Novel Method To Detect β-Galactosidase by a Dot-Blotting Assay on Nitrocellulose Membrane Using 6-Bromo-2-naphthyl β-D-Galactopyranoside as Substrate

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A novel method to detect β-galactosidase by dot-blotting samples on nitrocellulose membrane and staining the membrane with azo dye coupling technique using 6-bromo-2-naphthyl β-D-galactopyranoside as substrate is presented. The enzymatic reaction to split the substrate is pH-dependent, and the sensitivity of this method could be increased by more than 10-fold with an additional treatment of 0.01 M sodium carbonate after the coupling of diazo-blue B with 6-bromo-2-naphthyl released by β-galactosidase. As low as 0.0015 unit of β-galactosidase could be detected within 10 min. This improved β-galactosidase staining method may be used for a native or IEF gel to show the β-galactosidase active band and may also detect other hydrolases using substrates for the azo dye coupling technique.

Keywords: *β-Galactosidase; dot blotting; chromogenic*

INTRODUCTION

Peanut peroxidases are heme and calcium proteins and glycoproteins. Plant peroxidases play important roles in the synthesis and degradation of phytohormones that modulate cell differentiation and growth (Sanchez-Bravo et al., 1989; Kende, 1989; Biggs and Fry, 1987). We reported (Wan and van Huystee, 1993a) that the amino acid sequence of the peptide chain of cationic peanut peroxidase (C.PRX) corresponds to the base sequence of cDNA clone prxPNC1 (Buffard et al., 1990). Of its five potential N-glycosylation sites, only three are used (Wan and van Huystee, 1993a). A study on the structure of glycans of C.PRX revealed the occurrence of heterogeneous glycosylation of C.PRX at specific glycosylation sites (Wan and van Huystee, 1993a). Further investigation indicated that the heterogeneity of glycans of C.PRX is caused by a cosecreted β-galactosidase (Wan and van Huystee, 1994). When we purified β-galactosidase from the cell culture medium, a commercial colorimetric substrate, 6-bromo-2-naphthyl β-D-galactopyranoside (BNG), was used to detect active β-galactosidase. It was found that the enzymatic reaction is pH-dependent and the sensitivity of this method could be increased more than 10-fold with an additional treatment of 0.01 M sodium carbonate after the coupling of diazo-blue B with 6-bromo-2-naphthyl released by β-galactosidase. A combination of the dot-blotting assay with the improved enzyme staining contributes a sensitive, simple, and rapid method to detect β-galactosidase. As little as 0.0015 U of β-galactosidase could be detected within 10 min.

MATERIALS AND METHODS

Reagents. NC membranes (BA 85, 0.45 μm; PH79, 0.1 μm) were purchased from Schleicher and Schuell (Keene, NH); BNG, diazo-blue B, *p*-nitrophenyl β-D-galactopyranoside, *p*-nitrophenol, and Pharmalyte (pH 3–10) were from Sigma Chemical Co. *Escherichia coli* β-galactosidase was from Boehringer Mannheim.

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Peanut Cell Culture and β-Galactosidase Purification. Peanut (*Arachis hypogaea* L. var. Virginia 56R) cells were routinely cultured in a modified Linsmaier and Skoog medium for 14 days (Hu et al., 1987). The first steps in the procedure for the purification of β-galactosidase are the same as those for peroxidases (Sesto and van Huystee, 1989). Briefly, the 14 day culture was filtered through Whatman No. 1 filter paper, and the spent medium was brought sequentially to 70% acetone and to 80% ammonium sulfate with intermittant solubilizations of the pellet in 0.02 M sodium acetate, pH 5. After dialysis against the same buffer, the crude medium proteins were applied to a cationic exchange column, CM Sephadex 50, and fractionated into three peroxidase fractions with a gradient of sodium acetate buffer, pH 5.0. The anionic peroxidase (A.PRX) does not bind. The weakly bound fraction is C.PRX, and the tightly bound fraction, designated CPz, contains also the β-galactosidase activity (Wan and van Huystee, 1994). The pooled CPz was precipitated with 70% acetone, redissolved in 0.5 mL of 7% 2-propanol, and applied to a Sephadex G75 column (2.5 × 80 cm, Pharmacia). The flow rate was 0.3 mL/min, and the eluent was monitored at both 280 and 405 nm. Fractions of 4.5 mL were collected, and the β-galactosidase activity of each fraction was analyzed using a dot-blotting assay. The pooled β-galactosidase activity-containing fractions were lyophilized and subjected to preparative isoelectrofocusing.

Preparative Isoelectrofocusing. Preparative isoelectrofocusing was performed using a Rotofor cell (Bio-Rad). The β-galactosidase preparation from the G75 column was dissolved in 50 mL of Milli-Q water containing 20% glycerol and 1.5% Pharmalyte, pH 3–10, and loaded into the focusing chamber. The focusing was run at a constant power, 12 W, for 5 h at 4 °C. The contents of the focusing chamber were collected into 20 fractions, and each fraction was analyzed for pH, β-galactosidase activity, and absorbances at 280 nm.

Dot-Blotting Assay of β-Galactosidase Using BNG as Substrate. The NC membrane was wetted with distilled water. The excess water was removed with a filter paper. Five microliters of sample solution was dot-blotted on the membrane. After air-drying, the membrane was stained to show β-galactosidase activity using an improved staining method (Erickson and Steers, 1970). The membrane was incubated in 0.025% BNG in 10% methanol in a buffer (McIlvaine buffer pH 3.5, for culture medium β-galactosidase; 0.1 M sodium phosphate buffer, pH 8.0, for commercial *E. coli* β-galactosidase) containing 0.01 M NaCl and 0.01 M $MgCl_2$ for 5 min at room temperature, followed by 2 min in diazo-blue B (1 mg/mL double-distilled water). To enhance the development of

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the purple spots, the membrane was immersed additionally in 0.01 M sodium carbonate for 30 s. The reaction was terminated in 7.5% acetic acid.

β-Galactosidase Assay Using *p*-Nitrophenyl β-D-Galactopyranoside as Substrate. A modified method (Montreuil et al., 1986) was employed to measure β-galactosidase activity. Ten microliters of each fraction from preparative IEF was incubated with 200 μL of McIlvaine buffer, pH 3.5, and 200 μL of 10 mM aqueous *p*-nitrophenyl β-D-galactopyranoside solution at 37 °C for 30 min. Then, 1.25 mL of 1 M sodium carbonate aqueous solution was added to the incubant. The absorbance at 400 nm was measured on a Shimadzu UV-160 spectrophotometer.

One unit (U) of β-galactosidase is empirically defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol from *p*-nitrophenyl β-D-galactopyranoside in 30 min at 37 °C and pH 3.5 (for medium β-galactosidase) or pH 8.0 (for commercial *E. coli* β-galactosidase). A range of *p*-nitrophenol concentration was used to calculate the amount of β-galactosidase.

RESULTS AND DISCUSSION

The spent peanut cell culture medium has proved to be an enriched source of peroxidases. The cells secrete around 30 peptides into the medium, among which 2 were identified as peroxidase isozymes, A.PRX and C.PRX (Hu et al., 1990). A.PRX and C.PRX have yields of 5 mg of C.PRX and 0.5 mg of A.PRX per liter of spent medium (O'Donnell and van Huystee, 1992). There are two forms of C.PRX in the medium, namely CP− and CP+, based on their Con A binding property (O'Donnell et al., 1992). CP− and CP+ have the same peptide chain and the same number of *N*-glycans but differ in the peripheral structure at the glycans (Wan and van Huystee, 1993a, 1994). This heterogeneity of glycans is most likely caused by a cosecreted β-galactosidase (Wan and van Huystee, 1994). During the purification of the medium β-galactosidase, a novel method to screen β-galactosidase using BNG as substrate was established.

In an attempt to develop a histochemical method to detect glucuronidase, Seligman synthesized 6-bromo-2-naphthyl β-D-glucopyranosidase (Seligman et al., 1954). It was expected that the naphthol would remain at the site of enzymatic hydrolysis during a long period of incubation and that these sites would be detected by coupling with diazo-blue B to form blue azo dye (Seligman et al., 1954). As the purple color of the azo dye is intense and the color lasts for long time, this azo dye coupling technique was also used to detect other hydrolases, such as sulfatase, glucosidase, aminopeptidase, and galactosidase (Yarborough et al., 1967; Erickson and Steers, 1970), and the substrates are commercially available.

The amount of β-galactosidase is empirically calculated by the ability to release *p*-nitrophenyl from β-D-galactopyranoside, and *p*-nitrophenol was used as standard in the range 20−120 nmol. The crude medium contained 0.3 U/mL. The hydrolysis of BNG by β-galactosidase is pH-dependent, and the pH optimum for the culture medium β-galactosidase is around 3.5 extending to 5.5. An additional treatment with 0.01 M sodium carbonate enhances the development of the purple spots and increases the sensitivity of this staining method more than 10-fold (Figure 1). At least 0.0015 U of either medium or *E. coli* β-galactosidase could be detected. A wide range of 0.001−0.1 M sodium carbonate improves the sensitivity of the staining. Directly dissolving diazo-blue B in 0.01 M sodium carbonate causes a brown precipitant and does not help the color development. Probably a basic environment after coupling favors the color development of the coupled product.

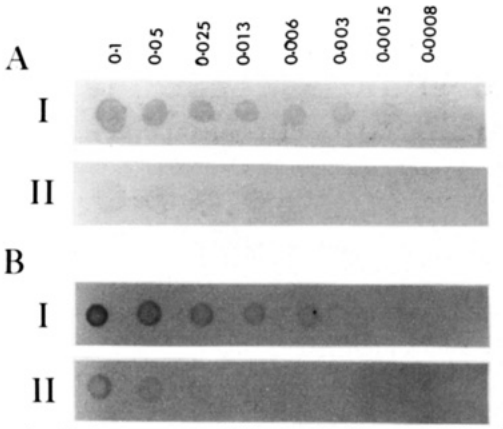

Figure 1. Enhancement of β-galactosidase staining by the treatment of 0.01 M sodium carbonate. Five microliters of aqueous solution containing different amounts of either medium or commercial *E. coli* β-galactosidase was dot-blotted on NC membrane (0.45 μm). The initial amount of both β-galactosidases was 0.1 U and was diluted as 2-fold series. The membrane was incubated in 0.025% BNG in 10% methanol in buffer (McIlvaine buffer, pH 3.5, for medium β-galactosidase; 0.1 M sodium phosphate buffer, pH 8.0, for commercial *E. coli* β-galactosidase) containing 0.01 M NaCl and 0.01 M $MgCl_2$ for 5 min, followed by 2 min in diazo-blue B (1 mg/mL double-distilled water) and with or without an additional treatment of 0.01 M sodium carbonate for 30 s. The reaction was terminated in 7.5% acetic acid. (A) Medium β-galactosidase; (B) commercial β-galactosidase; (I) with an additional treatment of 0.01 M sodium carbonate; (II) without the treatment of 0.01 M sodium carbonate.

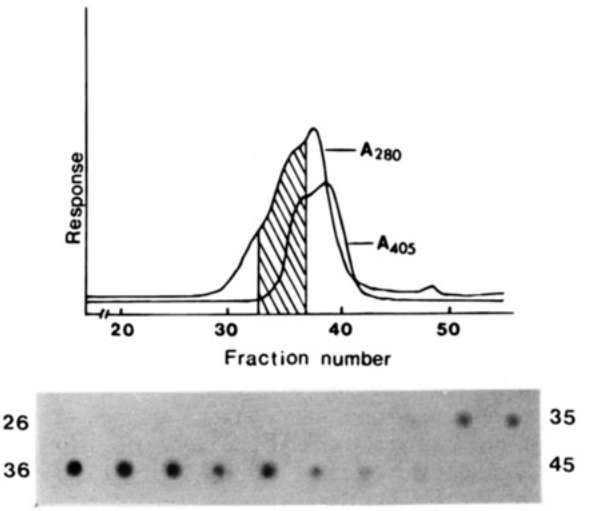

Figure 2. Preliminary purification of β-galactosidase on Sephadex G75 column. β-Galactosidase activity containing fractions (CPz) from a CM column were precipitated with 70% acetone, redissolved in 0.5 mL of 7% 2-propanol, and applied to a G75 column (2.5 × 80 cm). (A, top) Elution profile. The flow rate is 0.3 mL/min, and the eluent was monitored at both 280 and 405 nm. Fractions of 4.5 mL were collected. (B, bottom) Dot-blotting assay of β-galactosidase. Five microliters of each fraction (fractions 26−45) was dot-blotted on NC membrane for β-galactosidase staining. Fractions 33−36 showed purple dots and fractions 37−43, brown dots on membrane. The β-galactosidase-containing fractions are cross-hatched on the elution profile.

Figure 2 shows the screening of β-galactosidase-containing fractions from the G75 column using this improved staining technique. Absorbance at 405 nm is for the heme Soret band. Fractions 33−36 showed purple dots and fractions 37−43, brown dots on mem-

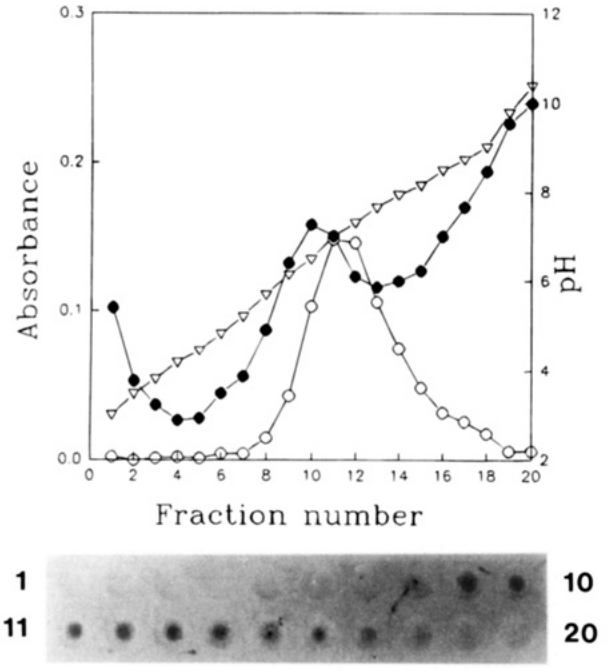

Figure 3. Preparative isoelectrofocusing of Sephadex G75 purified β-galactosidases. (A, top) 10 μL of each fraction was used to measure β-galactosidase activity using *p*-nitrophenyl β-D-galactopyranoside as substrate (A_{400}, ○; A_{280}, ●; and pH, ▽). (B, bottom) 5 μL of each fraction was dot-blotted on NC membrane and stained with β-galactosidase activity using BNG as substrate.

brane due to the occurrence of heme protein impurity. Figure 3 shows the application of dot-blotting assay of β-galactosidase after preparative IEF. Fractions 9–17 present purple dots on NC membrane to indicate the occurrence of β-galactosidase. As various buffer reagents do not prevent the binding of proteins onto NC membrane (Wan and van Huystee, 1993b), this dot-blotting assay supplies a sensitive, simple, and rapid method for the screening of β-galactosidase-containing fractions after chromatographies. Also, the improved β-galactosidase staining method could be used for native gel or IEF gel to show the β-galactosidase active band (Wan and van Huystee, 1994). It is possible to extend the above improved β-galactosidase staining method to detect other hydrolases using substrates for azo dye coupling technique.

ABBREVIATIONS USED

NC, nitrocellulose; C.PRX, cationic peanut peroxidase; A.PRX, anionic peanut peroxidase; BNG, 6-bromo-2-naphthyl β-D-galactopyranoside; CM, carboxymethyl; CPz, strongly bound fractions on CM column; Con A, concanavalin A; IEF, isoelectrofocusing; U, unit.

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