Biochem. J. (1971) **125**, 209–211
Printed in Great Britain

Biosynthesis of Levan and a New Method for the Assay of Levansucrase Activity

By M. CESKA
Department of Biochemistry, Pharmacia AB, Uppsala, Sweden

(*Received* 25 *June* 1971)

The polysaccharide levan was synthesized in a solidified agar medium containing sucrose as a source of fructose. The biosynthesis was achieved by the enzyme levansucrase (2,6-fructan–D-glucose 6-fructosyltransferase, EC 2.4.1.10), a small quantity of which was placed in circular wells cut in the agar gel. The enzyme slowly diffused through the agar–sucrose medium and the synthesis of levan was observed as circular white areas, the size of which was dependent on the time of incubation and the concentration of enzyme used.

It has been established previously by many authors that various hydrolytic enzymes are able to hydrolyse substrates incorporated into solidified agar media. Methods based on such diffusion phenomena are often called 'cup-plate' methods. The course of hydrolysis can be especially easily followed by the use of insoluble and coloured substrates (Ceska, 1971*a*,*b*). A visible gradient is readily observable between the circular area of hydrolysed substrate and the unhydrolysed substrate. The size of the area of hydrolysis in agar–substrate gel was found to be, under a given set of experimental conditions, proportional to the enzymic activity.

The present paper describes the use of the 'cup-plate' method to demonstrate and assay enzymic synthesis quantitatively. As a model system, the biosynthesis of levan was studied. The substrate sucrose and partially purified levansucrase from *Streptococcus mutans* were used (Carlsson, 1970). A uniform and solidified layer of agar–sucrose was prepared. Diffusion of the enzyme through the solidified medium resulted in the formation of levan, observable as a white circular area.

EXPERIMENTAL

Materials. Agar Noble was purchased from Difco Laboratories, Detroit, Mich., U.S.A. The partially purified enzyme, levansucrase, was a gift from Dr J. Carlsson, University of Umeå, Sweden, and was produced by strain JC2 of *Streptococcus mutans* (Carlsson, 1967). The enzyme was partially purified from the supernatant fluid of a glucose–broth culture by adsorption on hydroxyapatite at pH 6.0 and was eluted with 0.5M-potassium phosphate buffer (Carlsson, Newburn & Krasse, 1969; Carlsson, 1970). The levansucrase activity of this preparation was 20 units/ml. One unit of levansucrase is defined as the amount liberating 1 μmol of glucose/min 37°C at pH 6.0.

Sucrose (ribonuclease-free), lot no. TA-2076, was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. Plastic Petri dishes were purchased from Nune Co., Copenhagen, Denmark.

Methods. Sucrose, at various concentrations, was mixed with hot (about 85°C) agar solution (1% in 0.05M-sodium phosphate buffer, pH 6.0). Petri dishes (9cm diam.) were coated on a levelling table with measured portions of warm agar–sucrose mixture, giving a layer approx. 0.8mm thick. After solidification and cooling of the agar–sucrose, the plates were covered with lids to which wetted sponges were attached. Portions (5μl) of various dilutions of the levansucrase solution were placed in circular wells punched out of the solidified layer of agar–sucrose. The Petri dishes were then incubated in an air thermostat at 37°C for various times.

The contrast of the white circular areas that developed was improved by applying absolute ethanol to the top of the solidified layer. The diameter of each white circle was then taken as the mean of two measurements at right angles to each other with a caliper ruler, which measured the diameter with a precision of 0.005cm. Each experiment was made in duplicate.

The white circular areas (containing the synthesized levan) in solidified agar gel medium are shown in Fig. 1.

RESULTS

Effect of dilutions of enzyme. Levansucrase solution (20 units/ml) at three different dilutions (1:1, 1:4, 1:32) was transferred in 5μl portions to circular wells punched into the agar gel layer. The sucrose concentration was 0.05M and the incubation was at 37°C for various periods of time. The increase in size of the circular white areas with increasing time of incubation is seen in Fig. 2. Experiments performed at a lower as well as at a higher sucrose concentration gave similar results to those shown in Fig. 2. Hence the diameter of the white circle of levan is dependent on the time of incubation.

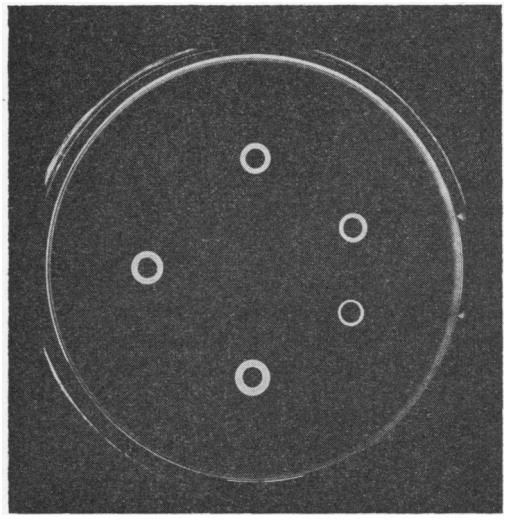

Fig. 1. Photograph of the experimental agar–substrate plate. The white circles are the areas containing synthesized levan. The size of the circles reflects the enzyme concentration used for the synthesis.

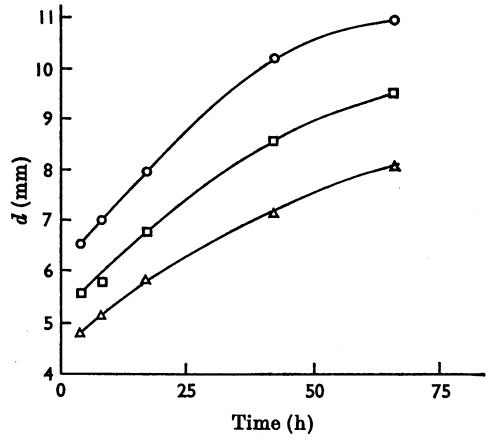

Fig. 2. Dependence of the diameter of the circular white areas on the time of incubation at 37°C. Levansucrase preparation (20 units/ml) was used at three dilutions: ○, 1:1; □, 1:4; △, 1:32.

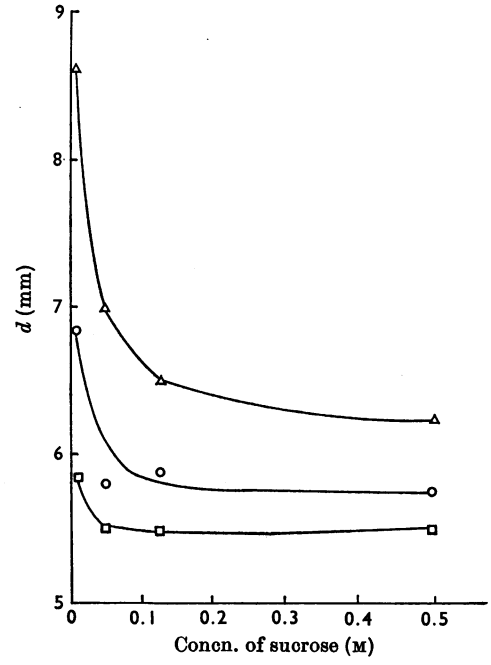

Fig. 3. Effect of various sucrose concentrations on the diameters of the circular white areas. The time of incubation at 37°C was 8h and levansucrase preparation (20 units/ml) at three dilutions was used: △, 1:1; ○, 1:4; □, 1:8.

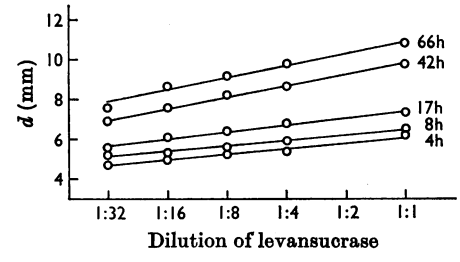

Fig. 4. Relationship of the size of the circular white areas and the serial dilution of levansucrase preparation (20 units/ml). The incubation was performed at 37°C for 4, 8, 17, 42 or 66h.

Effect of concentration of substrate. In this experimental system it was observed that the synthesis of levan was inversely proportional to the substrate concentration. The higher the sucrose concentration in agar gel, the smaller the size of the circular white area. This was tested at three different dilutions (1:1, 1:4, 1:8) of levansucrase (20 units/ml). This effect (Fig. 3) was shown to be dependent additionally on the enzyme activity. For any one sucrose concentration, under similar conditions, the size of the circular white area was found to increase with the increasing enzyme activity.

Fig. 4 shows the linear–logarithmic plot of the diameters of the white circles versus the serial dilution of levansucrase. The incubation was performed at 37°C for 4, 8, 17, 42 or 66h.

The increase in the size of the white circles as a function of enzyme concentration is shown at two substrate concentrations in Fig. 5. The higher the

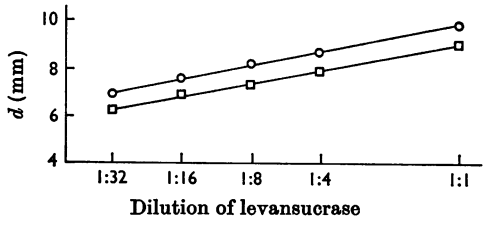

Fig. 5. Effect of serial dilution of levansucrase preparation (20 units/ml) on the diameter of the circular white areas with ○, 0.125 M-sucrose and □, 0.5 M-sucrose.

concentration of sucrose in agar gel medium, the lower the amount of levan obtained.

DISCUSSION

Levansucrase is an enzyme produced by many bacteria (Hestrin & Avigad, 1958). In some of them it is inducible and exocellular and in others it is constitutive and endocellular. The enzyme has been extensively studied in *Bacillus subtilis* and *Aerobacter levanicum* (Hestrin & Avigad, 1958; Ebert & Stricker, 1964) and its mechanism of action has been described (Ebert & Schenk, 1968). Levansucrase activity was also found in *Streptococcus mutans*, which forms colonies on the teeth (Carlsson, 1967). *Streptococcus mutans* has been shown to produce levan as well as dextran from sucrose (Wood & Critchley, 1966; Dahlquist, Krasse, Olsson & Gardell, 1967; Guggenheim & Schoeder, 1967; Gibbons, & Nygaard, 1968).

The strain (JC2) of *Streptococcus mutans* producing the enzyme used in the work described in this paper has been isolated from human dental plaque (Carlsson, 1967). The enzyme, which was partially purified, mediated the synthesis of a polymer that was eluted from a Sepharose 4B column in the void volume. Partial hydrolysis of this polymer by dilute hydrochloric acid yielded fructose (Carlsson, 1970).

Levansucrase activity may be determined in a number of ways, e.g. by measuring the release of glucose or other reducing sugars or by the formation of the polysaccharide. The present method, based on the last alternative, measures the amount of levan synthesized in an unique way. Sucrose is incorporated into agar gel and serves as the substrate (it is not necessary to initiate the synthesis by a primer). The levansucrase sample applied to the agar–sucrose medium diffuses through the medium and levan is synthesized.

The synthesized levan is seen as a turbid area, which can be visualized in the form of a solid white circle by applying ethanol to the top of the agar layer. It was observed that the size of the circular white area is dependent on the enzyme concentration. Higher levansucrase concentration yielded a larger area of levan synthesis. The size of the circular white area is inversely proportional to the concentration of substrate in the agar gel. This is probably due to the fact that the diffusion rate of the enzyme throughout the agar medium depends on the amount of the solid as well as soluble substances present in agar medium.

The specificity of the enzymic reaction towards the substrate was also studied. The following sugars when incorporated at various concentrations into solidified agar gel did not serve as a substrate for levansucrase: lactose, maltose, glucose, fructose. It is known that only molecules having a non-substituted fructosyl group linked to an aldosyl group by a C-1–C-1 bond, as in sucrose, may act as a donor. Sucrose and raffinose were the first known substrates for levansucrase.

The present method is very simple and rapid. The method is therefore very convenient for the assay of a large number of samples. Such a situation may, for example, be encountered during levansucrase purification. Another advantage of this method is that the dried agar–substrate layer can directly be stored as a record.

The separation of the solids from the enzyme samples is not necessary. Since the particles are stationary in the agar and are retained at the place of the sample application they therefore do not interfere with the assay. This fact also adds to the simplicity of the method as there is no need to centrifuge the enzyme-containing samples.

It is expected that this technique can also be applied to other biopolymers and that the activity of polymer-synthesizing enzymes, e.g. RNA- and DNA-polymerases of various kinds, may be measured by this very simple technique.

REFERENCES

Carlsson, J. (1967). *Odont. Revy*, **18**, 55.
Carlsson, J. (1970). *Caries Res.* **4**, 97.
Carlsson, J., Newburn, E. & Krasse, B. (1969). *Archs oral Biol.* **14**, 469.
Ceska, M. (1971*a*). *Clinica chim. Acta*, **33**, 135.
Ceska, M. (1971*b*). *Clinica chim. Acta*, **33**, 147.
Dahlquist, A., Krasse, B., Olsson, J. & Gardell, S. (1967). *Helv. odont. Acta*, **11**, 15.
Ebert, K. H. & Schenk, G. (1968). *Adv. Enzymol*, **30**, 179.
Ebert, K. H. & Stricker, H. (1964). *Z. Naturf.* **19b**, 23.
Gibbons, R. J. & Nygaard, M. (1968). *Archs oral Biol.* **13**, 1249.
Guggenheim, B. & Schoeder, H. E. (1967). *Helv. odont. Acta*, **11**, B1.
Hestrin, S. & Avigad, G. (1958). *Biochem. J.* **69**, 388.
Wood, J. M. & Critchley, P. (1966). *Archs oral Biol.* **11**, 1039.