Carbohydrate Research, 168 (1987) 120–124
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

(1→3)-α-D-Glucan synthase from *Streptococcus mutans* AHT (serotype *g*) does not synthesise glucan without primer

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(Received March 10th, 1987; accepted for publication, May 1st, 1987)

Extracellular glucosyltransferases from *Streptococcus mutans* catalyse the hydrolysis of sucrose and the synthesis of a glucan which is sticky and water-insoluble. When formed *in situ*, the glucan allows *S. mutans* and other bacteria to adhere to the surface of the tooth[1–3] and acts as a diffusion barrier for acids produced by bacteria[4]. Therefore, glucosyltransferases are key enzymes in the cariogenicity of *S. mutans*. Synthesis of the water-insoluble glucan requires the co-operation of these glucosyltransferases and, in order to elucidate the mechanism, each glucosyltransferase must be isolated and purified, and its properties determined.

S. mutans serotype *g* secretes four kinds of glucosyltransferase[5], namely, one water-insoluble glucan synthase [(1→3)-α-D-glucan synthase, EC 2.4.1.-] and three water-soluble glucan synthases [SGTases, (1→6)-α-D-glucan synthases, EC 2.4.1.5]. We now report on the purification of (1→3)-α-D-glucan synthase and its role in the insolubilisation of water-soluble glucan (SG).

Table I summarises the steps in the purification of the (1→3)-α-D-glucan synthase. PAGE**, SDS-PAGE, and IEF indicated the purified enzyme to be homogeneous and a double immunodiffusion test gave a single precipitin band. In IEF, the purified enzyme was isofocused at a position between those of the soybean trypsin inhibitor (pI 4.55) and β-lactoglobin A (pI 5.20) and had a pI value of ~5.1. Ultracentrifugation on a density gradient of glycerol indicated the purified enzyme to be larger than alcohol dehydrogenase (mol. wt. 148,000) and to have a mol. wt. of ~150,000. The enzyme had an optimum pH of ~6.2 in the McIlvain buffer, the K_m value for sucrose was ~4.17mM in the presence of 0.25 mg/mL of DT10, and the K_m value for DT10 was ~0.83mM glucose equivalent. Smith degradation

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**PAGE, Polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; IEF, isoelectric focusing.

0008-6215/87/$ 03.50

TABLE I

PURIFICATION OF THE (1→3)-α-D-GLUCAN SYNTHASE

Fraction	*Volume (mL)*	*Total activity (U)*	*Total protein (mg)*	*Specific activity (U/mg)*	*Purification (fold)*	*Yield (%)*
Ammonium sulfate	1620	464	6026	0.077	1	100
DEAE-cellulose first	108	34.0	220	0.155	2.0	7.3
second	21	10.3	65.6	0.157	2.0	2.2
Bio-Gel A-5m	21	6.0	37.1	0.162	2.1	1.3
Octyl-Sepharose CL-4B	14	3.0	9.69	0.310	4.0	0.65
Mono Q (FPLC)	1.4	1.65	2.44	0.676	8.8	0.36
TSK-gel G3000SW (FPLC)	2.5	1.39	0.685	2.029	26.4	0.30
TSK-gel phenyl 5PW (FPLC)	1.8	0.83	0.171	4.854	63.0	0.18

showed that the glucan synthesised contained ~79% and ~21% of (1→3) and (1→6) linkages, respectively.

The glucosyltransferases of *S. mutans* serotype *g* comprise four isozymes which synthesise glucans with different properties. (1→3)-α-D-Glucan synthase, which produces a water-insoluble glucan, has been purified[6–8] and a marked primer effect was reported although glucan was reported to be synthesised in the absence of primer. Since the more the (1→3)-α-D-glucan synthase was purified, the greater was the primer effect, it seemed likely that, on complete purification, glucan would be synthesised only in the presence of primer. This expectation was realised with the highly purified enzyme and, in the absence of primer, very little glucan was synthesised but sucrose hydrolysis was catalysed. In the presence of (1→6)-α-D-glucan as primer, some of the sucrose was hydrolysed but most of the glucose residues were transferred to the primer with the formation of (1→3) linkages. The final insoluble glucan contained 79% of such linkages.

EXPERIMENTAL

Culture conditions. — *S. mutans* AHT (serotype *g*, subculture AHT-k)[9], kindly provided by Dr. M. Inoue (Kagoshima University Dental School), was cultured anaerobically for 18 h at 37° in Trypticase, tryptose, and yeast extract–D-glucose broth[10] in 20 5-L batches.

The activity of the enzyme was determined by incubating a reaction mixture (0.9 mL) in a 100mM sodium acetate buffer (pH 6.0), containing 5% of sucrose, 5 mg/mL of dextran T10 (DT10), 5mM sodium fluoride, and enzyme solution at 37°. The insoluble glucan (IG) was collected by centrifugation (1,500*g*, 30 min), washed twice with distilled water, and quantified by a modification of the anthrone method[11].

One unit of glucosyltransferase activity is defined as the amount of enzyme catalysing the transfer of 1 μmol of D-glucose from sucrose to glucan per min in the above mixture.

Purification of (1→3)-α-D-glucan synthase. — All procedures were carried out at 0–4° except for fast protein liquid chromatography (f.p.l.c.) and h.p.l.c. The culture broth (100 L) was centrifuged at 7,000*g* for 20 min and the cell-free supernatant solution was 50% saturated with ammonium sulfate. The resulting precipitate was collected by centrifugation (11,000*g*, 60 min), suspended in a 20mM Tris-HCl buffer (pH 7.5), and dialysed against the same buffer; the final volume was 1,620 mL. The crude enzyme solution (540 mL) was applied to a column (9 × 36 cm) of DEAE-cellulose equilibrated with the Tris-HCl buffer until no absorbance at 280 nm was detected in the eluate. The column was then eluted with a linear gradient of 0→0.4M NaCl in the Tris-HCl buffer (6 L). The procedure was repeated thrice. Fractions with IG-synthesising activity were concentrated by ultrafiltration using an Amicon PM10 filter. The procedure was repeated thrice, yielding 108 mL of a DEAE-cellulose (first) fraction. A portion (54 mL) of the first fraction was applied to a column (2.6 × 35 cm) of DEAE-cellulose equilibrated with the Tris-HCl buffer and eluted by a linear gradient of 0→0.3M NaCl in the same buffer (1 L). The fractions containing IG-synthesising activity were combined. The procedure was repeated twice, yielding 21 mL of the second fraction. A portion (5.25 mL) of the second fraction was chromatographed on a column (2.6 × 100 cm) of Bio-Gel A-5m (Bio-Rad) equilibrated with a 50mM sodium acetate buffer (pH 6.0) containing 0.1M NaCl. The fractions containing IG-synthesising activity were combined. The procedure was repeated four times, yielding 21 mL of the Bio-Gel A-5m fraction.

Ammonium sulfate was added to a portion (10.5 mL) of the Bio-Gel A-5m fraction to 25% saturation, and the fraction was chromatographed on a column (1.6 × 15 cm) of Octyl-Sepharose CL-4B (Pharmacia) equilibrated with a 50mM sodium acetate buffer (pH 6.0) containing 25% of ammonium sulfate. After washing the column with the buffer, it was eluted with a linear gradient involving decrease of the ammonium sulfate concentration in 400 mL of the buffer to 0% and increase of the ethylene glycol concentration from 0 to 60%. The procedure was repeated twice. The active fractions were combined and dialysed against 20mM Tris-HCl buffer (pH 7.5), yielding 14 mL of an Octyl-Sepharose CL-4B fraction.

A portion (2 mL) of the Octyl-Sepharose CL-4B fraction was chromatographed using a Mono Q column (Pharmacia). After washing the column thoroughly with the Tris-HCl buffer (pH 7.5), the column was eluted by a linear gradient of 0→0.3M NaCl in 20 mL of the Tris-HCl buffer. The procedure was repeated seven times, yielding 1.4 mL of the Mono Q fraction.

A portion (100 μL) of the Mono Q fraction was chromatographed on a TSK-gel G3000SW column (h.p.l.c., ToyoSoda, Japan) equilibrated with 50mM sodium acetate buffer (pH 6.0) containing 0.2M NaCl and 0.02% of NaN_3. The active fractions were combined, yielding 2.5 mL of the TSK-gel G3000SW fraction.

Ammonium sulfate was added, to 25% saturation, to a portion (500 μL) of the TSK-gel G3000SW fraction, which was then chromatographed on a TSK-gel phenyl 5PW column (h.p.l.c., ToyoSoda), equilibrated with a 50mM sodium acetate

buffer (pH 6.0) containing 25% of ammonium sulfate and 0.02% of NaN_3. The column was washed with the same buffer and then eluted with a linear gradient of decreasing ammonium sulfate concentration to 0% and of increasing ethylene glycol concentration to 60%. The active fractions were dialysed against the 50mM sodium acetate buffer (pH 6.0), yielding 1.8 mL of a final enzyme sample.

By using the foregoing procedures, 171 μg of purified (1→3)-α-D-glucan synthase was obtained from 100 L of culture broth.

Polyacrylamide gel electrophoresis (PAGE). — The method of Davis[12] was used with 3.8 μg of purified enzyme and staining with Coomassie Brilliant Blue R-250. After electrophoresis, a solution of the enzyme (3.8 μg) in 100mM sodium acetate buffer (pH 6.0) containing 5% of sucrose and 5mM sodium fluoride was stored at 37°. A second solution also contained 5 mg/mL of DT10. After 1 h with the latter mixture, a white opaque band of the glucan had been formed. After 20 h with the former mixture, a faint white band appeared.

Sodium dodecyl sulfate(SDS)-PAGE. — The method of Weber and Osborn[13] was used on a sample of enzyme (4.75 μg) which had been pretreated for 3 min at 100° in a 10mM sodium phosphate buffer solution containing 5% of 2-mercapto-ethanol and 1% of SDS. Coomassie Brilliant Blue R-250 was used for staining.

Isoelectric focusing (IEF). — The method of Mukasa *et al.*[14] was used. A 0.8-mm thick polyacrylamide gel containing 6.3% of Pharmalyte (pH 4–6.5) was employed with 40mM glutamic acid and 200mM histidine as the anolyte and catholyte, respectively. After preliminary electrophoresis for 90 min at 200 V, the sample (1.9 μg) was added to the gel surface, and electrophoresis was carried out for 180 min at 600 V. The pH of the gel surface was then measured by using a surface pH electrode (Fuji Kagaku Keisoku, Tokyo). An isoelectric-focusing calibration kit (Pharmacia) was used with staining with Coomassie Brilliant Blue R-250.

Ultracentrifugation. — The purified enzyme fraction was loaded onto linear 10→30% glycerol gradients, which were then centrifuged and fractionated as described by Coleman *et al.*[15]. Alcohol dehydrogenase and catalase were used as molecular-weight markers.

Protein assay. — Protein concentrations were determined by the method of Bradford[16] with bovine serum albumin as the standard.

Linkage analysis. — A solution of the purified (1→3)-α-D-glucan synthase was mixed with 100mM sodium acetate buffer (pH 6.0), containing 5% of sucrose, 5mM sodium fluoride, and 5 mg/mL of DT10, and was stored at 37° for 48 h. The mixture was diluted with distilled water and centrifuged (1,500*g*, 30 min), and the IG was collected, washed with distilled water, and dried at 40° under reduced pressure. The IG was subjected to Smith degradation[17].

Optimum pH and K_m value. — The reaction mixture containing an adequate amount of the purified enzyme added to four-fold diluted McIlvain buffer containing 5% of sucrose and 0.25 mg/mL of DT10 was stored at 37°. The IG-synthesising activity and the optimum pH were determined.

Reaction mixtures containing 0.25 mg/mL of DT10 and various concentrations of sucrose in the 100mM sodium acetate buffer (pH 6.0) were incubated for 90 min at 37°. The IG produced was quantified by the anthrone method. The K_m value for sucrose was obtained by the Lineweaver–Burk method.

The K_m value for DT10 was calculated by the same method. Reaction mixtures containing 5% of sucrose and various concentrations of DT10 in the 100mM sodium acetate buffer (pH 6.0) were incubated and the IG produced was quantified.

Antiserum against the purified enzyme and immunodiffusion analysis. — Purified (1→3)-α-D-glucan synthase (TSK-gel G3000SW column fraction, 58.5 μg of protein) was mixed with an equal volume of Freund's incomplete adjuvant and injected intradermally into a rabbit. The animal received three injections of antigen at intervals of 2 weeks and antiserum was collected from an ear vein 10 days after the final injection.

The antiserum was partially purified as the IgG fraction by using a Zeta Prep 15 DEAE Disk (AMF, U.S.A.) and was recovered by saturation to 50% with ammonium sulfate.

Ouchterlony double-immunodiffusion analysis was carried out in a 1% agarose gel.

Action of the purified (1→3)-α-D-glucan synthase on sucrose. — A 5% solution of sucrose in 100mM sodium acetate buffer (pH 6.5) containing 5mM sodium fluoride was incubated for 1 h at 37° with purified enzyme. H.p.l.c., using an SCR 101N column (Shimadzu) after filtration through a column guard (pore size, 0.45 μm, Nihon Millipore Ltd.), revealed the formation of glucose and fructose but no glucan.

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