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Fluorimetric Assays for Cathepsin B and Cathepsin H with Methylcoumarylamide Substrates

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Benzyloxycarbonyl-phenylalanyl-arginine 4-methyl-7-coumarylamide was found to be an excellent substrate for the fluorimetric assay of cathepsin B, and arginine 4-methyl-7-coumarylamide for cathepsin H. Procedures were developed that are very convenient, and avoid the hazards associated with the use of naphthylamides.

Cathepsin B, the best known of the lysosomal cysteine proteinases, has normally been assayed with either Bz-DL-Arg-NPhNO$_2$ (Otto, 1971; Barrett, 1972) or Bz-DL-Arg-2-NNap (Barrett, 1972, 1976) as substrate. The naphthylamide assay is much the more sensitive, because the substrate is more susceptible to the enzyme and the product is detectable at lower concentrations either by diazo-coupling (Barrett, 1972, 1976) or by direct fluorimetry (McDonald *et al.*, 1970).

A substrate far better still than Bz-Arg-2-NNap is Z-Arg-Arg-2-NNap, since the additional argininyl residue in P_2 (in the terminology of Schechter & Berger, 1967) increases $k_{cat.}/K_m$ over 100-fold (Table 1), and also increases the water-solubility of the substrate (McDonald & Ellis, 1975; Barrett, 1977, 1980). Z-Val-Lys-Lys-Arg-2-NNapOMe is an even more efficient substrate (MacGregor *et al.*, 1979).

The abbreviations used for substrates are based on the standard conventions for amino acids and *N*-terminal blocking groups [*Biochem. J.* (1972) **126**, 773–780]. The *C*-terminal leaving groups are: NMec, 4-methyl-7-coumarylamide; NNap, 2-naphthylamide; NNapOMe, 4-methoxy-2-naphthylamide; NPhNO$_2$, nitroanilide.

Cathepsin H is usually assayed with benzoyl-arginine 2-naphthylamide [and hence the enzyme is sometimes called 'BANA hydrolase' (Järvinen & Hopsu-Havu, 1975; Singh & Kalnitsky, 1978)], but this substrate offers no discrimination from cathepsin B, and leucine 2-naphthylamide or arginine 2-naphthylamide is preferable (Kirschke *et al.*, 1977*b*).

Increasingly, the use of naphthylamide substrates is causing concern on the grounds of their probable carcinogenicity, and so it is fortunate that an alternative type of derivative has been discovered, the 4-methyl-7-coumarylamides. The product, 7-amino-4-methylcoumarin, is an extremely efficient fluorogen (6.7 times more fluorescent than 4-methoxy-2-naphthylamine under typical assay conditions) (Castillo *et al.*, 1979).

I now describe direct fluorimetric assays based on the use of Z-Phe-Arg-NMec for cathepsin B, and Arg-NMec for cathepsin H, that are more sensitive than any previously described, safe and very convenient. The methylcoumarylamide substrates have previously been used for the assay of rat muscle enzymes, but without any attempt to optimize conditions or determine specificities (Hardy & Pennington, 1979).

Table 1. *Kinetic constants of some substrates of cathepsin B*

The values given were obtained at about pH 6.0 and 37°C with human cathepsin B (except where stated). The values for Bz-Arg-2-NNap are calculated for the L-isomer from data obtained with the racemate. Key to references: [1], C. G. Knight (personal communication); [2], MacGregor *et al.* (1979), for pig cathepsin B: [3], the present study.

	$k_{cat.}$ (s^{-1})	$10^3 K_m$ (M)	$k_{cat.}/K_m$ ($s^{-1} \cdot M^{-1}$)	Reference
Bz-Arg-2-NNap	9	4.3	2.1×10^3	[1]
Z-Arg-Arg-2-NNap	61	0.19	3.21×10^5	[1]
Z-Val-Lys-Lys-Arg-2-NNapOMe	68	0.14	4.86×10^5	[2]
Z-Phe-Arg-NMec	91	0.285	3.16×10^5	[3]

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Materials and Methods

Z-Phe-Arg-NMec and most other peptide methylcoumarylamides were purchased from the Peptide Research Foundation, Osaka, Japan; Arg-NMec, Z-Phe-Arg-NMec and 7-amino-4-methylcoumarin were from Bachem Feinchemikalien A.G., CH-4416 Bubendorf, Switzerland, and sodium monochloroacetate and Brij 35 from BDH Chemicals, Poole, Dorset, U.K. Human cathepsin B and cathepsin H were kindly provided by Dr. W. N. Schwartz, the cathepsin B having been purified essentially as described (Barrett, 1973) and the cathepsin H by an original method (W. N. Schwartz & A. J. Barrett, unpublished work). Rat cathepsin L (Kirschke *et al.*, 1977*a*) was kindly given by Dr. H. Kirschke.

The concentrations of enzyme solutions were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Values for specific activity and $k_{cat.}$ of the enzymes were calculated on the assumption that the preparations were fully active, and are therefore minimal estimates.

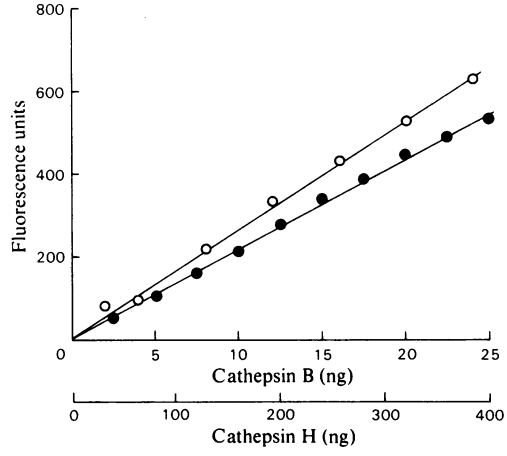

Fig. 1. *Typical standard curves for the assay of cathepsin B with Z-Phe-Arg-NMec* (●) *and cathepsin H with Arg-NMec* (○)

Experimental details are given in the text. The fluorimeter was adjusted so that 1000 arbitrary units corresponded to the release of 1 nmol of product.

Results and Discussion

Standard assay procedure for cathepsin B

The stock assay buffer contained KH_2PO_4 (352 mM), Na_2HPO_4 (48 mM) and disodium EDTA (4 mM), pH 6.0; cysteine (base) to 8 mM was added freshly before use. The substrate, Z-Phe-Arg-NMec, was dissolved in dimethyl sulphoxide as a 10 mM solution, which was stored at 4°C. For use, the substrate stock was freshly diluted to 0.02 mM with 0.1% Brij 35.

Tubes (PT 12/60; Luckam Ltd., Burgess Hill, Surrey RH15 9QN, U.K.) containing 500 μl of enzyme sample, diluted as necessary with 0.1% Brij 35 solution, and 250 μl of stock buffer, were prewarmed in a bath at 40°C for 2 min. The assays were started by introduction of 250 μl of 0.02 mM substrate solution into each tube with vigorous mixing. The final substrate concentration was thus 5 μM.

After 10 min incubation at 40°C the reaction was stopped by addition of 1 ml of 100 mM-sodium chloroacetate in a buffer containing sodium acetate (30 mM) and acetic acid (70 mM), pH 4.3.

Fluorescence was measured in a Locarte Mk. 4 single-sided fluorimeter fitted with an LF/2 filter (340–380 nm) in the excitation light-path, the emission monochromator being set to 460 nm. The instrument was zeroed against water and set to read 1000 arbitrary units (1.0 V output) with 0.5 μM-7-amino-4-methylcoumarin in the buffered sodium chloroacetate solution. A typical standard curve obtained with 2.5–25 ng of cathepsin B is shown in Fig. 1. [By contrast, 0.2–2 μg of cathepsin B would typically be used in the assay with Bz-DL-Arg-2-NNap, as described by Barrett (1972).]

One munit of activity is defined as that quantity releasing 1 nmol of aminomethylcoumarin/min. For the 10 min assay, a reading of 1000 therefore corresponds to 0.1 munit of activity in the tube. The specific activity of pure human cathepsin B was found to be 2.2 units/mg, under the conditions described.

Procedure for cathepsin H

The only changes made for cathepsin H were the substitution of Arg-NMec for Z-Phe-Arg-NMec, and a change in the composition of the assay buffer (to 200 mM-KH_2PO_4/200 mM-Na_2HPO_4) to adjust the pH to 6.8. It has been found that pH 6.8 is optimal for the action of human cathepsin H on this and most other synthetic substrates (W. N. Schwartz & A. J. Barrett, unpublished work).

A typical standard curve for 40–400 ng of cathepsin H is shown in Fig. 1. The specific activity of pure human cathepsin H was 0.14 unit/mg.

Continuous rate assays

Although the 'stopped' assays described above would be our choice for the determination of activity in large numbers of samples (column fractions etc.), continuous rate assays are preferable for kinetic studies. Such assays were made with reaction mixtures composed exactly as described above, in a thermostatically controlled (40°C) cell of a Farrand Optical Co. (New York) Mk. I spectrofluorimeter,

with excitation and emission monochromators set at 350nm and 460nm respectively. Good linear rates of increasing fluorescence were recorded so long as only 10% or so of the substrate was consumed.

Kinetics of Z-Phe-Arg-NMec hydrolysis by cathepsin B

To conserve substrate, kinetic parameters were determined with assays in which the volume of the incubation mixture was decreased 10-fold from the standard procedure, i.e. to 100μl. In the first experiment, 1ng of cathepsin B (0.04pmol) was used with substrate concentrations in the range 0.05–1.0mM. The measured reaction rates were analysed by the method of Wilkinson (1961), and gave vaues of K_m 0.285 ± 0.026 (S.E.) mM and $k_{cat.}$ 90.7 ± 0.36 (S.E.)s^{-1} ($k_{cat.}/K_m = 3.18 \times 10^5$ $s^{-1} \cdot M^{-1}$).

In a further experiment, assays were made with 4ng of cathepsin B/tube, and a range of substrate concentrations from 0.01 to 0.05mM. The slope of the linear relationship between initial rate of reaction and substrate concentration divided by enzyme concentration, which corresponds to $k_{cat.}/K_m$ (Knight, 1977), was $3.14 \times 10^5 s^{-1} \cdot M^{-1}$, in excellent agreement with the ratio calculated directly.

The sensitivity of the assay in this form has been found ample for most applications. The substrate concentration is far below K_m, however, so sensitivity can be greatly increased if necessary by increasing the concentration of substrate (with or without decrease in reaction volume). This applies also to the assay of cathepsin H.

Testing of other methylcoumarylamides as substrates for cathepsin B

Other methylcoumarylamides tested for susceptibility to hydrolysis by cathepsin B were Arg-NMec, Z-Arg-NMec, Pro-Phe-Arg-NMec, Boc-Val-Pro-Arg-NMec, Glt-Gly-Arg-NMec and Boc-Ile-Glu-Gly-Arg-NMec.

Pro-Phe-Arg-NMec was approximately half as sensitive as Z-Phe-Arg-NMec under the same conditions, but the other compounds were hydrolysed only to a very slight extent.

Both Z-Phe-Arg-NMec and Pro-Phe-Arg-NMec are excellent substrates of the kallikreins, which are serine proteinases (Morita *et al.*, 1977). It seems likely that their sensitivity to cathepsin B is mainly due to the phenylalanyl residue at P_2. This is what has been found previously for papain (Schechter & Berger, 1968), and for bovine cathepsin B with regard to inhibition by diazomethanes (Watanabe *et al.*, 1979).

Selectivity of the assay procedures

As has been mentioned, Z-Phe-Arg-NMec is a substrate of the kallikreins, but, in various human tissue extracts that I have assayed, activity not sensitive to thiol-blocking reagents has been barely detectable under my conditions. Nevertheless, it is recommended that controls with iodoacetate or chloroacetate be used to exclude interference by serine proteinases. Arg-NMec would be expected to be hydrolysed by any enzyme with aminopeptidase B-like specificity, and interference from such enzymes should be watched for. Tissue arylamidases tend not to be thiol-dependent, and are typically inhibited by 0.1mM-puromycin (Barrett & Poole, 1969), which has little action on cathepsin H (W. N. Schwartz & A. J. Barrett, unpublished work).

Bz-Arg-2-NNap is hydrolysed by both cathepsin B and cathepsin H about equally readily, and therefore does not allow selective assays (Kirschke *et al.*, 1977*b*; W. N. Schwartz & A. J. Barrett, unpublished work). I determined the activity of cathepsin H in my assay for cathepsin B, and vice versa. In each case, the activity was so low as not to be measurable with great accuracy, but it was clear that each enzyme gave less than 0.1% of the hydrolysis given by an equal weight of the enzyme for which the assay was designed. I also found that rat cathepsin L had very little or no activity in the assays, so they can be regarded as completely specific among the lysosomal cysteine proteinases for all normal purposes.

Conclusions

I conclude that the new fluorimetric assays for cathepsins B and H have sufficient advantages to justify their adoption for routine use in place of diazo-coupling assays with the naphthylamides, such as were described previously (Barrett, 1973, 1976). The methylcoumarylamide assays are not more expensive, although the substrates cost much more per milligram, because the quantity of substrate per assay is decreased from 4.5μmol/assay to 5nmol. In terms of safety, 2-naphthylamine is classified as a major carcinogenic hazard (Cater & Hartree, 1977). Few data are available for the aminomethylcoumarins, but they are unlikely to be carcinogenic, since they contain only a single aromatic ring (Cater & Hartree, 1977), and in any case they are used in almost 1000-fold smaller amounts, as is mentioned above.

I have found (not shown) that the fluorescence of aminomethylcoumarin is unaffected by pH over the range 4–7. This contrasts very favourably with the behaviour of 2-naphthylamine, and makes the methylcoumarylamides particularly suitable for experiments on the pH-dependence of enzymic activity.

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