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Detection of β-Glucosidase Activity in Polyacrylamide Gels with Esculin as Substrate

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β-Glucosidase can be located after nondenaturing polyacrylamide gel electrophoresis by incubating the gel with 0.1% esculin and 0.03% ferric chloride. The esculetin released from esculin by β-glucosidase action reacts with ferric ion to produce a black band, corresponding to the β-glucosidase, against the transparent background.

The ability to identify the position of an enzyme after polyacrylamide gel electrophoresis greatly facilitates the purification and characterization of the enzyme. Several zymogram techniques have been developed for the detection of β-glucosidase (EC 3.2.1.21) in polyacrylamide gels. However, they have some drawbacks in application (2, 3). For example, the position of β-glucosidase could be located as a yellow band of *p*-nitrophenol by incubating gels in *p*-nitrophenyl-β-D-glucoside solution (2). The contrast of the band, however, is not good enough to locate the exact position of the enzyme, and the sharpness of the band gradually decreases because of the highly diffusible nature of *p*-nitrophenol. Rissler (10) has detected β-glucosidase by incubating gels with 6-bromo-2-naphthyl-β-D-glucoside as a substrate and diazonium salt as a coupling agent. The bromonaphthol, a product of the action of β-glucosidase, forms a red precipitate with diazonium salt. Unfortunately, this technique requires fastidious handling procedures because of the photosensitivity of diazonium salt. Another method, using 4-methylumbelliferyl-β-D-glucoside as a fluorogenic substrate, needs UV light for observation (5).

In this report, we present a method in which esculin (6, 7-dihydroxycoumarin 6-glucoside) can be used as a substrate for a convenient zymogram technique to locate β-glucosidase in polyacrylamide gels. The rationale behind this technique is that a natural β-glucoside, esculin, is split into esculetin (6, 7-dihydroxycoumarin) and glucose by the action of β-glucosidase, and then the esculetin reacts with ferric ion to form a black precipitate (9).

All chemicals used in this study were reagent grade. Crude β-glucosidase solution was prepared from the culture filtrate of *Aspergillus nidulans* FGSC 168 (Fungal Genetic Stock Center, Arcata, Calif.) by ultrafiltration (PM10; Amicon), and used for the evaluation of the activity staining method. Details of β-glucosidase production in *A. nidulans* were described previously (7). Nondenaturing polyacrylamide gel electrophoresis in an 8.5% (wt/vol) polyacrylamide gel was carried out at pH 8.3 as described by Hames (4). Upon completion of electrophoresis, the gel was cut into three strips longitudinally. One strip was used for activity staining (Fig. 1c), and another strip was used for protein staining with Coomassie blue R-250 (Fig. 1d). The other strip was sliced into 2-mm-thick pieces, from which proteins were eluted by diffusion after homogenization in 50 mM sodium acetate buffer (pH 5.0). The eluate from each gel piece was assayed spectrophotometrically for β-glucosidase activity with *p*-nitrophenyl-β-D-glucoside (2 mM) as substrate (Fig. 1a). One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of *p*-nitrophenol or glucose per min. Activity staining of β-glucosidase was performed as follows. After electrophoresis, the gel was soaked in 0.2 M sodium acetate buffer (pH 5.0) for 10 min at room temperature in order to exchange the buffer system. The gel was then incubated in 0.2 M sodium acetate buffer containing 0.1% (wt/vol) esculin (Sigma) and 0.03% (wt/vol) ferric chloride for 5 min at 50°C. During incubation, the black bands corresponding to the β-glucosidase appeared against a transparent background. The reaction was stopped by immersing the gel in a 10% (wt/vol) aqueous solution of glucose, an inhibitor of β-glucosidase. The intensity of the bands was quantified by using an Ultrascan laser densitometer (LKB) (Fig. 1b). The two peaks in Fig. 1b from the densitometric scanning of the activity-stained gel are consistent with the peaks identified by conventional activity assay after elution of proteins from the gel (Fig. 1a). The two active bands correspond to the 125- and 50-kDa species of two β-glucosidase components of *A. nidulans* (7). This result indicates that our method is suitable for the localization of β-glucosidase activity in nondenaturing polyacrylamide gels.

The sensitivity of this technique was determined by using the purified β-glucosidase. Purification of β-glucosidase from *A. nidulans* was described previously (7). As little as 0.004 U of activity, which corresponds to only 10 ng of protein, can be readily detected with 15 min of incubation at 50°C in the reaction buffer described above (data not shown). Such a level of sensitivity allows the detection of enzyme activity from a culture filtrate without concentration.

This technique can be applicable to the detection of β-glucosidases from other organisms—if required, with minor modification of the reaction conditions (e.g., buffer pH and reaction temperature) for optimal enzyme activity. Crude and purified β-glucosidase preparations from well-known sources of fungi, bacteria, and yeast were used in order to evaluate the technique further. Samples containing purified β-glucosidases from *Trichoderma koningii* ATCC 26113 (6) and *Trimorphomyces papilionaceus* (100- and 56-kDa species, respectively [unpublished data]) and culture filtrates of *Saccharomyces cerevisiae* ATCC 26787 (11), *Trichoderma reesei* ATCC 26921 (11), and *Bacillus circulans* ATCC 21783 (8) were subjected to the zymogram technique described above (Fig. 2). Each β-glucosidase preparation showed a unique active band, while the

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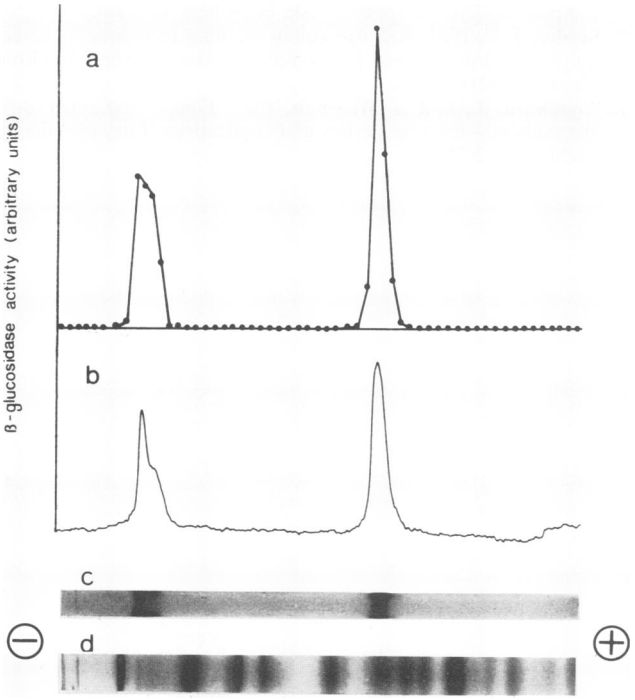

FIG. 1. Activity staining of β-glucosidase after nondenaturing polyacrylamide gel electrophoresis on an 8.5% polyacrylamide gel. The same amounts of crude β-glucosidase preparations of *A. nidulans* were applied to three wells of a gel. After electrophoresis, the gel was cut longitudinally into three strips. One strip was sliced into 2-mm-thick pieces, from which proteins were eluted and assayed for β-glucosidase activity with *p*-nitrophenyl-β-D-glucoside as substrate (a). Another strip was used for activity staining (c), and densitometric scanning of the activity-stained gel was performed (b). The other strip was used for protein staining with Coomassie blue (d). For activity staining, the gel strip in panel c was washed in 0.2 M sodium acetate buffer (pH 5.0) and incubated in the same buffer containing 0.1% (wt/vol) esculin and 0.03% (wt/vol) ferric chloride for 5 min at 50°C.

culture filtrate of *S. cerevisiae* showed broad bands. The broad bands might be attributed to the multiplicity of β-glucosidase components. Overall, our technique successfully detected different β-glucosidases from various sources.

The present technique facilitates the purification of β-glucosidase from the culture filtrate or cell lysate. Previously, we have applied the technique to the purification process of β-glucosidase from *A. nidulans* (7). After preparative electrophoresis of the crude β-glucosidase preparations with a large-dimension nondenaturing polyacrylamide gel, the enzyme was eluted from the activity-stained gel. The staining procedure was the same as that described above except for the incubation temperature and time. A temperature lower than the optimal reaction temperature of the β-glucosidase was introduced in order to minimize heat deactivation of the enzyme. The reaction was stopped just after the color began to appear, and then the active band was sliced. The β-glucosidase contained in the band could be eluted easily by conventional methods such as diffusion and electrophoretic elution (4). In our case, the sliced active band was macerated with a homogenizer in buffer and incubated overnight at 4°C with mild shaking. After removal of the polyacrylamide gel particle by centrifugation, the supernatant obtained contained β-glucosidase with considerable purity. Traces of low-molecular-weight contaminants (e.g., ferric-esculetin complex, esculin, glucose) in the eluent

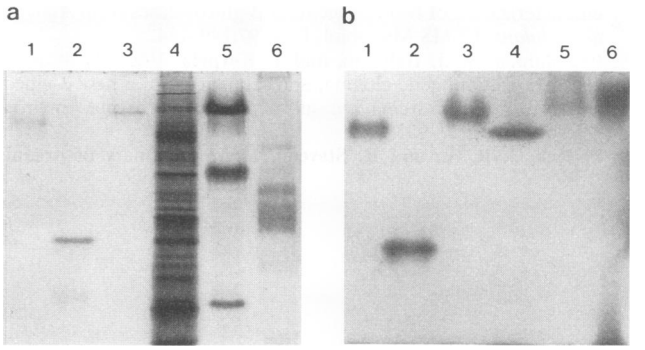

FIG. 2. Application of activity staining to detect β-glucosidases from various organisms. Purified β-glucosidases from *T. papilionaceus* (100-kDa species, lane 1; 56-kDa species, lane 2) and *T. koningii* (lane 3) and culture filtrates of *B. circulans* (lane 4), *T. reesei* (lane 5), and *S. cerevisiae* (lane 6) were subjected to nondenaturing polyacrylamide gel electrophoresis. Coomassie blue staining (a) and activity staining (b) were performed with duplicated gels. The conditions for activity staining were the same as those in Fig. 1.

were eliminated from the enzyme by gel filtration on a Sephadex G-25 desalting column. Subsequent ion-exchange chromatography enabled us to purify the enzyme to homogeneity (7).

With sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the present method also worked successfully (data not shown) after removal of sodium dodecyl sulfate from the gel by treatment with 25% aqueous isopropanol according to the method described by Blank et al. (1).

The detection of enzymatically produced esculetin with ferric ion can be adapted for the detection of some other hydrolases in polyacrylamide gels. If there are other coumarin derivatives that are substrates for some hydrolases, the enzymatically produced esculetin may be used for their activity staining as described above.

In conclusion, the use of esculin with ferric chloride provides a zymogram technique for locating β-glucosidase with high resolution and sensitivity that can be performed easily, rapidly, and at a minimal cost.

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