Journal of Helminthology (2001) **75,** 57–59 DOI: 10.1079/JOH200035

Purification and partial characterization of proteinase activity in eggs of *Dicrocoelium dendriticum*

C. Giménez-Pardo*, C. de Armas-Serra and F. Rodríguez-Caabeiro

Parasitology Laboratory, Faculty of Pharmacy, Alcalá University, Crtra. Madrid–Barcelona Km 33.600, 28871 Alcalá de Henares (Madrid), Spain

Abstract

A preliminary purification has been carried out by continuous elution electrophoresis of a 49.5 kDa protease of crude extracts from *Dicrocoelium dendriticum* eggs. The enzyme showed a high capacity to degrade the collagen derivative azocoll at acidic pH. Although it is necessary to carry out further experiments to confirm any physiological role, this protease could be implicated in penetration mechanisms.

Introduction

Proteases from eggs of *Dicrocoelium dendriticum* have previously been characterized and are proposed to play important roles in parasite nutrition, the hatching mechanism or in penetration through the molluscan tissues to the hepatopancreas (Armas-Serra *et al.*, 1993). Sung & Dresden (1986) purified two proteases from eggs of *Schistosoma mansoni*, which originated from glands in the miracidium, though its implication in any miracidial activities was impossible to elucidate. To our knowledge, no previous studies on the purification of proteolytic enzymes in *D. dendriticum* eggs have been undertaken so, in the present investigation, we report the purification by a single and one-step method and the partial characterization of a protease from crude extracts of *D. dendriticum* eggs.

Material and methods

Eggs of *D. dendriticum* were obtained from adult trematodes which were collected from the gallbladder and bile ducts of sheep at the municipal slaughterhouse in Alcalá de Henares (Spain). The cytosolic fractions were obtained by following the method described by Armas-Serra *et al.* (1993) and purified by continuous-elution electrophoresis (CEE), as applied by Armas-Serra *et al.* (1995). Gels, prepared in a 28 mm gel tube of the Model 491 Prep Cell (Bio Rad), comprised 1 cm of 4% acrylamide stacking gel and 3 cm of 15% acrylamide running gel. The protein sample (2.6 mg) diluted 3:1 in 30% glycerol was loaded onto the stacking gel, and then separated electrophoretically at constant current of 40 mA for 2 h. Samples were applied with or without sodium dodecyl sulphate (SDS). The running buffer was Tris-Glycine-SDS (pH 8.8), and the same buffer without SDS was used as the elution buffer. Proteins migrating from the bottom of the gel were electroeluted through a dialysis membrane (6 kDa). The proteins electroeluted from the gel (0.5 ml min^{-1}) were collected into a fraction collector (5 ml fractions collected) and samples (40 fractions eluted) were monitored from the protein content at 280 nm. Gyrocent-M membranes with a 3 kDa size exclusion were used to concentrate each separated fraction but previously, to eliminate the sodium azide from the membranes, these were washed with 0.1 N NaOH for 20 min and then with 2 ml deionized water for 20 min to 7000×*g*. Each fraction was then centrifuged at 7000×*g* for 20 min at 4°C and dialysed with Bio-Spin columns at 4°C, 1100×*g* for 5 min. Protein determination was assayed by micro Bradford assay (Bradford, 1976) in a spectro U-2001 (Hitachi) using bovine seroalbumin as standard.

The purity assessment of each fraction was performed by one-dimensional 15% polyacrylamide gel electrophoresis (SDS–PAGE) in the presence or absence of 2-mercaptoethanol according to Weber & Osborn (1969). Gels were silver stained and markers used for stimating

* Fax: +34 1 885 46 63
E-mail: consuelo.gimenez@uah.es

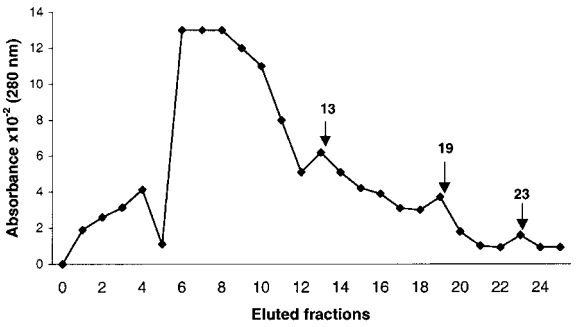

Fig. 1. Elution profile obtained at 0.5 ml min^{-1} from a 28 mm (inside diameter) gel tube of a model 491 Prep Cell containing a 1 cm-high layer of 15% acrylamide running gel for the crude extracts of *Dicrocoelium dendriticum* eggs. Fractions of 5 ml were collected and the optical density was measured at 280 nm. Arrows indicate the 13, 19 and 23 eluted fractions.

the molecular mass of the enzyme were included (phosphorylase B 106 kDa, bovine serum albumin 80 kDa, ovalbumin 49.5 kDa, carbonic anhydrase 32.5 kDa, soybean trypsin inhibitor 27.5 kDa and lysozyme 18.5 kDa).

Proteolytic activity of these three eluted fractions was determined by 15% SDS–PAGE copolymerized with 0.15% (w/v) gelatin as substrate. The samples (10 μg) were diluted 3:1 in sample buffer and electrophoresed at 70 v per gel. The gels were then washed in 2.5% Triton X-100 for 1 h to remove SDS and incubated in 0.1 M citrate buffer (pH 3 and 5) and 2 mM $CaCl_2$ for 8 h at 37°C and stained with Coomassie blue. After destaining, proteases were located as clear bands in a blue background. Proteinase assays were also carried out by colorimetric measurement by hydrolysis of azo-dye-couple hide powder (azocoll) as proposed by Monroy *et al.* (1989). Incubation time was 24 h at 37°C, and buffers used were 0.1 M citric acid/sodium citrate pH 3 and 5 in the presence of 5 mg ml^{-1} of azocoll as protein substrate. After incubation, the Eppendorf tubes were centrifuged at 1000×*g* for 5 min and the supernatant was removed for spectrophotometric measurement at 540 nm. All assays were performed simultaneously with their respective controls and experiements were repeated ten times.

Results and Discussion

As we have previously demonstrated, eggs of *D. dendriticum* have proteolytic activity (Armas-Serra *et al.*, 1993). In the present paper, despite the difficulty in obtaining a high protein content, due to the fact that not all eggs are embryonated at the same time, we have demonstrated the purification of a 49.5 kDa proteolytic enzyme from eggs of *D. dendriticum* with a single one-step purification method.

The purification profile comprised three eluted fractions with protein content 13, 19 and 23 (fig. 1) and were 38.5 μg ml^{-1} from the 13-eluted fraction, 67 μg ml^{-1} from the 19-eluted fraction and 27.6 μg ml^{-1} from the 23-eluted fraction. After electrophoresis and Rf was calculated, molecular weights of 27.5 kDa from the 13-eluted fraction, 32.5 kDa from the 19-eluted fraction and 49.5 kDa from the 23-eluted fraction were obtained. Results after 2-mercaptoethanol treatment revealed that only in the case of the 23-eluted fraction the mobility was different due to the subunits being linked by disulphide bonds (fig. 2). The results demonstrated that proteolytic activity only appeared when the 23-eluted fraction was applied onto gel at pH 5 (fig. 3).

This enzyme showed activity against gelatin and the collagen derivative azocoll. The pH optimum for proteinase activity was 5 in both azocoll (azocoll degradation percentage was 6.47%) and gelatin probes. Though activity appeared at pH 3 in azocoll (azocoll degradation percentage was 2.59%) and gelatin assays, it was lower than that observed at pH 5 and faint bands that could not be photographed appeared in SDS–PAGE-gelatin gels. We have only assayed acidic pH values as

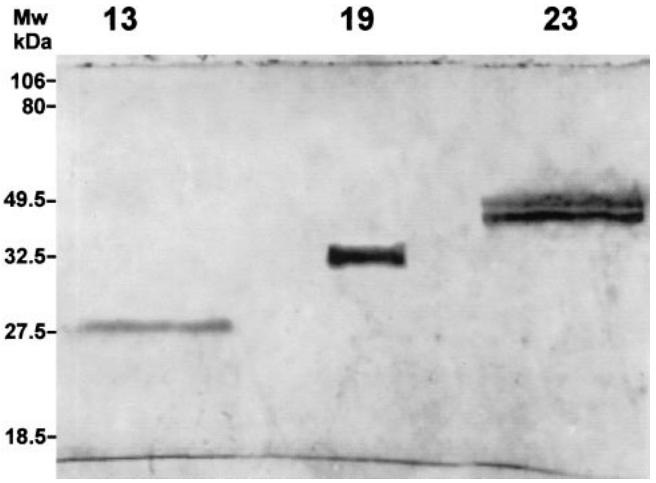

Fig. 2. 15% electrophoresis in polyacylamide–SDS gels of the three eluted fractions (13, 19 and 23) under reducing conditions from eggs of *Dicrocoelium dendriticum*. Molecular-mass markers (kDa) are shown in the left track.

https://doi.org/10.1079/JOH200035 Published online by Cambridge University Press

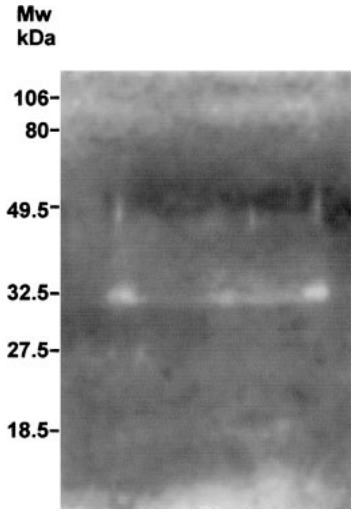

Fig. 3. Identification of the proteolytic activity of the 49.5 kDa protein from *Dicrocoelium dendriticum* eggs by a 15% gelatin–SDS–PAGE substrate gel and 8 h after incubation at pH 5. Protease activity appears as a clear band.

previously observed with the egg cytosolic fractions (Armas-Serra *et al.*, 1993). This acidic activity has also been observed in the purified thiol proteases from eggs of *S. mansoni* (Sung & Dresden, 1986; Yoshino *et al.*, 1993). When SDS was not added to the sample in the CEE purification method we neither obtain these three eluted fractions nor proteolytic activity in the 23-eluted fraction. This could be due without SDS to only negatively charged proteins being recovered and our purified protease has either a positive or neutral charge.

Additionally, the activity observed in gels was very strong. It should be noted that the protein concentration of the eluted fraction which showed activity was 27.6 $\mu g\ ml^{-1}$, to each gel 10 μg of protein was incorporated and it only took 8 h to degrade the gelatin protein substrate, an incubation time that was shorter than that observed with other helminths (Criado-Fornelio *et al.*, 1992; Armas-Serra *et al.*, 1995; Vázquez-López *et al.*, 2000). However, this method was always used as a qualitative method to determine proteolytic activity only and not the molecular weight of the enzyme as proposed by Hunter *et al.* (1992).

With respect to the physiological role of this protease, further information is needed about its characterization, but its activity at acidic pH on collagen makes it likely that this protease could assist eggs in passing through the wall of the intestine or to assist hatched miracidia to penetrate the muscle extracellular matrix as occurs with *S. mansoni* (Pino-Heiss *et al.*, 1985; McKerrow & Doenhoff, 1988). Further research is being planned at our laboratory to implicate this protease in any physiological activity of *D. dendriticum* eggs.

Acknowledgements

This research was supported by funds provided by Project PM96-0014 (Ministry of Education and Science, Spain), and Project E095/2000 (Alcalá University, Spain).

References

Armas-Serra, C. de., Giménez-Pardo, C. & Rodríguez-Caabeiro, F. (1993) Presence of proteolytic enzymes in *Dicrocoelium dendriticum* eggs. *International Journal for Parasitology* **23**, 681–684.

Armas-Serra, C. de, Giménez-Pardo, C., Jiménez-González, A., Bernadina, W.E. & Rodriguez-Caabeiro, F. (1995) Purification and preliminary characterization of a protease from the excretion-secretion products of *Trichinella spiralis* muscle-stage larvae. *Veterinary Parasitology* **59**, 157–168.

Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Criado-Fornelio, A., Armas-Serra, C. de, Gimenez-Pardo, C., Casado-Escribano, N., Jiménez-González, A. & Rodriguez-Caabeiro, F. (1992) Proteolytic enzymes from *Trichinella spiralis* larvae. *Veterinary Parasitology* **45**, 133–140.

Hunter, C., Lockwood, B.C. & Coombs, G.M. (1992) Hydrolysis of L-leucine methyl ester by *Leishmania mexicana mexicana* amastigote cysteine proteinases. *International Journal for Parasitology* **22**, 711–717.

McKerrow, J.H. & Doenhoff, M.J. (1988) *Schistosoma* proteases. *Parasitology Today* **4**, 334–340.

Monroy, F.G., Cayzer, C.J.R., Adams, J.H. & Dobson, C. (1989) Proteolytic enzymes in excretory/secretory products from adult *Nematospiroides dubius*. *International Journal of Parasitology* **19**, 129–131.

Pino-Heiss, S., Brown, M. & McKerrow, J.H. (1985) *Schistosoma mansoni*: degradation of host extracellular matrix by eggs and miracidia. *Experimental Parasitology* **59**, 217–221.

Sung, C.K. & Dresden, M.H. (1986) Cysteinyl proteinases of *Schistosoma mansoni* eggs: purification and partial characterization. *Journal of Parasitology* **72**, 891–900.

Vazquez-López, C., Armas-Serra, C. de, Pérez-Serrano, J., Giménez-Pardo, C. & RodrÍguez-Caabeiro, F. (2000) Localization of a 24-kDa collagenase in the *Gymnorhynchus gigas* plerocercoid. *Journal of Helminthology* **74**, 183–187.

Weber, K. & Osborn, M. (1969) The reliability of molecular weight determinations by dedecyl-sulphate-polyacrylamide gel electrophoresis. *Journal of Biological Chemistry* **224**, 4406–4412.

Yoshino, T.P., Lodes, M.J., Rege, A.A. & Chapell, C.L. (1993) Proteinase activity in miracidia transformation excretory-secretory products and primary sporocysts of *Schistosoma mansoni*. *Journal of Parasitology* **79**, 23–31.

(Accepted 22 June 2000)