Journal of Chromatography, 110 (1975) 174–177

CHROM. 8273

Note

A rapid and sensitive detection of proteolytic enzymes after electrophoresis

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(Received January 15th, 1975)

N-Substituted amino acid nitroanilides are widely used substrates for the quantitative and qualitative determination of proteolytic enzymes[1,2]. Recently, Gertler *et al.*[3] have published a qualitative method for the detection of trypsin and chymotrypsin after cellulose acetate electrophoresis using benzoyl-DL-arginine- and acetyl-L-tyrosine-*p*-nitroanilide, respectively, as substrate. However, no staining of the strips could be achieved and therefore no quantitative determination of the enzymes could be made. In our experiments on insect proteases, usually only obtainable in milligram amounts, we found that the sensitivity of this method can be further enhanced, the results better documented, and a quantitative determination of the enzymes made, if the nitroaniline produced during the enzymatic reaction is diazotized and then coupled with naphthylethylenediamine, as described by Bratton and Marshall[4].

This method is applicable not only for cellulose acetate electrophoresis but also for acrylamide and agarose gels normally used in disc and immunoelectrophoretic experiments.

METHODS

Cellulose acetate electrophoresis was performed on Macherey and Nagel (Düren, G.F.R.) membranes (25.5 × 145 mm) using 24 m*M* Veronal–HCl buffer (pH 8.6). The proteins were separated at 200 V for 30 min. Protein was stained with Coomassie Brilliant Blue. For clearing and scanning, the strips were treated as described by Perl and Voggel.[5] Disc electrophoresis was carried out in 40 × 1 mm capillaries at pH 4.3. The method of Reisfeld *et al.*[6] was slightly modified in so far as the separation gel contained 1% Triton X-100 and the stacking gel 0.5%, and the ammonium peroxydisulfate concentration was reduced to 90 mg/100 ml water. The gels were kept in a moist chamber for 12 h before use. For electrophoresis, 1 μl of the protein solution and 0.2 μl of methylene blue (0.2%) were applied to the gel, and the separation was carried out with a current of 0.1 mA/gel for 1 h. Scanning of the gels was performed with a Gilford spectrometer at 578 nm.

Substrate-staining solution

In all the experiments, the substrates (benzoyl-L-arginine- and acetyl-L-tyro-

sine-*p*-nitroanilide (E. Merck, Darmstadt, G.F.R.) were used in a concentration of 5 m*M* (final concentration) dissolved in 0.2 *N* Tris–HCl buffer (pH 8.4) containing 5% (v/v) dimethylformamide. Before use, a solution of $NaNO_2$ and N-α-naphthylethylenediamine (Serva, Heidelberg, G.F.R.) was added to the substrate solution to give final concentrations of 0.1% (w/v) and 0.5% (w/v), respectively.

RESULTS AND DISCUSSION

Disc electrophoresis gels were incubated directly in the substrate-staining solution at 35° for 10–30 min. After incubation the gels were washed with water for 1 min to remove the excess of substrate and then treated with an aqueous solution of 12.5% trichloroacetic acid (TCA) (w/v) for 30 sec, during which time a purple colour became visible. (A longer treatment with TCA causes precipitation of the substrate in the gel, and no quantitative determination is then possible.) The substrate which precipitated on the surface of the gel was washed out with 0.2 *N* Tris–HCl buffer (pH 8.4) until the gel became transparent again. Typical staining patterns of bovine chymotrypsin, trypsin (Worthington), hornet chymotrypsin, and honey-bee trypsin are shown in Fig. 1. Under our conditions with a 30-min incubation time at 35°, as little as 20 ng of trypsin were detectable after staining with benzoyl-L-arginine-*p*-nitroanilide; however, the best results were achieved by applying 60–80 ng trypsin/gel. Honey-bee trypsin, which has a higher specific activity than bovine trypsin, can be determined in a concentration of 6 ng/gel. Using acetyl-L-tyrosine-*p*-nitroanilide as substrate it was possible to stain 120 ng of bovine chymotrypsin and 30 ng of hornet chymotryp-

Fig. 1. Demonstration of proteolytic activities. Electrophoresis at pH 4.3, 20% gels, incubation at 35° for 30 min. From left to right: 426 and 213 ng bovine chymotrypsin; 184 and 94 ng hornet chymotrypsin (acetyl-L-tyrosine-*p*-nitroanilide used as substrate); 178 and 92 ng bovine trypsin; 38 ng and 76 ng honey-bee trypsin (benzoyl-L-arginine-*p*-nitroanilide used as substrate).

sin. For a comparison of the sensitivity of this method with that formerly used in these laboratories, *i.e.* staining proteolytic enzymes with glutaryl-L-phenylalanine-*β*-naphthylamide[7], two series of tests were performed. In the first experiment glutaryl-L-phenylalanine-*p*-nitroanilide was used as substrate, and in the second the naphthylamide substrate (50 m*M* in 0.2 *N* Tris–HCl buffer (pH 8.4)) was used. After 30 min of incubation in the nitroanilide substrate, 0.5 *μ*g of bovine chymotrypsin was detectable, whereas 2.6 *μ*g was the minimum detectable amount of the enzyme after a 2-h incubation in the naphthylamide substrate.

The quantitative determination of bovine chymotrypsin activity in electrophoresis gels is shown in Fig. 2. The enzyme was subjected to electrophoresis and scanned at 578 nm after staining with acetyl-L-tyrosine-*p*-nitroanilide. The linear dependence of the integrated peak area on the incubation time and the quantity of the enzyme applied indicates that only very small amounts of the diazotized nitroaniline were washed out during the washing processes.

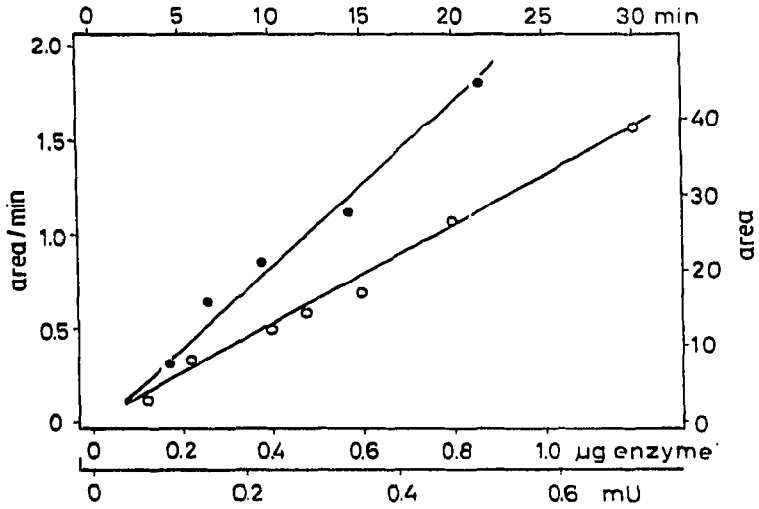

Fig. 2. Determination of the bovine chymotrypsin activity using acetyl-L-tyrosine-*p*-nitroanilide as substrate after electrophoresis and incubation at 35°. ○, Time dependent activity, 0.63 *μ*g chymotrypsin; ●, activity dependent on enzyme concentration, 30 min incubation.

This method can also be performed on a macroscale, but then, because of the acidity of the gel buffer, it is necessary to neutralize the gels before incubation to prevent precipitation of the substrate. Slight diffusion of the bands during this procedure is inevitable. As staining the cellulose acetate strips by incubating them in the substrate-staining solution was unsuccessful, the substrate-staining solution was dissolved in 0.1% agarose and then poured out as a thin layer onto a microscopic slide. After electrophoresis the strips were laid on the agarose layer and incubated at 35° for 10 min. The cellulose acetate membranes were then dipped into 12.5% TCA until the yellow nitroaniline had changed to a bright purple colour. Excess of substrate and acid were removed by washing with water. Fig. 3 shows an electrophoretic pattern of 14 *μ*g of porcine pancreatic crude extract (autolyzed) stained with Coomassie Brilliant

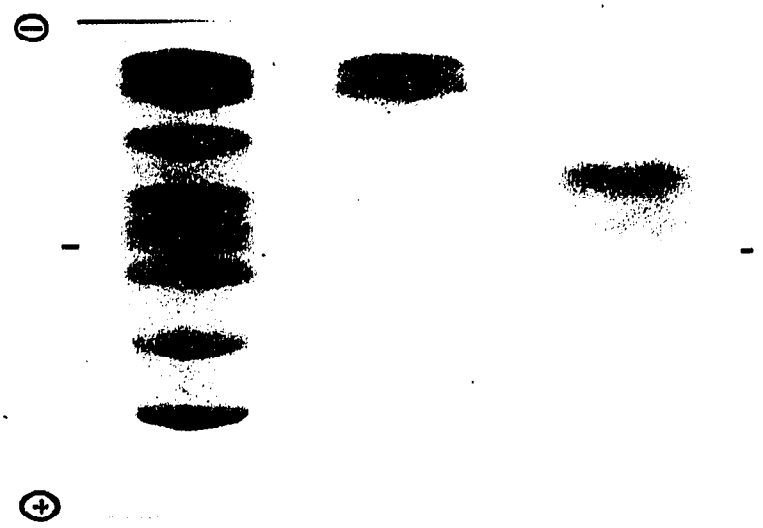

Fig. 3. Cellulose acetate electrophoresis of a crude porcine pancreas extract at pH 8.6. From left to right: protein staining; staining for trypsin activity with benzoyl-L-arginine-*p*-nitroanilide; staining for chymotrypsin activity with acetyl-L-tyrosine-*p*-nitroanilide.

Blue, and of acetyl-L-tyrosine- and benzoyl-L-arginine-*p*-nitroanilide. Clearing the stained strips permits absorptiometric scanning at 578 nm.

We have shown, therefore, that incubating the cellulose acetate membranes (as well as all kinds of gels used for electrophoresis) in an amino acid nitroanilide medium, and finally diazotizing and coupling the nitroaniline with naphthylethylenediamine, is a very useful method for identifying and determining proteolytic enzymes (endopeptidases as well as exopeptidases) in the applied sample. Not the least advantage of this simple and rapid staining procedure is its high sensitivity: the purple colour appears even when no yellow nitroaniline is visible on the gel with the naked eye.

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