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Enzymes released during hatching of *Globodera rostochiensis* and *Meloidogyne incognita*

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Summary — An analysis of lipases released during hatching of *Meloidogyne incognita* and *Globodera rostochiensis* showed that hatching of *M. incognita* was accompanied by considerable lipase activity which correlated with the percentage hatch. By contrast, no lipase activity was detected during the hatching of *G. rostochiensis*. The protein substrates azocasein and azocoll revealed marked enzymic activity during hatching of *M. incognita*. The presence of leucine aminopeptidase could not be correlated with the hatching of either species. The results are discussed in the context of the contrasting hatching mechanisms of the two species.

Résumé — ***Enzymes libérés pendant l'éclosion de* Globodera rostochiensis *et de* Meloidogyne incognita** — L'analyse des lipases libérées pendant l'éclosion de *Meloidogyne incognita* et de *Globodera rostochiensis* montre que l'éclosion de *M. incognita* est accompagnée d'une activité lipasique très importante, en corrélation avec le taux d'éclosion. Au contraire, aucune activité lipasique n'a été détectée pendant l'éclosion de *Globodera rostochiensis*. Les substrats protéiniques azocaséine et azocoll révèlent une activité enzymatique marquée pendant l'éclosion de *M. incognita*. La présence d'une aminopeptidase leucinique n'a pu être mise en corrélation avec l'éclosion de l'une ou de l'autre espèce. Les résultats sont discutés dans le contexte des mécanismes différents d'éclosion chez les deux espèces.

Key-words : *Globodera, Meloidogyne,* enzymes, hatching.

The involvement of enzymes in hatching of nematodes has been postulated for a number of species. Usually the evidence is indirect, being based on observations of changes in the structure and the flexibility of the eggshell immediately before eclosion. Much of this work was reviewed by Perry and Clarke (1982).

In relation to possible enzyme involvement, the hatching mechanisms of *Meloidogyne incognita* and *Globodera rostochiensis* differ markedly. Wallace (1966, 1968) considered that the lipid layer of the eggshells of *M. incognita* could be altered either by emulsification or by the secretion of enzymes. Ultrastructural studies of *M. javanica* eggs (Bird, 1968) indicated that the lipid layer was hydrolysed by enzymes which appeared to be synthesised in the subventral oesophageal glands of the juvenile. The eggshell becomes flexible and distorts in response to juvenile movements.

By contrast, the eggshell of *G. rostochiensis* remains inflexible during the hatching process. In this species, hatching factors in potato root diffusate (PRD) may bind to or replace Ca^{2+} in the lipid layer of the eggshell causing a structural alteration at the molecular level resulting in a change of permeability (Clarke *et al.*, 1978; Clarke & Perry, 1985). Although the oesophageal glands of the unhatched juveniles become full of secretory granules before hatch (Doncaster & Shepherd, 1967; Perry *et al.*, 1989), no secretory material was observed to be voided into the oesophagus or the intestine and Perry *et al.* (1989) considered it unlikely that enzymes from the oesophageal glands were involved in eclosion. The unusual behavioural sequence of coordinated stylet thrusts leading to the formation of a slit through which the juvenile escapes (Doncaster & Seymour, 1973) is probably dictated by the rigid eggshell and is further indication of the likely absence of enzyme activity (Perry, 1986).

However, the role of enzymes in hatching remains unclear and inferences from behavioural and ultrastructural investigations cannot be conclusive. The Ca^{2+}-mediated structural changes in the eggshell of *G. rostochiensis* need not preclude enzyme involvement in hatching. As discussed previously (Perry & Clarke, 1982; Perry, 1986), there are possible sources of enzymes other than from the juvenile; enzymes may be present in the egg fluid and kept inactive either by an inhibitor or by separation from their substrate by the lipid membrane. In either case, it is possible that a structural alteration in the lipid layer causing a permeability change would allow enzymes to erode, or soften the outer layers of the eggshell.

In this work, we have examined whether enzymes, particularly lipases, are released during the hatching process of *G. rostochiensis* and *M. incognita*.

Materials and methods

Nematode material

M. incognita was obtained from pot cultures of tomato cv. Pixie infected for twelve weeks. The entire root ball plus soil was immersed in water and the soil gently removed from around the roots before the egg masses were picked off individually with fine forceps under a dissecting microscope. Cysts of *G. rostochiensis* Ro1, grown on potato cv. Arran Banner in pots, were taken from a single generation harvested in 1985 and stored dry at room temperature (20 °C) after extraction from the soil. Cysts were soaked for one week in glass distilled water (GDW) prior to experimentation.

Hatching tests

For each hatching test and enzyme bioassay, 25 cysts of *G. rostochiensis* were cut open and as many eggs as possible freed. Each suspension of eggs was rinsed in GDW and then transferred to an excavated glass block containing 1.5 ml of potato root diffusate (PRD) at 20 °C. PRD was obtained by the method of Fenwick (1949) and diluted with GDW 1 in 4 by volume. Eggs were exposed to PRD for a total of 3 weeks and counts were taken of hatched juveniles at 1, 3, 5, 7, 14 and 21 days. At each time period the PRD was removed for enzyme analysis and 1.5 ml of fresh PRD was added. At the end of each test the number of unhatched eggs was counted and the percentage hatch determined. The procedure was similar for *M. incognita* except that 50 egg masses were used for each test and hatching was carried out in GDW.

Enzyme determinations

The PRD or GDW removed at each time interval was analysed for enzyme activity using an I. L. Multistat III microcentrifugal analyser (Instrumentation Laboratory, Cheshire, UK). Substrates were purchased from Sigma Chemical Co., Poole, Dorset, UK. Unless otherwise stated, buffers used in the determinations of enzyme activities were 0.1 M citrate phosphate (pH 5) and 0.1 M Tris/HCl (pH 8) both containing penicillin (500 U ml^{-1}) and streptomycin (5 mg ml^{-1}). All assays were performed at room temperature. Results are expressed as change in absorbance (mAbs units, 0.5 cm light path) and are corrected for the sample : reagent dilution in the assay protocols.

Lipase assay

Hydrolysis of the lipid substrate, naphthyl laurate, was detected by measuring the increase in absorbance due to the reaction of liberated 2-naphthol with Fast Blue RR salt (Seligman & Nachlas, 1963). Reaction mixtures usually comprised 50 µl sample/blank, 200 µl buffer, 50 µl substrate (5 mg ml^{-1} naphthyl laurate in acetone) and 3 µl Fast Blue RR salt (20 mg ml^{-1} in acetone). Following incubation for 18 h, colour was extracted with an equal volume of ethyl acetate and absorbance at 540 nm determined.

Proteolytic activity

Hatching media were assayed for proteolytic activity using azocasein, a non-specific protein substrate, and azocoll, a collagen-based substrate favouring collagenase activity (Knox & Jones, 1990). Sample/blank (50 µl) was incubated with 150 µl buffer and 50 µl protein substrate (5 mg ml^{-1} in water) for 18 h. Undigested protein was removed by centrifugation (azocoll) or by acid precipitation (Knox & Jones, 1990) and the absorbance (405 nm, azocasein; 520 nm, azocoll) of the supernatant was determined. Leucine aminopeptidase (LAP) activity was assayed using Boehringer kit number 124877 (Boehringer Mannheim UK, Lewes, East Sussex, UK), used according to the manufacturer's instructions.

Results

Hatching

The hatching patterns of *G. rostochiensis* and *M. incognita* were consistent for each trial. Hatching curves from the lipase assay tests are shown in Fig. 1. The majority of juveniles of *G. rostochiensis* hatched by the end of the second week; after 3 weeks the total percentage hatch was 68.2 % which was equivalent to 4535 eggs hatching per 25 cysts. Similarly, the majority of eggs from egg masses of *M. incognita* had hatched by the end of the second week. The total percentage hatch after 3 weeks was 64.4 %, equivalent to 2577 eggs hatching per 50 egg masses.

Enzyme activity

There was a marked difference between the species in the lipase activity detected in the hatching medium. No activity was detected during the entire three-week hatching period of *G. rostochiensis* at either pH 5 or 8 (Fig. 1 A). By contrast, lipase activity increased during the hatching of *M. incognita* (Fig. 1 B) at both pHs. For *M. incognita*, cumulative lipase activity appears from Fig. 1 B to correlate with the number of juveniles which hatch and this is especially true for hatching at pH 5 where the correlation coefficient between percentage hatch and lipase activity was 0.986 (Fig. 2).

The data for the analysis of azocaseinolytic, azocollytic and LAP-hydrolysing activity after 7, 14 and 21 days hatching are presented in Table 1. Results for the two species are not directly comparable because they have not been corrected for differences in numbers of eggs; total numbers of eggs hatching for *G. rostochiensis* was approximately 1.7 times greater than for *M. incognita*. However, expressing the data on a unit-hatch basis would only accentuate the higher enzyme activity during hatching of *M. incognita* (Table 1).

Hatching of *G. rostochiensis* was not accompanied by any significant increase in LAP activity compared to the

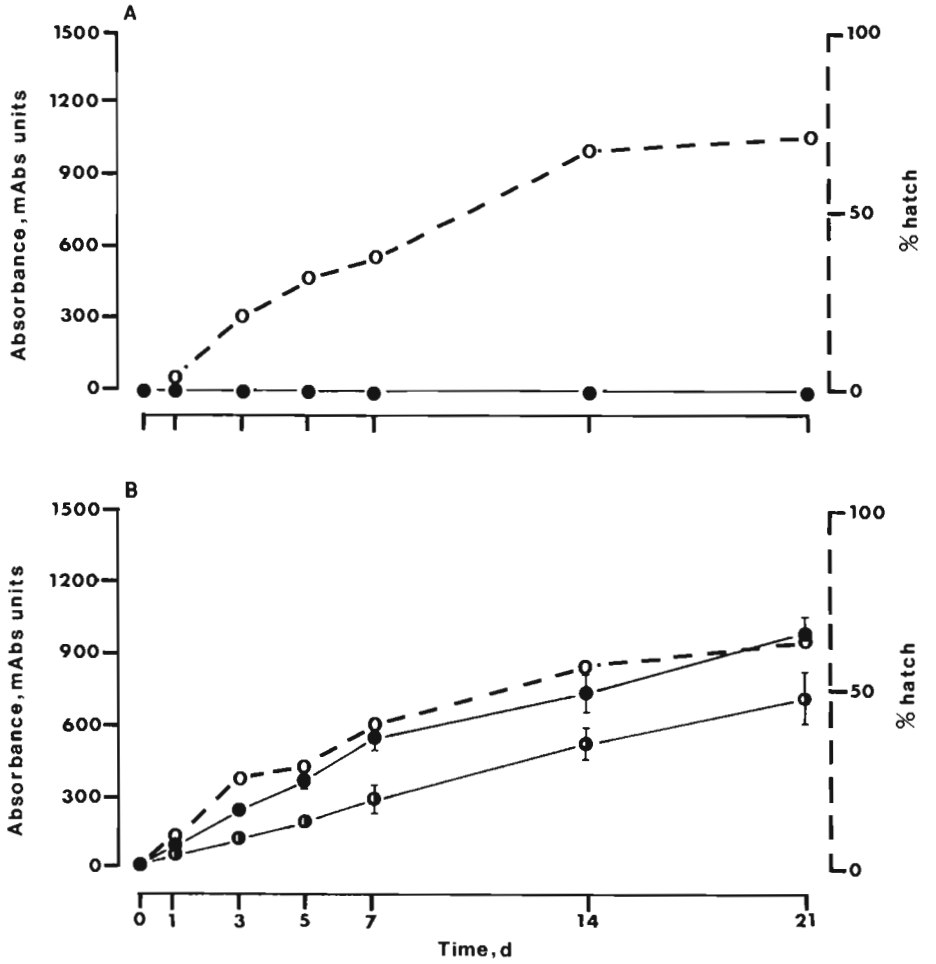

Fig. 1. Cumulative lipase activity, assayed at pH 5 (●) and 8 (○), and percentage hatch (◑) of (A) *Globodera rostochiensis* in potato root diffusate and (B) *Meloidogyne incognita* in glass distilled water over a three week period. Enzyme activity is expressed in absorbance units as mean ± S.E.M. of three separate analyses.

PRD control ($P > 0.05$) and the only indication of enzyme activity was a slight increase in azocasein activity at pH 8 and in azocoll activity at both pH 5 and 8.

By contrast, there was considerable enzyme activity during the hatching of *M. incognita* (Table 1). Azocasein activity increased significantly ($P < 0.01$) over the 3 week hatching period, especially at pH 5. Azocoll activity was not detected at pH 8 but increased significantly ($P < 0.01$) at pH 5. LAP activity was detected after the first week of hatching but subsequent increases during the second and third weeks were not significant ($P > 0.05$).

Discussion

An initial step in the hatching sequence of many plant and animal parasitic nematodes is a change in the permeability of the eggshell (Perry & Clarke, 1982). The permeability characteristics of the eggshell derive mainly from the lipid layer and this layer has been found in *M. incognita* (Bird, 1968) and *G. rostochiensis* (Perry *et al.*, 1982). However, the hatching mechanism of the two species and the nature of the permeability change differs.

M. incognita hatches well in water and is not dependent on stimulus from host root diffusates. Before hatching, the eggshell becomes pliable, the juvenile head protrudes in the distended and flexible eggshell and stylet thrusts eventually cause a tear that is extended by the escaping juvenile (Wallace, 1968). As juveniles of *M. incognita* are fully hydrated when they leave the egg (Ellenby, 1974), increase in egg size (Wallace, 1968) may be due to increased hydration of the unhatched juvenile after alteration of the permeability of the eggshell.

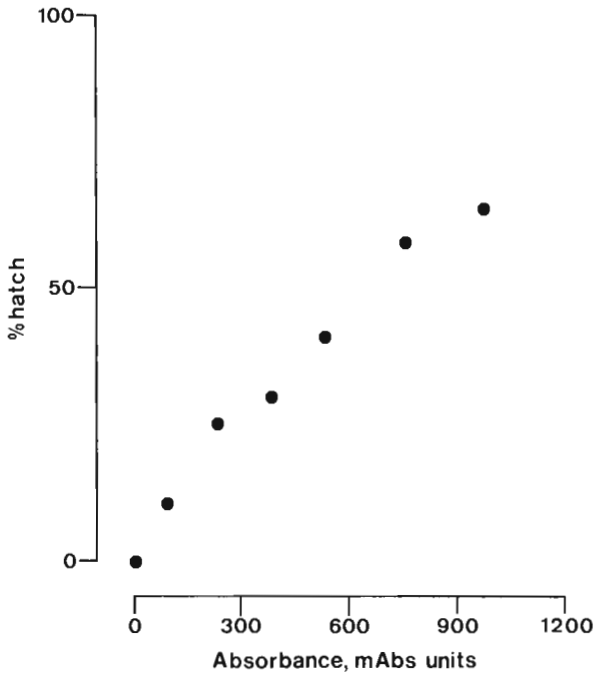

Fig. 2. The relationship between lipase activity (assayed at pH 5 and expressed in absorbance units) and the percentage hatch of *Meloidogyne incognita* over a three-week period (correlation coefficient = 0.986).

Ultrastructural investigations (Bird, 1968) indicate that enzymes are involved in the erosion of the eggshell and, in particular, the lipid layer.

G. rostochiensis is dependent on PRD for substantial hatch and active hatching factors in PRD may change lipoprotein membrane structure from a semi-permeable to a permeable state allowing leakage of trehalose out of the egg (Clarke *et al.*, 1978; Clarke & Perry, 1985). This removes the osmotic stress on the juvenile with a concomitant increase in juvenile water content (Ellenby & Perry, 1976); the juvenile is then able to cut its way out of the rigid eggshell. The eggshell remains rigid throughout the hatching sequence and imposes a physical constraint on the juvenile for it is not until eclosion is completed that the juvenile can become fully hydrated (Ellenby, 1974).

Analysis of enzymes released during hatching of *M. incognita* and *G. rostochiensis* concentrated primarily on the difference in lipase activity. The results support and extend inferences from behavioural and ultrastructural observations. Tests with *M. incognita* showed that lipase activity was very marked; increased lipase activity was positively correlated with the increase in percentage of eggs which hatched over the three-week period. By contrast, no lipase activity was detected during the same hatching period of *G. rostochiensis* which resulted in a total hatch of 68.2 %. There is no evidence of lipase involvement in the change in permeability of the lipid layer of *G. rostochiensis* egg-shells.

Table 1. Cumulative enzyme activities of hatching media during the three week hatching period of *Globodera rostochiensis* and *Meloidogyne incognita*. Enzyme activities are corrected for assay dilution and expressed in absorbance units as mean ± S.E.M. of three separate analyses (except for *G. rostochiensis*/azocasein which is based on two analyses).
N.D. = no activity detected. LAP = leucine aminopeptidase.

Media	*G. rostochiensis*			*M. incognita*		
	day 0 (PRD alone)	day 7	day 14	day 21	day 7	day 14	day 21
Azocasein							
pH 5.0	20.4 ± 6.1	11.5 ± 3.7	13.3 ± 0.7	15.4 ± 2.7	317.7 ± 6.8	446.7 ± 7.1	575.0 ± 11.9
pH 8.0	2.2 ± 2.2	1.4 ± 0.6	11.2 ± 6.5	26.7 ± 15.9	180.5 ± 45.5	245.0 ± 49.0	317.0 ± 54.0
Azocoll							
pH 5.0	4.6 ± 2.4	21.9 ± 2.6	31.7 ± 4.5	37.6 ± 8.3	49.0 ± 10.0	77.0 ± 1.0	140.5 ± 29.5
pH 8.0	1.6 ± 1.6	7.3 ± 1.4	10.3 ± 4.0	13.3 ± 2.7	N.D.	N.D.	N.D.
LAP							
pH 7.0-7.5	22.5 ± 22.5	15.3 ± 11.8	24.5 ± 18.3	51.3 ± 10.0	89.3 ± 20.3	119.0 ± 26.0	150.0 ± 31.5

The pHs for determinations of enzyme activity were chosen on the basis of previous experience of nematode enzyme pH optima. The inability to detect enzyme activity does not completely eliminate the possibility that *G. rostochiensis* has enzymes of radically different pH optima to those previously reported : e.g. Tefft and Bone (1985*a*) found the optimum pH for LAP activity in *Heterodera glycines* was over 8.5. However, the normal range for PRD activity is between pH 2-8; above pH 8 the PRD is inactivated. If lipase activity were to be present in hatching fluid from *G. rostochiensis* after hatch stimulation by PRD, then it is likely that some activity would have been detected even if neither pH 5 nor 8 is the optimum.

The flexibility of eggs of *M. incognita* before hatch indicates activity of enzymes in addition to lipases. The nematode eggshell primarily comprises collagen and chitin and, thus, the marked increase in asocaseinolytic and, particularly, azocollytic activity during hatching of *M. incognita* is interesting. Azocasein is a general proteinase substrate which should detect virtually all proteinases, whereas azocoll favours detection of collagenolytic activity. The lack of apparent correlation between azocoll and azocasein determinations could indicate the presence of multiple proteolytic enzymes, possibly including chitinase.

Chitin is a smaller constituent of eggshells than collagen (Clarke *et al.*, 1967), but chitin gives the eggshell its rigidity. Preliminary analysis of chitinase activity was carried out using the chitin based substrate chitin-azure; there was no significant activity during the 3-week hatching of *G. rostochiensis*, whereas with *M. incognita* there was some activity at pH 5.0 but very marked activity at pH 6.8 by week 3 (Perry *et al.*, unpbl.). The involvement of chitinases in hatching of species of plant parasitic nematodes and the link with increased flexibility of the eggshell is the subject of further studies.

The role of LAP in nematode hatching remains unclear. LAP has been implicated in hatching and exsheathment of the animal-parasitic nematode, *Haemonchus contortus* (Rogers & Brooks, 1977). Tefft and Bone (1984) considered that a zinc-dependent enzyme mediates hatching in *H. glycines* and found LAP activity in egg supernatants (Tefft & Bone, 1985*a*), although host-root diffusate did not directly increase LAP activity (Tefft & Bone, 1985*b*). Results from the present study indicate that LAP is unlikely to be involved in the hatching of *G. rostochiensis*. Although LAP activity was detected in the hatching medium of *M. incognita*, there was no significant increase during the three-weeks hatching period.

This work demonstrates differences in the hatching mechanism of *M. incognita* and *G. rostochiensis*. Although enzymes, particularly lipases, are implicated in the structural changes of the egg and subsequent hatching of *M. incognita*, hatching of *G. rostochiensis* appears not to involve lipases and other enzymes may play only a minor role. It would be interesting to examine whether inhibiting lipase activity prevents hatch of *M. incognita*.

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