Journal of Chromatography, 463 (1989) 212–215
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 019

Note

Detection of proteolytic enzymes in fractions after liquid chromatography

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(First received May 16th, 1988; revised manuscript received September 30th, 1988)

Proteinases are among the most intensively studied of enzymes. Many papers have been published about their chromatographic isolation and purification. During this process it is usually necessary to identify the fractions containing the desired proteolytic enzymes. Usually the standard assays for the determination of proteolytic activity are used for this purpose[1,2]. When a great number of fractions is to be tested, it may be time consuming and laborious.

A simple procedure for rapid detection of proteinases is described in this note. It is based on the hydrolysis of a very thin layer of proteinaceous insoluble chromolytic substrate (gelatin cross-linked with glutaraldehyde in the presence of a suitable dye[3,4]) by proteinases present in fractions after chromatography. Proteinase positive and negative fractions can clearly be distinguished in a short period of time.

EXPERIMENTAL

Materials

Gelatin for bacteriology, water-soluble nigrosin, test combination for the determination of trypsin activity and other chemicals were from Lachema (Czechoslovakia). Glutaraldehyde was from Fluka (Switzerland).

Trypsin (specific activity 35 nkat/mg using N-α-tosyl-L-arginine-4-nitroanilide as substrate) was obtained from Léčiva (Chechoslovakia). Alkaline bacterial proteinase produced by an alkalophilic strain of *Bacillus* sp. (declared specific activity 220 000 D.U. per g) was obtained from the Research Institute of Fat Industry, Rakovník, Czechoslovakia. Lysozyme from chicken egg white was from Drůbežářský průmysl (Czechoslovakia). GelBond film was from Marine Colloids Division, U.S.A.

The glass plates used for the preparation of gelatin plates were thoroughly washed in a detergent solution, rinsed with water and dried in a dust-proof chamber.

Preparation of gelatin plates

Gelatin (2 g) and water-soluble nigrosin (1 g) were dissolved in 100 ml of water with heating in a bath of boiling water. The warm solution was clarified by centrifugation and supernatant was poured on glass plates or hydrophilic plastic sheets (GelBond film). Usually 5 ml of solution were used to cover 8 cm × 8 cm plates or

0021-9673/89/$03.50

sheets. After approximately 30 min, the solution was poured off from the plates or sheets; only a very thin layer of the solution should remain on them. Plates were allowed to dry in a perfectly horizontal position at laboratory temperature overnight. Dry plates were immersed in 1–2% glutaraldehyde solution for 30–60 min to cross-link the gelatin molecules, thoroughly washed with water and dried at ambient temperature. The prepared gelatin plates can be stored at room temperature for at least 6 months without any damage.

Fast protein liquid chromatography

The fast protein liquid chromatography was performed on a FPLC system (Pharmacia, Sweden) equipped with a MONO S HR 5/5 column using 0.05 mol dm^{-3} phosphate buffer, pH 7.0, as a mobile phase A, and the same buffer containing 1 mol dm^{-3} sodium chloride as a mobile phase B. The flow-rate was 1 ml/min, and 0.5-ml fractions were collected. The distribution of total proteins in the effluent was monitored at 280 nm.

Detection of proteinases in eluted fractions

Two simple procedures can be used, depending on the pH of the effluent.

pH of effluent is close to the pH optimum of detected proteinase. A 10-μl volume of solution was taken from each fraction and pipetted onto the surface of a gelatin plate to form a small drop. The incubation was carried out at ambient temperature or in a thermostat for 10–20 min after the application of the last drop. The plate was then thoroughly rinsed with running water so that the gelatin fragments originating from the action of proteolytic enzymes were washed away. To speed up this procedure, hot (50–60°C) running water can be used without any risk. Colourless spots indicate the presence of proteinases.

pH of effluent is far away from the pH optimum of detected proteinase. First 10-μl drops of a suitable buffer were placed on the surface of a gelatin plate and then 10-μl aliquots from fractions were added to the buffer drops. The subsequent procedure was as described above.

RESULTS AND DISCUSSION

A 500-μl volume of a model mixture containing 0.5 mg of trypsin and 0.5 mg of lysozyme was used for chromatography on a column of cation exchanger. Fig. 1 shows the distribution of total proteins. Thirty fractions containing 0.5 ml of effluent were collected. The detection of proteolytic activity was performed as described in the Experimental; 10-μl aliquots were applied directly to the surface of a gelatin plate. Hydrolysis of the gelatin layer was observed in spots corresponding to fractions 12–14 (see Fig. 2). These fractions corresponded exactly to the trypsin peak (see Fig. 1, arrows indicate the presence of proteolytic activity).

The sensitivity of the detection was verified using standard solutions of trypsin and alkaline bacterial proteinase in 0.05 mol dm^{-3} Tris–HCl buffer, pH 8.5, containing 0.01 mol dm^{-3} calcium chloride. The properly prepared gelatin plates (thickness of gelatin layer measured by an optical microscope was 0.003–0.010 mm on different plates; on a single plate the layer was homogeneous) were hydrolyzed within 10–20 min

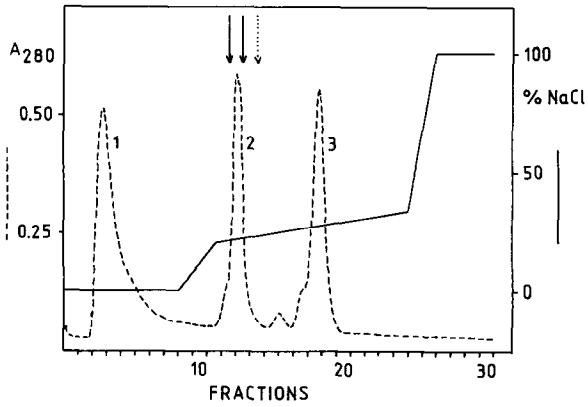

Fig. 1. Fast protein liquid chromatography of 500 μl of a model mixture containing 0.5 mg of trypsin and 0.5 mg of lysozyme on a MONO S HR 5/5 column. Buffers: A, 0.05 mol dm^{-3} phosphate buffer, pH 7.0; B, 0.05 mol dm^{-3} phosphate buffer, pH 7.0, and 1 mol dm^{-3} sodium chloride. Flow-rate 1 ml/min. --------- = Absorbance at 280 nm (protein content); ——— = gradient of sodium chloride; ——→ = presence of strong proteolytic activity; ·····→ = presence of weak proteolytic activity. Peaks: 1 = unidentified proteins; 2 = trypsin; 3 = lysozyme.

at ambient temperature by solutions containing 20 μg of trypsin per ml or 200 μg of bacterial alkaline proteinase per ml. The sensitivity depends, of course, on the thickness of the gelatin layer; the 0.070 mm thick gelatin layer (prepared only for comparison) was not hydrolyzed in 60 min by a solution containing 100 μg of trypsin

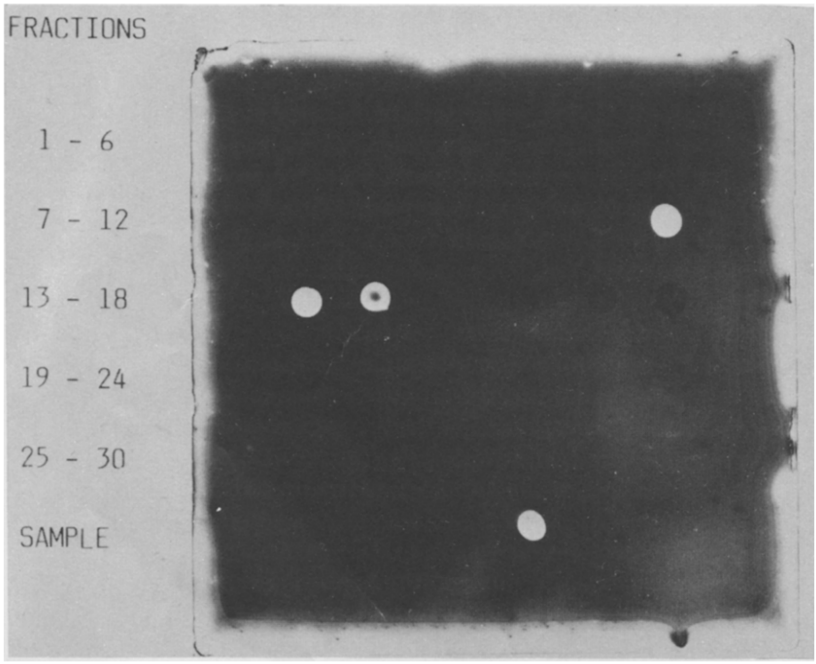

Fig. 2. Gelatin glass plate after detection of proteolytic activity in fractions. Trypsin is present in fractions 12–14.

per ml. No differences were observed between the properties of gelatin glass plates and gelatin plastic sheets.

Other proteinases, *e.g.*, chymotrypsin, various bacterial and mould extracellular proteinases hydrolyzed the gelatin layer and can be detected, too.

The pH of the effluent may play an important rôle in the detection of proteinases. When it is far away from the pH optimum of the detected proteinase, it can be adjusted to the appropriate value by mixing a drop of a suitable buffer with a drop of tested solution directly on the gelatin plate.

It is advisable to see first whether the sample to be separated contains proteinases which are capable of hydrolyzing the gelatin layer.

The procedure described can significantly shorten the time necessary for evaluation of fractions and thus speed up the whole separation process.

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