Isoelectric Focusing Studies on the β -Fructofuranosidases and α -Glucosidases of *Streptococcus mitis*

By MARIE-LOUISE SUND, LARS LINDER AND CARITA ANDERSSON

Department of Oral Microbiology, Karolinska Institute, Stockholm, Sweden

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Extracts of *Streptococcus mitis* ATCC 903 were analysed for β -fructofuranosidase and α -glucosidase activities by isoelectric focusing in thin-layer polyacrylamide gels combined with zymogram procedures. Three bands of activity were visualized in the gels after incubation with sucrose (pI 4.05, 4.25 and 4.85) and three other bands after incubation with *p*-nitrophenyl α -D-glucopyranoside (pI 3.90, 4.45 and 4.65). The enzymes responsible for the reaction with sucrose were identified as β -fructofuranosidases (EC 3.2.1.26) for the following reasons: identical enzyme bands were visualized in the gels after incubation with raffinose; no enzyme bands appeared in the gel after incubation with the α -glucosides maltose, turanose, trehalose and melezitose; and the soluble fraction hydrolysed sucrose to equimolar amounts of glucose and fructose.

INTRODUCTION

Streptococcus mitis comprises a significant proportion of the bacteria of dental plaque and saliva and on the buccal mucosa (Gibbons & van Houte, 1975). It is capable of sucrose metabolism, as are all other oral viridans streptococci (Facklam, 1977). However, in contrast to *S. mutans*, *S. sanguis* and *S. salivarius*, *S. mitis* is generally devoid of glucosyl- and fructosyltransferase activities (Facklam, 1977). Apparently *S. mitis* utilizes other enzymic reactions to bring sucrose into the glycolytic pathway.

Two types of enzymes hydrolysing the glycosidic bond in sucrose have been demonstrated in micro-organisms (Myrbäck, 1960): β -fructofuranosidases (β -D-fructofuranoside fructohydrolase, EC 3.2.1.26; invertase) and α -glucosidases (α -D-glucoside glucohydrolase, EC 3.2.1.20). Sucrose phosphorylase (sucrose:orthophosphate α -glucosyltransferase, EC 2.4.1.7), which catalyses the phosphorylytic decomposition of sucrose, has been found in some bacteria (Silverstein *et al.*, 1967).

In the present investigation the soluble fraction of extracts of *S. mitis* was analysed by isoelectric focusing in thin-layer polyacrylamide gels combined with zymogram procedures in order to separate and visualize enzymes active on β -fructofuranosidic and α -glucosidic linkages. The zymogram patterns showed three enzyme bands with substrate specificities characteristic of β -fructofuranosidase and three enzyme bands with α -glucosidase activity.

METHODS

Growth medium and cultivation technique. *Streptococcus mitis* ATCC 903 was grown in proteose/peptone medium (Linder, Holme & Frostell, 1974) which had been sterilized at 121 °C for 15 min. Carbohydrates and vitamins were sterilized separately by filtration (0.22 μ m pore size; Millipore) and added aseptically to the sterile medium. Conditions of pH, anaerobiosis, temperature, and stirring in a 1 litre fermenter (FL 101; Biotec, Stockholm, Sweden) were described earlier (Linder *et al.*, 1974). The gas was N₂/CO₂ (90 : 10, v/v).

Preparation of extracts. Bacteria were harvested 1 h after the exhaustion of the energy source or at the

end of the exponential growth phase and washed twice in cold 0.01 M-sodium phosphate buffer, pH 7.0. The washed cells were suspended (approx. 60 mg dry wt ml⁻¹) in 40 ml 0.05 M-sodium phosphate buffer, pH 7.0, and disrupted by freeze-pressing in an X-press (Edebo, 1960). The homogenates were thawed at room temperature and then deoxyribonuclease and ribonuclease were added (final concentrations 24 and 4 U. ml⁻¹, respectively). After 20 min at room temperature, the homogenates were centrifuged at 37000 *g* for 20 min at 4 °C. The resulting supernatant fractions or 'extracts', containing 3 to 7 mg protein ml⁻¹, were stored at 7 °C and used for the isoelectric focusing and for assays of enzyme activities.

Enzyme assays. β -Fructofuranosidase and α -glucosidase activities were determined by incubating extracts with 0.5 M-substrate and 0.1 M-sodium phosphate buffer, pH 7.0, in a total volume of 0.4 ml. After incubation for 30 min at 37 °C, the reactions were stopped by heating for 5 min in boiling water. Samples were then assayed for glucose with glucose oxidase and for total reducing sugars (Park & Johnson, 1949). Appropriate controls containing boiled enzyme fractions were run in parallel for each substrate.

α -Glucosidase activity was also determined by incubating the extracts with 0.025 M-*p*-nitrophenyl α -D-glucopyranoside (pNPG) and 0.05 M-sodium phosphate buffer, pH 7.2, in a total volume of 0.5 ml. After incubation for 15 min at 37 °C, the reactions were stopped by the addition of 2 ml 0.6 M-K₂B₄O₇, pH 10. The liberated *p*-nitrophenol was then measured at 400 nm using a Zeiss PMQ II spectrophotometer. Controls, as described above, were assayed in parallel.

Sucrose phosphorylase activity was assayed by measuring the amount of glucose 1-phosphate formed in 30 min at 37 °C in a 1.0 ml reaction mixture containing 0.1 M-sodium phosphate buffer, pH 6.8, 1 M-sucrose and extract. Glucose 1-phosphate was determined according to Bergmeyer & Klotzsch (1965).

Enzyme activities were expressed in katal, i.e. the amount of enzyme activity transforming 1 mol substrate s⁻¹ under the standard assay conditions.

Isoelectric focusing in gels. Isoelectric focusing in thin-layer polyacrylamide gels was performed on a Multiphor LKB 2117 (LKB, Bromma, Sweden). Preparation of the gels and the isoelectric focusing procedures were according to the instructions of the manufacturer, except that glycerol was used instead of sucrose as the stabilizing agent. Ampholine carrier ampholytes were used to give pH gradients of 3.5 to 9.5 and 2.5 to 6.0. The electrode strips were soaked in 1 M-NaOH and 1 M-H₃PO₄. For the narrow pH gradient, Ampholine pH 5 to 7 (0.5 %, v/v) was used instead of 1 M-NaOH. The composition of the polyacrylamide gels was T = 5 % (w/v) and C = 3 % (w/v).

About 0.5 ml of the extract was absorbed into a long strip (200 × 10 mm) of filter paper (Paratex III/80; Lohman KG, Fahr/Rhein, West Germany). The paper was applied to the gel about 1 cm from the cathode. At the start of the focusing, the voltage and current were 400 V and 75 mA, respectively. At the end of the run, the corresponding values were 900 V and 30 mA. The power supply was set to give a maximum voltage of 900 V and a maximum power of 30 W. Focusing time was 2 h and the paper strip was removed from the gel after 0.5 h. The temperature of the cooling water was 15 °C. After termination of the focusing, the pH was determined across the gel at 5 mm intervals with a surface electrode (Ingold LOT 403-30; W. Ingold, Zürich, Switzerland), calibrated at pH 5.0.

Zymogram procedures. Slices of about 10 mm were cut out of the gel perpendicular to the electrode strips and incubated for 30 min at 37 °C in 0.1 M-sodium phosphate buffer, pH 7.0, containing various substrates at 0.2 M. Staining methods for ketoses and aldoses were used to locate the enzyme activities in the gel sections as described by Gabriel & Wang (1969). After staining, the gels were fixed in 7.5 % (v/v) acetic acid. For detection of enzymes active on pNPG, slices were incubated in 0.025 M-pNPG/0.05 M-sodium phosphate buffer, pH 7.2, for about 20 min at 37 °C. The enzyme bands appeared without further treatment, but to increase the intensity of the yellow colour, 0.6 M-K₂B₄O₇ was added after incubation with pNPG.

Chemicals. Mono-, di- and trisaccharides, deoxyribonuclease type I and ribonuclease type I-A (both from bovine pancreas), 2,3,5-triphenyltetrazolium chloride and yeast α -glucosidase were obtained from Sigma. Carrier ampholyte solutions (Ampholine) were purchased from LKB; acrylamide and *N,N'*-methylene-bisacrylamide from BDH; Glox test from Kabi, Stockholm, Sweden; and pNPG from Koch-Light.

RESULTS

Isoelectric focusing in thin-layer polyacrylamide gels

Enzyme staining of the thin-layer polyacrylamide gel after isoelectric focusing in the narrow pH gradient 2.5 to 6.0 (Fig. 1) revealed three bands active with sucrose as substrate, three bands active with raffinose, three bands active with pNPG and two bands active with isomaltose. The positions of the bands corresponding to the enzymes active with sucrose and raffinose were identical. The enzymes active with isomaltose were in identical positions to the two cathodic enzymes active with pNPG. One gel slice was incubated in standard

Fig. 1. Zymogram pattern of glycosidic activities developed in thin-layer polyacrylamide gel after isoelectric focusing of an extract of *S. mitis* ATCC 903. Isoelectric focusing was done in a pH gradient of 2.5 to 6.0 for 2 h at 30 W. The gel was cut into slices and the enzyme bands were developed after incubation of the gel slices in substrate solutions. □, Yellow band; ■, red band.

sucrose buffer containing 0.025 M-pNPG. The six enzyme bands visualized in this gel slice confirmed the non-identity of the enzymes active with sucrose and with pNPG. Enzyme bands did not appear in the gel after incubation with the substituted β -fructofuranoside melezitose, or with the α -glucopyranosides trehalose, turanose, maltose, methyl α -glucoside, kojibiose and palatinose, or with the β -glucopyranoside cellobiose. The pI values of the three enzymes active with sucrose and raffinose were 4.05 ± 0.07 , 4.25 ± 0.06 and 4.85 ± 0.06 , and the pI values of the three enzymes active with pNPG were 3.90 ± 0.07 , 4.45 ± 0.04 and 4.65 ± 0.06 . These are mean values $\pm$ s.d. based on five independent experiments where the extracts were examined by isoelectric focusing within 24 h of preparation. Small deviations in the pH gradients between experiments were observed, but the relationships between the positions of the enzyme bands were identical and highly reproducible. It was consistently observed with extracts prepared more than 48 h prior to electrofocusing and stored at 7 °C that the sucrose-active band at pI 4.85 and the raffinose-active bands at pI 4.05 and 4.85 were absent. In contrast, all three bands active with pNPG were visualized in gels even after storing the extract at 7 °C for 1 month. Freezing and thawing of the extract caused the disappearance of the same bands as described above. No additional bands were seen with any of the mono-, di- or trisaccharide substrates after prolonged storage of the extract at 7 °C. The zymogram pattern obtained after focusing of extracts prepared from cells grown on sucrose did not differ from that obtained with extracts from cells grown on glucose. Nor did extracts from stationary phase cells produce different zymogram patterns from extracts of exponential phase cells. The ketose-staining procedure, when applied to gel slices incubated with sucrose or raffinose as substrates, produced sharper enzyme bands than the aldose-staining procedure.

The narrow pH gradient (2.5 to 6) was preferred because it gave higher resolution of the bands without concomitant loss of sharpness. However, the patterns observed after focusing in the broad (pH 3.5 to 9.5) and narrow pH gradients were identical in all respects except

Table 1. *Hydrolysis of sugars by extracts of S. mitis* ATCC 903

Rates of hydrolysis were measured in extracts by determining the formation of total reducing sugars (sucrose and raffinose), *p*-nitrophenol (pNPG) or glucose (all other substrates).

Substrate	Rate of hydrolysis [nkatal (mg protein) ⁻¹]	Substrate	Rate of hydrolysis [nkatal (mg protein) ⁻¹]
Sucrose	25	Isomaltose	4
Raffinose	4	Trehalose	0
Melezitose	0	Turanose	0
pNPG	9	Melibiose	5
Maltose	0	Methyl α -D-glucoside	0

Fig. 2. Zymogram pattern of glycosidic activities developed in thin-layer polyacrylamide gel after isoelectric focusing of a commercial preparation of yeast α -glucosidase (Sigma). Conditions were as described in Fig. 1. □, Yellow band; ■, red band.

resolution. The filter paper was applied at different positions on the gel to optimize the detection of enzyme activities. Application at a point near the cathode, corresponding to a pH of about 5.3 after focusing, gave the sharpest enzyme bands.

Enzyme assays

Extracts were quantitatively assayed for enzyme activities on β -fructosidic and α -glucosidic sugars. The substrate specificities found in the extracts (Table 1) were in accordance with the zymogram pattern except for melibiose, which was hydrolysed by the extract but no corresponding enzyme bands were observed in the electrofocusing studies. The determination of sucrose hydrolysis by measurement of both liberated glucose and reducing sugars revealed that sucrose hydrolysis yielded equimolar amounts of glucose and fructose. Sucrose phosphorylase activity could not be demonstrated in the extract.

Zymogram pattern of partially purified yeast α -glucosidase

The zymogram pattern of the extract was compared with that of a commercial preparation of partially purified yeast α -glucosidase (Sigma). A solution of α -glucosidase (0.1 mg

ml⁻¹) in 0.01 M-sodium phosphate buffer, pH 7.0, was used for isoelectric focusing in a narrow pH gradient. Enzyme-staining techniques revealed four bands active with sucrose (Fig. 2). The most anodic of these was broad and intensely stained. This band coincided with the single band detected in the gel incubated with raffinose as substrate. The other three bands, located in the pH range 4.6 to 5.1, focused in identical positions to the three pNPG bands, the two turanose bands and two of the three melibiose bands. The gel slice incubated with trehalose as substrate had one weakly stained band located parallel to the most anodic of the sucrose bands.

DISCUSSION

The enzymes active on sucrose were identified as true invertases (β -fructofuranosidases) because they hydrolysed raffinose which has an unsubstituted β -fructofuranoside moiety, but not melezitose, a substituted β -fructofuranosyl α -glucoside, and could not hydrolyse any of the α -glucosidic sugars. The hydrolysis of sucrose by the crude extract to equimolar amounts of glucose and fructose was consistent with β -fructofuranosidase activity.

The enzymes hydrolysing pNPG were inactive on all natural saccharides tested except isomaltose, a (1 $\rightarrow$ 6)- α -linked disaccharide of glucose. Under the conditions of the focusing experiments, the enzymes could not cleave the (1 $\rightarrow$ 1)- α linkage of trehalose, the (1 $\rightarrow$ 2)- α linkage of sucrose and melezitose, the (1 $\rightarrow$ 3)- α linkage of turanose or the (1 $\rightarrow$ 4)- α linkage of maltose. The pNPG-active enzymes in *S. mitis* ATCC 903 thus resemble in their substrate specificities the isomaltase of *Saccharomyces cerevisiae* (Gorman & Halvorson, 1966), the α -glucosidase of a strain of *Bacillus thermoglucosidius* (Suzuki *et al.*, 1976) and the dextran-glucosidase isolated from *S. mitis* strains described by Walker & Pulkownik (1973).

The pNPG-active enzymes in *S. mitis* ATCC 903 may be tentatively designated as α -glucosidases. The definitive classification of these enzymes and the characterization of both the β -fructofuranosidases and the α -glucosidases in *S. mitis* ATCC 903 will be made using purified enzymes. It seems probable that the three enzyme bands detected for each of the *S. mitis* glycosidases studied represent multiple molecular forms of the enzymes rather than being artefacts caused by the run conditions, in spite of the fact that such artefacts have been reported with the isoelectric focusing technique. Both the pH at the site of sample application and the formation of Ampholine-protein complexes have been reported to induce artefacts (Frater, 1970; Graesslin, Trautwein & Bettendorf, 1971; Lewin, 1970). However, in the present investigation the same number of enzyme bands was visualized in the gel irrespective of the position of sample application, and the relationships between the enzyme bands were highly reproducible.

Discordant results were obtained with the α -galactosidic sugar melibiose. No enzyme bands were visualized in the gel with melibiose although enzyme activity with this substrate was demonstrated in the extract. A possible explanation is that the enzyme(s) responsible for the hydrolysis of melibiose were inactivated in the gel, e.g. by unfavourable pH conditions at their isoelectric points.

The zymogram pattern of the commercial preparation of α -glucosidase revealed the presence of both a β -fructofuranosidase and at least three α -glucosidases. These α -glucosidases, in contrast to those of *S. mitis*, also had invertase activity as they hydrolysed sucrose.

Using the zymogram technique in combination with isoelectric focusing in a thin-layer gel, it may be possible to identify enzyme activities in various extracts. We conclude that the hydrolysis of sucrose in the presence of the *S. mitis* extract is solely catalysed by β -fructofuranosidases whereas hydrolysis of sucrose in the presence of the yeast extract is catalysed by two types of enzymes, a β -fructofuranosidase and α -glucosidases.

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