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EXTRACELLULAR β-D-FRUCTOFURANOSIDASE ELABORATED BY *Streptococcus salivarius* STRAIN 51 PREPARATION, AND MODE OF ACTION ON A LEVAN AND ON HOMOLOGUES OF INULOBIOSE

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ABSTRACT

Streptococcus salivarius strain 51 produces an extracellular β-D-fructofuranosidase when grown in a levan-containing medium The enzyme hydrolyses levan, and inulo-biose, -tetraose, -pentaose, and -hexaose, but has negligible effects on inulin, inulotriose, and inuloheptaose The branching in *S salivarius* strain 51 levan is through a single (2→1)-β-D linkage

INTRODUCTION

The ability to hydrolyse fructans seems to be widespread among human cariogenic streptococci[1,2] However, few reports have been concerned with such aspects of the responsible enzymes as purification and substrate specificity, although β-D-fructofuranosidases from other sources[3,4] receive unabated attention Our interest in fructan hydrolases arises from our work[5,6] on the structure of levans elaborated by oral bacteria, and stems from the observation that the amount of extracellular levan present in cultures of several strains of *Streptococcus salivarius*, with sucrose as substrate, reached a maximum between 50–60 h and thereafter decreased The amount of extracellular fructan in cultures of other bacteria, *e g*, *Odontomyces viscosus*[7], was similarly affected The subsequent hydrolysis of the fructans is probably due to the action of induced hydrolases Induction of a fructan hydrolase by a streptococcus when grown on a fructan was first noted by DaCosta and Gibbons[1] As β-D-fructofuranosidases can play a role[8] in structure determination of levans, we now report on the induction of such an enzyme by *S salivarius* strain 51 and its action on several substrates

RESULTS AND DISCUSSION

S salivarius strain 51 grew readily in a medium containing levan as the main substrate The protein precipitated with ammonium sulphate readily hydrolysed levan, fructose being the sole product detectable by p c and by electrophoresis in several

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electrolytes[9] Partial purification of the β-D-fructofuranosidase was achieved by gel filtration on Sephadex G-200, when the enzymically active protein was eluted with the void volume of the column, *i e*, its apparent M_r was $>2 \times 10^5$–6×10^5 Gel electrophoresis of the product revealed several proteinaceous materials with $M_r < 2 \times 10^5$, and subsequent attempts[10] to purify this enzyme preparation by ion-exchange chromatography resulted in gradual denaturation Such ionic materials as sodium dodecyl sulphate and cetylpyridinium chloride rapidly and irreversibly inactivate[11] β-D-fructofuranosidases from other sources In this case, therefore, gel

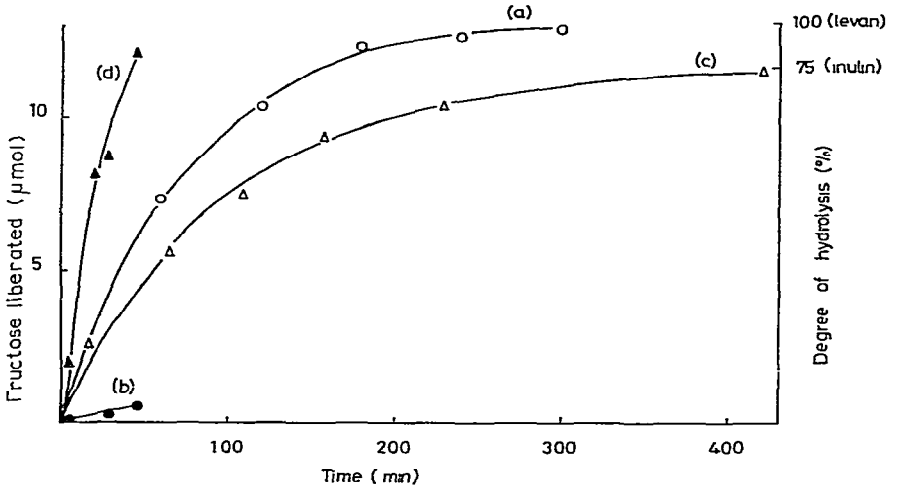

Fig 1 Action of *S salivarius* and *Candida utilis* β-D-fructofuranosidases on levan and inulin —○—, *S salivarius* enzyme (~ 0 12 U) and levan (2 1 mg), —●—, *S salivarius* enzyme (~0 12 U) and inulin (2 5 mg), —△—, *Candida utilis* enzyme (2 mg) and levan (2 1 mg), —▲—, *Candida utilis* enzyme (0 5 mg) and inulin (2 5 mg)

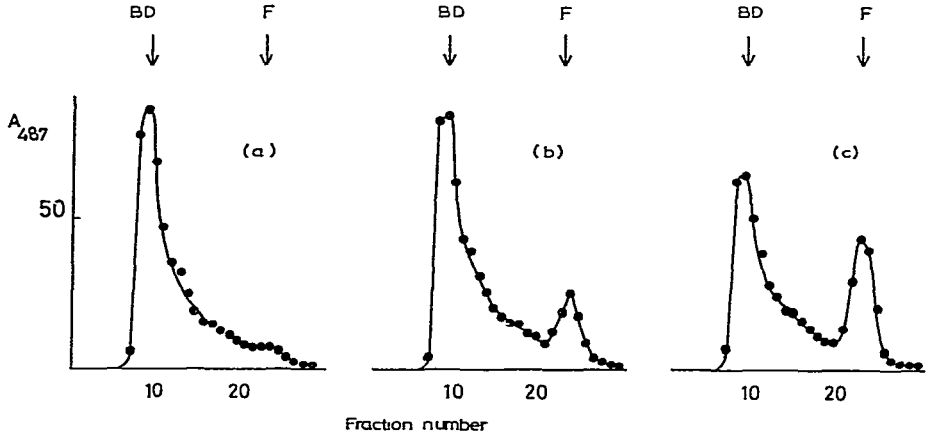

Fig 2 Chromatography on Sephadex G-200 of levan treated with *S salivarius* β-D-fructofuranosidase (a) native levan (*t* 0 h), (b) *t* 24 h, and (c) *t* 96 h, BD, Blue Dextran 2000, F, D-fructose Arrows indicate elution volumes of BD and F

electrophoresis may not be diagnostic of homogeneity Hence, we have used the enzyme preparation (*S s* -51), freeze-dried with the buffer used for the gel filtration, without further purification

The levan elaborated by the same organism[5] (when sucrose is the substrate) is completely hydrolysed by enzyme *S s* -51 (Fig 1a) The activity of the enzyme was 3 3 U/mg of protein or $2\ 4 \times 10^{-2}$ U/mg of freeze-dried solid (U = μmol of D-fructose released/min) Gel filtration of the levan and its partial enzymic hydrolysates showed (Fig 2) that the relative molecular-mass distribution of the polysaccharide remained essentially unaffected by the release of fructose We therefore conclude that the enzyme *S s*.-51 acts by the exo-mode and is indeed a β-D-fructofuranosidase

Enzyme *S s* -51 had only negligible effects on inulin and methyl β-D-fructofuranoside, even when the concentration of enzyme was greater than those shown in Fig 1

This finding is in contrast to the β-D-fructofuranosidase of *Candida utilis* (invertase), which readily hydrolysed inulin (Fig 1d), sucrose, and methyl β-D-fructofuranoside, as well as levan (Fig 1c)

The levan used[5] in this work has a branched structure possessing the structural units **1**, **2**, and **3** in the ratios ~1 7 1 Thus, enzyme *S s* -51 can hydrolyse the (2→1)-β-D linkages in levan but not the contiguous (2→1)-β-D linkages in inulin We have therefore investigated the effect of enzyme *S s* -51 on the di- and oligo-saccharides obtained by partial hydrolysis of inulin with acid Fig 3 shows that the di- and tetra-saccharides are readily hydrolysed, the increase in reducing-saccharide content of the digest containing the disaccharide (d p 2) was partly due to sucrose, from which inulobiose is not easily freed[12] The decrease in activity towards the penta-, hexa-, and hepta-saccharides is consistent with the activity of other exo-glycosidases Monitoring of the enzymic hydrolysates by p c revealed the products shown in Table I These results are complementary to those shown in Fig 3, and the fact that the disaccharide was not a detectable product confirms that the trisaccharide is not readily hydrolysed

TABLE I

PRODUCTS OF HYDROLYSIS BY *S salivarius* β-D-FRUCTOFURANOSIDASE OF DI- AND OLIGO-SACCHARIDES OBTAINED FROM INULIN

D p of substrate	*D p of product*
2	Fructose (and glucose[a])
3	None
4	3 and fructose
5	4, 3, and fructose
6	5, 4, 3, and fructose
7	None

[a]See text

β-D-Fruf-(2→

1

→6)-β-D-Fruf-(2→

2

→6)-β-D-Fruf-(2→
1
↑

3

D-Fru
1
↑
2
(β-D-Fruf)$_{1-4}$

4

→6)-β-D-Fruf-(2→
1
↑
2
(β-D-Fruf)$_n$

5

→6)-β-D-Fruf-(2→
1
↑
2
→6)-β-D-Fruf

6

→6)-β-D-Fruf-(2→
1
↑
2
→6)-β-D-Fruf
1
↑
2
→6)-β-D-Fruf
1
↑

7

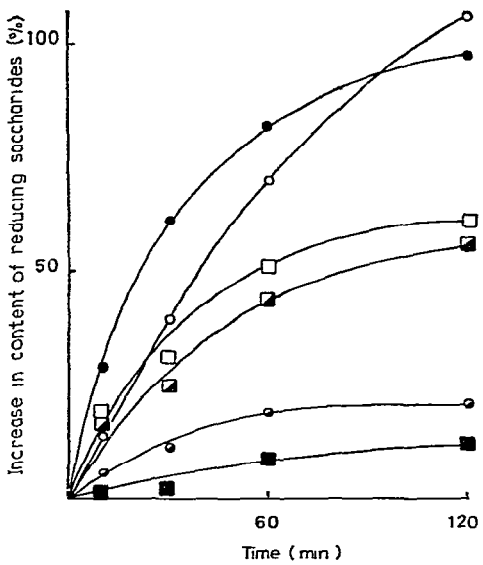

Fig 3 Action of *S salivarius* β-D-fructofuranosidase on oligosaccharides obtained from inulin —○—, d p 2, —◒—, d p 3, —●—, d p 4, —□—, d p 5, —◪—, d p 6, —■—, d p 7

Further evidence that inulobiose is hydrolysed by enzyme *S s*-51 is the fact that all the di- and oligo-saccharides obtained[5] by partial hydrolysis of the levan with acid were completely hydrolysed by the enzyme, fructose being the only final product Each of these saccharides contains an appreciable amount of a component in which the reducing D-fructose residue is linked only through C-1 (**4**) As enzyme *S s*-51 acts by the exo-mode, the hydrolysis of inulobiose is therefore the last step in these degradations

The unusual substrate-specificity of enzyme *S s* -51 cannot be commented upon until its homogeneity is ascertained. Nevertheless, the observations made here permit conclusions about the structure of the levan elaborated by *S salivarius* strain 51 Its branches through the (2→1)-β-D linkage contain[5] several D-fructofuranose residues (**5**, n = 1–4, or more) As the levan is smoothly hydrolysed to give only D-fructose, but inulotriose is essentially unaffected, we conclude that the branching is through a single (2→1)-β-D linkage (**6**), and that it is unlikely that the levan possesses segments where (2→1)-linked β-D-fructofuranosyl residues form a contiguous chain (**7**)

EXPERIMENTAL

Materials and methods — The levan used was that[5] elaborated by *S salivarius* strain 51, and inulin was a commercial sample (Sigma) The di- and oligo-saccharides from levan were those obtained[5] by partial, acid hydrolysis of the levan elaborated by *S salivarius* strain 51 The di- and oligo-saccharides from inulin were obtained in an analogous manner *Candida utilis* β-D-fructofuranosidase was a commercial sample (Grade X, Sigma)

P c was performed with 1-butanol–ethanol–water (40 11 19), with detection by silver nitrate in acetone–ethanolic sodium hydroxide

The carbohydrate content of samples was determined by the phenol–sulphuric acid method[13], and the reducing saccharides were determined by the Nelson method[14], using D-fructose as the standard Where appropriate, enzymes were deactivated before such determinations by immersion of the sample in boiling water for 15 s

Preparation of Streptococcus salivarius *β-D-fructofuranosidase* — Medium (500 cm^3) containing levan (1 %), D-glucose (0 1 %), dipotassium hydrogenphosphate (0 3 %), Tryptone (Difco, 1 %), and yeast extract (Oxoid, 0 5 %) was inoculated with a freshly sub-cultured suspension of *S salivarius* strain 51 and kept at 37° for 48 h The culture fluid was centrifuged at 4° and 8000*g* for 20 min The supernatant solution was dialysed for 48 h against an equal volume of saturated ammonium sulphate[15] Ammonium sulphate (22 5 g/100 cm^3 of dialysate) was added to the dialysate and the whole kept at 4° for 24 h The solid product was collected by centrifugation at 8000*g* for 20 min, and its solution in 0 1M citrate–phosphate buffer (pH 5.6) was dialysed against the same buffer at 4° for 48 h and concentrated in an Amicon Model 52 concentration cell (UM10 membrane) to ~5 cm^3 It was then fractionated on Sephadex G-200 (Pharmacia column K15/90, calibrated with Blue Dextran) by elution with buffer (pH 5 6, see above) The absorbance at 280 nm of fractions (3 cm^3) was recorded with an LKB Autoanalyser

Levan (0 5 %, 0 5 cm^3) and buffer (pH 5 6, 2 cm^3) were added to aliquots (0 2 cm^3) of fractions, the digest being kept at 37° for up to 7 h Determination of the release of reducing saccharides showed that Fractions 12–19, eluted with the void volume of the column, exhibited enzymic activity They were combined and freeze-dried (0 3 g, protein, 0 74 %) The bulk of the proteinaceous material was eluted with Fractions 30–50

Action of S salivarius *β-D-fructofuranosidase on various substrates* — (*a*) A mixture of levan (0 2%, 0 5 cm^3), dialysed enzyme preparation (~0 01 U, 0 5 cm^3), and 0 1M citrate–phosphate or 0 1M phosphate buffer (1 cm^3) was kept at 37° for 15 h, and the liberation (maximum at pH ~6 7) of reducing saccharides was determined

(*b*) A mixture of levan (0 42%, corrected for moisture and D-fructose content, 0 5 cm^3), dialysed enzyme preparation (from 5 mg in 1 cm^3 of H_2O), and 0 1M phosphate buffer (pH 6 7, 1 5 cm^3) was kept at 37°. The liberation of reducing saccharides was monitored (Fig 1a)

(*c*) Digests of concentrations similar to those above, but containing the oligosaccharides obtained from levan, sucrose, methyl β-D-fructofuranoside[16], inulin, or the oligosaccharides obtained from inulin, were incubated similarly, and processed as above and/or examined by p c (Figs 1 and 3)

(*d*) A mixture of levan (2 5%, 1 cm^3), enzyme preparation (~0 12 U), and 0.1M phosphate buffer (pH 6 7, 1 cm^3) was kept at 37° Samples (0 3 cm^3) were withdrawn at 0, 24, and 96 h, and fractionated on Sepharose 2B (Pharmacia column K9/30, calibrated with Blue Dextran and D-fructose) by elution with 1% sodium chloride The carbohydrate content of fractions (0 6 cm^3) was determined by the phenol–sulphuric acid method[13] (Fig 2)

Action of Candida utilis *β-D-fructofuranosidase on various substrates* — Digests containing levan, inulin, or methyl β-D-fructofuranoside (0 25–0 9%, 1 cm^3), enzyme preparation (~0 1–0 2%, 1 cm^3), and 0 1M phosphate buffer (pH 6 65, 3 cm^3) were kept at 37° and processed, at intervals, for determination of the content of reducing saccharides (Fig 1)

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