Japan. J. Trop. Med. Hyg., Vol. 11, No. 1, 1983, pp. 7–15

ENZYME HISTOCHEMISTRY OF *BRUGIA PAHANGI*

1. Localization of Acid Phosphatase Activity in Developing Larvae in Mosquito (*Aedes aegypti*)

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Received for publication October 15 1982

Abstract: Histochemical localization of acid phosphatase was reported on the microfilariae isolated from the peripheral blood of the dog and their developing larvae in mosquitoes in comparison with the localization in those from the jird's peritoneal cavity and their larvae. Whole body of microfilariae stained red with dark red excretory vesicle and anal vesicle. The sheaths were negative in those from the dog, while positive in most microfilariae from the jird. During the first two days after the intake unstained larvae were frequently found in the mosquitoes fed jird microfilariae in contrast to those fed on the dog. From the third day on, positive developing intestine was observed. After the first molt the esophagus appeared as a red double line. On the 7th day strong activity was found at the esophagus, intestine, developing anus and amphids, while the nerve ring was negative. Positive reaction was detected at the hypodermis and anus of infective larvae.

INTRODUCTION

A considerable amount of research has been reported on the acid phosphatase activity in parasitic helminths. The enzyme is supposed to play an important role in the absorption of nutrients and excretion of metabolites by the parasites. Reports that two species of microfilariae which could hardly be differentiated from each other by the morphological characteristics (Aoki *et al.*, 1976) were easily classified by the different localization of acid phosphatase (Chalifoux and Hunt, 1971; Redington *et al.*, 1975) gave a new light on this enzyme from a stand point of taxonomic importance.

When the infective larvae of *Brugia pahangi* were inoculated into the peritoneal cavity of the jird (*Meriones unguiculatus*), adults and microfilariae were recovered from the peritoneal cavity (McCall *et al.*, 1973). The adults and microfilariae localized in the peritoneal cavity seem to follow an aberrant mode of development, although the microfilariae from the peritoneal cavity have been proved to reach stage III (*i.e.* infective stage) in mosquitoes as those from the peripheral blood of the dog which is a normal host of *B. pahangi* (Chuang *et al.*, 1979).

In this paper, a report is made on the localization of acid phosphatase in the developing stages of *B. pahangi* from the microfilariae in the canine peripheral blood

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to the infective larvae in mosquitoes. Also described is the localization of the same enzyme in *B. pahangi* microfilariae produced in the peritoneal cavity of the jird and their developing larval stages in mosquitoes in comparison with those from the dog.

Materials and Methods

The microfilariae from the canine peripheral blood and those from the peritoneal cavity of the jird were smeared on coverslips. The mosquitoes used were Liverpool strain of *Aedes aegypti* maintained in our laboratory. A group of mosquitoes were fed on the infected dog with *B. pahangi* microfilaremia for about 15 minutes. Another group of mosquitoes were fed on the artificial feeding apparatus in which microfilariae from the peritoneal cavity of the jird were suspended in Dulbecco's phosphate-buffured saline without calcium or magnesium added with adenosine 5'triphosphate, 10^{-3} M (Chuang *et al.*, 1979). The mosquitoes from each group were then dissected in a drop of physiological saline on cover slips 2 hours and every 24 hours after the infecting feed. The preparations were then air-dried and stored at −20 C until the use. The stored preparations were used within 2 weeks with no recognizable decrease of the enzyme activity. Frozen sections of thoracic muscles of infected mosquitoes were made on 7th, 9th and 11th day after the infecting feed.

For the demonstration of acid phosphatase activity, the technique of Barka was used following the description by Chalifoux and Hunt (1971), in which naphthol AS-BI phosphate was a substrate and pararosanilin was a capturing agent. The pH of incubation medium was adjusted to 5.0. The time of incubation was from 60 to 90 minutes at 37 C. Localization of acid phosphatase was recognized as red to dark red precipitations of azo dye, depending on the strength of the reaction. Stained frozen sections were mounted according to the method of Wharton (1957).

Some preparations were processed without the substrate as a control. Sodium fluoride, 10^{-2} M was also used as a specific inhibitor of the enzyme.

Results

I. Direct Feeding on the Infected Dog

The whole body of microfilaria in the peripheral blood was stained red with two distinct dark red spots, excretory vesicle and anal vesicle (Figure 1). The inner body showed slightly weaker reaction in most of the microfilariae. With a few exceptions, microfilarial sheaths were negative for acid phosphatase. Occasionally the enzyme activity was recognized at the regions of amphids and phasmids.

Two hours after feeding, in the stomach of the mosquito, microfilariae showed the same staining pattern as in the peripheral blood.

Exsheathed first stage larvae were becoming shorter and thicker in the thoracic muscles of the mosquito 24 hours after feeding. The red excretory vesicle and anal vesicle were clearly seen in a light red tint of the larval body surface (Figure 2). On the second day, the strongest red reaction was found at the anal vesicle (Figure 3). In the light pink color of larval surface, the excretory vesicle became indistinguishable

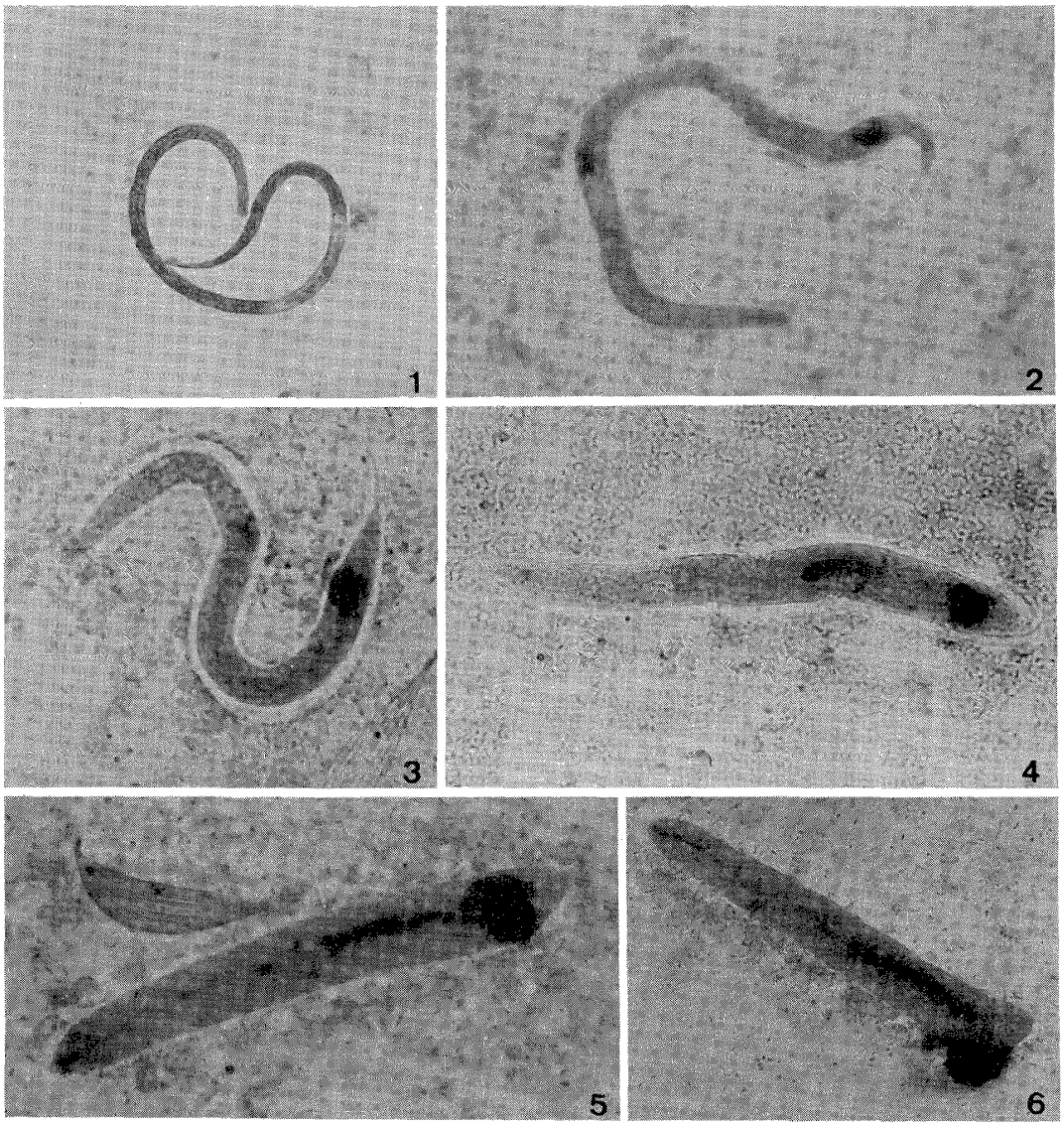

Figure 1 The microfilaria from dog peripheral blood stained red with dark red excretory vesicle and anal vesicle. Note the negative sheath.

Figure 2 Body surface of exsheathed 1st stage larva stained light red. Excretory and anal vesicles showed strong reaction.

Figure 3 Reaction of anal vesicle became stronger while that of excretory vesicle decreased on 2nd day.

Figure 4 Anal vesicle stained deep red, while excretory vesicle was indistinct in 3rd day larva. Note the positive developing intestine.

Figure 5 The positive intestine reached anal vesicle after 1st molt. Note the weak reaction at buccal cavity and esophagus.

Figure 6 Vague red staining was sometimes found at excretory vesicle on 4th and 5th day.

in about 40% of the larvae.

The larva became shortest on the third day with increasing redness on the body surface. The anal vesicle was prominent with strong red color, but the excretory

vesicle was indistinct. Around at this time a dark red linear spot, which must be a developing intestine, first appeared in the region formerly occupied by the inner body and gradually grew backward to the anal vesicle (Figure 4).

During the 4th and the 5th day of development, when the first molt occurred, the red intestine reached the anal vesicle. Buccal cavity began to show weak activity of acid phosphatase and then esophagus appeared as a light red double line (Figure 5). In this stage, a vague red dot was found at the place of excretory vesicle in some larvae (Figure 6).

After the sixth day, when all larvae might be in the second stage, a daily rapid growth in larval size with strong phosphatase activity on the body surface began to disturb close observation of the internal structures in most of whole body preparations (Figures 7, 8).

Frozen sections of the 7th and 9th day larvae showed strong enzyme activity in the esophagus, intestinal cells and developing anus (Figure 9). The amphids were clearly positive (Figure 10), but nerve ring was negative. We failed to find the phasmids in the sections.

At the 10th day, whole body of third stage larva was deep red and the internal structures eluded observation, but ano-rectal area was still prominent with a strong enzyme activity, especially at the anterior wall of the rectum (Figure 11).

A mature infective larva (11 day old) had an open anus and whole body was evenly stained in deep red in whole body preparation. Frozen section of this stage showed positive enzyme reaction at main bulky parts of hypodermis which constitute lateral, dorsal and ventral cords, and very week reaction in muscle tissues (Figure 12). Cuticle seemed to be negative, although it was not easy to distinguish cuticle and its underlying hypodermis under light-microscopical examination.

III. Artificial Feeding of Microfilariae from Peritoneal Cavity

The microfilariae from the peritoneal cavity of the jird were stained more clearly than those in the peripheral blood of the dog. The whole body except the inner body was pure red and the two vesicles were distinguished as dark red dots. Interestingly, most microfilarial sheaths (about 80%) were stained to variable extent from thin pink to strong red. Occasionally red small granules and/or red amorphous masses were observed in the space between the microfilaria and its sheath (Figure 13).

Acid phosphatase staining of the filarial larvae during the first two days after the infecting feed with artificial apparatus was unstable. Some larvae were stained fairly well but others were negative or poorly stained even at excretory and anal vesicles. After the second day, however, the enzyme activity of the larvae was found to be almost the same as in the direct feeding on the dog.

Control slides without substrate were all negative. Sodium fluoride (10^{-2} M) inhibited the reaction completely except a deep interior portion of anal region of third stage larva in whole body preparations. Most probably it resulted from a cuticular barrier against the penetration of the inhibitor.

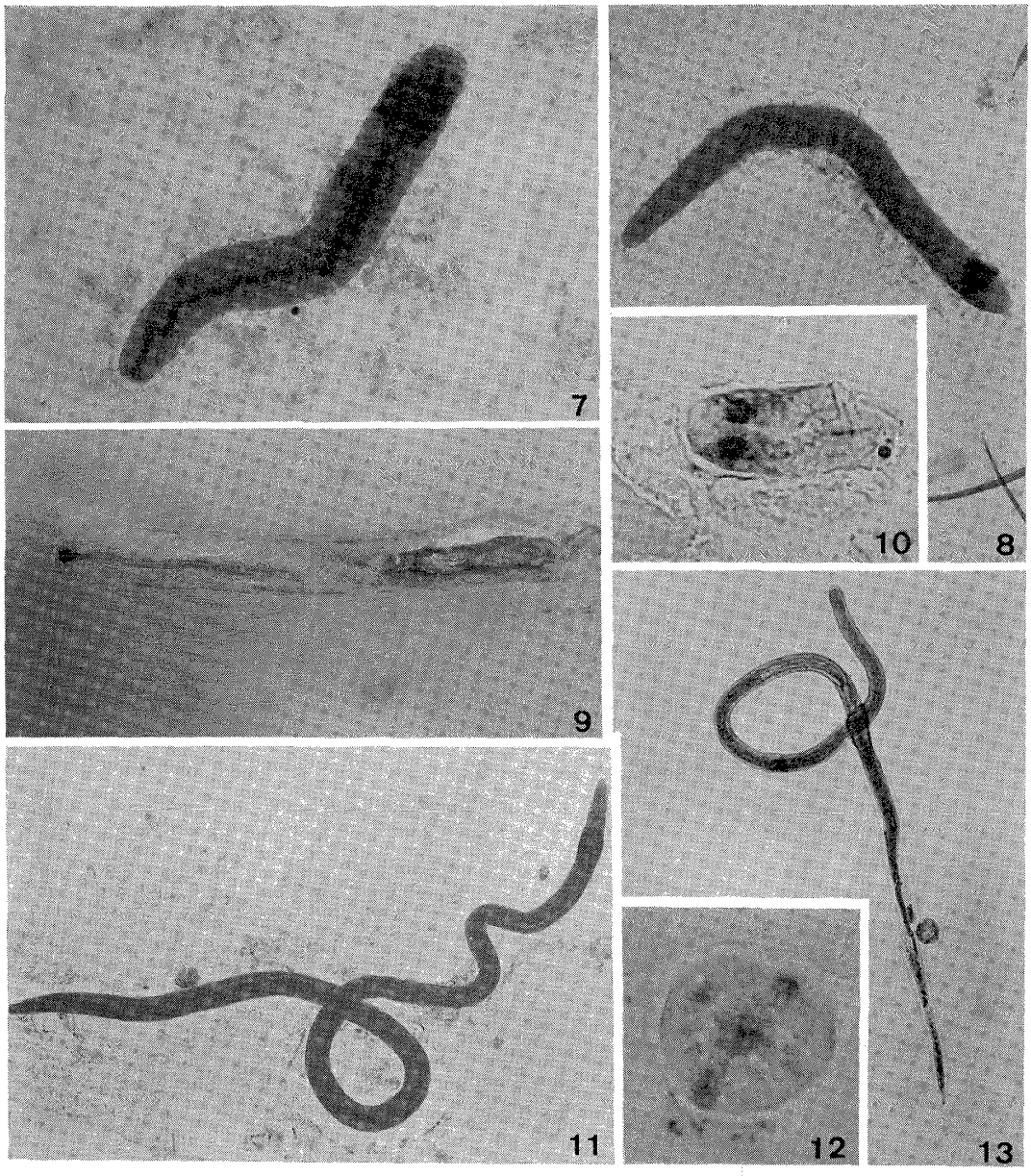

Figure 7 The positive esophagus and intestine were still visible in early 2nd stage larva.

Figure 8 Internal structures except developing anus became invisible due to strong activity of body surface accompanied with rapid growth of 2nd stage larva in the whole body preparation.

Figure 9 Note the strong reaction at amphids, esophagus and intestinal cells in frozen section of 7th day larva.

Figure 10 Note the strongly positive amphids in frozen section of 9th day larva.

Figure 11 Deep red stained whole body preparation of 10th day larva prevented observation of internal structures except strongly reacted ano-rectal area.

Figure 12 Note the positive reaction at intestinal cells as well as lateral, ventral and dorsal cords in the cross section of infective larva.

Figure 13 The microfilaria from jird peritoneal cavity stained well except the inner body. Note the positive granules in the space between the positive sheath and the body.

Discussion

Many studies have been reported on the distribution of phosphatases and the functional significance in parasitic helminths. In earlier days, Rogers (1947) found alkaline phosphatase activity at intestinal cells of *Ascaris lumbricoides* and at cuticle of *Moniezia expansa*, and supposed that the enzyme might have relationship with carbohydrate absorption by the parasites. Erasmus (1957) showed the existence of both acid and alkaline phosphatases in *Taenia pisiformis* and related the enzymatic function to an active transport of materials. Robinson (1961) studied alkaline phosphatase of *Schistosoma mansoni* and speculated the possibility of carbohydrate absorption through cuticle. More recently, Parshad *et al.* (1977) showed the activities of phosphatases in excretory system of several different kinds of parasites. Stood *et al.* (1977) recognized acid phosphatase activity in the cuticle and hypodermis of *Haemonchus contortus* and considered that these are metabolically active sites. Maki and Yanagisawa (1979a) studied *Angiostrongylus cantonensis* electronmicroscopically and showed extralysosomal acid phosphatase which may suggest the transport of substances through the cuticle. Although there are few reports which show clearly the definite function of acid phosphatase in parasites, it is widely accepted that the enzyme plays an important role in absorbing or excreting substances through the mechanism of active transport. From this view, the excretory and anal vesicles must be main places of metabolism in *B. pahangi* microfilaria and the presence of acid phosphatase activity on the filarial body surface may suggest a transport of substance through the cuticle, although we could not clearly define the localization of the enzyme among the surface structures. Interestingly, Redington *et al.* (1975) reported that the body surface of *B. malayi* showed negative reaction for the enzyme.

In the early first stage, anal vesicle seems to be the most active place of metabolism, whereas the enzyme activity of excretory vesicle and body surface decreased rapidly. In the late first stage, partial development of buccal cavity, esophagus and intestine is observed (Laurence and Simpson, 1971), which will be followed by functional maturation on the 6th and the 7th day, although it may not be complete at this time. This is partially supported by the present findings and our observation that acetylcholinesterase activity appears in glandular region of esophagus from the 6th day of development (in preparation), and also by some electronmicroscopical studies by Buckett *et al.* (1970) and Tongu *et al.* (1978), who showed mitochondria of mosquito host in the larval intestine of *B. pahangi* on the 6th and 7th day, respectively.

In the third stage larvae, the esophago-intestinal system is the positive structure of the enzyme as in the earlier stages, indicating its absorptive function. Functional importance of enzyme activity in hypodermal cells has been discussed by many authors. It may work for transport of substance through the cuticle or production and maintenance of the cuticle (Dusanic, 1959; Sood, 1977). Recently Maki and Yanagisawa (1979b, 1980a, b, c) reported that gastro-intestinal nematodes showed high enzymic activity in the intestine and low in the body wall, and that tissue nematodes including filarial worms showed high activity in the body wall. They speculated that the body wall of tissue nematodes may play a role comparable to that of the

intestine of the gastro-intestinal nematodes.

Microfilariae from the peritoneal cavity of Mongolian jird showed a characteristic staining pattern of the phosphatase. It is very interesting that most of the filarial sheaths were stained to variable extent from pink to deep red, making a clear contrast to microfilariae from the peripheral blood of the dog. Acid phosphatase was also found in the space between the microfilaria and its sheath in a shape of small granules and/or amorphous masses. Omar (1977) studied the localization of acid phosphatase activity in the larval stages of *B. pahangi* in *Aedes togoi* and stressed the positive reaction of the enzyme in microfilarial sheath. But Redington *et al.* (1975) did not mention the acid phosphatase activity in the sheath of *B. pahangi*, in spite of the same technique as of Omar. Our present results made these conflicting reports more complicating, although the substrate used in our study was different from those of Redington and Omar. Our suggestion is that environmental factors may influence the metabolism of parasites and change the distribution or intensity of enzyme activity even in the same species. Moore and Halton (1976) reported a pronounced change in acid phosphatase distribution and its isozyme pattern between starved and nourished *Fasciola hepatica*. Edwards *et al.* (1971) and Pavlov *et al.* (1975) also reported the influence of host immunity on the parasitic acid phosphatase. At the same time, we have to keep in mind a fact that microfilariae in the peritoneal cavity are a mixture of young and old microfilariae (Chuang *et al.*, 1979). Aging, if any, may cause changes in staining pattern of the enzyme. In case of artificial feeding, many larvae were stained poorly during the first two days after feeding. The finding may also be related to the fact that the artificial feeding produces less infective larvae than the direct feeding (Chuang *et al.*, 1979).

Omar (1977) observed acid phosphatase activity at the phasmids and the nerve ring of *B. pahangi* larvae on the 6th and 7th day. Alkaline phosphatase was also found to be positive in the nerve ganglion of *Fasciola hepatica* (Humiczewska, 1975) and the nerve cord of *Ligula intestinalis* (Arme, 1966). However, we could not detect any positive reaction at the nerve ring, although the amphids and phasmids were positive in microfilarial stage and amphids were stained red on the 7th and 9th day larvae.

Acknowledgment

Grateful acknowledgment is made to Dr. D. Katamine for his constant interest and guidance in this investigation. Thanks are tendered to Mrs. T. Miyazaki for raising vector mosquitoes.

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Brugia pahangi の酵素組織化学

1. 蚊 (*Aedes aegypti*) 内発育幼虫における酸性フォスファターゼ活性の局在

木村　英作・中島　康雄[1]・青木　克己

イヌ末梢血中ならびにスナネズミ腹腔中のミクロフィラリアと，それらの蚊体内で発育中の幼虫について，酸性フォスファターゼの局在を組織化学的に比較した。ミクロフィラリアは全体が赤く染まり，excretory vesicle と anal vesicle が暗赤色を呈する。イヌのミクロフィラリアでは鞘は陰性であるのに対し，スナネズミのものは大部分が陽性である。イヌを吸血した蚊とは対照的に，スナネズミのミクロフィラリアを摂取した蚊では，最初の2日間に頻回に染色されない幼虫を認める。3日目以降，陽性の発育中の腸管を見る。1回目の脱皮後に，食道は赤色の複線として見える。7日目に，食道，腸管，発育中の肛門，amphid は強い活性を示すが，神経輪は陰性である。感染幼虫の角皮下層と肛門に陽性反応を認める。

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