Adsorbed soluble [^3H]elastin as substrate for proteinase activity

A new microassay technique

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A microassay for elastase activity has been designed. Microtubes are coated with soluble elastin substrate by passive adsorption to the tube walls. On contact with enzymes in test samples, radioactivity is released into the sample buffer, which is then transferred to vials containing scintillation fluid without further processing. Porcine pancreatic elastase (EC 3.4.21.11) is easily detected in concentrations down to 8 $\mu\text{g/litre}$. The interassay coefficient of variation (CV) was below 10% for values above 50 $\mu\text{g/litre}$. The assay is rapid, precise, economical and sensitive.

The ability of proteins to be adsorbed to the surface of test tubes has been applied in immunological assays. Immunoglobulins used as a coating material react selectively with appropriate antigens from buffered solutions of biological samples (Wide, 1978).

Applying these observations to the substrate [^3H]elastin, which in soluble form will adhere to plastic, a new method has been developed which circumvents the problems of separating undigested material from enzyme-digested products.

Markers of enzyme digestion of the natural substrate elastin (Sandberg *et al.*, 1981) include dyes, fluorescence (Bieth, 1978; McGillivray *et al.*, 1981), marker enzymes (Saunders *et al.*, 1982) and radioactive labelling (Takahashi *et al.*, 1973; Bielefeld *et al.*, 1975; Yu & Yoshida, 1979). A particularly high sensitivity has been achieved by ^3H -labelling of soluble elastin (Bieger & Scheele, 1980).

The method presented here permits immediate measurement of the enzyme–substrate mixture, since undigested substrate is adsorbed to the tube walls, whereas cleavage products are released into the buffer, permitting a simple, rapid and precise microassay.

Experimental

Substrate

Soluble elastin was prepared by the method of Keller & Mandl (1971) and labelled with ^3H by the method of Bieger & Scheele (1980).

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Elastin (Sigma) was boiled with oxalic acid. The cooled supernatant was precipitated with trichloroacetic acid and resuspended in NaOH. The soluble elastin was then ^3H -labelled by reductive methylation with formaldehyde and NaB^3H_4 , the method of Bieger & Scheele (1980) being used.

Enzyme

Standard porcine pancreatic elastase (Sigma, 120 EC units/mg) was prepared in 0.1M-Tris buffer, pH 7.8. In some samples CaCl_2 (5mM) was added to the buffer to permit measurements of macrophage elastase.

Assay

First step. Microtubes (NUNC Immunoplates; Type I; 96 wells) were incubated with 350 μl of a dilution of [^3H]elastin in phosphate buffer. Substrate concentrations were varied from 0.75 to 7.5 mg/ml to assess the most economic and precise conditions. A standard procedure was then adapted which involved incubating the microtubes overnight at 4°C in a solution of [^3H]elastin containing 2.5 mg/ml (875 ng per tube). This permitted a firm adsorption of soluble [^3H]elastin to the walls of the test tubes that was independent of variations in pH from 7.4 to 8.5, in protein concentrations from 1 to 50 g/litre, and ionic strength from 0.1 to 1.0M-NaCl. Subsequent washing three times with water decreased the radioactivity by 20%. In the third washing only 0.1% of the applied radioactivity was found.

Control experiments in silicone-treated glass tubes were performed with non-adsorbed substrate. They showed the expected decrease in

radioactivity in the supernatant when undigested soluble [^3H]elastin was precipitated as described by Bieger & Scheele (1980). However, at low concentrations of radioactive substrate, a decrease in buffer radioactivity of 15%/h was noted even in silicone-treated tubes, unless detergent was added or the protein content increased.

Second step. A portion (350 ml) of the enzyme sample in the Tris buffer described above was added to the substrate-coated tubes and incubated at 37°C for various periods. The final standard procedure involved a 90 min incubation. A 250 ml portion of the reaction mixture was then transferred into 5 ml of scintillation fluid (Pico-Fluor 30; Packard) and counted for radioactivity in a β -radiation counter.

In the tubes coated with soluble [^3H]elastin, incubation with enzyme increased the radioactivity in the buffer to extents dependent on incubation time and enzyme concentration. Precipitation with trichloroacetic acid did not decrease the amount of ^3H in the supernatant.

Results and discussion

The initial experiments with substrate-coated tubes included a variation in enzyme concentration from 62.5 to 100 mg/litre and reaction times from 60 to 240 min (Fig. 1). The analyses were performed in quadruplicate on the same day with the same enzyme standard for each concentration. A decrease in enzyme activity was seen when 20% of the applied substrate was digested. This could be caused by the decrease in contact between enzyme and substrate, since only the enzyme is in free solution (heterogeneous reaction). Furthermore, prolonged incubation of proteolytic enzymes at 37°C will, by autolysis, decrease their activity.

Dilution of the enzyme/substrate components in the assay was avoided because the substrate/buffer was discarded and the coated tubes rinsed with water immediately before the enzyme samples were added. The enzyme reaction was stopped by substrate deprivation when transferred into the scintillation fluid. No further steps or sample dilutions were needed.

The final assay procedure was then as follows: (i) the tubes were coated by overnight adsorption at 4°C of 350 ml of [^3H]elastin (2.5 mg/ml); (ii) the tubes were rinsed with water three times; (iii) tubes were incubated with enzyme for 90 min at 37°C.

This permitted measurement of porcine-pancreatic elastase from 7 to 400 mg/l. The background radioactivity in an enzyme-free incubation with Tris buffer as above plus bovine albumin (1 mg/ml) yielded less than 2% of the total radioactivity applied.

In test samples the average coefficient of

Fig. 1. Adsorbed [^3H]elastin hydrolysed by pancreatic elastase

The amount of hydrolysed radioactive [^3H]elastin released from the walls of the coated tubes is shown as a function of the reaction time from 60 to 240 min. The Figure presents results obtained with the same enzyme solutions. Porcine-pancreatic-elastase (Sigma) concentrations (mg/litre): E₁, 1000; E₂, 500; E₃, 250; E₄, 125; E₅, 62.5.

variation of released radioactivity by the enzyme digestion in double estimations was 3.2% in 100 samples. Recovery was 96%.

Fig. 2 shows an 'average' standard curve based on eight standard curves which were constructed over a period of 11 weeks. Means $\pm$ S.D. are calculated for each concentration.

A previously described assay (Bieger & Scheele, 1980) involved the separation of the elastase-released products of low M_r from the remaining undigested substrate by addition of non-radioactive substrate, precipitation by trichloroacetic acid and centrifugation, before measurement of the released radioactivity. The released radioactivity was diluted by the addition of substrate (1 mg in 1 ml of buffer) and trichloroacetic acid.

In order to evaluate the two methods, precision profiles were calculated on the basis of 32

determinations in duplicate from four assays for each method (see Fig. 3). The detection limits estimated from Fig. 3 as $3 \times \text{s.d.}$ at zero concentra-

Fig. 2. An 'average' standard curve for porcine pancreatic elastase

The mean values are based on eight standard curves performed over a period of 11 weeks. The means $\pm$ s.d. are indicated for each enzyme concentration. The results are based on eight duplicate measurements. The assay time of 90 min used in these experiments gives a sensitivity of 8 mg of porcine pancreatic elastase/litre.

tion were 12 mg/l for the trichloroacetic acid method and 8 mg/litre for the adsorption assay.

The comparison of the two methods by pool dilutions gave the following relationship (where x is the result from the trichloroacetic acid method and y is the result from the adsorption method):

$$x = 5.0 + 0.83y$$

The precision and sensitivities are thus comparable for the two methods, the adsorption assay which has a detection limit of 8 mg of porcine pancreas elastase/litre, having a small advantage. The practicality favours the adsorption technique, since enzyme addition and measurement of resulting hydrolysis of [^3H]elastin are carried out by simple pipetting directly into the test tubes and transfer to the scintillation fluid. No further steps are needed before counting the released ^3H radioactivity.

The [^3H]elastin adsorption methods described here measure elastase activity and circumvent the problems of separating digested products from the remaining substrate. The reaction time can be decreased from the 90 min used in this assay with only a small loss of sensitivity at low concentrations. Incubation of substrate in the tubes for less than 20 h will give less adsorption, but can be compensated for by increasing the substrate concentration.

The micro-assay can be used to measure elastase concentrations in human granulocyte and monocyte cell preparations as well as in biological samples and is of increasing interest in studying

Fig. 3. Precision profiles for (a) the [^3H]elastin adsorption assay and (b) the [^3H]elastin trichloroacetic acid precipitation assay. The s.d. (----) and coefficient of variation (CV) (%) $\{ = (100 \times \text{s.d.})/[\text{elastase}] \}$ is shown for each method based on 32 determinations in duplicate from four assays. The detection limit was estimated as $3 \times \text{s.d.}$ at zero concentration. (a) 8 mg/litre; (b) 12 mg/litre.

diseases such as emphysema, atherosclerosis and arthritis.

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