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Note

Rapid assay of proteolytic enzymes on film material

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Animal, vegetable and microbial proteases, their characteristics and their numerous applications in medicine and food technology are of considerable interest. In general, fractionation by column chromatography and the determination of activity by conventional methods[1–3] are time consuming and also expensive if automatic equipment is used[4]. For the detection of proteases on electropherograms, it has been suggested[5–8] that a gelatinous layer of film material can be used.

In most instances, analytical or preparative column chromatography of mixtures of proteinases produces numerous samples to be investigated. According to our experience, the use of certain film material can be very useful. The basis of this method is the partial degradation of the gelatinous layer of films; the fundamental film layer must be coloured so as to make it possible to distinguish visually the variations and to evaluate the results without further measurements, for instance with a photometer.

EXPERIMENTAL AND RESULTS

At first, we used X-ray film or document film made by VEB Filmfabrik Wolfen (G.D.R.). We found that a suitable material must have a gelatinous layer with a matt surface and a thickness of more than 30 μm. Additionally, the fundamental layer must be coloured. Consequently, in further experiments we used a specially produced film material that consists of one layer support. On one side, X-ray emulsion is deposited with a thickness of 33 μm, approximately double the size of the usual layers, and the reverse side supports a coloured NC layer well known from the literature. The variations are best distinguished with a green colour, and we obtained better results with X-ray emulsion than with a hardened gelatinous layer. The film strip is fixed on a frame by four screws (Fig. 1). A dying box with moist cellulose on the bottom is used to incubate the sample.

The assay is carried out as follows. In each instance, 10 μl of the enzyme solution are spotted on the gelatine surface at a distance of about 1 cm apart. The incubation time depends on the properties of the proteolytic enzyme; we chose 20–30 min in general. The reaction is stopped by dipping the strip into a 6% aqueous solution of trichloroacetic acid. The strip is rinsed thoroughly with running tap-water and dried with warm air. The time of drying can be shortened by dipping the strip into

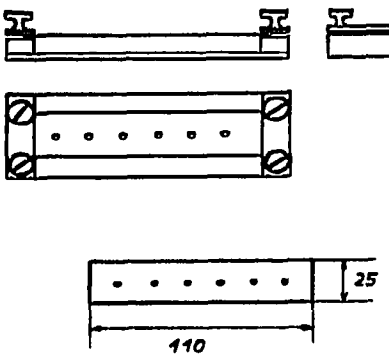

Fig. 1. Film strip and frame. The dimensions of the film strip are 25 × 110 mm.

Fig. 2. Equilibration graph for film strip proteolytic activity.

ethanol. In order to obtain semi-quantitative results, it is necessary to construct an equilibration graph (Fig. 2), for instance on the basis of the Anson method[1,2]. For this purpose, the enzyme is diluted. The lowest and highest activities should differ within a factor of 10, according to the enzyme and incubation temperature, in the ranges 1–10 μg, 5–50 μg of tyrosine per minute or $5.5 \cdot 10^{-3}$–$5.5 \cdot 10^{-2}$, $2.7 \cdot 10^{-2}$–$2.7 \cdot 10^{-1}$ μmole of tyrosine per minute. Exactly 10 μl were spotted using a Marburg pipette, and the strips were incubated for at least 30 min and further treated as described above.

According to the proteolytic activity, the gelatine layer is removed partially or completely and the coloured fundamental layer becomes visible. The spots are compared with those of the test run.

Figs. 3 and 4 show fractionations of microbial proteinases on Sephadex G-100 and CM-cellulose, respectively. The proteolytic activity of the fractions is evident, including those with a low activity. In this method, which permits the rapid detection of proteinases, it is not necessary to examine numerous samples of the eluate. In our experience, semi-quantitative and under certain circumstances quantitative determinations are possible with proteinases of mesophilic microorganisms. Of course, reproducible degradations will be achieved only if the equilibration graph is constructed for the same microorganism. Obviously, the standard deviation will be lower if the visual comparison is carried out skilfully; the mean standard deviation was ± 15%. The calculation by Student's t-test of values from four test runs with three different microbial proteases and thirty single values in each instance did not show significant differences for random tests. The sign-test was carried out in order to establish whether the deviation was positive or negative. The values of several test runs visually estimated by five skilled persons showed no significant deviation. With persons without special experience, differences in most cases were positive ($p < 0.05$). It should be mentioned that only a limited number of samples (not more than ten) should be spotted. If the incubation time was 30 min or more, the time necessary for spotting (about 1–2 min) could be neglected.

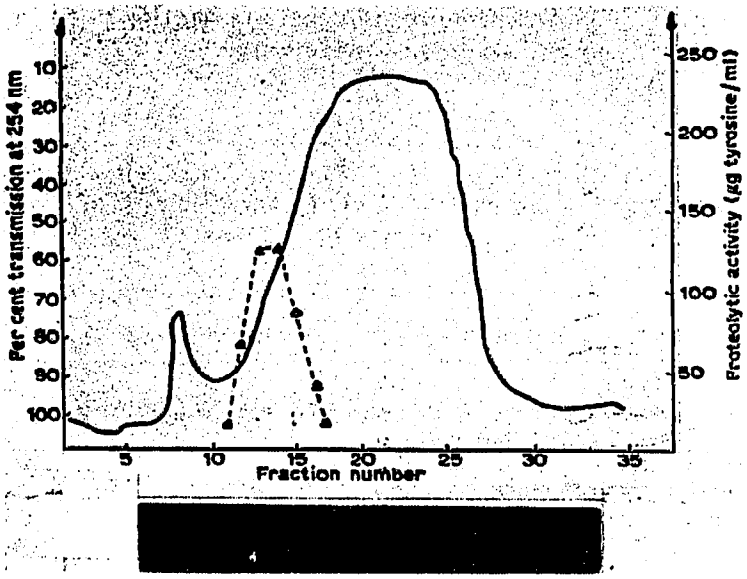

Fig. 3. Column chromatographic purification of a microbial protease of *Bacillus megaterium* on Sephadex G-100. ———, transmission at 254 nm; – – – –, proteolytic activity.

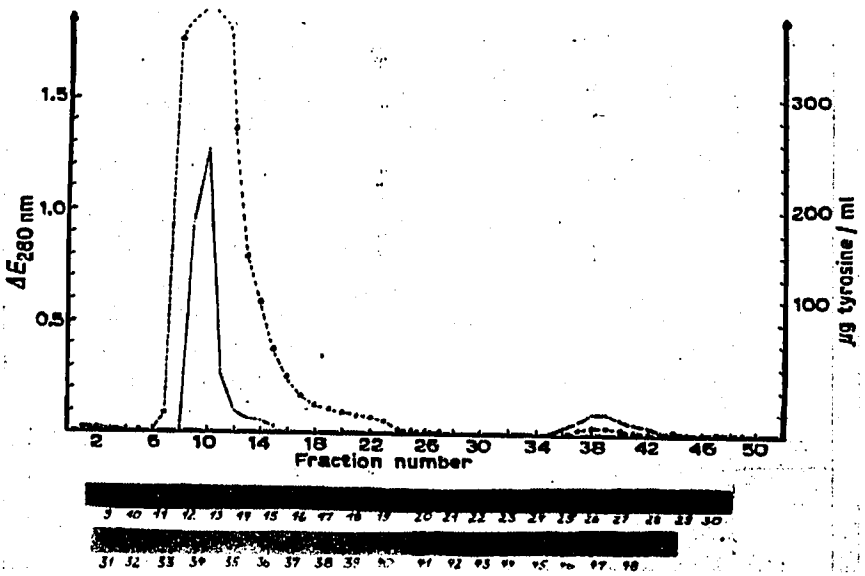

Fig. 4. Column chromatographic fractionation of Bacillus proteases (on CM-Cellulose). ———, $\Delta E_{280\,nm}$; – – – –, proteolytic activity.

The measurable range is from 1 to 10 μg of tyrosine per minute for proteinases from *Bacillus megaterium* and from 5 to 50 μg of tyrosine per minute for proteinases from Cytophaga sp. The range is dependent on the specificity of the enzyme towards gelatine and on the kinetics of hydrolysis, even if the temperature is constant.

The method is also useful for use in a screening programme, but for this purpose modifications must be made in order to expand the measurable range.

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