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Initial Characterization of Sucrose-6-phosphate Hydrolase from Streptococcus mutans and its Apparent Identity with Intracellular Invertase

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SUMMARY

An intracellular enzyme catalyzing the hydrolysis of sucrose-6-phosphate to glucose-6-phosphate and fructose has been identified in extracts of Streptococcus mutans 6715-10. The preparation was purified chromatographically and found to have an apparent molecular weight of 42,000. The enzyme has as a K_m for sucrose-6-phosphate of 0.21 mM, a pH optimum of 7.1, is quite stable and requires no added cofactors or metal ions. Sucrose is a competitive inhibitor of sucrose-6-phosphate hydrolysis (K_i = 8.12 mM). A previously described intracellular invertase co-purifies with the enzyme and could not be separated from it by disc gel electrophoresis. It is concluded that intracellular invertase is a sucrose-6-phosphate hydrolase with a low catalytic activity for hydrolysis of sucrose.

Streptococcus mutans has been implicated as a prime etiologic agent in the development of dental caries (1). When cultured in the presence of sucrose, S. mutans produces adherent extracellular glucans thought to play a role in the formation of dental plaque (1). However, the majority of the sucrose is metabolized by glycolysis with the accumulation of lactic acid in the culture media (2). This finding implies that both the glucosyl and fructosyl moieties of sucrose are primarily metabolized via glycolysis, thus indicating the need for an enzyme capable of hydrolyzing sucrose. Both extracellular (3) and intracellular invertases (4) have

Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; S6'P, α-D-glucopyranosyl-1→2-β-D-(6'-O-phosphoryl)-fructofuranoside; S6P, 6-O-phosphoryl-α-D-glucopyranosyl-1→2-β-D-fructofuranoside; G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); HK, hexokinase (EC 2.7.1.1); HEPES, N-2-hydroxyethylpiperazine-N-2'-ethanesulfonic acid; MES, 2-(N-morpholino)-ethanesulfonic acid; PEP, phosphoenolpyruvic acid; PTS, phosphotransport system; FDP, fructose-1,6-diphosphate; DTT, dithiothreitol; DE52, DEAE cellulose.

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been identified in various strains of S. mutans. The intracellular invertases are characterized by a strikingly high K_m for sucrose (30-140 mM) which is lowered (ca 30 mM) in the presence of phosphate (5,6).

Recently (7,8), evidence has been presented that S. mutans can transport sucrose by the action of a PEP dependent sucrose-PTS. Sucrose-PTS activity results in the intracellular appearance of S6P which can be hydrolyzed by permeabilized whole cells to liberate G6P (7,8). These results suggested the presence of an enzyme which would catalyze the hydrolysis of sucrose-6-phosphate. The present study reports the first isolation of S6P hydrolase from S. mutans 6715-10, and data indicating its apparent identity with the previously described intracellular invertases of S. mutans.

MATERIALS AND METHODS

F6P, G6P and G6PDH were obtained from Sigma Chemical Co. HEPES, MES, HK, ATP, $NADP^+$ and Aquicide III were products of Calbiochemical Corp. DE52 and Ultrogel AcA 54 were obtained from Whatman and LKB, respectively. Bio-Rad protein assay kit, pre-cast gels and pH 8.9 electrophoresis buffer were purchased from Bio-Rad Laboratories. S6'P was prepared enzymatically according to Leloir and Cardini (9). S6P was prepared by direct phosphorylation of sucrose with cyanoethyl phosphate and dicyclohexylcarbodiimide in pyridine, essentially as described for sucrose hepta-acetate by Buchanan et al. (10). The product was purified to homogeneity by Dowex-1-borate chromatography following the method of Leloir and Cardini (9).

Growth of Bacteria and Preparation of Extracts: S. mutans 6715-10 (obtained from Dr. Jacob Donkersloot, NIDR) was maintained in fluid thioglycolate broth. Large batches of cells were cultured in Jordan's broth containing 30 mM sucrose as described previously (11). Cells were broken by ultrasonic disruption for 9 min (Branson W350) in 50 ml 0.1 M HEPES pH 7.5 - 5 mM DTT. The debris was removed by centrifugation at 43,000 x g for 30 min and the resulting supernatant fluid was dialyzed overnight against 5 mM HEPES pH 7.5 - 5 mM DTT (4 liters).

S6P Hydrolase Assay: G6P resulting from the enzymatic hydrolysis of S6P was assayed spectrophotometrically at 340 nm (Gilford 2400) following $NADP^+$ reduction by G6PDH. Cuvettes contained 5 IU G6PDH, 0.2 M MES pH 7.1, 2 mM S6P, 1 mM $NAPD^+$ and sample in a final volume of 0.5 ml. A unit (U) is 1 µmole product formed/min at 22° C.

Invertase Assay: Hydrolysis of sucrose was measured by a G6PDH based spectrophotometric assay that used HK and ATP to convert glucose to G6P. Each cuvette contained 5 IU HK, 5 IU G6PDH, 10 mM ATP, 10 mM $MgCl_2$, 0.2 M potassium phosphate buffer pH 6.5, 100 mM sucrose, 1 mM $NADP^+$ and sample in a 0.5 ml final volume. A unit is 1 µmole product formed/min at 22° C.

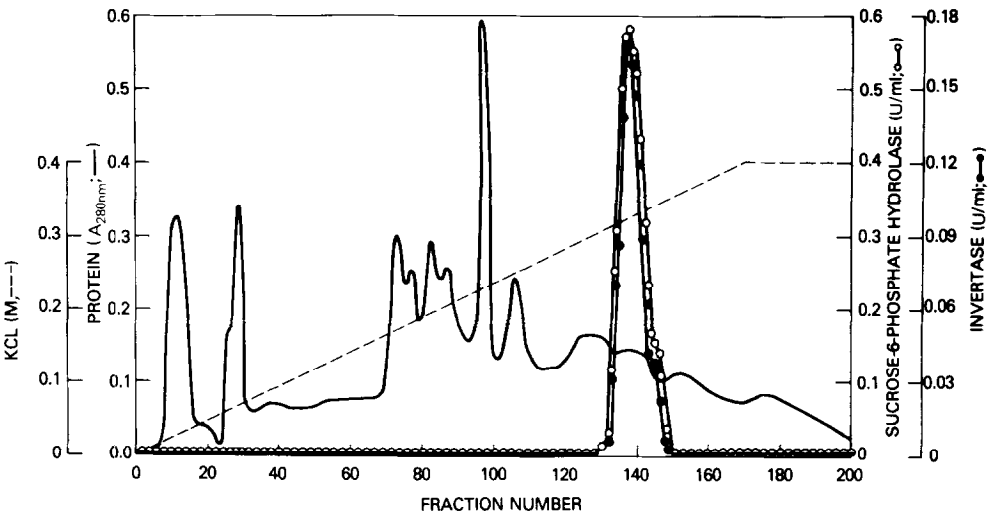


Fig. 1. Chromatography of S. mutans 6715-10 extract on DE52. A 2.5 x 40 cm column equilibrated with 0.1 M HEPES pH 7.5-5 mM DTT was loaded with 43 ml crude extract (1 ml/min; 7.5 ml/fraction). The column was developed with an ascending gradient to 0.4 M KCL in the same buffer. Protein elution was determined by monitoring absorbance at 280 nm (—). The S6P hydrolase (O-O) and invertase (●-●) activity was determined on an aliquot of each fraction.

RESULTS

Assay of unfractionated, dialyzed extracts of S. mutans 6715-10 revealed the presence of S6P hydrolase activity. The crude broken-cell supernatant had a S6P hydrolase activity of 1.14 U/ml; invertase activity was 0.33 U/ml. A partial purification of S6P hydrolase was achieved by chromatography on DE52 (Fig. 1). Invertase activity co-chromatographed with S6P hydrolase activity; the ratio of the two activities was the same for each fraction. Fractions 134-144, which contained the majority of the S6P hydrolase activity, were pooled and concentrated to 10 ml with an Amicon pressure cell (PM 10 membrane). The pool was further concentrated to 1.5 ml by Aquicide III dehydration of the pooled fractions contained in a dialysis casing. The concentrated fractions were chromatographed on Ultrogel AcA 54 (Fig. 2). The peak activity emerged in fraction 60, corresponding to a MW of 42,000. The S6P hydrolase activity

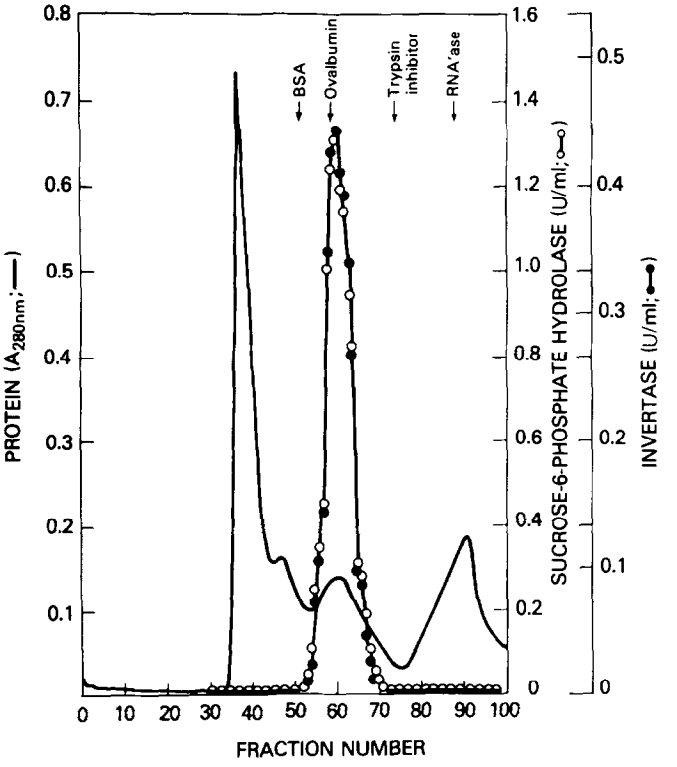


Fig. 2. Ultrogel AcA 54 chromatography of partially purified S6P hydrolase. A 1.4 x 60 cm column of Ultrogel AcA 54 equilibrated with 0.1 M HEPES pH 7.5-5 mM DTT was overlaid with 1.5 ml of the concentrated pool from DE-52 chromatography (flow rate 0.2 ml/min; 1 ml fractions). The protein elution profile was determined by monitoring absorbance at 280 nm (—). S6P hydrolase (o—o) and invertase (●—●) activities were determined by assay of aliquots of each fraction. The arrows indicate the elution positions observed for proteins of known molecular weight which were used to estimate the MW of S6P hydrolase.

again co-chromatographed with invertase activity; the ratio of the two activities was the same for each fraction. A summary of the purification is presented in Table I. The final S6P hydrolase preparation represented a 56% recovery of a 71-fold purified enzyme. A similar degree of purification and recovery was achieved for invertase; the ratio of S6P hydrolase to invertase activity remained essentially constant throughout the purification (Table I).

In order to assess the purity of the final preparation and to further examine the apparent identity of S6P hydrolase with the previously

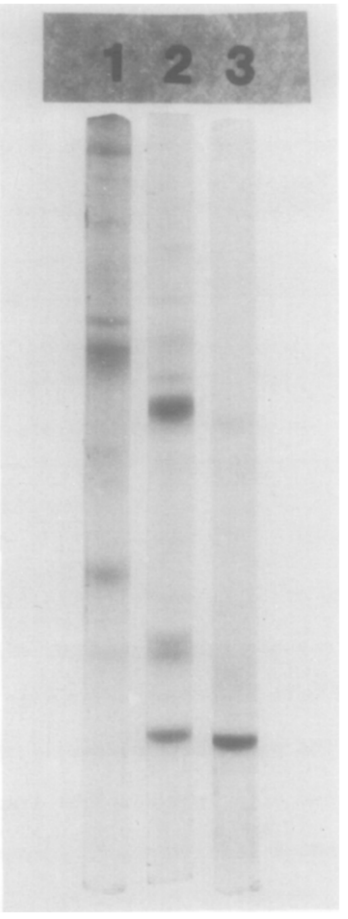
TABLE I

Co-purification of S6P hydrolase and invertase

Fraction	Vol. (ml)	Protein (mg/ml)	Specific Act. (U/mg protein) S6P hydrolase	inver-tase	Ratio: S6P hydrolase / invertase
Crude	43	3.15	0.36	0.10	3.50
DE52 pool	4	2.10	3.93	1.09	3.59
Ultrogel	10	0.10	25.56	7.38	3.46

reported invertase activity of S. mutans extracts, the pooled and concentrated preparations were subjected to disc gel electrophoresis. A photograph (Fig. 3) of the gels electrophoresed with aliquots of preparations at each step in the purification reveals the near homogeneity of the S6P hydrolase obtained after the Ultrogel step. When unstained gels were cut into slices and assayed for S6P hydrolase and invertase activity, the activity/slice profiles for the two enzymes were superimposable (Fig. 4). Direct activity staining of the gels (data not shown) confirmed this observation.

The purified preparation required no added metal ions or cofactors to express S6P hydrolase activity and was not inhibited by EDTA. The pH optimum for S6P hydrolysis was determined over the range of pH 5.5 to 9.5 in 0.2 M MES (pH 5.5-7.1), HEPES (pH 6.7-8.3) and TRIS (pH 7.9-9.5) buffers. At a concentration of 2 mM S6P, the enzyme exhibited the highest activity in 0.2 M MES buffer at pH 7.1. ATP, FDP and PEP, tested at 10 mM as potential regulators of activity, were without effect. The K_m for S6P was 0.21 ± 0.02 mM. Sucrose was examined as a potential inhibitor of S6P hydrolysis by measuring the rate of S6P utilization over the range of 0.1 to 1.5 mM S6P in the presence of 0.1 M sucrose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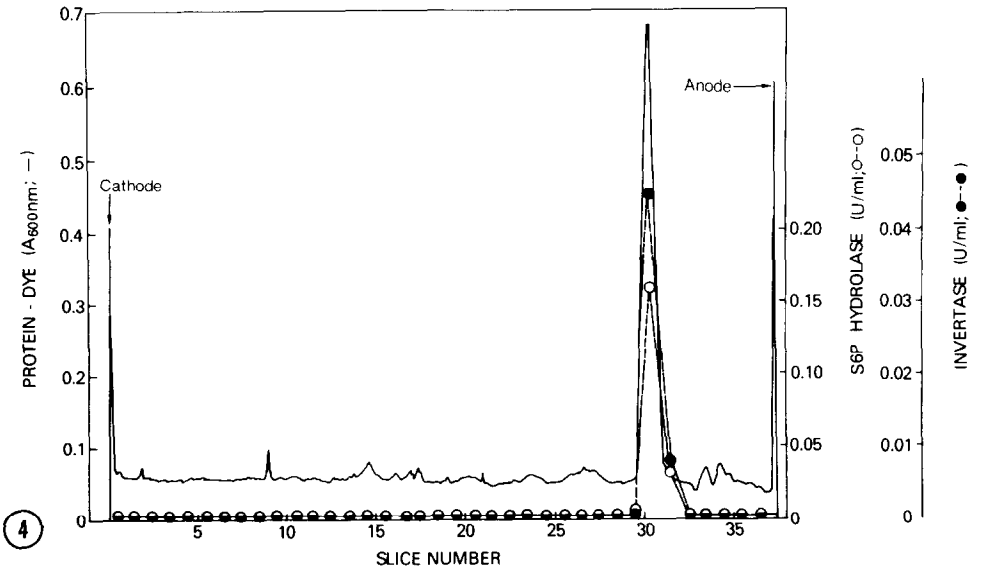
PROTEIN - DYE (A600nm; —)
S6P HYDROLASE (U/ml;O--O)
INVERTASE (U/ml; ●--●)
Cathode
Anode
SLICE NUMBER
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.20
0.15
0.10
0.05
0
0.05
0.04
0.03
0.02
0.01
0
5
10
15
20
25
30
35

4

and 0.1 M potassium phosphate buffer pH 7.1. Under these conditions, sucrose was a competitive inhibitor of S6P hydrolysis with a K_i of 8.12 ± 3.10 mM. S6'P was not a substrate for the enzyme. The preparation was stable for several days at room temperature and for months on storage at 4° C.

DISCUSSION

The S6P hydrolase reported here has not been previously isolated and characterized in S. mutans. A sucrase using S6P as the preferred substrate has been identified in Bacillus subtilis (13). These S6P hydrolases are functionally analogous to the phospho-β-galactosidases which hydrolyze lactose phosphate formed by the action of PEP-dependent lactose phosphotransport systems in Staphylococcus aureus (14) and in S. mutans (15). As is the case with phospho-β-galactosidase, S6P hydrolase is easily purified since it is both stable and comprises more than 1% of the soluble cellular protein.

Based on the co-purification of S6P hydrolase and invertase activities, it is concluded that a single enzyme with dual specificity for S6P and sucrose is present intracellularly in S. mutans. This conclusion is further supported by the observed co-electrophoresis of the two activities as well as the constant ratio of activities throughout purification and the competitive inhibition of sucrose against S6P hydrolysis. The previously described intracellular invertases of S. mutans (4,5,6)

Fig. 3. Disc gel electrophoresis of S6P hydrolase preparations at each stage of purity. Gel 1 was loaded with 50 µg crude extract; gel 2, 50 µg DE-52 pool concentrate; gel 3, 25 µg Ultrogel pool in the Tris-glycine BIOPHORE system described by Bio-Rad. Gels were stained with 125 µg/ml Coomassie brilliant blue in 12.5% Trichoroacetic acid and destained in 5% acetic acid (12).

Fig. 4. Densitometric scan and activity profile in disc gel of purified S6P hydrolase. The destained gel 3 described in the legend to Fig. 3 was scanned at 600 nm with a linear gel transport mounted on a Gilford spectrophotometer (—). A duplicate unstained gel was cut into 2 mm slices and the slices were eluted by standing overnight in 0.2 ml 0.1 M HEPES pH 7.5--5 mM DTT at 4°. The eluate of each slice was assayed for S6P hydrolase (O--O) and invertase (●--●) activity.

should probably be reclassified as S6P hydrolases since S6P is a better substrate than sucrose. It appears that the low K_m extracellular invertase reported by Chassy et al. (3) constitutes the true invertase of S. mutans.

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