RESOLUTION OF *STREPTOCOCCUS MUTANS* GLUCOSYLTRANSFERASES INTO TWO COMPONENTS ESSENTIAL TO WATER-INSOLUBLE GLUCAN SYNTHESIS

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1. Introduction

Streptococcus mutans has been implicated as a significant factor in the development of human dental plaque and subsequent caries formation [1,2]. The cariogenicity of this organism is mainly dependent on its ability to convert dietary sucrose to adhesive water-insoluble glucans (WIG) which give a high proportion of α-(1 → 3) to α-(1 → 6) linkages [3–5]. Many bacterial species produce extracellular glucosyltransferases (EC 2.4.1.5 'dextran-sucrase') which catalyze the formation of water-soluble glucans (WSG), consisting of α-(1 → 6) glucosidic linkages primarily [5–9]. The dextran-sucrase from several strains of *S. mutans* have been purified and characterized [8–10]. In contrast, attempts to purify the water-insoluble glucan-synthesizing glucosyltransferases (WIG-GTase) have consistently failed either because the enzyme remains in high M_r aggregates [9–13], or because the activity disappears during purification procedures [14,15]. Therefore, the mechanism of WIG synthesis by *S. mutans* as well as the number of proteins required for its synthesis are still unknown.

Here, we describe the isolation of the disaggregated WIG-GTase from the culture fluid of *S. mutans* strain B-13 (serotype *d*), and show an evidence that adhesive WIG are synthesized through an overall reaction by 2 protein components.

2. Materials and methods

2.1. *Organism and growth conditions*

S. mutans strain B-13, which does not have fructosyltransferases, was provided from our own culture collection. Strain B-13 was originally obtained from Dr Shklair (Naval Dent. Res. Inst., Great Lakes). The organism was grown aerobically at 37°C for 20 h in a partially defined midium M4 [16] with 0.5% glucose as carbon source.

2.2. *Purification procedures*

The culture supernatant fluid obtained from 600 ml culture by centrifugation (1800 × *g*, 30 min) was treated with 2/3rds vol. cold absolute ethanol (−80°C) to make a final concentration of 40%. The precipitate was suspended in 30 ml 5 mM triethanolamine and the suspension was dialyzed against the same solution for 24 h. The insoluble material was removed by centrifugation (13 000 × *g*, 10 min). The supernatant was applied to a Bio-Gel A-1.5 m column (1.7 × 100 cm) equilibrated with 50 mM phosphate buffer (pH 7.2), and eluted at room temperature with the same buffer. The fractions containing the activity were pooled, concentrated by solid polyethylene glycol, and dialyzed against 50 mM phosphate buffer (pH 6.5). This preparation was applied to a hydroxylapatite column (1.2 × 7 cm) equilibrated with 50 mM phosphate buffer (pH 6.5). After the column was washed with the same buffer, the adsorbed enzyme was eluted with 200 ml of a linear 50–500 mM phosphate buffer gradient.

2.3. *Enzyme assays*

Turbidimetric assay for WIG-forming activity: The assay was done as in [16]. The reaction mixture (0.3 ml) containing 50 mM sucrose, 100 mM acetate buffer (pH 5.5) and enzyme was incubated at 25°C in a microcuvette. The increase in adsorbance at 340 nm was recorded automatically by connecting a recorder to a Hitachi UV-VIS spectrophotometer model 100. The activity (ΔA_{340}/min) was determined from the slope at linear part of the time course curve.

Colorimetric assay for WIG- and WSG-forming activities: A 3-fold vol. of the above reaction mixture was incubated at 25°C for 10 min in the small test tube. The reaction was terminated by the addition of 30 μl of 0.6 M sodium dodecyl sulphate. Reaction mixture was then centrifuged at 10 000 × *g* for 3 min to separate WIG and WSG. The pellet was washed 3 times with 50% ethanol in 50 mM acetate buffer (pH 5.5). WSG in the supernatant were precipitated with 3 vol. ethanol at 4°C overnight. The precipitate was collected, and washed 3 times with 75% ethanol in 50 mM acetate buffer (pH 5.5). The washed polysaccharide were determined by a phenol–sulphuric acid method [17] with glucose as standard. One unit of the activity was defined as amount of enzyme required to incorporate 1.0 μmol glucose from sucrose into glucans per minute under the standard assay.

2.4. *Other methods*

Polyacrylamide gel electrophoresis (PAGE) was performed as in [18] using a 3.5% gel at pH 8.9. The gels were run at 1 mA/tube for 4.5 h. Protein was stained with 0.1% Coomassie brilliant blue. Activity was stained as in [19]. Protein and carbohydrate were measured as in [20] and [17], respectively.

3. Results and discussion

3.1. *Disaggregation of the enzyme*

S. mutans B-13 produced high levels of extracellular WIG-GTase when grown in M4 medium. One ml of the cell free culture fluids had an ability to synthesize ~0.7 μmol WIG from sucrose/min at 25°C. The enzyme in culture fluids was concentrated by ethanol precipitation and then dialyzed against triethanolamine solution. The 40% ethanol fraction was filtered through Bio-Gel A-1.5 m. Typical effluent patterns of WIG- and WSG-forming activities, protein and carbohydrate are shown in fig.1.

The majority of the WIG-forming activity was found in the second protein peak which had an effluent volume corresponding to M_r 220 000. A part of the activity was also eluted in the void volume of the column as high M_r aggregates. No significant WIG-forming activity was observed in all fractions.

The disaggregated enzyme in the second peak (fractions 29–36) was recovered with an overall yield of 34% and a specific activity of 23 U/mg protein. PAGE pattern of this preparation showed 2 main protein bands (fig.3). Both bands were evidently positive when the activity was detected by staining for fructose liberated from sucrose. The second peak was almost free from carbohydrate (fig.1). This finding is distinct from [9–12,21], suggesting that the enzyme may be a glycoprotein. Its discrepancy seems to be due to the enzyme purity.

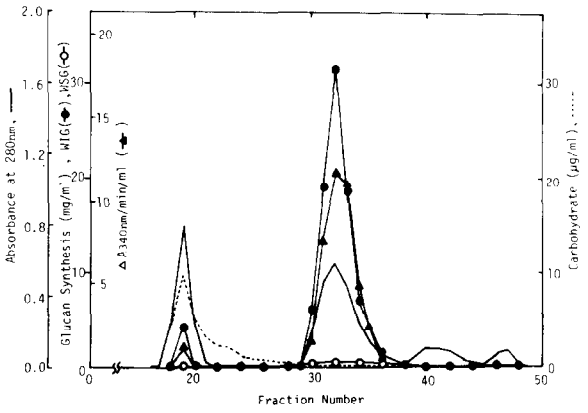

Fig.1. Effluent profile of a 40% ethanol fraction on Bio-Gel A-1.5 m. Gel filtration was performed as in section 2. Flow rate 24 ml/h. Fraction volume 5 ml.

When a $(NH_4)_2SO_4$ precipitation was used in the place of the ethanol precipitation, the majority of the activity was eluted in the void volume of the same column. Although many investigators have used a $(NH_4)_2SO_4$ fractionation to prepare their enzymes, this treatment may be undesirable for isolation of the disaggregated form.

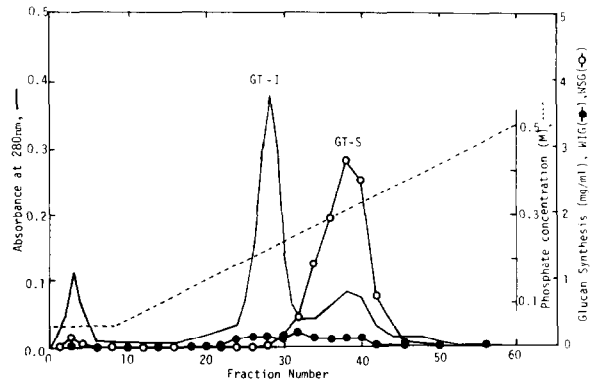

Fig.2. Effluent profile of a Bio-Gel fraction on hydroxylapatite. Column chromatography was performed as in section 2. Flow rate 12 ml/h. Fraction volume 3.8 ml.

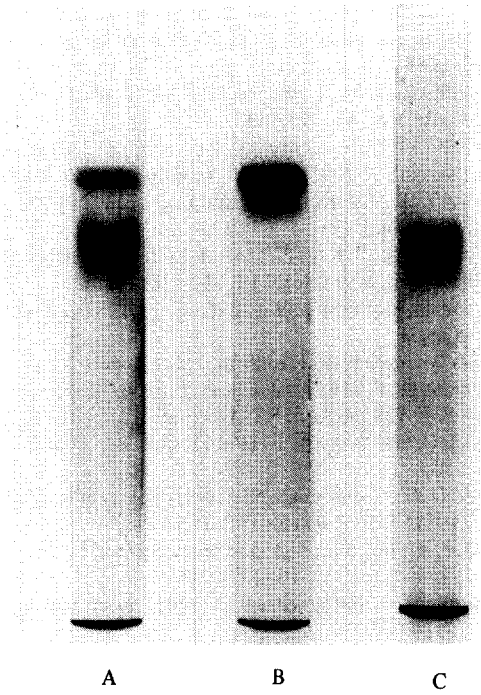

Fig.3. Polyacrylamide gel electrophoretic patterns of fractions obtained by Bio-Gel and hydroxylapatite chromatographies. Bio-Gel fractions(A), GT-I(B) and GT-S(C) were stained for protein with Coomassi brilliant blue.

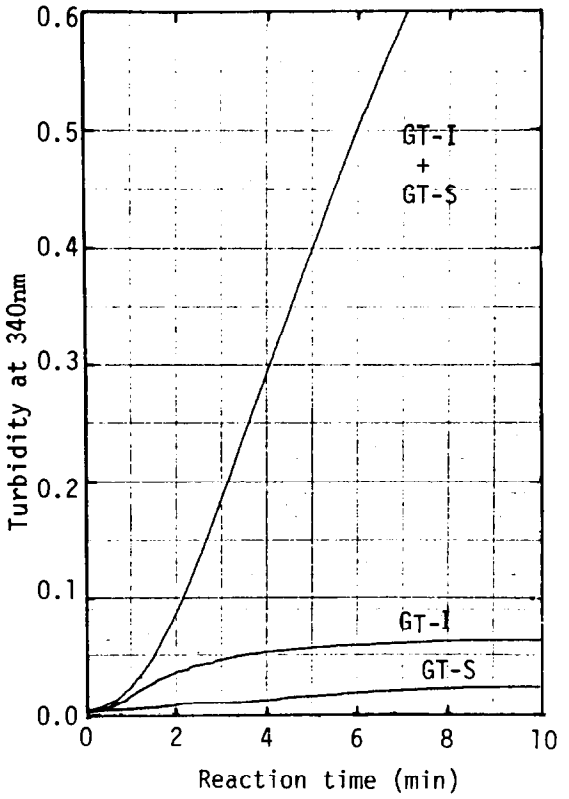

Fig.4. Initial reaction patterns of WIG synthesis by GT-I and/or GT-S. Activity was measured turbidimetrically as in section 2, using 5.2 μg GT-I and/or 1.8 μg GT-S.

3.2. *Resolution into two components*

The enzyme in the second peak was subjected to a hydroxylapatite column chromatography. Fig.2 shows its typical effluent profile. Two catalytically active components (GT-I and GT-S) were eluted at around 0.22 M and 0.31 M phosphate, respectively. On a gel of PAGE, GT-I (fractions 25–30) gave a single broad band which coincided in position with the under band of Bio-Gel enzyme, whereas GT-S (fractions 35–41) gave 1 main band coinciding with the upper band and 2 minor bands (fig.3). GT-I synthesized slightly non-adhesive WIG from sucrose. In contrast, GT-S synthesized a large amount of WSG and a little WIG from sucrose. Only 0.8% of the applied WIG forming activity was recovered in both peaks. Though the bulk of the activity disappeared through this treatment, it was restored by the mixing of all fractions containing both components.

Fig.4 shows the initial reaction patterns of WIG synthesis from sucrose by GT-I and/or GT-S. The formation of turbid materials by GT-S alone was extremely slight. On the other hand, the WIG-formation by GT-I alone proceeded obviously during the first 2–3 min. But the reaction rate fell into decay immediately and no change of the turbidity was attained after 8 min. Addition of GT-S to this reaction mixture resulted in marked stimulation of WIG formation and no decay of the reaction rate. The WIG thus synthesized was adhesive on the glass surface of the microcuvette.

These results suggest strongly that adhesive WIG synthesis is mediated by a cooperative action of GT-I and GT-S.

These data support the idea that WIG may be synthesized by 2 or more proteins, including a dextransucrase which catalyzes the formation of α-(1 → 6) linkages and a second enzyme which catalyzes the formation of α-(1 → 3) linkages [22–24]. They disagree with [13,14] suggesting that WIG and WSG syntheses are catalyzed by interconvertible forms of the same protein.

Experiments to characterize GT-I and GT-S, and to clarify these role in the WIG synthesis are in progress.

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