PRELIMINARY ANALYSIS OF THE PROTEOLYTIC ENZYMES IN THE EXCRETORY-SECRETORY PRODUCTS OF THE ADULT STAGES OF THE DOG HOOKWORM *UNCINARIA STENOCEPHALA*

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Summary :

The paper describes an introductory characterisation of proteinases present in the excretory-secretory products (ESP) of adult *Uncinaria stenocephala*. In SDS-PAGE gelatine substrate gels ESP resolved as a six bands of proteolytic activity, with a molecular weight of 182, 159, 98, 50, 39 and 26 kDa. The 98 and 39 kDa components were serine proteinases. The 50 kDa band was sensitive to a metalloproteinase inhibitor. The 26 kDa component was highly sensitive to cysteine proteinase inhibitors and was also partially inhibited in the presence of EDTA. The bands of 182 and 159 kDa were sensitive to a Zn-metalloproteinase inhibitor.

The enzymes present in ESP showed the highest proteolytic activity at pH 8-9. Quantitative analysis revealed maximum proteolytic activity of the polypeptides of 159 and 182 kDa at pH 7; 98 and 26 kDa at pH 8 while the 50 kDa and 39 kDa components showed the highest activity at pH 9.

KEY WORDS : hookworms, excretory-secretory products, proteinases, *Uncinaria stenocephala*.

Résumé : ÉTUDE PRÉLIMINAIRE DES CARACTÉRISTIQUES DES PROTÉASES PRÉSENTES DANS LES PRODUITS D'EXCRÉTION-SÉCRÉTION D'*UNCINARIA STENOCEPHALA* ADULTE

Uncinaria stenocephala est un parasite des canidés appartenant à la famille des ankylostomes. On ignore toujours la composition et la fonction des produits d'excrétion-sécrétion (ESP). Cet essai démontre les caractéristiques préliminaires des protéases présentes dans les ESP d'*U. stenocephala* adulte.

En utilisant la méthode de l'électrophorèse SDS-PAGE, on a découvert six protéines présentant des activités protéolytiques différenciées, de masse moléculaire : 182, 159, 98, 50, 39 et 26 kDa dans le gel-substrat. Les épreuves avec les inhibiteurs spécifiques des protéases ont démontré que les composants de masse 98 et 39 kDa appartiennent aux protéases à sérine. La bande 50 kDa a des propriétés de métalloprotéase. La bande 26 kDa démontre des caractères de protéase à cystéine. Les composants 182 et 159 semblent être des métalloprotéases à zinc. Les enzymes découvertes dans les ESP montrent une plus grande activité à pH 8-9.

MOTS CLÉS : ankylostome, produits d'excrétion-sécrétion, protéases, *Uncinaria stenocephala*.

INTRODUCTION

Nematodes release a variety of proteins with enzymatic properties which, *in vivo*, are believed to play important roles in the host-parasite relationship. Among the specific functions attributed to the excretory-secretory products (ESP) of nematodes are invasion and migration through host tissues, facilitation of feeding and evasion of host immune responses (Tort *et al.*, 1999). Some proteinases secreted by nematodes have been shown to be inhibited by host antibodies. This is particularly important because it indicates that proteinases of parasite origin constitute targets for the host's immune responses and suggests a way through which immunotherapeutic intervention may protect the host from disease (Tort *et al.*, 1999). *Uncinaria stenocephala* is a canine hookworm which is prevalent in dogs all over the world (Arene, 1984;

Blake & Overend, 1982; Gorski *et al.*, 1996; Vanparijs *et al.*, 1991). Attempts to understand a molecular pathobiology of hookworm infections have largely concentrated on *Ancylostoma caninum* in dogs and on the human parasites (Brown *et al.*, 1995; Hotez *et al.*, 1995; Fumidge *et al.*, 1995; Pritchard *et al.*, 1990). In contrast, *U. stenocephala* has received scarce attention and very little is known about molecular interactions in this host-parasite relationship, including enzymes secreted by the parasite. In this report we describe the range of proteolytic activities present in the ESP of adult *U. stenocephala* and we provide a preliminary characterization of the principal enzymes involved.

MATERIAL AND METHODS

COLLECTION OF THE EXCRETORY-SECRETORY PRODUCTS (ESP)

Adult nematodes were isolated from the intestines of experimentally infected dogs, washed extensively in 0.15 M sodium chloride containing 100 i.u./ml penicillin and 100 µg/ml streptomycin and

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then left over a period of 3 h at 37°C in RPMI 1640 containing 100 i.u./ml penicillin and 100 µg/ml streptomycin in order to permit loss of any remaining host tissues from the worms' intestines (Brown *et al.*, 1995). Following this "cleaning" period adult worms were cultured in sterile RPMI 1640, enriched with antibiotics (as above), for a further 24 h at 37°C. As assessed by monitoring worm motility, the parasites remained viable in culture. The culture fluid containing ESP was dialysed against distilled water pH 7.0, freeze dried and reconstituted in distilled water prior to use (Brown *et al.*, 1995).

MEASUREMENT OF PROTEINASE ACTIVITY

Proteinases were detected following their electrophoretic separation using gelatin-SDS PAGE assay system (Hotez *et al.*, 1990; Hawdon *et al.*, 1995) which consisted of 12 % w/v acrylamide gels containing 0.2 % w/v gelatin, TRIS-HCl buffer pH 8.8 and 0.1 % (w/v) SDS. Typically 10 µg of total protein was separated per lane of gels. Following electrophoresis gels were incubated at 25°C in 25 % v/w Triton X-100 for 1 h and then in glycine buffer (0.05 M, pH 6-8) with or without proteinase inhibitors at 37°C.

CHARACTERIZATION OF PROTEINASES USING PROTEINASE INHIBITORS

The proteinase inhibitors were obtained from Sigma-Aldrich Germany and applied at concentrations reported by Kumar & Pritchard (1992), Hawdon *et al.* (1995) and Cordova *et al.* (1997). The inhibitors used were as follows: 1,10 phenanthroline (2 mM; Zn-metalloproteinase inhibitor); ethylenediaminetetracetic acid (EDTA; 10 mM; metalloproteinase inhibitor); phenylmethanesulphonyl fluoride (PMSF; 10 mM & 1 mM, serine proteinase inhibitor); pepstatin (70 µM, aspartic proteinase inhibitor),

N-ethylmaleimide (NEM; 10 or 5 mM, cysteine proteinase inhibitor); iodoacetamide (IA; 5 mM, cysteine proteinase inhibitor). The assays were conducted at pH 7.0.

ESTIMATION OF THE pH OPTIMA FOR PROTEINASE ACTIVITY

Assays were conducted with a pH range from 3 to 11. After electrophoresis, gels were immersed in different buffers prepared as described by Dowd *et al.* (1994) and incubated at 37°C. After the incubation, the gels were scanned then area of proteolysis were measured and compared using a computer program "Diversi-One™" version 1.3 (*pdi*). The programme calculates the proteolytic activity index by measuring the area and intensity of substrate degradation.

RESULTS

Six bands of proteolysis at 182, 159, 98, 50, 39 and 26 kDa were observed in substrate gels incubated without inhibitors (Fig. 1, lane A). Several proteinase inhibitors affected the proteolysis associated with components of differing molecular weight (Fig. 1B-G, Table I). The 182, 159, 50 and 26 kDa bands were absent from gels incubated in the presence of EDTA (metallopro-

Proteinase inhibitor	Proteolytic activity					
	182 kDa	159 kDa	98 kDa	50 kDa	39 kDa	26 kDa
No inhibitor	+	+	+	+	+	+
EDTA						
10 mM	-	-	+	-	+	+/-
1,10 phenanthroline						
2 mM	-	-	+	+	+	+
PMSF						
10 mM	-	-	-	-	-	-
1 mM	+	+	-	+	-	+
Pepstatin						
70 mM	+	+	+	+	+	+
N-ethylmaleimide						
10 mM	+	+/-	+/-	+	+	-
5 mM	+	+	+	+	+	+
Iodoacetamide						
5 mM	+	+	+	+	+	+

Table I. – Sensitivity of proteinases secreted by adult *U. stenocephala* to inhibitors.

Fig. 1. – Characterization of gelatinolytic proteinases in the excretory-secretory products (ESP) of adult *Uncinaria stenocephala* using proteinase inhibitors.

Lanes: M - molecular weight markers (kDa); A - the SDS-PAGE profile of gelatinolytic proteinases in *U. stenocephala* ESP (no inhibitor); B - metalloproteinase inhibitor (EDTA); C - Zn-metalloproteinase inhibitor (1,10-phenanthroline); D - serine proteinase inhibitor (phenylmethanesulphonyl fluoride); E - aspartic proteinase inhibitor (pepstatin); F - cysteine proteinase inhibitor (N-ethylmaleimide); G - cysteine proteinase inhibitor (iodoacetamide).

teinas inhibitor, Fig.1B) while Zn metalloproteinase inhibitor (1,10 phenanthroline) caused deprivation of the proteolytic activity of 182 and 159 kDa components (Fig. 1C). In the presence of 10 mM PMSF only 182 and 159 bands exhibited a weak activity (Fig. 1D). However, when the incubation buffer contained 1 mM PMSF only 98 and 39 kDa bands were inhibited (Table I). None of the ESP proteinases were affected by the aspartic proteinase inhibitor (Fig. 1E). 10 mM NEM blocked the enzymatic activity of the 26 kDa and attenuated 159 kDa and 98 kDa bands (Fig. 1F). When lower concentration of the inhibitor was used only the proteolytic activity of 26 kDa declined (Fig. 1G, Table I). The optimal pH conditions for proteolysis by ESP were evaluated and quantitative analysis revealed maximum proteolytic activity of the polypeptides of 182 and 159 kDa at pH 7.0; those of 98 and 26 kDa at pH 8 while the 50 kDa and 39 kDa components showed higher activity at pH 9. No proteolytic activity was observed at pH 10 and 11. The results are shown in Figure 2.

DISCUSSION

Our experiments demonstrated that excretory-secretory products of adult *U. stenocephala* include a heterogeneous mixture of proteolytic

activities. To our knowledge, there have been no reports on proteinases of *U. stenocephala*. Reports concerning proteolytic activities found in the ESP of other hookworms are limited and among those which have been described so far, differences in structure and function have been reported (Dowd *et al.*, 1994; Hotez *et al.*, 1985; Kumar & Pritchard, 1992; Pritchard *et al.*, 1990; Tort *et al.*, 1999).

Results obtained using various proteinase inhibitors strongly suggested lack of aspartic proteinases in the ESP of adult *U. stenocephala*. The 26, 50, 159 and 182 kDa components lost their proteolytic activities in the presence of EDTA (metalloproteinase inhibitor), while 1,10 phenanthroline which is a strong Zn atoms chelator, inhibited only 159 and 182 kDa bands. Because EDTA is much less effective against Zn and more effective in chelating Ca, the results seem to suggest that the 159 and 182 kDa bands contain Zn metalloproteinases, whereas the 50 and 26 kDa components include enzymes utilising Ca or another cofactor (Bond & Butler, 1987). An activity of metalloproteinases has been detected in ES products of other hookworm species. For instance, Hawdon *et al.* (1995) found metalloproteinases of 50 and 90 kDa in ES products of *A. caninum*. Diverse results were obtained in the present experiment using serine proteinase inhibitor (PMSF). We initially used the 10 mM concentration of PMSF since

Fig. 2. – Results of computer analysis of effects of pH on proteolytic activity of enzymes present in excretory-secretory products of adult *U. stenocephala*. PA = proteolytic activity.

Kumar & Pritchard (1992) reported that only 10 mM but not 1 mM or 5 mM PMSF inhibited ESP proteinases of *Necator americanus*. However, the 10 mM PMSF blocked entirely the proteolytic activity of ESP of *U. stenocephala*. Therefore we repeated experiments using 1 mM PMSF and found that proteolytic activity of only 39 and 98 kDa components was inhibited. The complete inhibition of proteolytic activity of the ESP observed in presence of 10 mM PMSF resulted possibly from a non-specific inhibition (Bond & Butler, 1987). Similarly, a non-specific inhibition has probably also occurred in test using 10 mM NEM. The 10 mM NEM affected proteolytic activity of three components (159, 98 and 26 kDa) while 5 mM concentration of the inhibitor blocked activity only of the 26 kDa component. The 26 kDa band was also sensitive to IA and EDTA inhibition. On the strength of the fact that both IA and NEM, which are cysteine proteinase inhibitors, caused similar effect and that EDTA is a strong metal chelator, we can tentatively conclude that the 26 kDa component belongs to cysteine class of proteinases and possesses a metal cofactor. The metal ions are often required for the activity of other than metalloproteinases classes (Bond & Butler, 1987). The proteolytic activity of the 26 kDa component was most pronounced at pH 8 although a clear activity was also observed at pH 7 and 9. Similar pH values have been reported by Dowd *et al.*, (1994) and Brown *et al.* (1995) for cysteine proteinases secreted by adult *A. caninum*. The results of our preliminary analysis of the ESP of adult *U. stenocephala* suggest that serine and metalloproteinases account for the most pronounced proteolytic activity in the ESP of the nematode. However, further investigations of the proteinases released by *U. stenocephala* are needed in order to characterise their role in pathogenesis and immunobiology of the nematode infection.

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