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Expression, Purification, and Characterization of an Exo-β-D-Fructosidase of *Streptococcus mutans*

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A genetic library of *Streptococcus mutans* GS-5, constructed in an *Escherichia coli* plasmid vector, was screened for cells which could utilize sucrose as the sole carbon and energy source. The recombinant plasmid pFRU1, containing a 4.2-kilobase pair insert of *S. mutans* DNA, was shown to confer this phenotype. Further characterization of the gene product encoded by pFRU1 revealed that the enzyme was a β-D-fructosidase with the highest specificity for the β(2→6)-linked fructan polymer levan. The enzyme could also hydrolyze inulin [β(2→1)-linked fructan], sucrose, and raffinose with 34, 21, and 12%, respectively, of the activity observed for levan. The gene (designated *fruA*) appeared to be expressed under its own control in *E. coli*, as judged by the lack of influence on gene product activity of induction or repression of the β-galactosidase promoter adjacent to the insertion site on the cloning vector. The protein was purified to homogeneity, as judged by silver staining of purified protein in denaturing and reducing conditions in polyacrylamide gels, from sonic lysate of *E. coli*, as well as from culture supernatants of *S. mutans* GS-5 grown in a chemostat at low dilution rate with fructose as the sole carbohydrate source. Both purified proteins had an apparent molecular mass of 140,000 daltons in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, were immunologically related and comigrated in sodium dodecyl sulfate-polyacrylamide gel electrophoresis as determined by Western blotting with antisera raised against the cloned gene product, and were identical in all physical and biochemical properties tested. The pH optimum of the enzyme acting on fructan polymers was 5.5, with a significant amount of activity remaining at pH 4.0. The optimum pH for sucrose degradation was broader and lower, with a peak at approximately 4.5. Enzyme activity was inhibited almost completely by Hg^{2+} and Ag^{2+}, inhibited partially by Cu^{2+}, not inhibited by fluoride ion or Tris, and slightly stimulated by Mn^{2+} and Co^{2+}. Fructan polymers were attacked exohydrolytically by the enzyme, fructose being the only product released. With sufficient time, both levan and inulin were degraded to completion, with no evidence of product inhibition.

Several bacteria which normally inhabit the human mouth, including *Streptococcus mutans* (2, 3, 12, 18, 38), *S. salivarius* (3, 12, 13, 33), *S. sanguis* (24), and *Actinomyces viscosus* (3, 49), can synthesize polymers of D-fructose. The structures of the fructans produced by many of these organisms have been analyzed, and it appears as though *S. salivarius* (3, 12, 13, 32) and the actinomycetes (3, 49) produce a levan-type fructan, consisting predominantly of β(2→6)-linked fructosyl units with a significant degree of branching in the 1 position (13, 32). *S. mutans*, on the other hand, produces an inulin-type fructan which is made up almost exclusively of β(2→1) linkages with some branching in the 6 position (2, 3, 12, 38). In some cases, the physical properties of the fructan polymers have been examined, and generally they are high-molecular-weight polysaccharides (10^6 to 10^7). Their large size, probable spherical shape, and solution viscosity properties (3, 13) would indicate that these polymers would probably not diffuse from within the plaque matrix. Thus, it has been proposed that fructans could serve as storage polysaccharides (9, 31).

The actual synthesis and accumulation of fructans by human dental plaque samples in vitro (22) as well as the accumulation of fructans in plaque in vivo in studies with human volunteers fed a diet rich in sucrose have been demonstrated (16). Following a sucrose intake, fructans were shown to accumulate rapidly and the level of keto sugar slowly decreased with time. More recently, studies by Rølla et al. (36) indicated that the enzymes which participate in the synthesis of fructans are present in significant quantities and are able to actively synthesize fructans in early acquired enamel pellicle, the complex mixture of proteins and other macromolecules which coats the tooth surface after a thorough cleaning.

Manly and Richardson (31) have described the hydrolysis of levan catalyzed by human plaque samples, and the ability to hydrolyze both inulin- and levan-type fructans by human plaque streptococci has been observed by DaCosta and Gibbons (11) and van Houte and Jansen (44). Limited data are available on fructan hydrolase activity from *S. mutans*, but the existing data suggest a number of interesting properties with respect to regulation of the expression of the gene(s) for fructan degradation. An activity (or activities) from *S. mutans*, which is able to degrade β(2→6)- and β(2→1)-linked polymers of D-fructose, has been shown to be inducible by substrate or fructose and repressed in the presence of glucose (9, 23, 47, 48). Maximal synthesis of the enzyme(s) was observed at low growth rates, while little activity was produced by cultures grown at high dilution rates in continuous culture (23, 47, 48). Regulation of fructanase in this manner could have the net effect of allowing fructan reserves to accumulate in plaque during periods of dietary sucrose intake, as has been observed (16, 21). Then, at a later time, when exogenous sources of carbohydrate were exhausted, induction of synthesis of fructan hydrolase activity would permit degradation of these polymers in plaque. Logically, this could prolong the period of exposure of the host tissue to organic acids, which is a prime factor in the induction and progression of dental

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caries. It is quite reasonable to suggest that the fructanase activity of *S. mutans* may therefore represent an important virulence determinant for this organism.

Little is understood at the molecular level with respect to the protein(s) involved in the degradation of fructans, the multifaceted genetic regulation of the expression of the gene product(s), or the relative contribution of fructans and fructan hydrolase activity to dental caries. To begin to address these issues, we have sought to analyze fructanase activity from *S. mutans* genetically and biochemically. In a preliminary report (5), we have described the cloning of a fructanase gene of *S. mutans* GS-5. In this report, we describe in more detail the characteristics of expression of the *S. mutans* GS-5 fructanase gene in *Escherichia coli*. Also, the fructanase enzyme from sonic lysates of *E. coli* harboring the cloned fructanase gene, as well as the fructanase enzyme from culture supernatants of *S. mutans* GS-5, has been purified and compared, and some of the relevant physical and enzymatic properties of the enzymes have been elucidated.

(This work is in partial fulfillment of the requirements for the Ph.D. degree for R.A.B. at the University of Rochester School of Medicine and Dentistry.)

MATERIALS AND METHODS

Bacterial strains and growth medium. *S. mutans* GS-5 was maintained on Todd-Hewitt agar plates. In chemostat studies and in batch cultures, *S. mutans* GS-5 was grown in 3% tryptone–0.1% yeast extract medium supplemented with 0.5% fructose (TFY). For DNA preparations, this organism was grown in brain heart Infusion broth (Difco Laboratories) containing 20 mM D,L-threonine (6). *E. coli* JM83 [*ara* Δ(*lac-proAB*) *rpsL thi* φ80d*lacZ*Δ*M15*] (45), *E. coli* JM107 [*endA1 gyrA96 thi hsdR17 supE44 relA1* λ⁻ Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}*Z*Δ*M15*)] (50) and *E. coli* HB101 (F⁻ *hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ⁻) were maintained on Luria (L) broth plates. Ampicillin-resistant (Ap^r) *E. coli* organisms were selected on MacConkey agar containing lactose and 30 μg of ampicillin per ml. Sucrase-positive colonies of *E. coli* were selected on a minimal sucrose-containing medium (SPAM) (4). Liquid cultures of *E. coli* were grown in L broth with 30 μg of ampicillin per ml when necessary.

S. mutans GS-5 was grown in continuous culture in a New Brunswick model C32 chemostat equipped with a 2-liter vessel. The cells were grown in TFY medium at a dilution rate of 0.075 per h. The pH was maintained at 6.5 by addition of KOH. The culture was allowed to begin to equilibrate for 2 days after inoculation before collection of effluent began, and during this time enzyme activity was monitored as described below.

DNA manipulations. *S. mutans* DNA was isolated and prepared for construction of a genetic library as previously described (4, 30). Briefly, partially digested (*Sau*3A1) chromosomal DNA was enriched for 5-kilobase pair fragments by sucrose gradient centrifugation. Plasmid pMK5 was purified from cleared lysates of *E. coli* JM83 by CsCl-buoyant density centrifugation (15) and was prepared as vector by complete digestion with *Bam*HI, followed by treatment with bacterial alkaline phosphatase. Ligations were carried out with T4 DNA ligase. *E. coli* organisms were transformed by the procedure of Mandel and Higa (29). Restriction endonuclease mapping was done with the appropriate restriction enzymes and Bal31 exonuclease. All enzymes were from Bethesda Research Laboratories and were used according to the recommendations of the supplier.

Preparation of 100,000 × *g* soluble fractions. *E. coli* harboring the recombinant plasmid or control plasmid was grown in L broth with ampicillin for 16 h at 37°C with vigorous aeration. Cultures (500 ml) were cooled for 5 min on ice and cells were collected by centrifugation at 7,000 × *g* for 20 min at 4°C. Cells were washed in 30 ml of ice-cold 50 mM potassium phosphate buffer (KPB), pH 6.5, suspended in 6.0 ml of ice-cold KPB, and sonically disrupted with 8 15-s pulses (300 W), using a Braun-sonic 1500 sonicator. The resulting lysate was centrifuged at 100,000 × *g* for 30 min at 4°C. The supernatant fluid was recovered as the 100,000 × *g* soluble fraction and was used for enzymatic assays and for starting material for purifications. Samples were divided into aliquots and frozen at −20°C. No significant decrease in activity was observed for as long as 4 months. Protein concentrations were determined by the method of Lowry et al. (27).

Enzymatic assays. Unless otherwise noted, all assays were carried out in 50 mM potassium citrate buffer (KCit), pH 5.5. Levan (from *Aerobacter levanicum*), inulin (from chicory root), dextran T70, or starch (all from Sigma Chemical Co.) or mutan, prepared as described by Robrish et al. (35), was used at a concentration of 2.0 mg/ml. Free reducing sugar was measured by using the protocol of Luchsinger and Cornesky (28), with fructose, glucose, or a mixture of the two sugars as standards. Free glucose was assayed as described previously (40). The synthesis of polymer from sucrose was measured with [U-^{14}C]sucrose (35). In the case of polymeric substrates, 1 U of activity was defined as the amount of enzyme required to liberate 1 μmol of reducing equivalent per min. For sucrose or raffinose, 1 U of activity was that amount of enzyme required to hydrolyze 1 μmol of substrate per min. Sucrose-6-phosphate was a gift from Bruce Chassy (National Institutes for Dental Research) and sucrose-6-phosphate hydrolase activity was assayed as previously described (8).

Localization of the cloned gene product in *E. coli* was done by modifying the procedure of Hazelbauer and Harayama (20). Cultures (50 ml) were grown in L broth with ampicillin overnight. The cells were collected by centrifugation at 10,000 × *g* at 15°C for 10 min. The cells were washed twice in 5 ml of 10 mM KPB (pH 6.5)–30 mM NaCl and suspended in 1 ml of 10 mM KPB (pH 6.5)–0.58 M sucrose–0.1 mM EDTA (Na_2). After resuspension, the suspension was left at room temperature for 10 min. The cells were collected by centrifugation at 10,000 × *g* and 4°C for 5 min and suspended in 3 ml of ice-cold 0.5 mM $MgCl_2$. The cells were then centrifuged for 5 min at 10,000 × *g* and 4°C. The supernatant fraction was recovered as the shock fluid, and the pellet was suspended in 2 ml of KPB, pH 6.5. The cells were sonicated for 60 s at 300 W, and the lysate was centrifuged at 37,000 × *g* for 30 min at 4°C. The supernatant was recovered as the cytoplasmic fraction. The pellet was suspended in 2 ml of KPB, and all fractions were assayed for fructanase and β-galactosidase activity.

Preparation of antiserum. Fructanase was partially purified from *E. coli* 100,000 × *g* soluble fractions by the following protocol. Protein (60 mg) was applied to a Bio-Gel A1.5m column (40.0 by 1.3 cm) equilibrated with 20 mM KPB and eluted at a flow rate of 20 ml/h. The fraction containing the most activity was concentrated by ammonium sulfate precipitation, suspended in 20 mM KPB, pH 6.5, and dialyzed against 1,000 volumes of 20 mM KPB. This material was applied to a DEAE–Bio-Gel column (25.0 by 2.5 cm) and the column was washed with 5 volumes of 20 mM KPB. Material was eluted with a linear 0.0 to 0.5 M NaCl gradient

(300 ml) in 20 mM KPB. Fractions with activity were concentrated by ammonium sulfate precipitation and rechromatographed in Bio-Gel A1.5m as before. The fraction containing the most activity after this step was concentrated, emulsified in complete Freund adjuvant, and used to immunize a female New Zealand White rabbit. A 100-μg portion of this antigen was injected intramuscularly every 2 weeks for 8 weeks. A final injection with 200 μg of antigen was given in week 10. After 10 days, the animal was killed by cardiac puncture and blood was collected. Serum from the animal was stored frozen at −20°C, and this material was used for Western blot experiments.

Purification of fructanase enzyme. The cloned gene product was purified from 100,000 × *g* soluble fractions of *E. coli* JM83 harboring the recombinant plasmid pFRU1 as follows. One milliliter (11.30 U) of 100,000 × *g* soluble fraction was applied to a Bio-Gel A1.5m column (40.0 by 1.3 cm) which was equilibrated with 20 mM KPB, protein was eluted at a flow rate of 24 ml/h, and 4.0-ml fractions were collected. A single fraction containing the most activity (1.807 U) was applied, with multiple injections, onto a monoQ-DEAE column (10.0 by 0.5 cm; Pharmacia) equilibrated with 20 mM KPB (pH 7.0) in a Perkin-Elmer series 4 high-pressure liquid chromatograph (HPLC) with an LC85B detector. The column was washed with approximately 5 column volumes of equilibration buffer, and the protein was eluted with a linear 0.0 to 1.0 M NaCl gradient over 45 min, in 20 mM KPB (pH 7.0), at a flow rate of 0.9 ml/min. An aliquot (400 μl) of the fraction containing the most activity from the HPLC ion-exchange step was then subjected to HPLC gel permeation chromatography over a Bio-Sil TSK250 (TSK-3000SW) column (30.0 by 7.5 cm; Bio-Rad Laboratories) equilibrated with 20 mM KPB (pH 6.5), and protein was eluted at a flow rate of 0.9 ml/min with equilibration buffer.

The fructanase enzyme was purified from *S. mutans* GS-5 supernatants as follows. Effluent from the chemostat culture was collected in 500-ml aliquots on ice. The precipitate from a 70% saturation ammonium sulfate fractionation, designated *S. mutans* LGR-sup (low-growth rate supernatant), was suspended in a minimal volume of 20 mM KPB. LGR-sup was dialyzed against 1,000 volumes of 20 mM imidazole-HCl buffer (pH 7.4)–1 mM phenylmethylsulfonyl fluoride (PMSF)–10 mM hexanoic acid. This material was applied to a Bio-Gel A1.5m column, as described before, which had been previously equilibrated with 20 mM KPB (pH 7.0)–0.5 M NaCl–1 mM PMSF and eluted with equilibration buffer at a flow rate of 24 ml/min. Fractions (3.4 ml) were collected, and three fractions containing maximal activity from this separation were concentrated in an Amicon concentrator with a 30,000-M_r cutoff. This concentrate was then diluted to a final NaCl concentration of 0.075 M in imidazole-HCl (pH 7.4)–1 mM PMSF and loaded, by multiple injection, onto the DEAE-monoQ column equilibrated with imidazole-HCl (pH 7.4)–1 mM PMSF. The column was washed with 5 volumes of equilibration buffer, and the proteins were eluted with a linear 0.0 to 1.0 M NaCl gradient (45 min) in equilibration buffer at a flow rate of 0.9 ml/min. As with the cloned gene product, an aliquot (400 μl) of the fraction with optimal activity was applied to the TSK-3000SW column, equilibrated with imidazole-HCl (pH 6.5)–150 mM NaCl–1 mM PMSF, and eluted in this buffer.

The TSK-3000SW column was calibrated by eluting protein molecular weight standards (Pharmacia) in the appropriate buffer. The molecular weight of the fructanase enzymes was calculated from a plot of $\log_{10}$ of the molecular weight versus elution time.

Protein electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel (10%) electrophoresis (SDS-PAGE) was performed as described previously (26). Proteins were visualized by silver staining, using the protocol of Görg et al. (17), with protein molecular weight standards from Bio-Rad and Sigma.

For Western blotting (42), proteins from SDS-PAGE were transferred to nitrocellulose (Schleicher & Schuell BA85). Antiserum for Western blotting was adsorbed with a fivefold protein excess of 100,000 × *g* soluble fractions *E. coli* JM83 harboring the vector, which was brought to a concentration of 1 mg/ml in Tris-buffered saline (10 mM Tris-hydrochloride, pH 7.4, 0.9% NaCl). This mixture was incubated at room temperature for 1 h with gentle rocking and appropriately diluted in Tris-buffered saline to be used directly in Western blotting. Immune reactivity was detected by using a secondary antibody (goat anti-rabbit Fc) conjugated to horseradish peroxidase (Worthington Diagnostics). Prestained molecular weight standards were from Bethesda Research Laboratories.

Identification of reaction end products. Samples of levan or inulin (2.0 mg/ml) in KCit were incubated with various fructan hydrolase preparations. At 10-min intervals, 200 μl of the sample was removed to a boiling-water bath. Residual fructanase activity could not be detected after this treatment. The sample was then deionized with a mixed-bed resin (Bio-Rad AG 501 X-8). The sample was desiccated and suspended in 20 μl of distilled water. Samples were applied to Whatman 3MM paper and subjected to ascending paper chromatography in ethyl acetate-pyridine-water (10:4:3). Sugars were detected by staining with $AgNO_3$-saturated acetone (43). Fructose, glucose, sucrose, or mild acid hydrolysates of levan or inulin were used as standards.

RESULTS

Cloning, detection, and identification of the GS-5 fructanase. A genetic library of *S. mutans* GS-5, consisting of 5,000 recombinants with an average insert size of 4.5 kilobase pairs, was constructed in plasmid pMK5. pMK5 is a 4.9-kilobase pair plasmid constructed from pUC9 (45) and pC194 (14) which replicates in and confers Ap^r and chloramphenicol resistance (Cm^r) to *E. coli*. This library was screened for clones which were able to grow with sucrose as the sole carbohydrate source, as previously described (5). Seven clones, which were detected after 48 h of incubation, were isolated and toluenized cells from an overnight culture of each were tested for sucrolytic activity (4). All were positive and the clone harboring plasmid pFRU1 (Fig. 1) was chosen for further study.

Because *S. mutans* elaborates a number of enzymes capable of hydrolyzing sucrose, which can be differentiated on the basis of substrate specificity or reaction end products or both, a series of biochemical assays was undertaken to determine the identity of the cloned gene product. It was observed that, under the conditions tested, the enzyme had the highest specificity for the predominantly β(2→6)-linked fructan levan. Inulin was hydrolyzed by the cloned gene product with approximately 34% of the activity of that observed for levan. The unsubstituted β-fructofuranosides sucrose and raffinose were attacked with 21 and 12%, respectively, of the activity observed on levan. There was no detectable activity on a number of other substrates, including the substituted β-fructofuranoside melizitose or the α-glucosides dextran, mutan, and starch. When the cloned gene product was incubated with [U-^{14}C]sucrose and as-

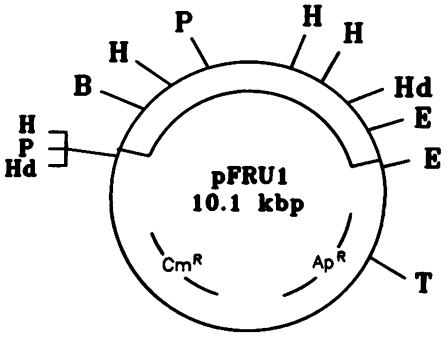

FIG. 1. Recombinant plasmid pFRU1 harboring the 4.2-kilobase pair (kbp) insert of *S. mutans* GS-5 DNA (double line), which encodes the fructanase enzyme. pMK5, the vector, was constructed by fusing fragments from pUC9 and pC194. This plasmid confers Ap^r and Cm^r and also contains the polylinker region and α-complementation of the β-galactosidase gene from pUC9. The insert of *S. mutans* DNA is in the *Bam*HI site in the pUC9 portion of the plasmid. B, *Bgl*II; E, *Eco*RI; H, *Hin*cII, Hd, *Hin*dIII; P, *Pst*I; T, *Taq*I.

sayed for ability to synthesize polymer by the procedure of Robrish et al. (35), no detectable polymer could be precipitated. The enzyme was unable to hydrolyze sucrose-6-phosphate, a characteristic of the intracellular invertase of *S. mutans* (8). This type of substrate specificity profile is typical of β-fructosidases (for examples, see references 33 and 41).

The level of activity of expression of the cloned gene product was such that approximately 200 U of levan hydrolase activity were produced per liter of *E. coli* JM83(pFRU1) (Table 1). The level of expression of this enzyme was not influenced by the induction of the *lacZ*-OP on pMK5 by IPTG (isopropyl-β-D-thiogalactoside), nor was it influenced by the presence of the $lacI^q$ mutation. Deletion of the small *Hin*dIII-*Eco*RI fragment resulted in complete loss of detectable activity (data not shown). These data suggested that the gene was probably transcribed and translated via its own regulatory sequences. By biochemical analysis, the enzyme was also shown to behave as an invertase, releasing equimolar quantities of glucose and fructose from sucrose (Table 1).

No fructanase activity was detected in the supernatant fraction or periplasmic space in a cold osmotic shock experiment; all assayable activity was found associated with the cellular fraction (Table 2). Even though a small amount of the cytoplasmic enzyme β-galactosidase could be found in the shock fluid, no fructanase was ever detected in this fraction. When the cells were then fractionated as described in Materials and Methods, approximately 21% of all assayable activity was associated with the cell pellet (Table 2). Collectively, these data suggest that the fructanase may be associated with the cytoplasmic membrane, although the extent of this association cannot be determined without an in-depth analysis.

TABLE 2. Localization of the *fruA* gene product in *E. coli*[a]

Cell compartment	% Fructanase activity	% β-Galactosidase activity
Culture supernatant	0.0	0.9
Periplasm	0.0	11.1
Cytoplasm	79.3	85.1
Cytoplasmic membrane	20.7	2.9

[a] Cultures (50 ml) of *E. coli* HB101(pFRU1) were grown to the mid-exponential phase of growth in L broth plus 30 μg of ampicillin per ml. Cold osmotic shock experiments and fructanase and β-galactosidase assays were performed as described in Materials and Methods.

Purification of the fructanase enzyme. The cloned gene product was purified from 100,000 × *g* soluble fractions of *E. coli* JM83 harboring the recombinant plasmid pFRU1 as detailed in Materials and Methods. Levan and inulin hydrolase eluted in a single peak in the void volume from the Bio-Gel A1.5m column, suggesting that the enzyme was highly aggregated. No activity could be detected in other fractions (data not shown). When the most active fraction was subjected to HPLC ion-exchange, levan and inulin hydrolase activity eluted in a single activity peak at an NaCl concentration of approximately 0.25 M (Fig. 2A). This material from the monoQ-DEAE column was then subjected to HPLC-gel permeation chromatography. Levan and inulin hydrolase activity eluted as a single peak with a calculated M_r of 260,000 (Fig. 2B; migration of M_r standards not shown). This protocol afforded a 315-fold purification of the fructanase enzyme from *E. coli*, with 8.5% of the activity recovered in the purified preparations (Table 3). When total recoveries of activity in all fractions was considered, 44.7% of the activity was recovered.

One microgram of material from the fraction containing the activity from the TSK column was subjected to electrophoresis in SDS-PAGE (10%) and stained with silver for two cycles as described in Materials and Methods. A single band with an apparent molecular weight of 140,000 was detected (Fig. 3A). No other bands were detected in this preparation by silver staining.

TABLE 1. Expression levels of the cloned gene product[a]

E. coli strain	Substrate	μg of reducing sugar released/min	μg of glucose released/min	Sp act (U/mg of protein)	Fructose/glucose
JM83(pMK5)	Levan	ND	ND		
JM83(pFRU1)	Levan	14.17	ND	0.654	
JM83(pFRU1) + IPTG	Levan	14.08	ND	0.650	
JM107(pMK5)	Levan	ND	ND		
JM107(pFRU1)	Levan	12.60	ND	0.580	
JM107(pFRU1) + IPTG	Levan	12.49	ND	0.575	
JM83(pMK5)	Sucrose	ND	ND	ND	
JM83(pFRU1)	Sucrose	5.94	3.00	0.137	0.98

[a] Either levan (2 mg/ml) or sucrose (100 mM) in KCit (pH 5.5) was incubated with protein (appropriately diluted) from 100,000 × *g* soluble fractions of the indicated *E. coli* strain prepared as described in Materials and Methods. Reactions were incubated at 37°C for 60 min, at which time the samples were immersed in boiling water. Reducing sugar or free glucose was measured as described before (28, 40). For induction with IPTG, cells were grown in L broth plus 30 μg of ampicillin per ml with vigorous aeration. At an optical density at 600 nm of 0.4, IPTG was added to a final concentration of 5 mM. Cells were harvested in stationary phase. Uninduced cultures were treated identically except no IPTG was added. Protein concentration was determined by the method of Lowry et al. (27). ND, None detected.

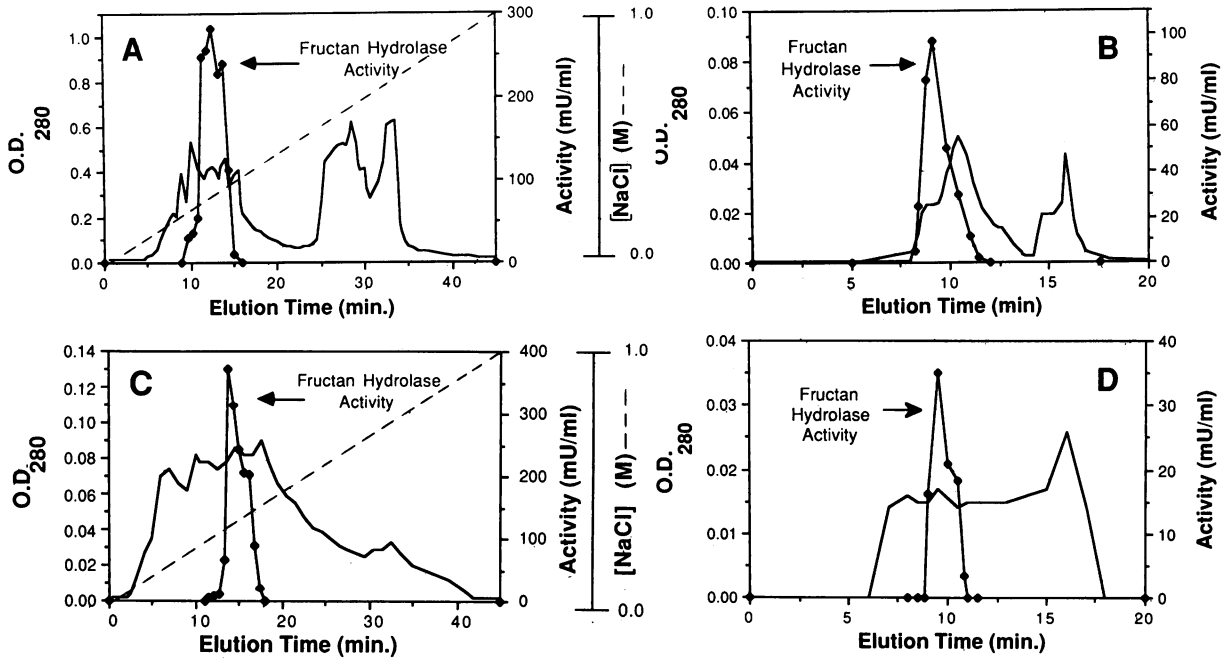

FIG. 2. Protein (——) and activity (-◆-) elution profiles of the cloned fructanase enzyme and the fructan hydrolase from *S. mutans* GS-5 LGR-sup. The first column step in both cases was a gel filtration step (Bio-Gel A1.5m; not shown). Details of the purification are described in the text. Migration of protein molecular weight standards (from Bio-Rad) are not shown. (A, B) *E. coli*-produced enzyme. (A) A single fraction from gel filtration of *E. coli* JM83(pFRU1) 100,000 × *g* soluble fractions was injected onto a DEAE-monoQ column and the proteins were eluted with a 0.0 to 1.0 M NaCl (- - -) gradient in 20 mM KPB, pH 7.0. (B) A single fraction with fructan hydrolase activity (400 μl) was injected in HPLC onto a TSK-3000SW column and eluted isocratically in 20 mM KPB, pH 6.5. (C, D) *S. mutans*-produced enzyme. (C) Fractions from the initial gel filtration step of LGR-sup were treated as described in the text and injected onto a DEAE-monoQ column. Protein was eluted with a linear 0.0 to 1.0 M NaCl gradient in 50 mM imidazole-HCl (pH 7.4)–1 mM PMSF. (D) A single fraction (400 μl) was injected onto a TSK-3000SW column equilibrated with 50 mM imidazole-HCl (pH 6.5)–1 mM PMSF and eluted isocratically. $O.D._{280}$, Optical density at 280 nm.

It had been reported that the synthesis by *S. mutans* of the activity which could degrade fructans was optimal when the cells were grown in a chemostat at low growth rates, with fructose as the sole carbohydrate source (23). Attempts, in batch culture, to generate sufficient enzyme activity to begin purifications were unsuccessful. Consequently, *S. mutans* GS-5 was grown in continuous culture under the conditions outlined in Materials and Methods. When the culture had equilibrated, strain GS-5 produced 211 U of fructan hydrolase activity per liter of supernatant as compared to only 0.5 to 5 U/liter observed for batch cultures grown in the same medium. These observations were consistent with those made by Jacques et al. (23) for a serotype *c S. mutans* (strain Ingbritt). The starting material for purification, LGR-sup, was prepared as described in Materials and Methods. It was found that this material was quite unstable due to degradation by protease(s). Breakdown, as measured by loss of activity or Western blotting, could be inhibited by a number of well-documented protease inhibitors including PMSF, hexanoic acid, pepstatin, benzamindine, soybean trypsin inhibitor, and ethylmaleimide. These inhibitors were effective individually or in combinations, such as hexanoic acid and trypsin inhibitor. However, they were largely ineffective in preventing breakdown when included in column buffers during the purification (data not shown). Consequently, purification of the fructanase proved difficult because much or all of the activity would be lost at various steps.

TABLE 3. Purification and recovery of fructanase activity from *E. coli* JM83(pFRU1)

Fraction[a]	Total protein (mg)	U	Sp act (U/mg)	Yield (%)	Purification (fold)
100,000 × *g* supernatant	17.40	11.3	0.654	100.0	1.00
Bio-Gel LC column (GPC)	1.005	1.82	1.807	16.1	2.76
HPLC DEAE-monoQ	0.0225	1.003	44.59	8.9	68.60
HPLC TSK-3000SW (GPC)	0.0056	0.961	204.5	8.5	314.60

[a] GPC, Gel permeation chromatography.

The fructanase enzyme was purified from *S. mutans* GS-5 supernatants as described in Materials and Methods. In the initial gel filtration step, activity eluted as a single peak in the void volume. No other levan or inulin hydrolase activities were observed (data not shown). In the HPLC ion-exchange step, fructan hydrolase activity eluted as a single peak at an NaCl concentration of approximately 0.25 M (Fig. 2C). As was observed for the cloned gene product in the HPLC gel filtration step, the fructan hydrolase activity from *S. mutans* eluted as a single peak with a calculated M_r of 260,000 (Fig. 2D). The yields in this experiment were not nearly as high as in the purification from *E. coli*, with 2.3% of the activity recovered in the purified preparation (Table 4), but only 18.5% of the activity could be recovered in all fractions.

It should be pointed out that when the *S. mutans* enzyme was purified by the same protocols as the cloned gene product, the two proteins behaved identically, eluting with virtually the same retention times. The only difference in the

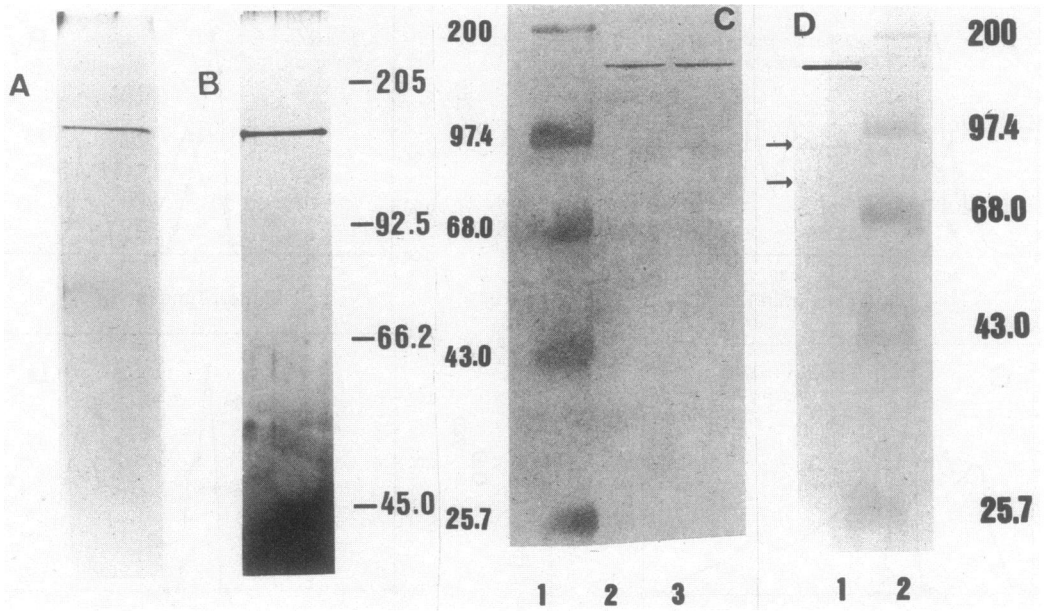

FIG. 3. SDS-PAGE and Western blotting. (A) Silver-stained protein (1 μg) from the final step (TSK-3000SW) in the purification of the fructanase enzyme from *E. coli*. (B) Silver-stained protein (1 μg) obtained from the final step (TSK-3000SW) in the purification of the fructanase from LGR-sup from *S. mutans* GS-5. Protein standards were from Bio-Rad (low molecular weight; 45,000 to 92,000) and myosin (205,000; Sigma). (C) Western blot with fructanase antiserum: lane 1, prestained molecular weight standards (Bethesda Research Laboratories); lane 2, protein (5 μg) from the final step in the purification of the enzyme from *E. coli*; lane 3, protein (5 μg) from a single fraction off the DEAE-monoQ column step in the purification of the enzyme from *S. mutans* LGR-sup. (D) Western blot of crude LGR-sup, dialyzed against 10 mM hexanoic acid, with fructanase antiserum as described in the text. Arrows indicate the faint 90,000- and 74,000-Da proteins. Lane 1, LGR-sup (25 μg) reacted with fructanase antiserum; lane 2, prestained molecular weight standards (Bethesda Research Laboratories).

two purifications were the recoveries of enzyme activity. This identical behavior for the two enzymes was also observed in liquid chromatography purifications, which were undertaken prior to HPLC purification (R. A. Burne, unpublished observation).

One microgram of the material from the fraction containing the most activity from the HPLC gel filtration step was concentrated by ethanol precipitation and subjected to SDS-PAGE. A single band was visualized by silver staining with an apparent molecular mass of 140,000 daltons (Da) (Fig. 3B).

The cloned gene product and the protein from *S. mutans* supernatants were also shown to be the same apparent molecular weight in SDS-PAGE by Western blotting with fructanase antiserum prepared as described in Materials and Methods. Material (5 μg) from the monoQ column (in the case of *S. mutans*) and material (5 μg) from the final step in the purification of the enzyme from *E. coli* were electrophoresed in SDS-PAGE and transferred to nitrocellulose membranes as described before (42). It can be observed (Fig. 3C) that the two proteins comigrate in this system. These data, coupled with the expression (Table 1) and purification (Fig. 3A to D) data, strongly suggest that the two proteins are identical, and therefore the entire coding sequence for the *S. mutans* fructanase enzyme is present on pFRU1.

TABLE 4. Purification and recovery of *S. mutans* fructanase activity from LGR-sup

Fraction[a]	Total protein (mg)	U	Sp act (U/mg)	Yield (%)	Purification (fold)
Ammonium sulfate cut	6.0	4.102	0.68	100.0	1.0
Bio-Gel LC column (GPC)	0.535	1.054	1.97	25.7	2.89
HPLC DEAE-monoQ	0.009	0.372	41.3	9.07	60.6
HPLC TSK-3000SW (GPC)	0.0014	0.093	66.4	2.30	97.5

[a] GPC, Gel permeation chromatography.

As a control for the antiserum, 25 μg of crude LGR-sup, which had been dialyzed in the presence of 10 mM hexanoic acid–1 mM PMSF, was subjected to electrophoresis in 10% SDS-PAGE and Western blotted as above. The antibody reacted strongly with the 140,000-Da fructanase (Fig. 3D). The antibody also reacted, albeit weakly, with a number of other proteins, including proteins with approximate molecular weights of 90,000 and 74,000. Whether these are breakdown products of the fructanase which are coprecipitated in the ammonium sulfate step or are proteins which share epitopes has not been determined.

Biochemical characteristics of the *S. mutans* fructanase and the cloned gene product. The substrate specificity of the enzyme was the first biochemical property to be examined with the purified enzymes. It was determined that the substrate specificity profiles of the *S. mutans* enzyme from LGR-sup and the enzyme isolated from *E. coli* were identical regardless of the degree of purity. In fact, in all cases examined, the biochemical properties of the fructanases, regardless of the source or the extent to which they were purified, were identical. Therefore, the biochemical data presented below represent cumulative results obtained from using a variety of enzyme preparations from both *E. coli* (pFRU1) and *S. mutans* GS-5 LGR-sup.

The pH optima of the enzymes were determined (Fig. 4A). The peak activity for the enzyme acting on levan or inulin

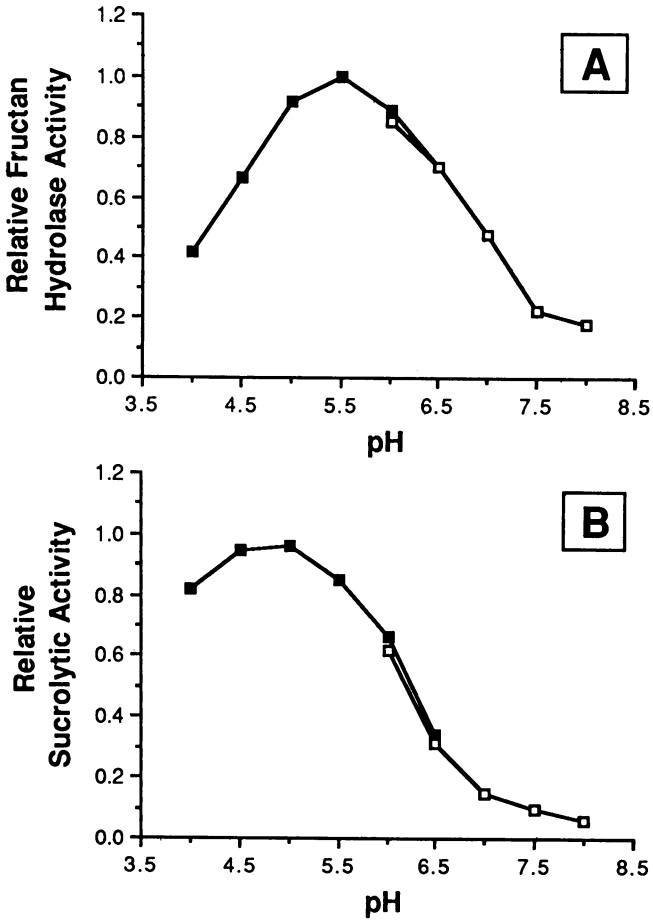

FIG. 4. pH optimum. Appropriate samples of protein with fructan hydrolase activity were tested for their activity at varying pH values. Protein samples were preincubated for 5 min at 37°C in either 100 mM KCit (■) or 100 mM KPB (□). Reactions were started by addition of substrate to 2 mg/ml for levan or 100 mM for sucrose. The reactions were terminated by immersion of the tubes in boiling water, and reducing sugar or free glucose was measured. The pH of the reaction was tested at the beginning and end of each experiment. (A) pH optimum of the enzyme acting on levan or inulin. (B) pH of the enzyme acting on sucrose.

was approximately pH 5.5. In contrast to previous reports (48), a significant amount (40%) of enzymatic activity remained at pH 4.0. Little fructanase activity was detected at or above pH 7.0.

Because this enzyme behaved as an invertase, liberating equilmolar quantities of glucose and fructose from sucrose (Table 1), it was of interest to determine if the pH optimum for sucrose was the same as that observed for the fructan polymers. These results (Fig. 4B) indicated that the pH optimum for sucrose (100 mM) was broader and significantly lower (≅pH 4.5) than that for levan or inulin degradation.

A number of well-documented enzyme inhibitors were tested for their ability to impair the activity of the two enzymes. Ag^{2+} (1 mM) and Hg^{2+} (0.01 mM) almost completely inhibited enzyme activity. Cu^{2+} (1 mM) and Mg^{2+} (1 mM) inhibited enzyme activity about 20%. Mn^{2+} appeared to augment enzyme activity (20%), while Ca^{2+} (1 mM) had little effect. Tris, an inhibitor of glucosyltransferase activity (9), had no effect, nor did fluoride ion influence activity, even at concentrations as high as 10 mM.

The substrate specificity of the enzyme indicated that it was a β-D-fructofuranosidase. To determine whether the enzyme attacked the fructan substrate endo- or exohydrolytically, levan and inulin were subjected to a timed, limited, enzymatic hydrolysis, using the cloned gene product or the parent enzyme as described in Materials and Methods. Regardless of the polymeric fructan substrate or the enzyme preparation used, identical results were obtained. Fructose appeared immediately and was the only sugar released during the course of the experiment, suggesting that the enzyme attacked polymers of D-fructose exohydrolytically. A typical result from these experiments is shown in Fig. 5, when the purified cloned gene product was used to hydrolyze inulin. The identity of fructose as the end product released from hydrolysis of levan or inulin was confirmed by using an HPLC ion-exchange column (Bio-Rad HPX-87H) with 20% acetonitrile as eluant (data not shown). The column was calibrated with glucose, fructose, and sucrose standards. The liberation of only fructose as the product indicated that the enzyme was an exo-β-D-fructofuranosidase, which released fructose from the end of polymeric substrates. It should be pointed out that glucose can be detected at later times in small quantities in these experiments when the chromatograms are overloaded (data not shown), reflecting the fact that, for synthesis of fructan polymers, sucrose often acts as the acceptor in catalysis of fructosyl transfer.

To determine whether the enzyme could completely degrade polymeric substrates, samples of levan or inulin were dried to constant weight in vacuo in the presence of P_2O_5. A known quantity of each was then hydrolyzed to completion in diluted acetic acid, and total reducing sugar was determined. The dried levan and inulin were then treated with enzyme, and reducing sugar was assayed at various times. Fructose was liberated linearly with time (Fig. 6), and with sufficient time the enzyme was able to degrade the β(2→6)-linked fructan levan to completion. The enzyme also de-

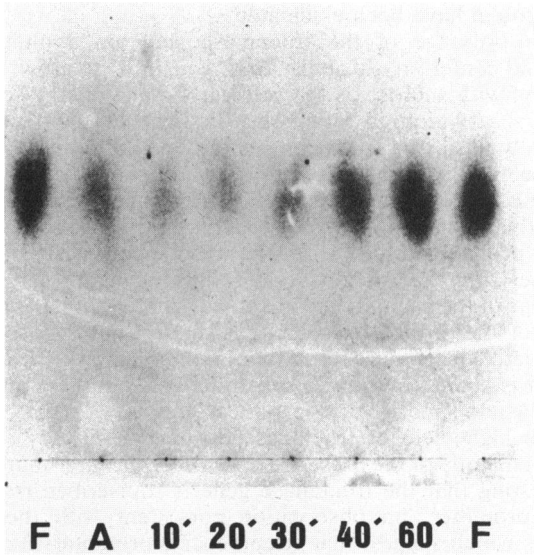

FIG. 5. Paper chromatogram of reaction end products from a limited enzymatic hydrolysis of inulin by enzyme purified from *E. coli* as previously described. Samples were removed at the indicated intervals and treated and detected as described in Materials and Methods. F, Fructose; LA, a complete acid hydrolysate of *Aerobacter levanicum* levan.

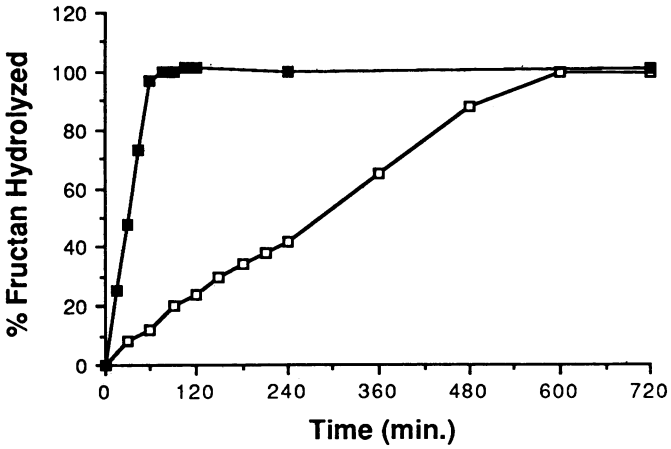

FIG. 6. Timed enzymatic hydrolysis of fructan polymers by the enzyme. In this case, approximately 15 mU of levan hydrolase activity was incubated in 50 mM KCit, pH 5.5, with either levan (■) or inulin (□) (200 μg of each) which had previously been dried to constant weight in the presence of P_2O_5. The dried levan was also hydrolyzed completely in mild acetic acid, and the total reducing sugar was determined. This figure was used as a reference for available substrate to determine the percentage of substrate hydrolyzed.

graded inulin to completion, but as expected, the rate was significantly slower (Fig. 6) than that observed for levan. There was no evidence of product inhibition.

DISCUSSION

A fructanase gene from *S. mutans* GS-5 has been cloned and expressed in *E. coli*. The cloned gene product from *E. coli* JM83 harboring the recombinant plasmid containing the fructanase gene, as well as the protein from culture supernatants of the parent organism, has been purified, and some of the relevant physical and biochemical characteristics of the protein have been elucidated.

The presence of the fructanase gene on a multicopy plasmid conferred the ability to *E. coli* JM83 to grow, albeit poorly, with sucrose as the sole carbon and energy source. This observation is interesting in light of the fact that virtually all of the fructanase activity could be localized to the cellular fraction in a cold osmotic shock experiment. Since *E. coli* lacks a mechanism to transport sucrose into the cell, this ability to grow with sucrose as the sole carbohydrate probably results from the spontaneous lysis of cells synthesizing the fructanase and subsequent hydrolysis of sucrose in the surrounding medium. An alternative explanation for the growth on sucrose is that the enzyme may be associated with the cytoplasmic membrane in such a way that it is able to hydrolyze substrate which diffuses into the periplasmic space.

The high yield of fructanase enzyme in *E. coli* results independently of the presence of IPTG or the *lacI*^{q} mutation, suggesting that the fructanase gene is transcribed from its own promoter, an observation consistent with those of others which suggest that streptococcal promoters function well in *E. coli* (10). The fructanase enzymes isolated from the recombinant *E. coli* and from the parent organism appear to be identical by a number of criteria. First, they comigrate in SDS-PAGE, as determined by silver staining the purified proteins. The reactivity of the antiserum, which was raised against the cloned gene product in Western blots, confirmed that the proteins were the same molecular weight in SDS-PAGE and, importantly, demonstrated the immunological relatedness of the two proteins. The enzymes are also identical biochemically with regard to pH optima, substrate specificity, reaction end products, etc. Also, although the data are not shown, when the two crude enzyme preparations were subjected to the same purification regimens, the two proteins always chromatographed identically. That the cloned gene product and the parent enzyme are identical by the above criteria, as well as the enzyme's apparent association with the cytoplasmic membrane, suggests that *E. coli* may be removing the streptococcal signal sequence, assuming, of course, that this protein is synthesized with a signal sequence. N-terminal analysis of both proteins will be necessary to confirm this, but the correct processing of other gram-positive secreted proteins by *E. coli* has been observed (34). Analysis of the DNA sequence of the entire insert will be used to verify that the entire coding sequence for the GS-5 fructanase is present on pFRU1.

During purification of the enzyme from *E. coli* recoveries were good, with little, if any, breakdown of the enzyme observed. There was, however, a significant (50.3%) loss of activity after the initial gel filtration step. Concentration of the enzyme enhances activity, and perhaps the observed loss of activity was due to dilution of the enzyme at this step. Purification of the enzyme from GS-5 supernatant fluid was complicated by the breakdown of the enzyme and subsequent loss of the activity, which was most likely due to an as yet uncharacterized streptococcal protease since spontaneous breakdown of the enzyme was not observed when it was purified from *E. coli*. Inclusion of a variety of protease inhibitors in crude LGR-sup preparations augmented activity. Also, dialysis of the LGR-sup in the absence of inhibitors, followed by Western blotting with fructanase antiserum, revealed a variety of immunologically related lower-molecular-weight species (data not shown), while dialysis in the presence of protease inhibitors yielded a single major band at 140,000 Da (Fig. 3D). Interestingly, a number of very faint bands could be visualized in these Western blots of samples which were dialyzed with protease inhibitors, including bands of approximate molecular masses of 90,000 and 74,000 Da, similar in M_r to fructosyltransferase and glucan-binding protein, respectively (37). Whether these bands represent breakdown of the fructanase or shared epitopes among exoproteins of *S. mutans*, as described by Russell and Gilpin (37), will require further analysis.

The fructanase enzyme elutes from the first column step (Bio-Gel A1.5m) in the excluded volume of this column, suggesting that the enzyme is in a highly aggregated form. Inclusion of 0.5 M NaCl in elution buffers to overcome some electrostatic interactions did not significantly alter the interactions with the Bio-Gel column. Migration in the TSK-3000SW column in HPLC suggests that the active form of the enzyme may be multimeric, perhaps a dimer. This possibility is being investigated more thoroughly.

The biochemical properties of the enzyme were examined by using a variety of enzyme preparations from both *S. mutans* and *E. coli*. By using this approach, influences from external sources (e.g., culture medium, bacterial products, differential processing, or specific or nonspecific protein-protein interactions) which could influence activity would be ruled out. Therefore, the biochemical data presented here should accurately reflect the characteristics of the *S. mutans* GS-5 fructanase.

The preference of the enzyme for β(2→6)-linked fructan is interesting in the sense that the fructan polymer produced by

S. mutans is almost uniquely β(2→1) linked (2, 12). The primary source of fructans in plaque is currently unknown. A likely candidate would be *S. salivarius*, which is normally present in high numbers in saliva and on soft tissue in the mouth (18), although other oral streptococci, as well as other oral bacteria, make a levan-type polymer (3, 12, 13, 32, 49). If a large amount of β(2→6)-fructan synthase is present in plaque or pellicle, this could explain the preference for fructans with this linkage. Further characterization of plaque fructosyltransferase and its source, as well as the linkages of the fructans in plaque, will be necessary to obtain a better assessment of the accuracy of this theory.

The pH optimum of the enzyme is approximately 5.5, but contrary to other published reports (48) for fructanase from a serotype *c S. mutans*, which indicated that the enzyme was inactive at low plaque pH, a significant amount of activity (40%) remained at a pH of 4.0, generally considered to be the lower limit for plaque. This difference in pH profiles may be attributable to differences in the method of preparation of enzyme or the differences between the two strains (Ingbritt versus GS-5). Since fructans do accumulate in plaque in vivo (16, 22), fructan hydrolase must not be active or must be present in limiting amounts. Because the enzyme is still active at low-plaque pH, it would seem more likely that the degradation of fructans is controlled on the genetic level rather than at the protein level. A model for fructan utilization by *S. mutans* has been proposed by Manly and Richardson (31) which is consistent with the biochemical data presented in this report as well as with the regulation of synthesis of fructanase presented by others (23, 48).

The presence of an extracellular invertase in culture supernatants of *S. mutans* has been described previously (7, 33). We have previously postulated (5), based on substrate specificity and molecular weight, that this enzyme may represent the extracellular invertase of GS-5. The expression of the gene for fructanase appears to be highly regulated and requires fructose, low glucose concentration, and low growth rates for maximal expression (23). The extracellular invertase, described previously (7, 33), seemed to be expressed efficiently in batch cultures and did not appear to be as stringently controlled as fructanase expression; however, this does not rule out that the fructanase and invertase are identical. The protein preparations used to assess "extracellular invertase" activity were not purified significantly. The methodology used to identify extracellular invertase activity considered, but did not eliminate, the possible contribution of sucrase activities from other sources, nor was the contribution of glucose from the action of dextranase(s) on polyglucan considered (7, 33). In support of the theory that the fructanase and the extracellular invertase are the same protein, gel filtration of the extracellular invertase activity indicated that it was highly aggregated, as was observed for the fructanase. The activity of the enzyme on sucrose is relatively low compared to levan, but if the contribution by fructanase to total sucrolytic activity were considered, *S. mutans* GS-5 fructanase enzyme would account for 50 U of invertase per liter of culture supernatant under the conditions used in the chemostat. This represents a considerable amount of invertase activity. The determination of the relationship of these two enzymes will contribute to understanding the physiological and environmental aspects of the utilization of sucrose and the pathogenesis of *S. mutans*.

The substrate specificity data coupled with the reaction end product and timed hydrolysis experiments indicate that it is an exo-β-D-fructofuranosidase, an enzyme which will release fructose from the end of levan- and inulin-type polymers and digest these polymers to completion. Because no other fructanase activities were detected in LGR-sup, it appears that *S. mutans* can synthesize and regulate the expression of one polypeptide capable of degrading fructans. This enzyme should enable *S. mutans* to degrade the fructans produced by itself, other oral streptococci, and oral *Actinomyces* sp., thus providing bacteria in the plaque matrix with a source of fermentable hexose.

This enzyme, although it is considerably higher in molecular weight in SDS-PAGE, is similar to the exo-β-D-fructosidase purified from culture supernatants of *S. salivarius* KTA19 (41). Interestingly, the *S. mutans* enzyme has a lower pH optimum and is considerably more pH resistant. The possible relationship of these two enzymes is currently under investigation, using the DNA probe and the antifructanase serum.

The profile of inhibition of enzyme activity by those inhibitors tested indicates further similarities between the *S. mutans* and *S. salivarius* KTA-19 enzymes. The enzymatic activity of fructanase is dramatically affected by the presence of Ag^{2+} (1 mM) and Hg^{2+} (10 μM) and to a lesser extent by Cu^{2+} (1 mM). The enzyme was not affected by fluoride ion even at 10 mM. It appears that the insensitivity to fluoride is not an unusual property of the sucrolytic enzymes of *S. mutans* and may be an important factor in the contribution of these potential virulence factors to disease in geographical areas where individuals are exposed to high fluoride levels.

Isolation of the fructanase gene from *S. mutans* will now facilitate the construction of a fructanase-deficient mutant of *S. mutans* by protocols which have already been successful (1). It will then be possible to assess the cariogenicity of such a mutant in the rat model. Also, the role of fructans as storage polysaccharides and their potential role as structural polymers or antigens (21, 39) can be examined. An *S. mutans* strain deficient in fructanase production should be an ideal mutant for examining the relative contribution of fructans to oral disease.

Many of the proposed virulence determinants of the oral streptococci are influenced by growth rate, substrate, and catabolite repression, including GTF-I (25, 48), fructosyltransferase (23), and structural surface antigens which are thought to play a role in adherence and aggregation (19; Drake et al., J. Dent. Res. **66**:226, abstr. 955, 1987). By using the *fruA* gene as well as other proposed virulence determinants from *S. mutans* as models for gene expression, we hope to gain a more thorough understanding of gene regulation and the genetic control of virulence determinants in the gram-positive bacteria.

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