Volume 243, number 2, 343–346 FEB 06750 January 1989

Evidence for the presence of two distinct sites of sucrose hydrolysis and glucosyl transfer activities on 1,3-α-D-glucan synthase of *Streptococcus mutans*

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Received 7 November 1988

1,3-α-D-Glucan synthase of *Streptococcus mutans* catalyzes both the hydrolysis of sucrose to glucose and fructose, and the glucosyl transfer to glucosyl polymers to yield water-insoluble glucan. The enzyme catalyzes only sucrose hydrolysis, however, in the absence of 1,6-α-D-glucan as an acceptor. In the present study, we found that glucosyl transfer activity was completely inhibited by the antiserum against isolated 1,3-α-D-glucan synthase but that the sucrose hydrolysis activity was not. The antiserum did not impair the binding of the enzyme to the acceptor. These findings indicate that sucrose hydrolysis and glucosyl transfer occur at two distinct sites on the enzyme.

Glucan synthase, 1,3-α-D-; (*S. mutans*)

1. INTRODUCTION

In synthesizing polysaccharides, many glucosyltransferases utilize a sugar nucleotide as a high-energy donor for glucosyl transfer [1–3]. However, glucosyltransferases secreted by microorganisms such as *Streptococcus mutans* and *Leuconostoc mesenteroides* utilize the energy derived from the hydrolysis of sucrose to glucose and fructose for glucosyl transfer [4]. In the latter case, the sucrose hydrolysis and glucosyl transfer activities are thought to occur at two distinct sites on the enzyme, but there has been no report directly supporting this hypothesis. *S. mutans* secretes not only 1,6-α-D-glucan synthase, but also 1,3-α-D-glucan synthase related to water-insoluble glucan formation [5,6]. We have reported that 1,3-α-D-glucan synthase of *S. mutans* serotype *g* requires 1,6-α-D-glucan for glucosyl transfer and that, in its absence, glucosyl transfer will not occur with only sucrose hydrolysis taking place [5]. The enzyme is believed to form a branch of the 1,3-α-D-glucoside linkage on the 1,6-α-D-glucan chain at the C-3 position and extend the 1,3-α-D-glucan chain, resulting in the formation of a water-insoluble glucan [5,6]. 1,6-α-D-Glucan acts as an acceptor for glucosyl transfer rather than a primer, as suggested by Cote and Robyt [7].

Here, using antiserum against 1,3-α-D-glucan synthase, we report that the active site of the enzyme from *S. mutans* serotype *g* is composed of at least two components: the sucrose hydrolysis site and the glucosyl transfer site.

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2. MATERIALS AND METHODS

2.1. *Purification of 1,3-α-D-glucan synthase and preparation of antiserum against the enzyme*

Purification of 1,3-α-D-glucan synthase from an AHT strain of *S. mutans* (serotype *g*, subculture AHT-k) and preparation of the antiserum against the enzyme were carried out as in [5]. Control serum was obtained by bleeding the rabbit prior to immunization. The antiserum and control serum were purified as the IgG fraction using an Affi-gel protein A MAPS-II kit (Bio-rad) and the concentration of each fractionated antibody was adjusted to 2.8 mg/ml protein.

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

2.2. *Assay for water-insoluble glucan synthesis, sucrose hydrolysis and glucosyl transfer activities of 1,3-α-D-glucan synthase*

150 μl of an assay mixture, containing 28 mM sucrose (reference grade, Phanstiehl Labs) and 5 mM NaF in 0.1 M sodium acetate buffer (pH 6.0) with or without 1 mg/ml of dextran T10 as an acceptor was prepared. After incubation the reaction was stopped by heating for 5 min at 85°C, and water-insoluble glucan synthesis activity was assayed as in [5]. Sucrose hydrolysis activity was defined as the amount of release of free fructose derived from sucrose, and the glucosyl transfer activity was calculated using the following equation:

glucose transferred to glucan = free fructose − free glucose

The amounts of free glucose and fructose in the reaction mixture were measured using an F-kit, a biochemical assay of fructose/glucose (Boehringer Mannheim, FRG).

2.3. *Analysis of the reaction products of 1,3-α-D-glucan synthase by HPLC*

After incubation, the reaction of the enzyme was stopped by heating and the mixture centrifuged at 8000 × *g* for 15 min to remove the water-insoluble glucan. The resulting supernatant was desalted using Amberlite MB-3 (Rohm and Haas, USA) and filtered through a Millipore column guard (0.22 μm). The filtered solution was subjected to HPLC on a Shimadzu SCR-101N column and carbohydrates were then detected using a refractometer (RI-8; TOSOH, Tokyo). The reaction mixture, containing the enzyme with 28 mM raffinose in the same buffer specified for sucrose, was analyzed as described above.

2.4. *Other procedures*

Protein determination and electrophoresis was carried out as described [8].

3. RESULTS AND DISCUSSION

The reaction mechanisms of glucosyltransferase secreted by microorganisms have been examined in previous studies [9–12] while that of 1,3-α-D-glucan synthase has only been investigated by Robyt and Martin [10]. Although it was reported that the addition of 2 mg/ml final concentration of dextran resulted in a more than 20-fold increase in 1,3-α-D-glucan synthase activity [6], their enzyme showed only a 1.6-fold increase in enzyme activity at 1.7 mg/ml dextran [10]. Since the enzyme used in the present study synthesized almost no glucan in the absence of the acceptor, as shown in fig.1C, the rate of increase in enzyme activity on addition of acceptor could not be calculated. It is believed that the enzyme used in the study of Robyt and Martin [10] contained a considerable amount of water-soluble glucan synthase. Furthermore, their method of using immobilized enzyme, while correct in evaluating the reaction mechanism

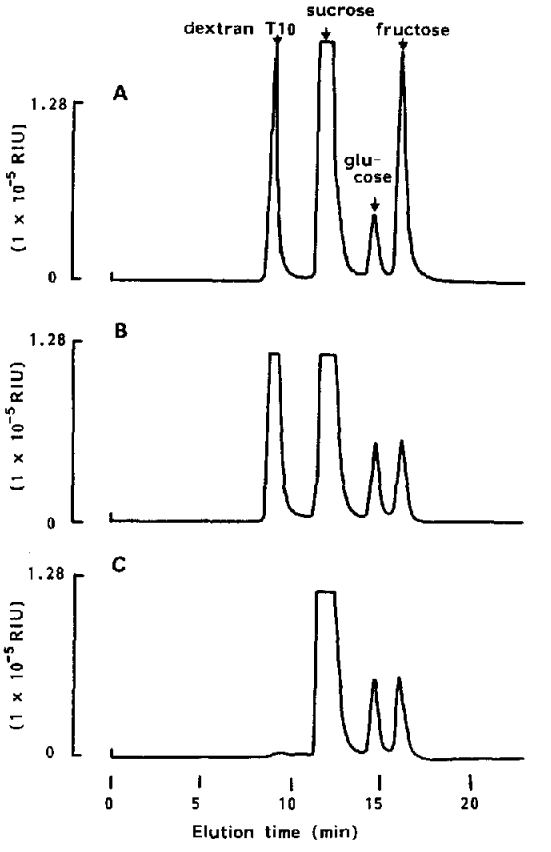

Fig.1. Analysis of the reaction mixture of 1,3-α-D-glucan synthase by HPLC. Each reaction mixture was incubated for 1 h at 37°C, and the products in the reaction mixture were analyzed using HPLC as described in the text (0.5 ml/min flow rate at 60°C). (A) Enzyme (0.25 μg) was incubated in the reaction mixture with 1 mg/ml dextran T10. (B) Enzyme (0.25 μg) was incubated in the same buffer with 20 μl antiserum against purified 1,3-α-D-glucan synthase. (C) Enzyme (0.25 μg) was incubated in the same buffer without dextran T10 in the absence of antiserum. Arrows beneath dextran T10, sucrose, glucose and fructose indicated the corresponding elution times.

of water-soluble glucan synthase, is unreasonable in terms of evaluating that of water-insoluble glucan synthase, since it is impossible to separate water-insoluble glucan from the immobilized enzyme using either centrifugation or filtration at a neutral pH.

In the present study, we found that the sucrose hydrolysis activity of 1,3-α-D-glucan synthase continued in the presence of the antiserum, while the glucosyl transfer activity disappeared (fig.1B). Similar results were observed for the reaction mix-

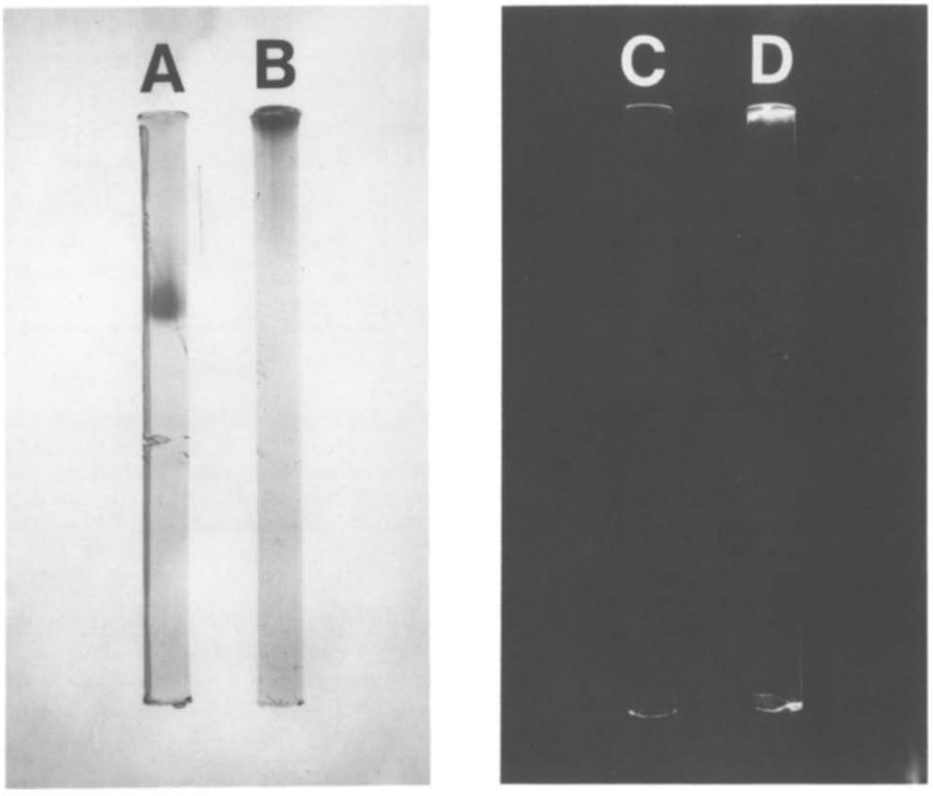

Fig.2. Effect of preincubation with acceptor on the polyacrylamide gel electrophoretic pattern of the enzyme. Enzyme (1 μg) was applied to a 7.5% polyacrylamide gel and electrophoresis was carried out at 4 mA per gel for 3 h at 4°C in 5 mM Tris/glycine buffer (pH 8.4) (A,C). Enzyme (1 μg) was preincubated in 1 mg/ml of dextran T10 for 30 min at 25°C and subjected to electrophoresis under the same conditions as described above (B,D). After electrophoresis, gels A,B were stained with Coomassie brilliant blue R-250. Water-insoluble glucan synthesis activities of gels C,D were detected by incubation of the gels in 0.1 M sodium acetate buffer (pH 6.0) containing 28 mM sucrose in the absence of the acceptor.

ture without the acceptor (fig.1C). From these findings, three hypotheses are proposed: (i) the purified enzyme is contaminated by invertase; (ii) the antiserum interrupts the binding of the enzyme

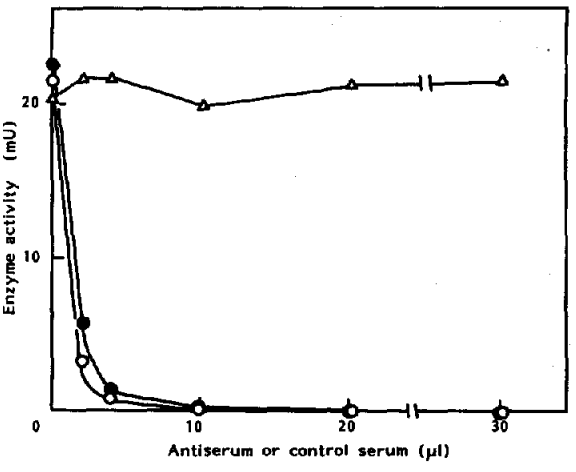

Fig.3. Effects of preincubation of the enzyme with the acceptor on the inhibitory effect of the antiserum. Enzyme (1 μg) was preincubated with 1 mg/ml of dextran T10 for 1 h at 37°C and then incubated with various amounts of antiserum against 1,3-α-D-glucan synthase (●) or control serum (△) for 1 h at 37°C. (○) Enzymes (1 μg) were preincubated with various amounts of antiserum prior to incubation with acceptor, and then incubated with the acceptor under the above conditions. 20 μg bovine serum albumin was added to each sample to avoid inactivation of the enzyme during incubation. After 1 h of incubation at 37°C, water-insoluble glucan synthesis activity was measured as described in the text.

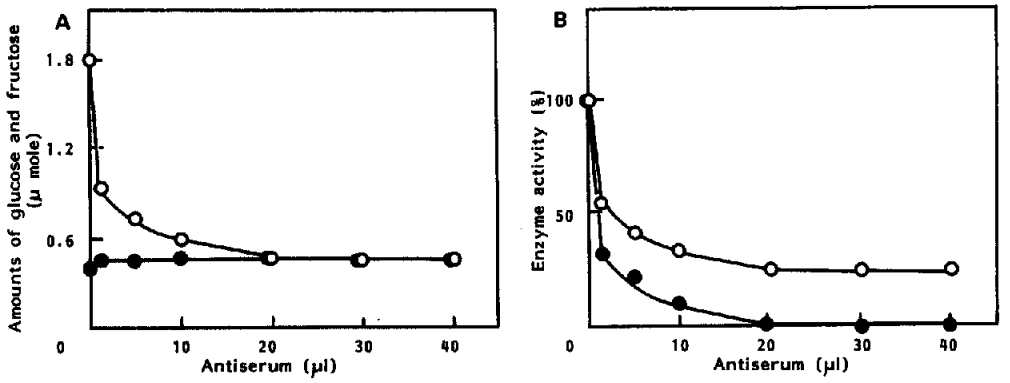

Fig.4. (A) Effects of antiserum on the releasing activities of free glucose (●) and fructose (○). Reaction mixtures of the enzyme (1 μg) containing various amounts of antiserum were incubated for 1 h at 37°C. After incubation, the reaction was stopped, and the amounts of free glucose and fructose in the reaction mixtures were determined as described in the text. (B) Inhibitory patterns of glucosyl transfer (●) and sucrose hydrolysis (○) activities of the enzyme with the antiserum. Values are given as percentages, calculated as rates, of the corresponding reaction mixtures without the antiserum. Glucosyl transfer and sucrose hydrolysis activities are defined in the text.

with an acceptor; (iii) the enzyme has two distinct active sites and their responses to the antiserum differ from each other.

The hydrolysis activity of raffinose examined in the purified enzyme preparation was not detected by HPLC (not shown), thus invalidating the first hypothesis.

The enzyme which was preincubated with a primer exhibited a delay in migration and a white band corresponding to a protein band in electrophoresis (fig.2B,D). These findings suggest a tight covalent binding of the enzyme with the acceptor even in the absence of sucrose. Preincubation of the enzyme with the acceptor did not prevent inhibition of enzyme activity by the antiserum (fig.3). The antiserum added to the reaction mixture during the reaction also inhibited the enzyme activity (not shown). These results invalidate the second hypothesis, demonstrating that the antiserum exerts an inhibitory effect on enzyme activity regardless of whether the enzyme binds to the acceptor, and that the antiserum affects the catalytic site directly.

A quantitative analysis of the effects of antiserum on the enzyme activities showed two distinct inhibitory patterns of sucrose hydrolysis and glucosyl transfer activities, as illustrated in fig.4B. While glucosyl transfer activity was completely inhibited by the antiserum, sucrose hydrolysis activity resisted antiserum inhibition. From these findings, the last hypothesis seems to be the most acceptable. Furthermore, it has been proven that 1,3-α-D-glucan synthase from *S. mutans* serotype *g* has two distinct sites for glucan synthesis: one is the sucrose hydrolysis site, the other being the glucosyl transfer site.

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