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ACTION OF *ARTHROBACTER UREAFACIENS* INULINASE II ON SEVERAL OLIGOFRUCTANS AND BACTERIAL LEVANS

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Summary

1. *Arthrobacter ureafaciens* inulinase II which converts inulin to di-D-fructofuranose 1,2′ : 2,3′ dianhydride (difructose anhydride III) leaving a small amount of oligosaccharides, was investigated in order to characterize its mode of action.

2. After the enzymatic reaction on the glucose-terminated inulin molecules had been completed, the oligosaccharides left in the enzyme digest were isolated, and identified to be the fructose-glucose oligosaccharides; *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside (1-kestose), *O*-β-D-fructofuranosyl-[(2→ 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopyranoside and *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_3$ α-D-glucopyranoside. The difructose anhydride formation from the three fructose-glucose oligosaccharides in the separate reaction system with an increased substrate concentration was observed only with the latter two substrates, but not with the first one.

3. The difructose anhydride formation with several (2 → 1)-β-linked fructose oligosaccharides and bacterial (2 → 6)-β-fructans was examined. The (2 → 1)-β-linked fructose oligosaccharides were effective as substrates for the enzyme with the exception of inulobiose, but the (2 → 6)-β-fructans remained unaffected.

4. It was concluded that the enzyme attacks (2 → 1)-β-linked fructan molecules from the nonreducing fructose ends and requires the presence of at least two adjacent (2 → 1)-β-fructofuranosyl linkages.

Introduction

In the previous papers [1,2], it was reported that a strain of *Arthrobacter ureafaciens* isolated from soil produces an extracellular enzyme which converts inulin to the dianhydride and a small amount of other oligosaccharides. Since the enzyme produces the dianhydride from inulin without producing free D-fructose, it was presumed to be an intramolecular transfructosylase, and was

designated arbitrarily as inulinase II to be distinguished from the common inulinase, β-2,1-fructan fructanohydrolase (EC 3.2.1.7). However, the characterization of the enzymic reaction was incomplete and requires further studies to show the mode of enzyme action.

In the present paper, the following two points are investigated. (1) From which direction does the enzyme attack inulin molecules? For this experiment, existence of a glucose residue in the oligosaccharide molecules left in the enzyme digest of the glucose-terminated inulin molecules was examined. (2) What kind of relationship exists among the substrate molecules available for the enzymic reaction? Clear-cut results on this respect were expected to support the intramolecular transfructosylation reaction of the mode of enzyme action previously proposed [1,2]. For this experiment, several fructose-glucose oligosaccharides with (2 → 1)-β-linked fructose residues, (2 → 1)-β-linked fructose oligosaccharides and some bacterial (2 → 6)-β-fructans (levans) were examined for their availabilities as substrates.

Materials and Methods

Substrates

Inulin; a commercial preparation (Wakō Pure Chemical Co. Ltd,) was used after alkaline treatment according to Rutherford and Deacon [3] to remove the contaminating glucose-free inulin molecules. The inulin obtained was essentially composed of glucose-terminated inulin molecules. The average degree of polymerization, which was determined by estimating the molar ratio of fructose to glucose after acid hydrolysis, was 21.

Inulobiose, inulotriose and inulotetraose were prepared by partial acid hydrolysis of the commercial inulin, 5 g, with 50 ml of 0.2% citric acid at 90° C for 8 min and by fractionation on an active carbon [4] after neutralization with 0.1 M NaOH. The identification was carried out by the estimations of degrees of fructose polymerization and the specific rotations (Table I).

Acetobacter acetigenum and *Acetobacter xylinum* (2 → 6)-β-fructans were obtained through the courtesy of Dr E.T. Reese, U.S. Army Natick Laboratories.

Enzyme

The enzyme used in the previous study [2], which had been stored for 7 months in a refrigerator as solution, was employed to identify the oligosaccharides left in the enzyme digest of inulin. The corresponding enzyme was prepared anew, but the result obtained by the Sephadex G-100 gel-filtration of the acetone precipitate showed large difference not only in the elution pattern (Fig. 1) but also in the specific activity of enzyme. The G-100 eluate was purified in the following way. The fraction indicated by the bar were combined (62.4 units/96 ml), and dialyzed in a cellophane bag under toluene against 10 l of deionized water for 20 h, and lyophilized. The lyophilized material was dissolved in 3 ml of 0.05 M acetate buffer (pH 6.0) and subjected to gel-filtration on a Sephadex G-200 column. The resulting elution pattern of enzyme relative to that of protein is shown in Fig. 2. The fractions indicated by the solid line (14.5 units/66 ml) and dotted line (13.8 units/60 ml) were separately

TABLE I

CHARACTERIZATION OF THE PREPARATIONS OF INULOBIOSE, INULOTRIOSE AND INULOTETRAOSE

0.5 ml of 0.024% fructose oligosaccharide solution was incubated with 0.5 ml 0.2 M acetate buffer (pH 5.0) and 1.0 ml 0.1% β-fructosidase at 30°C for 3 h. The reaction was stopped by heating the reaction mixture in boiling water bath for 3 min. Reducing sugar was measured by the method of Somogyi and Nelson [5].

Probable oligosaccharide	Degree of polymerization	
	Reducing sugar after β-fructosidase hydrolysis/ Reducing sugar before hydrolysis	Total ketohexose/ Reducing sugar before hydrolysis
Inulobiose	2.04	
Inulotriose	2.39	2.86
Inulotetraose	3.55	3.61
	($[\alpha]_D$ of the fructose oligosaccharide in water)	
	Present work	Reported
Inulobiose	−61.2°	−32.5° [6], −72.4° [7] −39.0° [8], −26.3° [9] −26° → −61° [10]
Inulotriose	−57.4°	−41.0° [8], −58.4° [10]
Inulotetraose	−53.6°	−43.7° [10]

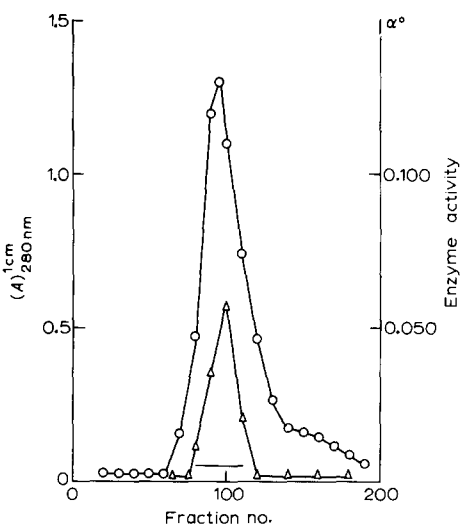

Fig. 1. Gel-filtration of the acetone precipitate on a Sephadex G-100 column. A 50 mg portion of the acetone precipitate was dissolved in 2.0 ml of 0.05 M acetate buffer (pH 6.0), and applied to a Sephadex G-100 column (3.2 × 65 cm, Pharmacia, Uppsala, Sweden; particle size, 40—120 μm) pre-equilibrated with 0.05 M acetate buffer (pH 6.0). The column was eluted with the same buffer, ascending. The flow rate was 10 ml/h, and 3 ml fractions were collected. △———△, enzymatic activity (assayed under the conditions described previously [2]). ○———○, absorbance at 280 nm.

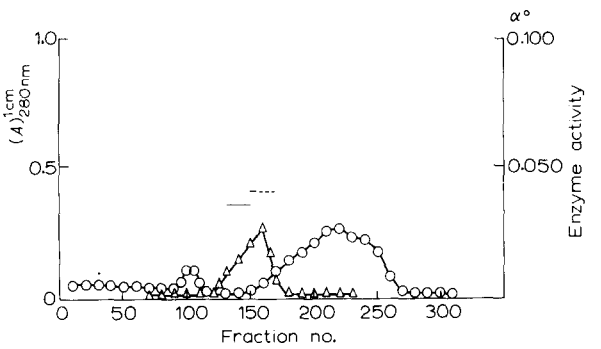

Fig. 2. Gel-filtration of the G-100 eluate on a Sephadex G-200 column. Size of column, 3.2 × 100 cm; ^———^, enzymatic activity; ○———○, absorbance at 280 nm. The elution was carried out in the same way as described in the Fig. 1.

pooled. The specific activity is expressed in units/mg protein [2] of the two fractions was 80 and 64, respectively, and no β-fructosidase activity was found in either fraction. Only the former enzyme fraction was used as Sephadex G-200 eluate for the enzyme assays on oligofructans and bacterial (2 → 6)-β-fructans.

Assay of inulinase II activity on oligosaccharides

The enzyme activity on oligosaccharides was examined by following the change of optical rotation in the reaction mixture due to the formation of the dianhydride [2]. It was composed of 0.5 ml of enzyme solution, 1.0 ml of 6 mM aqueous oligosaccharide solution and 0.5 ml of 0.2 M acetate buffer (pH 6.0), and incubated at 37° C. Estimation of the optical rotation was carried out by using a Jasco DIP-180 type polarimeter with 1.0 dm light path. In controls, the enzyme solution was replaced by the same volume of distilled water, and the enzymic change of the optical rotation was obtained by subtracting the change of the control from that of the complete reaction system at every estimation time.

Sugar assay

Reducing sugar was determined by the method of Somogyi and Nelson [5]. Total ketohexose was determined by the method of Roe [11], and free glucose by using glucose oxidase (Glucostat, Worthington Biochemical Corporation, Freehold, N. J.) according to the method of Papadopoulas [12]. Protein was determined by phenol reagent [13] using bovin serum albumin as standard. β-Fructofuranosidase used for the hydrolysis of oligosaccharides was purchased from Boehringer Mannheim GmbH (Catalogue No. 15067).

Charcoal column chromatography

1 : 2 mixture of Tokusei Shirasagi (Takeda Pharma. Indust. Co. Ltd) and Celite No. 535 (Johns Manville Co. Ltd) was used. Size of the column and its elution were varied by each experiment. The procedures were principally similar to those described earlier [4].

Thin-layer chromatography

Methods for thin-layer chromatography were described earlier [2]. The fructose-glucose oligosaccharides used for comparison were: 1-kestose (kindly supplied by Prof. F. Kurasawa, Department of Agriculture, University of Niigata), *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopyranoside, *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_3$ α-D-glucopyranoside and *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_4$ α-D-glucopyranoside by Prof. M. Kōmoto and Dr H. Tsuchida, Department of Agriculture, University of Kōbe. These four oligosaccharides showed $R_{fructose}$ 0.59, 0.48, 0.37, and 0.35 respectively under conditions used. Part of this hexasaccharide sample was also used for the enzyme substrate.

Results

Isolation and identification of fructose-glucose oligosaccharides left in the inulinase II digest of inulin

Isolation and identification of oligofructan from a glucose-terminated (2 → 1)-β-linked fructose chain, were expected to show from which direction the enzyme acts on the inulin molecule. For this reason, the substrate inulin was purified from reducing fructans by the alkaline treatment of a commercial preparation. When the enzyme (Sephadex G-100 elutate 10.9 units) was incubated with 2% of the purified inulin in 300 ml of 0.1 M acetate buffer (pH 6.0) at 37°C, the optical rotation rose to +0.25° (light path 0.1 dm) in 72 h and remained unchanged for a further 50 h. Examination of reaction products by thin-layer chromatography showed in addition to the spot of the dianhydride ($R_{fructose}$ = 1.38), three oligosaccharide spots with lower mobilities. Each oligosaccharide was isolated by submitting the concentrated mixture to active carbon column chromatography. The column was eluted successively with increased concentrations of ethanol from 5 to 50%. The resulting elution pattern determined for ketohexose is presented in Fig. 3. The fractions of each peak indicated by the bar were combined, concentrated and lyophilized. Thin-layer chromatographic analysis showed that Peak A corresponded to the dianhydride, and Peaks B, C and Peak D to the oligosaccharides described above. Peak D appeared to be contaminated by a trace of Peak C sugar. Peak E and Peak F remained at the origin. Purification of Peak D was performed by rechromatography of an active carbon column (1 × 44 cm) using successive elution with 7.5% ethanol and 10% ethanol. Each sugar sample of B, C and D was purified by dissolving in water and precipitation with methanol. Their yields were B, 32 mg; C, 82 mg; D, 47 mg.

After the complete hydrolysis of each sugar with yeast β-fructofuranosidase, the ratios of reducing sugar to glucose, and ketohexose to glucose were determined. The ratios of B, C, D sugars coincided with the estimates of *O*-β-D fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside (1-kestose), *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopyranoside and *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_3$ α-D-glucopyranoside respectively (Table II). Each specific rotation of the three sugars also agreed approximately with that of *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside, *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$

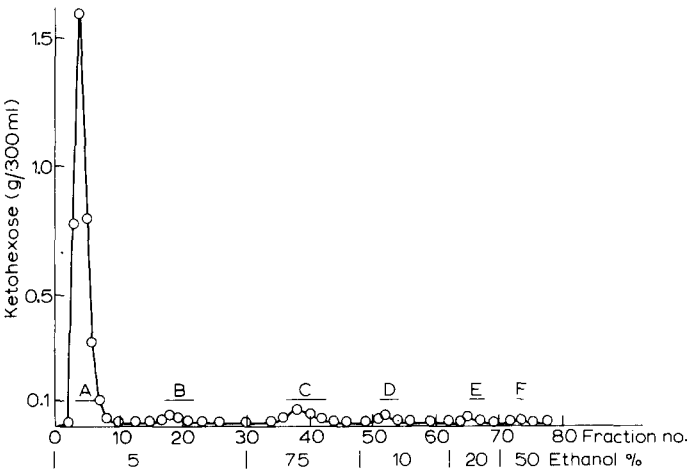

Fig. 3. Separation of the oligosaccharides produced from the purified inulin with active carbon. A 250 ml portion of the heat inactivated reaction mixture was concentrated in vacuo at 40° C to about 50 ml. The resulting solution (containing 4300 mg of ketohexose as D-fructose) was applied to a charcoal column (4.5 × 42 cm) which had been pre-washed with 2 l of 50% ethanol and 3 l of distilled water. After the column had been eluted with 2 l of water, oligosaccharides were eluted with 5% ethanol (9 l), 7.5% ethanol (5.4 l), 10% ethanol (4.2 l), 20% ethanol (2.4 l) and 50% ethanol (1.4 l) successively. The flow rate was 150 ml/h. The effluent was collected in 300-ml fractions. The yields of recovered oligosaccharides were determined by the method of Roe [11] using D-fructose as a standard.

TABLE II

CHARACTERIZATION OF THE FRUCTOSE-GLUCOSE OLIGOSACCHARIDES PRODUCED

Hydrolysis of the fructose-glucose oligosaccharide was carried out as follows. A 3-ml portion of 0.024% sugar solution was incubated with 6 ml of 0.1% β-fructosidase and 3 ml of 0.1 M acetate buffer (pH 5.0) at 30°C for 50 h. The reaction was stopped by heating the solution at 100°C for 3 min. The reducing sugar liberated was determined by Somogyi-Nelson's method [5] using D-fructose as a standard. Glucose was determined by the glucose oxidase method [12] and ketohexose by the method of Roe [11].

Probable fructose-glucose oligosaccharide	Degree of polymerization Total reducing sugar after β-fructosidase hydrolysis/Glucose	Ketohexose/ Glucose
B *O*-β-D-Fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside	3.30	2.17
C *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopyranoside	4.12	3.08
D *O*-β-D-Fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_3$ α-D-glucopyranoside	5.24	3.94

	($[\alpha]_D$ of the fructose-glucose oligosaccharides in water)	
	Enzymic product	Authentic sugar
O-β-D-Fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside	+27.1°,	+25.4° [14] +29.2° [15]
O-β-D-Fructofuranosyl-[(2 → 1)-*O*-β-D-fructofurabosyl]$_2$ α-D-glucopyranoside	+10.4°,	+10.6° [16] +10.0° [18] + 8.9° [17]
O-β-D-Fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_3$ α-D-glucopyranoside	− 1.2°	− 2.3° [19]

TABLE III

YIELDS OF THE OLIGOSACCHARIDES

Amount of each peak is based on the ketohexose content.

Sugar	D-Fructose (mg)	%
Peak A	3666.4	85.5
Peak B	179.4	4.18
Peak C	298.1	6.93
Peak D	78.2	1.82
Peak E	68.8	1.66
Peak F	6.1	0.14
Total	4297.0	

α-D-glucopyranoside and *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D fructofuranosyl]$_3$ α-D-glucopyranoside (Table II).

Though, as mentioned above, some of the separated sugars in the first column chromatography were not chromatographically pure, the amounts calculated on the basis of ketohexose content probably represent their approximate yields (Table III). The total recovery of the sugars accounted for 99.9% of the applied sample as D-fructose. This fact indicates that the whole oligosaccharide components produced from the inulin molecules by the inulinase II actions have been fractionated without loss. Peak A comprised 85% of the original inulin. The sum of the three B, C and D sugars comprised 88% of the residual oligosaccharide fractions.

Inulinase II activities on fructose-glucose oligosaccharides

In the preceding experiment, the main fructose-glucose oligosaccharides left in the inulinase II digest of the purified inulin were found to be *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside and *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopyranoside. However, their concentrations in the reaction mixture were assumed to be very low, so that the susceptibility of these sugars to the enzyme can not be decided precisely. To determine the real limit fructose-glucose oligosaccharide unavailable to the enzyme action, the enzyme activity to several fructose-glucose oligosaccharides including both sugars were examined. These fructose-glucose oligosaccharides used were the enzymic products from the purified inulin, in the preceding experiment, except *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_4$ α-D-glucopyranoside [19] which was a gift of Dr Kōmoto. In these experiments, the molarity of each fructose-glucose oligosaccharide in the reaction mixture was the same as inulin of DP = 21. Inulinase II activities on these oligosaccharides are illustrated in Figs 4 and 5. The *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_4$ α-D-glucopyranoside was attacked by the enzyme at approximately the same rate as inulin in the initial phase, but the reaction rapidly slowed down due to the consumption of the former substrate. The *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_3$ α-D-glucopyranoside and *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopy-

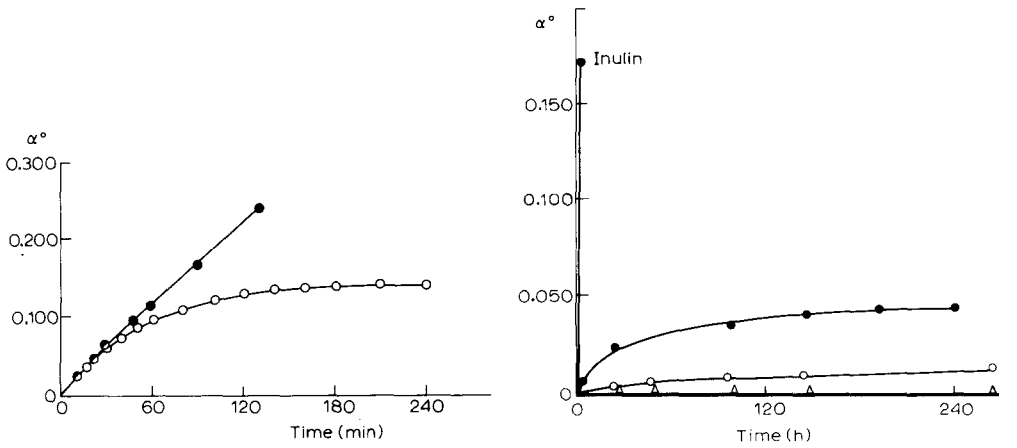

Fig. 4. Time course of the enzyme action on a fructose-glucose oligosaccharide. The reaction mixture composed of 0.5 ml of enzyme solution, 1.0 ml of 6 mM substrate sugar and 0.5 ml of 0.2 M acetate buffer (pH 6.0) was incubated at 37°C for 240 min. The reaction was stopped by heating at 100°C for 3 min. ●——●, inulin; ○——○, *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_4$ α-D-glucopyranoside.

Fig. 5. Time course of the enzyme action on fructose-glucose oligosaccharides. Reaction conditions were the same as described in Fig. 4, except that the time of reaction was varied. ●——●, *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_3$ α-D-glucopyranoside; ○——○, *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D fructofuranosyl]$_2$ α-D-glucopyranoside; △——△, *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl-α-D-glucopyranoside.

ranoside were attacked very slowly, but *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside remained unaffected in the reaction mixture after 264 h incubation. After the enzyme reaction had been reached to the final equilibrium, the reaction mixture was heated in a boiling water bath for 3 min to stop the reaction, and the products were examined by thin-layer chromatography after treatment with ion-exchange resins (Amberlite IR-120 B H-form and IR-45 OH-form). The resulted chromatogram showed that the oligosaccharides produced in the dianhydride formation from the hexasaccharide, the pentasaccharide and the tetrasaccharide were *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopyranoside, *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside and sucrose respectively, but no products were obtained from *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D fructofuranosyl α-D-glucopyranoside.

Inulinase II activities on (2 → 1)-β-linked fructose oligosaccharides

Reactivity of inulinase II on (2 → 1)-β-linked fructose oligosaccharides was similarly estimated using inulobiose, inulotriose nad inulotetraose as the substrates. Reactivity of enzyme on inulotetraose was very similar to that of the *O* β-D-fructofuranosyl-[(2 → 1)-*O*-β-D fructofuranosyl]$_4$ α-D-glucopyranoside of fructose-glucose oligosaccharide type, that is, it was attacked by the enzyme at the apparently the same of rate as inulin during the initial phase (Fig. 6). Inulotriose was also attacked by the enzyme very slowly but inulobiose was resistant to the enzymic action (Fig. 7). Thin-layer chromatography of the reaction mixtures showed that, when the tetraose (R_{fructose} = 0.38) was used

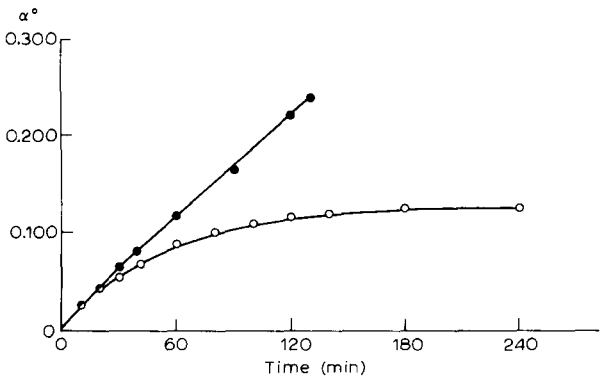

Fig. 6. Time course of the enzyme reaction on inulotetraose. Reaction conditions were the same as described in Fig. 4. ●———●, inulin; ○———○, inulotetraose.

as the substrate, the formation of the dianhydride and inulobiose ($R_{fructose}$ = 0.64) were observed, while in the case of inulotriose ($R_{fructose}$ = 0.50), the formation of the dianhydride and free fructose were observed. In the latter case, an additional spot ($R_{fructose}$ = 0.81) which moved slightly faster than sucrose ($R_{fructose}$ = 0.77) was detected on the chromatogram. No products were detected from inulobiose.

Action of inulinase II on bacterial (2 → 6)-β-fructans

Acetobacter acetigenum and *Acetobacter xylinum* (2 → 6)-β-fructans were subjected to the action of the enzyme. If the (2 → 6)-β-fructans can be attacked by the inulinase II, the formation of another dianhydride involving a (2 → 6)-β-linkage in the molecule would be expected. In the reaction mixture, the final concentration of (2 → 6)-β-fructans was 0.15%, similar to the experiment of Loewenberg and Reese [20] for the (2 → 6)-β-fructosanase assay. The results showed that the enzyme was inactive against these (2 → 6)-β-fructans by estimation of optical rotation as well as thin-layer chromatographic methods.

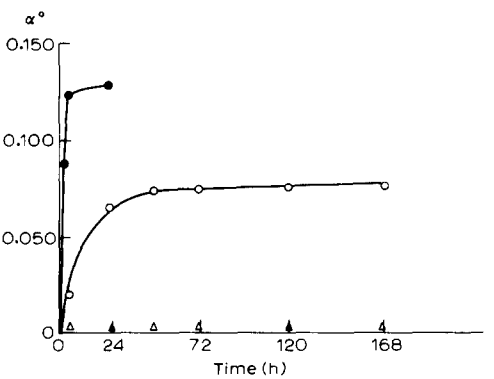

Fig. 7. Time course of the enzyme reaction on inulotetraose, inulotriose and inulobiose. Reaction conditions were the same as described in Fig. 4, except the time of reaction was varied. ●———●, inulotetraose; ○———○, inulotriose; △———△, inulobiose.

Discussion

The gel-filtration pattern of the enzyme preparation of acetone precipitate on Sephadex G-100 was different from the result reported previously, and was of lower specific activity [2]. Further purification of this preparation by gel-filtration with Sephadex G-200, did not increase the specific activity beyond that of the previous Sephadex G-100 eluate. The yield of the acetone precipitate from 2 g of the $(NH_4)_2SO_4$ precipitate, varied from 25 mg to 75 mg but the recoveries of enzyme activity were almost constant [2]. This fact suggests that the variation in the specific activity depends not on the $(NH_4)_2SO_4$ contamination in the crude enzyme as described previously [2], but on some variation in the proteins formed in the cultured medium. The different elution pattern of protein of the Sephadex G-100 gel-filtration from the previous one is probably due to this variation. Prior to the gel-filtration on Sephadex G-200, the G-100 eluate was lyophilized after dialysis against deionized water. The G-100 eluate was found to have lost 30% of the original activity after freezing and subsequente thawing at room temperature. In fact, the yield of enzyme after Sephadex G-200 gel-filtration was only 45%. These finding suggests that the low specific activity of the Sephadex G-200 eluate probably depends not on the contaminated protein but is largely due to partial inactivation of the enzyme by the lyophilization. However, some of the present experiments were performed using this enzyme preparation, and its substrate specificity is thought to be essentially the same with that of the native enzyme.

When the inulin molecules with a glucose residue at the reducing end of (2 → 1)-β-linked fructose chain were reacted with inulinase II, a small amount of oligosaccharides accompanied the dianhydride formation. These oligosaccharides all contained a glucose residue and the major components were identified as *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside and *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopyranoside. From these results it is clear that the inulinase II attacks the inulin molecules from their non-reducing fructose ends as an exo-enzyme. Reactivity of the enzyme toward these fructose-glucose oligosaccharides was examined with their concentrated solutions in the separate reaction system. The results showed that *O*-β-D-fructofuranosyl-[(2 → 1)-*O*-β-D-fructofuranosyl]$_2$ α-D-glucopyranoside as well as the other fructose-glucose oligosaccharides with a longer (2 → 1)-β-linked fructose chain length could be utilized as the substrate, but *O*-β-D-fructofuranosyl-(2 → 1)-*O*-β-D-fructofuranosyl α-D-glucopyranoside was not attacked. The enzyme activity toward several (2 → 1)-β-linked fructose oligosaccharides showed that inulotriose as well as other (2 → 1)-β-fructose oligosaccharides with a longer chain length could be utilized for the formation of the dianhydride, but inulobiose was not attacked.

These results together with the result of fructose-glucose oligosaccharides indicates that, the compounds containing at least three (2 → 1)-β-linked fructofuranose residues are essential for the substrate of the enzyme. This common feature of the substrates available for the action strongly supports that the reaction catalyzed by the enzyme is an intramolecular transfructosylation reaction. This conclusion can be also supported by the fact that the reaction systems used in the experiments are free from any energy-rich compounds such

as ATP, sugar nucleotides and phosphorylated sugars. The energy used for the new formation of a (2 → 3)-fructofuranosyl linkage in the dianhydride molecule would be supplied from the second (2 → 1)-β-fructofuranosyl linkage of the substrate itself.

When the enzyme was reacted with inulotriose, a disaccharide-like product, which differs from inulobiose in the mobility on the thin-layer chromatogram, was detected in addition to the dianhydride. This compound was hydrolyzed to free fructose by yeast β-fructofuranosidase. The sugar could not be detected in any other digest of the oligosaccharides. The previous results on inulobiose characterization have been confused. Several inulobiose samples obtained from partial hydrolyzates of inulin or aqueous-ethanol extract of Jerusalem artichoke tubers were considerably different from one another with respect to their specific rotation in water (Table I). Identification of this sugar which is under study, will provide a further important finding with regard to the mechanism of the inulinase II action.

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References

1 Tanaka, K., Uchiyama, T. and Ito, A. (1972) Biochim. Biophys. Acta 284, 248—256
2 Uchiyama, T., Niwa, S. and Tanaka, K. (1973) Biochim. Biophys. Acta 315, 412—420
3 Rutherford, P.P. and Deacon, A.C. (1972) Biochem. J. 129, 511—512
4 Putman, E.W. (1957) in Methods in Enzymology (Colowick, S.P. and Kaplan, N.O., eds), Vol. 3, pp. 57—61, Academic Press, New York
5 Nelson, N. (1944) J. Biol. Chem. 153, 375—380
6 Pazur, J.H. and Gordon, A.L. (1953) J. Am. Chem. Soc. 75, 3458—3460
7 Schlubach, H.H. and Scheffler, A. (1954) Ann. 588, 192—194
8 Feingold, D.S. and Avigad, G. (1956) Biochim. Biophys. Acta 22, 196—197
9 Strepkov, S.M. (1959) Doklady Akad. Nauk. S.S.S.R. 124, 1344—1346
10 Mizuno, T. (1961) Report of the Faculty of Agriculture, Shizuoka University No. 11, 121—128
11 Roe, J.H., Epstein, J.H. Goldstein, N.P. (1949) J. Biol. Chem. 178, 839—845
12 Papadopoulas, N.M. and Hess, W.C. (1960) Arch. Biochem Biophys. 88, 167—171
13 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
14 Kurasawa, H., Yamamoto, Y., Igaue, I. and Nakamura, Y. (1956) J. Agric. Chem. Soc. Jap. 30, 624—630
15 Barker, S.A. and Carrington, T.R. (1953) J. Chem. Soc. 3588—3593
16 Binkley, W.W. and Altrnburg, F.W. (1965) Int. Sugar J. 67, 110—112
17 Tsuchida, T., Fujii, S. and Kōmoto, M. (1966) Agric. Biol. Chem. Jap. 30, 429—433
18 Kamerling, J.P., Vliegenthart, J.F.G., Kahl, W., Roszkowski, A. and Zurowska,. (1972) Carbohydr. Res. 25, 293—297
19 Kōmoto, M. and Tsuchida, H. (1968) Seitōgijitsukenkyu-kaishi 20, 29—38
20 Loewenberg, L.R. and Resse, E.T. (1957) Can. J. Microbiol. 3, 643—650