A convenient zymogram stain for cellulases

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(Received 28 May 1981/Accepted 30 June 1981)

The release of dye from phosphoric acid-swollen cellulose azure by endocellulases and exocellulases is the basis of a technique for locating these enzymes on gels.

The enzymic hydrolysis of crystalline cellulose requires the co-operative action of endocellulase (endo-1,4- β -glucanase, EC 3.2.1.4), exocellulase (exo-cellobiohydrolase, EC 3.2.1.91) and cellobiase (β -D-glucosidase, EC 3.2.1.21). Investigation of the cellulases is complicated by the fact that each enzyme exists in a number of forms (see, e.g., the review by Gong & Tsao, 1979). Indeed, the lack of a convenient technique for locating and identifying the various forms has slowed progress considerably. Eriksson & Pettersson (1973, 1975) developed a technique whereby flat-bed isofocusing gels after completion of a run were sprayed with a viscous solution of carboxymethylcellulose. The reducing-sugar products were soaked into an overlaid paper and the spots corresponding to the location of the enzymes were detected with a reagent for reducing sugars. Unfortunately this technique locates only the endocellulases, since exocellulases are without effect on carboxymethylcellulose. Nummi *et al.* (1980) developed an immunoelectrophoretic technique whereby cellulolytic activity was located by incubating dried immunoplates in contact with plates containing amorphous cellulose in agarose. Cellulase activity was detected '... as clear zones in the turbid gels ...'. The drawback in this technique is that antisera have to be prepared and the incubation period for producing clear zones is 24 h. Other workers have separated cellulases by polyacrylamide-disc-gel electrophoresis, then slicing the unstained gels eluting the enzyme and assaying the eluate for activity (e.g. Umezurike, 1979; Tong *et al.*, 1980; A. McHale & M. P. Coughlan, unpublished work). Although this method may have to be used for providing definitive confirmation of enzyme identity, it is laborious and unsuited to routine investigation. The method described in the present paper is suitable for the detection of cellulases on slab or disc gels and is more convenient than existing techniques.

Materials and methods

The thermophilic fungus *Talaromyces emersonii* CBS 814.70 was obtained from the Centraal Bureau voor Schimmelcultures, Baarn, The Netherlands. The organism was grown on a medium containing

corn-steep liquor, NH_4NO_3 and cellulose as described by Folan & Coughlan (1981). The culture filtrate was obtained at 48–50 h, at which time β -glucosidase activity is very low, and the 20–80%-satd.-(NH_4) $_2\text{SO}_4$ fraction was collected and freeze-dried (McHale & Coughlan, 1980). The freeze-dried material was dissolved in running buffer and portions were subjected to polyacrylamide-disc-gel electrophoresis in 5% (w/v) gels with the buffer system described by Reisfeld *et al.* (1962). The cathode was at the top and the anode at the bottom. Gels were pre-run for 2 h at 5 mA/tube. The sample was then applied in 10% (w/v) sucrose in the electrode buffer. Bromocresol Purple (0.4% w/v) was used as marker dye. Gels were stained for protein with 0.1% (w/v) Coomassie Blue in 50% (w/v) trichloroacetic acid, and for glycoprotein with the periodic acid/Schiff reagent described by Zacharius *et al.* (1969).

The location of endocellulase and exocellulase activities was determined as follows. Completed disc gels were placed in tubes and surrounded by 0.2 M-sodium acetate buffer, pH 5.0, containing 2% (w/v) agar (Special Agar-Noble; Difco Laboratories, Detroit, MI, U.S.A.) and 5% (w/v) phosphoric acid-swollen cellulose azure (see below). The agar was then solidified by cooling and, when solidified, the mixtures were incubated at 45°C for 1 h. During incubation, dye released from the cellulose azure containing agar overlay diffuses into the disc gel at the locations corresponding to the enzymes (i.e. the endo- and exo-cellulases) that effect its release. The purple bands corresponding to these enzymes were readily observed by taking the disc gels from the surrounding agar, rinsing with water to remove excess of agar and placing in tubes containing water. However, for photographic or for scanning purposes it is better to place the gels in 0.05 M-NaOH so that the bands are coloured blue. Gels stained for protein, for glycoprotein and for activity as above were, for convenience, all scanned at 540 nm by using a Pye-Unicam SP. 800 spectrophotometer especially adapted for the purpose. The cellulose azure (C 8647) used in the present studies was kindly provided by Mr. Ken Humphries [Head, Enzyme Production, Sigma (London)

Fig. 1. Densitometric tracing of culture filtrate of *T. emersonii* after polyacrylamide-gel electrophoresis (a) protein stain; (b) glycoprotein stain; (c) activity stain. Abbreviations used: Endo, endocellulase; Exo, exocellulase.

Chemical Co., Poole, Dorset, U.K.]. It was made by a method based on that of Fernley (1963). Swelling in phosphoric acid was performed as described previously (McHale & Coughlan, 1980).

Results and discussion

Talaromyces emersonii when grown on the medium described in the Materials and methods section produces an extracellular cellulolytic enzyme system (Folan & Coughlan, 1981). In addition to β -glucosidases this system is comprised of at least two endocellulases and at least four exocellulases (A. McHale & M. P. Coughlan, 1980; unpublished work). The bands corresponding to these activities are stained for protein in Fig. 1(a). Fig. 1(b) shows that each of these enzymes is a glycoprotein. The lack of proportionality between the intensity of staining of a band for protein (Fig. 1a) and the corresponding band for glycoprotein (Fig. 1b) is due no doubt to the fact that each protein does not contain the same amount of carbohydrate. Indeed differences in carbohydrate content rather than in primary structure may well explain the multiplicity of forms of each enzyme (see e.g. Gong & Tsao, 1979). Fig. 1(c) shows that each of the bands staining for protein also stains for activity. Once again the lack of proportionality in the intensity of staining of the corresponding bands may readily be explained. On the one hand, one would expect to find variations in substrate specificity and in catalytic-centre-activity values for each enzyme. Secondly, the mode of binding of the dye to the cellulose may be such as to exclude linearity between bonds cleaved and dye released. The zymogram staining technique described here is more convenient than those already published. It should be mentioned, however, that this method does not distinguish between endocellulase and exocellulase activities. For much work this will not be a drawback, as the enzymes in question would have been separated in earlier stages of

purification by techniques such as ion-exchange chromatography. In any event, combination of the technique described here and that of Eriksson & Pettersson (1973) would provide a method for distinguishing between activities. A number of other staining techniques that might be of use in identifying cellulases come to mind. For example, cello-tetraose, which is acted on by both endo- and exo-cellulases (Mandels *et al.*, 1976), could be used as substrate and activity located by staining with a solution containing cellobiase, glucose oxidase, peroxidase and a suitable dye.

We are grateful to Professor P. F. Fottrell for helpful discussion and to Mr. T. J. Cussen who converted the spectrophotometer to a gel scanner. A. McH. acknowledges receipt of a maintenance grant from the Department of Education, Ireland.

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