A Radiochemical Titrant for the Determination of the Operational Molarity of Solutions of Acid Proteinases

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N-Diazoacetyl-L-phenylalanine 3-phenyl[2,3-³H]propylamide was synthesized and shown to inhibit pepsin A (EC 3.4.23.1) and cathepsin D (EC 3.4.23.5) irreversibly and stoichiometrically in the presence of Cu²⁺. Quantitative separation of the inhibited enzyme from excess reagent by gel filtration followed by measurement of the radioactivity of the protein peak provided a method for determining the operational molarity of these enzymes. Several other putative active-site-directed irreversible inhibitors were synthesized, but were inactive. Data on the synthesis of these compounds have been deposited as Supplementary Publication SUP 50096 (4 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) 169, 5.

Numerous reagents have been developed for determining the operational molarity of solutions of the serine proteinases and thiol proteinases by spectrophotometry and spectrofluorimetry. No such methods are available for the acid proteinases. Moreover, although Santi *et al.* (1974) developed a radiochemical method of titrating thymidylate synthetase based on the stoichiometric formation of an isolatable complex with 5-fluoro-2'-deoxy[6-³H]uridylate, radiochemical titrants for enzymes have yet to be fully exploited. With the knowledge that *N*-diazoacetyl derivatives of amino acid esters in the presence of Cu²⁺ inhibit pepsin irreversibly (Kozlov *et al.*, 1967; Rajagopalan *et al.*, 1966; Lundblad & Stein, 1969; Bayliss *et al.*, 1969) by reaction at Asp-215 and that the most sensitive substrates of pepsin have aromatic amino acids on both sides of the scissile bond (Fruton, 1974), we have synthesized *N*-diazoacetyl-L-phenylalanine 3-phenylpropylamide (I) and we have shown that it irreversibly inhibits pepsin, cathepsin D and chymosin (rennin; EC 3.4.23.4). The tritiated analogue, *N*-diazoacetyl-L-phenylalanine 3-phenyl[2,3-³H]propylamide, was found to be a useful radiochemical titrant. The inhibited enzyme was separated from excess inhibitor by gel filtration and the ³H content was determined by liquid-scintillation counting.

In view of reports that cathepsin D is secreted by some malignant cells (Keilova *et al.*, 1968; Kazakova *et al.*, 1972), several other compounds (II–IV) were synthesized as potential irreversible inhibitors, but none was active.

A preliminary account of this work has been published (Irvine & Elmore, 1976).

Experimental

Materials

Pig pepsin (twice crystallized, batch no. PM 34 M 609) was purchased from Worthington Biochemical Corporation (Cambrian Chemicals Ltd., Croydon, Surrey, U.K.). Cathepsin D was isolated from chicken livers by the method of Barrett (1970). Analytical isoelectric focusing of the purified enzyme in polyacrylamide gels showed only three bands representing the α -, β - and γ -isoenzymes (Barrett, 1970). The enzyme preparation that was used for titration was further purified by gel filtration at 4°C on a column

(60 cm × 3 cm) of Sephadex G-100 in 0.05 M-sodium acetate buffer, pH 5.1, with a flow rate of 20 ml/h. Activities of pepsin and cathepsin D were measured by the steady-state assay methods of Bayliss *et al.* (1969) and Barrett (1970) respectively, with bovine haemoglobin type II (Sigma Chemical Co., St. Louis, MO, U.S.A.) as substrate. Chymosin (rennin) (Sigma) was assayed by a method similar to that used for cathepsin D, except that the incubation temperature was 35 instead of 45°C. The increase in $A_{280}^{1\text{cm}}$ up to a value of 0.3 was found to be proportional to the enzyme concentration.

The following compounds were synthesized as described in the literature: cinnamylamine hydrochloride (Ishibashi, 1965), 3-phenylpropylamine hydrochloride (Shapiro *et al.*, 1959), *N*-t-butoxycarbonylglycine (Anderson & McGregor, 1957), *N*-t-butoxycarbonyl-L-phenylalanine (Schnabel, 1967), *N*-diazoacetyl glycine ethyl ester (Curtius & Darapsky, 1906), *N*-diazoacetyl-L-phenylalanine methyl ester (Bayliss *et al.*, 1969).

N-t-Butoxycarbonyl-L-phenylalanine cinnamylamide. A solution of *N*-t-butoxycarbonyl-L-phenylalanine (7.95 g) and *N*-methylmorpholine (3.3 ml) in *NN*-dimethylformamide (50 ml) was kept at -15°C during the addition of isobutylchloroformate (3.9 ml). The mixture was stirred at -10°C during 10 min. A cold solution of cinnamylamine hydrochloride (5.07 g) and *N*-methylmorpholine (3.3 ml) in *NN*-dimethylformamide (50 ml) was then added. The mixture was stirred for 2 h at -10°C and then allowed to warm slowly to room temperature in melting ice. Solvent was removed under reduced pressure and the residue was dissolved in a mixture of ethyl acetate and water. The organic phase was washed successively with 5% (w/v) citric acid solution, saturated NaHCO₃ solution and water, then dried (over anhydrous MgSO₄). The product (82%) was crystallized by addition of light petroleum (b.p. 40–60°C). After recrystallization from ethyl acetate/light petroleum (b.p. 40–60°C), it had m.p. 128.0–128.5°C (Found: C, 72.8; H, 7.4; N, 7.1; C₂₃H₂₈N₂O₃ requires C, 72.6; H, 7.4; N, 7.4%).

L-Phenylalanine cinnamylamide hydrochloride. A saturated solution (50 ml) of dry HCl gas in dry diethyl ether was added to a solution of *N*-t-butoxycarbonyl-L-phenylalanine cinnamylamide (1.5 g) in dichloromethane (5 ml) and the mixture was stirred for 2 h at room temperature. After cooling to 0°C, the product (95%) was collected, when it had m.p. 195.5–197.5°C (Found: C, 68.3; H, 6.8; N, 8.7; C₁₈H₂₁ClN₂O requires C, 68.2; H, 6.7; N, 8.8%).

N-t-Butoxycarbonylglycyl-L-phenylalanine cinnamylamide. This compound (91%) was prepared from *N*-t-butoxycarbonylglycine and L-phenylalanine cinnamylamide hydrochloride in a manner similar to that used for *N*-t-butoxycarbonyl-L-phenylalanine cinnamylamide. After recrystallization from ethyl

acetate/light petroleum (b.p. 40–60°C), it had m.p. 134–136°C (Found: C, 68.5; H, 7.3; N, 9.5; C₂₅H₃₁N₃O₄ requires C, 68.6; H, 7.4, N, 9.6%).

Glycyl-L-phenylalanine cinnamylamide hydrochloride. This compound (94%) was prepared from the *N*-t-butoxycarbonyl derivative in a manner similar to that used for L-phenylalanine cinnamylamide hydrochloride. After recrystallization from methanol/diethyl ether, it had m.p. 243–244°C (Found: C, 63.8; H, 6.6; N, 11.1; C₂₀H₂₄ClN₃O₂ requires C, 64.2; H, 6.5; N, 11.2%).

Glycyl-L-phenylalanine 3-phenylpropylamide hydrochloride. A solution of glycyl-L-phenylalanine cinnamylamide hydrochloride (1 mmol) in freshly distilled *N*-methylpyrrolid-2-one (10 ml) was hydrogenated at atmospheric pressure over 10% palladium on charcoal (5 mg). After removal of catalyst and solvent, the product (90%) was crystallized from ethanol/diethyl ether and then from methanol/diethyl ether, when it had m.p. 193–195°C (Found: C, 63.7; H, 7.2; N, 11.1; C₂₀H₂₆ClN₃O₂ requires C, 63.9; H, 7.0; N, 11.2%).

The corresponding 3-phenyl[2,3-³H]propylamide hydrochloride was prepared as above, except that the reduction was carried out with gaseous ³H₂ at The Radiochemical Centre, Amersham, Bucks., U.K. The uptake of ³H was 20 Ci/mmol. A portion of the product was co-crystallized with the unlabelled analogue to constant specific radioactivity and m.p. 193–195°C. After storage for several months, this compound was found to contain a small amount of labelled impurity, which appeared at the void volume during gel filtration on Sephadex G-25 in water. Consequently, it was purified by gel filtration on a column (27 cm × 1.5 cm) of Sephadex G-10 in 0.05 M-ammonium formate buffer, pH 3.2, and collected in 3 ml fractions. The purified peptide emerged in fractions 20–30, which were pooled and freeze-dried.

N-Diazoacetyl-L-phenylalanine 3-phenylpropylamide. Solutions of NaNO₂ (0.15 g) in water (2 ml) and glycyl-L-phenylalanine 3-phenylpropylamide hydrochloride (0.5 g) in methanol (15 ml) were added in alternate portions to a solution of acetic acid (0.1 ml) in 2 M-sodium acetate (3 ml) at 0°C during 30 min. The solution was stirred at 0°C for 2 h, treated with acetic acid (0.1 ml) and left overnight at 0°C. A solution of NaNO₂ (0.1 g) in water (0.5 ml) and acetic acid (0.1 ml) was added and the mixture was left for 48 h at 0°C. Water (20 ml) was added and the mixture was left overnight at 0°C. The product (45%) was collected by filtration, washed with cold water and dried over P₂O₅ under vacuum. After crystallization from dichloromethane/diethyl ether, it had m.p. 157–158°C and the i.r. spectrum had a strong peak at 2100 cm⁻¹ (diazo group) (Found: C, 68.5; H, 6.3; N, 15.7; C₂₀H₂₂N₄O₂ requires C, 68.5; H, 6.3; N, 16.0%).

The corresponding 3-phenyl[2,3-³H]propylamide was prepared similarly and recrystallized from methanol/water to constant specific radioactivity, when it had m.p. 157–158°C.

Data on the synthesis of other putative irreversible inhibitors mentioned in the present paper have been deposited as Supplementary Publication SUP50096 (4 pages) at the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1978) **169**, 5.

Methods

Counting of radioactivity. Liquid-scintillation counting of ³H radioactivity was carried out on an Intertechnique SL30 spectrometer, with PPO (2,5-diphenyloxazole; 0.4%, w/v) in toluene/Triton N-101 (2:1, v/v) as scintillation fluid. An aqueous solution of the sample (0.05 or 0.5 ml) was mixed with the scintillation fluid (5 ml). When the radioactivity gave <10000 c.p.m. the sample was counted for 10 min. Background radioactivity was determined by using control samples from which the radioactive material had been omitted and was subtracted from the radioactivity of the samples. Calibration curves relating c.p.m. to concentration of *N*-diazooacetyl-L-phenylalanine 3-phenyl[2,3-³H]propylamide were constructed by counting the radioactivity of serial dilutions of a solution of known concentration. All measurements were made in triplicate.

Radiochemical titration of solutions of pepsin. A solution of *N*-diazooacetyl-L-phenylalanine 3-phenyl[2,3-³H]propylamide (0.5 mg) in methanol (0.5 ml) was mixed with 0.1 M-cupric acetate (50 μ l) and incubated for 10 min. A portion (10 μ l) of this solution was then added to a solution (200 μ l) of pepsin (20–500 μ g) in 0.05 M-sodium acetate buffer, pH 5.5, and the mixture was incubated at 20°C for 10 min. Then 1 M-HCl (10 μ l) was added and a portion (100 μ l) of the solution was subjected to gel filtration on a column (15 cm $\times$ 0.7 cm) of Sephadex G-25 in water. Fractions (0.5 ml) were collected manually into vials containing scintillation fluid (5 ml) and counted for radioactivity.

Radiochemical titration of solutions of cathepsin D. 0.1 M-Cupric acetate (20 μ l) was added to a solution (400 μ l) of cathepsin D (28–140 μ g) in 0.05 M-sodium acetate buffer, pH 5.1, and the mixture was incubated at 20°C for 10 min. A 4.0 mM solution (10 μ l) of titrant in methanol was added and the solution was incubated at 40°C for 15 min. A further portion (10 μ l) of the titrant solution was added and incubation at 40°C was continued for a further 15 min. Then 0.7 M-HCl (20 μ l) was added and a portion (100 μ l) of the solution was subjected to gel filtration on a column (15 cm $\times$ 0.7 cm) of Sephadex G-25 in water/*t*-butyl alcohol (49:1, v/v). Fractions (0.5 ml) were collected manually into vials containing scintillation fluid (5 ml) and counted for radioactivity.

Results and Discussion

Incubation of solutions of pepsin or cathepsin D with solutions of *N*-acetyl(*p*-bromoacetamido)-L-phenylalanine methyl ester (II) or *N*-methoxycarbonyl-L-phenylalanyl-L-phenylalaninol methanesulphonyl ester (III) did not result in any loss of enzyme activity. Likewise *N*-chloroacetyl-L-phenylalanine 3-phenylpropylamide (IV) did not inhibit pepsin. *N*-Diazooacetyl-L-phenylalanine 3-phenylpropylamide (I), however, inactivated pepsin and, more slowly, cathepsin D in the presence of Cu²⁺. To achieve a strictly stoichiometric labelling of pepsin by *N*-diazooacetyl-L-phenylalanine methyl ester, reaction conditions must be rigidly controlled (Bayliss *et al.*, 1969). Factors affecting the rate of inhibition of pepsin by *N*-diazooacetyl amino acid esters include pH, temperature, the concentrations of inhibitor and Cu²⁺ ions and the order in which these and the enzyme solution are mixed (Lundblad & Stein, 1969). The effect of varying these parameters on the rate of inactivation of pepsin and cathepsin D by *N*-diazooacetyl-L-phenylalanine 3-phenylpropylamide was therefore examined in order to determine the optimum conditions for inactivation.

The presence of Cu²⁺ (0.2 mM) in a solution of pepsin (0.15 mg/ml) in 0.05 M-sodium acetate buffer, pH 5.0, at 20°C had no measurable effect on the activity of the enzyme. Similar treatment of a solution of cathepsin D (0.01 mg/ml) caused a decrease in activity of about 5%. Incubation of a solution (2.5 ml) of pepsin (0.15 mg/ml) in 0.05 M-sodium acetate buffer, pH 5.0, with a solution (0.1 ml) of *N*-diazooacetyl-L-phenylalanine 3-phenylpropylamide (1 mg/ml) in methanol at 20°C for 30 min caused a decrease in activity of 13%. Similar treatment of a solution of cathepsin D (0.01 mg/ml) resulted in a decrease in activity of 12%. The small degree of inactivation of both enzymes by either Cu²⁺ or *N*-diazooacetyl-L-phenylalanine 3-phenylpropylamide alone is in accord with the results obtained for pepsin with *N*-diazooacetyl-L-phenylalanine methyl ester (Bayliss *et al.*, 1969) and for cathepsin D with *N*-diazooacetyl-DL-norleucine methyl ester (Keilova, 1970). In the presence of 200 μ M-Cu²⁺, however, *N*-diazooacetyl-L-phenylalanine 3-phenylpropylamide rapidly inactivated pepsin and cathepsin D and further experiments were carried out to optimize the conditions.

The extent of inactivation of pepsin and cathepsin D under otherwise standard conditions as a function of pH is shown in Fig. 1. A similar profile was obtained if the solution of Cu²⁺ and pepsin was mixed before the addition of *N*-diazooacetyl-L-phenylalanine 3-phenylpropylamide.

Inactivation of pepsin proceeded rapidly at 20°C. Although a solution of pepsin (0.15 mg/ml) containing 0.2 mM-Cu²⁺ at pH 5.2 and 20°C is only 70%

Fig. 1. Effect of pH on the inhibition of pepsin and cathepsin D by *N*-diazoacetyl-L-phenylalanine 3-phenylpropylamide

0.1 M-Cupric acetate (0.05 ml) was added to a solution (0.95 ml) of inhibitor (○, 0.37 mg/ml; ●, 0.74 mg/ml) in methanol and the mixture was incubated for 10 min at 20°C. A portion (0.1 ml) of this solution was then added to a solution (1.9 ml) of pepsin (0.15 mg/ml) or cathepsin D (0.02 mg/ml) in 0.05 M-sodium acetate buffer of the required pH, and the mixture was incubated for 4 min at 12°C for pepsin or 10 min at 20°C for cathepsin D before assay. ○, Pepsin; ●, cathepsin D.

inhibited in 5 min in the presence of 10 μ M-*N*-diazoacetyl-L-phenylalanine 3-phenylpropylamide, elevation of inhibitor concentration to 50 μ M resulted in complete inactivation under the same conditions. Provided that the inhibitor concentration exceeded that of enzyme, these conditions sufficed to inhibit pepsin solutions containing up to 3 mg of protein/ml. At this latter enzyme concentration, 330 μ M-inhibitor solution was used. In contrast, cathepsin D was inactivated relatively slowly at 20°C and it was more convenient to carry out the inhibition at 37 or 40°C. Also, inhibition was incomplete when the enzyme solution contained >0.05 mg/ml even with inhibitor concentrations as high as 0.2 mM. Cathepsin D in concentrations up to 0.35 mg/ml could be completely inactivated within 30 min at 40°C by a second addition of inhibitor (Fig. 2). This phenomenon was not due to enzymic hydrolysis of the inhibitor at the phenylalanine bond, since no 3-phenylpropylamine, detectable with fluorescamine, was formed on incubation of inhibitor with either pepsin or cathepsin D. It is possible that cathepsin D in concentrated solution exists as a less reactive form, possibly as an oligomer.

A comparison of the rates of inactivation of pepsin and cathepsin D by equimolar concentrations of *N*-diazoacetyl-L-phenylalanine 3-phenylpropylamide is shown in Fig. 3. The last compound was most effective, presumably because of the presence of two aromatic groups in the molecule, which thus simulates a good substrate. Indeed, *N*-diazoacetyl-L-phenylalanine 3-phenyl-

Fig. 2. Inactivation of cathepsin D by a double addition of *N*-diazoacetyl-L-phenylalanine 3-phenylpropylamide

0.1 M-Cupric acetate (0.02 ml) was added to a solution (0.4 ml) of cathepsin D (0.35 mg/ml) in 0.05 M-sodium acetate buffer, pH 5.1, and the mixture was preincubated for 10 min at 40°C. Additions of a portion (0.01 ml) of a solution of inhibitor (1.4 mg/ml) in methanol are shown by the arrows. Addition of a single portion (0.02 ml) of the solution of reagent at zero time inhibited only 77% of the enzyme after 30 min.

propylamide appears to be a better inhibitor of pepsin than is 1,1-bis(diazoacetyl)-2-phenylethane, which Husain *et al.* (1971) considered to be 'the most effective pepsin inhibitor hitherto described'.

To quantify the reaction of *N*-diazoacetyl-L-phenylalanine 3-phenyl[2,3-³H]propylamide with pepsin, a solution of the inhibited enzyme was purified from low-molecular-weight radioactive components by gel filtration (Fig. 4). The concentration of protein in the enzyme fractions was determined by measuring A_{280} , by assuming $A_{280}^{0.1\%, 1\text{cm}}$ 1.47 (Perlmann, 1959). The recovery of material absorbing at 280 nm was quantitative. The molarity of the enzyme-bound inhibitor was calculated from the radioactivity of the reagent measured under the same conditions (14 900 c.p.m./nmol in this experiment). The uptake of inhibitor was 0.97–1.03 mol/mol of enzyme. Addition of HCl to the incubation mixture containing enzyme and inhibitor was necessary, since in previous experiments when acidification was omitted the uptake of inhibitor was ~1.2 mol/mol of enzyme. Measurement of A_{250} showed that the inhibitor was rapidly destroyed at pH 3.1 or less, and this technique prevented reaction of the inhibitor at sites other than the active centre. In separate experiments it was shown that pepsin that had been stoichiometrically

Fig. 3. Comparison of the rates of inactivation of pepsin and cathepsin D by three related inhibitors

Inactivation of pepsin (a) was carried out as follows: 0.1 M-cupric acetate (0.05 ml) was added to solutions (0.95 ml) of *N*-diazoacetyl-glycine ethyl ester (0.18 mg/ml) (●), *N*-diazoacetyl-L-phenylalanine methyl ester (0.27 mg/ml) (□) or *N*-diazoacetyl-L-phenylalanine 3-phenylpropylamide (0.37 mg/ml) (○) in methanol, and the mixture was incubated for 10 min at 20°C. A portion (0.05 ml) was then added to a solution (0.95 ml) of pepsin (0.16 mg/ml) in 0.05 M-sodium acetate buffer, pH 5.5, and the mixture was incubated at 20°C. Samples were withdrawn periodically for assay. Inactivation of cathepsin D (0.02 mg/ml) (b) in 0.05 M-sodium acetate buffer, pH 5.1, was carried out by a similar procedure, except that the concentration of each inhibitor was twice that used in the experiment with pepsin. The smooth curves in (a) were computed by using a least-squares fit to the equation $[E] = [E]_0 e^{-kt}$ for pseudo-first-order kinetics. First-order kinetics were not observed with cathepsin D (b), possibly because the enzyme exists as an oligomer in solution.

Fig. 4. Gel filtration on Sephadex G-25 of pepsin inhibited by *N*-diazoacetyl-L-phenylalanine 3-phenyl[2,3-³H]propylamide

0.1 M-Cupric acetate (0.1 ml) was added to a solution (10 ml) of pepsin (1.6 mg/ml) in 0.05 M-sodium acetate buffer, pH 5.0, and the solution was incubated for 10 min at 20°C. A portion (0.4 ml) of a solution (8.6 mM) of inhibitor in methanol was then added. Incubation of this solution for 10 min at 20°C resulted in the complete loss of enzyme activity. 1 M-HCl (0.5 ml) was then added and the inhibited pepsin was purified by gel filtration on a column (25 cm × 1.5 cm) of Sephadex G-25 in water. Fractions (2.0 ml) were collected at a flow rate of 30 ml/h. Samples (50 μl) were withdrawn for liquid-scintillation counting. □, A_{280} ; ●, radioactivity; △, uptake of inhibitor by the inhibited enzyme [mol/mol (inset scale)].

This also proved that no exchange occurred with inhibitor that had already been incorporated.

The foregoing experiments enabled us to establish a protocol, detailed under 'Methods', for determining the operational molarity of solutions of pepsin. It was further shown that the radioactivity associated with the pepsin peak during gel filtration of the inactivated enzyme was proportional to the amount of enzyme. Calculation by the least-squares method of the linear regression curve relating radioactivity of the pepsin peak to amount of pepsin for six solutions of enzyme gave a line for which the ratio (s.d. of slope)/slope was 0.3%. Moreover, the ordinal and abscissal intercepts were not significantly different from zero by the *t* test (5%). By using reagent with a specific radioactivity of 6000 c.p.m./nmol, it was possible to titrate accurately amounts of pepsin down to 300 pmol.

The development of a reliable method for titrating solutions of cathepsin D was more difficult. Complete inactivation could be achieved only with enzyme concentrations ≤ 0.35 mg/ml, giving an upper limit of about 600 pmol for the titration. It was not possible, therefore, to demonstrate a stoichiometric uptake of inhibitor by the method used with pepsin. Moreover, it was found that, with low concentrations of cathepsin D, the labelled inactivated enzyme was substantially retained on columns of Sephadex G-25. This latter difficulty, which was probably due to hydrophobic binding, was overcome by incorporating 2% (v/v) of *t*-butyl alcohol in the eluting solvent. By using the protocol detailed under 'Methods', it

inactivated by unlabelled inhibitor would incorporate a further 0.01 mol/mol of enzyme during a 10 min incubation with labelled inhibitor at pH 5.5. No further incorporation, however, occurred when the second exposure to inhibitor was carried out at pH 2.3.

was shown that the radioactivity found in the void volume after gel filtration was directly proportional to the amount of enzyme used. Calculation by the least-squares method of the linear regression curve relating radioactivity of the cathepsin D peak to amount of cathepsin D for nine solutions of enzyme gave a line for which the ratio (s.d. of slope)/slope was 2.8%. Moreover, the ordinal and abscissal intercepts were not significantly different from zero by the *t* test (5%).

A preliminary study of the inactivation of chymosin by *N*-diazooacetyl-L-phenylalanine 3-phenylpropylamide

Fig. 5. Inactivation of chymosin by *N*-diazooacetyl-L-phenylalanine 3-phenylpropylamide

0.1 M-Cupric acetate (0.02 ml) was added to a solution (5 ml) of chymosin (0.13 mg/ml) in 0.05 M-sodium acetate buffer, pH 5.0, and the mixture was incubated for 10 min at 20°C. A solution (0.2 ml) of inhibitor (1.1 mg/ml) in methanol was then added and the solution was incubated at 20°C. Portions (0.5 ml) were withdrawn at intervals for assay.

mid was carried out with a commercial preparation of the enzyme. The rapid inactivation of the enzyme (Fig. 5) suggests that the labelled inhibitor could be used to titrate solutions of this and other acid proteinases.

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