Biochimica et Biophysica Acta, 1076 (1991) 149–151

ADONIS 0167483891000788

BBAPRO 30277

BBA Report

Photometric or fluorometric assay of cathepsin B, L and H and papain using substrates with an aminotrifluoromethylcoumarin leaving group

Jean Roger Tchoupé [1], Thierry Moreau [2], Francis Gauthier [2] and Joseph G. Bieth [1]

[1] *INSERM Unité 237, Université Louis Pasteur de Strasbourg, Illkirch and* [2] *CNRS URA 1334, Laboratoire des Protéines des Liquides Biologiques, Université François Rabelais, Tours (France)*

(Received 3 July 1990)

Key words: Cathepsin; Fluorometry; Papain; Cysteine proteinase; Lysosomal enzyme

***N*-trifluoromethylcoumarinylamide derivatives of benzyloxycarbonyl-Arg-Arg, benzyloxycarbonyl-Phe-Arg and Arg are convenient chromogenic and fluorogenic substrates of cathepsin B, L and H, respectively. Benzyloxycarbonyl-Phe-Arg-*N*-trifluoromethylcoumarinylamide is also a highly sensitive substrate for papain.**

Cathepsin B, L and H are lysosomal enzymes that belong to the papain superfamily of cysteine proteinases. They play a key role in intracellular protein turnover [1]. They are also thought to be involved in tumor invasion and metastasis [2]. Investigation of their physiological and pathological functions requires their assay in tissues and biological fluids. This is best done with synthetic substrates which are more convenient and more specific than denatured proteins, such as azocasein. Substrates with a 2-napthylamine leaving group have been widely used in the past. Most frequently, the release of 2-napthylamine was quantitated colorimetrically after coupling with a diazonium salt. Such assays are convenient because they are sensitive and do not need special equipment. They are still in use [2]. They do not, however, allow continuous rate measurements to be made.

One decade ago, novel sensitive substrates were introduced with 7-amino-4-methylcoumarin as a fluorogenic leaving group [1]. Z-Arg-Arg-NMec was found to be a very sensitive and specific substrate for cathepsin B while Z-Phe-Arg-NMec was hydrolyzed by both cathepsin B and L but not by cathepsin H. Further, Arg-NMec was a moderately sensitive, but highly specific substrate for cathepsin H [1]. These compounds are now widely used because they provide very sensitive assays of lysosomal cathepsins and allow reliable continuous rate measurements to be made. However, tissue extracts or biological fluids that are assayed for cathepsin activity may contain compounds that quench the fluorescence of the leaving group. There is thus still a need for chromogenic substrates whose hydrolysis may be followed continuously and which would have the same specificity as the above fluorogenic compounds. We thought of two colored leaving groups, *p*-nitroaniline ($\epsilon_{410nm} = 8800\ M^{-1} \cdot cm^{-1}$) and 7-amino-4-trifluoromethylcoumarin [3] ($\epsilon_{380nm} = 12600\ M^{-1} \cdot cm^{-1}$) as potential candidates for such chromogenic substrates. We therefore designed Arg-X, Z-Arg-Arg-X and Z-Phe-Arg-X (X = pNA or AFC) and tested them on cathepsin H, B and L. Papain was included for the sake of comparison.

Arg-NMec, Z-Arg-Arg-NMec and Z-Phe-Arg-NMec came from Bachem (Bubendorf, Switzerland). The corresponding pNA and AFC derivatives were synthesized for us by Bachem and Enzyme System Products (Livermore, CA), respectively. These new substrates are now commercially available from the corresponding companies. E-64 was from the Peptide Institute (Osaka, Japan).

Rat liver cathepsin B, L and H were purified as described previously [4]. The molarities of active enzyme solutions were determined by active site titration with E-64 [5]. Papain (Sigma) was further purified by FPLC chromatography on a Mono S column (Pharmacia, Paris). A 2 mg/ml solution of papain was prepared in a buffer containing 33 mM phosphate, 0.3 mM EDTA and 0.7 mM dithiothreitol (pH 6.8) and loaded

Abbreviations: Z, benzyloxycarbonyl; pNA, *p*-nitroanilide; NMec, *N*-methylcoumarinylamide; AFC, *N*-trifluoromethylcoumarinylamide; E-64, L-3-(trans-carboxyoxiran-2-carbonyl)-L-leucine-agmatine.

Correspondence: J.G. Bieth, INSERM Unité 237, Université Louis Pasteur de Strasbourg, F-67400 Illkirch, France.

onto the column. A small peak of inactive proteins was eluted upon washing the column with the above buffer. Two major overlapping peaks containing papain activity and one minor inactive protein peak appeared following salt gradient elution (0–0.5 M NaCl) of the adsorbed material. The active fractions were pooled, diluted and rechromatographed using a less steep gradient which sharply separated the two protein peaks. Active papain eluted as a symmetrical peak at an ionic strength of about 0.25 M NaCl. It migrated as a single band of $M_r = 25000$ on sodium dodecyl sulfate polyacrylamide gel electrophoresis [6]. Active site titration with E-64 [5] showed that the preparation contained more than 80% active papain, whereas the commercial compound was only about 6% active.

Initial velocities of enzymatic reactions were measured either spectrophotometrically at 410 or 380 nm for pNA and AFC substrates, respectively, or spectrofluorometrically for the NMec substrates ($\lambda_{ex} = 350$ nm, $\lambda_{em} = 460$ nm). Stock solutions of the enzymes were diluted into the buffer contained in a cuvette thermostated at 30°C. After thermal equilibration, the reaction was started by addition of an aliquot of substrate dissolved in dimethylformamide or dimethylsulfoxide and the release of product was recorded. A total of 6–9 substrate concentrations were used to measure the kinetic parameters k_{cat} and K_m. The composition of the reaction media is given in Tables I and II. The substrate/velocity data were fit by nonlinear regression analysis to the Michaelis-Menten rate equation (ENZFITTER program). This yielded the best estimates of k_{cat} and K_m as well as their standard error.

We designed the *p*-nitroanilides as potential substrates for cathepsin B, L and H with the hope that they would have enzyme detection limits close to those of the corresponding aminomethylcoumarinylamides. Our hypothesis was based on the observation that a number of serine proteinases exhibit higher k_{cat} values for peptide-pNA than for peptide-NMec substrates [7–9]. We therefore hoped that the lower photometric detection of *p*-nitroaniline as compared with the fluorometric detection of aminomethylcoumarin could be compensated for by significantly higher k_{cat} values of the chromogenic substrates. Table I shows that this is by far not the case: except for cathepsin H, k_{cat} (pNA) is lower than k_{cat} (NMec), thus rendering the *p*-nitroanilide substrates quite unsuitable for the sensitive chromogenic detection of cathepsin activity.

We then designed substrates with a 7-amino-3-trifluoromethylcoumarin leaving group which have the advantage of being both chromogenic (yellow color with $\epsilon_{380nm} = 12600$) and fluorogenic ($\lambda_{ex} = 400$ nm, $\lambda_{em} = 505$ nm) [3]. Table I shows that these substrates have k_{cat} values similar to or higher than those of the NMec compounds. Surprisingly however, substitution of the 4-methyl group of the NMec substrates by a 4-trifluoromethyl group in AFC substrates leads to a 5–7-fold decrease of the affinity of cathepsin B and L for their corresponding substrate. This effect is not observed with cathepsin H nor with serine proteinases of the trypsin family [3]. The proteolytic coefficients k_{cat}/K_m for Z-Arg-Arg-AFC and Z-Arg-Arg-NMec are of the same order of magnitude, while that of Z-Phe-Arg-AFC is 8-fold lower for than that of Z-Phe-Arg-NMec. This unfavorable effect apparently renders Z-Phe-Arg-AFC less sensitive than Z-Phe-Arg-NMec for the detection of cathepsin L activity. This drawback may be circumvented by using substrate concentrations close to or higher than the K_m of the AFC derivative, since the two substrates have similar k_{cat} values on the one hand, and both leaving groups yield about the same fluorescence intensity [3] on the other hand.

The specificity of the AFC substrates was found to be comparable to that of the NMec derivatives [1,10]. For instance, Z-Phe-Arg-AFC is also a good substrate for cathepsin B ($k_{cat} = 38$ s^{-1}, $K_m = 0.13$ mM). Substrates with P1 = Arg may also be cleaved by trypsin-like enzymes, such as kallikrein. These proteinases are however insensitive to heavy metals and oxidants. If cysteine proteinase activity is to be measured in complex biological fluids or tissues, it is advisable to run a control without EDTA and dithiothreitol in order to detect and substract possible trypsin-like activity.

The AFC substrates have several advantages over the NMec derivatives. Firstly, they are also chromogenic and can thus be used in spectrophotometric assays. The sensitivity of the latter is about 50-fold lower than that of fluorometric assay. Yet, the activity of 1 nM cathepsin L can easily be measured using 60 μM Z-Phe-Arg-AFC ($\Delta A_{380nm} \approx 0.015$ per min.). Secondly, the

TABLE I

Action of rat liver cathepsin B, L and H on substrates with different leaving groups

The buffer was 100 mM phosphate, 1 mM EDTA, 2 mM dithiothreitol pH 6.0 (cathepsin B and L) or pH 6.8 (cathepsin H). The final reaction medium contained 5% (v/v) dimethylformamide. The temperature was 30°C throughout. To avoid overcrowding of the table, the errors on the kinetic parameters are not given and all values are rounded up. The standard errors on k_{cat}, K_m and k_{cat}/K_m were equal or lower to 13, 21 and 34%, respectively

Enzymes	Substrates	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Cathepsin B	Z-Arg-Arg-pNA	15	1.00	15
	Z-Arg-Arg-NMec	23	0.23	100
	Z-Arg-Arg-AFC	42	1.10	40
Cathepsin L	Z-Phe-Arg-pNA	20	0.036	550
	Z-Phe-Arg-NMec	33	0.006	5500
	Z-Phe-Arg-AFC	28	0.042	660
Cathepsin H	Arg-pNA	13	0.2	65
	Arg-NMec	8	0.1	80
	Arg-AFC	10	0.1	110

TABLE II

Action of papain on cathepsin B and L substrates with different leaving groups

The buffer was composed of 100 mM phosphate, 1 mM EDTA, 2 mM dithiothreitol, pH 6.8. The reaction medium contained 5% (v/v) dimethylsulfoxide and the temperature was 30°C. The standard errors are within the limits given in Table I and the data are rounded up

Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Z-Arg-Arg-pNA	0.3	3.20	0.1
Z-Arg-Arg-NMec	0.2	0.36	0.5
Z-Arg-Arg-AFC	0.6	2.60	0.2
Z-Phe-Arg-pNA	33	0.14	240
Z-Phe-Arg-NMec	34	0.08	430
Z-Phe-Arg-AFC	9	0.06	150

peptide-AFCs are blue in fluorescence while free AFC has a yellow-green fluorescence. In contrast, both free and acylated NMec fluoresce in the blue region with overlapping spectra. Hence, fluorescent detection of enzyme activity in cells or on electrophoretic gels is impossible with the NMec substrates, but quite easy with the AFC substrates [3]. Thirdly, working with high NMec substrate concentrations strongly decreases the sensitivity of the proteinase assay, since λ_{ex} and/or λ_{em} have to be shifted to avoid unmanageable interference with the substrate. It is also worthwhile mentioning that the present AFC substrates have been successfully used to monitor the inhibition of rat liver cathepsin by fluorescent mansyl-peptides whose excitation spectra overlap those of the NMec substrates, but not those of the AFC derivatives [11].

Table II shows that Z-Phe-Arg-AFC may also be used as a convenient substrate for papain. In the fluorometric detection mode, it is about 3-fold less sensitive than Z-Phe-Arg-NMec, but unlike this substrate, it allows a sensitive spectrophotometric assay of papain: the activity of 2 nM papain can easily be measured using 1 mM Z-Phe-Arg-AFC ($\Delta A \approx 0.015$ per min). Various pNA substrates have been proposed in recent years for the assay of papain [12–14]. None of these chromogenic substrates allows papain activity to be measured with a sensitivity comparable to that provided by Z-Phe-Arg-AFC used in its photometric assay mode.

The authors thank the Association pour la Recherche sur le Cancer (ARC, Paris) and the Ligue Nationale contre le Cancer (Colmar) for financial support.

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