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Purification and Preliminary Characterization of Exo-β-D-Fructosidase in *Streptococcus salivarius* KTA-19

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***Streptococcus salivarius* fructosidase (β-D-fructan fructohydrolase, EC 3.2.1.80) was purified to homogeneity. The molecular weight of the fructosidase was estimated to be 83,000 to 85,000 by gel filtration and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The pH optimum of the enzyme was 7.0, and the isoelectric point was pH 4.7. The purified enzyme preparation hydrolyzed levan, inulin, and several 2-β-linkage-containing oligosaccharides such as sucrose and raffinose, but not melezitose, dextran, and pseudonigeran. The fructosidase was inhibited by Fe^{3+}, Cu^{2+}, Hg^{2+}, and Ag^{+}, but not by Ca^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+}, at a concentration of 10^{-3} M. Mn^{2+} was particularly effective in stimulating activity at the same concentration. The presence of either EDTA or KCN also increased fructosidase activity by 20 to 30%. The enzyme was susceptible to sulfhydryl reagents since *p*-chloromercuribenzoate (10^{-7} M) produced 63% inhibition of the activity. However, this inhibition was overcome in the presence of cysteine. This enzyme acts as an exofructosidase since thin-layer chromatographic analysis revealed that D-fructose was formed from levan or inulin by the action of the enzyme.**

A renewed interest in the fructan-hydrolyzing enzymes produced from oral bacteria has resulted from the realization that these enzymes potentially participate in dental plaque metabolism of the oral environment (2, 8, 10, 14, 26, 30). The fructan-hydrolyzing enzymes from *Actinomyces viscosus*, *Actinomyces naeslundii*, *Streptococcus mutans*, *Streptococcus salivarius*, and other dental plaque organisms have been reported previously but not fully characterized: although a fructan-hydrolyzing enzyme has been partially purified from *A. viscosus* by Miller and Somers (19), biochemical properties and reaction mechanisms of the fructan-hydrolyzing enzyme are notably lacking (2, 14, 18, 26, 27, 30). Our previous investigation showed that the supernatant of the culture of *S. salivarius* KTA-19 contained two types of fructan hydrolases: one was a levanase showing an activity specific for levan (24), and the other was a nonspecific fructan hydrolase (K. Takamori, F. Mizuno, N. Takahashi, and T. Horikawa, Jpn. Div. Int. Assoc. Dent. Res., Osaka, abstr. no. 61, 1982). In this communication, we report the biochemical properties of a nonspecific fructan-hydrolase (fructosidase) present in the culture supernatant of *S. salivarius* KTA-19.

MATERIALS AND METHODS

Bacterial strains. *S. salivarius* KTA-19 for enzyme purification and KT-12 for levan preparation were isolated from dental plaque in our laboratory. These organisms were cultivated as described previously (24).

Substrate. Levan, a β-(2,6) fructan, was prepared from the supernatant of the culture of *S. salivarius* KT-12 in a brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with 5% sucrose, as described by Feingold and Gehatia (4). Inulin, a β-(2,1) fructan, and other substrates (sucrose, raffinose, and melezitose) were purchased from Sigma Chemical Co., St. Louis, Mo. Acid hydrolysis of the levan and inulin preparations detected only fructose by thin-layer chromatography.

Assay methods. The fructosidase activity was measured by monitoring the release of reducing sugar after incubation of the enzyme with the substrate by the method of Somogyi (22). Unless otherwise noted, the reaction was performed by the addition of 0.5 ml of an appropriately diluted enzyme solution into 0.5 ml of 20 mM sodium phosphate buffer (pH 7.0) containing 0.2% inulin at 37°C for 30 min. One unit of fructosidase activity was defined as the amount of enzyme that liberated 1 μmol of reducing sugar, equivalent to fructose from inulin, per min under standard conditions. Levan-hydrolyzing activity was estimated in the same way as above by using 0.2% levan solutions in place of inulin (21). Hydrolysis of sucrose, raffinose, and melezitose was assayed by the formation of reducing sugars from the respective substrates by the method of Somogyi (22) and by the formation of D-glucose with glucose oxidase reagent by the method of Sund and Linder (23). Protein was determined by the method of Lowry et al. (13), with bovine serum albumin as the standard. The protein in the eluates from column chromatography was monitored by recording the absorbance at 280 nm with an LKB Uvicord or Shimadzu double-beam spectrophotometer (model UV-200).

Polyacrylamide gel electrophoresis. Polyacrylamide disc gel electrophoresis was performed with the discontinuous buffer system of Davis (3). After electrophoresis, some gels were stained with Coomassie brilliant blue, and the other gels were qualitatively stained by the 2,3,5-triphenyltetrazolium chloride method of Gabriel and Wang (6). The extruded polyacrylamide gels were washed several times with distilled water and incubated for 2 h at 37°C in 20 mM phosphate buffer (pH 7.0) containing 10 mg of levan or inulin. The gels were then rinsed thoroughly with water, transferred to a freshly prepared solution containing 0.1% 2,3,5-triphenyltetrazolium chloride in 1.0 M NaOH, and kept in this solution for 15 min at room temperature in the dark. The gels were removed from the staining mixture when the formation of a

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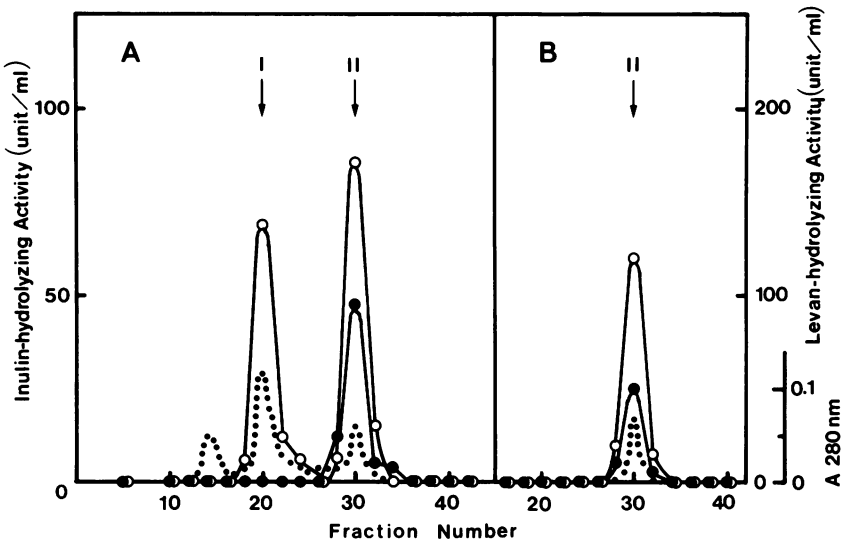

FIG. 1. Gel chromatography on Bio-Gel A1.5m. (A) Fractions with fructosidase activity eluted from a DEAE-cellulose chromatography were pooled, concentrated, and applied to gel filtration chromatography (2.5 by 70 cm) that was previously equilibrated with 20 mM phosphate buffer (pH 7.0) and eluted with the same buffer. Fractions (5 ml) were collected at a flow rate of 15 ml/h. Peak I comprises fractions 19 to 21 as reported previously (24), and peak II contains fractions 29 to 31. (B) Bio-Gel A1.5m rechromatography. Material pooled from the second fructan-hydrolyzing peak II eluted from A was rechromatographed by the same column which was used with the same elution conditions. Symbols: (○) levan-hydrolyzing activity; (●) inulin-hydrolyzing activity; and (. . .) absorbance at 280 nm.

discrete pink band on a diffuse background was observed, and they were thoroughly washed with 7.5% acetic acid until the excess 2,3,5-triphenyltetrazolium chloride was removed. The resulting gels were stored in 7.5% acetic acid. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and calculation of molecular weights were performed by the method of Weber and Osborn (28) with 7.5% polyacrylamide-2% SDS gels. Sample solutions (10 μg of protein in 50 μl) were prepared by heating at 100°C for 2 min in 20 mM phosphate buffer (pH 7.0) containing 1% SDS (wt/vol) and 1% β-mercaptoethanol (vol/vol).

Isoelectric focusing. Isoelectric focusing was performed at 4°C in an LKB 8101 electrofocusing column (LKB Produkter AB; pH range of 3.5 to 10.0) with glycerol as the stabilizing agent. Purified enzyme solution (2 ml) was applied to the column and focused for 40 h at ca. 4 W. Fractions (2.5 ml) were collected from the gradient, and the pH of each fraction was measured with a microelectrode (Horiba, Ltd., Kyoto). Fructosidase activity was measured as described above.

Thin-layer chromatography. Samples were applied to an Avicel SF (microcrystalline cellulose-acetate film) plate (Funakoshi Pharmaceutical Co. Ltd., Tokyo) and developed with *n*-butanol–pyridine–water (6:4:3 [vol/vol/vol]) as described previously (24). The sugar spots were visualized by spraying alkaline silver nitrate solution on the plate.

RESULTS

Purification of enzyme. All procedures for enzyme purification were carried out at 4°C. Column resins and protein samples were initially equilibrated with 20 mM phosphate buffer (pH 7.0). The cell extract was obtained from the culture supernatant of *S. salivarius* KTA-19 precipitated with ammonium sulfate and was followed by acetone fractionation as reported previously (24). The extract (629 mg of protein) was dissolved in 10 ml of the buffer and submitted to chromatography on a Bio-Gel A5m column (2.5 by 100 cm; Bio-Rad Laboratories, Richmond, Calif.). Flow rate was 20 ml/h, and 7-ml fractions were collected. All fractions containing over 5 U of fructan-hydrolyzing activity per ml were pooled and used for the next purification step. The enzyme activity could be readily purified ca. 100-fold with a 25% yield in three steps from the cell extract. Fractions having fructan-hydrolyzing activities were pooled, concentrated by ultrafiltration in an Amicon pressure cell equipped with a Diaflo PM-10 membrane (Amicon Corp., Lexington, Mass.), and then applied to a DEAE-cellulose column (DE-52; 1.0 by 25 cm; Whatman Biochemical Ltd., Kent, England). The column was eluted with 200 ml of phosphate buffer, followed by a 200-ml linear gradient of 0 to 1.0 M sodium chloride in phosphate buffer at a flow rate of 25 ml/h (5-ml fractions were collected). The most active fractions of fructan-hydrolyzing activity were pooled, concentrated by ultrafiltration, and then applied to a Bio-Gel A1.5m column (2.5 by 70 cm). The column was eluted with the buffer at 15 ml/h. This final purification step resulted in the separation of two fructan hydrolases: levanase and nonspecific fructan hydrolase (fructosidase), which were distinguishable by their different specificity for the substrates. Levanase comprised fractions 19 to 21 as described previously (24). The activity present in fractions 29 to 31, which possessed both inulin- and levan-hydrolyzing activity, was further investigated (Fig. 1A). The enzyme activity rechromatographed on the Bio-Gel A1.5m column showed a single symmetrical peak coincident with the absorbance at 280 nm which corresponded to a molecular weight of ca. 84,000 ± 1,000 (Fig. 1B). The purification data of the enzymes are summarized in Table 1. Seven percent of the activity of the initial extract was recovered from this column in a volume of ca. 5 ml.

Enzyme purity. Purified enzyme was electrophoresed under nondenaturing conditions at 4°C, and the gel was stained for fructosidase activity by the procedure of Gabriel and Wang (6) and for protein with Coomassie brilliant blue. A single band appeared in each gel with either stain. The band possessing enzyme activity and that of protein staining migrated at the same distance on the gel (Fig. 2A and B). A single band was also observed when purified enzyme was

TABLE 1. Purification of fructosidase from *S. salivarius* KTA-19

Purification step	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Yield (%)	Purification (-fold)
Crude enzyme	8,600	3,354	0.39	100	1
Ammonium sulfate precipitation	2,112	3,119	1.5	93	4
Acetone fractionation	629	2,750	4.4	82	11
Bio-Gel A5m	22.7	839	37.0	25	95
DEAE-cellulose	3.5	637	182.0	19	447
Bio-Gel A1.5m	0.55	238	433.0	7	1.110

electrophoresed in the presence of SDS. On the basis of these observations, fructosidase purified in the present report was found to be electrophoretically homogeneous.

Molecular weight. The molecular weight of the native protein was ca. 83,000 to 85,000 as estimated by gel filtration with Bio-Gel A1.5m. Analysis of the purified enzyme by electrophoresis under denaturing conditions yielded a molecular weight of 85,000 (Fig. 3).

Isoelectric focusing. When subjected to preparative isoelectric focusing between pH 3.5 and 10.0, purified enzyme was obtained at a pI value of 4.7 (Fig. 4).

Enzymatic properties. Under standard assay conditions, the effect of pH on fructosidase activity was tested with acetate and phosphate buffers of pH values ranging from 3.0 to 8.0. The pH activity profile (Fig. 5) indicated a pH optimum of 7.0. The enzyme was stable at 37°C for 30 min at pH 7.0, but it gradually became inactivated below pH 5.0 and lost almost all its activity at pH 4.5. The temperature dependence of the maximum reaction rate was examined in the range of 20 to 70°C. The optimum activity was obtained at 50°C. The enzyme was heat labile; it retained ca. 80% of the activity up to 55°C but rapidly became inactive over 60°C. When stored at 4°C as a diluted solution (20 μg/ml) in 20 mM phosphate buffer (pH 7.0), the enzyme was stable at least for several days and thereafter gradually became inactive; ca. 90% of the activity was lost by 2 weeks. Storage of the enzyme solution at −20°C rendered it more stable than at 4°C, but repeated freezing and thawing accelerated the inactivation. The purified enzyme showed high activity against levan [β-(2,6) fructan] and inulin [β-(2,1) fructan], but it was inactive for dextran [α-(1,6) glucan] and pseudonigeran [α-(1,3) glucan] (Table 2). The enzyme was also tested with a number of substrates containing the 2-β-linkages of D-fructose, including sucrose, raffinose, and melezitose. The enzyme hydrolyzed sucrose but hydrolyzed raffinose to a lesser extent. With each of these substrates, the release of fructose was confirmed by thin-layer chromatography. No activity could be detected with melezitose as a substrate. The enzyme activity was inhibited by Fe^{3+}, Cu^{2+}, Hg^{2+}, and Ag^{+}, but not by Ca^{2+}, Co^{2+}, Mg^{2+}, and Zn^{2+}, at

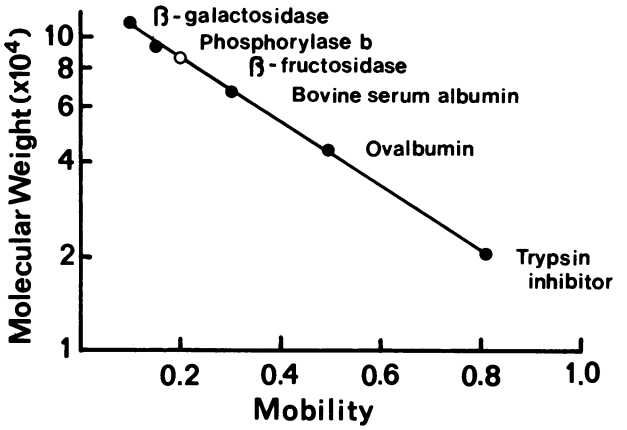

FIG. 3. Molecular weight determination of *S. salivarius* fructosidase by SDS-polyacrylamide gel electrophoresis. The molecular weights were estimated from the following protein standards: β-galactosidase, 116,000; phosphorylase b, 94,000; bovine serum albumin, 67,000; ovalbumin, 43,000; and trypsin inhibitor, 20,100.

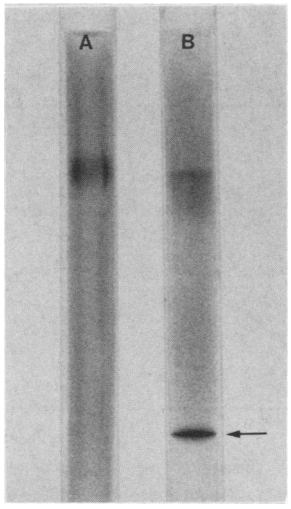

FIG. 2. Disc gel electrophoresis of the Bio-Gel A1.5m fraction. The protein (40 μg) was electrophoresed for 3 h at 4°C. The fructosidase activity of the left gel (A) was obtained as described in the text. The right gel (B) was stained for protein with Coomassie brilliant blue. The position of the dye front is indicated by the arrow.

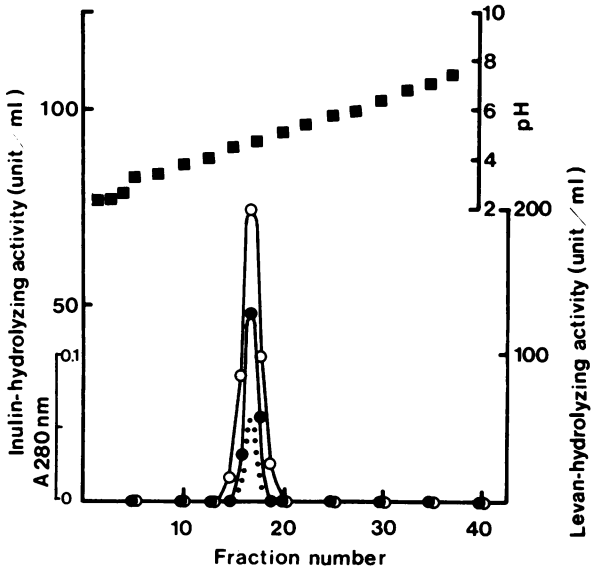

FIG. 4. Determination of homogeneity and isoelectric point (pI) of *S. salivarius* fructosidase. The enzyme (20 μg) from the Bio-Gel A1.5m column was applied to an isoelectric focusing column. Levan- (○) and inulin- (●) hydrolyzing activity, pH gradient (■), and protein (. . .) were measured as described in the text.

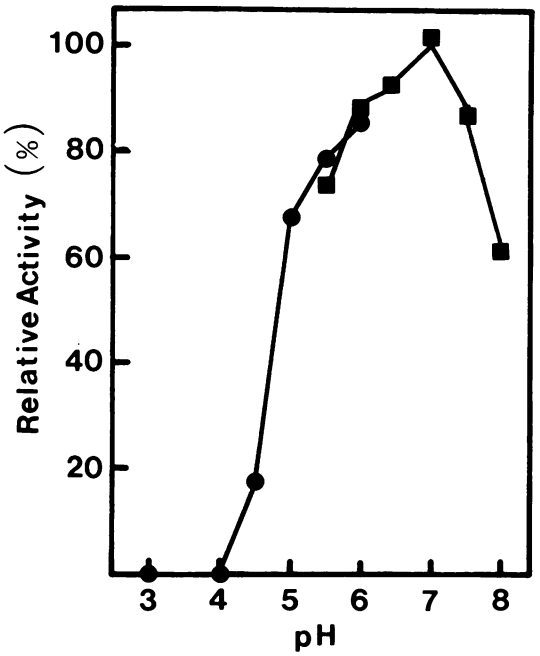

FIG. 5. The effect of pH on the activity of *S. salivarius* fructosidase. The enzyme was assayed with buffers of various pH under the conditions described in the text. Buffers used were as follows: CH_3COOH-CH_3COONa buffer for pH 3.0 to 6.0 (●) and Na_2HPO_4-NaH_2PO_4 buffer for pH 5.5 to 8.0 (■).

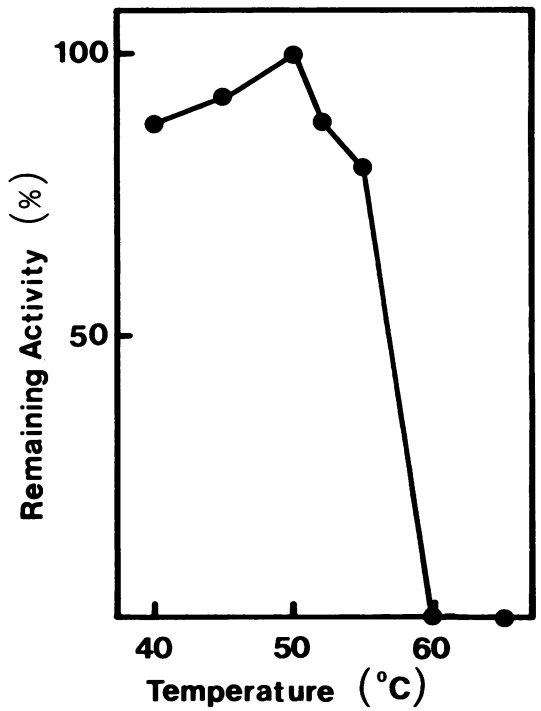

FIG. 6. Heat stability of *S. salivarius* fructosidase. Enzyme samples dissolved in 20 mM phosphate buffer (pH 7.0) were heated from 15 min at various temperatures, and the remaining activity was determined as described in the text.

a concentration of 10^{-3} M. Mn^{2+} stimulated its activity (ca. 70%) at the same concentration. The presence of either EDTA or KCN showed a slightly stimulative effect after 30 min of incubation with fructosidase. The enzyme was particularly susceptible to a sulfhydryl reagent, *p*-chloromercuribenzoate, showing 63% inhibition of enzyme activity at a concentration of 10^{-7} M. In contrast, another sulfhydryl reagent, iodoacetate, produced a negligible inhibition at this concentration. The presence of cysteine completely protected the enzyme activity against *p*-chloromercuribenzoate inactivation. However, the addition of cysteine after the inactivation of fructosidase by *p*-chloromercuribenzoate showed no effect for the recovery of activity. Tris gave 25% inhibition at a concentration of 10^{-3} M. *S. salivarius* fructosidase was able to hydrolyze both inulin and levan. Throughout the reaction, thin-layer chromatographic analysis revealed that D-fructose was the only product from both polysaccharides (Fig. 6). These results suggest that *S. salivarius* fructosidase has a typical exohydrolytic nature.

TABLE 2. Substrate specificity of fructosidase from *S. salivarius* KTA-19

Substrate and concn[a]	Reducing sugar (μmol/ml)
Sucrose (10 mM)	27
Raffinose (10 mM)	15
Melezitose (10 mM)	0
Levan (2 mg/ml)	65
Inulin (2 mg/ml)	43
Dextran T-2000[b] (2 mg/ml)	0
Pseudonigeran[c] (2 mg/ml)	0

[a] Each substrate was incubated at the concentrations indicated for 30 min in standard reaction mixtures.
[b] From Pharmacia Fine Chemicals (Uppsala, Sweden).
[c] From *Aspergillus niger* ATCC 16888 as described by Hasegawa et al. (9).

DISCUSSION

In a previous paper, we described a levanase isolated from the culture supernatant from *S. salivarius* KTA-19 which showed specific activity for levan (24). Avigad and Bauer (1) have described three types of fructan hydrolase. The patterns of action of these enzymes fell in one of the following categories: (i) a nonspecific β-fructofuranosidase which hydrolyzes 2-β-linkage-containing poly- or oligosaccharides;

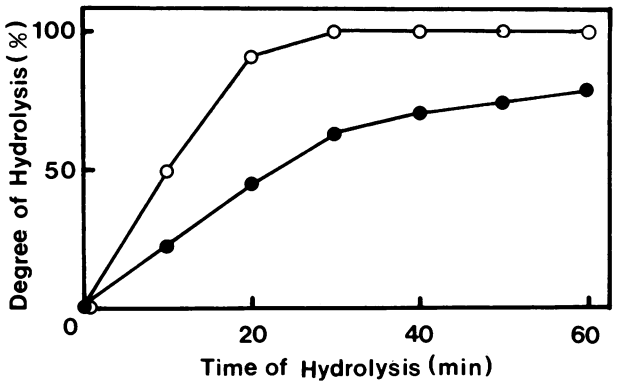

FIG. 7. Comparison of the time course of hydrolysis of levan and inulin by *S. salivarius* fructosidase. Fructosidase was incubated with levan (○) or inulin (●) in 20 mM phosphate buffer (pH 7.0) at 37°C. At the appropriate time, samples were withdrawn from the reaction mixture, and the amount of reducing sugar was determined as described in the text.

TABLE 3. Effect of various metal ions and chemicals on purified fructosidase activity[a]

Additions	Concn (M)	Relative activity (%)	Additions	Concn (M)	Relative activity (%)
None	0	100	None	0	100
Ca^{2+}	10^{-3}	100	KCN	10^{-3}	119
Co^{2+}	10^{-3}	100	Disodium EDTA	10^{-3}	132
Mg^{2+}	10^{-3}	100	Tris	10^{-3}	74
Zn^{2+}	10^{-3}	100	pCMB[b]	10^{-5}	0
Mn^{2+}	10^{-3}	171		10^{-6}	12
Fe^{3+}	10^{-3}	45		10^{-7}	63
Cu^{2+}	10^{-3}	0			
Ag^{+}	10^{-3}	10			
Hg^{2+}	10^{-5}	17			
	10^{-6}	50			
	10^{-7}	90			

[a] All of the metals and chemicals were tested at a final concentration of 10^{-3} M, with the exception of Hg^{2+} and pCMB. The enzyme solutions were preincubated with each of the metal ions or chemicals (1/500 M) at 37°C for 30 min before measurement of activities. After preincubation, an equal volume of levan solution in phosphate buffer (pH 7.0) was added and incubated for 30 min.

[b] pCMB, p-Chloromercuribenzoate.

(ii) an enzyme which specifically hydrolyzes β-(2,6) linkage (levanase); or (iii) an enzyme which specifically hydrolyzes β-(2,1) linkage (inulinase). In the present report, we described the purification and properties of a nonspecific β-fructofuranosidase (fructosidase) in *S. salivarius* KTA-19, which could be categorized as a nonspecific β-fructofuranosidase. The final purification was 1,110-fold, with a recovery of 7%. The low overall yield during purification was mostly due to the sacrifice of fractions showing less than maximum specific activity, particularly after DEAE-cellulose and gel filtration chromatography. The enzyme preparation seems to be homogeneous, as indicated on polyacrylamide gel electrophoresis in the presence and absence of SDS and on isoelectric focusing. By polyacrylamide gel electrophoresis, the protein band also coincided with fructosidase activity. The purified enzyme also showed a single protein peak on Bio-Gel A1.5m column chromatography. The homogeneous *S. salivarius* fructosidase indicated low substrate specificity. It is shown to be a nonspecific fructan-hydrolyzing enzyme which catalyzes 2-β-linkage-containing poly- or oligosaccharides to D-fructose. This fructosidase hydrolyzed unsubstituted β-fructofuranoside such as sucrose and raffinose, but not that of substituted β-fructofuranoside such as melezitose. Moreover, it hydrolyzed poly-β-fructofuranoside such as levan and inulin, although it did not hydrolyze poly-α-glucoside such as dextran and pseudonigeran. Therefore, we assume that *S. salivarius* fructosidase is a nonspecific type of β-fructofuranosidase capable of attacking both β-(2,6) and β-(2,1) fructofuranoside linkages, and its hydrolytic function is thought to cleave the terminal fructosyl unit from the chain ending. Oral streptococcal (5, 7, 12, 15, 16, 25) and *A. viscosus* (11) invertases have similar specificity and hydrolyze the unsubstituted β-fructofuranoside raffinose as well as sucrose. However, *S. salivarius* KTA-19 appeared to produce an undetectable level of invertase activity after growth with levan. Since the results were obtained with the purified enzyme preparation, it is likely that this fructosidase functions in a manner similar to the fructan hydrolase produced by oral streptococci as reported by DaCosta and Gibbons (2) and by *A. naeslundii* as reported by Miller (18). The fructosidase from *S. salivarius* KTA-19 can be identified as exo-β-D-fructosidase (β-D-fructan fructohydrolase, EC 3.2.1.80), based on substrate specificity properties.

The biological role of the fructan was investigated in its relation to dental disease. Although *S. salivarius* is not normally found in large numbers in dental plaque, other organisms predominantly inhabiting dental plaque, such as *A. viscosus* and *A. naeslundii* (18, 19), produce fructan-hydrolyzing enzyme. Fructans formed in plaque would serve as an exogenous carbohydrate source for plaque bacteria (17, 20) and would become gradually hydrolyzed by such bacteria, resulting in continued acid production in plaque (14, 29). In the presence of the fructan-hydrolyzing enzymes, a rapid decline in plaque fructan concentration would influence the production of polysaccharides and therefore have a profound effect on plaque accumulation and metabolism. It is possible that the enzymes produced and secreted into human saliva by *S. salivarius* may also play a role in plaque metabolism.

LITERATURE CITED

1. **Avigad, G., and S. Bauer.** 1966. Fructan hydrolase. Methods Enzymol. **8**:621–628.
2. **DaCosta, T., and R. J. Gibbons.** 1968. Hydrolysis of levan by human plaque streptococci. Arch. Oral Biol. **13**:609–617.
3. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404–427.
4. **Feingold, D. S., and M. Gehatia.** 1959. The structure and properties of levan, a polymer of D-fructose produced by cultures and cell-free extracts of *Aerobacter levanicum*. J. Polym. Sci. Part D Macromol. Rev. **23**:783–790.
5. **Fukui, K., Y. Fukui, and T. Moriyoma.** 1974. Purification and properties of dextransucrase and invertase from *Streptococcus mutans*. J. Bacteriol. **118**:796–804.
6. **Gabriel, O., and S.-F. Wang.** 1969. Determination of enzymatic activity in polyacrylamide gels. I. Enzymes catalyzing the conversion of nonreducing substrates to reducing product. Anal. Biochem. **27**:545–554.
7. **Gibbons, R. J.** 1972. Presence of an invertase-like enzyme and a sucrose permeation system in strains of *Streptococcus mutans*. Caries Res. **6**:122–131.
8. **Gold, W., F. B. Preston, M. C. Lache, and H. Blechman.** 1974. Production of levan and dextran in plaque in vivo. J. Dent. Res. **53**:442–446.
9. **Hasegawa, S., J. H. Nordin, and S. Kirkwood.** 1968. Enzymes that hydrolyze fungal cell wall polysaccharides. I. Purification and properties of an end-α-D-(1-3)-glucanase from *Trichoderma viride*. J. Biol. Chem. **244**:5460–5470.
10. **Higuchi, M., Y. Iwami, T. Yamada, and S. Araya.** 1970. Levan synthesis and accumulation by dental plaque. Arch. Oral Biol. **15**:563–567.
11. **Kiel, R. A., J. M. Tanzer, and F. N. Woodiel.** 1977. Identification, separation, and preliminary characterization of invertase and β-galactosidase in *Actinomyces viscosus*. Infect. Immun. **16**:81–87.
12. **Kuramitsu, H. K.** 1973. Characterization of invertase activity from cariogenic *Streptococcus mutans*. J. Bacteriol. **115**: 1003–1010.
13. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
14. **Manly, R. S., and D. T. Richardson.** 1968. Metabolism of levan by oral samples. J. Dent. Res. **47**:1080–1086.
15. **Maynard, M. T., and H. K. Kuramitsu.** 1979. Purification and antigenic properties of intracellular invertase from *Streptococcus mutans*. Infect. Immun. **23**:873–883.

16. **McCabe, M. M., E. E. Smith, and R. A. Cowman.** 1973. Invertase activity in *Streptococcus mutans* and *Streptococcus sanguis*. Arch. Oral Biol. **18**:525–531.
17. **McDougall, W. A.** 1964. Studies on the dental plaque. IV. Levans and the dental plaque. Aust. Dent. J. **9**:1–5.
18. **Miller, C. H.** 1974. Degradation of sucrose by whole cells and plaque of *Actinomyces naeslundii*. Infect. Immun. **10**:1280–1291.
19. **Miller, C. H., and P. J. B. Somers.** 1978. Degradation of levan by *Actinomyces viscosus*. Infect. Immun. **22**:266–274.
20. **Parker, R. B., and H. R. Creamer.** 1971. Contriburion of plaque polysaccharides to growth of cariogenic microorganisms. Arch. Oral Biol. **16**:855–862.
21. **Snyder, H. E., and H. J. Phaff.** 1960. Studies on a beta-fructosidase (inulinase) produced by *Saccharomyces fragilis*. Antonie van Leeuwenhoek J. Microbiol. Serol. **26**:433–452.
22. **Somogyi, M.** 1952. Notes on sugar determination. J. Biol. Chem. **195**:19–23.
23. **Sund, M.-L., and L. Linder.** 1980. Cell-surface bound β-fructofuranosidase (invertase) of the oral bacterium *Streptococcus mitis*. Arch. Oral Biol. **25**:573–578.
24. **Takahashi, N., F. Mizuno, and K. Takamori.** 1983. Isolation and properties of levanase from *Streptococcus salivarius* KTA-19. Infect. Immun. **42**:231–236.
25. **Tanzer, J. M., A. T. Brown, M. F. McInerney, and F. W. Woodiel.** 1977. Comparative study of invertase of *Streptococcus mutans*. Infect. Immun. **16**:318–327.
26. **Van Houte, J., and H. M. Jansen.** 1968. Levan degradation by streptococci isolated from human dental plaque. Arch. Oral Biol. **13**:827–830.
27. **Walker, G. J., M. D. Here, and J. G. Morrey-Jones.** 1983. Activity of fructanase in batch cultures of oral streptococci. Carbohydr. Res. **113**:101–112.
28. **Weber, K., and M. Osborn.** 1969. The reliability of molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.
29. **Wood, J. M.** 1964. Polysaccharide synthesis and utilization by dental plaque. J. Dent. Res. **43**:955.
30. **Wood, J. M.** 1967. The amount, distribution and metabolism of soluble polysaccharides in human dental plaque. Arch. Oral Biol. **12**:849–858.