Natural anti-proteases in rainbow trout, *Oncorhynchus mykiss* and brook charr, *Salvelinus fontinalis* and the *in vitro* neutralization of fish α2-macroglobulin by the metalloprotease from the pathogenic haemoflagellate, *Cryptobia salmositica*

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SUMMARY

Natural anti-proteases (α1-protease inhibitor (α1-PI; α1-antitrypsin) and α2-macroglobulin (α2-M)) were found in the blood of rainbow trout, *Oncorhynchus mykiss* and brook charr, *Salvelinus fontinalis*. The α2-M inhibited *Cryptobia salmositica* proteases and was significantly higher in brook charr than in rainbow trout. Under *in vitro* conditions it took longer for the same number of parasites to neutralize the α2-M in charr than in trout blood. The haemolysis which occurred when *C. salmositica* was incubated in the blood of rainbow trout was due to neutralization of α2-M. This *in vitro* study also showed that it was the metalloprotease of *C. salmositica* that lysed red blood cells and the plasma of the two species of fishes initially prevented haemolysis by inhibiting the proteolytic activity. We suggest that the natural plasma α2-M plays an important role in defence against cryptobiosis in fishes.

Key words: *Cryptobia salmositica*, *Oncorhynchus mykiss*, *Salvelinus fontinalis*, α2-macroglobulin, α1-protease inhibitor, protease.

INTRODUCTION

Cryptobia salmositica Katz (Sarcomastigophora: Kinetoplastida) is a pathogenic haemoflagellate of salmonids on the west coast of North America (Woo, 1994; Woo & Poynton, 1995). Infected rainbow trout (*Oncorhynchus mykiss*) develops anaemia, exophthalmia, splenomegaly, abdominal distension with ascites and general oedema (Woo, 1979) and anorexia (Li & Woo, 1991; Thomas & Woo, 1992). However, infected brook charr (*Salvelinus fontinalis*) with very high parasitaemias do not suffer from cryptobiosis (Ardelli, Forward & Woo, 1994; Forward, Ferguson & Woo, 1995).

The pathogenic strain of *C. salmositica* has both cysteine and metallo-proteases and loses its metalloprotease when it becomes avirulent; the avirulent strain of *C. salmositica* and the non-pathogenic *C. catostomi* of white suckers (*Catostomus commersoni*) have only cysteine protease and their overall proteolytic activities are much lower than those in the pathogen (Zuo & Woo, unpublished observations).

Natural anti-proteases have been detected in fishes; an analogous protein to the human α1-PI was found in the muscle and serum of cod (*Gadus morhua*) (Hjelmeland, 1983) and a homologue of the human α2-M was demonstrated in the plasma of plaice (*Pleuronectes platessa* L.) (Starkey, Fletcher & Barrett, 1982) and rainbow trout (*Oncorhynchus mykiss*) (Ellis, 1987). However, little is known about their functions.

Our working hypothesis is that proteases secreted by *C. salmositica* are neutralized by host anti-proteases, and that the inhibitory activity of natural anti-proteases is much higher in brook charr than in rainbow trout.

The main objectives of the present study were to (1) identify the natural anti-proteases in the blood of rainbow trout and brook charr; (2) determine the *in vitro* neutralization of fish anti-proteases by *C. salmositica* proteases and (3) determine the effects of the metalloprotease of *C. salmositica* on fish red blood cells.

MATERIALS AND METHODS

Parasites

The pathogenic *C. salmositica* was isolated from the blood of an infected rainbow trout and cultured in a modified minimum essential medium at 12 °C (Woo & Li, 1990; Li & Woo, 1991) for no more than 2 months to obtain a large number of parasites free of host cells. The parasite on short-term culture (about 2 months) is still pathogenic to trout (Woo & Thomas, 1991). Parasites harvested from the me-

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dium were washed 3 times in sterile cold-blooded vertebrate Ringer's solution (CBVR) by centrifugation (7000 ***g***, at 4 °C for 10 min) and washed parasites were inoculated into sterile culture bottles with fish blood.

In vitro *culture of* C. salmositica *in fish blood*

Heparinized fish blood was obtained aseptically via caudal vein puncture (Forward *et al*. 1995). Blood from 3 individual rainbow trout or brook charr, were pooled as the blood medium. Five million *C. salmositica* were inoculated aseptically into 10 ml of the blood medium and incubated at 12 °C in duplicate. The controls were the same blood without parasites and were maintained under the same conditions.

Blood (1 ml) was withdrawn at 3, 6, 12, 18, 24 and 30 days from each of the culture flasks. The number of parasites was counted using a haemocytometer (Archer, 1965) before the blood was centrifuged at 1350 ***g*** for 10 min at 4 °C. The supernatant (or fish plasma) was used fresh or stored at −100 °C for less than 6 months. Our preliminary study indicated that low temperature storage did not affect the effectiveness of plasma anti-proteases.

Detection of α2-macroglobulin (α2-M) in fish plasma (from culture)

The method used was as described by Ellis (1990) with some modifications. Briefly, 100 μl of trypsin (bovine pancreas type III, Sigma; 100 μg/ml of 0·1 M Tris–HCl, pH 8·2) were incubated with 50 μl of fish plasma for 10 min at 22 °C. Tris buffer (0·1 M, pH 8·2) was added to bring the volume to 2·5 ml, and 2 ml of Na-benzoyl-DL-arginine-*p*-nitroanilide HCL (BAPNA, Sigma) were added and then incubated at 22 °C for 25 min. The reaction was terminated by the addition of 0·6 ml of 30 % (v/v) acetic acid. The fine precipitates were removed by filtration through a 0·22 μm Millipore filter, and the optical density of the filtrate was measured at 410 nm using a spectrophotometer against an appropriate blank prepared individually by adding acetic acid to the enzyme/plasma mixture prior to addition of the substrate.

Detection of α1-protease inhibitor (α1-PI) in fish plasma (from culture)

The method was according to Ellis (1990) with some modifications. Briefly, 100 μl of trypsin (100 μg/ml) were incubated with 50 μl of fish plasma for 10 min at 22 °C. Sodium caseinate (2·5 mg/ml PBS) was added to a final volume of 2·5 ml and incubated for 15 min at 22 °C. The reaction was terminated with the addition of 1·2 ml of 10 % trichloroacetic acid (TCA). The sample was centrifuged to remove protein precipitates. The absorbance of the supernatant was recorded using a spectrophotometer at 280 nm against an appropriate blank prepared individually by adding the TCA to the enzyme/plasma mixture prior to addition of the substrate.

The inhibitory capacity of anti-protease was expressed as its inhibition on trypsin activity, i.e. a percentage of the difference between the control activity (A1) (without the anti-protease) and the activity remaining (A2) (after incubation with the anti-protease) in the control activity (A1).

$$\text{Inhibitory capacity } (\%) = \frac{\text{A1} - \text{A2}}{\text{A1}} \times 100\,\%.$$

Detection of the in vitro *release of haemoglobin in fish plasma (from culture)*

The released haemoglobin in fish blood was determined using the cyanmethaemoglobin method (Wedemeyer & Yasutake, 1977) with slight modifications. Briefly, 940 μl of Drabkin's reagent (Sigma) were added to 60 μl of plasma sample in a 1·5 ml centrifuge tube. It was shaken with a pipette shaker for 10 sec. After standing for 15 min, the absorbancy reading of the sample was recorded using a spectrophotometer at 540 nm against a reagent blank. The amount of haemoglobin was calculated using a standard haemoglobin curve (Bovine haemoglobin, Sigma).

Isolation of the metalloprotease from C. salmositica *lysate*

Crude intracellular proteases of *C. salmositica* were prepared from lysed parasites and the enzyme was isolated using ion-exchange chromatography according to the method described by Yu & Greenwood (1994). Briefly, the crude enzyme sample was applied to a diethylaminoethyl (DEAE)–agarose column (1·5 × 20 cm) (Bio-Rad Laboratories). The two types of intracellular proteases (metallo- and cysteine proteases) were eluted with a linear increasing NaCl gradient (0–400 mM). The fractions (2 ml/tube) were collected and those with metalloprotease activity were detected using the azocasein in the presence of cysteine protease inhibitor, E-64. The fractions (tubes 11–14) with high metalloprotease activity were pooled and confirmed as belonging to the metallo class of protease by using EDTA, a metal-chelating reagent (Polzer & Taraschewski, 1993).

Effects of commercial anti-proteases and fish plasma on the activity of the C. salmositica *metalloprotease*

A volume (50 μl) of human plasma α1-protease inhibitor (α1-PI, Sigma) or of bovine plasma α2-macroglobulin (α2-M, Boehringer) (both at 1 mg/ml in 0·1 M citrate buffer, pH 6·5) or a volume

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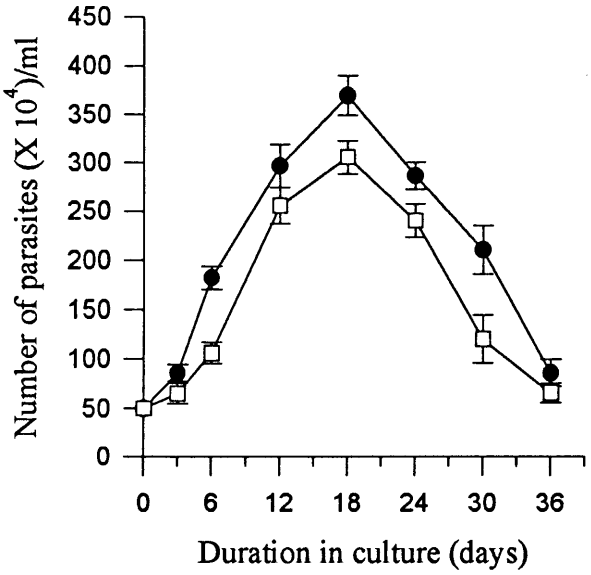

Fig. 1. *Cryptobia salmositica* in blood culture: (●) in the blood of rainbow trout; (□) in the blood of brook charr. Each point is mean ± standard deviation ($n = 4$).

(50–100 μl) of fish plasma was added to 10 μl of the metalloprotease from *C. salmositica* (1 mg protein/ml). Citrate buffer was then added to make the volume up to 200 μl and the mixture of enzyme/anti-protease was pre-incubated at 22 °C for 10 min. Controls were conducted under the same conditions but the enzyme was pre-incubated with the buffer instead of the anti-proteases or fish plasma. The mixture was then incubated with its substrate (haemoglobin) at 22 °C and proteolytic activity was measured as described by Polzer & Taraschewski (1993). The haemoglobin, a protein substrate, was extracted and prepared from the blood of an uninfected rainbow trout (Knox, Redmond & Jones, 1993).

In vitro *lysis of fish erythrocytes by the parasite metalloprotease and the inhibition of haemolysis by fish plasma*

A 5 % (v/v) suspension of red blood cells (Frbc) was prepared from an uninfected rainbow trout (Thomas & Woo, 1988). A volume (10 μl) of the parasite metalloprotease (1 mg protein/ml) was added to 25 μl of fish plasma, and CBVR was then added to make the volume up to 100 μl. The metalloprotease/plasma mixture was pre-incubated at 22 °C for 10 min before 900 μl of 5 % suspension of Frbc were added and incubated at 12 °C for 12 h. The positive control was the same as above but the metalloprotease was pre-incubated with a buffer instead of fish plasma, while the negative control was the same amount of Frbc incubated in the buffer only. Haemolysis was monitored by the determination of released haemoglobin using a spectrophotometer calibrated for the cyanmethaemoglobin method (see above).

Statistical analysis

The *t*-test (Wardlaw, 1985) was used to determine the difference between control and experiment assays. Results were considered significant if $P < 0{\cdot}05$.

RESULTS

Culture of C. salmositica *in fish blood*

The parasites multiplied readily in the blood of both rainbow trout and brook charr at 12 °C. The highest numbers were at 18 days in both blood media, but a significantly higher number ($P < 0{\cdot}05$) was detected in the blood of rainbow trout at 18 days after culture (Fig. 1). However, there was no significant difference ($P > 0{\cdot}05$) in numbers between the two blood media at 3, 12 and 36 days of culture (Fig. 1).

Changes of α2-M and α1-PI in blood media during culture of C. salmositica

Brook charr had a significantly higher α2-M inhibitory capacity than rainbow trout at the start of the experiment and inhibitory capacities in the blood media decreased with increased parasite numbers (Fig. 2). After 6 days of culture, the inhibitory capacities of α2-M in the experimental blood media (with parasites) were significantly lower than those in the controls (without parasites) (Fig. 2). This indicates that the α2-M was depleted and probably neutralized by proteases secreted by *C. salmositica*. However, it took longer for the parasite to completely neutralize the α2-M in charr (30 days) than in trout (18 days) blood under similar *in vitro* conditions (Fig. 2).

The inhibitory capacities of α1-PI in the blood also decreased in cultures (Table 1). However, no significant difference was found between the control (without parasites) and the experimental blood (with parasites); this indicates that the α1-PI of fish was not neutralized by the protease secreted by *C. salmositica*. The decrease of α1-PI inhibitory activity was perhaps due to normal degradation of this anti-protease under *in vitro* conditions.

The release of haemoglobin during culture of C. salmositica *in blood media*

Haemoglobin was detected in the blood media 6 days after culture (Table 2). Amounts of released haemoglobin increased with time and were significantly higher ($P < 0{\cdot}05$) in the experimental blood media (with parasites) than in the controls (without parasites) at 12, 18, 24 and 30 days after culture. Released haemoglobin indicates that the parasite caused lysis of red blood cells. Also, the amount of released haemoglobin in trout blood was significantly higher ($P < 0{\cdot}05$) than that in charr blood during the same period.

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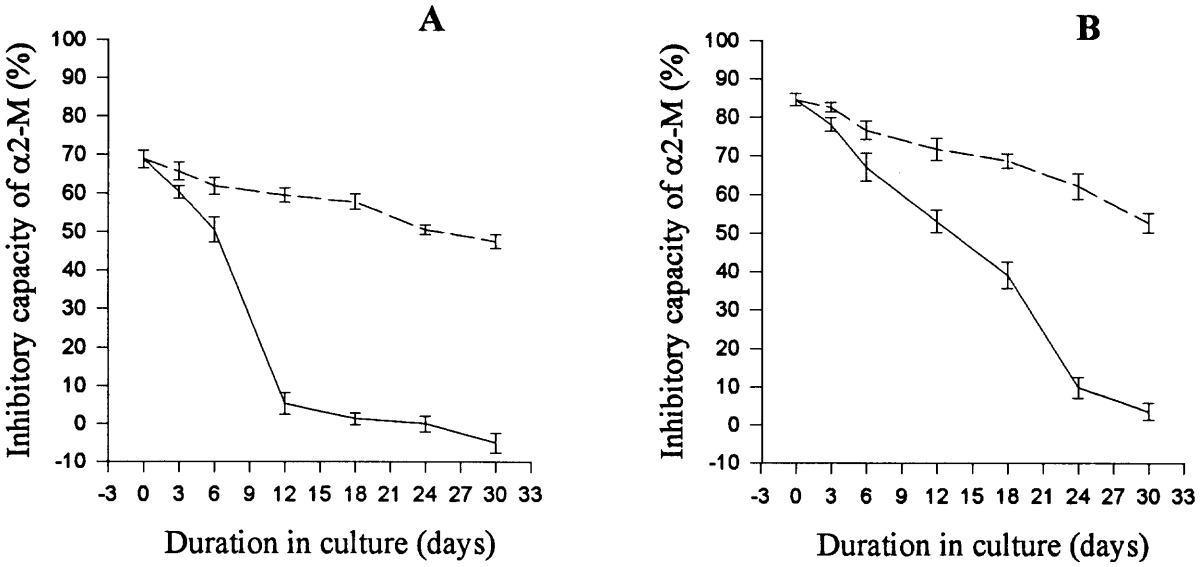

Fig. 2. *In vitro* neutralization of α2-M in blood culture of *Cryptobia salmositica* (A) in the blood of rainbow trout; (B) in the blood of brook charr. (– – –) Blood without parasites; (——) blood with parasites. Each point is mean ± standard deviation ($n = 4$).

Table 1. *In vitro* inhibitory capacity of α1-PI (%) in fish blood during culture of *Cryptobia salmositica*

(Results are mean ± standard deviation ($n = 4$). No significant difference ($P > 0{\cdot}05$) between blood without parasites and blood with parasites.)

Duration in culture (days)	Rainbow trout		Brook charr	
	Blood without parasites	Blood with parasites	Blood without parasites	Blood with parasites
0	72·3 ± 2·6	72·3 ± 2·1	81·3 ± 2·6	80·9 ± 2·1
3	66·0 ± 1·7	63·4 ± 2·6	74·5 ± 1·7	71·1 ± 2·1
6	58·3 ± 4·3	56·5 ± 3·0	63·8 ± 1·3	57·5 ± 3·4
12	46·0 ± 2·6	47·7 ± 3·4	56·6 ± 2·1	49·5 ± 4·3
18	32·3 ± 3·0	26·8 ± 5·1	36·6 ± 3·8	31·9 ± 5·5
24	25·5 ± 3·8	20·9 ± 4·7	31·1 ± 3·8	20·4 ± 4·7
30	14·9 ± 4·3	14·0 ± 5·5	20·0 ± 4·7	17·0 ± 5·1

Table 2. The release of haemoglobin (g/100 ml) during culture of *Cryptobia salmositica* in the fish blood

(Results are mean ± standard deviation ($n = 4$). N.D., No released haemoglobin was detected. *, ** Significantly higher ($P < 0{\cdot}05$) than those in blood without parasites; ** significantly lower ($P < 0{\cdot}05$) than * in the rainbow trout.)

Duration in culture (days)	Rainbow trout		Brook charr	
	Blood without parasites	Blood with parasites	Blood without parasites	Blood with parasites
0	N.D.	N.D.	N.D.	N.D.
3	N.D.	N.D.	N.D.	N.D.
6	0·087 ± 0·027	0·114 ± 0·023	0·079 ± 0·023	0·069 ± 0·023
12	1·181 ± 0·046	2·485 ± 0·027*	1·045 ± 0·046	1·340 ± 0·065**
18	1·605 ± 0·041	4·673 ± 0·046*	1·581 ± 0·037	1·983 ± 0·079**
24	2·731 ± 0·096	6·305 ± 0·059*	2·875 ± 0·074	4·095 ± 0·122**
30	3·500 ± 0·100	6·550 ± 0·120*	3·670 ± 0·110	5·910 ± 0·150**

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Table 3. Effects of commercial anti-proteases and fish plasma on the metalloprotease isolated from *Cryptobia salmositica*

(Results are mean ± standard deviation ($n = 3$). † Hydrolysis unit (HU) was defined as the amount of substrate required to give an absorbance of 1·0/mg of protein/h of assay time. *, ** Significantly lower ($P < 0·05$) than that in control; ** significantly lower ($P < 0·05$) than * those by using same amount of trout plasma.)

Regents	Protease activity (HU)†	Activity remaining (%)
None (control)	0·234 ± 0·016	100
Human α1-antiprotease (0·25 mg/ml)	0·223 ± 0·013	95·3
Bovine α2-macroglobulin (0·25 mg/ml)	0·035 ± 0·006*	15·0
Plasma of rainbow trout 25 % (v/v)	0·142 ± 0·011*	60·7
50 % (v/v)	0·062 ± 0·012*	26·5
Plasma of brook charr 25 % (v/v)	0·052 ± 0·008**	22·2
50 % (v/v)	0·012 ± 0·004**	5·1

Table 4. Effects of fish plasma on haemolysis caused by metalloprotease from *Cryptobia salmositica*

(Results are mean ± standard deviation ($n = 3$). Frbc: red blood cells of rainbow trout; MP: metalloprotease of *C. salmositica*; P1: plasma of rainbow trout; P2: plasma of brook charr. *, ** Significantly lower ($P < 0·05$) than positive control; ** significantly lower ($P < 0·05$) than * that by using same amount of P1.)

Reagents	Released haemoglobin (g/100 ml)	Positive control (%)
CBVR + Frbc (negative control)	0	0
MP + Frbc (positive control)	0·122 ± 0·010	100
MP + 25 % P1 + Frbc	0·042 ± 0·015*	34·4
MP + 25 % P2 + Frbc	0·010 ± 0·005**	8·2

The effects of commercial anti-proteases and fish plasma on the C. salmositica *metalloprotease*

The activity of *C. salmositica* metalloprotease was significantly inhibited by bovine plasma α2-M (0·25 mg/ml). However, human α1-PI at the same concentration did not significantly decrease the activity of the metalloprotease. The activity of the *C. salmositica* metalloprotease was also significantly inhibited by plasma of rainbow trout and brook charr. Inhibitions were higher in larger volumes of plasma, and inhibitions by charr plasma were significantly higher ($P < 0·05$) than those by trout plasma (Table 3).

In vitro *lysis of fish red blood cells by the metalloprotease and inhibition of haemolysis by fish plasma*

No haemoglobin was detected when fish erythrocytes were incubated with CBVR only. Significant amounts of haemoglobin were released after fish erythrocytes were incubated with the parasite metalloprotease (Table 4). However, the haemolysis was significantly reduced by pre-incubation of the enzyme with either charr or trout plasma. This indicates that fish plasma neutralized the protease and thus protected red blood cells from lysis. Also, the protection was higher by charr than by trout plasma as the released haemoglobin was significantly lower when the protease was incubated with charr plasma ($P < 0·05$) than with trout plasma.

DISCUSSION

The major plasma anti-proteases in mammals are α1-PI and α2-M, which are glycoproteins synthesized in the liver and secreted into the blood. While α1-PI only inhibits serine proteases, α2-M is a non-specific protease inhibitor which forms complexes with all major classes of proteases (Andus *et al*. 1983). The most convenient way of quantifying plasma anti-proteases is to measure inhibition of trypsin activity (Ellis, 1990).

In the present study we have shown that rainbow trout and brook charr have similar natural anti-proteases. Plasma α2-M, but not α1-PI, in the two species of fishes, were neutralized by secreted *C. salmositica* proteases under *in vitro* conditions. Activity of the metalloprotease was inhibited by bovine plasma α2-M, but not by human plasma α1-PI. Anti-protease, α1-PI, was not neutralized by the *C. salmositica* proteases and this appears to confirm our other investigation in that the parasite does not have serine proteases (Zuo & Woo, unpublished observations).

In the present study, much lower plasma α2-M activity was found in rainbow trout than in brook charr, although the numbers of parasites were similar in both cultures. We suggest that the lower level of α2-M in rainbow trout resulted in significant haemolysis in the blood. For example, at day 12 of the culture when the numbers of parasites in the blood media were similar, the released haemoglobin was significantly higher ($P < 0·05$) in trout blood, and this coincided with significantly lower α2-M inhibitory capacity in the blood of rainbow trout ($P < 0·05$) compared to that in brook charr.

The neutralization of α2-M in the blood medium was very closely related to significant haemolysis, indicating that secretion of proteases is one of the causes of anaemia in infected fish. Also, the isolated metalloprotease caused *in vitro* lysis of fish red blood cells and α2-M in the blood prevented haemolysis

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until the anti-protease was neutralized. When fish were infected with *C. salmositica* the metalloprotease was in the blood; this was either secreted by live parasites (Zuo & Woo, unpublished observations) or was released from lysed parasites as a result of complement lysis (Li & Woo, 1995). We suggest that the metalloprotease of *C. salmositica* may be one of the factors which cause anaemia in cryptobiosis and that it is likely to be the secreted haemolysin detected in earlier studies (Thomas & Woo, 1988, 1989).

Preliminary studies suggest that α2-M contributes to the defence against invasive pathogens. It is believed that proteases produced by *Pseudomonas aeruginosa*, in burned and other compromised patients, are virulence factors, and human α2-M greatly reduces the virulence of this bacterium by inhibiting its proteolytic activity (Holder & Haidaris, 1979). The protease secreted by the bovine protozoan *Tritrichomonas foetus* cleaves some of the host defence proteins, and the host α2-M inhibits the action of the parasite protease (Talbot, Neilsen & Corbeil, 1991). *Aeromonas salmonicida*, a pathogenic fish bacterium, produces protease which contributes to its pathogenesis in rainbow trout and the protease is neutralized by fish α2-M (Ellis, Hastings & Munro, 1981; Hastings & Ellis, 1985).

Compared to rainbow trout, brook charr had significantly higher α2-M activity and it seemed to be more effective in neutralizing the parasite protease. We suggest that the high activity and rapid production of α2-M in brook charr prevents the fish from cryptobiosis. This was confirmed in our *in vivo* study on cryptobiosis where there was no anaemia as long as the α2-M remained high during the infection (Zuo & Woo, unpublished observations). However, in infected rainbow trout, the inhibitory effects of α2-M were significantly reduced at the time when the fish had high parasitaemia and severe anaemia.

C. salmositica causes disease (e.g. anaemia) in trout but not in charr although their parasitaemias are just as massive (Ardelli *et al*. 1994; Forward *et al*. 1995). One of our current projects is to focus on this difference in susceptibility to disease. The findings of this study may also be useful to scientists who are trying to understand the difference in susceptibility to disease between zebu cattle and trypanotolerant animals (e.g. wildlife) in African trypanosomiasis; the mechanism is not well understood (Logen-Henfrey, Gardiner & Mahmoud, 1992).

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