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Veterinary Parasitology 107 (2002) 73–83

veterinary parasitology

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A preliminary analysis of proteolytic activity of excretory–secretory products from Cyathostominea

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Received 24 September 2001; received in revised form 28 February 2002; accepted 4 April 2002

Abstract

The excretory–secretory product (ESP) derived from Cyathostominea in vitro was assessed, in terms of subunit composition, and proteolytic activity using as substrates azocasein and two synthetic fluorogenic peptides. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) resolved 13 subunits, and the presence of the protein cysteine proteinase activator dithiothreitol (DTT) revealed 21 subunits. DTT also enhanced azocaseinolysis, and hydrolysis of carbobenzoxy-phenylalanyl-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-NHMec) and carbobenzoxy-arginyl-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-NHMec). At the optimum pH of 5.5, hydrolysis of Z-Phe-Arg-NHMec was three-fold greater than that of Z-Arg-Arg-NHMec suggesting that the proteolytic specificities of the ESP are more like those of papain or cathepsin L, rather than cathepsin B. In SDS-PAGE gelatin gels, DTT was a requirement for proteolysis by the ESP. Optimum resolution was at pH = 5.5, resolving six bands ranging from 114–20 kDa. Cysteine proteinase inhibitors abolished all gelatinolytic activity at the pH values tested. Such data indicate the presence of cysteine-class proteinases in the ESP of Cyathostominea. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Cyathostominea; Excretory–secretory product (ESP); Cysteine proteinases

1. Introduction

Nematodes of the subfamily Cyathostominea are now regarded as the principal parasitic pathogens of the horse, *Equus caballus* (Langrova, 1998). The marked decline in prevalence of large strongyle infections has not dramatically reduced the occurrence of

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PII: S0304-4017(02)00087-0

strongyle-associated disease, and clinical attention is now focusing on the pathogenicity of cyathostomins. More than 50 species of Cyathostominea have been documented (Lichtenfels et al., 1998) and infected horses often harbour burdens of tens of thousands of cyathostomins, mostly comprising 5–10 common species (Gawor, 1995).

Adult worm burdens are subclinically associated with general debilitation, weight loss, poor appetite, and disordered intestinal motility (Matthews and Morris, 1995). Cyathostomins have also been associated with a non-specific mild equine colic (Uhlinger, 1990), and specific forms of colic, including non-strangulation infarction (Mair and Pearson, 1995), caecocaecal intussusception (Mair et al., 2000), and caecal tympany (Murphy et al., 1997). These parasites are further associated with a distinctive and acute clinical syndrome known as larval cyathostominosis. The syndrome has a case fatality rate of 50% (Love and McKeand, 1997), and is a potentially fatal enteritis which is directly associated with the synchronous mass emergence of developing larvae from the caecal and colonic mucosa (Abbott, 1998).

There is a paucity of information surrounding the detailed biology and pathogenic mechanisms of the cyathostomins. In particular, there seems to be no quantification of proteolytic activities in Cyathostominea and their role in pathogenicity.

Proteinases may play a crucial role in the pathogenicity of cyathostomin infections as shown for other parasites such as *Anisakis simplex*, *Schistosoma mansoni* and *Fasciola hepatica* (Morris and Sakanari, 1994; Smith et al., 1994; Halton, 1997, respectively). Their biological functions may include digestion of host protein for acquisition of nutrients, facilitation of migration through tissues, and inactivation of host immune effector molecules (Gotz and Klinkert, 1993; Song and Kim, 1994; Smith et al., 1993).

Furthermore, proteinases also serve as potential targets for serodiagnosis and vaccine development, as has been shown for *F. hepatica*, *S. mansoni*, *Paragonimus westermani* and *Haemonchus contortus* (Cornelissen et al., 2001; Grogan et al., 1997; Ikeda et al., 1996; Jasmer et al., 2001, respectively). This study presents a preliminary analysis of proteolytic activity in the excretory–secretory product (ESP) of Cyathostominea. The identities, prevalence, and intestinal distribution of Cyathostominea in Ireland, will be the subject of a future report (Kinsella, Lichtenfels and Ryan, in preparation).

2. Materials and methods

2.1. Preparation of excretory–secretory products (ESP) from Cyathostominea

On 14 separate occasions between November 1997 and August 1998, Cyathostominea were removed from the intestinal contents, caecal walls, and ventral and dorsal colon of 63 horses, freshly slaughtered at the Irish Horse Abattoir Trading Co. Ltd., (Straffan, Co. Kildare Ireland). The parasites were removed with the aid of fine forceps and horse hair paintbrushes, placed in RPMI-1640 medium (Dutch Modification), pH = 7.3, containing 200 IU ml^{-1} streptomycin, 200 $\mu g\,ml^{-1}$ penicillin, 20 $\mu g\,ml^{-1}$ gentamicin, 4 $\mu g\,ml^{-1}$ amphotericin B, and 0.5% (w/v) glucose at 37 °C and immediately transported to the laboratory. The worms were washed extensively in RPMI-1640 at 37 °C, pH = 7.3, prior to the collection of ESP.

Batches of worms incubated at 37 °C in the above medium (15–50 per 5 ml) were checked every 8 h to remove non-motile worms. The medium was replaced after 24 h and incubations usually continued for 48 h. The collected medium was sterile-filtered through a 0.2 μm millipore filter, and concentrated 20-fold over a YM10 flat ultrafiltration membrane, cut-off at 10 kDa, in a Model 8010 ultrafiltration cell at 4 °C. ESP was frozen in liquid nitrogen and stored at −70 °C. The ESP from the 14 occasions was pooled for this study and was concentrated a further 15-fold using microconcentrators, cut-off at 3 kDa (Amicon). ESP was again frozen in liquid nitrogen and stored at −70 °C. The nematodes were cleared in phenol–alcohol (80% melted phenol crystals and 20% absolute ethanol) and stored in 70% ethanol. Identifications were made with the aid of a light microscope using the keys of Lichtenfels (1975) and Lichtenfels et al. (1997); names of the cyathostomin species follow the recommendations of Lichtenfels et al. (1998). The Bradford method (Bradford, 1976) was used to ascertain protein concentrations.

2.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE was carried out in mini slab gels (ATTO corporation). Samples incorporating 10 μl ESP and 5 μl sample buffer (187.5 mM Tris–HCl (pH = 6.8 at 25 °C), 6% (w/v) SDS, 30% glycerol and 0.03% (w/v) phenol red), were boiled for 1 min in the presence or absence of 5 mM dithiothreitol (DTT; Sigma). Samples were applied to a 3% stacking gel and resolved through a 12.5% resolving gel (Laemmli, 1970), over 3 h at room temperature. After electrophoresis, gels were fixed and stained using the Biorad® Silver Staining procedure, and were analysed using GeneTools (Syngene).

Molecular weight markers (Sigma) were as follows: MBP-ß-galactosidase (175 kDa), MBP-paramyosin (83 kDa), glutamic dehydrogenase (62 kDa), aldolase (47.5 kDa), triphosphate isomerase (32.5 kDa), ß-lactoglobulin A (25 kDa), lysozyme, (16.5 kDa) and aprotinin (6.5 kDa).

2.3. Azocasein as a substrate

Activities of proteinases in solution were estimated using azocasein as a substrate. Assays at 37 °C incorporated 10 μl ESP (1.76 μg protein), and 500 μl of a designated buffer, 0.1 M sodium citrate (pH = 3–5), 0.1 M sodium phosphate (pH = 6 and 6.5) and 0.1 M Tris–HCl (pH = 7–9) containing 0.25% (w/v) azocasein (Sigma). DTT when added, was at a final concentration of 5 mM. The reaction was stopped by adding an equal volume of 10% (w/v) trichloroacetic acid (TCA). Centrifugation followed at 13,000 rpm for 10 min in a Heraeus Sepatech microcentrifuge, to remove undigested material. Supernatant absorbances at 366 nm were determined in a Beckman DU® 650 spectrophotometer, and activity was expressed as μmol azopeptides released min^{-1} mg^{-1} protein. $E^{1\%}_{366}$ of azopeptides was taken as 40.

2.4. Synthetic fluorogenic substrates

Proteinase activity was measured fluorometrically using the synthetic peptides, carbobenzoxy-phenylalanyl-arginine-7-amido-4-methylcoumarin (Z-Phe-Arg-NHMec) and

carbobenzoxy-arginyl-arginine-7-amido-4-methylcoumarin (Z-Arg-Arg-NHMec, Sigma). The reaction mixture contained 102.5 μl of a designated buffer and 2.5 μl (0.44 μg) ESP pre-incubated for 10 min at 37 °C. The reaction was started by introducing one of the peptide substrates (Z-Phe-Arg-NHMec) or (Z-Arg-Arg-NHMec), bringing the final substrate concentration to 35 μM. When present, DTT was at a final concentration of 5 mM. Reactions were stopped by the addition of iodoacetic acid (IA) to a final concentration of 10 mM following incubation at 37 °C, for 12 min in the presence of DTT and for 20 min in its absence. Fluorescence was measured in a final volume of 2 ml (made up with dH_2O) using a Sequoia-Turner fluorometer (model 450; excitation 360 nm, emission 460 nm). Enzyme activity was expressed as nmol NMec released min^{-1} mg^{-1} ESP, as calculated from a standard curve constructed with free NMec.

2.5. Gelatin-substrate SDS-PAGE

Gelatin-substrate SDS-PAGE was adapted from the method described by Heussen and Dowdle (1980). Both 3% stacking gels and 10% resolving gels were prepared as described (Laemmli, 1970) but with the addition of 0.1% bovine skin gelatin (Sigma) to the resolving gels. Samples incorporating 10 μl (1.76 μg) ESP and 5 μl sample buffer were applied to the stacking gel. Electrophoresis over 4–5 h, at a constant current of 22 mA, was performed at 4 °C.

Following electrophoresis, gels were sliced and then washed for 90 min at room temperature in two changes of buffer containing 0.1% (v/v) Triton X-100. Gels were then incubated for 16 h at 37 °C in a similar buffer, devoid of detergent; with one change after 2 h. Buffers included 0.1 M sodium citrate (pH = 3), 0.1 M sodium phosphate (pH = 5.5), and 0.1 M Tris–HCl (pH = 8). Gel slices were stained overnight in methanol: acetic acid:dH_20 (40:10:50) containing 0.06% (w/v) Coomassie Brilliant Blue R-250 (Sigma), and were destained for approximately 1 hr in methanol:acetic acid:dH_2O (40:10:50).

For inhibition studies, the proteinase inhibitors trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64), iodoacetic acid (IA; Sigma), and ethylenediaminetetraacetic acid (EDTA) from British Drug House were employed. Prior to use, E-64 was dissolved in 10% (v/v) dimethylsulphoxide (DMSO). Proteinase inhibitors, at a final concentration of 0.1 mM E-64, 1 mM IA and 10 mM EDTA were pre-incubated with ESP for 30 min prior to the addition of sample buffer and were also incorporated in the washing and incubation buffers. Gelatinolytic activity was quantified using gel analysis software, GeneTools (Syngene). Molecular weight markers were as described in Section 2.

3. Results

3.1. Species composition

The species composition and relative abundance of the collected cyathostomins is represented in Table 1.

Table 1
The relative abundance of Cyathostominea species from which ESP was collected ($n = 2467$)

Species	%
Coronocyclus coronatus	17
Coronocyclus labiatus	1
Cyathostomum catinatum	14
Cyathostomum pateratum	1
Cylicocyclus nassatus	9
Cylicocyclus leptostomum	1
Cylicocyclus insigne	3
Cylicocyclus ashworthi	1
Cylicostephanus calicatus	8
Cylicostephanus minutus	6
Cylicostephanus longibursatus	16
Cylicostephanus goldi	9
Parapoteriostomum mettami	1
Petrovinema poculatum	12
Larvae	1
Damaged/unidentifiable	2

Both male and female cyathostomins are included.

3.2. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Some 13 subunits were resolved between 175 and 6.5 kDa following electrophoretic separation of 10 μl (1.76 μg) ESP under non-reducing conditions. In the presence of 5 mM DTT, some 21 subunits were resolved within the same range.

3.3. Azocaseinolysis

In the absence of DTT, azocaseinolysis by cyathostomin ESP was optimal at pH = 4.0 with 8.13 μg azopeptides released min^{-1} mg^{-1} protein. 47% of this activity was retained

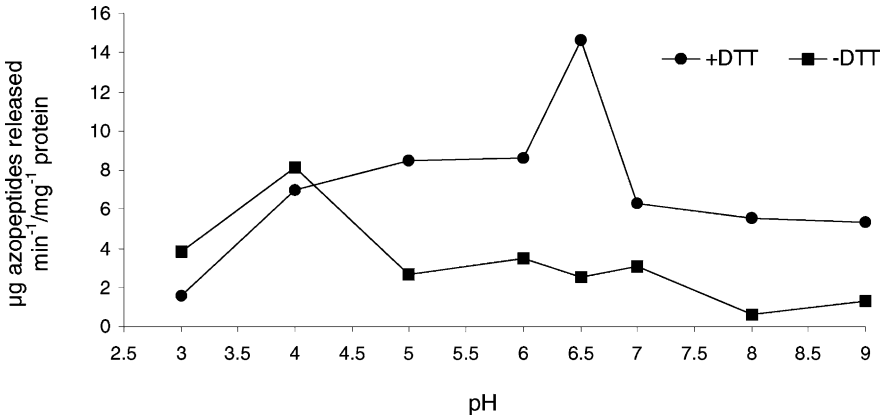

Fig. 1. pH profile of azocaseinolysis by cyathostomin ESP in the presence of 5 mM DTT (●) and in its absence (■).

at pH = 3.0, with 32, 43, 31, 38, 8, and 16% persisting at pH = 5.0, 6.0, 6.5, 7.0, 8.0, and 9.0, respectively. Azocaseinolytic activity was enhanced six-fold to a maximum of 14.64 μg azopeptides released min^{-1} mg^{-1} protein in the presence of 5 mM DTT at pH = 6.5. At pH = 3.0, 11% of maximal activity was retained with 47, 58, 59, 43, 38, and 37% activity persisting at pH = 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0, respectively (Fig. 1).

3.4. Hydrolysis of synthetic substrates

In the absence of DTT, hydrolysis of Z-Phe-Arg-NHMec by the ESP was optimal at pH = 5.5 with 2.28 nmol NMec released min^{-1} mg^{-1} protein. 3% of this activity was retained at pH = 3.0, with 82, 61, 90, 87, 74, 69, and 97% persisting at pH = 4.0, 5.0, 6.0, 6.5, 7.0, 8.0, and 9.0, respectively. Activity was enhanced at all pH values by the presence of 5 mM DTT culminating in a six-fold increase to a maximum of 12.97 nmol NMec released min^{-1} mg^{-1} protein at pH = 5.5. At pH = 3.0, 73% of activity was retained with 78, 80, 87, 82, 89, 81, and 62% persisting at pH = 4.0, 5.0, 6.0, 6.5, 7.0, 8.0, and 9.0, respectively.

Hydrolysis of Z-Arg-Arg-NHMec was optimal at pH = 4.0 in the absence of DTT with only 1.64 nmol NMec released min^{-1} mg^{-1} protein, which is similar to that released with Z-Phe-Arg-Arg-NHMec at the same pH. Hydrolysis of Z-Arg-Arg-NHMec increased in the presence of 5 mM DTT at all pH values except pH = 4.0, demonstrating 2–20-fold greater activity to a maximum of 4.88 nmol NMec released min^{-1} mg^{-1} protein at pH = 5.5. This is approximately three-fold less than with Z-Phe-Arg-NHMec under the same conditions. In the presence of 5 mM DTT, Z-Phe-Arg-NHMec was a superior substrate with a 3–30-fold higher release rate of NMec (Fig. 2). All activities were abolished by IA at a final concentration of 10 mM.

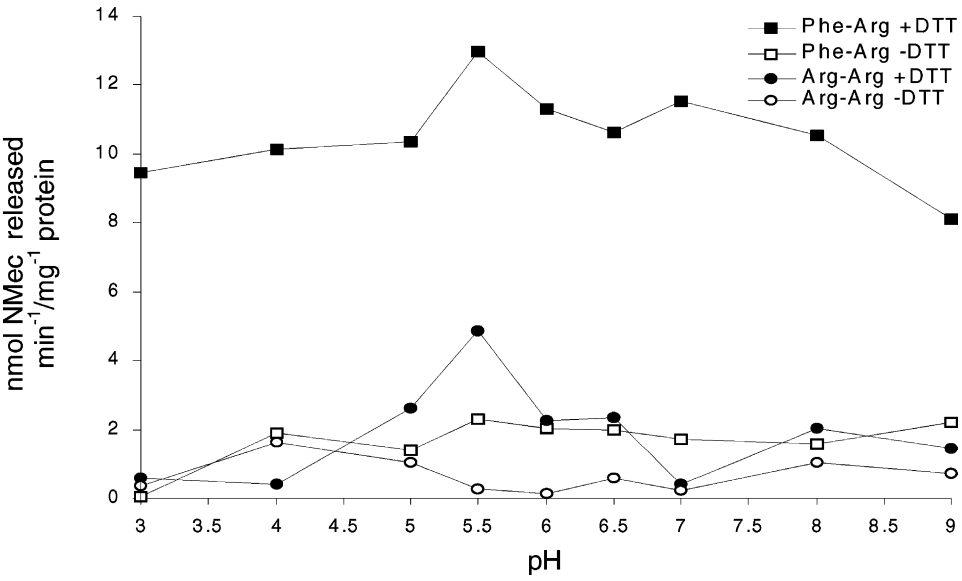

Fig. 2. pH profile of hydrolysis by ESP of the fluorogenic peptide substrates Z-Phe-Arg-NHMec (■□)and Z-Arg-Arg-NHMec (●○) in the presence (closed symbols) and absence of 5 mM DTT (open symbols).

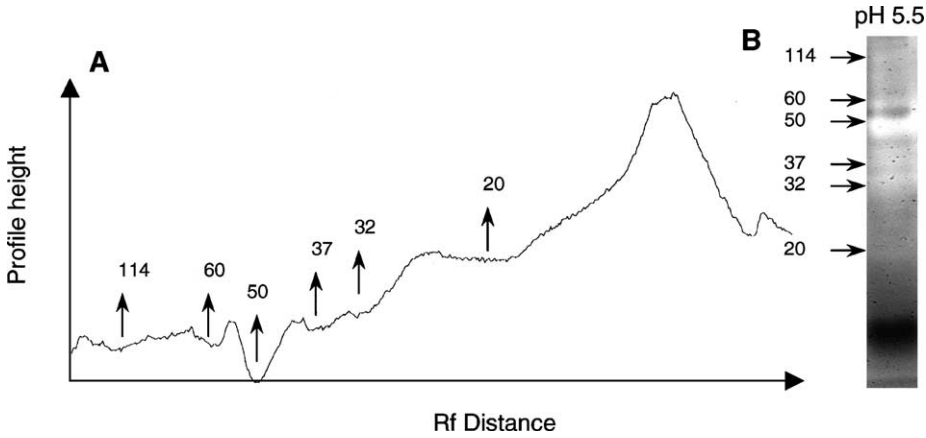

Fig. 3. Gel and densitiometric scan of proteolysis by ESP in gelatin-substrate SDS-PAGE at the optimal pH of 5.5 in the presence of 5 mM DTT. Troughs indicate gelatinolysis and profile height values represent percentage values for gelatinolysis. (A) Gelatin gel; (B) densitometric scan. Molecular weights were calculated using a molecular weight standard and GeneTools software.

3.5. Gelatin-substrate SDS-PAGE

In the absence of DTT, no gelatinolytic activity was detected at pH = 3.0, 5.5, and 8.0. The presence of 5 mM DTT elicited activity at both pH = 5.5 and 8.0 but not at pH = 3.0. The optimum pH was 5.5, resolving six bands ranging from 114 to 20 kDa; maximum activity was at 50 kDa (Fig. 3, Table 2). At pH = 8.0 four bands were resolved, of which those at 114 and 50 kDa demonstrated lower activity than at pH = 5.5; two additional bands were also detected at 80 and 73 kDa (Table 2, Control).

Inhibitor studies were conducted at pH = 3.0, 5.5, and 8.0 in the presence of 5 mM DTT. EDTA enhanced activity at both pH = 5.5 and 8.0, such that at pH = 5.5 maximum enhancement was exhibited at 32 kDa, followed by 114 and 37 kDa, but with no enhancement at 60, 50, and 20 kDa. At pH = 8.0, the EDTA was less effective in respect of these bands as none exhibited maximal activity although activities of 114 and 80 kDa were enhanced, however, new bands were resolved at 37 and 20 kDa. The faint band detected at 50 kDa in the absence of EDTA was not detected. EDTA had no effect at pH = 3.0. E-64 and IA abolished all activity at all pH values (Table 2).

3.6. Discussion

Cyathostomin communities comprise mixed populations and the species composition presented (Table 1) is typical of that recorded during the course of this study. Accordingly, the ESP documented is representative of a mixed cyathostomin infection. To understand the contribution of individual species, mono-specific culturing techniques will be necessary.

This study indicates the presence of cysteine-class proteinases in the ESP of Cyathostominea. Firstly, DTT (5 mM), a cysteine proteinase activator enhanced azocaseinolysis in the pH range 5.0–9.0, culminating in a six-fold increase to a maximum at pH = 6.5.

Table 2
pH profile of proteolytic activity by ESP as resolved by gelatin-substrate SDS-PAGE in the presence of 5 mM DTT

M_W (kDa)	Control			+10 mM EDTA			+0.1 mM E-64			+1 mM IA		
	pH = 3	pH = 5.5	pH = 8	pH = 3	pH = 5.5	pH = 8	pH = 3	pH = 5.5	pH = 8	pH = 3	pH = 5.5	pH = 8
114	−	++	+	−	+++	++	−	−	−	−	−	−
80			+			++						
73			+			+						
60	−	++	−	−	++	−	−	−	−	−	−	−
50	−	+++	+	−	+++	−	−	−	−	−	−	−
37	−	++	−	−	+++	+	−	−	−	−	−	−
32	−	+	−	−	+++	−	−	−	−	−	−	−
20	−	+	−	−	+	+	−	−	−	−	−	−

Activity scored from densitometric scans. Scores (+++) 80–100% digestion, (++) 50–80% digestion, (+) 5–50% digestion and (−) 0–5% digestion.

The addition of 5 mM DTT also enhanced hydrolysis of the synthetic substrate Z-Phe-Arg-NHMec at all pH values, culminating in a six-fold increase to a maximum at pH = 5.5. Hydrolysis of Z-Arg-Arg-NHMec was increased 17-fold to a maximum at pH = 5.5 in the presence of 5 mM DTT, approximately three-fold less than with Z-Phe-Arg-NHMec under similar conditions. DTT was also required for gelatinolysis following substrate gel electrophoresis. The cysteine proteinase inhibitors E-64 (0.1 mM) and IA (1 mM) abolished all gelatinolytic activity in substrate gels. Furthermore, the metal chelator EDTA (10 mM), a cysteine proteinase activator, enhanced gelatinolytic activity at pH = 5.5 and 8.0.

Cysteine proteases are instrumental in various biological processes including cell death (Yuan et al., 1993), and virulence (Mottram et al., 1996). The use of the substrate Z-Phe-Arg-NHMec may monitor cysteine proteinase activity as expressed by cathepsins B, L, and papain (Barrett and Kirschke, 1981; Zucker et al., 1985). The substrate Z-Arg-Arg-NHMec may be used to selectively assay for cathepsin B (Barrett and Kirschke, 1981). The present data indicate that there was proteolysis of both Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec, suggesting that this cyathostomin ESP includes cathepsin L-like, and papain- and cathepsin B-like activities. Z-Phe-Arg-NHMec was preferentially degraded over Z-Arg-Arg-NHMec and in the presence of 5 mM DTT, at the optimum pH of 5.5 maximal hydrolysis of Z-Phe-Arg-NHMec was three-fold greater than that of Z-Arg-Arg-NHMec under similar conditions. This indicates that their proteolytic specificities are predominantly papain- or cathepsin L-like rather than cathepsin B-like. The high hydrolysis rate of Z-Phe-Arg-NHMec also indicates that cathepsins H, N, and S are not involved as cathepsin H does not hydrolyse this substrate, and it is a poor substrate for cathepsins N and S (Barrett and Kirschke, 1981; Maciewicz and Etherington, 1988).

Lysosomal cathepsins L and B are rapidly inactivated at pH = 7.0 and above (Kirschke and Barrett, 1987). The present data indicate that, the pH optimum of the ESP is 5.5–6.5. In the presence of 5 mM DTT, activities against the substrates azocasein, Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec, were 38, 81, and 42%, respectively of maximal at pH = 8.0, and 37, 62, and 30%, respectively at pH = 9.0. Furthermore, at pH = 8.0 considerable gelatinolytic activity was detected in substrate gels both in the presence and absence of EDTA. As such, it seems that the proteolytic activity of the ESP is more like that of papain which displays activity well into the alkaline pH range (Zucker et al., 1985), and has a pH optimum of 5.0–7.0 (Boller, 1986). Such proteolytic activity by the ESP seems appropriate as the pH of equine caecal contents may vary between pH = 5.9 and 7.8, depending on both diet and time after feeding (Goodson et al., 1985).

Cysteine-class proteinases have been documented for many other parasitic helminths including *Strongylus vulgaris*, *Nippostrongylus brasiliensis*, and *Strongyloides ratti* (Caffrey and Ryan, 1994; Kamata et al., 1995; Harrop et al., 1995, respectively). In *H. contortus* cysteine proteinases degrade the matrix of host connective tissue (Rhoads and Fetterer, 1996), inhibit the clotting of sheep blood, and hydrolyse haemoglobin; their activity was characterised as cathepsin L-like (Rhoads and Fetterer, 1995). The dominant intestinal transcripts were represented by cathepsin B-like cysteine proteinase genes (cbls) (Jasmer et al., 2001) and substrate based histological analysis located their products in the intestinal microvilli (Shompole and Jasmer, 2001). Cysteine proteinases are proven vaccine immunogens in helminthiases, as immunization with cathepsin L-like proteinases of *F. hepatica* elicited more than 50% protective immunity in cattle (Dalton et al., 1996). Furthermore, the

potential of cysteine protease inhibitors in the therapeutic control of parasites has elicited much interest. Peptidyl fluoromethyl ketones and peptidyl methyl ketones effectively inhibit haemoglobin degradation, which results in the death of schistosomules both in vitro and in vivo (Wasilewski et al., 1996). The present data are derived from assemblages of Cyathostominea and thus represent a first approximation. The use of ESP constituents as leads in immunological or chemical control of these parasites will require characterization of ESP from at least the principal individual species.

Acknowledgements

We thank Dr. Wen-Yuan Chung for technical assistance and Mr. Ray Magee of the Irish Horse Abattoir Trading Co. Ltd.

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