100 eluate).

Enzyme assay

Since the reaction product, difructose anhydride III, is nonreducing but has a high specific rotation, the measurement of enzymatic activities was polarimetrically conducted. Unless otherwise stated, the reaction mixture contained 2.0 ml of enzyme solution, 1.0 ml of 0.2 M acetate buffer (pH 6.0) and 1.0 ml of 4.0% inulin. The inulin was purchased from Wako Pure Chemical Co. These solutions, preincubated at 37 °C for 10 min, were combined in a test tube. The mixture was immediately transferred into a cuvette with a 0.1-dm light path and the cuvette was quickly equipped within a Jasco DIP-180 type polarimeter. At once the dial was set at 0.000 °. The reaction was continued in the cuvette at 37 °C, and the change of optical rotation was estimated automatically. In controls, the inulin or enzyme solution was replaced by the same volume of water in the reaction mixture. One enzyme unit was defined as the enzyme amount producing the +0.001 ° increase in the optical rotation per min under the described conditions. This value was derived from the fact that the optical rotation of 1.0 μmole difructose anhydride III corresponds to about +0.001 ° in the 0.1-dm light path. Specific enzyme activity was expressed as units/mg protein. Protein was determined by phenol reagent using bovine serum albumin as standard[2].

Gel electrophoresis

Polyacrylamide disc gel electrophoresis was carried out by the method of Davies[3] after slight modification. One set of two equal running gels (tube size: 6 mm × 65 mm) were prepared, one was used to examine protein locality, and the other to examine enzyme locality. The test solution of the acetone precipitate was prepared by dissolving it in 0.06 M Tris–HCl buffer (pH 6.7) at a concentration of about 40 μg per 0.1 ml. The test solution of the Sephadex G-100 eluate was prepared as follows. A 10-ml portion of the enzyme solution was dialyzed in a cellophane bag against 3 l of deionized water at room temperature for 24 h and the dialyzed solution

was lyophilized. The material obtained was dissolved in a small volume of the Tris–HCl buffer and diluted to the concentration of about 30 μg per 0.1 ml with the same buffer. The protein concentration of these test solutions was determined by phenol reagent as described above. Gelatinization of these test solutions was performed by the addition of cellulose hydroxyethylcellosize (Cellosize Q P – 15000, Futaba Shoji Co.) at about 2% concentration[4]. The electrophoresis was carried out at 2.5 mA/tube for 2 h at 5 °C. Protein was stained with amido black. To examine the enzyme locality, the gel was cut into 2.5-mm sections, and the enzyme was extracted by incubating each section in 1.0 ml of 0.05 M acetate buffer (pH 6.0) for 20 h at 5 °C.

Thin-layer chromatography

Thin-layer chromatography to examine the products of enzymatic reaction on inulin in the test of inulase I contamination was carried out using Avicel SF plates (Funakoshi Chemical Co.). The ascending development employing the solvent mixture, *n*-butanol–pyridine–water (6:4:3, v/v/v)[5] was doubled. Location of sugars on the plate was detected by spraying with anthrone–phosphoric acid reagent[6] and heating at 115 °C for 5 min.

RESULTS AND DISCUSSION

Determination of enzymatic activity

Inulase II activity was estimated by the polarimetric determination of difructose anhydride III produced by the enzymatic action on inulin, utilizing the property of high specific rotation of the product ($[\alpha]_D$ + 136 ° in water). In the estimation, it was assumed that all the partially degraded inulin molecules from the formation of difructose anhydride III have the same specific rotation as the original inulin molecule.

Effects of enzyme amounts and time of reaction on the formation of difructose anhydride III were studied under the conditions described, except that the time was varied. In these experiments, the $(NH_4)_2SO_4$ precipitate was used after inactivation of the contaminating inulase I by the thermal treatment as described later. Fig. 1 shows the results of the experiments when the enzyme amount was varied from 0.23 to 0.92 unit, and the time of reaction from 5 to 60 min. It indicates that there is a inear relationship between the increase of optical rotation and the length of reaction

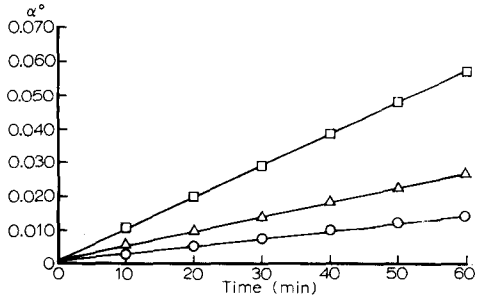

Fig. 1. Dependence of reaction rate on enzyme concentration. Reaction conditions were the same as described in the text except that the time of reaction was varied. ○—○, 0.23 unit; △—△, 0.46 unit; □—□, 0.92 unit.

time and that the rate of increase of the rotation increased proportionally to the enzyme amounts used. Since the controls in which inulin or enzyme was omitted in the reaction mixture showed no increase of rotation during the experimental periods, the control experiments were omitted in further experiments.

Purification of enzyme

In the previous study[1], the enzyme in the cultured liquid was precipitated with $(NH_4)_2SO_4$, dialyzed against deionized water and lyophilized. In the present study, purification of this enzyme preparation was performed by acetone fractionation and Sephadex G-100 gel filtration. In the estimation of enzymatic activity, each preparation was examined for contamination of inulase I, which interferes with the estimation of inulase II activity. The test was carried out in the following way. After the optical rotation had been reached to the constant value (approximately $+0.128°$), a small portion of the reaction mixture was withdrawn, concentrated to one tenth of the original volume at 40 °C, and the solution was examined as to the products formed by thin-layer chromatography; detection of free fructose on the chromatogram indicates the contamination of inulase I. By this test, the acetone precipitate was found to still contain a trace of inulase I. Inactivation of inulase I of this preparation was attempted by heating at 65 °C for 10 min at pH 6.0 as described in the previous paper[1]. The complete inactivation of the inulase I occurred but was accompanied by a partial loss of the inulase II activity. The degree of loss was estimated to be about 20% by the second similar thermal treatment of the inulase I-inactivated enzyme solution. Inulase II activity of the original solution was calculated in correction of the activity of the enzyme solution obtained after the first thermal treatment by this value. Enzymatic activities of the culture supernatant and $(NH_4)_2SO_4$ precipitate were calculated in the same way using this value. The purity and recovery of enzyme in each step of purification are summarized in Table I. The yield of the

TABLE I

PURIFICATION OF INULASE II

Enzymatic activity was measured under the standard conditions of assay

Fraction	*Activity (units)*	*Protein (mg)*	*Specific activity (units/mg)*	*Yield (%)*
Culture supernatant (1 l)	49.9	47.0	1.06	100
$(NH_4)_2SO_4$ precipitate	35.3	4.55	7.76	70.8
Acetone precipitate	26.4	0.541	48.8	52.9
Sephadex G-100 eluate	21.6	0.133	162.4	43.3

$(NH_4)_2SO_4$ precipitate from the culture supernatant was about 70%, and the enzyme was purified about 8-fold in the specific activity. The yield of the acetone precipitate from the $(NH_4)_2SO_4$ precipitate on the basis of dry material varied widely, but the recovery of enzyme was almost constant, probably depending upon the degree of contamination of $(NH_4)_2SO_4$ by the incomplete dialysis before lyophilization. The enzyme was further purified about 6-fold and the yield was about 53% of that of the culture supernatant. The Sephadex G-100 gel filtration was found to be useful in

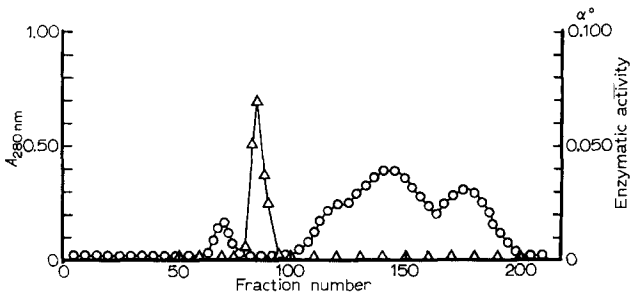

Fig. 2. Gel filtration of inulase II on a Sephadex G-100 column. Size of column; 3.2 cm × 60 cm, elution with 0.05 M acetate buffer (pH 6.0) ascending. △—△, enzymatic activity (assayed under the standard conditions described); ○—○, absorbance at 280 nm.

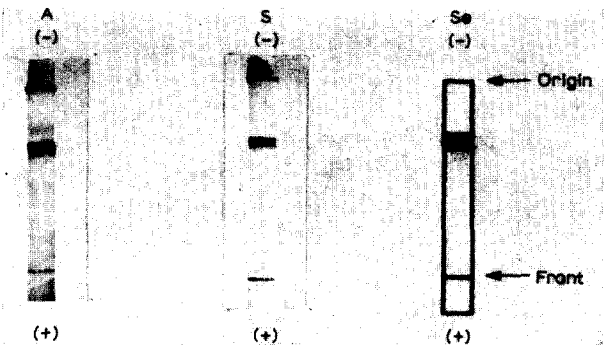

Fig. 3. Polyacrylamide disc gel electrophoresis of the Sephadex G-100 eluate and the acetone precipitate. A, the acetone precipitate (stained with amido black); S, the Sephadex G-100 eluate (stained with amido black); Se, diagrammatic representation of the locality of enzymatic activity when the Sephadex G-100 eluate was examined.

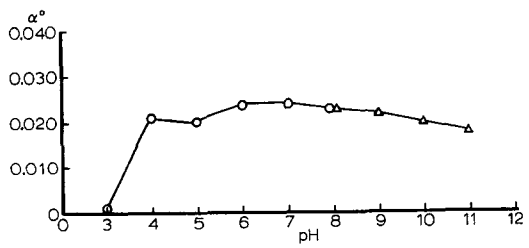

Fig. 4. pH-stability dependence of inulase II. A 14-ml portion of the Sephadex G-100 eluate was dialyzed against 3 l of deionized water under toluene for 20 h at room temperature. 1.0 ml of the dialyzed enzyme solution was added to 1.0 ml of 0.02 M buffer of the various pH values as indicated and the mixture was incubated at 30 °C for 2 h. A 2.0-ml portion of the enzyme solution was withdrawn, and incubated with 2.0 ml of 2% insulin dissolved in 0.4 M acetate buffer (pH 6.0) at 37 °C for 60 min and the optical rotation was measured. ○, 0.02 M citrate buffer (pH 3.0 to 8.0; △, 0.02 M borate buffer (pH 8.0 to 11.0).

removing inactive proteins in the foregoing preparation. The elution patterns of protein (absorbance at 280 nm) and the enzyme are illustrated in Fig. 2. The enzyme was eluted in the Fractions 81 to 90, which showed almost no absorbance at 280 nm. These fractions were combined and the solution was examined on its purity. In the specific activity, the enzyme was purified more than 3-fold from the acetone precipitate, and about 150-fold from the culture supernatant with the recovery of 43%. This purified enzyme showed no contamination of inulase I.

Homogeneity of the Sephadex G-100 gel eluate was examined by polyacrylamide disc gel electrophoresis under the conditions described. The results are shown in Fig. 3. The acetone precipitate, which was used for comparison, gave 6 protein bands, and the purified enzyme gave a single protein band, stained with amido black. The enzyme activity was found to be located exclusively in the segments corresponding to this single protein band.

Properties of purified enzyme

Several properties of the purified enzyme were investigated. Fig. 4 shows the stability of the enzyme against different pH values, when examined using a citrate (pH 3.0 to 8.0)–borate (pH 8.0 to 11.0) buffer system. The enzyme was stable within a broad pH range (pH 4 to 11), the optimum range was at about pH 6 to 7. The thermal stability of the enzyme at pH 6.0 is shown in Fig. 5. The enzyme was stable below 50 °C, but was rapidly inactivated beyond 60 °C. At pH 6.0 and under toluene, the enzyme could be stored in a refrigerator for a few months without appreciable loss of activity. pH dependence of the enzymatic activity was estimated using an acetate (pH 4.0 to 6.0)–sodium phosphate (pH 6.0 to 8.5) buffer system. The results, Fig. 6, indicate that the enzyme was optimally reactive at around pH 6.0. Activity–temperature dependence of the enzyme was estimated at pH 6.0. In this experiment, the enzyme reaction was carried out in test tubes and stopped by adding $HgCl_2$ at a concentration of $1 \cdot 10^{-3}$ M, since $HgCl_2$ was found to inhibit the enzymatic action completely as mentioned later. Then the mixture was submitted to the polarimetric measurement. The enzyme was most reactive at 50 °C (Fig. 7). Effects of chemicals on the enzymatic activity were investigated. Various divalent cations, which were used as the chloride forms, cysteine and EDTA were examined. The results are presented in Table II. Except for the heavy metal salts, the other chemicals used showed no effect on the activity. $HgCl_2$, $CaCl_2$ and $PbCl_2$ showed an inhibitory effect. $HgCl_2$ especially was a potent inhibitory substance. It almost completely inhibited the enzymatic activity at a concentration of $1 \cdot 10^{-4}$ M (Fig. 8). No visible precipitation was formed in the reaction mixture by the addition of these heavy metal salts.

The polarimetric estimation of inulase II activity was based on the assumption that the partially degraded inulin molecules from the formation of difructose anhydride III have the same specific rotation as the original inulin molecule. Though the oligosaccharides produced were not characterized in the present study, they could be assumed to be glucofructans, $[O\text{-}\beta\text{-D-fructofuranose } (2 \rightarrow 1)]_n\text{-D-glucopyranose}$, if pure inulin of the glucofructan type had been used as the substrate. With reference to such oligosaccharides, the following four compounds have been characterized with respect to specific rotation. 1'-β-Fructofuranosyl-sucrose (isokestose), $[\alpha]_D + 25.4°$ (ref. 7); 1'-β-inulobiofuranosyl-sucrose, $[\alpha]_D - 2°$ (ref. 8), $[\alpha]_D + 17.9°$ (ref. 9), $[\alpha]_D + 10.6°$ (ref. 10); 1'-β-inulotetraofuranosyl-sucrose, $[\alpha]_D - 32.6°$ (ref. 11); and

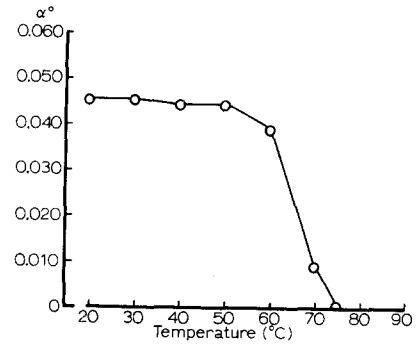

Fig. 5. Thermal stability of inulase II. The Sephadex G-100 eluate was heated at the temperatures indicated for 30 min. After cooling to 20 °C, 1.0 ml of enzyme solution was withdrawn and incubated with a solution composed of 1.0 ml of distilled water, 1.0 ml of 4% inulin and 1.0 ml of 0.2 M acetate buffer (pH 6.0) at 37 °C for 60 min, and the optical rotation was measured.

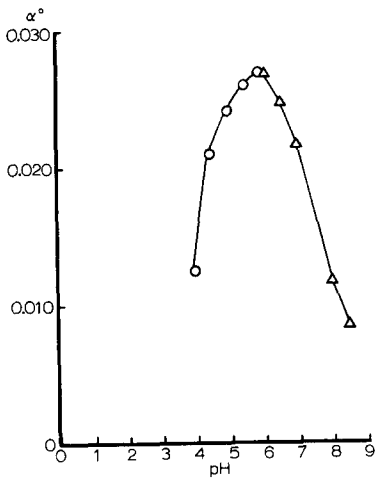

Fig. 6. Effect of pH on inulase II activity. 1.0 ml of the dialyzed enzyme solution prepared as described in the legend attached to Fig. 4 was incubated with a solution composed of 1.0 ml of distilled water, 1.0 ml of 4% inulin and 1.0 ml of buffer at 37 °C for 60 min, and the optical rotation was measured. ○, 0.2 M acetate buffer (pH 4.0 to 6.0); △, 0.066 M phosphate buffer (pH 6.0 to 8.5).

1′-β-inuloheptaofuranosyl-sucrose, $[\alpha]_D$–37.0 ° (refs 12, 13). There seems to be a tendency for the specific rotation to increase as the chain length becomes shorter. The last two compounds show very close specific rotation to that of inulin ($[\alpha]_D$— 40 °). Therefore, if the estimation of enzymatic activity was carried out at an early stage of the degradation of inulin molecules, the observed increase in the rotation represents the real rotatory value of the amounts of difructose anhydride III produced. Fig. 1 shows that the presently described polarimetric method and the reaction conditions were suitable for the determination of the enzymatic activity.

It was surprising that in the Sephadex G-100 gel filtration, the enzyme fractions did not show absorbance at 280 nm. This may be related to a lower content of aromatic amino acid residues in the enzyme molecule in comparison with most proteins.

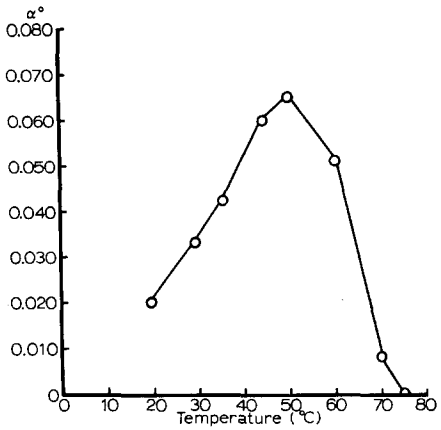

Fig. 7. Effect of temperature on inulase II activity. The reaction mixture composed of 1.0 ml of enzyme solution, 1.0 ml of distilled water, 1.0 ml of 4% inulin and 1.0 ml of 0.2 M acetate buffer (pH 6.0) was incubated for 60 min at the temperatures indicated. The reaction was stopped by the addition of 0.2 ml of 0.021 M $HgCl_2$ solution. The optical rotation expressed was obtained by correction of the observed rotation by the degree of dilution of the original reaction mixture.

TABLE II

EFFECT OF VARIOUS CHEMICALS ON INULASE II ACTIVITY

The reaction mixture composed of 1.0 ml of enzyme solution, 1.0 ml of 0.2 M acetate buffer (pH 6.0), 1.0 ml of 4% inulin, and 1.0 ml of the test solution to be examined, was incubated at 37 °C for 60 min, and the optical rotation was measured

Chemicals	*Concentration (mM)*	*Activity found (α °)*	*(%)*
None		0.046	100
$CaCl_2$	1.0	0.045	98
$CoCl_2$	1.0	0.046	100
$MgCl_2$	1.0	0.046	100
$MnCl_2$	1.0	0.046	100
$ZnCl_2$	1.0	0.046	100
$PbCl_2$	1.0	0.033	73
$CuCl_2$	1.0	0.016	36
$HgCl_2$	1.0	0.000	0
Cysteine	1.0	0.046	100
EDTA	1.0	0.046	100

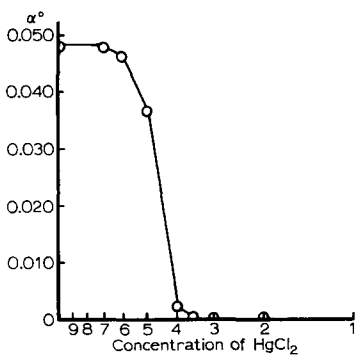

Fig. 8. Effect of $HgCl_2$ concentration (shown as $-\log$ M on the abscissa) on inulase II activity. The reaction mixture composed of 1.0 ml of enzyme solution, 1.0 ml of $HgCl_2$ solution, 1.0 ml of 4% inulin and 1.0 ml of 0.2 M acetate buffer (pH 6.0) was incubated at 37 °C for 60 min, and the optical rotation was measured.

In the previous paper, it was assumed that the inulase II probably acts on inulin to produce difructose anhydride III by an intramolecular transfructosidation reaction. The results obtained on the homogeneity of the purified enzyme by polyacrylamide disc gel electrophoretic studies strongly support this view.

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