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ACTIVITY OF FRUCTANASE IN BATCH CULTURES OF ORAL STREPTOCOCCI*

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ABSTRACT

Several strains of oral streptococci produced fructanase when grown in the absence of D-fructan in a complex medium supplemented with D-glucose. The major part of the activity was extracellular, and only 1–5% was associated with the cells. Release of fructanase began early in the exponential phase and the enzyme was stable in the stationary phase for several h if the pH did not fall below 6. Among the strains of *Streptococcus mutans*, serotypes *a*, *d*, and *g* released the highest amount of fructanase, and the low level of enzyme produced by strains of serotype *c* was increased when D-fructose replaced D-glucose as carbon source for growth. Fructanase of *S. mutans* readily hydrolysed (2→6)-β-D-fructans, but (2→1)-β-D-fructans and inulin were more resistant. Adsorption of fructanase to (2→6)-β-D-fructan, or inhibition with Tris buffer, provided effective means of eliminating fructanase activity from culture filtrates. This procedure should permit a more accurate determination of fructosyltransferase activity of *S. mutans* strains.

INTRODUCTION

Streptococci are known to be a major source of the fructanase activity observed in human dental plaque[1]. The induction of fructanase in oral streptococci grown in the presence of D-fructan has been demonstrated in several studies[1–3], and 13 out of 28 strains tested in one study[2] were able to hydrolyse D-fructan. There have been no reports of fructanase production by oral streptococci grown in a D-glucose medium.

The amount of D-fructan in dental plaque is low (0–1% of plaque dry weight[4,5]), and it was thus of interest to determine whether any streptococci released fructanase when growing in the absence of D-fructan. For this purpose, 36 strains of oral streptococci were grown in media supplemented with either D-glucose or D-fructose. The work forms part of our continuing study of the occurrence in oral streptococci of enzymes involved in the synthesis and degradation of the polysaccharides produced from sucrose.

*Part VI of the series Metabolism of the Polysaccharides of Human Dental Plaque.

METHODS

Microorganisms. — Fifteen strains were selected at random from a collection of streptococci isolated from the dental plaque of New South Wales (NSW) school children, and the biochemical and physiological reactions of the NSW strains were recorded[6]. *Streptococcus mutans* strains K21 and NG112 were isolated by Dr. R. G. Schamschula from New Guinea aborigines. In addition, 19 representative organisms were chosen as reference strains of *S. salivarius* (NCTC8606 and ATCC13419), *Streptococcus sanguis* (804 and ATCC10588), *Streptococcus mitis* (S3, 439, RB1633), and *Streptococcus mutans* (AHT, BHT, FA-1, JC2, GS-5, Ingbritt, OMZ176, B13, K1-R, LM7, OMZ175, and OMZ65). The organisms were grown in 350 mL of medium[7] under anaerobic conditions (19:1 nitrogen + carbon dioxide) at 37° in a 1-L fermenter (Multigen Model F-1000, New Brunswick Scientific Co. Inc., New Brunswick, NJ, U.S.A.). The pH of the cultures was controlled, usually at pH 6, by the automatic addition of sodium hydroxide.

Fractionation of cultures. — The culture fluid was cooled to 4° and centrifuged for 10 min at 12,000*g*. Portions of the supernatant solution were dialysed against sodium citrate buffer (0.05M, pH 6.0) with two changes of buffer over a 24-h dialysis period. The cells were washed twice with the same buffer and then resuspended in one-seventh of their original volume. When a cell-bound fraction or intracellular extract was required, a suspension of washed cells containing ~12 mg dry weight mL^{-1} was shaken with pre-cooled glass beads (Glasperlen 0.10–0.11 mm diameter, from B. Braun, Melsungen, W. Germany) in a cell homogeniser (MSK, B. Braun). The suspension was continuously cooled with liquid carbon dioxide and shaken at 4,000 r.p.m. Treatment for 1.5 min was suitable for the release of cell-associated enzymes without breaking the cells, whereas shaking for 4 min gave complete disruption. The resulting suspensions were filtered through a sintered-glass filter (Quickfit, porosity 3), and the filtrates were centrifuged at 35,000*g* for 20 min. The supernatant solutions were dialyzed against 0.05M sodium citrate buffer, pH 6.0.

Determination of fructanase activity. — Fructanase activity of dialysed cell-free filtrates, cell-bound fractions, and intracellular extracts was determined from the release of reducing sugar in digests (1 mL) that contained *Streptococcus salivarius* NCTC8606 D-fructan (3 mg), sodium citrate buffer, pH 6 (10mM), and an appropriate volume of enzyme, usually 0.2 mL. Samples were taken at intervals up to 24 h for the determination of reducing power with neocuproine reagent[8]. One unit of activity is defined as the amount of enzyme that releases 1 μmol of reducing power per min under the conditions of the assay.

Fructanase activity of the cells was determined in digests (4 mL) containing *S. salivarius* D-fructan (8 mg), 2 mL of washed cell-suspension, and 0.2 mL of toluene. At intervals, portions (0.5 mL) were withdrawn, cooled in ice–water, and centrifuged at 12,000*g*. The supernatant solution was boiled for 1 min and then assayed for reducing power with neocuproine reagent. All enzyme activities were determined at 35°.

Dry weight of cells. — Triplicate portions (5 mL) of the culture were centri-

fuged and washed once with sodium citrate buffer pH 6.0. The pellets were then washed with water and dried to constant weight at 90°. Units of fructanase activity of the cell-bound fraction, cell suspension, and cell-free filtrate were converted to units per g of dry weight of the cells with which they were associated.

Fructans. — The *S. salivarius* D-fructan used for routine fructanase assays was synthesised from sucrose by the extracellular fructosyltransferase of *S. salivarius* NCTC8606 as previously described[1]. Fresh solutions of D-fructan were boiled before use. Inulin was purchased from Sigma Chemical Co., and grass D-fructan[9] was prepared from dried, milled Italian rye-grass. *Aspergillus sydowi* fructan[10,11] was a gift from Dr. H. Taniguchi.

Determination of apparent glucosyl- and fructosyl-transferase activity. — Multiple reaction-mixtures containing sucrose (10 mg), sodium citrate buffer pH 6.5 (10mM), and cell-free filtrate in a volume of 0.25 mL were incubated at 35° in centrifuge tubes. At appropriate times (usually 1, 3, 6, and 24 h), 3 volumes of ethanol were added. The polysaccharides which precipitated on being kept for 1 h at 4°, were centrifuged at 2000*g* for 10 min, washed 3 times with 75% ethanol, and then dissolved in dilute acid. The weight of D-glucan and D-fructan was determined with anthrone reagent[12,13]. One unit of activity is the amount of enzyme that transfers 1 μmol of sugar from sucrose to polysaccharide in one min.

Determination of apparent invertase activity. — The hydrolysis of sucrose was estimated from the release of reducing sugars in the transferase-activity digests. Reducing power that could not be accounted for by the release of monosaccharides during polysaccharide synthesis was assumed to be due to invertase activity. One unit is defined as the amount that hydrolyses 1 μmol of sucrose in one min. The invertase activity of the culture filtrate of *S. mutans* OMZ176 constituted 14% of the total activity, most of the remainder being located in the intracellular extract.

RESULTS AND DISCUSSION

Distribution of fructanase in cell fractions of Streptococcus mutans. — *S. mutans* OMZ176 was grown for 18 h at pH 6.0 in medium supplemented with 2% of D-glucose. Cell-bound and intracellular extracts were prepared as described under Methods, but these did not hydrolyse D-fructan. Fructanase activity (45 units per litre) was found only in the cell-free filtrate.

The ability of washed-cell suspensions of *S. mutans* strains K1-R, OMZ176, and Ingbritt to hydrolyse D-fructan was then compared with that of their cell-free-filtrates. In all cases, >90% of the total activity was extracellular. Further investigations on fructanase activity of oral streptococci were therefore performed with cell free filtrates.

Effect of growth conditions on extracellular fructanase activity. — The release of fructanase by *S. mutans* strains Ingbritt and OMZ176 was determined at stages during batch growth under different conditions (Figs. 1 and 2). During growth at pH 6.0 in a D-glucose medium, the fructanase activity in the OMZ176 culture reached

a value (77 units per litre) that was 8-fold higher than that in the Ingbritt culture (9.5 units per litre). When the organisms were grown without pH control, the differences between the two strains became greater. Fructanase was barely detected in the Ingbritt culture, whereas OMZ167 fructanase was relatively stable for long periods in the stationary phase, whether the pH was 7.0, 6.0, or had fallen below pH 5. Maximum fructanase activity of strain OMZ176 (units per g dry wt. of cells) was 33 and 34 at pH 6 and pH 7, respectively, and 68 for the uncontrolled culture. In a further experiment (not shown) *S. mutans* Ingbritt was grown at pH 6 for 8 h, and the supply of sodium hydroxide was stopped so that the pH fell to 4.2. Fructanase activity in the cell-free filtrate after 8 h without pH control was 3 units per litre. The results shown in Figs. 1 and 2 indicate that two factors should be considered when oral streptococci are surveyed for their ability to hydrolyse D-fructan. These are: (*i*) reliable values may be obtained only when the organisms are grown with pH control, and (*ii*) increased production of fructanase may occur in a D-fructose medium.

Fructanase activity of oral streptococci. — Streptococci isolated from dental plaque, including representative strains from different serotypes, were grown for

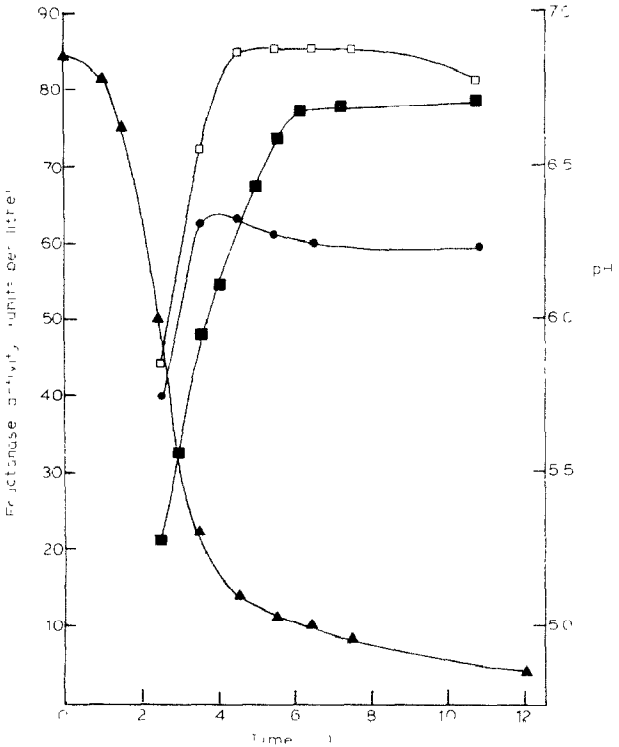

Fig. 1. Fructanase activity of filtrates of *S. mutans* OMZ176 grown at pH 6 (■), pH 7 (●), and without control of pH (▲) in medium supplemented with D-glucose (2% w/w): (▲), pH of the uncontrolled culture. Samples were withdrawn at intervals for determination of culture density (E_{600}) and dry weight of cells (not shown).

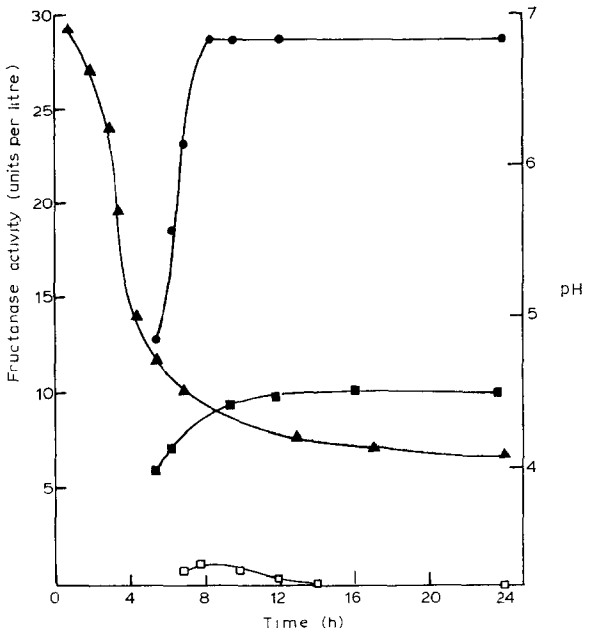

Fig. 2. Fructanase activity of filtrates of *S. mutans* Ingbritt grown at pH 6 in D-glucose medium (■) and in D-fructose medium (●). A low release of fructanase in D-glucose medium (□) without control of pH (▲) is also shown.

18 h in batch culture at pH 6.0 in medium supplemented with 2% D-glucose. Extracellular fructanase activity (Table I) varied considerably within species, revealing differences greater than found previously between *S. mutans* strains Ingbritt and OMZ176. The fructanase activity of *S. mutans* serotypes *c*, *e*, and *f* (genotype I) was barely detectable, strain Ingbritt being an exception with values of 6–10 units per litre. Strains of *S. mutans* serotype *b* (genotype II) gave intermediate levels of fructanase (10–12 units per litre), and the highest activities (25–900 units per litre) were released by *S. mutans* serotypes *a*, *d*, and *g* (genotypes III and IV). None of the *S. mitis* or unclassified NSW strains released the enzyme, whereas both of the *S. salivarius* strains, and one of the eight *S. sanguis* strains, produced high levels of fructanase. Thus, 18 of the 36 strains surveyed released fructanase when grown in a D-glucose medium. All of the strains that produced fructanase were also capable of synthesising extracellular polysaccharide from sucrose (compare Da Costa & Gibbons[1]).

Comparison of fructanase activity released by S. mutans *strains in a* D-*glucose- and* D-*fructose medium.* — Several strains of *S. mutans* were grown for 18 h in batch culture at pH 6.0. Although the faster growers spent a longer time in the stationary phase, it was possible to make valid comparisons between strains because of the stability of the fructanase (Figs. 1 and 2). With few exceptions, more fructanase (up to 3.8 fold) was released when D-glucose in the medium was replaced by D-fructose

TABLE I

EXTRACELLULAR FRUCTANASE ACTIVITY OF ORAL STREPTOCOCCI

Species	*Strain*	*Serotype*	*Fructanase[a] (units per litre)*
S. mutans	AHT	a	430, 944
	BHT	b	12
	FA1	b	11
	NSW1	c	1
	NSW47	c	0
	GS5	c	1
	JC2	c	0, 1.4
	Ingbritt	c	6, 10
	K21		13
	NG112		5
	OMZ176	d	17, 30
	B13	d	25, 30
	K1-R	d/g	63, 103
	LM7	e	2
	OMZ175	f	1
	OMZ65	g	44
S. sanguis	G9B	I	0
	804	I	0
	ATCC10558	II	704
	NSW13		2
	NSW10,23,44,57		0
S. salivarius	NCT8606	I	380
	ATCC13419	II	37
S. mitis	S3, 439, RB1633		0
	NSW16,22,32		0
Unclassified	NSW4,18,30,41,43		0

[a]The medium was supplemented with D-glucose (2%).

TABLE II

EFFECT OF CARBOHYDRATE MEDIUM ON EXTRACELLULAR FRUCTANASE ACTIVITY OF *S. mutans* STRAINS

Strain	*Serotype*	*Fructanase (units per g. dry wt. of cells)*	
		Glc (2%)	*Fru (2%)*
AHT	a	549	810
BHT	b	5.0	8.3
FA1	b	3.8	3.9
GS5	c	0.7	2.8
JC2	c	1.0	3.3
Ingbritt	c	6.7	13.17
B13	d	18	19
LM7	e	4.4	6.3
OMZ175	f	0.6	5.4
OMZ65	g	31	57

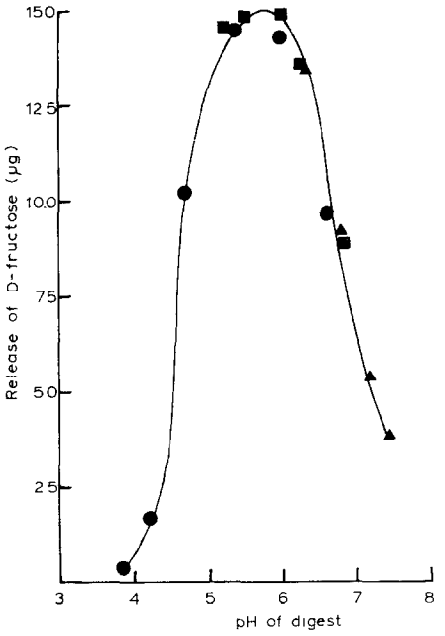

Fig. 3. Effect of pH on the extracellular fructanase activity of *S. mutans* Ingbritt. The digests, which contained citrate–phosphate buffer (●), citrate buffer (■), or phosphate buffer (▲) at a concentration of 25mM, were incubated for 60 min at 35°.

(Table II). This effect was consistently shown by serotype *c* strains, and was high in the single serotype *f* strain, which belongs to the same genetic group. No evidence was obtained that D-fructose promoted increased production of fructanase in *S. mutans* B13 (serotype *d*). Nor did D-fructose (0.5%) induce increased production of fructanase in strains K1-R (serotype *d/g*).

Effect of various conditions on fructanase activity. — The extracellular filtrates were dialysed immediately after harvesting the cells. The dialysis step not only lowered the reducing power of the filtrate, but also gave increased values for fructanase activity. Dialysis of *S. mutans* OMZ176 culture-filtrates raised the fructanase activity by 45 and 65% after dialysis for 1 and 24 h, respectively. Fructanase activity of the dialysed filtrates did not alter during storage at 5° for up to 10 weeks, and after 10 months, those from *S. mutans* OMZ176 and K1-R still retained 55 and 70% respectively, of their original activity. The activity of culture filtrates was always determined within 2 weeks of harvesting.

Dialysed, cell-free filtrates were incubated with *S. salivarius* D-fructan at various pH values. The pH for optimal activity of the fructanase was between pH 5.5 and 6.0 (Fig. 3).

The effect of Tris on the activity of *S. mutans* AHT fructanase was determined by including various concentrations of Tris–HCl buffer in the standard-activity digests. The lowest concentration tested, 5mM, gave 85% of inhibition, and 50mM

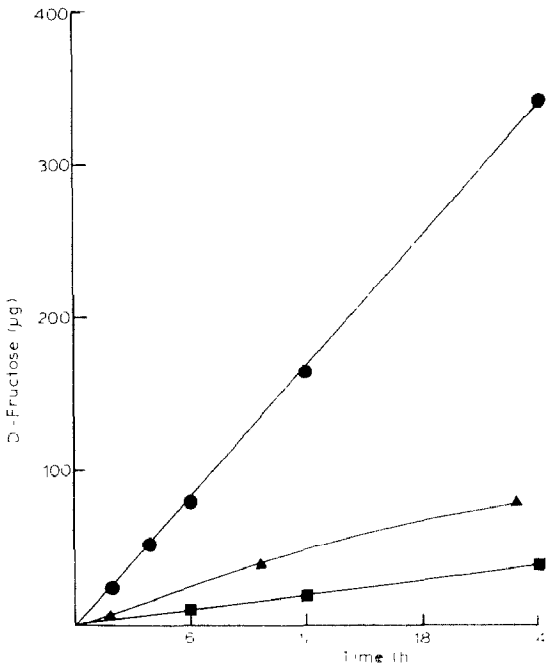

Fig. 4. Hydrolysis of *S. salivarius* fructan (●), inulin (▲), and *A. sydowi* D-fructan (■) by washed suspensions of *S. mutans* K1-R cells. The standard-activity digest (4 mL) contained toluene (0.2 mL).

TABLE III

ACTIVITY OF EXTRACELLULAR FRUCTANASE OF *S. mutans* STRAINS ON VARIOUS D-FRUCTANS

Fructan	*Major linkage*	*Fructanase (units per litre)*		
		Ingbritt	*OMZ176*	*K1-R*
S. salivarius	β-(2→6)	6.4	65.6	242
Rye grass	β-(2→6)		54.0	
Aspergillus sydowi	β-(2→1)	2.4		7.8
Inulin	β-(2→1)	0.49	5.0	26

Tris gave complete inhibition. Clearly, a more-accurate determination of fructosyltransferase activity should be possible when fructanase is inhibited. As expected, the synthesis of D-fructan by *S. mutans* NG112 was greatly increased (>3-fold) when Tris buffer (20mM) replaced citrate buffer in the standard reaction with sucrose.

Hydrolysis of fructans from various sources — The substrate specificity of extracellular fructanase from *S. mutans* strains was studied in digests containing D-fructans from different sources. The results (Table III, Fig. 4) indicated that (2→1)-β-D-fructans (*Aspergillus sydowi* fructan[10,11] and inulin[14,15]) were poor substrates for fructanase from strains K1-R and OMZ176, whereas rye-grass fructan[15] and

S. salivarius fructan[16-18], which have the same major linkage [β-(2→6)], were hydrolysed rapidly and at comparable rates. Although strain Ingbritt fructanase showed the highest relative rate of hydrolysis of *A. sydowi* D-fructan, its action on inulin was as low as that of the serotype *d* fructanases. These results indicated that the fructanase was most likely a (2→6)-β-D-fructan fructohydrolase.

An induced, extracellular enzyme of *S. salivarius* strain 51, named β-D-fructofuranosidase[3], was also more active towards (2→6)-β-D-fructan than towards inulin or methyl β-D-fructofuranoside. The specificity of this enzyme is therefore similar to the constitutive fructanase of *S. mutans*.

The affinity of fructanase for (2→6)-β-D-fructan was demonstrated in experiments in which dialysed culture-filtrates from *S. mutans* K21, *S. mutans* NG112, and *S. salivarius* ATCC13419 (12 mL) were treated with *S. salivarius* D-fructan (30 mg, 0.5 mL) for 15 min at 4°. The mixtures were then centrifuged for 1 h at 140,000*g* and the supernatant solutions were dialysed against 50mM sodium citrate buffer, pH 6.0. Determination of fructanase activity showed that, in all cases, the treatment with D-fructan had removed >90% of the fructanase from solution. Although this procedure[19] has been used for the purification of *Aerobacter levanicum* fructosyltransferase, there was no evidence that *Streptococcus sp.* fructosyltransferase was lost by adsorption to D-fructan under these conditions. This simple method for removing the bulk of fructanase activity from culture filtrates should therefore also facilitate the detection of fructosyltransferase activity. The results in Table IV indicate that fructosyltransferase activity is apparently absent from strains of *S. mutans* that produce fructanase. Nevertheless, it is possible that *S. mutans* strains AHT, K1-R, and B13 produce a D-fructan which, being a good substrate for their highly active indigenous D-fructanase, cannot readily be isolated. *S. mutans* OMZ176 is known to synthesise a (2→6)-β-D-fructan[20] from raffinose. The fact that synthesis of (2→1)-β-D-fructan by *S. mutans* was detected earlier than that of (2→6)-β-D-fructan is no doubt a consequence of the higher activity of *S. mutans* fructanase towards (2→6)-β-D-fructans. Those strains of *S. mutans* that are known to synthesise (2→1)-β-D-fructans (such as JC2[16], BHT[18], and Ingbritt[21]) produced the lowest amounts of fructanase in batch culture (Table IV). Moreover, the fructanase activity of strain Ingbritt fell to zero under conditions that are still often used for batch growth, for example without control of pH (Fig. 2).

Products of hydrolysis of S. salivarius *fructan.* — Chemical analyses of *S. salivarius* D-fructans[16-18] have shown that the linkage in the main chain is β-(2→6), and that 11–17% of β-(2→1) branch linkages may be present. Dialysed culture-filtrates of *S. mutans* strains K1-R, Ingbritt, and OMZ176 were incubated with *S. salivarius* NCTC8606 D-fructan, and the reactions were monitored to completion. The apparent conversion into D-fructose was >95%, and D-fructose was the only product detected by paper chromatography in 10:4:3 ethyl acetate–pyridine–water. This result indicated that the fructanase preparations were able to hydrolyse (2→1)-branch linkages.

D-Fructose was also the only product detected by p.c. when washed-cell sus-

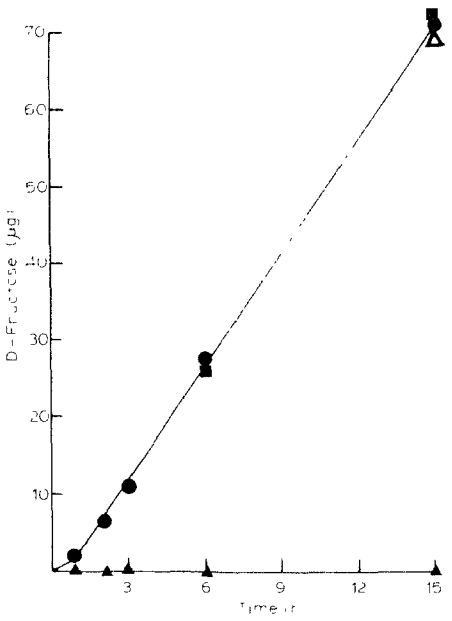

Fig. 5. Release of D-fructose from *S. salivarius* D-fructan by washed suspensions of *S. mutans* OMZ176 cells. The standard-activity digest contained either toluene (●), 5mM sodium fluoride ( ), or both toluene and fluoride (■). No D-fructose was detected (▲) in the absence of inhibitors.

pensions of *S. salivarius* or *S. mutans* were incubated with D-fructan (see Methods). No products could be detected when toluene or fluoride were omitted (Fig. 5), thus proving that the product (*D*-fructose) was utilized immediately. Either toluene or fluoride prevents the utilization of reducing saccharides by *S. mutans*[22], but these inhibitors of glycolysis were not required to demonstrate *endo*-dextranase activity of the same cell suspensions[23]. The end-products from dextran were oligosaccharides having d.p. $\geqslant 3$, and these accumulated in the digests.

It is pertinent to consider whether the extracellular fructanase of oral streptococci has the same specificity as the intracellular *S. mutans* and *S. sanguis* enzyme (named β-D-fructofuranoside fructohydrolase or invertase) that hydrolyses sucrose and raffinose[22,24]. Evidence to the contrary is provided by the following facts: (*i*) *S. mutans* intracellular extracts did not hydrolyse D-fructan, and (*ii*) the best producers of fructanase (*e.g. S. mutans* AHT and *S. sanguis* ATCC10558) fermented inulin but not raffinose, whereas strains having low D-fructanase activity (such as *S. mutans* JC2, NSW47, and *S. sanguis* 804) were able to ferment raffinose. The fermentation of raffinose and melibiose is correlated for most strains[25], and so the degradation of raffinose may produce sucrose. The indication is that invertase activity does not correspond with the release of fructanase. This conclusion may also be inferred from Table IV, where it is shown that *S. mutans* strains having low D-fructanase activity (6 units per litre, or less) have the highest apparent invertase activity (100–200 units per litre), whereas strains that release higher amounts of

TABLE IV

COMPARISON OF APPARENT FRUCTOSYL- AND GLUCOSYL-TRANSFERASE, FRUCTANASE, AND APPARENT INVERTASE ACTIVITY OF ORAL STREPTOCOCCI

Organism	*Major linkage in fructan*	*Enzymic activities (units per litre)*			
		FTF[a]	*Fructanase*	*Invertase*	*GTF*[b]
S. mutans					
NSW47		14	0	203	72
JC2	(2→1)	34	1.4	105	57
Ingbritt	(2→1)	0	6	166	81
B13		0	25	9	59
OMZ176	(2→6)	0	82	16	45
K1-R		0	130	9	108
AHT		0	430	n.d.	56
S. mitior					
S3		0	0	5	
S. sanguis					
ATCC10558		0	704	53	32
S. salivarius					
ATCC13419	(2→6)	83	37	136	72
NCTC8606	(2→6)	18	380	101	23

[a]FTF, fructosyltransferase. [b]GTF, glucosyltransferase.

fructanase (25 units per litre, or more) have far less invertase activity (9–16 units per litre).

The finding that 50% (18 out of 36) of the streptococci studied released a constitutive fructanase lends further support to previous suggestions[1,26,27] that D-fructan can function as a carbohydrate-storage compound. As the hydrolysis of D-fructan releases D-fructose, which is promptly converted into lactic acid, the ability of streptococci to produce D-fructan and fructanase can be considered to be a cariogenic property. It may be significant that *S. mutans* serotype *d* strains, which are frequently predominant in smooth-surface lesions of primary teeth[28], and in approximal plaque of adults having active caries[29], are among the best producers of fructanase.

ACKNOWLEDGMENTS

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