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FORMATION OF DI-D-FRUCTOFURANOSE 1,2′:2,3′ DIANHYDRIDE FROM INULIN BY AN EXTRACELLULAR INULASE OF *ARTHROBACTER UREAFACIENS*

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SUMMARY

1. A strain of *Arthrobacter ureafaciens* isolated from soil was shown to produce an extracellular enzyme which degrades inulin to a difructose anhydride and a small amount of oligosaccharides. The products resulting from enzymic hydrolysis are different from those produced by the common inulase (β-2,1-fructan fructanohydrolase, EC 3.2.1.7) of the β-fructofuranosidase type.

2. Characterization and identification of the difructose anhydride indicated that it corresponds to di-D-fructofuranose 1,2′:2,3′ dianhydride, difructose anhydride III, described by Jackson and McDonald in 1931 (*Bur. Stand. J. Res.*, 6 (1931) 709).

INTRODUCTION

Inulases (β-2,1-fructan fructanohydrolase, EC 3.2.1.7) from yeast[1], fungi[2,3] and higher plants[4,5] are known to be β-fructofuranosidases which split off the terminal fructose units from the inulin molecule. However, considering the wide distribution of inulin and related substances as carbohydrate reserves in plant tissues, and their efficient degradation, the existence of other types of inulase could be expected. We have tried to isolate bacterial strains which produce such an inulase. The present paper reports that a strain of *Arthrobacter ureafaciens* isolated from soil produces an extracellular enzyme which efficiently converts inulin to a difructose anhydride. As the first paper in a series on this enzyme, this paper describes the characterization and identification of the difructose anhydride.

MATERIALS AND METHODS

Cultivation

The strain used for the test was isolated from soil and identified as a strain of *A. ureafaciens*. The solid agar culture medium used for the isolation and storage of

bacteria was composed of 15 g inulin, 2 g $NaNO_3$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, a trace of $FeCl_3$, and 0.5 g KH_2PO_4 per l of distilled water, adjusted to pH 7 with 2 M NaOH. The liquid culture medium for the enzyme preparation had approximately the same composition as mentioned above with the exception of a lower inulin concentration (10 g per l). A loop of cultured bacteria was inoculated into a 150 ml aliquot of the medium in a 500 ml Rox flask and grown at 30 °C for 8 days.

Enzyme preparation

The cultured medium was centrifuged at 6500 × *g*, 5 °C, for 15 min to remove the bacteria, and the supernatant obtained was adjusted to pH 7 (bromothymol blue) with 0.2 M NaOH. Solid ammonium sulfate was added to a 1-l aliquot of the combined solution to 65% saturation, with continuous stirring, and the mixture was kept overnight in a refrigerator. The precipitate formed was collected by filtration on a Buchner funnel after the suspension had been mixed with 15 g hyflo super-cel. Enzyme was extracted by suspending the cake in 50 ml water and shaking the mixture for 15 min. After filtration, the residue was washed with 20 ml distilled water. The combined solution of filtrate and wash was dialyzed in a cellophane bag under toluene against 10 l of deionized water for 24 h, and lyophilized. Yields were 65 to 100 mg.

Isolation of difructose anhydride

Prior to experiments, the enzyme preparation described above was dissolved in 0.2 M acetate buffer (pH 6.0) and the mixture was heated at 65 °C for 10 min to inactivate a contaminating β-fructofuranosidase (EC 3.2.1.26). The reaction mixture containing 2% inulin and 30 mg of enzyme in 100 ml of 0.1 M acetate buffer (pH 6.0), was incubated at 30 °C under toluene for 6 days. The reaction was stopped by heating at 100 °C for 5 min. The reaction mixture was concentrated to about 20 ml at 40 °C under reduced pressure. To remove any fermentable sugars produced during the enzymic reaction, the mixture was subjected to a yeast fermentation. For this purpose, a 10 g portion of baker's yeast was washed with distilled water and then suspended in 50 ml distilled water. After incubation of the reaction mixture with the yeast suspension at 37 °C for 2 h, the yeast cells were removed by centrifugation. The supernatant was concentrated to 10 ml under reduced pressure. Isolation of difructose anhydride from the yeast digest was carried out by charcoal column chromatography (2.5 cm × 45 cm, with a mixture of 60 g celite No. 535 and 30 g Darco G-60). Prior to chromatography, the column was washed successively with 1 l of 50% ethanol and 1.5 l distilled water. After the yeast digest had been applied, the column was washed with 1.8 l distilled water and eluted with 5% (v/v) ethanol. The flow rate was 100 ml/h, and 15 ml-fractions were collected. The ketohexose content of the fractions was determined by the resorcinol–HCl method[6]. The fractions containing the difructose anhydride were combined and the solution (about 400 ml) was concentrated to about 15 ml at 40 °C under reduced pressure. Lyophilization yielded about 400 mg white solid material.

Moisture determination

Unless otherwise stated, samples of sugar and the derivatives were dehydrated *in vacuo* at 60 °C over P_2O_5 for 6 to 8 h until constant weights were obtained.

Chemical treatments

Acetylation of difructose anhydride. A solution of 2 ml acetic anhydride, 3 ml pyridine, and 200 mg of the dried sugar sample was kept at 0 °C for 24 h. Crushed ice was added with shaking, and the white viscid acetate was obtained. The mixture was allowed to stand for a few hours, thereafter the liquid layer was removed by decantation. The residual acetate was taken up in 5 ml chloroform, and the solution was washed with distilled water. The chloroform layer was dried over $CaCl_2$. After removal of $CaCl_2$ by centrifugation, chloroform was removed by evaporation under reduced pressure. The resulting colorless syrup was dried *in vacuo* at 40 °C over P_2O_5.

Methylation of sugars. Methylation was carried out as described by Hakomori[7] with the exception that the reaction time of the carbanion was prolonged for 4 to 5 h. Methylation of inulin was carried out under the same conditions, but the methylation procedure was repeated once more to insure complete methylation.

Hydrolysis of methylated sugars. Methylated difructose anhydride was dissolved in 72% sulfuric acid with cooling in an ice-water bath. The solution was then allowed to stand at room temperature for 1 h, and was diluted to 4% with regard to sulfuric acid concentration. The diluted solution was heated at 95 °C for 4 h, and neutralized with a saturated barium hydroxide solution. The precipitate of barium sulfate was removed by filtration and washed with water and ethanol. The combined solution of filtrate and wash was concentrated to a syrup under reduced pressure at 40 °C. Other methylated sugars used for comparison were hydrolyzed under the same conditions with the exception that the methylated sugar–72% sulfuric acid mixtures were diluted to 8% as to the acid concentration, and heated in boiling water for 4 h.

Tritylation of difructose anhydride. Tritylation was performed by the method of McDONALD AND JACKSON[8], yielding white crystalline needles of the tri-trityl ether. Recrystallization was repeated twice from a methanol–acetone mixture.

Preparation of difructose anhydride III by acid hydrolysis of inulin

The preparation of difructose anhydride III was carried out as described by Jackson and McDonald[9]. The crystallization of difructose anhydride II from the mixture of difructose anhydride II and III could not be accomplished in our hands. The isolation of difructose anhydride III was performed by ascending thin layer chromatography, using Avicel SF plates. The developing solvent was *n*-butanol–acetone–water (4:5:1, v/v/v)[10]. After a third development with the same solvent, plates were dried at room temperature. The cellulose powder of the area containing the corresponding sugar was scraped off, and extracted with water. The extract was concentrated to a small volume by evaporation under reduced pressure, and lyophilized. The dried material was taken up in a small volume of absolute ethanol. A small insoluble portion was removed by centrifugation, and the supernatant was lyophilized to dryness.

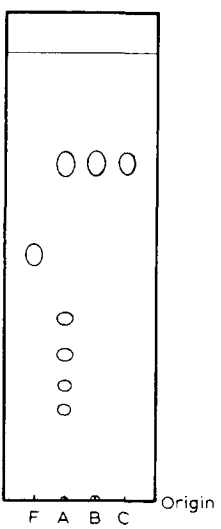
Chemicals

Inulin used for the culture media was purchased from Wako Pure Chemicals Co., and that for the enzyme reactions was obtained from Nakarai Chemicals Ltd. Reference sugars used for paper chromatography and Bio-Gel filtration and the standard sugars for methylation studies were of the highest purity grades commercially available.

RESULTS AND DISCUSSION

Isolation of difructose anhydride

Reaction products of the action of the bacterial enzyme on inulin were examined by paper chromatography, using alkaline silver nitrate as a spray reagent, and fructose as a reference sugar. As indicated in Fig. 1, in addition to spots of oligosaccharides, a spot showing a high migration, compared to fructose, was detected. When sprayed with the alkaline silver nitrate reagent[12], the reaction of this fast moving material was initially weak, but after a prolonged time on standing at room temperature, it became markedly strong. In order to isolate the corresponding substance, charcoal column chromatography was performed, using a 5–15% ethanol gradient system. When checked by the resorcinol test, elution of a large peak containing the main product, was found to begin at about 5% ethanol concentration. The oligosaccharides eluted shortly thereafter (Fig. 2). Fractions from the peak were



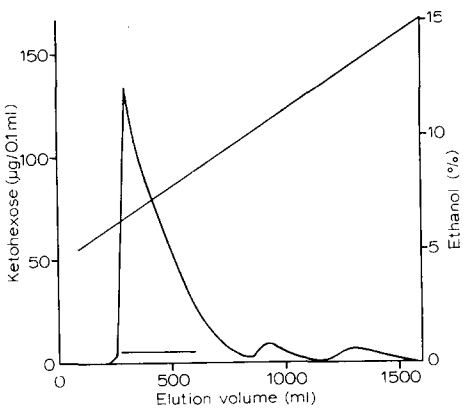


Fig. 1. Diagrammatic illustration of a paper chromatogram showing the enzymic formation of a difructose anhydride from inulin and its isolation by the procedures of yeast fermentation and rechromatography on a charcoal column. Toyo filter paper No. 51 A. Solvent system, *n*-butanol–pyridin–water (6:4:3, v/v/v)[11], ascending. Spray reagent, alkaline silver nitrate reagent. F, D-fructose; A, reaction products in the enzyme digest of inulin; B, yeast-fermented reaction mixture; C, purified sample obtained after rechromatography on a charcoal column of Sample B.

Fig. 2. Separation of the main enzymic product by column chromatography on active carbon. Chromatography was carried out in the same way as described in the text, with the exception of the use of a 5–15% ethanol gradient system as the eluent. Ethanol concentration was measured by estimation of specific gravity. Total ketohexose was determined by the resorcinol method using D-fructose as a standard. The fractions indicated by the bar were combined, concentrated, and submitted to yeast fermentation.

combined and treated with baker's yeast in order to remove oligosaccharides. Yeast fermentation completely removed the oligosaccharides produced by the inulase hydrolysis. However, a contaminant was formed during the fermentation process. On paper chromatography the contaminant remained at the origin (Fig. 1). Purification

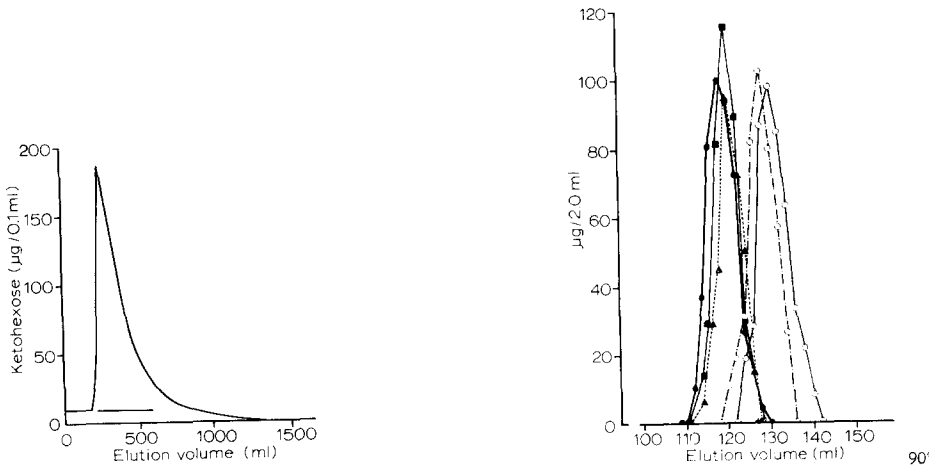


Fig. 3. Isolation of the enzymic product by charcoal column chromatography. (The details are given in the text.) The fractions indicated by the bar were combined, concentrated, and lyophilized.

Fig. 4. Determination of the degree of polymerization of the enzymic product by filtration on Bio-Gel P-2. Column, Bio-Gel P-2 200–400 mesh (1.5 cm × 90 cm); eluent, water; flow rate, 10 ml/h. 2 ml-fractions were collected. 1.0 ml solution containing 500 μg of each sugar was applied. Determination of sugar was carried out by the resorcinol–HCl method for D-fructose (○—○), and enzymic product (●—●), and by the anthrone–H_2SO_4 method[13] for D-glucose (○·-·○), maltose (▲---▲), and sucrose (■—■).

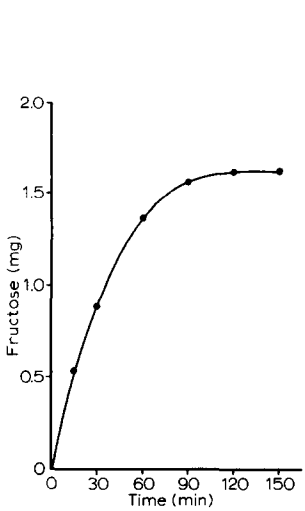
of the yeast-fermented sample was performed by charcoal chromatography using 5% ethanol as the eluent. The dianhydride was obtained in a chromatographically pure form (Fig. 1). However, most of the difructose anhydride used for identification was prepared as described before, that is, the enzymic digest of inulin was concentrated, and subjected to the yeast fermentation, followed directly by charcoal column chromatography, using 5% ethanol as the eluent (Fig. 3).

The fact that the main product was eluted at 5% ethanol concentration suggested that it might be a disaccharide. Its degree of polymerization was determined by gel-filtration on a Bio-Gel P-2 (Bio-rad Laboratories Calif.) column, using glucose, fructose, sucrose and maltose as reference sugars. The resulting patterns are shown in Fig. 4. The results strongly suggest that the sample sugar has the properties of a disaccharide. Analysis of the sugar was carried out after dehydration at 65 °C *in vacuo* over P_2O_5 for 20 h. It had: C, 44.10; H, 6.13; $C_{12}H_{20}O_{10}$ requires C, 44.45; H, 6.20. The sugar had no reducing power and was hydrolyzable up to 82% by 0.1 M HCl at 100 °C for 150 min (Fig. 5). Paper chromatography of the acid hydrolyzate showed that it consisted solely of D-fructose (Fig. 6). In addition, we identified the phenylosazone of the hydrolyzed product, as that of D-fructose, m.p. 202 °C, (found: C, 60.21; H, 6.30; N, 15.71; $C_{18}H_{22}N_4O_4$ requires C, 60.32; H, 6.19; N, 15.63).

The data described above indicate that the extracellular enzyme of this bacterial strain produces a difructose anhydride as the main product from inulin.

Identification of difructose anhydride

With reference to difructose anhydrides, the following seven compounds have been reported; difructose anhydride I, di-D-fructofuranose 1,2′:2,1′ dianhydride[15]



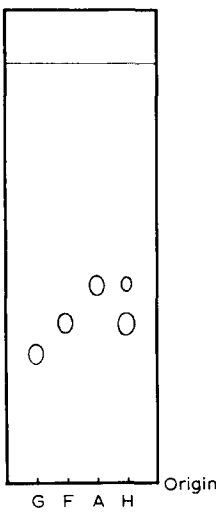


Fig. 5. Acid hydrolysis of the enzymic product. A 14 mg portion of the enzymic product was dissolved in 7.0 ml of 0.1 M HCl solution, and heated at 100 °C. After hydrolysis for 15 to 30 min intervals, 1.0 ml aliquots were withdrawn, neutralized with 0.1 M NaOH, and the reducing sugar formed was determined by Somogyi's reagent[14].

Fig. 6. Diagrammatic representation of a paper chromatogram showing formation of D-fructose in the acid hydrolyzate of enzymic product. Chromatography was carried out as described in Fig. 1, with the exception that *n*-butanol–ethanol–water (13:8:4, v/v/v)[12] was used as the solvent system. G, D-glucose; F, D-fructose; A, enzymic product; H, acid hydrolyzate of the enzymic product.

TABLE I

POLARIMETRIC PROPERTIES OF DIFRUCTOSE ANHYDRIDES AND THEIR DERIVATIVES

Accompanied by melting point of tri-trityl difructose anhydride III. (W), in water; (C), in chloroform.

Trivial name	[α]D		
	Free sugar	*Hexa-methyl*	*Tri-trityl*
Difructose anhydride I	+ 27° (W)[15]	+ 50.4° (W)[8] + 23.7° (C)[8]	
Difructose anhydride II	+ 14° (W)[9]	+ 6.0° (W)[8] − 28.2° (C)[8]	
Difructose anhydride III	+136° (W)[9]	+164.5° (W)[8] +157.9° (C)[8]	+ 64.2° (C)[8] m.p. 127°C[8]
Diheterolevulosan I	− 46° (W)[18]	− 49° (C)[22]	
Diheterolevulosan II	− 39° (W)[18]	− 22.5° (C)[21]	
Diheterolevulosan III	−179° (W)[19]		
Diheterolevulosan IV	−309° (W)[20]	−243° (C)[20]	

difructose anhydride II, di-D-fructofuranose 2,1′:4,2′ dianhydride[9,16]; difructose anhydride III, di-D-fructofuranose 1,2′:2,3′ dianhydride[8,9]; diheterolevulosan I, di-D-fructopyranose 1,2′:2,1′ dianhydride[17]; diheterolevulosan II, D-fructopyranose-D-fructofuranose 1,2′:2,1′ dianhydride[18]; diheterolevulosan III, di-D-fructopyranose 1,2′:2,3′ dianhydride[19] and diheterolevulosan IV, anomer of diheterolevulosan I[20]. However, difructose anhydride II and diheterolevulosan III are considered probably

to be anomers of difructose anhydride III and diheterolevulosan II respectively[19]. Polarimetric properties of these free difructose anhydrides and their derivatives are summarized in Table I.

The enzymic product had $[\alpha]_D^{15}$ + 123.8° (*c* 3.19 in water) close to the value described for difructose anhydride III (+136°). This slightly lower specific rotation was checked by comparison with difructose anhydride III which was prepared from inulin after acid hydrolysis[9], +126.0° (*c* 2.34 in water). Our low values presumably depend on incomplete dehydration conditions in the moisture determination. Further identification of the enzymic product was carried out by preparing the hexa-acetate, hexamethyl, and tri-trityl derivatives, and by comparing their $[\alpha]_D$, and melting point of the last compound with the results reported.

The hexa-acetate of the enzymic product could not be crystallized from common solvents, and was obtained as a liquid showing $[\alpha]_D^{30}$ + 82.8° (*c* 1.21 in ethanol), (found: C, 49.76; H, 5.69; $C_{24}H_{32}O_{16}$ requires C, 49.99; H, 5.59).

For preparation of the hexamethyl derivative, a 189 mg of the sample difructose anhydride was methylated; the yield of methylated product being 177 mg. The liquid product had $[\alpha]_D^{15}$ + 161.9° (*c* 0.82 in water), +156.8° (*c* 1.48 in chloroform); (found: C, 52.78; H, 8.15; $C_{18}H_{32}O_{10}$ requires C, 52.93; H, 7.90). McDonald and Jackson have shown[8] that the hexamethyl derivative of difructose anhydride III produced from inulin by acid hydrolysis, showed $[\alpha]_D^{20}$ + 164.5° (*c* 2.23 in water) and $[\alpha]_D^{20}$ + 157.9° (*c* 3.95 in chloroform).

The tri-trityl derivative of the enzymic product had m.p. 127 °C; $[\alpha]_D^{29}$ + 65.0° (*c* 1.20 in chloroform).

Hydrolysis of the hexamethyl ether and the identification of the hydrolyzed products by thin layer chromatography were carried out. Fig. 7 indicates that two methyl esters produced from the hexamethyl derivative of the difructose anhydride

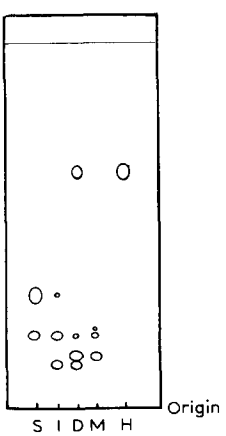


Fig. 7. Identification of the hydrolyzed products of the methylated difructose anhydride by thin layer chromatography. A silica gel plate (Spotfilm Tokyo Kasei). Solvent system, a top layer of the mixture of benzene–ethanol–water–ammonium hydroxide (spec. gravity 0.9) (200:47:15:3.76, by vol.) ascending, modified as to the concentration of ammonia[23]. Spray reagent, naphthoresorcinol–H_2SO_4 reagent[24]. S, hydrolyzed products of methylated sucrose. I, hydrolyzed products of methylated inulin. M, hydrolyzed products of methylated melezitose. D, hydrolyzed products of methylated sample sugar. H, hexamethyl derivative of sample sugar.

correspond to 3,4,6-trimethyl fructose and 1,4,6-trimethyl fructose which were prepared from methylated inulin and melezitose respectively.

These properties of the free and derivative forms of the enzymic product indicate that the difructose anhydride was identical with di-D-fructofuranose 1,2′: 2,3′ dianhydride, difructose anhydride III.

The results described so far indicate that the present enzyme produces difructose anhydride III as the main reaction product from inulin molecules; the product was different from that normally obtained by the action of the common inulase. Since the purification of the enzyme is incomplete, it is not clear whether the reaction is catalyzed by one enzyme component or by the collaboration of different enzyme components. However, we presume that the present enzyme probably acts on inulin to form difructose anhydride III by means of an intramolecular transfructosidation reaction. We arbitrarily designate the common inulase as inulase I and the present inulase as inulase II.

Schlubach and Knoop[25] suggested the existence of difructose anhydride III in the tubers of Jersalem artichoke, but reported that the sugar could not be obtained in a pure state in spite of a variety of different operations. A role for the present enzymic reaction in the formation of difructose anhydride III in plant tissue can be expected.

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