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Note

Bioautography of proteinase inhibitors of microbial origin

A simple enzymatic detection procedure on casein agar plates

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Bioautography of antimicrobial activities from thin-layer chromatograms is a common and versatile detection procedure in screening for new secondary metabolites. We have developed a similar detection method for proteinase inhibitors, based on the enzymatic hydrolysis of casein by proteolytic enzymes. This note describes a non-conventional detection procedure which allows us to detect proteinase inhibiting activity directly on cellulose acetate strips or thin-layer plates.

Cellulose acetate, which has been used for years in membrane filters, has gained popularity as a supporting medium for electrophoresis[1] and partition chromatography[2,3], because of its ease of use, reproducibility and low cost. Cellulose acetate strips are produced by a number of manufacturers and come packaged ready for use, requiring only equilibration with buffer for a few minutes.

EXPERIMENTAL

Test samples used in this study are of microbial origin. Antipain[4] and chymostatin[5] were purchased from Protein Research Foundation, Osaka, Japan. α-Microbial alkaline proteinase inhibitor (α-MAPI)[6] and unknown inhibitors have been isolated in our laboratory from culture filtrates of streptomycetes and a fungus, respectively. The crude material was purified by adsorption chromatography, counter-current distribution and reversed-phase high-performance liquid chromatography. The structures of the compounds have been identified by amino acid analysis and fast atom bombardment mass spectrometry.

All separations were carried out on a Horiphor E-200 electrophoresis apparatus (Innovativ-Labor, Adliswil, Switzerland). The polymethyl methacrylate (PMMA) tank measured 370 × 290 × 90 mm. The chromatophor E 510 (Innovativ-Labor) was used for chromatography.

Phthalate buffer (0.05 *M*, pH 4.0), acetate buffer (0.2 *M*, pH 4.65) and phosphate buffer (71 m*M* potassium dihydrogen phosphate, 10 m*M* sodium hydrogen phosphate, pH 6.00) were used. All buffers were diluted with an equal amount of doubly distilled water before use.

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Preparation of cellulose acetate strips, sample application and electrophoresis

Cellulose acetate strips (17 × 8 cm and 18 × 18 cm) were purchased from Cellogel, Milan, Italy. Trapping of air within the capillaries was avoided by first floating the strips on the surface of the buffer solution for 15 min. The strips were then submerged and stored in buffer at room temperature. Excess buffer was removed by blotting the strips gently between two sheets of filter paper. The samples were usually applied by gradual delivery of a volume of 2 μl with the help of a 10-μl Hamilton syringe. Electrophoresis was performed at 18 V/cm for 2 h. Heating of the strips was minimal under these conditions and consequently all runs were carried out at room temperature.

Casein agar plates

Casein agar contained 1% (w/v) vitamin-free casein and 0.75% (w/v) agar in 0.1 *M* sodium phosphate buffer, pH 7.5, 5 m*M* L-cysteine, and 2 m*M* EDTA. It was prepared by heating double strength buffer to 100°C, and adding casein gradually under constant stirring. This solution was then added to an equal volume of molten double strength agar. Then 150 ml of this mixture were poured into transparent polystyrol dishes (23 × 16 × 3 cm, Semadeni, Ostermundigen, Switzerland). The surface of the solidified agar was dried at 37°C for 2 h.

Detection of papain inhibitors on cellulose acetate strips

After electrophoresis or ascending chromatography, the cellulose acetate sheets were applied to the surface of the casein agar plates; care was taken to avoid trapping of air bubbles between the sheets and the agar surface. The plates were incubated for 30 min at 37°C. The cellulose acetate sheets were then removed and replaced by sheets of chromatography paper (Whatman 3MM) soaked in a 1% (w/v) solution of papain. The plates were again incubated for 60 min at 37°C. Inhibitory activities became visible as translucent spots on a milky background of partially hydrolysed casein. Contrast photography was used; therefore the translucent inhibition zones appear as dark spots on Figs. 1–3.

Chemicals

Vitamin-free casein was purchased from Fluka, Buchs, Switzerland. Papain, product No. 31610, was obtained from Serva, Heidelberg, F.R.G.

RESULTS AND DISCUSSION

The principle of our new technique is illustrated in Fig. 1. Active compounds are visible as dark inhibition zones on a milky background of partially hydrolysed casein. Physicochemical parameters, such as electrophoretic mobility and R_F values of active compounds, can be obtained directly from these plates. The technique using cellulose acetate strips as the gel support in electrophoresis can easily be applied to crude fractions from fermentation broths. It allows a rapid comparison of unknown activities with known inhibitors and eliminates time consuming and complex purification procedures at an early stage. The technique is not only rapid, but also very sensitive. Low-molecular-weight peptide aldehydes such as antipain or α-MAPI which inhibit the papain activity by 50% (IC_{50}) at a concentration of *ca.* 0.5 μg/ml

TABLE I

PHYSICAL CONSTANTS AND INHIBITORY VALUES OF VARIOUS ENZYME INHIBITORS

Thin-layer chromatography (TLC) solvents: I, *n*-butanol–acetic acid–water (4:1:5); II, chloroform–*n*-butanol–ethanol–conc. ammonia–water (20:40:50:30:13). NT = Not tested..

Inhibitor	*TLC R_F value*		*IC_{50} (μg/ml)*	
	Solvent I	*Solvent II*	*Papain*	*Cathepsin G*
Antipain	0.2	0.1	0.4	20
α-MAPI	0.6	0.4	0.6	5
Chymostatin	0.65	0.45	5	3
Inhibitor from fungal strain	0	NT	NT	NT

* Fig. 1.
** Fig. 2.

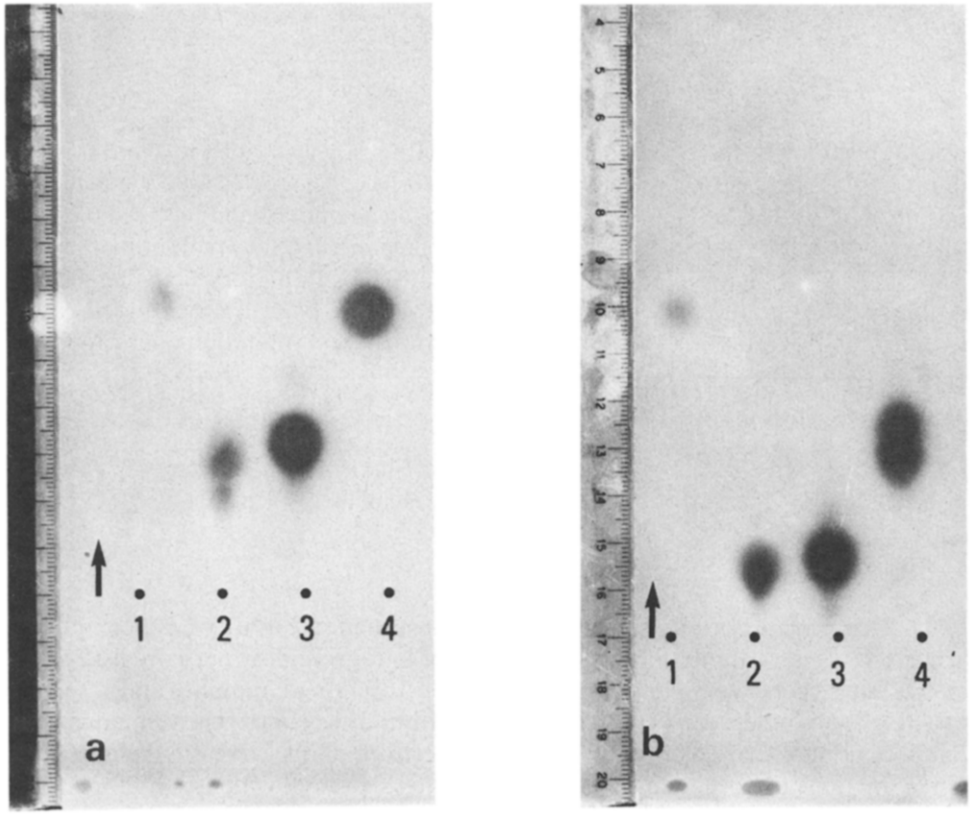

Fig. 1. Bioautography of proteinase inhibitors. Partition chromatography, 105 min on cellogel strips using (a) phthalate buffer (potassium hydrogen phthalate 0.05 *M*, pH 4.00) and (b) phosphate buffer [potassium dihydrogen phosphate (25 m*M*)–sodium hydrogen phosphate (25 m*M*), pH 6.88]. Spots: 1 = inhibitor from a fungal strain (100 μg crude); 2 = chymostatin (20 μg); 3 = α-MAPI (2 μg); 4 = antipain 2 μg.

*Partition chromatography (cm from origin)**		*Electrophoretic mobility (cm from origin)***	
pH 4.0	*pH 6.88*	*pH 4.65*	*pH 6.00*
4.5	6	5.5	5.8
1.7	3.2	2	2.5
1.2	3	1.8	2
7	6.5	6	6.5

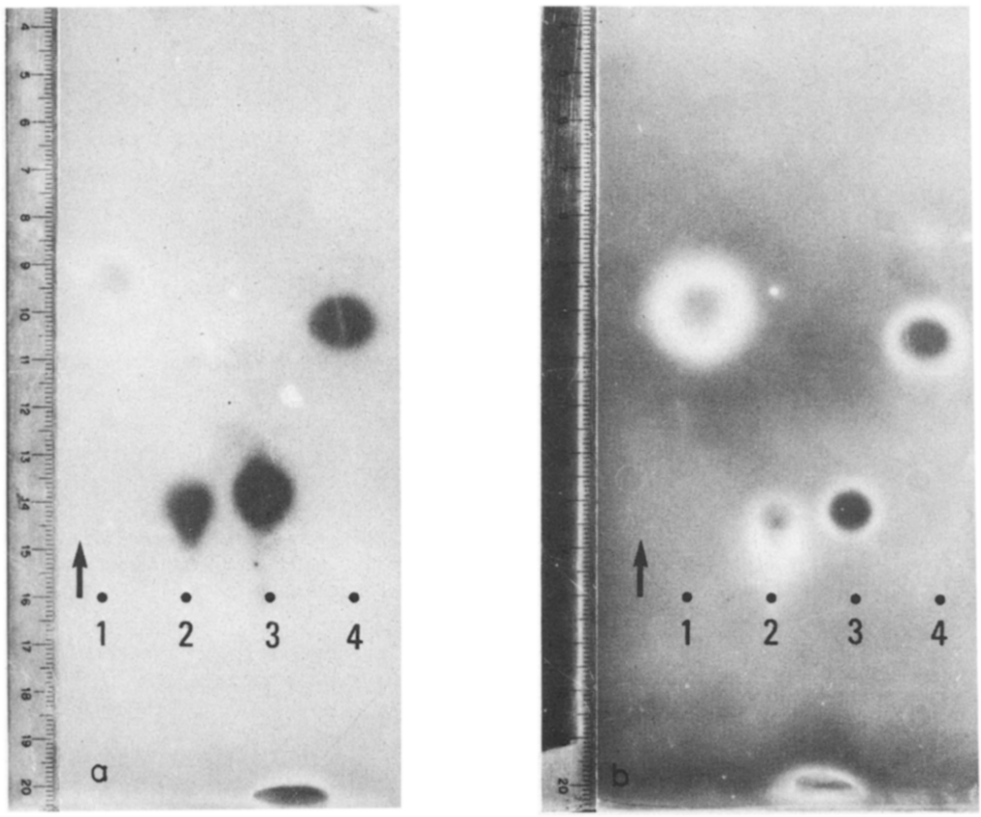

Fig. 2. Bioautography of proteinase inhibitors. Electrophoresis performed at 200 V for 120 min on cellogel strips using (a) phosphate buffer, pH 6.00 [potassium dihydrogen phosphate (71 m*M*)-sodium hydrogen phosphate (10 m*M*)] and (b) acetate buffer, pH 4.65 (acetic acid (0.1 *M*)-sodium acetate (0.1 *M*). Spots: 1 = crude inhibitor (100 μg); 2 = chymostatin (20 μg); 3 = α-MAPI (2 μg); 4 = antipain 2 μg.

(Table I), can be detected with nanomoles of active compound (Fig. 3). The method is therefore at least as sensitive as coloring reagents, such as 4,4-tetramethyldiaminodiphenylmethane (TDM), currently used in high-performance thin-layer chromatography[8].

Our technique with direct transfer of proteinase inhibitors to a casein agar biodetection support can also be applied to compounds separated by conventional thin-layer methods on sheets (MN Polygram Sil G, Macherey, Nagel & Co., Düren, F.R.G.) or plates. This enables a comparison of R_F values.

Papain has been used in our study as a lead enzyme to detect inhibitors of serine or thiol proteinases. It is cheap and easily obtainable from a variety of commercial sources. The principle of our assay, however, should also be applicable to other enzymes. We believe that we have developed a rapid, sensitive and accurate technique, which is one of the prerequisites for successful detection of novel compounds of microbial origin.

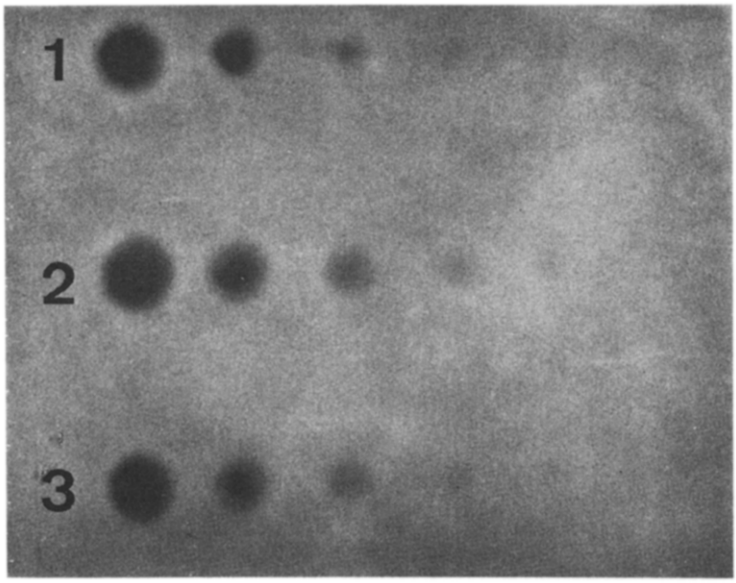

Fig. 3. Bioautography of proteinase inhibitors: sensitivity test. Spots 1 = α-MAPI (mol.wt. 595) 9 μg, 3 μg, 1 μg, 0.3 μg, 0.1 μg, 0.03 μg, 0.01 μg from a streptomycete; 2 = unknown inhibitor from a streptomycete, same amounts as 1; 3 = antipain (mol.wt. 604), same amounts as 1.

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