A β -D-Fructofuranosidase from *Claviceps purpurea*

By A. G. DICKERSON

Department of Biochemistry, Imperial College of Science and Technology, London S.W.7, U.K.

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Evidence suggests that sucrose is the main carbon source for growth of *Claviceps* spp. in the parasitic condition. The sucrose acts as substrate for an active β -fructofuranosidase, produced by the fungus, which in the first instance converts the disaccharide into glucose and an oligofructoside. In this way, 50% of the glucose, supplied as sucrose, is made available to the parasite for assimilation. Subsequent action of the enzyme on both sucrose and the oligofructoside leads to the release of more glucose and the formation of additional oligosaccharides. The structures of the main oligosaccharides formed have been elucidated and the interactions of each compound studied. In experiments with purified enzyme *in vitro* the interaction of the oligosaccharides is rapid but in culture they are assimilated only slowly; in each case some free fructose is liberated. Free fructose is not assimilated in the presence of glucose and, further, inhibits growth at concentrations which might be expected to occur in the parasitic condition. A dual role has been suggested for the enzyme, with sucrose as substrate, in which glucose is made available to the growing parasite, while at the same time transfer of the fructose to form oligosaccharides prevents it from accumulating at inhibitory concentrations. Ultimately, when glucose becomes limiting, the fungus will adapt to fructose assimilation.

Sucrose is a good carbon source for the growth of ergot fungi, including *Claviceps purpurea*, in shake culture. This disaccharide is rapidly converted into a series of oligosaccharides and a limited amount of monosaccharide (Perenyi *et al.*, 1968; Dickerson *et al.*, 1970; Arcamone *et al.*, 1970a). Glucose, liberated on fructose transfer to form the oligosaccharides, is preferentially assimilated and the oligosaccharides, together with limited amounts of free fructose, remain in the culture medium for a prolonged period of many days (Bassett *et al.*, 1972).

Arcamone *et al.* (1970b) suggest that the oligosaccharides act as a storage of glucose for later growth; however, liberation of the glucose also involves liberation of considerable quantities of fructose, which they claim retards growth. This would account for the fact that the oligosaccharides are only assimilated slowly.

When grasses are infected with *Claviceps* spp., a well-known early indication of the presence of the parasite is a viscous secretion called honeydew which exudes from the inflorescence. This has been shown to be composed of glucose, fructose, sucrose and oligosaccharides containing glucose and fructose (Fuchs & Pöhm, 1953). Infection normally takes place before the ovaries of the inflorescence are fertilized and, clearly, the tissues of the ovary could only support limited fungal growth. These observations suggest that important substrates for parasitic growth and development of the fungus are sugars, mainly sucrose, obtained via the phloem ducts of the host plant.

The properties of a purified β -D-fructofuranosidase ('sucrase') obtained from the fungus have been studied to establish its role in the carbon assimilation of the organism, in culture and in the parasitic condition, outlined above.

Methods

The presence of sucrase activity was noted in *Claviceps fusiformis* (Dickerson *et al.*, 1970) as well as *C. purpurea* but all the experiments were done with the latter.

Submerged-culture procedures

These were as described by Dickerson *et al.* (1970) with the following changes: the medium, modified after Stoll *et al.* (1954), was used for the seed stage only; a 2% (v/v) portion of this culture was used to inoculate a production-stage medium composed (% w/v) as follows: citric acid, 1.50; $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$, 0.10; $\text{KH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$, 0.025; KCl, 0.012; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0007; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0006. The carbon sources used were (% w/v): sucrose, 5, 10, 20 or 30; glucose, 5, 20 and 30; fructose, 5, 20 and 30; glucose+fructose, 2.5+2.5.

General methods

Determinations. Reducing sugar was determined by the method of Somogyi (1952). Fructose was determined by the method of Cole (see Bacon & Bell, 1948)

and free glucose by the glucose oxidase method of Fleming & Pegler (1963). Total carbohydrate was determined by the anthrone method of Morris (1948) or by use of the phenol-sulphuric acid reagent (Dubois *et al.*, 1951, 1956).

Protein in solution was estimated by determining the E_{260} and E_{280} of the sample. The concentration was then obtained from a nomograph compiled by Adams (California Corporation for Biochemical Research) from the data of Warburg & Christian (1942).

Ammonia in media samples was measured after distillation into 0.04M-HCl, by the method of Conway (1962); sodium borate buffer, pH 10.0, was used to liberate the ammonia instead of 1M-NaOH as this minimizes the hydrolysis of labile material, particularly, glutamine (<3% under these conditions). The distillates were Nesslerized and the E_{500} of the resulting solutions was measured.

Methylation. Methylation of the oligosaccharides was done by the method of Anderson & Cree (1966). I.r. spectra of the methylated oligosaccharides were obtained with a Unicam S.P. 200 G spectrophotometer and methylation was considered complete when there was no absorption at 3500cm^{-1} .

Silylation. The oligosaccharides were silylated in dry pyridine by the method of Sweeley *et al.* (1963) with trimethylchlorosilane and hexamethyldisilazane.

Chromatography. Paper and thin-layer chromatography were done by the methods described by Buck *et al.* (1968). In addition, methylated sugars were run on thin-layer plates coated with silica-gel (Merck, G) in benzene-ethanol (20:1, v/v) and detected with iodine vapour.

Electrophoresis. Paper electrophoresis was done on Whatman 3MM paper, at 15°C with a gradient of 40–45 V/cm in 50mm-sodium borate buffer, pH 9.3.

Mass spectrometry. Mass spectra of the methylated and silylated sugars were obtained with an AEI MS9 instrument. Samples were inserted on glass or ceramic probes into the ion source at the minimum temperature for obtaining a spectrum.

Enzymic procedure. Enzyme reactions were done at 30°C according to the recommendations of the International Union of Biochemistry. For further details, see Dickerson *et al.* (1970).

Assay of radioactivity. Products derived by enzyme action on ^{14}C -labelled substrates were separated by paper chromatography and the dried papers exposed to Kodirex No-screen X-ray film. The radioautograph obtained was used to determine the radioactive areas, which were removed, cut into small pieces ($10\text{mm} \times 10\text{mm}$) and immersed in 10ml of liquid scintillant [6g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole + 50g of naphthalene/l of toluene]. Samples in aqueous solution were placed in the above scintillant and converted into a single-phase system by adding 2-methoxyethanol (methyl cellosolve). The

radioactivity in the samples was determined in a Packard counter (model 3003). Corrections for variable efficiencies were made.

Preparation methods

Purification of oligosaccharides. The oligosaccharides arising from the various sources described above were separated by paper chromatography, identified by markers, eluted from the paper and re-chromatographed until single compounds only could be observed. [^{14}C]Oligosaccharides were isolated in the same way from [^{14}C]sucrose incubations with the sucrase and also those arising from the action of yeast invertase on sucrose (Edelman, 1954).

The oligosaccharides 6^G- and 1^F-fructosylsucrose [for nomenclature system see *Biochem. J.* (1956) **64**, 351–361] were separated by paper electrophoresis and by the method of Bacon (1954) on a column of charcoal and Celite ($1.4\text{cm} \times 14\text{cm}$) by using a linear gradient increasing from 0 to 50% ethanol (by vol. with water); the flow rate was between 40 and 60ml/h and $200 \times 5\text{ml}$ fractions were collected. The oligosaccharide 1^F-fructosylsucrose was also obtained from Jerusalem artichoke extracts by the method of Edelman & Dickerson (1966).

Enzyme purification. Although some β -D-fructofuranosidase had been noted in the extracellular enzymes of *C. purpurea* the major activity was associated with the cell (Dickerson *et al.*, 1970). The following procedure was therefore adopted to extract the enzyme in quantity and purify it.

Mycelium of *C. purpurea* 117/2 obtained from 7-day-old shake cultures was washed briefly in 10mm-sodium phosphate buffer, pH 6.0, with approx. 5ml/g wet weight; after filtration, the moist mycelium (110g) was crushed in an X-press (AB Biox, Nacka, Sweden). The enzyme was extracted by gently agitating the crushed tissue in 10mm-sodium phosphate buffer, pH 6.0, containing 5% (w/v) sucrose (approx. 5ml/g wet wt.) for 2h at room temperature. Subsequent steps were done at 4°C . The extracted material was centrifuged at $12000g$ and the supernatant cleared by four successive centrifugations at $28000g$; between each centrifugation, residue of low density was removed from the surface of the supernatant. The supernatant was then dialysed overnight against 10 litres of the above buffer. The dialysis residue was centrifuged at $12000g$, subjected to ultrafiltration in 0.63cm ($\frac{1}{4}\text{in}$) diam. Visking tubing which reduced the volume overnight from 600 to 100ml then finally freeze-dried. This preparation (1.25g) was resuspended in 12ml of water, centrifuged and dialysed as before. The final volume of the dialysis residue was 15ml, which on freeze-drying yielded an off-white, fluffy material (108mg). The protein content of this material was 77% and it had a specific

enzyme activity of 0.68 μ mol of sucrose utilized/min per mg of protein.

The freeze-dried crude enzyme preparation was dissolved in 5ml of water and dialysed overnight against 10 litres of 10mM-sodium phosphate buffer, pH 6.0. The dialysis residue was applied to a column (3cm $\times$ 46cm) of Sephadex G-100 previously equilibrated with 5 litres of the same buffer. The column was eluted with the same buffer at a flow rate of 20ml/h; fractions (5ml) were collected. The fractions were dialysed overnight against 1mM-sodium phosphate buffer, pH 6.0, and their protein content and enzymic activity were determined. This procedure resolved the protein into several distinct components. The enzyme activity was associated with the high-molecular-weight material that emerged from the column between fractions 4 and 10. These fractions were combined and freeze-dried. The residue (20mg) had a protein content of 80% and a specific enzyme activity of 3.9.

Cellulose powder (Whatman DE52; 50g) was treated according to the manufacturers general instructions to remove adsorbed CO₂. The suspension obtained was poured into a column (1.5cm $\times$ 36cm) and equilibrated with 5 litres of 5mM-sodium phosphate buffer, pH 6.0, which, together with the buffers used below, was made up in CO₂-free water.

The enzyme preparation from the Sephadex column was dissolved in 3ml of 5mM-sodium phosphate buffer, pH 6.0, and dialysed overnight against 10 litres of the same buffer. The dialysis residue was applied to the above column and eluted with a linear gradient of sodium phosphate buffer at pH 6.0, increasing from 5 to 150mM, prepared by the method of Bock & Ling (1954); the flow rate was approx. 30ml/h and 50 $\times$ 5ml fractions were collected. The fractions obtained were dialysed overnight against 1mM-sodium phosphate buffer, pH 6.0, and their protein content and enzymic activity were determined. This procedure resolved the protein into several components. Enzyme activity was found between fractions 23 and 37 (approx. 78–114mM on the gradient) with the peak between fractions 29 and 31. Fractions 23 to 37 inclusive were combined and freeze-dried. The residue (13.5mg) had a protein content of 28% and a specific activity of 17.3.

Results

Structure of the oligosaccharides

Evidence from paper chromatography suggested that the oligosaccharides formed in the culture medium after growth of *C. purpurea* on sucrose, those present in honeydew and those found in aqueous extracts of infected ovaries from the plant were the same; there were four main oligosaccharides and traces of at least two others.

Some of the properties of the main oligosaccharides, isolated from enzyme incubations while sucrose was still present, are shown in Table 1. The glucose/fructose ratio obtained by acid hydrolysis of the ¹⁴C-labelled compounds and by reducing action, where this occurred, indicated a disaccharide, two trisaccharides and a tetrasaccharide.

Partial acid hydrolysis in 0.05M-oxalic acid at 60°C yielded sucrose and oligosaccharide S₁ from oligosaccharides S₂ and S₄, but sucrose was absent from the acid hydrolysate of S₃ and from all of the enzyme hydrolysates (Table 2). In the latter case the absence of sucrose may be explained by its lability to the enzyme's action; also, the disparity of products can be attributed to further limited transfer as well as hydrolysis.

All the oligosaccharides were hydrolysed by yeast invertase (grade VI; sigma) but the rate was less than one third of that obtained with sucrose as substrate. The intermediates formed during the hydrolysis (Table 2) can be seen to be consistent with the structures proposed below for the oligosaccharides.

The individual oligosaccharides isolated by paper chromatography were chromatographed on a column of charcoal and Celite (Bacon, 1954). Comparison with the oligosaccharides obtained by the action of yeast invertase on sucrose, treated in the same manner, showed that both oligosaccharide S₁ and D-Fruf β 2 $\rightarrow$ 6D-Glcp α from the invertase incubation eluted between fractions 19 and 26 and that oligosaccharide S₂ and 6^G-fructosylsucrose eluted between fractions 39 and 46. The oligosaccharide 1^F-fructosylsucrose, which co-chromatographs with 6^G-fructosylsucrose on paper and is a product of the action of yeast invertase on sucrose (Bacon, 1954), eluted

Table 1. *General properties of the main oligosaccharides*

For details, see the Methods section. The solvent system used to measure R_{sucrose} was propan-1-ol-ethyl acetate-water (7:1:2, by vol.).

Symbol	R_{sucrose}	Glc/Fru ratio	$\frac{\text{Reducing wt.}}{\text{Total wt.}}$ (%)
S ₁	0.81	1:1.0	44.0
S ₂	0.63	1:2.0	2.0
S ₃	0.42	1:1.9	31.0
S ₄	0.35	1:3.0	1.5

Table 2. Action of acid, yeast invertase and the β -fructofuranosidase on the major oligosaccharides (S_1 – S_4)

Each oligosaccharide was hydrolysed in 0.05M-oxalic acid at 60°C for 30 min at a concentration of 15 mg/ml. For yeast invertase, each oligosaccharide was incubated at 30°C for 60 min with 5 μ g of enzyme/ml of 50 mM-sodium acetate buffer, pH 5.0; the amount of oligosaccharide/ml was 20 mg for S_1 , 30 mg for S_2 and S_3 and 40 mg for S_4 . The incubations with β -fructofuranosidase were similar to those of yeast invertase except that 0.1 mg of enzyme/ml of 50 mM-sodium phosphate buffer, pH 6.0, was used. For the enzyme incubations results, calculated from assay of radioactivity, are expressed as % (w/w) of total carbohydrate. +, Present in hydrolysate; –, not present in hydrolysate or enzyme incubation mixture.

Treatment	Oligosaccharide	Products of reaction						
		Fru	Glc	Sucrose	S_1	S_2	S_3	S_4
Acid	S_1	+	+	–	–	–	–	–
	S_2	+	+	+	+	–	–	–
	S_3	+	+	–	+	–	–	–
	S_4	+	+	+	+	+	+	–
Yeast invertase	S_1	6.4	6.1	–	87.5	–	–	–
	S_2	6.4	3.9	1.8	2.0	85.9	–	–
	S_3	5.0	1.1	–	10.7	–	83.2	–
	S_4	3.1	–	–	–	–	12.1	84.8
β -Fructofuranosidase	S_1	2.1	3.2	–	91.5	–	3.2	–
	S_2	29.0	6.5	–	31.0	27.0	6.5	–
	S_3	20.5	2.0	–	39.3	–	38.2	–
	S_4	34.4	3.1	–	21.7	6.4	8.4	26

between fractions 29 and 38. The proportion of 1^F - to 6^G -fructosylsucrose produced from sucrose by yeast invertase was in the ratio of 3:2. This method did not reveal the presence of a compound corresponding to the 1^F -fructosylsucrose reported by Perenyi *et al.* (1968) in the *Claviceps* series; however, small amounts of an oligosaccharide might not be detected because of dispersion amongst several fractions. The oligosaccharide S_2 was therefore subjected to paper electrophoresis together with 1^F - and 6^G -fructosylsucrose. This showed that oligosaccharide S_2 contained two components; one migrated at the same rate as 1^F -fructosylsucrose (2.8 cm/h towards the cathode) and the other at the same rate as 6^G -fructosylsucrose (3.8 cm/h towards the cathode). The proportion of 1^F - to 6^G -fructosylsucrose varied between 1:10 and 1:7 in the samples investigated. These conclusions were confirmed below by mass spectrometry.

Mass spectrometry provided further evidence for the structures of the oligosaccharides as follows. In the mass spectra of pertrimethylsilyl (trimethylsilyl) derivatives of hexoses an ion at m/e 437 is characteristic of 2-ketohexoses whereas an m/e of 435 indicates an aldohexose; also, an m/e of 217 is characteristic of furanose rings and an m/e of 204 of pyranose rings (Karady & Pines, 1970). Oligosaccharides showing these ions, or these ions supplemented by some multiple of 378 (where 378 is the mass increment of one trimethylsilylhexose unit), must therefore contain aldo- and keto-hexose units. The results for sucrose and the four main oligosaccharides (Table 3) clearly

indicate the presence of the 2-ketohexose furanose structure but the aldohexose pyranose structure is not generally so well defined.

A fragment ion at m/e 353 in the spectrum of a methylated sample of oligosaccharide S_1 , not present in the spectrum of octamethylsucrose, indicated an ($x \rightarrow 6$) linked disaccharide (Chizhov *et al.*, 1964); also, the spectrum of oligosaccharide S_1 was similar to that of authentic $\text{Fruf}\beta 2 \rightarrow 6\text{Glc}\alpha$ obtained by the action of yeast invertase on sucrose (Bacon, 1954). The spectrum of the trimethylsilyl derivative had an ion at m/e 583 which also indicated an ($x \rightarrow 6$) linked disaccharide (Chizhov *et al.*, 1967) and agreed with results published by Kamerling *et al.* (1971) for $\text{Fruf}\beta 2 \rightarrow 6\text{Glc}\alpha$.

The spectrum of a methylated sample of oligosaccharide S_2 had virtually no fragments between m/e 613 and 439 (i.e. m/e 409 + 204 and 235 + 204; where 204 is the mass increment of one trimethylhexose unit) which suggested that this compound was a sucrose derivative since sucrose has virtually no fragments between m/e 409 and 235. There was no peak at m/e 557 which would correspond to m/e 353 shifted by a trimethylhexose unit (i.e. 353 + 204) indicating that the terminal saccharide unit was not linked through the 6 position. Also, the spectrum was very similar to that of authentic 6^G -fructosylsucrose obtained from the yeast invertase incubation. The spectrum of the silyl derivative had no fragment at m/e 961 (i.e. m/e 583 + 378), which confirmed the absence of an ($x \rightarrow 6$) terminal link.

An ion fragment at m/e 557 in the spectrum of a

Table 3. *Characteristics of the mass spectra of silylated oligosaccharides (S₁–S₄) that indicate the presence of aldo- and keto-hexose units*

For details see the text. Abbreviations: ++, well-defined in spectrum; +, weakly defined in spectrum; –, absent from spectrum.

Compound	<i>m/e</i> Values	...	437	815	435	813	217	595	204
Sucrose			++		–		++		+
S ₁			++		+		++	+	–
S ₂			++	++	–	–	++	–	++
S ₃			++	++	+	++	++	+	+
S ₄			++	++	–	+	++	+	++

methylated sample of oligosaccharide S₃ indicated an (x→6) linked terminal saccharide unit; this was confirmed by a peak at *m/e* 961 in the spectrum of the silyl derivative.

The methylated derivative of oligosaccharide S₄ had no ion at *m/e* 761 (i.e. 353+408; where 408 is the mass increment of two trimethylhexose units) which again indicated that the terminal saccharide unit was not linked through the 6 position. The absence of fragments between *m/e* 817 and 643 (i.e. *m/e* 409+408 and 235+408) suggested that this compound was also a sucrose derivative.

The spectrum of methylated 1^F-fructosylsucrose did not correspond to any of the spectra of the methylated oligosaccharides.

From the above data, the enzymological evidence (see below) and the structures proposed for the oligosaccharides isolated by Arcamone *et al.* (1970a), the four major oligosaccharides here have structures consistent with the following:

S₁: D-Frufβ2→6D-Glcpα (abbreviation: F2→6G)

S₂: D-Frufβ2→6D-Glcpα1←2D-Frufβ (abbreviation: F2→6G1←2F 6^G-fructosylsucrose) + D-Frufβ2→1D-Frufβ2→1D-Glcpα (abbreviation: F2→1F2→1G 1^F-fructosylsucrose)

S₃: D-Frufβ2→1D-Frufβ2→6D-Glcpα (abbreviation: F2→1F2→6G)

S₄: D-Frufβ2→1D-Frufβ2→6D-Glcpα1←2D-Frufβ (abbreviation: F2→1F2→6G1←2F).

The tetrasaccharide (F2→1F2→1F2→6G) reported by Arcamone *et al.* (1970a) would agree with this series and also suggests the presence of a pentasaccharide F2→1F2→1F2→6G1→2F. The low *R*_{sucrose} values of two of the minor oligosaccharides observed indicate that both of these sugars are probably present in small amounts in incubations and cultures containing the major oligosaccharides.

Enzyme studies

The enzyme is inducible since activity could only be found in shake cultures where sucrose was the carbon

Table 4. *Effect of pH on the activity of the β-fructofuranosidase*

The incubation mixtures contained 20mg of sucrose and 0.5mg of the purified β-fructofuranosidase/ml of 50mM buffer at 30°C. Sodium acetate buffer was used at pH 3.6, 4.6 and 5.6 and sodium phosphate buffer at pH 5.6, 6.0, 6.6, 7.6 and 8.2. The ratio of free fructose to free glucose liberated was taken as a measure of the type of activity: ratio >1:2, mainly hydrolytic; ratio 1:2–1:5, mainly transfer; ratio <1:5, transfer.

pH of incubation	Sucrose utilized (μmol/min per mg of enzyme)	Type of activity
3.6	1.2	Mainly hydrolytic
4.6	2.4	Mainly transfer
5.6	9.8	Transfer
5.6	10.0	Transfer
6.0	11.4	Transfer
6.6	7.8	Transfer
7.6	5.5	Transfer
8.2	3.8	Transfer

source. Sucrose could not be replaced by glucose, fructose or a mixture of the two.

Sucrose, raffinose, 1^F- and 6^G-fructosylsucrose and the oligosaccharides S₁–S₄ were all substrates for the purified enzyme preparation.

Table 4 shows the variation of activity with pH when sucrose was substrate. Except at the lowest pH the enzyme acted as a transfructosylase (i.e. the reaction products were glucose and oligosaccharides).

When raffinose was substrate, hydrolysis (to melibiose and fructose) only was observed at all pH values; the rates were approximately half those for the corresponding sucrose incubation. The fact that no transfructosylation occurred to form 6^G- and 1^F-fructosylraffinose (Edelman & Jefford, 1964) supports the evidence for both fructose transfer to the 6 position of the glucose ring in 6^G-fructosylsucrose

Table 5. Action of the β -fructofuranosidase on 1^F- and 6^G-fructosylsucrose

Each incubation mixture contained 25 mg of trisaccharide and 0.1 mg of enzyme/ml of 50 mM-sodium phosphate buffer, pH 6.0, at 30°C. Results are expressed as % (w/w) of total carbohydrate. The reaction products S₁–S₄ had the same R_{sucrose} values as the oligosaccharides S₁–S₄.

Substrate	Time (min)	Product						
		Fru	Glc	Sucrose	S ₁	S ₂	S ₃	S ₄
1 ^F -Fructosylsucrose	0	—	—	—	—	100	—	—
	50	20.4	9.8	—	5.0	64.5	—	0.3
	120	36.2	19.1	—	9.2	35.3	—	0.2
6 ^G -Fructosylsucrose	0	—	—	—	—	100	—	—
	50	25.2	8.9	0.2	30.1	22.3	10.7	2.6
	120	33.4	12.1	0.4	35.0	10.6	7.8	0.9

Table 6. Action of the β -fructofuranosidase on sucrose

The incubation mixture contained 20 mg of sucrose and 0.5 mg of the purified β -fructofuranosidase/ml of 50 mM-sodium phosphate buffer, pH 6.0, at 30°C. Results are expressed as μ mol/ml of incubation mixture. The values in parentheses indicate the ratios of 6^G- to 1^F-fructosylsucrose. Abbreviations: S₁–S₄, oligosaccharides S₁–S₄.

Time (min)	Product						
	Fru	Glc	Sucrose	S ₁	S ₂	S ₃	S ₄
0	—	—	58.4	—	—	—	—
1	0.7	3.9	51.2	—	3.2 (9.5:1)	—	0.06
2	1.2	7.1	44.9	0.6	5.5 (9.2:1)	—	0.3
4	2.1	10.9	37.4	1.3	7.9 (8.2:1)	0.08	0.6
12	5.5	21.8	18.1	5.0	11.0 (5.0:1)	0.4	2.3
30	10.4	28.7	6.7	8.9	9.9 (2.1:1)	0.9	3.5
80	20.2	32.4	2.6	13.4	5.0 (0.4:1)	4.1	1.3

formation and the low rate of 1^F-fructosylsucrose synthesis with sucrose as substrate.

The low transferase activity of the enzyme with 1^F-fructosylsucrose as substrate is further indicated by comparison with the enzyme's activity towards 6^G-fructosylsucrose (Table 5). The products of the reaction in each case correspond in R_{sucrose} value to the oligosaccharides S₁–S₄.

Action of the enzyme on sucrose at pH 6.0 was not inhibited by equimolar free glucose or fructose, but equimolar F2→6G inhibited the sucrose breakdown by 10%.

The K_m for the enzyme with sucrose as substrate was 0.148 M at pH 6.0.

The formation of the main oligosaccharides (see above) took place in two series during enzyme incubation with sucrose as substrate (Table 6). The two oligosaccharides that appeared early (S₂ and S₄) slowly diminished whereas two others (S₁ and S₃) appeared later and slowly increased in amount together with some free fructose. In cultures with sucrose as carbon source, S₁ and S₃ were the main oligosaccharides detected in the culture medium after 2–3 days when

all the sucrose had been utilized; these remained in the medium for periods in excess of 10 days.

Discussion

Action of the *Claviceps* β -fructofuranosidase on sucrose gives rise to a number of oligosaccharides. The disaccharide S₁ has been established as a product of the action of *Aerobacter levanicum* on sucrose (Feingold *et al.*, 1956) and has been found among the oligosaccharides formed by the action of yeast invertase on sucrose (Bell & Edelman, 1954). Together with 1^F- and 6^F-fructosylsucrose, 6^G-fructosylsucrose has been shown to be a common product of the action of a number of fungal and higher-plant enzymes on sucrose (Allen & Bacon, 1956; Bacon, 1960; Hammer, 1969). In the majority of cases with mould and yeast invertases high concentrations of sucrose were used to obtain transfructosylation, as the action of these enzymes is primarily hydrolytic (Bacon & Edelman, 1950; Blanchard & Albon, 1950; White & Secor, 1952; Allen & Bacon, 1956).

With the *Claviceps* enzyme, hydrolysis is absent

initially; Table 6 shows that sucrose acts both as donor and acceptor and reaction (1) occurs:

As the concentration of oligosaccharide S_2 builds up, this also can act as an acceptor:

For reaction (2), three mol of sucrose are required and this gives rise to two of glucose; as the reaction progresses this situation is approached but not entirely reached because of further transfers and some hydrolysis of products. The latter two processes yield the oligosaccharides S_1 and S_3 and fructose which appear later during the reaction:

Incubating each oligosaccharide alone with the enzyme (Table 2) shows that oligosaccharides S_2 and S_4 are attacked at approximately the same rate as sucrose (Table 6), S_3 at 70% of the sucrose rate but S_1 at less than 8%; hence, very little glucose is liberated in any of these reactions. Due to the inertness of the ($F2 \rightarrow 6G$) linkage most of the free fructose liberated must be derived by hydrolysis of the sucrose or the ($F2 \rightarrow 1F$) linkage; the latter is clearly the case with S_3 . With oligosaccharides S_2 and S_4 , however, intermediates arise early in the reaction in significant amounts. Table 2 (1h) shows that, for every 100 mol of oligosaccharide S_2 broken down, 26.5 are completely hydrolysed to glucose and fructose, 64.6 give rise to S_1 and fructose and 8.9 form S_3 . The latter can arise by self-transfer:

also

Reaction (6) yields the same products as reaction (3) and it is impossible to distinguish directly by which reaction oligosaccharide S_1 arises. However, on the basis of the enzyme's action against raffinose, a fructose transfer to position 6 on the glucose ring would render the sucrose link more susceptible to direct hydrolysis by the enzyme. If this were not the case, a poly-repetitive action resulting in fructosans with a higher degree of polymerization could be expected. Hence reaction (5) indicates that 55.7% (64.6–8.9) of the S_2 molecules are directly hydrolysed

Table 7. *Transfer of fructose from sucrose to $F2 \rightarrow 6G$ by the β -fructofuranosidase*

The incubation mixture contained 10mg of sucrose, 10mg of [U - ^{14}C] $F2 \rightarrow 6G$ and 0.5mg of the purified β -fructofuranosidase/ml of 50mM-sodium phosphate buffer, pH6.0, at 30°C. Results are expressed as % of total radioactivity in each compound. Hydrolysis of the $F2 \rightarrow 1F2 \rightarrow 6G$ formed showed that the specific radioactivity of the glucose was twice that of the fructose, which indicates that reaction (8) and not self-transfer of $F2 \rightarrow 6G$ was occurring. Other reactions arising from the action of the enzyme on sucrose would also occur here but are not registered as the products are unlabelled.

Time (h)	Fru + Glc	$F2 \rightarrow 6G$	$F2 \rightarrow 1F2 \rightarrow 6G$
0	—	100	—
1	6.8	88.2	5.0
5	14.7	74.4	10.9
20	29.1	51.5	19.4

to oligosaccharide S_1 and fructose and 17.8% undergo self-transfer. These are minimum values since the totally hydrolysed S_2 molecules may have also undergone one of the above reactions initially.

For reactions (5) and (6) to occur a low K_m for the enzyme with the tetrasaccharide as substrate would be required since it was only detectable in small amounts (<1% of the total product). Therefore the following alternative for the origin of S_3 , starting from the oligosaccharides alone with the enzyme, is possible:

This is analogous to reaction (8):

Table 7 shows that with equimolar reactants the transfer is approximately equal to the rate of hydrolysis of oligosaccharide S_1 which is slow (Table 2). Thus if reaction (7) occurs it is unlikely to be significant until a substantial amount of oligosaccharide S_1 is formed by reaction (3). Similarly, when a sufficient amount of oligosaccharide S_1 is formed there will be a small contribution to the total amount of S_3 due to self-transfer with this disaccharide as substrate (Table 2).

In addition to the above reactions, 1^F -fructosyl-sucrose is also synthesized (Table 6), probably by a reaction analogous to (1) although other modes of formation can be envisaged. The amounts formed and the reactivity of the enzyme towards this

Fig. 1. Growth of *C. purpurea* in shake culture

Glucose (5%, w/v), fructose (5%, w/v) or glucose+fructose (2.5+2.5%, w/v) were present as carbon source. On each occasion one 500-ml Erlenmeyer flask, containing 100 ml of culture, was harvested and the total mycelial dry weight determined after a brief washing (10 ml of water/g wet wt. of mycelium) and desiccation to constant weight over CaCl_2 in vacuum. $\circ$, Glucose; $\bullet$, fructose; $\square$, glucose+fructose.

Fig. 2. Utilization of carbon source in shake cultures of *C. purpurea*

On each occasion one 500-ml Erlenmeyer flask, containing 100 ml of culture, was harvested and the medium assayed for glucose and/or fructose as described in the Methods section. $\circ$, Glucose; $\bullet$, fructose; Δ , glucose in the presence of fructose; $\blacktriangle$, fructose in the presence of glucose.

trisaccharide suggest that it plays a minor role in the *Claviceps* system.

Physiological role of the enzyme

In culture, the fungus grows equally well on 5% glucose or fructose as a sole carbon source (Fig. 1) although the presence of fructose completely suppresses sporulation. However, when the two are present together virtually all the glucose is assimilated before utilization of the fructose commences (Fig. 2).

The switch to fructose utilization is marked by a progressive rise in the pH of the medium from 4.5 to over 7; this is due to an increase in free ammonia (Fig. 3).

When the fungus is growing on its host, the young parasitic tissue is surrounded by a concentrated sugar solution [equivalent in many honeydew samples to over 40% (w/v) of sucrose]. The high K_m for the enzyme with sucrose as substrate (equivalent to a V_{max} at 10%, w/v) is indicative of these high concentrations. Sucrose accounts for over 90% of the total soluble carbohydrate in most samples of freshly

Table 8. Growth of *C. purpurea* on various concentrations of glucose or fructose as sole carbon source

On each occasion one 500-ml Erlenmeyer flask, containing 100ml of culture, was harvested and the total mycelial dry weight determined after a brief washing (10ml of water/g wet weight of mycelium) and desiccation to constant weight over CaCl₂ in vacuum.

Carbon source (%, w/v)	Time ...	Dry weight (mg/ml of culture)			
		42h	70h	115h	188h
None		0.03	0.11	0.17	0.22
Glucose (5)		2.6	7.8	9.5	15.5
(20)		1.6	7.9	13.4	18.5
(30)		0.5	1.4	14.0	20.8
Fructose (5)		2.6	6.9	10.6	16.4
(20)		0.6	2.2	3.2	10.5
(30)		0.02	0.6	1.3	8.2

exuded honeydew uncontaminated with fungal spores; oligosaccharides are only found when the honeydew has been in contact with parasitic fungal tissue for several hours, a condition which may be correlated with the presence of spores in the exudate. Preliminary experiments (A. G. Dickerson & P. G. Mantle, unpublished work) in which ¹⁴CO₂ is supplied to the ears of infected rye plants confirm that sucrose is the precursor of all soluble carbohydrates in the infected and uninfected ovaries.

Hence, the β-fructofuranosidase provides a means

for making over 50% of the glucose from the sucrose available to the fungus, in the first instance; free fructose does not accumulate because interconversions lead to the formation of oligosaccharides. If the enzyme's action was primarily hydrolytic (e.g. invertase: EC 3.2.1.26, β-D-fructofuranoside fructohydrolase), more glucose could be supplied to the parasite but high concentrations of free fructose would accumulate because, as noted above, fructose would not be utilized while glucose was available. Table 8 shows that the concentrations of fructose that might be expected, if oligosaccharide formation did not occur, could inhibit growth and sporulation. Whereas the oligosaccharides themselves might also be inhibitory, their effective molarity would be much lower than that of an equal concentration of fructose.

The formation of oligosaccharides, therefore, appears to be a mechanism that decreases inhibitory concentrations of free fructose in the first instance, and a minor role might be that they act as a reserve carbon source for later growth as suggested by Arcamone *et al.* (1970b).

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Fig. 3. Production of ammonia nitrogen by *C. purpurea*

C. purpurea was grown on glucose (5%, w/v), fructose (5%, w/v) or glucose+fructose (2.5+2.5%, w/v) as carbon source. On each occasion one 500-ml Erlenmeyer flask, containing 100ml of culture, was harvested, filtered and the ammonia nitrogen of the medium was determined as described in the Methods section. The lowest point represents a pH of 4.5 and the highest 7.4. ○, Glucose; ●, fructose; □, glucose+fructose.

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