A *Boophilus microplus* vitellin-degrading cysteine endopeptidase

A. SEIXAS[1,2], P. C. DOS SANTOS[1,2]†, F. F. VELLOSO[1], I. DA SILVA VAZ JR[1,3], A. MASUDA[1,4], F. HORN[1,5] *and* C. TERMIGNONI[1,2]*

[1] *Centro de Biotecnologia*, [2] *Departamento de Bioquímica*, [3] *Departamento de Patologia Clínica Veterinária*, [4] *Departamento de Biologia Molecular e Biotecnologia*, [5] *Departamento de Biofísica*, *Universidade Federal do Rio Grande do Sul*, *P.O. Box 15005*, *91501-970*, *Porto Alegre*, *RS*, *Brasil*

(*Received 16 July 2002; revised 8 October 2002; accepted 8 October 2002*)

SUMMARY

Here we describe the purification and characterization of a vitellin (VT) degrading cysteine endopeptidase (VTDCE) from eggs of the hard tick *Boophilus microplus*. A homogeneous enzyme preparation was obtained by chromatographic fractionation on ion-exchange and gel filtration columns and an autolysis step. This step consisted of incubation of a semi-purified enzyme (after the first ion-exchange chromatography) at pH 4·0 that dissociated the enzyme from VT, to which VTDCE is naturally tightly associated. The enzyme purity was confirmed by capillary and native gel electrophoresis, and SDS–PAGE suggested the enzyme is a dimer of 17 and 22 kDa. VTDCE was active upon several synthetic substrates, with a preference for a hydrophobic or a basic residue in P_1, and a hydrophobic residue in P_2. VTDCE also hydrolysed haemoglobin, albumin, gelatin and vitellin. VTDCE is inactive in the absence of DTT and was totally inhibited by E-64, indicating it is a cysteine endopeptidase. Our results suggest that VTDCE is a major enzyme involved in yolk processing during *B. microplus* embryogenesis.

Key words: tick, *Boophilus microplus*, cysteine endopeptidase, vitellin, embryogenesis.

INTRODUCTION

The embryo development of an arthropod requires that the proper nutrients be available at the right time. The bulk volume of the arthropod eggs are comprised of yolk granules or spheres, specialized organelles that contain reserve material and the enzymatic machinery necessary to make these reserves available. Yolk granules thus provide aminoacids, carbohydrates and lipids for the embryonic process.

The major protein contained within yolk granules is vitellin (VT), a heme-containing phosphoglycolipoprotein derived from a maternal serum protein precursor, vitellogenin (Canal *et al*. 1995). In many species, such as the tick *Ornithodoros moubata* (Fagotto, 1991), the cockroach *Blatella germanica* (Liu, McCarron & Nordin, 1996), the sea urchins *Strongilocentrotus purpuratus* and *Lytechinus pictus* (Mallya *et al*. 1992), the frog *Xenopus laevis* (Fagotto & Maxfield, 1994) and the mosquito *Aedes aegypti* (Cho *et al*. 1999), VT degradation is triggered by a developmentally regulated acidification of the yolk granules. This acidification, in turn, activates different proteinases involved in arthropod yolk digestion, among them acid cysteine proteinases from the cathepsin-like group, mainly cathepsin L-like (Fagotto, 1990) and cathepsin B-like (Medina, Leon & Vallejo, 1988).

A previous report has shown that acidification, and the consequent activation of an acid aspartic proteinase, is required for yolk degradation in the cattle tick *Boophilus microplus* (Logullo *et al*. 1998). In this work we report the presence of a (cathepsin L-like) cysteine endopeptidase, which is associated with vitellin, in eggs of *B. microplus* and that probably has a role in the VT degradation during *B. microplus* embryogenesis.

MATERIALS AND METHODS

Materials

All the 7-amido-4-methylcoumarin (MCA) fluorogenic substrates were purchased from Sigma Chemical Co. (St Louis, USA). The internal quenched fluorescence substrate, MCA-Gln-Ser-Ser-Arg-His-Arg-Arg-Ala-Leu-Gln-EDDnp (ethylenedinitrophenyl-N-[2.4-dinitrophenyl]-ethylenediamine) was synthesized and kindly supplied by Dr Luiz Juliano (Escola Paulista de Medicina, Universidade Federal de São Paulo, Brazil). Centricon-10 and Microcon-10 were from Amicon Inc. (Beverly, USA), the other filters were from Millipore Ind. Com. Ltda (Bedford, USA). *B. microplus* vitellin was prepared

* Corresponding author: Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, P.O. Box 15005, 91501-970, Porto Alegre, RS, Brasil. Tel: +55 51 33166092. Fax: +55 51 33167309. E-mail: trmgn@dna.cbiot.ufrgs.br

† Present address: Department of Biochemistry, Virginia Tech, Blacksburg, VA, USA 24061.

Parasitology (2003), **126**, 155–163. © 2003 Cambridge University Press
DOI: 10.1017/S0031182002002731 Printed in the United Kingdom

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press

according to the method described by Da Silva Vaz Jr, Osaki & Masuda (1994).

Ticks and eggs

Ticks from the Porto Alegre strain were reared on bovines, which were brought from a tick-free area and maintained in insulated individual boxes protected from contact with other ticks and insects. Bovines were infested with 15-day-old (from hatching) *B. microplus* larvae. After 22 days, engorged adult female ticks that had spontaneously detached from the host were collected and incubated at 28 °C and 85% relative humidity. Eggs from the 1st to the 12th day after oviposition were collected and stored at −70 °C until used.

Protease activity assays

Zymograms. The zymogram is a simple and sensitive technique used to detect proteolytic activity. It is particularly useful when the sample may contain more than 1 proteolytic activity. In this work zymograms were used to search for proteases in 3 different tick crude extracts and to evaluate the enzyme capacity to hydrolyse different proteins. Assays were done according to the method of Heussen & Dowdle (1980). Briefly, polyacrylamide gels (7·5%) copolymerized with 0·1% gelatin, 0·1% haemoglobin, 0·1% vitellin, 0·1% albumin or 0·1% ovalbumin were run under non-denaturing conditions (without sodium dodecyl sulfate (SDS)) in 50 mM Tris-glycine buffer, pH 8·3. After the electrophoretic separation, gels were washed and incubated overnight in 0·1 M sodium acetate buffer, pH 3·5, containing 5 mM dithiothreitol (DTT), at 37 °C. At the end of the incubation period, gels were washed with water and stained with Coomassie Brilliant Blue R-250.

Activity with fluorogenic substrates. Column fractions (10 μl aliquots) or other samples to be tested were incubated with 50 mM sodium citrate/sodium phosphate buffer, pH 3·5, and 10 mM DTT at 37 °C. After 10 min, the fluorogenic substrate *N*-Cbz-Phe-Arg-MCA (Cbz, carboxibenzoyl) was added to a final concentration of 1·4 μM in a total volume 100 μl. Hydrolysis was monitored at 11 sec intervals by fluorimetry in a *f*max Microplate Reader (Molecular Devices Corporation, California, USA). The wavelength pair for excitation and emission was 370 nm/460 nm (Oliveira *et al*. 1992). MCA concentrations were calculated using standard curves.

The enzyme activity was also tested with MCA-Gln-Ser-Ser-Arg-His-Arg-Arg-Ala-Leu-Gln-EDDnp (3·6 μM final concentration). This substrate, when hydrolysed in any one of its peptide bonds, emits fluorescence of the MCA group, otherwise quenched by the EDDnp group when the substrate is intact. This substrate was efficiently hydrolysed by the cysteine endopeptidase and was therefore used to determine the enzyme's optimum pH and optimum temperature. The optimum pH was determined in 50 mM sodium citrate/sodium phosphate buffer, 10 mM DTT at several pHs. Optimum temperature was determined in 50 mM sodium citrate/sodium phosphate buffer, pH 3·5, 10 mM DTT by incubating the enzyme for 30 min at different temperatures. Hydrolysis of the substrate was measured by reading the increase in fluorescence at 320/430 nm after the incubation.

Enzyme purification

Eggs from the 1st to the 12th day after oviposition were homogenized in a Potter-Elvehjem tissue grinder with 10 mM phosphate buffer, pH 7·2 (0·1 g of eggs/ml). The homogenate was centrifuged (16 000 ***g*** for 5 min) to remove the insoluble material and the supernatant fraction was filtered through a sequence of filters (AP glass fibre filter, 0·45 μm and 0·22 μm). The sample (7 ml) was then applied onto a 0·5 cm × 5·0 cm Mono Q HR 5/5 (Pharmacia, Uppsala, Sweden) column previously equilibrated with 10 mM sodium phosphate buffer, pH 7·2, and eluted with a 0–0·8 M NaCl gradient in the same buffer system at room temperature with a flow rate of 0·5 ml/min. Fractions of 1·0 ml were collected. Fractions containing activity (6–7 ml) were pooled and submitted to autolysis. In this step, the pooled fractions were acidified to pH 3·5 with 1·0 M citric acid and incubated at 37 °C for approximately 3 h, after which the sample was centrifuged at 3000 ***g*** for 15 min.

After centrifugation, the supernatant fraction was concentrated in a Centricon-10 and applied onto a 1·0 cm × 30 cm Superdex 75 HR (Pharmacia, Uppsala, Sweden) column equilibrated with 10 mM acetate buffer, pH 4·0, using a Fast-Purification-Liquid Chromatography system (FPLC) at room temperature with a flow rate of 0·3 ml/min. The pool of active fractions was then applied onto a second Mono Q HR 5/5 column (0·5 cm × 5·0 cm), previously equilibrated with acetate buffer 10 mM, pH 4·0. The enzyme was eluted with a 0–0·8 M NaCl gradient in the same buffer. Enzyme activity in the fractions was monitored with *N*-Cbz-Phe-Arg-MCA as substrate.

Protein determination

Protein concentration was estimated by reading the absorbance at 280 and 260 nm (Warburg & Christian, 1941) in a quartz microplate using a SpectraMax microplate reader (Molecular Devices Corporation, California, USA) or in a 1·0 cm quartz cuvette using a spectrophotometer (Pharmacia, Uppsala, Sweden). Protein concentration of column eluates was monitored by absorbance at 280 nm.

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press

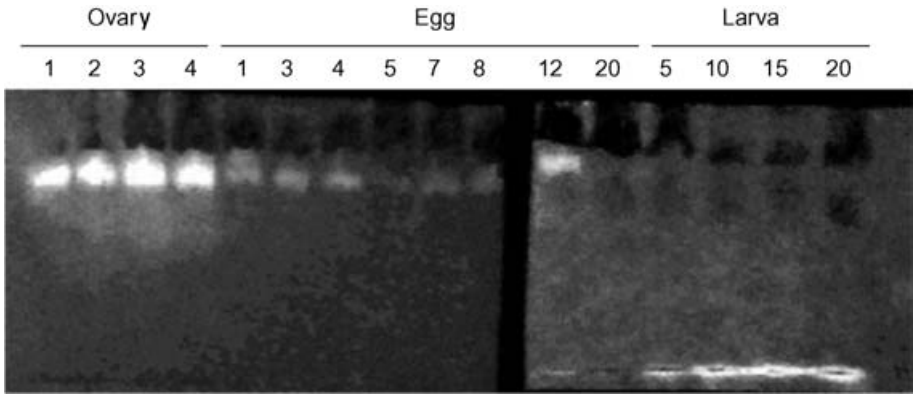


Fig. 1. Analysis by zymogram of the proteolytic activity present in the extracts of *Boophilus microplus* ovaries, eggs and larvae. The experiment was done using polyacrylamide (6%) gel electrophoresis containing co-polymerized gelatin (0·1%), stained with Coomassie R-250. Proteolytic activity is indicated by the translucent bands in the gels. Samples: ovaries dissected from females of 1, 2, 3 and 4 days after detachment from the bovine; extract of eggs 1, 3, 4, 5, 7, 8, 12 and 20 days after oviposition; and extract of larvae of 5, 10, 15 and 20 days after (egg) hatching.

Enzyme purity

The enzyme purity was checked by capillary electrophoresis as described (Kist, Termignoni & Grieneisen, 1994). Briefly, the sample was labelled with fluorescamine and applied to a capillary (75 μm i.d. × 52·35 cm) filled with 20 mM $Na_4B_2O_7$, pH 9·3, in methanol (15%, v/v), to which a potential of 13 kV (30 μA) was applied. The components were detected by a N_2 laser-induced fluorescence detection system. Fluorescamine was used in a very large excess in order to assure all primary amino groups of the protein were labelled and also to serve as an internal standard.

Molecular weight determination

Samples were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (14%) under non-denaturing and denaturing conditions (Laemmli, 1970). The electrophoresis was performed using a Bio-Rad Mini-Protean Cell II unit. Resolving and stacking gels were 14% and 5% polyacrylamide, respectively.

Sensitivity to inhibitors

The purified enzyme was incubated with 100 μM phenylmethylsulfonyl fluoride (PMSF), 10 mM, ethylenediaminetetraacetic acid (EDTA), 50 μM Na-*p*-tosyl-lysine chloromethyl ketone (TLCK), 1·4 μM pepstatin A, 100 μM chymostatin or 10 μM L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64) in 50 mM sodium citrate/sodium phosphate buffer, pH 3·5, for 15 min at room temperature. After addition of 10 mM DTT and a further 10 min incubation at 37 °C, the inhibitory activity of those compounds was determined by measuring the residual enzymatic activity upon *N*-Cbz-Phe-Arg-MCA (as described above).

RESULTS

Proteolytic activity in ovaries, eggs and larvae

Zymograms in native polyacrylamide gels with co-polymerized gelatin were used to verify the presence of proteolytic activity in *B. microplus* ovaries, eggs and larvae. Figure 1 shows that all extracts tested have proteolytic activity at acid pH, indicated by the translucent bands in the gels. Enzyme(s) with proteolytic activity from ovaries and eggs migrated less than the larval proteolytic activity (Fig. 1), suggesting that the endopeptidases present in eggs and ovaries can be bigger than the ones present in larvae, or/and they can associate strongly with itself or with other proteins. The egg peptidase was selected for further studies.

Purification of the egg peptidase activity

An homogeneous enzyme preparation was achieved in 4 steps. Crude egg extracts were applied onto a MonoQ column and eluted with a 0–0·8 M NaCl gradient (Fig. 2A). Almost all vitellin, the major reserve protein of tick eggs, was eluted in the flow-through, while the enzyme eluted at 0·15–0·25 M NaCl. Judging by the brownish colour, an amount of vitellin was still contaminating the active fractions. Vitellin was eliminated from the peptidase activity by the autolysis step. Figure 3 shows an electrophoretic analysis of the material after the autolysis, in the presence and absence of E-64, a specific cysteine endopeptidase inhibitor. The gels show that, as autolysis proceeds, egg proteins with high molecular weight are hydrolysed yielding intermediates of lower molecular weight (Fig. 3). After a 60 min incubation, the high molecular weight bands were hardly visible, and after 180 min they virtually disappeared. At this time, the only remaining visible protein bands were of ~55 and ~30 kDa. At any incubation time, hydrolysis was fully inhibited by E-64, indicating that the reaction is carried out by a cysteine endopeptidase.

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press

Table 1. Purification protocol and recovery

Purification step	Volume (ml)	Protein (mg)	Activity		Yield (%)	Purification fold
			Total (U)*	Specific (U/mg)		
Extract	5·7	272	68	0·25	—†	—
Mono Q	6·5	60	1688	28	100	1
Autolysis	5·9	14	1874	134	111	5
Superdex 75	2·8	3·7	1310	354	78	13
Mono Q	1·0	0·4	740	1947	44	70

* U = mFU/min.
† The activity present in the crude extract was not considered as 100% because enzyme activity is increased after the first purification step.

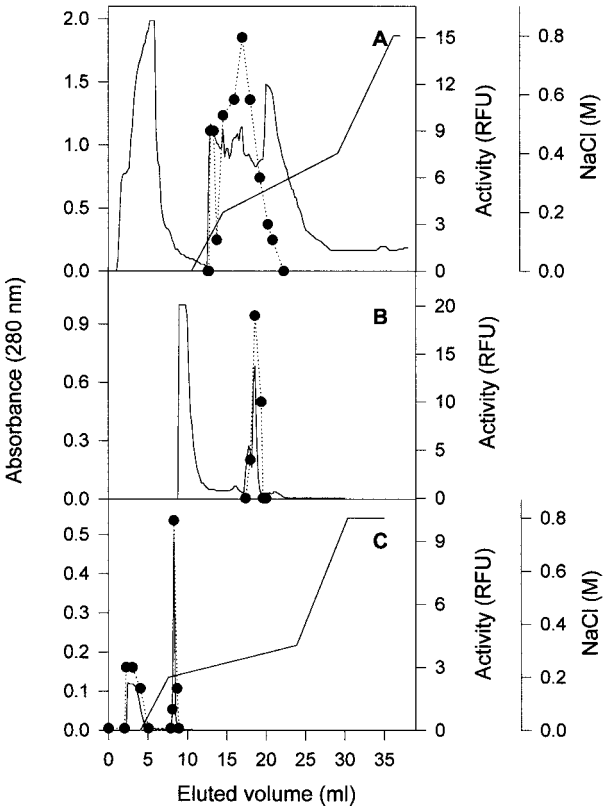


Fig. 2. Purification of the egg peptidase activity. (A) MonoQ1 – egg homogenate was applied onto a MonoQ column previously equilibrated with 10 mM sodium phosphate buffer, pH 7·2. Proteins were eluted with a 0–0·8 M NaCl gradient. (B) Superdex 75 – the supernatant from the autolysis step (see Materials and Methods section) was concentrated and applied onto a gel filtration chromatography column equilibrated with 10 mM acetate buffer, pH 4·0. (C) MonoQ2 – fractions from Superdex 75 showing activity were pooled and applied onto a MonoQ column previously equilibrated with 10 mM acetate buffer, pH 4·0; the sample was eluted with a 0–0·8 M NaCl gradient. Protein elution was monitored at 280 nm. Fractions were assayed upon the substrate *N*-carboxybenzoyl-Phe-Arg-MCA. The solid line represents protein elution, the dotted line, enzyme activity and the stepped line, the salt gradient.

After the autolysis step, the supernatant fraction containing proteolytic activity was applied onto a gel filtration column (Fig. 2B). The activity eluted at 17–19 ml, coinciding with 1 of the major protein peaks. These fractions were pooled and submitted to a second anion-exchange (Mono Q) column, equilibrated with sodium acetate buffer, pH 4·0. Under these conditions, some of the activity eluted in the flow-through and the retained activity eluted as a single sharp protein peak, at 0·3 M NaCl (Fig. 2C). An outline of the purification is presented in Table 1. The activity increased considerably after the first purification step (Mono Q), and this sample was thus taken as 100% activity to calculate yield and purification fold. The increase in activity after the first anion-exchange may be due to removal of vitellin excess that interferes with the enzyme activity.

The purity of the enzyme preparation is demonstrated in Fig. 4A, which shows a single protein band in native PAGE. To confirm this result, the preparation was also checked by free-zone capillary electrophoresis, a more sensitive method. Again, only 1 protein signal was observed (Fig. 4C). In SDS–PAGE (Fig. 4B), however, 2 protein bands, one of approximately 17 000 Da and another of 22 000 Da (Fig. 4B, lane 3), were observed, suggesting that the peptidase may be a dimer. Surprisingly, these 2 bands became 1 broad band, ranging from 13 000 Da to 16 000 Da, under reducing conditions (Fig. 4B, lane 2). This is an indication that the endopeptidase has peptide chains connected by disulfide bridges.

Optimum pH and temperature

Using a synthetic fluorogenic substrate, the cysteine endopeptidase showed maximal activity at pH 4·0 (Fig. 5A) and the optimum temperature was 50 °C (Fig. 5B).

Effect of inhibitors

The effect of a variety of compounds upon the activity of the purified proteinase is shown in Table 2. As the

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press

Table 2. Enzyme inhibition by proteinase inhibitors

(The purified *Boophilus microplus* egg enzyme was pre-incubated with the indicated inhibitors for 15 min at room temperature, and assayed for residual activity using *N*-Cbz-Phe-Arg-MCA as substrate.)

Inhibitor	Specificity	Concentration (mM)	Inhibition (%)
E-64	Cysteine	0·01	100
Leupeptin	Cysteine	0·01	100
TLCK	Serine/cysteine	0·1	100
Chymostatin	Serine/cysteine	0·1	100
PMSF	Serine/cysteine	0·1	33
Pepstatin A	Aspartic	0·001	0
EDTA	Metallo	0·5	0

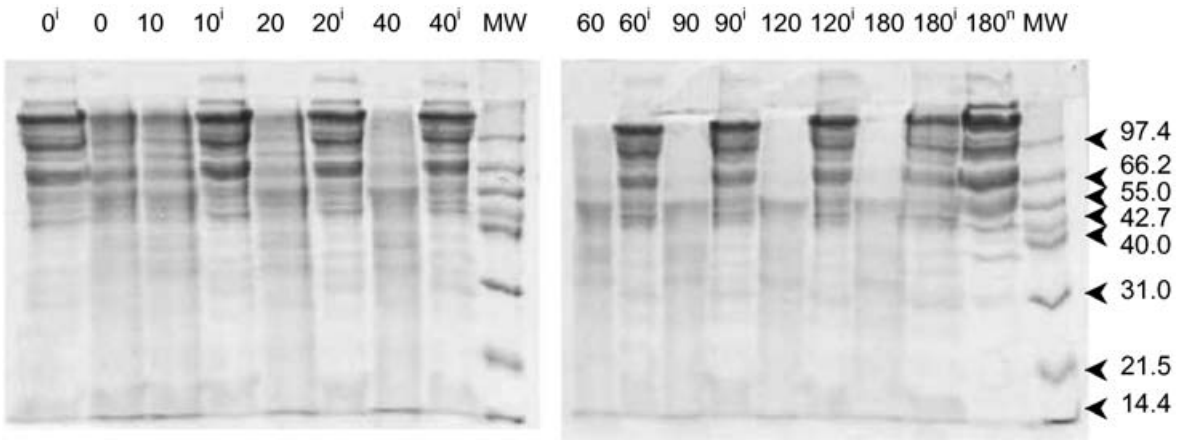


Fig. 3. Analysis by SDS–PAGE (12·5%) of autolysis products. Active fractions from MonoQ1 were incubated at 37 °C, in the presence or absence of E-64 (10 μM), after adjusting the pH to 3·5 with 1 M citric acid. At the indicated times, aliquots (67 μg) were taken and analysed by SDS–PAGE (12·5%). The presence of E-64 is indicated by ^{i}. 180^{n} shows an assay done in sodium phosphate buffer, pH 7·2 (n = neutral). MW, molecular weight standards.

enzyme has no activity in the absence of DTT, this compound was used to activate the enzyme prior to determination of the residual activity. E-64, a specific cysteine endopeptidase inhibitor, leupeptin, TLCK and chymostatin completely inhibited the enzyme activity. The enzyme was partially inhibited by PMSF and was insensitive to pepstatin A and EDTA. The requirement for DTT and sensitivity to E-64 strongly suggest that the enzyme is a cysteine endopeptidase.

Activity with synthetic peptides

The enzyme activity was evaluated upon a panel of synthetic substrates (Table 3). As, in these substrates, the hydrolysable amide bond is the one between the peptide and the fluorophorous group, we could identify the aminoacid residues at P_1 and P_2 subsites that are preferred by the enzyme. Among them, the higher activity was upon ε-NH_2-Cap-(SBzl)C-MCA and *N*-carboxibenzoyl-Phe-Arg-MCA. The enzyme also showed activity upon ε-NH_2-Cap-Leu-Phe-MCA and ε-NH_2-Cap-Leu-(OBzl)Thr-MCA. No activity upon the other peptidyl-methylcoumarin substrates was observed (Table 3). According to these results, the enzyme was more active upon substrates with a hydrophobic or a basic residue in P_1, and a hydrophobic reside in P_2. This specificity pattern, a basic residue in P_1 and a hydrophobic residue in P_2, was also observed in peptidyl-*p*-nitroanilide substrates (not shown).

Activity upon proteins

Zymograms showed that the partially purified enzyme was able to hydrolyse, in addition to vitellin, haemoglobin, gelatin and bovine serum albumin (BSA), whereas activity upon ovalbumin was barely observed (Fig. 6). Activities from crude extracts are also shown (Fig. 6, lane 3), although they were more difficult to visualize because most of the translucent bands were covered by the excessive amount of protein from the extract.

DISCUSSION

Our results demonstrate the presence of an endopeptidase activity in *B. microplus* unfed larvae, ingurgitated female ovaries and eggs. Differences in the electrophoretic migration of the endopeptidase activities may indicate the presence of distinct enzymes in each tick stage or organ. Since eggs are very

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press

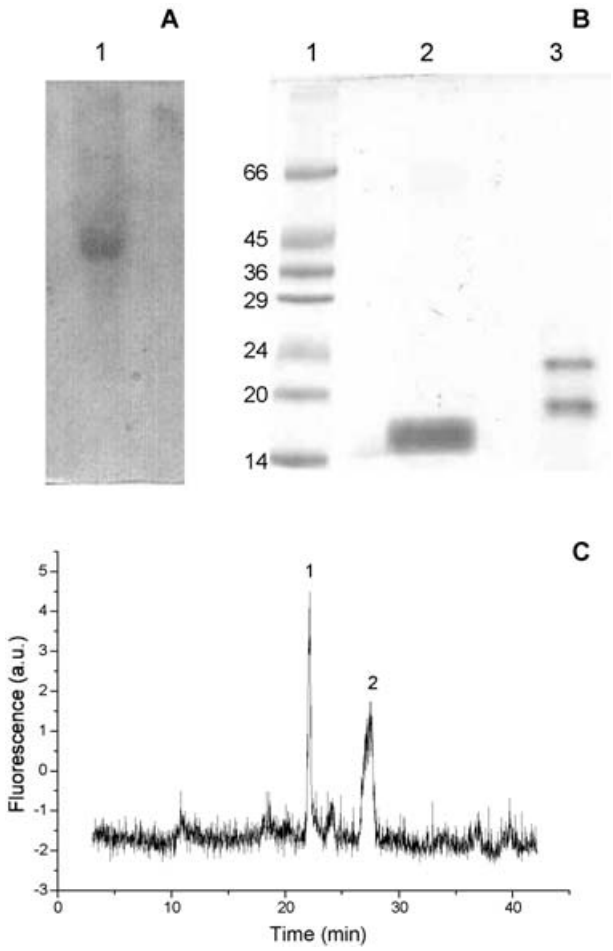


Fig. 4. (A) Electrophoresis of the purified cysteine endopeptidase (40 μg) in 1·5% native-polyacrylamide gel (without SDS). (B) Electrophoresis of the purified cysteine endopeptidase (50 μg) in SDS–PAGE (14%). (1) Molecular weight standards (Low-Range Sigma); (2) enzyme reduced by β-mercaptoethanol; (3) enzyme without reduction. Protein was detected by colloidal Coomassie staining. (C) Capillary electrophoresis of the purified enzyme (for experimental conditions see Materials and Methods section). Peak 1 purified enzyme, peak 2 excess of fluorescamine.

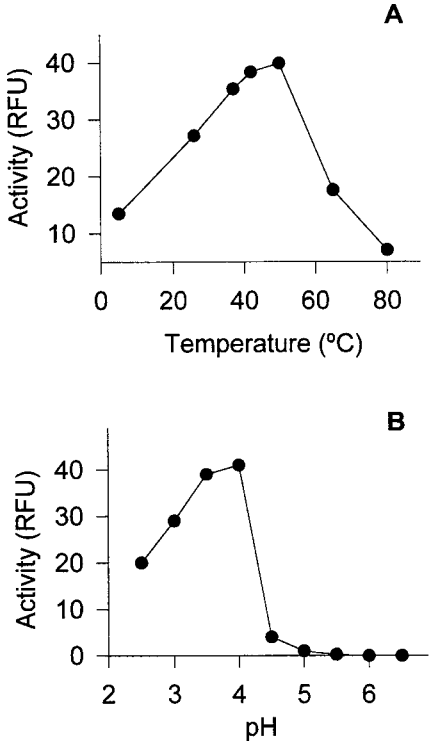


Fig. 5. Effect of temperature (A) and pH (B) on the activity of partially purified cysteine endopeptidase (after Superdex 75). The activity was tested upon MCA-Gln-Ser-Ser-Arg-His-Arg-Arg-Ala-Leu-Gln-EDDnp as described in the Materials and Methods section.

rich in one single protein, vitellin, 13–7·5 μg VT/egg, (Logullo *et al*. 2002) and its hydrolysis is of paramount importance for embryo nutrition, we decided to investigate the egg vitellin-degrading activity.

Tick oogenesis starts in the female ovary soon after blood feeding has begun. Vitellogenin produced in the fat body, and present in the maternal haemolymph, is matured to vitellin, which then accumulates in oocytes (Diehl, Aeschlimann & Obenchain, 1982). Proteins, mainly vitellin, lipids and sugars constitute the yolk granules, which are fundamental to embryo development and larvae survival until they attach to the bovine and start blood feeding. Certain peptidases are transferred by endocytosis to the oocyte during vitellogenesis (Diehl, Aeschlimann & Obenchain, 1982). In some species, they appear to be synthesized extraovarially as inactive pro-endopeptidases (Ribolla & De Bianchi, 1995; Nussenzveig, Oliveira & Masuda, 1992), which are associated with vitellin (VT) in the yolk granules and activated by acidification (Fagotto, 1995). This seems to be the case of the *B. microplus* egg endopeptidase now described. This enzyme is tightly associated with VT, whose contamination is easily detected by the brownish colour conferred from its high heme-binding capacity. Actually, *B. microplus* vitellin binds more than 40 heme molecules per vitellin molecule (Logullo *et al*. 2002). Several chromatographic procedures, including hydrophobic interaction and addition of 1 M glucose to the gel filtration buffer, were attempted before we could successfully free VTDCE from VT. Incubation of the enzyme preparation with endoglycosidase F was equally inefficient. Vitellin-free VTDCE was obtained by VT degradation achieved by acidification and subsequent incubation of the active material eluted from the first Mono Q column. The acidification required for VT degradation, which does not occur at neutral pH, coincides with VTDCE optimum pH. Clearly, pH alone is not sufficient to degrade VT, as inhibition of the enzyme activity with the cysteine inhibitor E-64 did not result in substrate degradation despite the acid pH. A similar example is a cathepsin B-like cysteine proteinase from *Drosophila* that is associated to yolk granules and blockage of acidification prevents yolk degradation (Medina *et al*. 1988). Degradation of VT

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press

Table 3. Activity of the purified enzyme upon fluorogenic peptide substrates

(Purified VTDCE (7·6 μg) was incubated with 50 mM sodium citrate/sodium phosphate buffer, pH 3·5, 10 mM DTT at 37 °C. After 10 min, the fluorogenic substrates were added (100 μM, final concentration) to a total volume of 100 μl. Hydrolysis was monitored at 370 nm/460 nm, in a *f*max Microplate Reader.)

Fluorogenic substrates	Activity (pmoles MCA/min)	Activity (%)
ε-NH_2-Cap-(SBzl)-C-MCA	14·2	100
N-Cbz-Phe-Arg-MCA	10·20	72
ε-NH_2-Cap-Leu-Phe-MCA	1·29	9
ε-NH_2-Cap-Leu-(Obzl)-Thr-MCA	0·88	6
N-t-Boc-Glu-Ala-Arg-MCA	0	0
N-t-Boc-Gln-Ala-Arg-MCA	0	0
N-t-Boc-Gly-Arg-MCA	0	0
N-t-Boc-Ile-Glu-Gly-Arg-MCA	0	0
N-α-benzoyl-Lys-Arg-MCA	0	0
N-t-Boc-Gln-Val-Pro-Arg-MCA	0	0

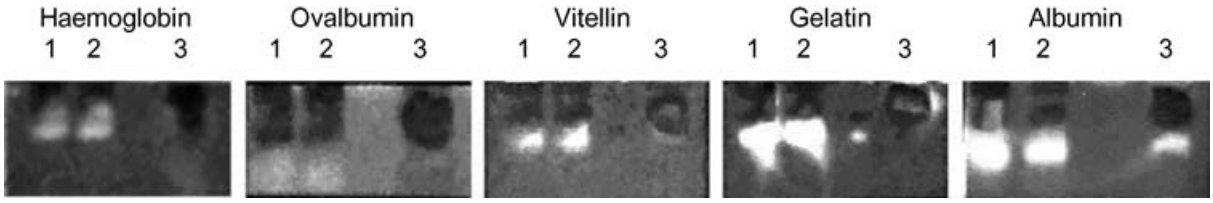


Fig. 6. Specificity of the partially purified enzyme upon natural substrates. A partially purified enzyme was electrophoresed in polyacrylamide gels (7·5%) co-polymerized with 0·1% haemoglobin, 0·1% ovalbumin, 0·1% vitellin, 0·1% gelatin or 0·1% albumin. After the electrophoretic separation, gels were incubated overnight in 0·1 M sodium acetate buffer, pH 3·5, containing 5 mM DTT, at 37 °C. After incubation, gels were washed with water and stained with Coomassie Brilliant Blue R-250. A see-through band indicates degradation of substrate by the enzyme. Lanes 1 and 2: partially purified enzyme after MonoQ1 and Superdex 75, respectively; lane 3: whole egg extract.

by VTDCE was confirmed when purified VT was incubated with purified VTDCE (results not shown).

This strong association between VTDCE and VT may maintain the enzyme saturated with VT and in this way increasing its hydrolysis. This could be of physiological relevance for VT utilization by the embryo. The VT would block the access of synthetic substrates to the active site, thus explaining the increase in the enzyme activity upon the *N*-Cbz-Phe-Arg-MCA after the first purification step.

Analysis by native PAGE and capillary electrophoresis show that VTDCE was obtained in a homogeneous form. On the other hand, SDS–PAGE (under non-reducing conditions) revealed 2 protein bands (22 kDa and 17 kDa), indicating that the enzyme is formed by 2 non-covalently bound subunits. Another possibility is that a fragment of VT remained bound to the enzyme and was dissociated by SDS during electrophoresis. This is unlikely, though, since the preparation was not recognized by a polyclonal anti-VT antibody (Da Silva Vaz Jr, Ozaki & Masuda, 1994); contamination, if present, would be less than 10^{-4} μg Vt/μg VTDCE. In addition, this preparation did not absorb at 400 nm (VT maximum absorbance wavelength). Under reducing conditions, only 1 diffuse protein band was observed, corresponding to a molecular range from 13 kDa to 16 kDa. This result suggests that each subunit observed in SDS–PAGE would have at least 2 peptide chains linked by S-S bridges. Heteropolymeric endopeptidases have been described in eggs of other arthropods. Eggs of the argaside tick. *O. moubata* has a cathespin L-like acid proteinase that presents one peptide chain in native-gelatin-copolymerised-gels and two chains (37 and 39 kDa) in gelatin-copolymerised SDS–PAGE (Fagotto, 1990). Likewise, *B. germanica* contains a cysteine protease with three polypeptides of approximately 29 kDa each (Liu *et al*. 1996).

The strict requirement of the purified enzyme for DTT and its inhibition by E-64 and leupeptin indicate VTDCE is a cysteine endopeptidase. Proteases of the cysteine endopeptidase subclass often participate in arthropod yolk degradation, as has been reported for *B. germanica*, *Drosophila melanogaster*, *O. moubata* and *Bombyx mori* (Liu *et al*. 1996; Medina *et al*. 1988; Fagotto, 1990; Kageyama & Takahashi, 1990).

VTDCE was able to hydrolyse the synthetic substrates ε-NH_2-Cap-(SBzl)-Cys-MCA (that has a hydrophobic aminoacid in P_1) and *N*-Cbz-Phe-Arg-MCA, which is a substrate highly susceptible to hydrolysis by cathepsin L but is also hydrolysed

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press

by cathepsin B (Medina *et al*. 1988). Based on this specificity and the optimum pH (pH 4·0), we can suggest that VTDCE is a cathepsin L-like enzyme. We exclude the possibility that VTDCE is *Bm*c11, a cathepsin L-like enzyme previously described in *B. microplus* larvae, because they differ in substrate specificity, sensitivity to inhibitors and optimum pH (Renard *et al*. 2000). Further support to this idea is that the synthetic substrates *N*-t-Boc-Glu-Ala-Arg-MCA, *N*-t-Boc-Gln-Ala-Arg-MCA, *N*-t-Boc-Gln-Val-Pro-Arg-MCA are hydrolysed by *Bm*c11 but not by VTDCE. The VTDCE, like most members of the papain family, hydrolyses substrates with hydrophobic aminoacids in P_1 and/or P_2, or with arginine or lysine residues in P_1.

VTDCE hydrolyses haemoglobin, albumin, gelatin and vitellin. This shows that VTDCE has a broad specificity, in agreement with its ability to fully degrade VT. Nevertheless, we believe that the physiological substrate for VTDCE is vitellin, since VT is the major protein of the yolk granule and is naturally associated with the enzyme.

Altogether, our results demonstrate that VTDCE is a major enzyme involved in yolk processing during *B. microplus* embryogenesis. Another important *B. microplus* enzyme implicated in this process is the aspartic proteinase BYC (*Boophilus* Yolk procathepsin) (Logullo *et al*. 1998). Ticks fed on BYC-vaccinated bovines presented functional bovine immunoglobulins circulating in haemolymph (Da Silva Vaz Jr *et al*. 1996). Moreover, cattle vaccinated with BYC presented a partially protective immune response against *B. microplus* infestation, mainly due to a decreased number of fertile eggs (Da Silva Vaz Jr *et al*. 1998). Considering that VT degradation results from the activity of both enzymes (BYC and VTDCE), VT degradation would be even more impaired if, in addition to antibodies against BYC, bovine antibodies against VTDCE could also be raised.

The authors are grateful to Dr Jair R. Chagas and Dr Luiz Juliano (Escola Paulista de Medicina, Universidade Federal de São Paulo, Brasil) for the synthetic fluorogenic substrate MCA-Gln-Ser-Ser-Arg-His-Arg-Arg-Ala-Leu-Gln-EDDnp. We also thank Dr Tarso B. Kist and Sandro Hillebrand for the capillary electrophoresis of VTDCE. This work was supported by grants from Financiadora de Estudos e Projetos (PRONEX-FINEP), Programa de Apoio ao Desenvolvimento Científico e Tecnológico (PADCT), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-PIBIC). A. S. and P. C. S. were recipients of PIBIC-CNPq scholarships.

REFERENCES

CANAL, C. W., MAIA, H. M., DA SILVA VAZ Jr, I., CHIES, J. M., FARIAS, N. A., MASUDA, A., GONZALES, A. J. C., OZAKI, L. S. & DEWES, H. (1995). Changing of vitellin-related peptides during development of the cattle tick *Boophilus microplus*. *Experimental and Applied Acarology* **19**, 325–336.

CHO, W. L., TSAO, S. M., HAYS, A. R., WALTER, R., CHEN, J. S., SNIGIREVSKAYA, E. S. & RAIKHEL, A. S. (1999). Mosquito cathepsin B-like protease involved in embryonic degradation of vitellin is produced as a latent extraovarian precursor. *Journal of Biological Chemistry* **274**, 13311–13321.

DA SILVA VAZ Jr, I., MARTINEZ, R. H., OLIVEIRA, A., HECK, A., LOGULLO, C., GONZALES, J. C., DEWES, H. & MASUDA, A. (1996). Functional bovine immunoglobulins in *Boophilus microplus* hemolymph. *Veterinary Parasitology* **62**, 155–160.

DA SILVA VAZ Jr, I., OZAKI, L. S. & MASUDA, A. (1994). Serum of *Boophilus microplus* infested cattle reacts with different tick tissues. *Veterinary Parasitology* **52**, 71–78.

DA SILVA VAZ Jr, I., LOGULLO, C., SORGINE, M., VELLOSO, F. F., ROSA DE LIMA, M. F., GONZALES, J. C., MASUDA, H., OLIVEIRA, P. L. & MASUDA, A. (1998). Immunization of bovines with an aspartic proteinase precursor isolated from *Boophilus microplus* eggs. *Veterinary Immunology and Immunopathology* **66**, 331–341.

DIEHL, P. A., AESCHLIMANN, A. & OBENCHAIN, F. D. (1982). Tick reproduction: oogenesis and oviposition. In *Physiology of Ticks* (ed. Obenchain, F. D. & Galun, R.), pp. 277–350. Pergamon Press, Oxford.

FAGOTTO, F. (1990). Yolk degradation in tick eggs: I. Occurrence of a cathepsin L-like acid proteinase in yolk spheres. *Archives of Insect Biochemistry and Physiology* **14**, 217–235.

FAGOTTO, F. (1991). Yolk degradation in tick eggs: III. Developmentally regulated acidification of the yolk spheres. *Development Growth and Differentiation* **33**, 57–66.

FAGOTTO, F. (1995). Regulation of yolk degradation, or how to make sleepy lysosomes. *Journal of Cell Science* **108**, 3645–3647.

FAGOTTO, F. & MAXFIELD, F. R. (1994). Yolk platelets in *Xenopus* oocytes maintain an acidic internal pH which may be essential for sodium accumulation. *Journal of Cell Biology* **125**, 1047–1056.

HEUSSEN, C. & DOWDLE, E. B. (1980). Electrophoretic analysis of plasminogen activators in polyacrilamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analytical Biochemistry* **102**, 196–202.

KAGEYAMA, T. & TAKAHASHI, S. Y. (1990). Purification and characterization of a cysteine proteinase from silkworm eggs. *European Journal of Biochemistry* **193**, 203–210.

KIST, T. B. L., TERMIGNONI, C. & GRIENEISEN, H. P. (1994). Capillary zone electrophoresis separation of kinins using a novel laser fluorescence detector. *Brazilian Journal of Medical and Biological Research* **27**, 11–19.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature, London* **227**, 680–685.

LIU, X., MCCARRON, R. C. & NORDIN, J. H. (1996). A cysteine protease that processes insect vitellin. Purification and partial characterization of the enzyme and the proenzyme. *Journal of Biological Chemistry* **271**, 33344–33351.

LOGULLO, C., DA SILVA VAZ Jr, I., SORGINE, M. H., PAIVA-SILVA, G. O., FARIA, F. S., ZINGALI, R. B., DE LIMA, M. F., ABREU, L., OLIVEIRA, E. F., ALVES, E. W., MASUDA, H., GONZALES, J. C., MASUDA, A. & OLIVEIRA, P. L. (1998). Isolation of an aspartic proteinase precursor from the egg of a hard tick, *Boophilus microplus*. *Parasitology* **116**, 525–532.

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press

LOGULLO, C., MORAES, J., DANSA-PETRETSKI, M., DA SILVA VAZ Jr, I., MASUDA, A., SORGINE, M. H. F., BRAZ, G. R. & OLIVEIRA, P. L. (2002). Binding and storage of heme by vitellin from the cattle tick. *Insect Biochemistry and Molecular Biology* **32**, 1805–1811.

MALLYA, S. K., PARTIN, J. S., VALDIZAN, M. C. & LENNARZ, W. J. (1992). Proteolysis of the major yolk glycoproteins is regulated by acidification of the yolk platelets in sea urchin embryos. *Journal of Cell Biology* **117**, 1211–1221.

MEDINA, M., LEON, P. & VALLEJO, C. G. (1988). *Drosophila* cathespin B-like proteinase: a suggested role in yolk degradation. *Archives of Biochemistry and Biophysics* **263**, 355–363.

NUSSENZVEIG, R. H., OLIVEIRA, P. L. & MASUDA, H. (1992). Identification of yolk platelet-associated hydrolases in the oocytes of *Rhodnius prolixus*. *Archives of Insect Biochemistry and Physiology* **21**, 253–262.

OLIVEIRA, M. C., HIRATA, I. Y., CHAGAS, J. R., BOSCHCOV, P., GOMES, R. A., FIGUEIREDO, A. F. & JULIANO, L. (1992). Intramolecularly quenched fluorogenic peptide substrates for human renin. *Analytical Biochemistry* **203**, 39–46.

RENARD, G., GARCIA, J. F., CARDOSO, F. C., RICHTER, M. F., SAKANARI, J. A., OZAKI, L. S., TERMIGNONI, C. & MASUDA, A. (2000). Cloning and functional expression of a *Boophilus microplus* cathepsin L-like enzyme. *Insect Biochemistry and Molecular Biology* **30**, 1017–1026.

RIBOLLA, P. E. & DE BIANCHI, A. G. (1995). Processing of procathepsin from *Musca domestica* eggs. *Insect Biochemistry and Molecular Biology* **25**, 1011–1017.

WARBURG, O. & CHRISTIAN, W. (1941). Isolierung und Kristallisation des Garungsferments enolase. *Biochemische Zeitschrift* **310**, 384–421.

https://doi.org/10.1017/S0031182002002731 Published online by Cambridge University Press