Biochemical and Biophysical Research Communications **277,** 79–82 (2000)
doi:10.1006/bbrc.2000.3633, available online at http://www.idealibrary.com on IDEAL®

Partial Characterization of a Novel Cathepsin L-like Protease from *Fasciola hepatica*

Susan J. Hawthorne,*[,1] Maurice Pagano,† David W. Halton,‡ and Brian Walker*

**Biomedicinal Chemistry Group, School of Pharmacy, Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, Northern Ireland, BT9 7BL; †Biochimie de Protéases, Faculté de Médecine, Broussais Hôtel-Dieu, Université Pierre et Marie Curie, 15 Rue de l'école de Médecine, 75270 Paris Cedex 06, France; and ‡School of Biology and Biochemistry, Queen's University of Belfast, Medical Biology Centre, 97 Lisburn Road, Belfast, Northern Ireland, BT9 7BL*

Received August 5, 2000

A 30-kDa protease, purified previously from *Fasciola hepatica,* was sequenced and the first 15 N-terminal residues were found to be 100% homologous to a region in the protein Fcp1c, which was cloned and expressed from *F. hepatica.* This terminal region was also 53 and 54% identical to two other cathepsin L-like proteases isolated from the same source. The 30-kDa protease demonstrated a specificity different from human cathepsin L when assayed with novel peptidyl enediones of the type Z-Phe-Ala-CH═CH_2-CO_2R (where R = Me/Et/Bu^t). The ethyl ester peptide was a more efficient inhibitor of the protease than the corresponding methyl ester. This is in contrast to bovine cathepsin B and human cathepsin L where both are more readily inhibited by the methyl, rather than the ethyl ester peptide. These differences in the inhibition of the novel parasite protease may allow it to be exploited as a chemotherapeutic target. © 2000 Academic Press

***Key Words: Fasciola hepatica;* cysteine protease; cathepsin L-like; inhibition.**

Fasciola hepatica, a parasitic trematode, is of substantial commercial importance in temperate agricultural countries where it parasitises livestock (1). It can infect a wide range of animals including sheep, goats and cattle, and even humans, leading to chronic infections (2). Due to its economic importance it has been the subject of many scientific investigations. It has been shown previously that adult *F. hepatica,* when maintained *in vitro,* secrete a battery of cysteine proteases and it is thought that these proteases assist the parasite in its migration through the liver parenchyma (3). Two proteases have previously been purified from the excreted/secreted products of *F. hepatica* and were found to have cathepsin L-like amino acid sequences as well as substrate preferences (4, 5).

This work involved the N-terminal sequencing and partial kinetic characterisation of a third protease released by *F. hepatica in vitro* (6). Partial kinetic characterisation of this protease was achieved using a novel group of cysteine protease-specific inhibitors, the enediones, based on the general sequence Z-Phe-Ala-CH═CH_2-CO_2R (where R = Me/Et/Bu^t) (7) (Fig. 1).

MATERIALS AND METHODS

Materials. Bovine cathepsin B was purchased from Sigma Chemical Co. (Poole, Dorset, England). Human cathepsin L was purified as described by Dalet-Fumeron, Guinec & Pagano (8). *F. hepatica* cathepsin L-like protease was purified as described by Hawthorne *et al.* (6). The peptidyl enediones were synthesised as described by Darkins (7). Polyvinylidene fluoride (PVDF) membrane (0.2 micron) was from BioRad Ltd. (Hemel Hempstead, Hertfordshie, England). Z-Arg-Arg-AMC and Z-Phe-Arg-AMC were purchased from Bachem (Bubendorf, Switzerland).

Protein sequencing. The method used for sample preparation and blotting was a modified version of that described by Matsudaira (9). To a sample of purified protease (40 μl, 4.2 μg) was added ×5 Laemmli treatment buffer (10) and the samples boiled. Reduced and denatured samples were then loaded onto a 5–15% gradient polyacrylamide gel and electrophoresed at 30 mA for 1.5 h. After electrophoresis, the gel was washed in transfer buffer, on an orbital shaker, for 5 min to reduce the amount of SDS in the gel. During this time the PVDF membrane was soaked in 100% methanol for 10 s and placed in transfer buffer for 5–10 min. The gel, sandwiched between the PVDF membrane and several layers of filter paper, was blotted for 10 min at 110 mA and then for 80 min at 90 mA. After blotting, the PVDF membrane was washed in water (3 × 5 min) and stained with Amido Black (isopropanol:water, 3:1; 0.1% Amido Black w/v) for 1 min and destained (isopropanol:water, 3:1) until the bands could be clearly seen and the background was reduced. After staining, the membrane was rinsed in water and air dried prior to sequencing.

Sequencing was carried out on an Applied Biosystems 476A Protein Sequencer at University of Leicester, Leicester, England.

Fluorimetric inactivation studies. The inhibitory effects of a variety of peptidyl enediones were tested against bovine cathepsin B, human cathepsin L, and the novel 30-kDa cathepsin L-like protease. An aliquot of the inhibitor in DMF (1 mM) was added to a solution of

[1] To whom correspondence should be addressed. Fax: +44 (0) 28 9024 7794. E-mail: s.hawthorne@qub.ac.uk.

0006-291X/00 $35.00

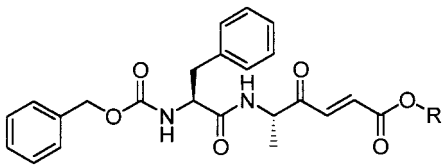

FIG. 1. Structure of the peptidyl enedione inhibitors, where R = Me/Et/Bu^t.

the enzyme being assayed (20–40 μl) in 100 mM sodium phosphate buffer, pH 6.4, containing 2 mM cysteine hydrochloride, 1 mM EDTA and 0.1% Brij 35 (final volume 1 ml), so that the final concentration of inhibitor was 5–200 μM, and the sample incubated at 37°C.

Aliquots (20 μl) were removed at 10 min intervals over a 30 min period and assayed for residual activity using Z-Phe-Arg-AMC for cathepsin L-like activity or Z-Arg-Arg-AMC for cathepsin B-like activity (50 μM final concentration). Hydrolysis of the substrates was monitored at 455 nm (exc. 383 nm). The dilution involved in setting up the assay stopped further reaction with the inhibitor. The resultant traces were used to determine the kinetic constants for each inhibitor. Fluorescence measurements were carried out on a Perkin Elmer Luminescence Spectrometer LS30 (Buckinghamshire, England).

RESULTS

N-Terminal Sequencing

The N-terminal sequence of the purified 30-kDa protease is shown in Fig. 2. When a database sequence search was carried out, it was found that the sequence was 100% homologous to a region of the cathepsin L-like Fcp1c, which was cloned and expressed from RNA isolated from adult *F. hepatica* by (11). The N-terminal sequence was also compared with N-terminal sequences of other cathepsin Ls and other related proteases (Table 1) and was found to be 53% identical to the cathepsin L isolated by Smith *et al.* (4) and 54% identical to that isolated by Dowd *et al.* (5). The novel 30-kDa protease sequence is also 53% identical to the N-terminal of liver cathepsin L from chicken-, rat-, human-, and bovine-species. In addition, it can be seen from Table 1, that residues 2, 6, 8, 13, and 14 are conserved in all the sequences.

Fluorimetric Inactivation Studies

Hydrolysis of Z-Phe-Arg AMC by human cathepsin L and the 30-kDa protease and Z-Arg-Arg-AMC by bovine cathepsin B could be blocked by prior incubation with the enediones. Figure 3 shows the inactivation of the 30-kDa protease activity by Z-Phe-Ala-CH═CH_2-CO_2Et, the most potent inhibitor of this enzyme. Table 2 shows the kinetic parameters for the inactivation of

1- VPESIDWRDYYYVTE -15

FIG. 2. N-terminal sequence of *F. hepatica* purified 30-kDa cathepsin L-like protease.

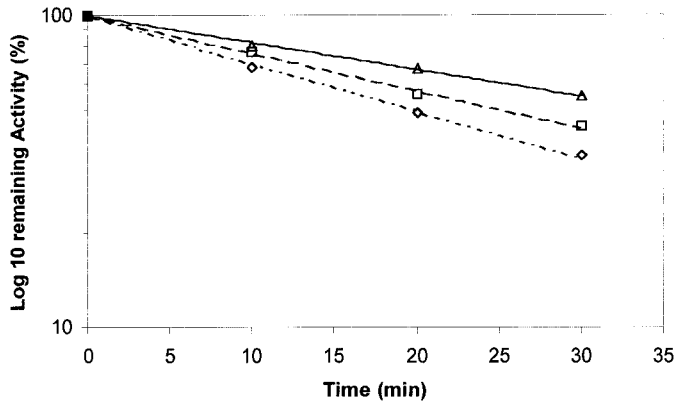

FIG. 3. Inactivation of *Fasciola hepatica*-purified 30-kDa cathepsin L-like protease by Z-Phe-Ala-CH═CH_2-CO_2Et at 5 μM (◇), 1 μM (□), and 0.1 μM (△) using the substrate Z-Phe-Arg-AMC (50 μM). For sake of clarity error bars have been omitted. Each point is the mean of 3 values.

bovine cathepsin B, human cathepsin L, and 30-kDa protease, by the peptidyl enediones.

DISCUSSION

The N-terminal of the 30-kDa protease purified from *F. hepatica* E/S products was shown to be 100% homologous to the N-terminal of Fcp1c (11). Fcp1c encodes an immature protein (proenzyme) of 326 amino acids with a molecular weight of 38 kDa. It is thought that the proenzyme is processed by the cleavage of the proregion, to produce an active enzyme (30 kDa). Although the 30-kDa mature cathepsin L-like protease was not purified by Heussler and Dobbelaere (11), they detected it on Western blots using antibodies raised against the recombinant 38-kDa proenzyme. The N-terminal sequence information suggests that the 30-kDa protease purified from *F. hepatica* concentrated conditioned media may be the processed (mature) form of the 38 kDa proenzyme. The fact that the 30-kDa protease also shows only limited N-terminal sequence homology to other cathepsin L-like proteases isolated from *F. hepatica* E/S products suggests that they are distinct.

Since the 30-kDa protease displayed a cathepsin L-like N-terminal sequence, we attempted to characterise it kinetically using a novel group of cysteine protease inhibitors, peptidyl enediones. Enediones, with the sequence Z-Phe-Ala-CH═CH_2CO_2Me/Et/Bu^t, were made as possible inhibitors of cathepsin B/L-like proteases and their potency tested against bovine cathepsin B, human cathepsin L, and the 30-kDa protease, by fluorimetry.

As can be seen from Table 2, the peptide Z-Phe-Ala-CH═CH_2CO_2Me is an efficient inhibitor of bovine cathepsin B with a second order rate constant of 85 mM^{-1} min^{-1}. This result implies that enediones are slightly

TABLE 1

Comparison of the N-Terminal Sequence of Cathepsin L from Different Sources (Residues 1–15)

Cathepsin L source	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
30-kDa protease	V	**P**	E	S	I	**D**	W	**R**	D	Y	Y	Y	**V**	**T**	E
F. hepatica (1)	V	**P**	D	K	I	**D**	P	**R**	E	S	G	Y	**V**	**T**	G
F. hepatica (2)	V	**P**	D	K	I	**D**	R	**R**	E	S	G	Y	**V**	—	—
Chicken liver	A	**P**	R	S	V	**D**	W	**R**	E	K	G	Y	**V**	**T**	P
Rat liver	I	**P**	R	S	V	**D**	W	**R**	E	K	G	Y	**V**	**T**	P
Human liver	A	**P**	R	S	V	**D**	W	**R**	E	K	G	Y	**V**	**T**	P
Bovine liver	L	**P**	D	S	V	**D**	W	**R**	E	K	G	G	**V**	**T**	P

Note. The N-terminal sequence of the 30-kDa protease was compared to the N-terminal sequence of *F. hepatica*-1 cathepsin L (4), *F. hepatica*-2 cathepsin L (5), chicken liver cathepsin L (14), rat liver cathepsin L (15), human liver cathepsin L (16), and bovine liver cathepsin L (17).

more potent inhibitors than the corresponding diazomethyl ketone, which inactivates bovine cathepsin B with a second order rate constant of 54 mM^{-1} min^{-1} (12). Z-Phe-Ala-CH═CH_2-CO_2Et is a slightly less effective inhibitor with a second order rate constant of 20 mM^{-1} min^{-1} and Z-Phe-Ala-CH═CH_2-CO_2Bu^t is a very poor inhibitor with a second order rate constant of less than 10 M^{-1} min^{-1}. These results demonstrate that as the ester side chain (R) increases in steric bulk from a methyl group to a *t*-butyl group, inhibitor efficacy is lost. There is only a slight drop in inhibitor potency between the methyl and ethyl ester peptides but the second order rate constant drops dramatically for the *t*-butyl ester peptide. This suggests that the *t*-butyl sidechain is much too large to fit into the active site of bovine cathepsin B thereby greatly reducing the effectiveness of the inhibitor.

Both Z-Phe-Ala-CH═CH_2-CO_2Me and Z-Phe-Ala-CH═CH_2CO_2Et are inhibitors of human cathepsin L, although they are less potent against this enzyme than they are against bovine cathepsin B (28.4 mM^{-1} min^{-1} and 2.34 mM^{-1} min^{-1}, respectively). This is to be compared with the exactly opposite findings employing diazomethyl ketone inhibitors. In this instance, the sequence Z-Phe-Ala-CHN_2 is a more potent inhibitor of L than B (13). This implies that it may well be possible to exploit differences in active-site topography in the S1 subsite in the cysteine proteases. The results suggest that the peptide sequence used is not suitable as an inhibitor for human cathepsin L. The effect of the ester sidechain bulk on the potency of the inhibitor mirrors that seen for bovine cathepsin B with the butyl ester peptide having no inhibitory effects towards human cathepsin L whatsoever.

Z-Phe-Ala-CH═CH_2-CO_2Me efficiently inactivates the *F. hepatica* cathepsin L-like protease, with a second order rate constant of 372 mM^{-1} min^{-1} and Z-Phe-Ala-CH═CH_2-CO_2Et is even more effective (500 mM^{-1} min^{-1}). This is in contrast to bovine cathepsin B and human cathepsin L where both are more readily inhibited by the methyl, rather than the ethyl ester peptide. The 30-kDa protease is also inactivated, although to a much lesser extent, by Z-Phe-Ala-CH═CH_2-CO_2Bu^t (0.8 mM^{-1} min^{-1}). These results suggest that it has a

TABLE 2

Kinetic Parameters for the Inactivation of Bovine Cathepsin B, Human Cathepsin L, and the 30-kDa Protease by Peptidyl Enediones of the General Sequence Type Z-Phe-Ala-CH═CH_2-CO_2R

Enzyme	Inhibitor	First-order rate constant k_i (min^{-1})	Steady-state inhibitor constant K_i (μM)	Second-order rate constant k_i/K_i (mM^{-1} min^{-1})
Cathepsin B	Z-Phe-Ala-CH═CH_2-CO_2Me	N.D.	N.D.	85
	Z-Phe-Ala-CH═CH_2-CO_2Et	N.D.	N.D.	20
	Z-Phe-Ala-CH═CH_2-CO_2Bu^t	N.D.	N.D.	<10 (M^{-1} min^{-1})
Cathepsin L	Z-Phe-Ala-CH═CH_2-CO_2Me	0.125	4.4	28.4
	Z-Phe-Ala-CH═CH_2-CO_2Et	0.095	40	2.34
	Z-Phe-Ala-CH═CH_2-CO_2Bu^t	—	—	—
30-kDa protease	Z-Phe-Ala-CH═CH_2-CO_2Me	0.067	0.18	372
	Z-Phe-Ala-CH═CH_2-CO_2Et	0.03	0.06	500
	Z-Phe-Ala-CH═CH_2-CO_2Bu^t	0.04	50	0.8

Note. N.D. = not determined.

much larger active site than both bovine cathepsin B and human cathepsin L and can accommodate the larger *t*-butyl sidechain. The ethyl ester peptide may be a better inhibitor than the methyl ester peptide because the ethyl sidechain occupies the active site more fully. Z-Phe-Ala-CH═CH_2-CO_2Et is a much more effective inhibitor of the novel 30-kDa protease than the DNP-labelled inhibitor tested previously (22.4 mM^{-1} min^{-1}) (6).

These results indicate that peptidyl enediones are potent inhibitors of cysteine proteases being more effective than the corresponding diazomethyl ketones. Moreover, the differences in inhibitor efficacy between the mammalian and parasite proteases may prove useful in the design of inhibitors as possible chemotherapeutic agents for use in parasite systems.

REFERENCES

1. Shubkin, C. D., White, W. W., Abrahamson, M. S., Rognile, M. C., and Knapp, S. E. (1992) A nucleic acid-based test for the detection of *Fasciola hepatica*. *J. Parasitol.* **78,** 817–821.
2. Milbourne, E. A., and Howell, M. J. (1993) Eosinophil differentiation in response to *Fasciola hepatica* and its excreted/secreted antigens. *Int. J. Parasitol.* **23,** 1005–1009.
3. Dalton, J. P., and Heffernan, M. (1989) Thiol proteases released by *Fasciola hepatica*. *Mol. Biochem. Parasitol.* **35,** 161–166.
4. Smith, A. M., Dowd, A. J., McGonigle, S., Keegan, P. S., Brennan, G., Trudgett, A., and Dalton, J. P. (1993) Purification of cathepsin L-like proteinase secreted by *Fasciola hepatica*. *Mol. Biochem. Parasitol.* **62,** 1–8.
5. Dowd, A. J., Smith, A. M., McGonigle, S., and Dalton, J. P. (1994) Purification and characterisation of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica*. *Eur. J. Biochem.* **223,** 91–98.
6. Hawthorne, S. J., Pagano, M., Halton, D. W., and Walker, B. (1998) The synthesis and utilisation of 2,4-dinitrophenyl-labeled irreversible peptidyl diazomethyl ketone inhibitors. *Anal. Biochem.* **261,** 131–138.
7. Darkins, P. (1995) Synthesis and enzyme inhibitory properties of novel proteinase inhibitors. Ph.D. thesis. Queen's University of Belfast.
8. Dalet-Fumeron, V., Guinec, N., and Pagano, M. (1991) HPLC method for the simultaneous purification of cathepsin B, cathepsin H and cathepsin L from human liver. *J. Chromat. Biomed. Appl.* **568,** 55–68.
9. Matsudaria, P. (1987) Sequence from picomole quantities of proteins electroblotted onto PVDF membranes. *J. Biol. Chem.* **262,** 10035–10038.
10. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature, London* **277,** 680–685.
11. Heussler, V. T., and Dobbelaere, D. A. E. (1994) Cloning of a gene family of *Fasciola hepatica* by the polymerase chain reaction. *Mol. Biochem. Parasitol.* **64,** 11–23.
12. McGinty, A. (1991) The design, synthesis and application of novel inhibitors of cysteine proteases. Ph.D. thesis. Queen's University of Belfast.
13. Barrett, A. J., and Kirschke, H. (1981) Cathepsin B, cathepsin H and cathepsin L. *Meths. Enzymol.* **80,** 535–561.
14. Dufour, E., Obled, A., Valin, C., and Bechet, D. (1987) Purification and amino acid sequence of chicken liver cathepsin L. *Biochem.* **26,** 5689–5695.
15. Ishidoh, K., Towatari, T., Imajoh, S., Kawasaki, H., Kominami, E., Katunuma, N., and Suzuki, K. (1987) Molecular cloning and sequencing of cDNA for rat cathepsin L. *FEBS* **223,** 69–73.
16. Mason, R. W., Walker, J. E., and Northrop, F. D. (1986) The N-terminal amino acid sequences of the heavy and light chains of human cathepsin L. *Biochem. J.* **240,** 373–377.
17. Turk, V., Brzin, J., Lenatcic, B., Locnikar, P., Popovic, T., Ritanja, A., Babnik, J., Bode, W., and Machleidt, W. (1985) *In* Intracellular protein catabolism (Khairallah, E. A., Bond, S., and Bird, J. W. C., Eds.).