

Proteases in pathogenic and nonpathogenic haemoflagellates, *Cryptobia* spp. (Sarcomastigophora: Kinetoplastida), of fishes

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ABSTRACT: Proteases were detected in *Cryptobia salmositica* (pathogenic and nonpathogenic vaccine strains), *C. bullocki*, and *C. catostomi* using azocasein and hide powder azure as substrates. Maximum activity occurred in acidic pH and the pathogenic strain of *C. salmositica* had the highest activity. Cysteine protease was found in pathogenic and nonpathogenic *Cryptobia* spp., but metallo-protease was only present in the pathogenic strain of *C. salmositica*. Five enzymatic bands were detected in the pathogenic *C. salmositica* using haemoglobin-SDS-PAGE: four of these were cysteine proteases (49, 60, 66 and 97 kDa) and the other was a metallo-protease (200 kDa). The pathogenic *C. salmositica* lost the metallo-protease after 10 mo of *in vitro* culture. We suggest that the metallo-protease of the pathogenic *C. salmositica* is related to pathogenicity of the parasite.

KEY WORDS: *Cryptobia salmositica* · *C. bullocki* · *C. catostomi* · Cysteine protease · Metallo-protease

INTRODUCTION

Cryptobia spp. have been described from the gills, body surface, digestive tract, and blood of freshwater and marine fishes. Although a few species are pathogenic to fish, many are not known to cause disease (Woo 1987, 1991, 1994).

Cryptobia salmositica is a pathogenic haemoflagellate of salmonids on the Pacific coast of North America (Woo 1987, 1994). Infected fish develop anaemia, anorexia, exophthalmia, abdominal distension with ascites, general edema, and splenomegaly (Woo 1979, Li & Woo 1991). Serial *in vitro* culture of *C. salmositica* in minimum essential medium resulted in a strain that produced no clinical disease but protected adult (Woo & Li 1990, Li & Woo 1995) and juvenile (Sitja-Bobadilla & Woo 1994) rainbow trout from disease. *Cryptobia bullocki* is a pathogenic haemoflagellate in estuarine and marine flat fishes along the Atlantic coast of North America. The clinical signs of the disease include lethargy, anaemia, abdominal distension with ascites, and splenomegaly (Burreson 1982a, b). *Cryptobia cato-*

stomi is a nonpathogenic haemoflagellate of white suckers in Ontario, Canada. Though high parasitaemias occur in naturally and experimentally infected fish, there are no clinical signs associated with the infection (Bower & Woo 1977a, b, Thomas & Woo 1992).

Proteases which hydrolyse peptide bonds are found in all organisms and are separated into 4 major classes [serine, cysteine (thiol), metallo, and aspartyl (carboxy) protease] based on the important chemical groups in their active sites. Parasite proteases are presumed to facilitate invasion of host tissues, allow parasites to digest host proteins, help parasites evade the host immune response, and prevent blood coagulation (McKerrow 1989). Nothing is known about the proteolytic enzymes of *Cryptobia*. The aims of the present study are to characterize and quantify the major protease(s) of pathogenic and nonpathogenic *Cryptobia* species.

MATERIALS AND METHODS

Cryptobia salmositica and *C. bullocki* were cultured as described earlier (Woo & Thomas 1991) and *C. catostomi* was cultured in TDL-15 medium supplemented

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with 2% white sucker serum (Li & Woo 1996). The blood form of *C. salmositica* (pathogenic strain) was isolated from an infected rainbow trout and subsequently cultured for no more than 2 mo, at which time the parasite is still pathogenic to trout (Woo & Thomas 1991). This was to obtain a large number of parasites free of host cells. *C. salmositica* (vaccine strain) and *C. bullocki* had been in continuous *in vitro* culture for about 7 and 8 yr, respectively. The *C. catostomi* was from the blood of a naturally infected white sucker and maintained in the laboratory by blood inoculation into laboratory raised suckers.

Sample preparation. All procedures, unless otherwise stated, were carried out at 4°C. Parasites harvested from the late log phase were washed 3 times with 0.25 M sucrose by centrifugation at $7000 \times g$ for 10 min. A washed pellet containing about 5×10^7 cells was suspended in 1 ml of 0.1 M phosphate buffer (pH 6.5) containing 0.2% Triton X-100 (v/v), and sonicated for 5 min, and followed by 3 freeze and thaw cycles. After centrifugation at $7000 \times g$ for 20 min, the supernatant was pooled and used immediately or stored at -100°C. Storage at -100°C did not decrease proteolytic activities.

Protein concentration of the samples was determined using the method of Bradford (1976).

Protease assays. Proteolytic activities were assayed using azocasein (AZC) (North & Walker 1984) and hide powder azure (HPA) (North & Whyte 1984) as substrates with slight modifications. Briefly, 100 µl of the cell lysate (containing 100 µg protein) was incubated with 0.5 ml of either AZC (Sigma, 10 mg ml⁻¹) or 0.5 ml HPA (Sigma, 10 mg ml⁻¹) and 0.5 ml buffer at 37°C. The incubation time was 2 h with AZC or 4 h with HPA; 0.2 ml of 50% trichloroacetic acid (TCA) was then added to terminate the reaction and the tube left to stand at 4°C for 30 min. The insoluble material was removed by centrifugation and the dye released was determined spectrophotometrically at 520 nm for the AZCase and 595 nm for the HPAase against the blank (the same incubation solution but with no cell lysate). The activity is given in unit (1 µg substrate protein hydrolysed per hour per mg protein of the lysate). An increase of 1 absorbency unit was caused by the hydrolysis of 0.71 mg AZC and 3.4 mg HPA under the reaction conditions (North & Walker 1984, North & Whyte 1984). The buffers used to determine pH effects on protease activity were 0.1 M phosphate buffer (pH 3.0 to 7.0) and 0.1 M Tris-HCl buffer (pH 7.5 to 9.0).

Inhibition of proteases. The method used was that described by North et al. (1983). All the inhibitors used in this study were purchased from Sigma Chemical Company, Mississauga, Canada. Briefly, cell lysate was preincubated with inhibitors for 30 min at 25°C before adding the substrate. The concentrations of in-

hibitors used for the preincubation were 10 to 20 µM E-64 (L-trans-epoxysuccinyl-leucylamido-4-guanidinobutane) or 1 to 2 mM of PMSF (phenylmethylsulphonyl fluoride), or phenanthroline, or IAA (iodoacetamide), or TLCK (N-α-p-tosyl-L-lysine chloromethyl ketone); 100 to 200 µg ml⁻¹ of pepstatin, or leupeptin, or chymostatin, or antipain. Stock solutions of inhibitors in methanol (phenanthroline, chymostatin, pepstatin, or PMSF) or in water (all other inhibitors) were prepared at 20 times the required concentration. Appropriate control was preincubated with either water or methanol (but with no inhibitor).

Activation of proteases. Protease hydrolysis was also assayed in the presence of each of the following cysteine protease activators (Sigma): 1.0 mM DTT (dithiothreitol), or 5.0 mM EDTA (ethylene diamine tetraacetic acid), or 1.0 µg ml⁻¹ cysteine (Garber & Lemchuk-Favel 1989). The activators were preincubated with the lysate as was done with the inhibitors.

Substrate SDS-PAGE. Polyacrylamide gel electrophoresis using SDS (sodium dodecyl sulfate)-discontinuous buffer system (SDS-PAGE) was performed as described by Hames (1981). The substrate-SDS-PAGE was performed as above except that the haemoglobin (0.2% w/v) was incorporated into the 7.5% running gel. The haemoglobin, a blood protein substrate, was extracted and prepared from the blood of an uninfected rainbow trout according to the method of Knox et al. (1993). A 20 µl volume of cell lysate (containing 20 µg protein) was mixed with 10 µl of SDS-sample buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol and 0.02% bromophenol blue) without a reducing agent and without boiling. The sample was then applied to the gel and electrophoresed using a BioRad Mini-protean system (BioRad Ltd, Canada) with a constant current of 12 mA per gel at 4°C. After electrophoresis the gels were immersed in 1 l of 2.5% (v/v) Triton X-100 for 1 h to remove the SDS and were washed once with incubation buffer (0.1 M phosphate buffer, pH 5.0 or 7.0). The protease bands were developed by immersing the gels in the incubation buffer for 12 h at 37°C.

To determine the effects of inhibitors on the development of the protease bands, some strips of the gels were incubated in the buffer containing either E-64 (20 µM) or phenanthroline (1 mM). Zones of proteolysis were clear bands against a blue background after Coomassie Blue staining. Molecular weight of individual protease was determined from their mobility relative to those of protein standards.

Statistical analysis. Student's *t*-test (Wardlaw 1985) was used to determine significant difference in protease activities between different *Cryptobia* spp. and between either inhibition or activation of proteases and controls. Results were considered significant if $p < 0.05$.

RESULTS

Effects of pH on proteolytic activity

Proteolytic activities were detected in cell lysates of all *Cryptobia* spp. using either AZC or HPA as the substrate (Fig. 1). In all cases, proteolytic activities were significantly higher at acidic pH than at either neutral or alkaline pH. All activities were maximal between pH 3.5 and 4.5, and the optimal pH differed with both substrate and species/strain of *Cryptobia* (Fig. 1).

The optimal protease activity varied between species and strains of *Cryptobia* (Fig. 1). At optimal pH, the *C. salmositica* (blood form) had much higher activity than the other species or strain ($p < 0.05$); it was nearly 2-fold higher than that in the vaccine strain.

Classification of major classes of proteases by using standard inhibitors

Proteolytic activities in *Cryptobia* spp. were not inhibited by the serine protease inhibitor, PMSF, nor by the aspartyl protease inhibitor, pepstatin (Table 1). However, proteolytic activities of all *Cryptobia* spp. were significantly inhibited by E-64 (Table 1), indicating that cysteine protease was present in all *Cryptobia* spp. E-64 is a powerful inhibitor and at only 20 μM it completely inhibited the proteolytic activities of the vaccine strain of *C. salmositica* and *C. catostomi*. Phenanthroline, an inhibitor of metallo-protease, significantly reduced the proteolytic activities of the blood form of *C. salmositica* but not those of the vaccine

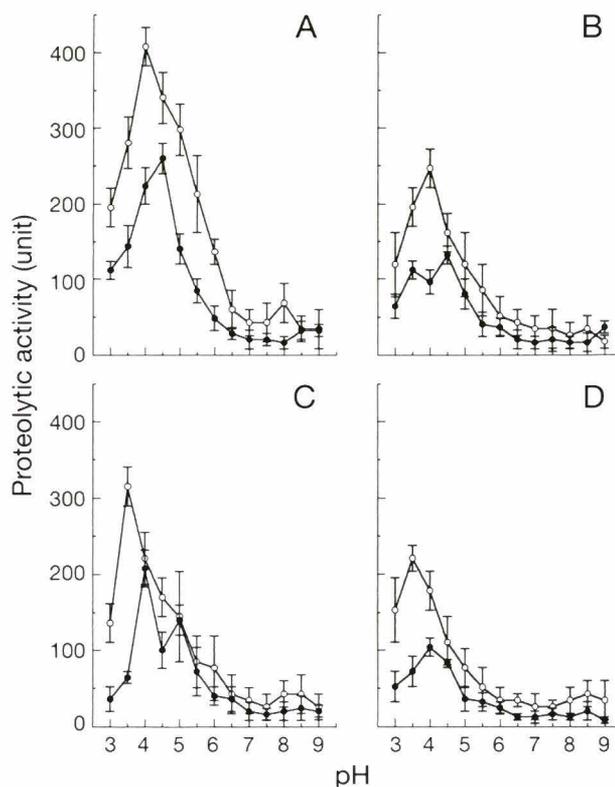


Fig. 1. Effects of pH on proteolytic activities of *Cryptobia* spp. (A) *C. salmositica* (blood form); (B) *C. salmositica* (vaccine strain); (C) *C. bullocki*; (D) *C. catostomi*. The buffers used were 0.1 M phosphate buffer (pH 3.0 to 7.0) and 0.1 M Tris-HCl buffer (pH 7.5 to 9.0). Each point is mean \pm standard deviation from 3 independent determinations. Proteolytic activity is given in units (μg substrate hydrolysed per hour per mg protein of enzyme). (●) using AZC as substrate; (○) using HPA as substrate

Table 1. Effects of protease inhibitors on proteolytic activity of *Cryptobia* spp. Activity remaining (with the inhibitor) was given as a percentage (%) of the control (without the inhibitor); mean \pm standard deviation from 3 independent determinations. PMSF: phenylmethylsulphonyl fluoride; E-64: L-trans-epoxysuccinyl-leucylamidi-4-guanidino-butane

| Inhibitors (protease class) | <i>C. salmositica</i> (blood form) | | <i>C. salmositica</i> (vaccine) | | <i>C. bullocki</i> | | <i>C. catostomi</i> | |
|--------------------------------|---------------------------------------|--------------------|------------------------------------|--------------------|--------------------|--------------------|---------------------|--------------------|
| | AZCase (pH 4.5) | HPAase (pH 4.0) | AZCase (pH 4.5) | HPAase (pH 4.0) | AZCase (pH 4.0) | HPAase (pH 3.5) | AZCase (pH 4.0) | HPAase (pH 3.5) |
| PMSF: (serine-) | | | | | | | | |
| 1 mM | 99.5 \pm 12.0 | 98.6 \pm 7.5 | 97.9 \pm 14.1 | 94.0 \pm 12.5 | 107.2 \pm 18.5 | 97.1 \pm 12.8 | 113.9 \pm 20.9 | 94.0 \pm 6.9 |
| 2 mM | 101.9 \pm 7.5 | 102.3 \pm 14.0 | 88.5 \pm 11.5 | 95.5 \pm 18.2 | 98.9 \pm 6.0 | 98.3 \pm 16.1 | 105.6 \pm 23.6 | 104.2 \pm 18.9 |
| Pepstatin: (aspartyl-) | | | | | | | | |
| 100 $\mu\text{g ml}^{-1}$ | 102.1 \pm 8.0 | 95.5 \pm 8.2 | 95.7 \pm 12.4 | 101.2 \pm 10.9 | 113.8 \pm 19.7 | 98.1 \pm 7.4 | 115.3 \pm 14.7 | 103.0 \pm 6.9 |
| 200 $\mu\text{g ml}^{-1}$ | 102.0 \pm 9.4 | 111.6 \pm 7.0 | 84.6 \pm 7.7 | 90.9 \pm 18.2 | 102.3 \pm 10.6 | 103.5 \pm 12.6 | 116.7 \pm 10.1 | 109.7 \pm 9.5 |
| Phenanthroline: (metallo-) | | | | | | | | |
| 1 mM | 73.3 \pm 5.0* | 70.9 \pm 7.5* | 91.4 \pm 6.7 | 82.1 \pm 12.9 | 84.4 \pm 11.2 | 79.0 \pm 7.2 | 89.7 \pm 10.5 | 84.7 \pm 6.4 |
| 2 mM | 61.0 \pm 7.1* | 66.0 \pm 8.5* | 86.7 \pm 6.6 | 88.0 \pm 10.0 | 82.2 \pm 11.1 | 84.0 \pm 16.0 | 90.5 \pm 9.5 | 90.0 \pm 10.0 |
| E-64: (cysteine-) | | | | | | | | |
| 10 μM | 30.4 \pm 5.4* | 27.7 \pm 8.5* | 10.0 \pm 6.6* | 16.0 \pm 8.0* | 20.0 \pm 6.7* | 25.0 \pm 6.3* | 14.3 \pm 4.8* | 10.0 \pm 5.0* |
| 20 μM | 23.2 \pm 7.1* | 21.3 \pm 6.4* | 0* | 0* | 8.9 \pm 2.2* | 9.4 \pm 3.1* | 0* | 0* |

*Activity was significantly lower ($p < 0.05$) than the control (without the inhibitor)

Table 2. Effects of cysteine protease inhibitors on proteolytic activity of *Cryptobia* spp. Activity remaining (with the inhibitor) was given as a percentage of the control (without the inhibitor); mean \pm standard deviation from 3 independent determinations. TLCK: N-a-p-tosyl-L-lysine chloromethyl ketone; IAA: iodoacetamide

| Inhibitors | <i>C. salmositica</i> (blood form) | | <i>C. salmositica</i> (vaccine) | | <i>C. bullocki</i> | | <i>C. catostomi</i> | |
|------------------------------|---------------------------------------|--------------------|------------------------------------|--------------------|--------------------|--------------------|---------------------|--------------------|
| | AZCase (pH 4.5) | HPAase (pH 4.0) | AZCase (pH 4.5) | HPAase (pH 4.0) | AZCase (pH 4.0) | HPAase (pH 3.5) | AZCase (pH 4.0) | HPAase (pH 3.5) |
| TLCK: | | | | | | | | |
| 1 mM | 17.5 \pm 4.2 | 35.6 \pm 7.0* | 10.4 \pm 4.7 | 13.1 \pm 7.4 | 33.4 \pm 4.5* | 30.4 \pm 7.4 | 9.7 \pm 6.4 | 18.2 \pm 4.6 |
| 2 mM | 18.9 \pm 7.5* | 27.9 \pm 9.3* | 0 | 0 | 15.9 \pm 6.8* | 17.2 \pm 6.9* | 0 | 0 |
| Leupeptin: | | | | | | | | |
| 100 μ g ml ⁻¹ | 32.8 \pm 5.8* | 40.9 \pm 10.0* | 25.8 \pm 6.5 | 48.8 \pm 12.5 | 30.4 \pm 4.6* | 40.0 \pm 8.6 | 14.9 \pm 5.3 | 27.8 \pm 8.7 |
| 200 μ g ml ⁻¹ | 30.4 \pm 7.1* | 31.9 \pm 8.5* | 0 | 0 | 15.6 \pm 4.4* | 18.8 \pm 6.3* | 0 | 0 |
| Chymostatin: | | | | | | | | |
| 100 μ g ml ⁻¹ | 37.5 \pm 6.4* | 56.8 \pm 10.4* | 25.0 \pm 6.3 | 25.0 \pm 10.7 | 32.6 \pm 7.8 | 46.1 \pm 10.3* | 16.7 \pm 11.1 | 24.2 \pm 6.9 |
| 200 μ g ml ⁻¹ | 28.3 \pm 5.7* | 34.9 \pm 9.3* | 0 | 0 | 9.1 \pm 3.2* | 17.2 \pm 4.5* | 0 | 0 |
| Antipain: | | | | | | | | |
| 100 μ g ml ⁻¹ | 18.9 \pm 5.9* | 28.4 \pm 6.4 | 8.6 \pm 4.9 | 22.6 \pm 9.0 | 55.6 \pm 8.9* | 60.9 \pm 9.2* | 5.7 \pm 5.2 | 19.4 \pm 6.4 |
| 200 μ g ml ⁻¹ | 17.9 \pm 5.4* | 21.3 \pm 6.3* | 0 | 0 | 15.6 \pm 2.2* | 15.6 \pm 3.1* | 0 | 0 |
| IAA: | | | | | | | | |
| 1 mM | 50.0 \pm 10.0* | 41.7 \pm 12.5* | 43.0 \pm 11.3 | 38.1 \pm 12.5* | 41.1 \pm 8.6 | 49.5 \pm 13.5* | 21.8 \pm 8.7 | 13.9 \pm 4.8 |
| 2 mM | 32.1 \pm 7.1* | 34.0 \pm 8.5* | 0 | 0 | 17.8 \pm 6.7* | 24.0 \pm 4.0* | 0 | 0 |

*Significantly higher ($p < 0.05$) than those of *C. catostomi*

strain of *C. salmositica*, *C. bullocki* and *C. catostomi* (Table 1), showing that metallo-protease is only present in the pathogenic strain of *C. salmositica*.

Effects of cysteine protease inhibitors on proteolytic activities

Proteolytic activities in all *Cryptobia* spp. were significantly inhibited by the following cysteine protease inhibitors: TLCK, leupeptin, chymostatin, antipain and IAA (Table 2), thus confirming that cysteine protease was present in all *Cryptobia* spp. tested. Proteases of the blood form of *C. salmositica* and *C. bullocki* were significantly less sensitive to these cysteine protease in-

hibitors than that of *C. catostomi* (Table 2). However, there were no significant differences ($p > 0.05$) between proteases of the nonpathogenic *C. catostomi* and of the vaccine strain of *C. salmositica* in their sensitivity to most inhibitors. Proteolytic activities in the vaccine strain of *C. salmositica* and *C. catostomi* were completely blocked by cysteine protease inhibitors at 2 mM or 200 μ g ml⁻¹, indicating that only cysteine protease was present in nonpathogenic *Cryptobia*.

Activations of the cysteine protease

Three cysteine protease activators (DTT, EDTA and cysteine) were used on protease assays at their optimal

Table 3. Effects of cysteine protease activators on proteolytic activity of *Cryptobia* spp. Activity remaining (with the activator) was given as a percentage (%) of the control (without the activator); mean \pm standard deviation from 3 independent determinations. DTT: dithiothreitol; EDTA: ethylene diamine tetraacetic acid

| Activators | <i>C. salmositica</i> (blood form) | | <i>C. salmositica</i> (vaccine) | | <i>C. bullocki</i> | | <i>C. catostomi</i> | |
|---|---------------------------------------|--------------------|------------------------------------|--------------------|--------------------|--------------------|---------------------|--------------------|
| | AZCase (pH 4.5) | HPAase (pH 4.0) | AZCase (pH 4.5) | HPAase (pH 4.0) | AZCase (pH 4.0) | HPAase (pH 3.5) | AZCase (pH 4.0) | HPAase (pH 3.5) |
| DTT (1.0 mM) | 239.3 \pm 12.9* | 279.8 \pm 18.0* | 268.8 \pm 24.7* | 301.2 \pm 38.5* | 268.9 \pm 19.0* | 299.0 \pm 11.5* | 279.3 \pm 31.0* | 344.4 \pm 31.6* |
| Cysteine (1.0 μ g ml ⁻¹) | 295.5 \pm 13.5* | 248.6 \pm 28.3* | 277.4 \pm 40.4* | 298.8 \pm 23.8* | 420.7 \pm 13.4* | 424.7 \pm 43.4* | 220.7 \pm 17.3* | 195.8 \pm 12.5* |
| EDTA (5.0 mM) | 99.4 \pm 8.4 | 108.3 \pm 11.6 | 138.7 \pm 11.1* | 127.4 \pm 6.3* | 123.3 \pm 8.0* | 125.3 \pm 7.2* | 123.0 \pm 8.0* | 127.8 \pm 8.7* |

*Activity was significantly higher ($p < 0.05$) than the control (without the activator)

pH (Table 3). DTT and cysteine significantly increased protease activity. However, EDTA only activated proteases from the vaccine strain of *Cryptobia salmositica*, *C. bullocki* and *C. catostomi* ($p < 0.05$) but not the protease from the blood form of *C. salmositica* ($p > 0.05$). EDTA is both an activator of cysteine protease and an inhibitor of metallo-protease; the blood form of *C. salmositica* had both cysteine and metallo-proteases (Table 1); hence there was no net increase in the activity of proteases by using EDTA.

Detection of pathogenic *Cryptobia salmositica* proteases using haemoglobin-SDS-PAGE

The banding pattern of the pathogenic *Cryptobia salmositica* proteases was studied using haemoglobin-SDS-PAGE (Fig. 2). There were 5 protease bands (200, 97, 66, 60 and 49 kDa) after the gel was incubated in the buffer alone (pH 5.0). However, 3 protease bands (66, 60 and 49 kDa) were not detected and the activity (or density) of the 97 kDa protease band was considerably reduced after the gel was incubated with E-64 (at 10 μ M in the incubation buffer) (Fig. 2); this identified the 4 bands as cysteine proteases. The band with the highest molecular mass (200 kDa) was not affected by E-64 but it was not detected after the gel was incubated with phenanthroline (at 1 mM in the incubation buffer) (Fig. 2); this identified it as a metallo-protease.

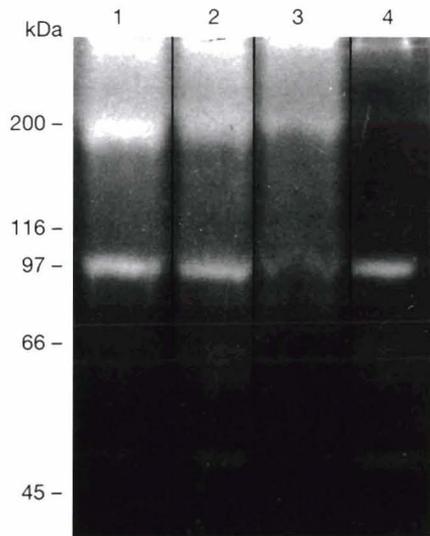


Fig. 2. Proteases of pathogenic *Cryptobia salmositica* detected using haemoglobin-SDS-PAGE. The protease bands were developed in 0.1 M phosphate buffer at pH 5.0. Lanes 1 and 2: the gel was incubated in buffer alone; Lane 3: the gel was incubated with E-64 (10 μ M in the buffer); lane 4: the gel was incubated with phenanthroline (1 mM in the buffer). Standard molecular mass markers (kDa) as indicated

Effects of prolonged *in vitro* culture on protease activity

Proteolytic activities (with either AZC or HPA as the substrate) of *Cryptobia salmositica* (blood form) at 6 mo were significantly lower ($p < 0.05$) than those at 2 mo of culture, and the activities at 10 mo were still significantly lower ($p < 0.05$) than those at 6 mo of culture (Fig. 3). This indicates that proteolytic activities of the pathogenic *C. salmositica* decreased significantly during 10 mo of continuous culture. However, there were no significant changes in proteolytic activities of the other *Cryptobia* spp. (Fig. 3).

Cysteine protease of the pathogenic *Cryptobia salmositica* remained with prolonged *in vitro* culture, since leupeptin significantly inhibited the protease activity ($p < 0.05$) throughout the 14 mo of culture (Table 4). However, the metallo-protease in the parasite was lost after 10 mo of culture. Phenanthroline only significantly reduced the protease activity at 2 and 6 mo of the culture ($p < 0.05$), but did not significantly reduce it at 10 and 14 mo of culture ($p > 0.05$) (Table 4).

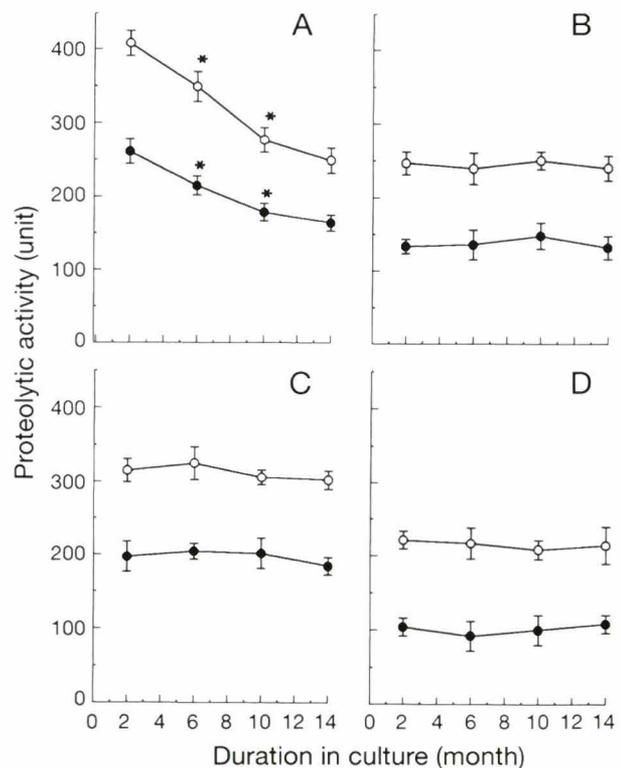


Fig. 3. Proteolytic activities of *Cryptobia* spp. during prolonged *in vitro* culture. (A) *C. salmositica* (blood form); (B) *C. salmositica* (vaccine strain); (C) *C. bullocki*; (D) *C. catostomi*. Each point is mean \pm standard deviation from 3 independent determinations. Proteolytic activity is given in units (μ g substrate hydrolysed per hour per mg protein of enzyme). (●) Using AZC as substrate; (○) using HPA as substrate. *Significantly lower ($p < 0.05$) compared with the previous point

Table 4. Effects of protease inhibitors on proteolytic activities of pathogenic *Cryptobia salmositica* during prolonged *in vitro* cultivation. Activity remaining after incubation with the inhibitor (either 100 $\mu\text{g ml}^{-1}$ leupeptin or 1 mM phenanthroline) is given as a percentage (%) of the control (without the inhibitor); mean \pm standard deviation from 3 independent determinations

| Duration in culture (mo) | AZCase | | HPAase | |
|--------------------------|-----------------|-----------------|-----------------|-----------------|
| | Leupeptin | Phenanthroline | Leupeptin | Phenanthroline |
| 2 | 36.2 \pm 6.4* | 72.4 \pm 6.2* | 49.2 \pm 7.3* | 69.7 \pm 7.0* |
| 6 | 27.1 \pm 7.8* | 69.9 \pm 4.3* | 44.7 \pm 5.5* | 71.9 \pm 6.6* |
| 10 | 27.5 \pm 7.6* | 84.2 \pm 8.8 | 42.2 \pm 8.4* | 84.4 \pm 8.4 |
| 14 | 21.1 \pm 4.6* | 89.8 \pm 5.4 | 35.8 \pm 5.6* | 85.2 \pm 7.4 |

*Activity was significantly lower ($p < 0.05$) than the control (without the inhibitor)

The protease banding pattern of the pathogenic *Cryptobia salmositica* during prolonged *in vitro* culture was studied using haemoglobin-SDS-PAGE. The activity of the metallo-protease was considerably enhanced and that of the cysteine proteases was reduced after the gel was incubated at pH 7.0 (Fig. 4) compared with those bands developed at pH 5.0 (Fig. 2); this indicates that the metallo- and cysteine proteases had a different optimal pH. At 0, 2, 6 mo of culture the pathogen had 5 protease bands (Fig. 4): 1 band was metallo-protease and 4 bands were cysteine proteases. However, the metallo-protease band (about 200 kDa) was not detected from sample after 10 mo of culture (Fig. 4); this confirms that the parasite lost the metallo-protease with prolonged *in vitro* culture (Fig. 3, Table 4). Although the activities (or den-

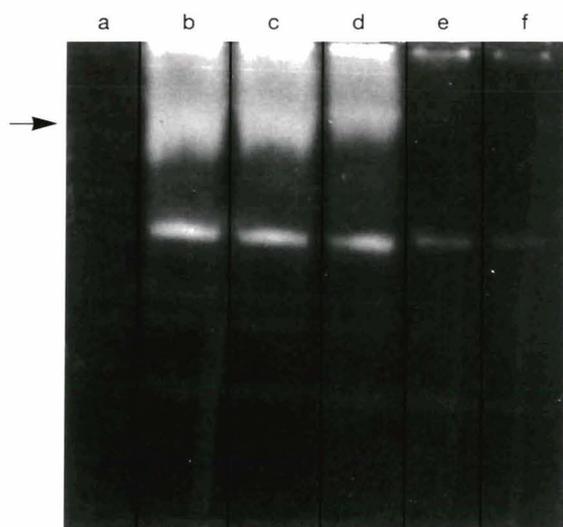


Fig. 4. Proteases of pathogenic *Cryptobia salmositica* from the different times of *in vitro* culture detected using haemoglobin-SDS-PAGE. Protease bands were developed at pH 7.0. Lane a: blood cells of an infected rainbow trout; lanes b, c, d, e and f: the parasite at 0, 2, 6, 10 and 14 mo of culture, respectively. Arrow indicates the metallo-protease band (about 200 kDa)

sity) of 4 cysteine protease bands also decreased, these protease bands were present during 14 mo of culture (Fig. 4). The sample (lane b, Fig. 4 at 0 mo of culture) was contaminated with proteins from host blood cells since the parasite was isolated directly from the blood of an infected rainbow trout. However, no protease band was detected from the blood cells of the rainbow trout (lane a, Fig. 4), indicating that the host blood cells did not affect the detection of *C. salmositica* proteases using the substrate gel electrophoresis.

DISCUSSION

Substrate-SDS-PAGE has been more widely used than native substrate-PAGE (without SDS) in zymograms of proteases, because in SDS-gels molecular weights of electrophoretically fractionated proteases can be determined (Lacks & Springhorn 1980, Sarath et al. 1989). The SDS in the gel denatures some proteases but the denatured enzymes appear to recover their activities after the SDS is diffused out of the gel (Lacks & Springhorn 1980). Using the haemoglobin-SDS-PAGE the intracellular proteases of *C. salmositica* appeared as 5 enzymatic bands (Fig. 2); the same number of protease bands were also detected using native substrate-PAGE (data not shown). We also found that SDS in the gel inhibited or reduced *C. salmositica* protease activities, because when SDS was not diffused out of the gel, 2 minor protease bands (60 and 66 kDa) were not seen and the other 3 protease bands (200, 97 and 49 kDa) were much weaker (visualized as less clear zone of proteolysis). Hence, we suggest that if SDS is used during electrophoresis, it should be routinely removed by washing the gel in 1 to 2.5% Triton X-100 for about 1 h to ensure maximum protease activity when the gel is in incubation buffer. Our study confirms the suggestions of earlier workers (Lacks & Springhorn 1980, Sarath et al. 1989).

Cysteine (thiol) proteases in parasitic protozoa have been assumed to have metabolic and physiologic roles in these parasites (North 1992). The major protease in *Trypanosoma cruzi*, a human pathogen in central and south America, is a cysteine protease with chemical properties similar to mammalian cathepsin L (Bontempi et al. 1984, Nazareth et al. 1992, Harth et al. 1993). The purified cysteine protease is able to degrade azocasein and blood protein substrates (e.g. haemoglobin, albumin) at acidic pH (Bontempi et al. 1984). Peptide-fluoromethyl ketone, a specific inhibitor of cysteine protease, arrests intracellular replication of

the parasite and intercellular transmission. This indicates that the protease functions in intracellular protein degradation and also in remodelling the parasite during transformation between stages (Harth et al. 1993). Peptidyl diazomethane derivatives, a class of irreversible inhibitors for cysteine protease, impair host cell invasion and block the intracellular development of *T. cruzi*; this suggests that the ability to infect and develop intracellularly in mammalian cells is dependent on the activity of its major cysteine protease (Nazareth et al. 1992).

In the present study, cysteine protease was detected in pathogenic and nonpathogenic *Cryptobia* spp. (Tables 1, 2 & 3). Nonpathogenic *C. salmositica* and *C. catostomi* had only cysteine protease and its activity remained unchanged with prolonged *in vitro* culture (Fig. 3). The total proteolytic activities in the pathogenic strain of *C. salmositica* decreased significantly with prolonged *in vitro* culture (Fig. 3). However, the cysteine protease at least remained unchanged, whereas the metallo-protease was lost after 10 mo of *in vitro* culture (Table 4, Fig. 4). The chemical properties (e.g. the sensitivity to inhibitor and activator, optimal pH) of the cysteine protease in *Cryptobia* spp. were similar to those of the cysteine protease in *Trypanosoma cruzi* (Bontempi et al. 1984). We suggest that cysteine protease in the *Cryptobia* spp., as in *T. cruzi* (Bontempi et al. 1984), is important in intracellular protein catabolism (e.g. in digesting exogenous host proteins to obtain free amino acids for protein synthesis); hence the level of the cysteine protease in *Cryptobia* is quite stable.

Metallo-protease has also been found in parasitic protozoa. The metallo-protease in promastigotes of *Leishmania* helps the parasite to invade mammalian macrophages by cleaving the host complement factor C3b to C3bi; this allows binding of the parasite via C3bi to the macrophage surface (Bouvier et al. 1989, 1990, Etges 1992). A metallo-protease in the trophozoite of *Entamoeba histolytica* degrades host collagen, suggesting that it plays an important role in the invasion of host tissue (Munoz et al. 1982, 1990). *Trypanosoma cruzi* also contains metallo-protease which is mainly located in the cell membrane (Greig & Ashall 1990, Bonaldo et al. 1991). Using gelatin gel electrophoresis, Greig & Ashall (1990) found multiple proteases in *T. cruzi* epimastigotes: 4 bands are cysteine proteases and 1 band was a metallo-protease. This protease pattern is quite similar to what we found in the pathogenic *Cryptobia salmositica* using haemoglobin gel electrophoresis (Fig. 2). *T. cruzi* trypomastigotes penetrate cells and tissues and a surface or secreted protease is involved in penetration of cells (Piras et al. 1985); it was proposed that the metallo-protease might play an important role in host cell invasion (Greig & Ashall 1990, Bonaldo et al. 1991).

The pathogenic strain of *Cryptobia salmositica* lost its virulence after 1 yr of serial *in vitro* cultivation (Woo & Li 1990); however, its virulence was not reduced on short-term (e.g. 2 mo) culture (Woo & Thomas 1991). In the present study there was a significant loss of metallo-protease activity after 10 mo of continuous *in vitro* culture (Figs. 3 & 4, Table 4). We suggest that the decrease in activity of the metallo-protease is related to the loss of virulence in the pathogenic strain of *C. salmositica*.

Two basic haemolytic components (a lytic component and an immune complex-forming component) in the pathogenic *Cryptobia salmositica* are responsible for the anaemia in rainbow trout (Thomas & Woo 1988). The lytic component lyses red blood cells independent of antibody/complement, whereas the immune complex-forming component attaches to red blood cells forming immune complexes with specific antibody and activates complement resulting in haemolysis (Thomas & Woo 1988). These are also secreted by *C. salmositica* under *in vitro* conditions (Woo & Thomas 1992).

The biochemical nature of the haemolytic components of *Cryptobia salmositica* is not clear, though it is believed to be proteinaceous in nature (Thomas & Woo 1989). In the present study metallo-protease was only found in the pathogenic strain of *C. salmositica* and the activity of metallo-protease significantly decreased with prolonged *in vitro* culture. In another investigation we (Zuo & Woo 1997) demonstrated that the purified metallo-protease from the pathogenic *C. salmositica* causes *in vitro* lysis of fish red blood cells and that the enzyme is secreted by the parasite into the blood of infected fish. We suggest that the metallo-protease is likely the 'secreted haemolytic component' detected in earlier studies (Thomas & Woo 1988, 1989, Woo & Thomas 1992). Although *C. bullocki* causes anaemia in flatfishes, metallo-protease was not found in our present strain probably because it has been in continuous *in vitro* culture for more than 8 yr in our laboratory. It is likely that our *C. bullocki* strain has lost its virulence with prolonged *in vitro* culture as did our *C. salmositica* strain. *C. catostomi* was freshly isolated and is not known to cause anaemia in fish (Thomas & Woo 1992), and no metallo-protease was detected in the nonpathogenic *C. catostomi* and the vaccine strain of *C. salmositica* (Table 1). Further research into the biochemical nature of the metallo-protease in virulent *C. salmositica* would provide us with a better understanding of the mechanism of the disease in salmonid cryptobiosis.

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