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Role of Chicken Pancreatic Trypsin, Chymotrypsin and Elastase in the Excystation Process of *Eimeria tenella* Oocysts and Sporocysts

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ABSTRACT The role of pancreatic proteolytic enzymes in the excystation process of *Eimeria tenella* oocysts and sporocysts was studied in vitro. Intact sporulated oocysts were preincubated in phosphate buffer, NaCl 0.9% (PBS) added with 0.5% chicken bile extract in a 5% $C0_2$ atmosphere for 30 minutes prior to exposure to either 0.25% (w/v) chicken trypsin, chymotrypsin, pancreatic elastase, or a 1% (w/v) crude extract of unsporulated and sporulated oocysts of *E. tenella* (Expt.1). No excystation was observed under these conditions. Sporocysts were also incubated under the same conditions without pretreatment in $C0_2$. Excystation was observed for sporocysts incubated with either trypsin, chymotrypsin or pancreatic elastase, the best percentage of excystation being recorded for the latter after 5 hours (Expt. 2). Crude extracts of *E. tenella* oocysts failed to bring about excystation of sporocysts at any time. In experiment 3, sporocysts were incubated with either trypsin, chymotrypsin, or pancreatic elastase alone, any combinations of 2 of these enzymes or with all 3 enzymes. The best percentage of excystation was observed after 5 hours with sporocysts incubated with trypsin and chymotrypsin (99%). The other combinations of 2 enzymes gave also comparable results.
Sporocysts incubated in the presence of the 3 enzymes excysted similarly well, although a significantly lower percentage ($P<0.05$) after 5 hours was recorded when compared to that in sporocysts incubated with trypsin and chymotrypsin. In most cases, the association of 2 or 3 enzymes had a synergistic effect on the percentage excystation of sporocysts in vitro.

INTRODUCTION

In vivo excystation of sporulated oocysts of avian *Eimeria* spp. proceeds in two distinct steps, the first one consisting in the mechanical rupture of the oocyst wall in the gizzard (Doran and Farr 1962; Farr and Doran 1962; Goodrich 1944) and the second one in the release of sporozoites from the sporocysts under the combined action of proteolytic enzymes (mainly trypsin and chymotrypsin) and bile salts (Doran 1966; Doran and Farr 1962; Hibbert et al. 1969; Smetana 1933).
Excystation is also obtained in vitro using a combination of trypsin and bile, after mechanical rupture of the oocyst wall (Doran and Farr 1962) or after pretreatment of intact oocysts with gases ($C0_2$, N_2, NO and H_2S) and reducing agents (Bunch and Nyberg 1970; Jensen et al. 1976; Nyberg et al. 1968).

Recently, excystation of intact *E. tenel1a* oocysts were observed after injection directly into the

intestine (Guyonnet et al. 1989). Under these conditions, the mechanical rupture of the oocyst walls by the gizzard did not occur, suggesting that non-mechanical factors are involved in the excystation process resulting in the release of sporozoites. It thus seems likely that excystation from intact sporulated oocysts should be reproduced in vitro. Since all attempts of in vitro excystation of *Eimeria* spp. of the chicken have been performed using proteolytic enzymes of bovine or porcine origin but never of avian origin, the present study was undertaken to determine the role of chicken pancreatic proteolytic enzymes in the excystation process of intact oocysts and sporocysts of *E. tenella*.

MATERIALS AND METHODS

Infective materials: Oocysts and sporocysts of Eimeria tenel1a were obtained as previously described (Long 1978).

Pancreatic proteolytic enzvmes and bile: Crude pancreatic juice was obtained from chicken pancreas and trypsin, chymotrypsin and elastase were purified as described (Guyonnet et al. 1990). Chicken bile extracts were purchased from Sigma Chemical Co. (St. Louis, MO).

Experiment 1: Intact oocysts of *E. tenella* were incubated in phosphate buffer, NaCl 0.9% (PBS) added with 0.5% (w/v) bile extract at 41 ^{O}C in 5% CO^2, for 30 minutes prior to addition of either the crude pancreatic extract, the combination of 0.25% (w/v) purified trypsin, chymotrypsin and elastase or a 1% (w/v) crude lyophilized extract of unsporulated or sporulated oocysts of *E. tenella.* Oocysts were examined at various times to detect any excysting forms.

Experiment 2: Sporocysts of *E. tenella* were incubated at 41 ^{O}C in PBS with 0.5% bile extract (w/v) and either trypsin, chymotrypsin, pancreatic elastase 0.25% (w/v) or a crude lyophilized extract of unsporulated or sporulated oocysts of *E.*
tenella. Microscopic examinations were carried out up to 5 hours to calculate the percentage of excystation (number of sporozoites counted/2 x number of sporocysts present initially).

Experiment 3: Sporocysts of *E. tenella* were incubated at 41 ^{O}C in PBS with 0.5% bile extract (w/v) and various enzymatic combinations: trypsin, chymotrypsin or elastase 0.25% (w/v) alone, any combination of 2 of these enzymes and trypsin, chymotrypsin and pancreatic elastase 0.25% (w/v) combined together. Microscopic examinations were carried out as described earlier.

Statistical analysis: Data were analyzed using the general linear model (Anonymus 1982) and means were analyzed using Duncan's multiple range test using a significance base of $P<0.05$.

RESULTS

Experiment 1: None of the different treatments were effective in producing excystation of intact sporulated oocysts at any time.

Experiment 2: Sporozoites of *E. tenella* were released from sporocysts excyst with all 3 pancreatic proteolytic enzymes used. The percentages of excystation over a 5-hour incubation are presented in Figure 1. After 60 minutes of incubation, the percentage excystation was significantly greater in sporocysts incubated with elastase and the rate of excystation remained the greatest for this group throughout the incubation period. Significant differences in the excystation rate were observed between sporocysts incubated with trypsin and chymotrypsin at 150 minutes and thereafter. No excystation was observed when the sporocysts were incubated with the crude lyophilized extracts of either sporulated or unsporulated oocysts of *E. tenella*.

Experiment 3: The percentages of excystation for the different treatment groups are presented in Figure 2. Excystation was observed for all treatments. The excystation rates were consistently lower for sporocysts incubated with a single proteolytic enzyme. Again, elastase gave significantly better results than trypsin or chymotrypsin alone. The best percentages of excystation were observed with sporocysts incubated with the combination of trypsin and chymotrypsin, reaching 99% after 5 hours. The other combinations of 2

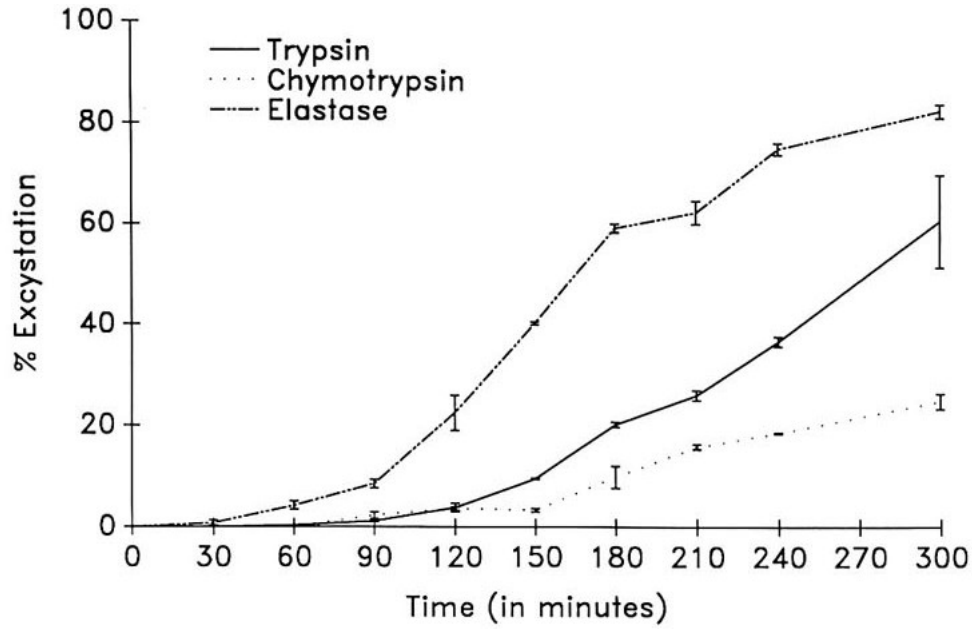

Figure 1. Percentage of excystation of sporocysts of *Eimeria tenella* after incubation at 41 ^{O}C in phosphate buffer, NaCl 0.9% (PBS) added with 0.5% (w/v) bile extract and 0.25% (w/v) of either chicken trypsin, chymotrypsin or pancreatic elastase. Percentage of excystation is equivalent to the number of sporozoites counted/ 2 x number of sporocysts present initially. The bars indicate the standard error.

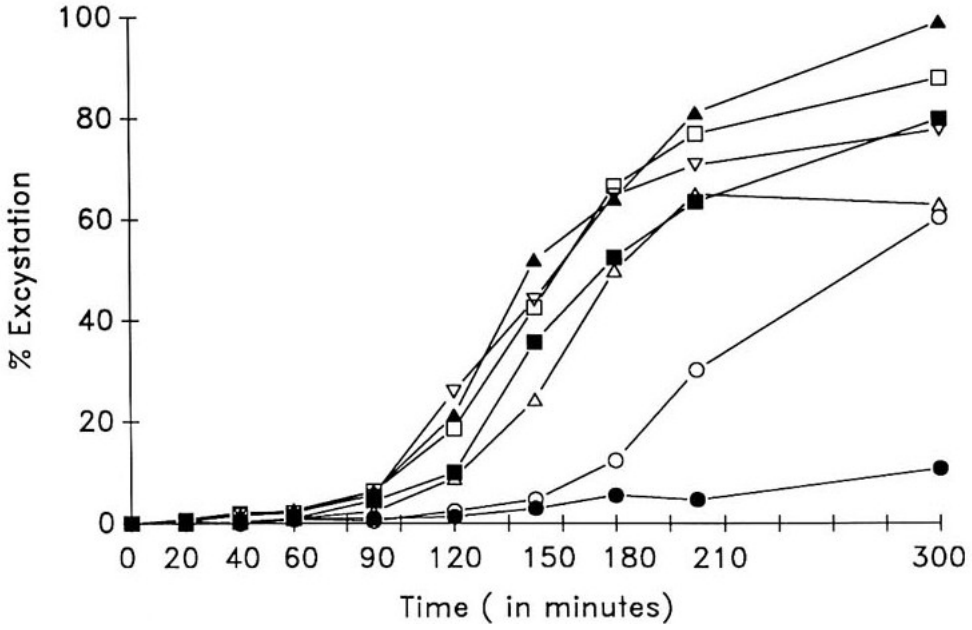

Figure 2. Percentage of excystation of sporocysts of *Eimeria tenella* after incubation at 41 ^{O}C in phosphate buffer, NaCl 0.9% (PBS) added with 0.5 % (w/v) bile extract and 0.25% (w/v) of either chicken trypsin (○), chymotrypsin (●), pancreatic elastase (A), trypsin and chymotrypsin (A), trypsin and elastase (□), chymotrypsin and elastase (■) or the 3 enzymes together (v). Percentage of excystation is equivalent to the number of sporozoites counted/ 2 x number of sporocysts present initially.

enzymes (trypsin + elastase and chymotrypsin + elastase) gave also comparable results. Sporocysts incubated in the presence of the 3 proteolytic enzymes excysted equally well, although a significantly lower percentage was observed after 5 hours when compared to that observed with sporocysts incubated with trypsin and chymotrypsin together.

DISCUSSION

Early studies on excystation have shown that proteolytic enzymes and bile salts are the major factors involved in the in vivo release of sporocysts and sporozoites from the intact oocysts (Levine 1942; Smetana 1933). Later, studies indicated that excystation was also observed in vitro without mechanical breakage of the oocyst wall, for both mammalian (Bunch and Nyberg 1970; Nyberg and Hammond 1964) and avian *Eimeria* spp. {Bunch and Nyberg 1970; Nyberg et al. 1968}. In our first experiment, we attempted to produce excystation of intact sporulated oocysts after a short preincubation period in a $C0_2$ atmosphere and bile, followed by the addition of either trypsin, chymotrypsin or pancreatic elastase of chicken origin. Under these conditions, no excystation from intact oocysts was observed. Although several workers reported in vitro excystation without mechanical breakage of the oocyst walls, the period of pretreatment in CO_2 (Bunch and Nyberg 1970; Nyberg et al. 1968) or reducing agents (Jensen et al. 19/6) appeared rather long and quite incompatible with observations of excystation made in vivo (Doran and Farr, 1962; Farr and Doran 1962). A shorter time of pretreatment was more likely to mimic the conditions in vivo but failed to prepare the oocysts for the second step of the excystation process. Likewise, the use of the pancreatic proteolytic enzymes of chicken origin was not sufficient to bring about the excystation of intact sporulated oocysts. Over the years, several authors have also suggested that some excystation factors may be present inside the oocysts (Hibbert and Hammond 1968; Lotze and Leek 1968; Ryley 1973; 1980) and more recently, some protease activity against azocasein was associated with sporozoites and merozoites of *Eimeria tenella* (Fuller and McDougald 1990). Therefore, we obtained some crude extracts of both sporulated and unsporulated oocysts of *E. tenella* and tested their effect on the excystation process. Again, no excystation was observed from intact oocysts at any time, confirming previous observations by Wang (1982).

Among several enzymes tested, only trypsin, chymotrypsin and papain were able to release sporozoites from sporocysts (Doran 1966; Doran and Farr 1962; Jackson 1962; Smetana 1933; Wang and Stotish 1975). In experiments 2 and 3, we confirmed the role of trypsin and chymotrypsin, and also established that pancreatic elastase was able to bring about excystation of sporocysts. Results on excystation rates were significantly better with elastase than with trypsin or chymotrypsin, indicating that at similar concentration (w/v), chicken pancreatic elastase was the most effective enzyme. The low excystation percentage observed with chymotrypsin, already observed by Chapman (1978), proved that chymotrypsin was not the essential enzyme for excystation, as opposed to previous report by Wang and Stotish (1975). Again, crude extracts of sporulated and unsporulated oocysts of *E. tenella* did not produce the release of sporozoites from sporocysts.

Because more than one pancreatic proteolytic enzyme is present in the lumen of the intestine, we studied also the effect of the combination of trypsin, chymotrypsin and elastase on the percentage of excystation. The combination of any 2 or 3 enzymes together produced better excystation percentages than any single enzyme. In all cases, the effect of the association of 2 or 3 enzymes was synergistic, since the percentage of excystation observed was superior to the sum of the percentages recorded for each enzyme. Considering the mode of activation of trypsin and chymotrypsin, such synergistic effects were expected to take place. Interestingly, the combination of the 3 enzymes did not produce the best results on excystation, although no statistically significant difference ($P<0.05$) were detected until 5 hours of incubation. The excystation was not observed after 5 hours of incubation since the viability of sporozoites usually decreased with time.

Although we were not able to bring about excystation from intact sporulated oocysts, we have demonstrated that another enzyme, the pancreatic elastase, is also involved in the excystation process. Additional work would be required to find the factors of excystation involved in the release of sporozoites from sporulated oocysts.

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