Some Paper-Chromatographic Studies with *Aspergillus niger* '152' Transfructosylase

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(*Received* 8 *November* 1957)

The transfructosylase of *Aspergillus niger* '152' normally acts on sucrose to give trisaccharides [mainly O-α-D-glucopyranosyl-(1 → 2)-O-β-D-fructofuranosyl (1 → 2)-β-D-fructofuranoside], glucose and fructose (Barker, Bourne & Carrington, 1954). In the presence of streptomycin it was shown that although the formation of the trisaccharide was virtually unaffected the ratio of fructose to glucose was markedly reduced (Barker, Bourne, Stacey & Ward, 1957). Since these results indicated that more than one enzyme (e.g. a sucrose hydrolase as well as a transfructosylase) was present, attempts were made to separate the enzymes by paper chromatography. The separation of the enzyme fraction into two activity bands and the unusual properties associated with them are discussed below.

EXPERIMENTAL

Enzyme-extract preparation. The extract (100 ml.) was prepared from freeze-dried *A. niger* '152' mycelium (3 g.) by the method of Barker & Carrington (1953). The extract was dialysed against running tap water for 48 hr. at room temperature, freeze-dried and redissolved in water (10 ml.). This dialysis before chromatography was necessary to remove small molecules (e.g. sugars) which appeared to impede the formation of discrete bands and caused high blank values in the activity assays.

Enzyme chromatography. The enzyme solution (0·2 ml.) was streaked across the base line of Whatman no. 1 paper (35 cm. × 15 cm.) and dried at room temperature, except where otherwise stated. The chromatograms were irrigated with water at 0° [20% and 50% (v/v) aqueous acetone were found to give similar separations] until the solvent front was 3–4 cm. from the lower edge (about 6 hr.). On assay (see below) the activity was found to be concentrated into two bands. The faster band was readily isolated by irrigating for double the period used above and freeze-drying the eluate in the presence of 0·05M-citrate buffer (pH 6·8; 1 ml.).

Activity assay. The chromatogram was dried at 0° and cut into twelve horizontal strips, each 2·5 cm. wide. Each strip was incubated at 30° for 24 hr. with 10% sucrose solution (5 ml.). The amount of activity present was ascertained by determination of the total reducing power liberated (Shaffer & Hartmann, 1920) and expressed in mg. of 'glucose'/5 ml. of digest. Blank incubations omitting first the paper strip and then the sucrose were also made.

RESULTS

The results of a typical separation (chromatogram 1) carried out as described above with retention of the faster-moving band on the paper are given in Table 1. Further separations were carried out with the following modifications; the results are given in Table 1.

Irrigation was commenced (*a*) without previously drying the enzyme on the paper (chromatogram 2), (*b*) after drying at 45° (chromatogram 3)

Table 1. *Activity distribution on chromatograms* 1–9

Activity is expressed as milligrams of reducing sugar liberated from sucrose (see Experimental section). Details of the chromatograms are given in the Results section. The arrow indicates the direction of flow.

Strip no.	Chromatogram no. 1	2	3	4	5	6	7	8	9
1	4·5	0·5	4·4	2·4	−0·2	1·5	3·9	0·3	8·1
2	2·1	0·6	2·2	0·4	0·2	0·8	2·0	0·4	3·2
3	1·3	0·5	0·6	0·5	0·3	0·4	1·5	0·6	1·4
4	1·1	0·3	0·4	0·8	−0·1	0·3	1·0	0·5	0·8
5	0·8	0·6	0·3	0·6	0·1	0·3	0·8	0·8	0·6
6	0·9	0·6	0·6	0·4	−0·2	0·3	0·5	0·7	0·6
7	1·0	0·5	0·8	1·0	−0·2	0·3	0·4	0·9	0·9
8	1·7	0·8	2·6	2·4	Nil	0·5	0·3	1·5	1·5
9	2·8	5·1	3·3	2·5	−0·1	1·0	0·4	8·3	7·2
10	8·1	11·3	8·1	8·1	Nil	3·0	0·6	10·5	16·3
11	3·4	4·1	2·4	4·6	0·1	0·7	0·6	4·1	3·1
↓ 12	Nil	0·2	0·2	0·1	Nil	0·1	0·7	0·2	Nil

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and (*c*) after drying at 0° (chromatogram 4). In chromatogram 5, developed after drying the enzyme on the paper at room temperature, each strip was heated with the sucrose solution at 95° for 30 min. before incubation and determination of activity. In chromatogram 6 the enzyme was applied and dried at room temperature; two-thirds of the enzyme had been previously inactivated by heating at 95° for 30 min. A chromatogram, in which the enzyme had been dried at room temperature on the paper, was run for a longer period and the faster-running fraction eluted. The activity remaining on the paper is shown in the results for chromatogram 7. The eluates from two such papers were re-applied to another paper, dried at room temperature and irrigated with retention of the faster-moving fraction (chromatogram 8). Chromatogram 9 was obtained by collecting the eluates from two longer-running chromatograms on which the enzyme had been dried at 0° before irrigation, and re-applying them to another paper, drying at room temperature and irrigating with retention of the faster-moving fraction.

Attempts to elute the enzyme of the slower band from chromatograms with various buffer solutions or with sucrose solution were unsuccessful.

The two bands of activity from a duplicate of chromatogram 1 were cut out and found to have no effect on inulin or levan when incubated separately on the strips with these two polysaccharides.

Sucrose was converted readily by the enzyme associated with both bands into a mixture of glucose, fructose and at least one trisaccharide. No difference could be detected by paper chromatographic analysis between the actions of the two bands.

The two bands of activity from a duplicate of chromatogram 1 were cut out. Each band of activity was incubated at 30° in 10% sucrose solution (5 ml.), after the pH had been adjusted to 6·5 with sodium hydroxide. Concurrently two similar incubations were made including streptomycin sulphate (0·5 g.) in the digests. The digests were examined periodically by paper-chromatographic analysis, and the amounts of sugars compared visually. The streptomycin caused a slight reduction in the amount of glucose and a marked reduction in the amount of fructose produced by the faster band (cf. whole enzyme). However, with the slower band the streptomycin totally inhibited the enzyme action.

The faster-moving fraction was prepared in bulk by drying the enzyme extract at room temperature (60 ml. from 18 g. of freeze-dried mycelium) on a suitable area of paper which was then cut up finely, packed into a column and eluted with water at 0° (3 l.). The eluate was freeze-dried in the presence of 0·02M-citrate buffer (pH 6·8, 25 ml.), redissolved in water and diluted to 28 ml.

The enzyme solution (28 ml.) was incubated at 30° for 90 hr. with 25% sucrose solution (110 ml.) and then inactivated by heating at 95° for 30 min. The inactivated digest was fractionated twice by the method of Whistler & Durso (1950) on charcoal–Celite no. 545 columns (45 cm. × 5 cm.), the ethanol-gradient (0–25%) technique developed by Lindberg & Wickberg (1954) being used. The fraction containing the predominant trisaccharide was freeze-dried to a powder (2·07 g.); $[\alpha]_D^{20}+30°$ in water (*c*, 2·0).

The trisaccharide had an infrared spectrum identical with that of *O*-α-D-glucopyranosyl-(1 → 2)-*O*-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranoside. The trisaccharide (0·469 g.) was heated at 80° for 5 hr. with 5 mN-sulphuric acid (23·5 ml.); the solution was cooled, neutralized with barium carbonate and filtered. The partial hydrolysate was fractionated by the method of Whistler & Durso (1950) on a charcoal–Celite no. 545 column (18 cm. × 3 cm.), the ethanol-gradient (0–15%) technique developed by Lindberg & Wickberg (1954) being used. The fraction containing a disaccharide was freeze-dried to yield 0·192 g. (60·3%). The disaccharide had $[\alpha]_D^{20}+66°$ in water (*c*, 0·77) and m.p. 183° alone or in admixture with authentic sucrose. The sucrose was further characterized as its octa-*O*-acetate, $[\alpha]_D^{20}+59°$ in chloroform (*c*, 0·3), m.p. 76°, alone or in admixture with an authentic specimen. Oxidation of the trisaccharide with periodate by the method described by Dyer (1956) showed that 4·09 mol. prop. of periodate was consumed and 1·07 mol. prop. of formic acid was liberated. Methylation (Albon, Bell, Blanchard, Gross & Rundell, 1953), hydrolysis and subsequent fractionation on a silica-gel column (Bell & Palmer, 1949) gave a tri-*O*-methylfructose and two tetra-*O*-methyl sugars. The latter were present in equal quantities and were chromatographically identical with 2:3:4:6-tetra-*O*-methyl-D-glucose and 1:3:4:6-tetra-*O*-methyl-D-fructose. The mixture had $[\alpha]_D^{20}+54°$ in water (*c*, 1·1) (calc. for an equimolar mixture of 2:3:4:6-tetra-*O*-methyl-D-glucose and 1:3:4:6-tetra-*O*-methyl-D-fructose, +56·4°) (Found: OMe, 51·9. Calc. for $C_{10}H_{20}O_6$: OMe, 52·6%). The tri-*O*-methyl sugar was characterized as 3:4:6-tri-*O*-methyl-D-fructose by its equilibrium specific rotation $[\alpha]_D^{20}+30°$ in water (*c*, 0·7), its methoxyl content (Found: OMe, 42·2. Calc. for $C_9H_{18}O_6$: OMe, 41·9%) and its conversion into 3:4:6-tri-*O*-methyl-D-fructose phenylosazone, m.p. 129–131°, alone or in admixture with an authentic specimen. Other confirmatory evidence was the formation of an *O-iso*propylidene derivative and the results of periodate oxidation (1·17 mol. prop. of periodate consumed, 0·92 mol. prop. of formaldehyde formed).

The enzyme associated with both the faster and the slower bands hydrolysed this trisaccharide to glucose, fructose and sucrose.

DISCUSSION

The present work has demonstrated some unexpected properties of the *A. niger* transfructosylase. Although a separation into two bands of activity was achieved when the enzyme was dried on the paper at room temperature or 45°, no separation was achieved if the enzyme had not been dried or only partial separation if it had been dried at 0°. Once 'complete' formation of the slow band of activity had been accomplished by chromatographing after drying at room temperature, the fast band of activity showed only one component after eluting and rechromatographing. Whenever 'incomplete' formation had occurred, e.g. when the drying had been carried out at 0° or when the enzyme had not been dried, rechromatographing the fast band after drying at room temperature resulted in the appearance of two bands of activity. From this it is evident that the original cause of the enzyme separation is a factor inherent in the enzyme and is not due to saturation of a property of the paper with excess of enzyme. A further possibility would be that there is an insufficiency of some factor in the enzyme extract, e.g. a coenzyme which enables the enzyme to be absorbed strongly on the paper or, conversely, enables the enzyme to avoid irreversible absorption. Some evidence against this was obtained when the enzyme extract was diluted with two volumes of inactive extract and then applied to a chromatogram. The distribution of activity between the fast and slow bands was the same as that obtained with the active extract alone (cf. chromatograms 1 and 6, Table 1).

During the separations described above there was no fractionation into a hydrolysing enzyme and a transfructosylase, which was expected from the quantitative examination of the effect of streptomycin on the whole enzyme fraction. Both the enzyme in the fast and the slow bands produced glucose, fructose and trisaccharides from sucrose. The two bands of activity could, however, be clearly distinguished by the effect of streptomycin. The presence of streptomycin appeared to inhibit completely the action of the slow band on sucrose. The fast band, however, showed an effect similar to that of the whole enzyme extract, inasmuch as the formation of the monosaccharides, and particularly fructose, was reduced. The summation of the effects of the antibiotic on the two enzyme fractions gives a result similar to that on the whole enzyme fraction.

The trisaccharides produced by either enzyme fraction from sucrose appeared identical. That formed by the faster fraction was rigorously characterized as O-α-glucopyranosyl-(1 → 2)-O-β-D-fructofuranosyl-(1 → 2)-β-D-fructofuranoside. The irreversible absorption of the slow band on the paper prevented sufficient quantities from being prepared for a similar characterization of its trisaccharide product. Although the characterization of the enzyme associated with the slow band is thus incomplete it would appear that enzymes occurring in the two bands are isodynamic. (Isodynamic enzymes are enzymes which catalyse the same chemical change. They may be differentiated only by other means, e.g. physical properties, susceptibility to inhibition.) If enzymes are often multiple in this manner, and in certain cases only one form is affected by an antibiotic, then a ready mechanism for the development of resistance is available. The organism has merely to develop greater reliance on the unaffected enzyme.

SUMMARY

1. Investigations of the transfructosylase of *Aspergillus niger* '152' showed that the activity-distribution pattern obtained on chromatography of the extract was extremely dependent on the method of application.

2. Under suitable conditions two enzyme fractions were obtained. These two fractions appeared isodynamic but differed in their susceptibility to streptomycin.

3. The trisaccharide produced by the chromatographically faster-moving component has been characterized.

One of us (R.B.W.) wishes to thank the British Rayon Research Association for the award of a Scholarship.

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