RELATIONSHIP BETWEEN EXSHEATHMENT AND ENZYME ACTIVITY (ALKALINE PHOSPHATASE AND LEUCINE AMINO PEPTIDASE) DURING AGEING OF TRICHOSTRONGYLUS COLUBRIFORMIS INFECTIVE LARVAE

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RELATIONSHIP BETWEEN EXSHEATHMENT AND ENZYME ACTIVITY (ALKALINE PHOSPHATASE AND LEUCINE AMINO PEPTIDASE) DURING AGEING OF TRICHOSTRONGYLUS COLUBRIFORMIS INFECTIVE LARVAE

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Résumé

RELATIONS ENTRE LE DÉGAINEMENT ET L’ACTIVITÉ ENZYMATIQUE (PHOSPHATASE ALCALINE ET LEUCINE AMINO-PEPTIDASE) AU COURS DU VIEILLISSEMENT DES LARVES INFESTANTES DE TRICHOSTRONGYLUS COLUBRIFORMIS. — Un stock de larves infestantes (L3) de T colubriformis a été conservé pendant 15 semaines à 24 °C. Toutes les trois semaines, un échantillon de L3 a été prélevé afin d’estimer leur taux de dégainement et leur activité enzymatique (leucine amino peptidase et phosphatase alcaline). Entre 0 et 9 semaines de conservation, une baisse d’activité enzymatique a été observée, corollée à une diminution de la vitesse du dégainement (pourcentage de L3 dégaines in vitro en 20 min); le taux maximum de dégainement (pourcentage de L3 dégaines in vitro en 180 min) était alors constant (75 %). Entre 9 et 15 semaines de conservation, l’activité enzymatique de même que la vitesse de dégainement furent faibles et constantes ainsi que le taux maximum (30 %). Le rôle des enzymes dans le mécanisme du dégainement est discuté.

Important physiological changes occur during ageing of trichostrongyle infective larvae. A loss in infectivity, correlated with an increased fecundity of adult female worms have been observed with Trichostrongylus colubriformis (Mallet and Kerbœuf 1985). A relationship has been recently demonstrated between the exsheathement rate and infectivity of the third stage larvae during ageing (Lesage and Mallet 1987). The possible role of enzymes in the phenomenon was then discussed.

An experiment was designed to determine the possible relationships between enzyme activity (leucine amino peptidase and alkaline phosphatase) and exsheathement rate during ageing of T colubriformis infective larvae.

Materials and Methods

Experimental design

A stock of 2 000 000 freshly collected third stage larvae (L3) was stored at 24 °C in water at a concentration of 5 000 L3/ml as in Mallet and Kerbœuf (1985). Aliquots were taken from this stock at three weeks intervals over the 15 weeks of storage. Four estimations of the exsheathement rate and of the enzyme activity were performed on each aliquot.

Exsheathing technique

Infective larvae were stimulated for exsheathment at 38 °C in 0.9 % NaCl acidified to pH 2 with 1N HCl and gassed with pure CO₂ (Lesage and Mallet 1987). The number of larvae exsheathed was estimated on subsamples taken after 10, 20, 40, 60, 120 and 180 min of incubation. Dead larvae were not taken into account for exsheathment estimations.

Biochemical techniques

Larval suspensions were cleaned using a Baermann apparatus to eliminate dead larvae and then adjusted to 20 000/ml of distilled water. Subsamples of 0.5 ml were stored at – 20 °C before the tests as previous unpublished studies have demonstrated that enzyme activity remained stable several months in these conditions. Just before testing, the samples were thawed and homogenized 1 min using a micro-potter running at 1 400 RPM and maintained in ice. For a better solubilisation, the extracts were stored half an hour at 4 °C before being centrifuged 10 min at 5 000 g.

Protein determinations were performed on supernatants according to the Lowry method (Lowry et al 1951) and using dilutions of bovine serum albumin as standards. The assay was performed on 0.1 ml samples of standard and test materials.

Leucine Amino Peptidase (LAP) activity was assayed using a kit supplied by Sigma Chemical Company for the...
determination of LAP activity in serum according to a modification of the colorimetric method of Goldbarg and Rutenburg (1958). In the present experimentation, a micro-method was adapted from the procedure outlined in Sigma Technical Bulletin N° 251 (1981). The amount of all reagents were divided by five and the incubation of test material (0.1 ml of supernatant) with substrat (0.02 mg of L Leucyl B Naphthylamide) was four hours at 37 °C. The colourless product of the reaction (β Naphthylamine) was then diazotized and combined with the dye. The optical density was read at 580 nm and results expressed in units of enzyme (amount of LAP releasing 1 μMole of β Naphthylamine in the conditions of the test).

Alkaline phosphatase (AP) activity was assayed using the method of Butterworth and Probert (1970) with some modifications: 0.5 ml of substrate (8.8 μMoles of p nitrophenylphosphate per ml of glycine buffer pH 8.8) were incubated four hours at 37 °C with 0.1 ml of supernatant. The reaction was stopped with 0.5 ml of 0.05N NaOH. The yellow coloured product of the reaction (p nitrophenol) was measured as optical density at 410 nm and results expressed in units of enzyme (amount of AP releasing 1 μMole of p nitrophenol in the conditions of the test).

Results

Exsheathment rate of infective larvae

The percentage of exsheathed larvae increased with incubation time to a maximum value at a rate inversely correlated with the time of storage. The maximum values were respectively 75 % and 30 % for the larvae stored from 0 to 9 weeks or from 9 to 15 weeks, as in a previous experiment (Lesage and Mallet 1987). The percentage of larvae exsheathed within 20 min of incubation, in the linear part of the graph, was defined as Exsheathment Potential (EP20) and used to characterize the exsheathment of the different batches of larvae (fig 1).

Protein contents of L3

A variance analysis showed significant differences (P ≤ 0.01) between the aliquots of larvae but no correlation between protein levels and age of larvae.

Enzyme activity

From 0 to 9 weeks of storage, a decrease was observed in LAP and AP activity to a minimum value which remained constant for up to the 15th week of storage (fig 2).

Significant correlations were noticed between the enzyme activity and the number of larvae exsheathed at different incubation times. A linear regression was observed between LAP activity and the larvae exsheathed after 20 min (EP20) (fig 3a) and between AP activity and the number of larvae exsheathed after 180 min (fig 3b).

Discussion

The percentage of exsheathed larvae increased with incubation time to a maximum at a rate function of the age of the larvae as recently demonstrated (Lesage and Mallet 1987) but with some differences. During the first nine weeks, both exsheathment potential (EP20) and maximum number of exsheathed larvae were lower in the present experiment. From 9 to 15 weeks, the exsheathment rate remained low and constant, and the maximum decreased from 75 % to 30 %. These differences may be explained by differences in experimental design as larvae obtained from
several faecal cultures were used in the previous experiment and only one batch of larvae from the same culture in the present work. Certainly, ageing of the larvae differs from one batch to another one.

In a previous experiment on infectivity and egg production of *T. colubriformis* (Mallet and Kerboeuf 1985), an increased egg production was noticed after nine weeks of ageing in similar experimental conditions. This time of storage might then correspond to an important period in worm physiology.

Protein contents of *Nippostrongylus brasiliensis* infective larvae have been proved to decrease during ageing (Wilson 1965); this was not observed in the present experiment as protein contents of the larvae were not correlated with the age. The significant differences observed in protein contents of each aliquot of larvae may be explained by fluctuations in the preparation of larval extracts. The enzyme activity was thus expressed in units per mg of protein in the extracts instead of units per number of L3.

The activity of the two enzymes tested was correlated with exsheathment. From 0 to 9 weeks of ageing, the enzyme activity decreased as well as the EP20. From 9 to 15 weeks, both enzyme activity and exsheathment rate were low and constant and the maximum number of exsheathed larvae decreased from 75 % to 30 %. A similar decrease in enzyme activity during ageing, correlated with infectivity, has been previously noticed with *Heligmosomoides polygyrus*, a mouse parasite (Mallet and Kerboeuf 1984).

The link between LAP activity and EP20 is plausible, though LAP activity was never correlated previously with exsheathment rate, the role of this enzyme in the phenomenon is generally accepted (Rogers 1965, 1970, 1982, Ozerol and Silverman 1972, Rogers and Brooks 1976, 1977, 1978a, 1978b). The role of AP is more difficult to understand as the activity of this enzyme is more correlated with the maximum value of exsheathment than with EP20. It may then express a physiological stage of the larvae without being directly involved in exsheathment mechanisms.

One hypothesis may explain the evolution of exsheathment during ageing of the larvae: from 0 to 9 weeks of ageing, 75 % of the larval population are able to exsheath. A decrease in enzyme activity of the larvae may induce a proportional decrease in exsheathment rate without reducing the maximum number of exsheathed larvae. From 9 to 15 weeks of ageing, the amount of enzyme activity has decreased under a critical level where half of the larval population cannot normally exsheath.

**Fig 3**——Correlation determined by the least squares method.

a) between the number of larvae exsheathed after 20 min and LAP (▼)
b) between the number of larvae exsheathed after 180 min and AP (●)

Abstract

A stock of *Trichostrongylus colubriformis* infective larvae (L3) was stored for up to 15 weeks at 24 °C. At three weeks intervals, samples of L3 were taken to assess exsheathment rate and enzyme activity Leucine Amino Peptidase (LAP) and Alkaline Phosphatase (AP). From 0 to 9 weeks of storage, a decrease in enzyme activity was observed correlated with a decrease in exsheathment rate (percentage of L3 exsheathed *in vitro* after 20 min); the maximum percentage of exsheathed larvae (L3 exsheathed after...
180 min) was then 75%. From 9 to 15 weeks of storage, both enzyme activity and exsheathment rate were low and constant; the maximum percentage of exsheathed larvae never exceeded 30%. The role of enzymes in the mechanism of exsheathment is discussed.

References


