The effects of acid pepsin pretreatment, bile acids and reductants on the excystation of *Clonorchis sinensis* metacercariae were studied. The velocities of metacercarial excystation, digestion of the metacercarial outer layer and tryptic BAEE hydrolysis were measured in various media. Pretreatment with acid or acid pepsin accelerated the excystation, but was not required under adequate tryptic activity. Taurocholate and deoxycholate evoked no excystation by themselves. However, in the trypsin medium with taurocholate and deoxycholate, they accelerated both the velocity of metacercarial excystation and of digestion of the outer layer, although the accelerative effect did not increase above 4\% deoxycholate. Taurocholate below 1\% and deoxycholate below 4\% did not affect the rate of tryptic hydrolysis, while this was depressed by deoxycholate over 4\%. Addition of deoxycholate over 4\% also depressed the final percentage of excystation, as well as worm motility. By themselves, cysteine and 2-mercaptoethanol (MCE) swelled and deformed the cyst wall and evoked metacercarial excystation without trypsin, however, ascorbate and Na dithionite evoked no excystation. Addition of ascorbate to the trypsin medium did not change the velocity of excystation, but this was completely inhibited by the addition of dithionite and was accelerated by the addition of cysteine or MCE. Although excystation was accelerated with an increase of cysteine concentration, this acceleration was depressed at over 0.01 M cysteine. The rate of tryptic hydrolysis was depressed at over 0.002 M cysteine. The present study suggests that the excystation facilitative effects of pretreatment of acid pepsin, bile acids and reductants (cysteine and MCE) in trypsin medium are due to denaturation of protein, detergent action and cleavage of SS-linkage, respectively, on the digestion step of the outer layer of the metacercariae and cessation of the facilitative effect at a high concentration of deoxycholate is related to the rupturing step of the inner layer by larval movement.

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**Abbreviations:** BAEE, benzoyl-arginine ethyl ester; TES, *N*-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid; MCE, 2-mercaptoethanol

**Keywords:** *Clonorchis sinensis*; Metacercaria; Acid pepsin pretreatment; Bile acid; Reductant; Excystation
1. Introduction

In the digenetic trematode metacercariae, various species adopt different factors to bring about excystations. In spite of their various mechanisms it is thought that the excystations would basically consist of digestion of the cyst wall and its rupture by larval movement.

In digenetic trematodes which parasitize mammals, many studies on in vitro excystation have been made in *Clonorchis sinensis* [1–3], *Fascioloida* spp. [4–8] and in *Paragonimus westermani* [9,10]. In these species, excystation occurs in the small intestine of the host. However, their excystations differ with regard to their reaction to host enzymes, i.e. *C. sinensis* requires the extrinsic host enzymes pepsin and trypsin, while *Fascioloida* spp. [4–8] and *P. westermani* do not.

In the *Fascioloida* spp. and *P. westermani*, there have been no reports on dominant digestive enzymes related to the excystation and many other factors have been examined, including bile, high CO₂ tension, cysteine, Na dithionite and low osmolarity, but their action mechanisms have not been clear. However, a recent report on *P. westermani* suggested that intrinsic digestive enzyme was related to the metacercarial excystation [11]. And the analyses of the digestive processes of excystations in these species has been required as well as the analyses of the activation processes of larval movements are.

On the contrary, in *C. sinensis*, due to the ease of in vitro excystation with peptic and tryptic treatment, there have been few reports on other factors, bile and acid pretreatment, whose effects are discrepant [1–3] and there have been no reports on the effect of reductants.

In this study using the in vitro excystation system of *C. sinensis* metacercariae which is easy to control excystation conditions, the effects of acid peptic pretreatment and bile acids were confirmed and the effects of reductants were elucidated. The action mechanisms of these factors would be common in other trematodes, therefore the present information is very important to understand the excystation of other trematodes.

2. Materials and methods

2.1. Preparation of metacercariae

Metacercariae of *C. sinensis* were prepared from cyprinid fishes, *Pseudorasbora parva* and *Gnathopogon elongatus elongatus*, caught in southern Okayama prefecture, Japan. The metacercarial cyst is encapsulated by the adventitious host-origin outermost layer and the proper cyst which consists of an outer layer approximately 3 μm thick and an inner layer comprised of a thin membrane [1]. Therefore to collect metacercarial cysts themselves without an adventitious host-origin layer, the pithed fish were chopped and treated with artificial gastric solution 0.1% pepsin in 0.7% conc. HCl at 37°C for 2 h, which made it easy to isolate metacercariae. For the pretreatment experiment, metacercariae were searched for in sliced fish flesh interposed between two glass slides under a binocular dissecting microscope and the host-origin layer around the metacercariae were removed by weakly pressing with a flat-tipped needle. Metacercariae were stored in physiological saline at 4°C and used for the experiments within 2 weeks.

2.2. General preparation of medium

The basic medium used for all of the experiments was TES–NaCl (0.3 M TES:0.15 M NaCl /1:9) buffer (pH 7.5). Various concentrations of trypsin (Type 3, 11250 BAEE unit/mg protein, Sigma, USA) solution stored in 10⁻³ M HCl (4°C) were added in a volume of 1% just before the experiment. The concentration of trypsin in the excystation medium was usually adjusted from 4⁻⁶ to 4⁻⁷%, at which 50% of metacercariae excyst after approx. 10 min and approx. 100% excyst within 20 min. The bile salts used were Na taurocholate and Na deoxycholate 4⁻⁵ to 1% (w/v) and the reductants used were Na ascorbate (0.01 M), Na dithionite (0.01 M), cysteine (0.0004–0.05 M) and MCE (0.01 M and 0.05 M). The reductants were added in the media just before use and pH was adjusted to pH 7.5 by 0.3 M NaOH if necessary.
2.3. Excystation experiments

The experiments were performed in an excystation cuvette (4 ml) which was set in a water bath at 37 ± 0.5°C. In each trial, 10–20 metacercariae were pre-incubated in the cuvette with TES-NaCl buffer (pH 7.5) for 5–10 min and transferred to an excystation medium which contained trypsin. Worms were observed at intervals of 30 or 60 s under a stereoscopic microscope for 10–30 min. The number of experimental trials was at least three (20 metacercariae per trial) or six (10 metacercariae per trial) times, except where otherwise stated. To deal with the wide range of the velocity of metacercarial excystation, it was expressed in terms of VE-50, as shown in Fig. 1. For the excystation experiments 4−7−4−6% trypsin was used to prevent rapid excystations except where otherwise stated (Fig. 2).

For the pretreatment experiments, metacercariae that had been mechanically isolated from fish flesh were treated in 0.9% NaCl solution for a control (at 37°C for 2 h), in acid solution (0.7% conc. HCl at 37°C for 2 h) or in acid pepsin solution (0.1% pepsin in 0.7% conc. HCl at 37°C for 2 h) and then washed in TES–NaCl buffer and transferred to each excystation medium.

2.4. Velocity of digestion of the outer layer of the cyst wall

The velocity of digestion of the outer layer of the cyst wall was calculated based on the change in the thickness of the outer layer. Both polar sites and both equatorial sites of the cyst wall under the microscope with a water immersion lens were photographed within 2 s at 5–90 s intervals which were measured accurately by a time clock superimposed on the films and the averages of thickness of the four sites were calculated in each time (see Fig. 3). The velocity of digestion was expressed as the reciprocal of the 50% swelling time, at which the outer layer was 50% swollen between the initial and maximum levels. The components of the medium were the same as in the excystation experiments, except that the concentration of trypsin was 4−5% and the temperature of the medium was 34°C.

2.5. Tryptic activity

For the measurement of tryptic activity, a modification of the method of Schwert and Takenaka [12] was adopted. The spectrophotometric changes in BAEE as a substrate were measured at 37°C,
Fig. 3. Morphological changes in the cyst wall of C. sinensis metacercariae that had been incubated in 4% trypsin medium (pH 7.5, 25°C) after pretreatment with acid pepsin. A: Metacercaria before exposure to the medium. B: Metacercaria after 3 min of exposure, note the swelling of the outer layer (OL). C: Metacercaria after 12 min of exposure, note the difference in the thickness of the outer layer (OL) at the upper and lower poles, due to floatation of the worm within the inner layer. D: Remaining inner layer (IL) after excystation of the worm and complete digestion of the outer layer at 17 min after exposure. Scale bars = 20 μm.

pH 7.5, with a Beckman DU-8. The final assay mixture contained 2.5 × 10⁻⁴ M or 10⁻³ M BAEE and other components were the same as in the excystation experiments, except that the concentration of trypsin was 4⁻⁷%.

2.6. Statistical analysis

For the excystation experiment, the trial data were expressed in terms of VE-50, while the generalized Wilcoxon’s test was used to evaluate differences in the times of excystation for all individuals. The results of the experiments on digestive velocity of the outer layer and trypsic activity were evaluated by Wilcoxon’s test and the t-test, respectively.

3. Results

3.1. Effects of concentration of trypsin

The velocity of metacercarial excystation of C. sinensis was accelerated by the increase of the concentration of trypsin (Fig. 2). In the media of 4⁻³, 4⁻⁴ and 4⁻⁷% trypsin the 50% of metacercariae excysted within 1.3 ± 0.1, 3.6 ± 0.4 and 12.0 ± 1.0 min, respectively and 100% of them within 5–30 min. The calculated VE-50 were 39.2 ± 11.8, 14.1 ± 1.5 and 4.2 ± 0.4%/min, respectively.

3.2. Morphological change of cyst wall

The outer layer of the cyst wall was swollen after exposure to the trypsin medium (Fig. 3). The typical change in the thickness of the outer layer at 34°C was shown in Fig. 4. The swelling was observed after pre-swelling period of 2.5–5.5 min. Then after the start of swelling the thickness reached its maximum within 1–2 min and then fluctuated by the worm compression to the cyst wall. The thickness before exposure to the excystation medium varied from 1.9 to 3.1 μm but there was no correlation between the initial thickness and the velocity of digestion of the outer layer. At the concentrations of bile acids which facilitate the velocity of cyst wall digestion as mentioned later, both the pre-swelling and swelling times were shorter than without bile acids.

3.3. Effects of acid pepsin pretreatment

No morphological differences were observed between non-pretreated metacercariae and those that had been pretreated with acid or acid pepsin. Metacercariae that had been pretreated with only 0.9% NaCl (at 37°C for 2h) did not excyst within 20 min in 4⁻⁶ 5% trypsin medium, while 98% and
Fig. 4. Typical example of the change in the thickness of the outer layer of *C. sinensis* metacercaria incubated in 4\textsuperscript{\%} trypsin medium (pH 7.5, 34°C) after pretreatment with acid pepsin. The thickness of the outer layer just before excystation could not be measured due to movement of the worm and/or disappearance of the outer layer.

88\% of those that had been pretreated with acid pepsin or acid, respectively, excysted within 25 min. But in 4\textsuperscript{\%} trypsin after 0.9\% NaCl pretreatment, all the metacercariae excysted within 2 min (Fig. 5).

3.4. Effects of bile acids

Metacercariae did not excyst in trypsin-free medium which contained bile acids. Taurocholate facilitated the velocity of excystation and the digestion of the outer layer of metacercariae in a concentration-dependent manner and the VE-50 value and the velocity of wall digestion reached 261\% and 253\% of the control (without taurocholate) at a concentration of 1\%, respectively, while the tryptic activity towards BAEE was hardly affected (Fig. 6). Deoxycholate in trypsin-containing medium hastened excystation and digestion of the outer layer at low concentrations. In particular, deoxycholate at 4\textsuperscript{\%} maximally increased the velocity of the excystation to 330\% of that in the control. Above 4\textsuperscript{\%} deoxycholate, the enhancement of the velocity of the excystation and of the digestion of the outer layer were stopped. Tryptic activity was relatively constant (84–109\% of the control) at a low concentration of deoxycholate, but above 4\textsuperscript{\%} deoxycholate it fell to 26\% of that in the control at 1\% deoxycholate (Fig. 6).

All of the metacercariae excysted at any concentration of taurocholate and at low concentrations of deoxycholate in the excystation medium. However, 10–15\% of the metacercariae did not excyst at 4\textsuperscript{\%} and 1\% deoxycholate (Fig. 7). When the metacercariae were exposed to excystation medium that contained 1\% deoxycholate for more than 10 min, excystation barely occurred and both the excysted and non-excysted worms showed no motility, except for rare and very weak movement.

3.5. Effects of reductants

The addition of 0.05 M cysteine and MCE without trypsin evoked slow excystations (VE-50 values were 2.8\%/min and 1.7\%/min, respec-
Fig. 6. Effects of taurocholate and deoxycholate on the velocity of excystation (VE-50) of *C. sinensis* metacercariae after pretreatment with acid pepsin. Vertical bars show S.D. 

Fig. 7. Excystation of *C. sinensis* metacercariae in medium containing different concentrations of sodium deoxycholate (0%, 4% and 1%) in trypsin medium after pretreatment with acid pepsin. Vertical bars show S.D.

Excystation rose to 151 ± 9%, while at 0.05 M it fell to 119 ± 17% of that in the control (Fig. 8). Tryptic activity fell slightly to 88 ± 3% at 0.002 M cysteine and fell sharply to 11 ± 1% at 0.01 M cysteine.

Morphological changes of the metacercariae in medium containing reductants are shown in Fig. 9 and Fig. 10. When metacercariae were exposed to 0.05 M cysteine or 0.05 M MCE without trypsin, the outer layer of the cyst wall gradually swelled and deformed (Fig. 9B,E), but the worm did not float. After actively moving, the worm finally excysted (Fig. 9C,F). Na dithionite alone did not swell the outer layer as well as ascorbate did. After exposure to excystation medium consisting of 0.01 M cysteine with trypsin, the outer layer was swollen and deformed (Fig. 10A), but the worm did not float and the inner and outer layers were broken down by the worm moving (Fig. 10B). After 150 min of exposure, the outer layer became more swollen. However, this layer did not disappear (Fig. 10C). After exposure to excystation medium consisting of Na dithionite with trypsin, swelling progressed very slowly. Swelling was recognized after 30 min and the worm moved, but excystation did not occur (Fig. 10D,E).
Fig. 8. Effects of cysteine on the velocity of excystation of *C. sinensis* metacercariae pretreated with acid pepsin and on tryptic activity. The concentration of trypsin in the excystation experiment was $4 \times 10^{-6.5} \%$. The tryptic activity at 0.05 M cysteine was not measured because of the extremely high absorption at the wavelength used to detect BAEE hydrolysis (253 nm). $^* P < 0.005$ (generalized Wilcoxon’s test); $^\dagger P < 0.003$; $^\ddagger P < 0.0001$ (Wilcoxon’s test) vs. the respective control. Vertical bars show S.D.

4. Discussion

In vitro excystation in *C. sinensis* metacercariae was first reported by Faust and Khaw [13], but Hsü and Khaw [1] later questioned whether these specimens were indeed *C. sinensis*. They then reported in rigorously identified specimens that the excystation of *C. sinensis* metacercariae consisted of three steps: digestion of the adventitious host-origin layer by pepsin; digestion of the outer layer and the remaining inner layer by trypsin; and disruption of the inner layer by movement of the larval worm.

Hsü and Khaw’s observation that trypsin did not digest the outer layer of this metacercaria without pretreatment with peptic juice [1] suggested that pretreatment with acid pepsin was necessary for excystation. In the present study, no metacercaria without acid pepsin pretreatment excysted in medium with a low concentration of trypsin, but excystation occurred under higher tryptic activity. Yokogawa and Kobayashi [3] reported that metacercariae did excyst with or without acid pepsin pretreatment, but both the facilitative and depressive effects of acid pepsin pretreatment were observed. The present results confirmed that pretreatment with acid or acid pepsin is not required for, but does facilitate the excystation of *C. sinensis* metacercariae. This facilitative effect may be due to facilitation of the second step of excystation (digestion of the outer layer) by the denaturation of trypsin-sensitive protein [14,15]. The present study also showed that nearly 100% of *C. sinensis* metacercariae excysted after acid pepsin pretreatment for 2 h. But in *Cyathocotyle bushiensis* and *Holostephanus luehei* the percentages of excystation after the same pretreatment were 40% and 0%, respectively, [16,17]. This characteristic of *C. sinensis* metacercariae may be due to the very low permeability (Ohyama, unpublished) and the pepsin-resistance of the inner layer [1]. These results show that metacercariae in the duodenum of the final host would be ready to be easily digested by the increased tryptic sensitivity of the outer layer as well as by removal of the outermost fish tissue.

Treatment of *C. sinensis* metacercariae with bile acid alone did not lead to excystation, which is consistent with the report of Hsü and Wang [2], but bile acids did facilitate the velocity of excystation in trypsin solution (Fig. 6). Yokogawa and Kobayashi [3] reported that bile acids did not enhance excystation. However, its excystation seems to have occurred too rapidly to detect differences in the percent rate of the excystation between the control and bile acid groups. Based on the present results with taurocholate and deoxycholate, it is apparent that their facilitative effects on excystation are due to the enhancement of cyst wall digestion (second step of *C. sinensis* excystation). The facilitation of cyst wall digestion could not be explained by the tryptic activity. Regardless of whether or not bile acids enhance tryptic activity, we can not neglect that bile acids have a detergent action. This enhancement of wall digestion can be explained by the accelerated washing out of fragments of polypeptides produced by tryptic hydrolysis within the outer layer. The difference in the enhancing ef-
Fig. 9. Morphological changes of *C. sinensis* metacercariae in the medium (trypsin-free, 34°C) containing 0.05 M cysteine and 0.05 M MCE after pretreatment with acid pepsin. A-D: Before exposure to the medium. B: 30 min after exposure, note the swelling of the outer layer. C: 50 min after exposure, note that the worm has already excysted. E: 20 min after exposure, note the swelling of the outer layer and deformation. F: 30 min after exposure, note that the worm has already excysted. The numeral in each figure indicates the number of minutes after exposure to the medium. Solid triangles show the outer later of the cyst wall. Scale bars = 20 μm.

Effects between deoxycholate and taurocholate at a high concentration would be due to the difference in their capacity for solubilization, as was pointed out by Helenius et al. [18]. At a high concentration of deoxycholate, BAEE hydrolysis was inhibited. Similar phenomena were observed with regard to plasmin activities toward N-tosyl-l-arginine methyl ester [19]. At a high concentration of deoxycholate, digestion of the cyst wall would be inhibited on one hand, while powerful solubilization of polypeptides would occur on the other. Based on the present experiment of *C. sinensis* metacercariae, one of the Lackie’s four categories of bile action in digenetic trematodes [20] was firstly confirmed, i.e. bile acid enhanced excystation by having a synergistic action with the host’s digestive enzyme of trypsin.

Bile acids may also affect the disruption of the inner layer by larval movement (the third step of *C. sinensis* excystation). In fact, when metacercariae were exposed to medium which contained a high concentration of deoxycholate for more than 10 min, both excysted and non-excysted worms hardly moved. This decrease in movement could be due to the wormicidal action of deoxycholate which has passed into the cyst through a small breach in the inner layer (Ohyama, unpublished). The wormicidal or movement-suppressing
Fig. 10. Morphological changes of *C. sinensis* metacercariae in the medium (4% trypsin, 34°C) containing 0.01 M cysteine (A,B,C) and 0.01 M sodium dithionite (D,E) after pretreatment with acid pepsin. A: 4 min after exposure, note the swelling and deformation of the cyst wall. B: 12 min after exposure, just before excystation. The movement of the worm has produced the opening in the cyst wall. C: 150 min after exposure, note the increase in the thickness of the outer layer. D: 5 min after exposure, note the lack of apparent swelling of the outer layer. E: 30 min after exposure, note the swelling of the outer layer and deformation. The numeral in each figure indicates the number of minutes after exposure to the medium. Solid triangles show the outer layer of the cyst wall. Scale bars = 20 μm.

As mentioned above, the permeability of the inner layer of the *C. sinensis* cyst wall is very low. Therefore in the present experiments, reductants would have an effect outside the inner layer in the second step of excystation. While cysteine inhibited the tryptic hydrolytic activity towards the outer layer as shown by the incomplete digestion of the outer layer (Fig. 10C) and the inhibition of BAEE hydrolysis, it strongly weakened the outer layer in which the disulphide bonds of the protein would be reduced. Consequently the increase in the velocity of excystation by cysteine would occur. The effect of MCE can be explained similarly to that of cysteine. The lack of an effect by ascorbate on excystation could be explained by its weak reducing ability. Grob [22] observed that ascorbate had no depressive effect on proteolytic activity. The inhibition of excystation by dithionite could be due to its very strong reducing effect towards trypsin. The slight deformation and swelling of the cyst wall in medium containing dithionite (Fig. 10) would be due to remaining of very weak tryptic activity. In this study, the use of dithionite alone did not swell the outer layer, while both cysteine and MCE did swell the outer layer. The reason might be due to the difference in the sensitivity of the protein comprising the outer layer to ionic and non-ionic reductants as reported by Ward and Lundgren [23]. In addition, they suggested that the reactivities of the non-ionic and ionic reductants towards disulfide bonds.

effects of deoxycholate have been reported in the metacercariae of *P. westermani* [8], *Probolocoryphe uca* and *Gynaecotyla adunca* [21].

are different in different parts of the protein structure. In fact, pretreatment with dithionite leads to swelling of the cyst wall in *Himasthla quissetensis* [24] and *Acanthoparyphium spinulosum* [25].

In this study the action mechanisms of acid pretreatment (denaturation of protein), bile acids (detergent action) and some reductants (cleavage action of SS-linkage) were firstly suggested in metacercarial excystation of *C. sinensis*. These mechanisms would be common in other trematodes, therefore this information is very important to understand the excystation of other trematodes such as *Fascioloida* spp. and *P. westermani* whose excystation mechanisms have not been unclear. It was firstly observed that cysteine and MCE solely evoked metacercarial excystations of *C. sinensis*. This means that the metacercariae can excyst without trypsin if the mechanical strength of the cyst wall become weak enough to rupture by any cause.

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