SIGNIFICANCE OF SOME ENZYMES AND METABOLITES DURING AGEING OF THE DIPTERAN FLESH FLY *SARCOPHAGA RUFICORNIS*

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ABSTRACT

In *S. ruficornis*, aspartate and alanine aminotransferase activity gradually increases during the larval as well as pharate-adult development, suggesting their role in histogenesis preparative to growth of larval structures and in adults, differentiation and formation of tissues. Acid protease activity (pH 5·6) participating in histolysis, decreases during larval development and traces a U-shaped pattern during pupal-adult development. High activity of these enzymes (acid proteases both during initial and final stages of pupal development suggests their participation in histolysis where transformation from larva → pupa → adult takes place.

The initial low activity of phenoloxidases in the young larva increases 10-fold on its maturation, totally disappears in the two day old pupa and after reappearing on the 5th day maintains a low level during pharate-adult development. Tyrosine accumulates gradually during larval growth and gets significantly utilized in the freshly ecdysed pupa (day 1). This suggests the importance of this aromatic amino acid in the process of sclerotization of cuticle.

Total free amino acids accumulate during larval period and subsequently get utilized by the pre-pupa. The metamorphosing pupa also accumulates amino acids till fly emergence. Total keto acids and aminotransferases reveal a parallel trend in their variation. Soluble proteins exhibit a reciprocal relationship to the protease activity in the metamorphosing insect.

INTRODUCTION

TRANSAMINATION is one of the chief mechanisms whereby the balance between amino acid pool and protein biosynthesis is regulated.

Several investigators[1, 2] have reported the participation of alkaline proteases in the digestion of food. However, literature consulted does not reveal much information regarding the existence of acid proteases and their role except for a few studies made during embryogenesis of insects[3, 4].

One of the most important biochemical processes in insects is the sclerotization which involves darkening and hardening of cuticle.

The present study was undertaken with a view to study the relationship between the free amino acid pool, transamination and protein synthesis, free amino acids and keto acids, proteins and proteases, tyrosine and phenoloxidases and their significance during ageing of *S. ruficornis*.

MATERIALS AND METHODS

Larvae of *S. ruficornis* were reared in the laboratory as described by Pant and Kumar[5].

Six hour starved larvae, pupae and adults were randomly selected from colonies of known age, washed with distilled water, air-dried, chilled, weighed and homogenized with ice-cold distilled water to 10% tissue concentration (w/v) in a Potter-Elvehjem type homogenizer which was kept immersed in crushed ice. The homogenate was strained through nylon cloth to remove tissue debris and chitin and centrifuged at 3,000 rpm for 5 min, at 0° C. The supernatant was employed for various enzymic assays and metabolite estimations.

Aspartate aminotransferase (EC 2·6·1·1, L-aspartate 2-oxoglutarate aminotransferase GOT) and alanine aminotransferase (EC 2·6·1·2, L-alanine: 2-oxoglutarate aminotransferase GPT) activities were determined by the method of Reitman and Frankel[6]. The optimum time determined for the GOT system was 60 min. while that for GPT was 30 min. Total free amino acids were estimated by Rosen's method[7] employing glycine as the standard. Total soluble protein was determined by the method of Lowry *et al.*[8] using bovine serum albumin as the standard protein. The homogenate was deproteinized with trichloroacetic acid (10%, w/v), refrigerated for 10 min and centrifuged. The residue after washing 2–3 times with trichloroacetic acid (5%, w/v) was dissolved in 0·1 N sodium hydroxide and used for protein determination.

Acid protease activity (pH 5·6) was assayed according to Pant and Morris[9] using casein as the substrate. Total keto acids were determined by the method of Friedemann and Haugen[10] employing pyruvic acid as the standard keto acid.

Phenoloxidase activity (EC 1·10·3·1, *o*-diphenol : oxygen oxidoreductase also referred as tyrosinase, catecholoxidase, phenolase, polyphenoloxidase) was assayed by Evan's method[11] using DL–3, 4 dihydroxy phenylalanine as the substrate.

For the estimation of tyrosine, the original tissue homogenate (3 ml, 10%, w/v) was repeatedly extracted with absolute ethanol (7 ml), till the supernatant after centrifugation was negative to ninhydrin test. The combined extract was employed for paper partition chromatography using *n*-butanol–acetic acid—water (12 : 3 : 5, v/v/v) as irrigating solvents. The position of tyrosine was located on the chromatogram by nitrosonaphthol reagent[12] and estimated according to Lee and Takahashi's method[13].

All assays were carried out in triplicates in three sets of experiments employing 10 insects in each lot at various developmental stages and the average values were employed for plotting the graphs.

Results and Discussion

During development of *S. ruficornis* both aspartate and alanine aminotransferase activity run parallel to one another, the former being more active than the latter. The activity increases in larva, declines in the pre-pupa and becomes maximally active before adult emergence (Fig. 1). Total keto acids follow a similar trend as the aminotransferases (Fig. 2). Previous findings in the lepidopteran *Philosomia ricini* revealed that while fat body and haemolymph both exhibited higher aspartate aminotransferase activity than alanine aminotransferase all through larval-pupal development, in the intestine the activity of these enzymes was in the reverse order[14]. Further, the activities of these enzymes also appeared to parallel the rate of protein synthesis in the fat body and haemolymph during larval development. These observations lend support to McAllen's findings that increase in the protein anabolic rate is parallel to the increased rate of glutamate-aspartate transformations in intestine, fat body and silk glands of silkworms[15]. However, in the present investigation the aminotransferases and soluble protein level do not record any relationship all through larval-pupal development. This could be explained by the fact that during larval growth and pharate-adult development, the high aminotransferase activity involves the synthesis of proteins which subsequent to their participation in the formation and differentiation of larval and adult structures get transformed into insoluble structural proteins. The observed gradual accumulation of insoluble proteins during larval development substantiates the above statement (under publication). This leads one to conclude that during histogenesis (growth and differentiation), the aminotransferases are highly active while during histolysis it is the reverse.

Further, the low activity of the aminotransferases during early pupal development in *S. ruficornis* as also observed in other insects[14,16] was probably due to an appreciable diversion of citric acid cycle substrates. This speculation is based on the observed decline in citrate concentration during the above periods[17]

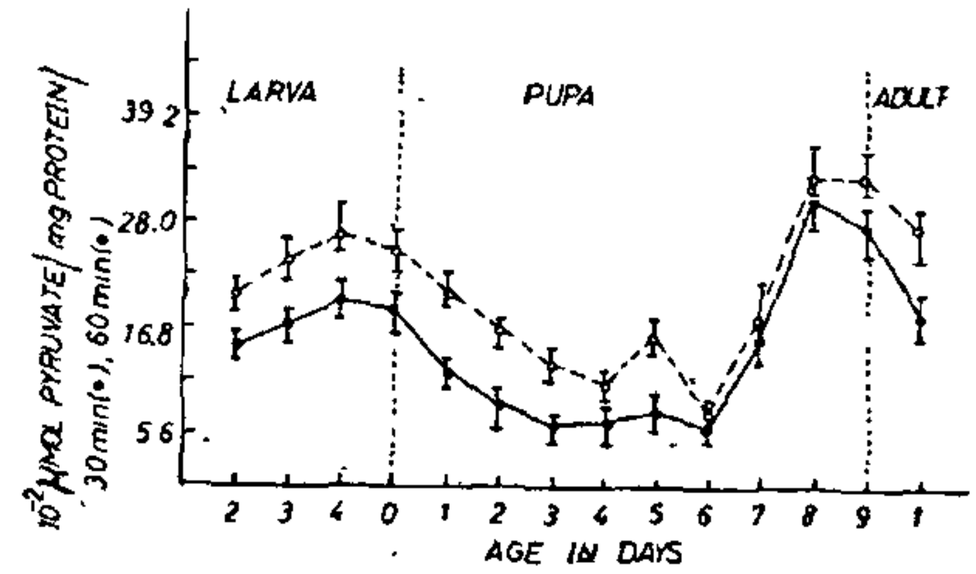

Fig. 1. Age-dependent changes in aspartate (○) and alanine (●) aminotransferase activity during development of *S. ruficornis*.

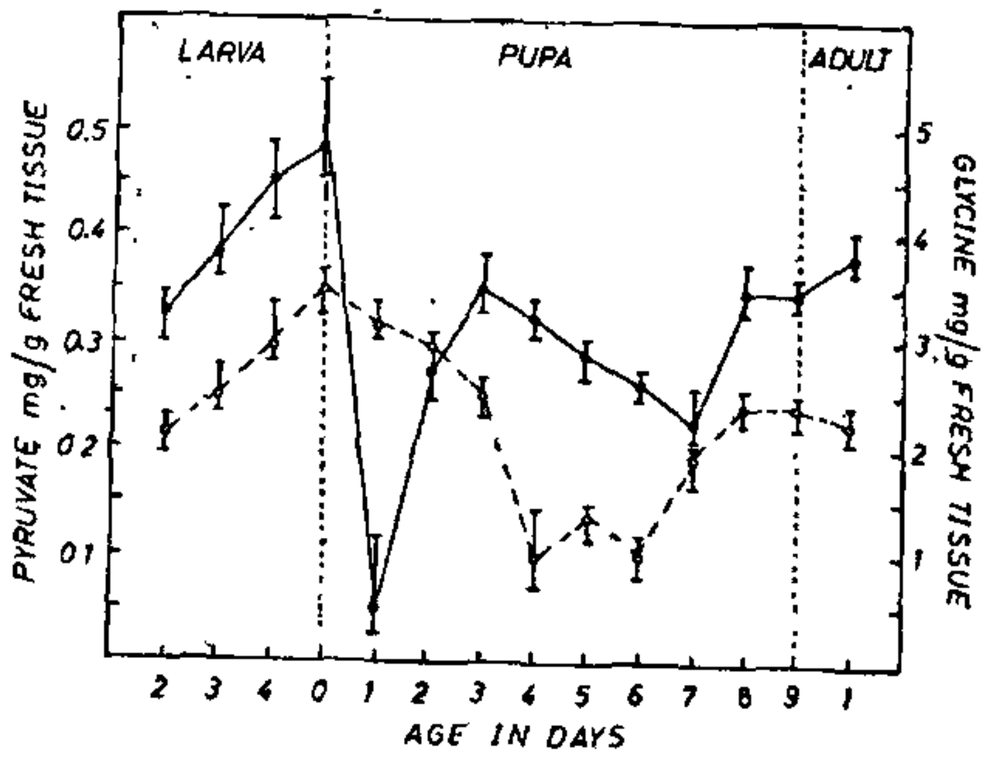

Fig. 2. Age-dependent changes in total free amino acids as "glycine equivalents" (●) and total keto acids as "pyruvate equivalents" (○) during development of *S. ruficornis*.

Total free amino acids increase during larval growth, significantly decline at the onset of pupation (day 0–1) and reveal a gradual increasing trend with intermittent fluctuations all through metamorphosis till adult emergence (Fig. 2). Similar observations were also made on other two dipteran flies *Musca nebula*[18] and *Musca domestica*[19]. The increase in free amino acids during larval development suggests the degradation of the ingested dietary proteins and their further utilization for the formation of larval structures. At the onset of pupation the significant depletion in free amino acids indicates their involvement in the synthesis of cuticular proteins for the formation of puparium. The subsequent gradual accumulation in free amino acids with intermittent fluctuations all through metamorphosis till fly emergence is suggestive of the gradual degradation of stored proteins into amino acids which eventually get involved in the formation of adult tissues. Besides this, amino acids have been reported to participate in energy meta-

bolism during metamorphosis of some insects[10,11]. However, in *S. ruficornis* this is yet to be established.

Total soluble proteins in *S. ruficornis* decline during larval growth whereafter they increase till day 4 in the metamorphosing pupa. Subsequently, during pharate-adult development a marked utilization was apparent in the soluble protein content till fly emergence (Fig. 3). During metamorphosis of several insects it has been reported that the conversion of soluble proteins into insoluble ones and the reverse process occurred respectively during histogenesis and histolysis[22, 23]. The observed decrease in the soluble proteins till day 4 of larval growth is probably indicative of its conversion into insoluble proteins during this period. However, the gradual increase in soluble protein content during early pupal development (days 0–4) appears to be directly associated with the conversion of insoluble larval cuticular and other tissue proteins into soluble ones suggesting histolysis while the decrease in the soluble protein during pharate-adult development (days 5–9) probably evolves histogenesis of adult tissues. The reciprocal relationship of the soluble and insoluble proteins during development of this insect lends support to the above view (under publication).

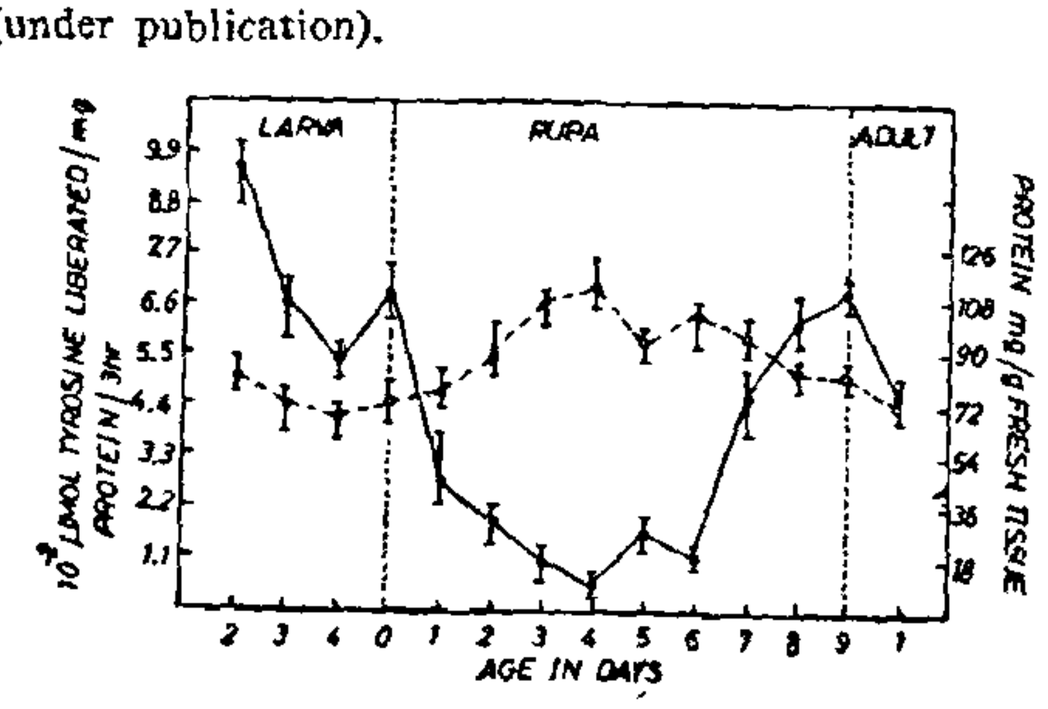

FIG. 3. Age-dependent changes in acid protease activity (●) and soluble proteins (○) during development of *S. ruficornis*.

In *S. ruficornis* during larval development, there is a gradual decrease in acid proteases till larval maturation on day 4 (Fig. 3). These results are in conformity with those obtained for *Philosamia ricini*[9]. Histolytic phenomena in anuran metamorphosis like tail regression, lysis of internal gills and skin, degeneration of intestinal mucosa cells and resorption of anal canal, etc., have been shown to be accompanied with manifold increase in the acid hydrolases, *viz.*, acid phosphatase, β-glucuronidase, ribonuclease, acid proteases and cathepsin[24–26]. The peak activities of all these hydrolases are marked by maximum histolysis. Thus the gradual decrease in the protease activity observed during larval growth reflects the low rate of histolysis occurring during this period.

Likewise, during metamorphosis the acid protease activity (Fig. 3) with a slight increase at the onset of larval-pupal transformation shows a somewhat U-shaped pattern indicating that during initial and final stages of metamorphosis high protease activity corresponds to the rate of histolysis of larval structures for reorganization of pupal tissues. The same phenomenon recurs during pupal-adult transformation.

In the developing larva phenoloxidase activity increases gradually and records the maximum activity during larval-pupal transformation (day 0). In the two day old pupa the enzyme hardly exhibits any activity and thereafter totally disappears in the five day old pupa. During pharate-adult development the enzyme once again reappears and then onwards maintains more or less a steady activity till adult emergence (Fig. 4). Free tyrosine (Fig. 4), on the other hand, exhibits a similar trend of variation as that of phenoloxidases except during pharate-adult development when it reveals a gradual increasing trend.

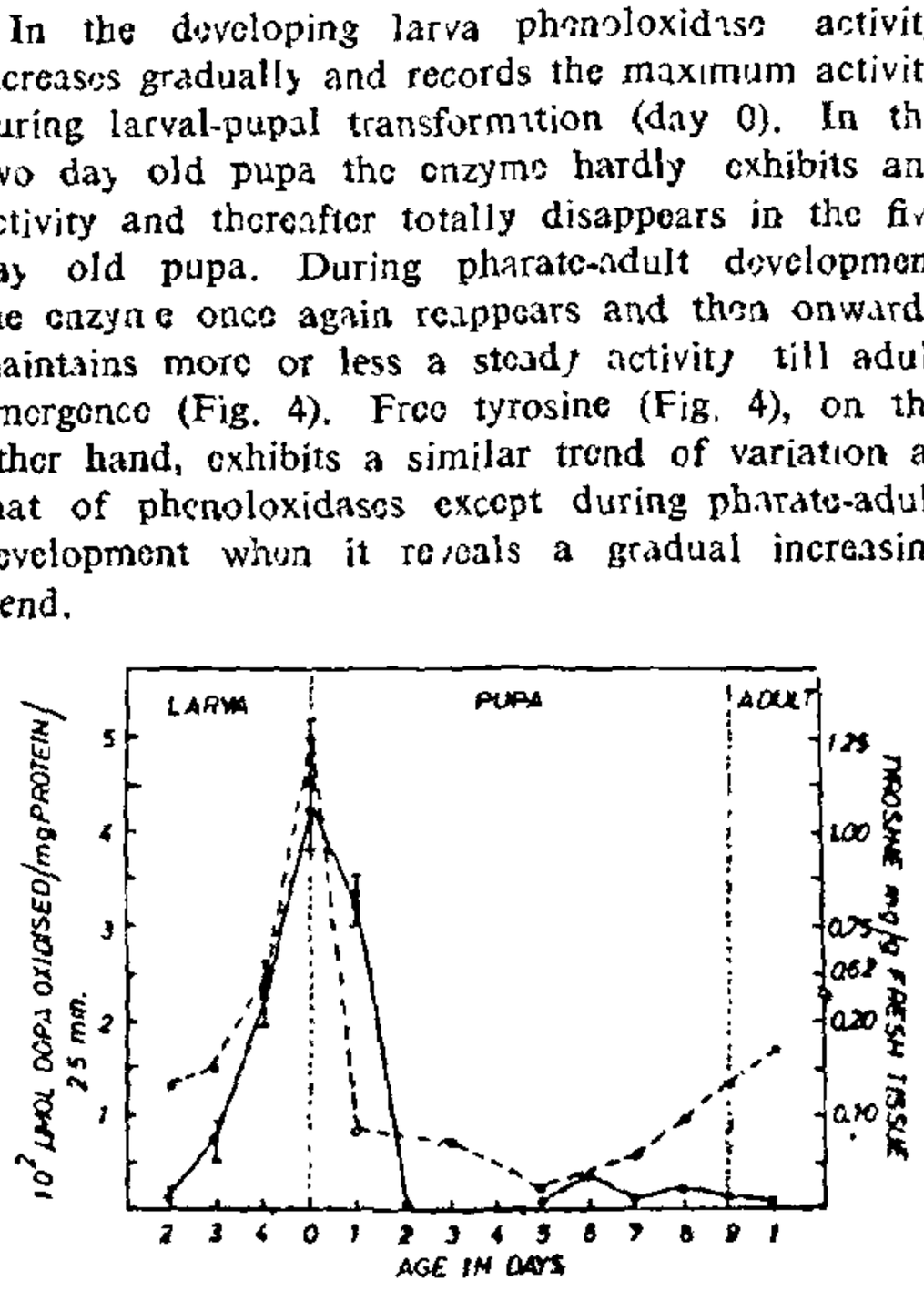

FIG. 4. Age-dependent changes in free tyrosine (○) and phenoloxidase activity (●) during development of *S. ruficornis*.

Despite the fact that both the enzymes (phenoloxidases) and the substrate (tyrosine) occur together in insect tissues, the enzymes become active only under certain conditions (sites of an injured integument, moulting fluid or haemolymph and in the newly secreted cuticle during tanning). The inactive state of phenoloxidases has been attributed to inhibition of the enzyme or to the segregation thereof from its substrate[27–29]. However, the evidence now available favours the occurrence of the enzymes as the inactive precursors, prophenoloxidases[30]. During larval growth, tyrosine level increases in tune with the activity of phenoloxidases throughout development. This suggests that either substrate and enzyme or the enzyme and its activators are localized in different places or the enzyme is present as an inactive precursor (prophenoloxidase) *in vivo* which gets activated *in vitro* during homogenization due to integumentary injury

or by contact with its activators. This means that during growth of the larva the enzyme proteins of phenoloxidases accumulate in the form of inactive precursors.

During larval-pupal transformation (white pupa) phenoloxidases and tyrosine record maximally. Subsequently, when the puparium becomes brown on day 1 of the metamorphosing pupa depletion by 25% in the former and 94% in the latter occurs. This suggests that the substrate enzyme reaction occurs only on day 1 and results in the catabolism of tyrosine into its metabolic products which subsequently participate in the hardening and darkening of the pupal cuticle.

The other aromatic amino acid phenylalanine does not reveal significant changes during the above period[31]. Karlson and Schlossberger-Raecke[32] reported that *Locusta* can incorporate injected labelled tyrosine and DOPA (dihydroxy phenylalanine) into their cuticle and N-acetyl dopamine is presumably an intermediate in the process of sclerotization[33]. These findings as also those in the present investigation suggest that tyrosine is the only major aromatic amino acid which is necessary for the sclerotization process to produce quinones for cross-linking and tanning of cuticular proteins[34]. Several other reports[35–37] also further confirm that free tyrosine concentration in insects increases prior to puparium formation and decreases rapidly after ecdysis.

According to Karlson and Sekeris[38] tyrosine metabolism is regulated by ecdysone which induces DOPA decarboxylase causing the metabolic shift towards the formation of N-acetyl dopamine. In view of this, it is likely that during larval growth high level of Juvenile Hormone (JH) inhibits phenoloxidases and on puparium formation the lowered JH level accompanied with simultaneous high ecdysone concentration facilitates this process causing thereby a significant depletion in tyrosine level (Fig. 4).

During pharate-adult development the phenoloxidase activity reappears and maintains a constant lavel till fly emergence. This could perhaps be ascribed to its involvement in the pigmentation required for the formation of adult structures such as cuticle, wings, etc. The gradual increase in tyrosine concentration during this period can well be explained by the high proteolytic activity and subsequent degradation of proteins and peptides resulting in the release of this aromatic amino acid.

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