Fluorogenic method for the assay of proteinase activity with the use of 4-methylumbelliferyl-casein

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A method for the preparation of casein labelled with the 4-methylumbelliferyl fluorophore is described, and the product was used as a fluorogenic macromolecular substrate for a sensitive assay of the activity of proteinases. Nanogram quantities of trypsin, chymotrypsin, elastase and cathepsin D can be detected, but the substrate is unaffected by cathepsin B.

The role of proteinases and their natural inhibitors in the biological control of tissue turnover is currently of great interest (Holzer & Heinrich, 1980). Although sensitive and selective low-molecular-weight synthetic substrates have been used with success in some cases (Zimmerman *et al.*, 1977), it is evident that some proteinases, e.g. those responsible for activation of lysosomal enzyme precursors, are significantly active only on macromolecular substrates. Haemoglobin or casein, variously modified with dyes, or labelled with radioisotopes, have been commonly used (Charney & Tomarelli, 1947; Drucker, 1972).

The labelling of antibodies with fluorescent markers has proved to be a successful alternative to radioisotope methods in some cases (Ekeke *et al.*, 1979), and has the advantages of cheapness and low hazard. More recently, the use of fluorescent labelled proteins as proteinase substrates has been reported, with a sensitivity comparable with that of radioisotope methods (Wiesner & Walter, 1982).

Fluorimetric assay of lysosomal enzymes with the use of conjugates of 4-methylumbelliferone as substrates is widely used, and we now describe the preparation and evaluation of a fluorescent derivative of casein, based on the same fluorophore, for the assay of proteinase activity.

The method is broadly applicable to the fluorescent labelling of proteins in general, and enzyme activity is measured by estimation of fluorescent peptides released into solution, after removal of precipitable protein after proteolysis.

Materials and methods

Materials

The fluorophore 4-methylumbelliferone-3-acetic acid was synthesized as described by Ekeke *et al.*

(1979). Purified casein, cathepsin D, bovine α -chymotrypsin and pig elastase were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.); trypsin was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.); pure cathepsin B was generously given by Dr. A. J. Barrett, Strangeways Laboratory, Cambridge, U.K.

Activation of fluorescent label

The *N*-hydroxysuccinimide ester of 4-methylumbelliferone-3-acetic acid was prepared as follows. To a solution of 233 mg of 4-methylumbelliferone-3-acetic acid and 138 mg of *N*-hydroxysuccinimide in 100 ml of tetrahydrofuran was added 247 mg of *NN'*-dicyclohexylcarbodi-imide, with cooling. The mixture was stirred overnight in the cold, and the dicyclohexylurea formed was then filtered off and the filtrate concentrated *in vacuo* to a yellow oil, which crystallized on the addition of diethyl ether. The *N*-hydroxysuccinimide ester so obtained was used without further purification.

Coupling to casein

Approx. 1 g of casein was left to dissolve overnight in 20 ml of 50 mM-sodium tetraborate buffer, pH 9.0. After removal of any undissolved particles by centrifugation, 99 mg of the above *N*-hydroxysuccinimide ester was added in three portions over a 1 h period with vigorous stirring. The reaction mixture was kept at room temperature for a further 5 h. The solution was then centrifuged, and the soluble fluorescent casein was precipitated by the addition of 0.5 M-sodium formate/HCl buffer, pH 4.0. The precipitate was filtered off and washed, first with the above buffer and then with 10 mM-phosphate-buffered saline (0.15 M-NaCl/10 mM-sodium phosphate buffer), pH 4.0, until the washings were substantially free of fluorescence. Final

traces of fluorescent contamination were removed by redissolving the 4-methylumbelliferyl-casein in 20 ml of 10 mM-phosphate-buffered saline, pH 9.0, and dialysing overnight against a large volume of 10 mM-phosphate-buffered saline, pH 7.2, before freeze-drying the product.

General assay method

The 4-methylumbelliferyl fluorophor generates maximum fluorescence at pH 10.0 when the 7-hydroxy group is fully ionized, and the method of assay involves incubation at the optimum pH for proteinase activity, precipitation of unhydrolysed casein and adjustment of a sample of the solution to pH 10.3 for fluorimetry. The quantities described below have proved to be convenient with the Locarte LMF fluorimeter used, but in no way extend the method to the limits of its potential sensitivity.

To 0.3 ml of the appropriate assay buffer, containing any required activators, is added 0.1 ml of enzyme solution. After equilibration at 37°C, 0.1 ml of a stock substrate solution is added (20 mg of 4-methylumbelliferyl-casein/ml in 10 mM-phosphate-buffered saline, pH 7.4), to give a final substrate concentration of 4 mg/ml. After incubation for up to 30 min, the reaction is stopped and unhydrolysed substrate is precipitated by the addition of 2 ml of 5% (w/v) trichloroacetic acid. After 5 min the mixture is filtered and a sample is diluted 10-fold by the addition of 0.2 M-glycine/NaOH buffer, pH 10.3, for fluorimetry.

Controls are performed by adding enzyme subsequent to incubation and trichloroacetic acid precipitation, and a suitable fluorescent standard is a

0.5 μ M solution of 4-methylumbelliferone-3-acetic acid in the above glycine buffer. Activation and emission wavelengths are 360 nm and 460 nm respectively. Control values indicate that more than 99.5% of the protein is precipitated.

Incubations at acid pH may cause isoelectric precipitation of the substrate, but this is without detrimental effect on the assay.

Detection of the products

The course of hydrolysis and the nature of the peptides formed can be followed visually by subjecting a 25 μ l sample of the assay mixture before trichloroacetic acid precipitation to sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by using standard procedures as described by Laemmli (1970). The gels are viewed and photographed under u.v. illumination at 350 nm.

Results and discussion

The effectiveness of the fluorescent-labelling procedure can be monitored by the appearance of a strong absorption band at 365 nm at pH 10.0 with solutions of the conjugated casein. Unconjugated casein has negligible absorbance under these conditions.

Assuming that the spectral characteristics of the fluorophore are not affected by conjugation, the conditions described above produce a degree of substitution of 0.1 μ mol of 4-methylumbelliferone-3-acetic acid/mg of protein, equivalent to approx. 2.5 basic amino acid residues being labelled on each molecule of casein.

Fig. 1. Sodium dodecyl sulphate/12%-polyacrylamide-gel electrophoresis of (a) digestion products of 4-methylumbelliferyl-casein with various concentrations of α -chymotrypsin after 30 min and (b) digestion products of 4-methylumbelliferyl-casein with α -chymotrypsin (2 μ g) after various times

Incubations were in 50 mM-Tris/HCl buffer, pH 7.8, under the general assay conditions described in the text. (a) Lane 1, control (unhydrolysed casein); lane 2, 1.2 μ g of α -chymotrypsin; lane 3, 2.8 μ g of chymotrypsin; lane 4, 8.0 μ g of chymotrypsin. (b) Lane 1, 10 min; lane 2, 20 min; lane 3, 30 min; lane 4, 40 min; lane 5, 50 min. (c) Progress curve for digestion of 4-methylumbelliferyl-casein with α -chymotrypsin (2 μ g) as a function of time at 37°C. Fluorescence liberated is given in arbitrary units, control values typically being 30–40 units.

Fig. 2. (a) Hydrolysis of 4-methylumbelliferyl-casein with various enzymes as a function of time at 37°C and (b) standard curve for digestion of 4-methylumbelliferyl-casein as a function of enzyme concentration at a fixed time of 30 min ●, Incubation with trypsin (300 ng in a) in 50 mM-Tris/HCl buffer, pH 8.2, containing 20 mM/CaCl₂; ▲, incubation with elastase (830 ng in a) in 50 mM-Tris/HCl buffer, pH 7.8; ■, incubation with α-chymotrypsin (500 ng in a) in 50 mM-Tris/HCl buffer, pH 7.8. Fluorescence liberated is given in arbitrary units, control values typically being 30–40 units.

Fig. 1(a) shows the sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic 'fingerprint' when fluorescent casein is allowed to react for the same length of time with three different concentrations of chymotrypsin. Fig. 1(b) shows the time course over a 50 min incubation period for a single chymotrypsin concentration. Fig. 1(c) shows the quantitative curve corresponding to Fig. 1(b).

The resistance of certain peptides to any further hydrolysis is clearly seen, and the heterogeneous nature of the commercial casein is evident.

When low concentrations of enzyme are used, the liberation of soluble fluorescent products is linear with time over a considerable period (Fig. 2a). These initial rates of liberation are proportional to enzyme concentration (Fig. 2b). The sensitivity of the method under the above conditions of assay is such that 75 ng of trypsin or 100 ng of chymotrypsin is sufficient to produce a soluble fluorescence twice that of the control after a 30 min incubation. By the same criteria, cathepsin D acting on insoluble 4-methylumbelliferyl-casein at pH 3.3 can be esti-

mated down to 0.05 Sigma haemoglobin unit [1 unit will produce an increase in A_{280} of 1.0/min per ml at pH 3.0 at 37°C, measured as trichloroacetic acid-soluble products, with haemoglobin as substrate (light-path 1 cm)] but cathepsin B at concentrations as high as 2 μg of pure enzyme/assay mixture had no significant effect after incubations of up to 2 h.

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