Blue biliprotein as an effective factor for cryptic colouration in *Rhodinia fugax* larvae

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Abstract

The fifth instar larva of the saturniid silkworm, *Rhodinia fugax*, is light yellowish-green on its dorsal surface and dark green on the ventral surface with a lateral demarcation between the two colours. The larva of *R. fugax* closely resembles the leaves of the host plant, *Quercus serrata*, in colour and shape. The spectral reflectance of the larval integument of *R. fugax* corresponds to that of the *Q. serrata* leaf. In the larval integument, there is more blue biliproteins (BPs) on the ventral surface than on the dorsal surface. Light intensity influences larval colouration. The larval integuments are green under light conditions (1000 lux), whereas larvae kept in dark conditions (10 lux) turn yellow. The BP-I content of the haemolymph is also affected by light intensity. The quantities of BP-I and its blue chromophore are higher under light conditions than under dark conditions. In contrast, there is little difference in the yellow chromophore content between the two light intensities. When larvae are kept in the light, the BP-I content in the cuticle is higher than under dark conditions in both the ventral and dorsal surfaces, and its chromophore content parallels the BP content. However, the amounts of BP-II and its chromophore in the epidermis show no change with the light intensity. Moreover, the quantity of yellow chromophore in the integument is also not affected by light intensity. Therefore, light stimulates the accumulation of BP-I and its chromophore in the haemolymph and cuticle, whereas the accumulation of BP-II and its chromophore in the epidermis are not influenced by light intensity. These results suggest that BPs and their chromophores determine the larval colouration and may play an important role in the cryptic colouration of *R. fugax* larvae. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

The caterpillars of phytophagous insects are frequently green, and both the haemolymph and integument are often green. The green colouration presumably serves as a protective camouflage (i.e. crypsis), helping caterpillars to avoid predation. The colour results from a combination of blue (e.g. bilins: biliverdin IXβ, phorcabilin and sarpedobilin) and yellow (e.g. carotenoids: β-carotene and lutein) pigments that are intimately associated with proteins (Kawooya et al., 1985; Kayser, 1985; Law and Wells, 1989).

The blue biliprotein insecticyanin (Ins) has been isolated from the haemolymph of the tobacco hornworm, *Manduca sexta* and characterised (Cherbas, 1973; Riley et al., 1984; Goodman et al., 1985; Holden et al., 1986, 1987). Ins is synthesised and stored in pigment granules in the epidermis and secreted into both the haemolymph and the cuticle (Riddiford, 1982; Riddiford et al., 1990). The bilin binding protein of *Pieris brassicae* has been crystallised (Huber et al., 1987a,b) and its complete amino acid sequence has been determined (Suter et al., 1988). Blue chromoproteins have also been isolated and characterised from the larval haemolymph of several lepidopteran insects: *Heliothis zea* (Hauenerland and Bowers, 1986), *Trichoplusia ni* (Jones et al., 1988), *Spodoptera litura* (Yoshiga and Tojo, 1995), *Agrius convolvuli* (Saito and Shimoda, 1997), *Attacus atlas* (Saito, 1997) and *Antheraea yamamai* (Yamada and Kato 1989, 1991; Saito et al., 1998). In *Samia cynthia ricini*, a biliverdin-binding protein (BBP) found in the moulting fluid during the larval–pupal ecdysis has been purified and character-
ised (Saito, 1993), and the N-terminal amino acid sequence of BBP has also been determined (Saito, 1994). Recently, two BBPs were found in the larval haemolymph of S. cynthia ricini (Saito, 1998a).

In *Rhodinia fugax*, blue biliproteins (BPs) are found in the haemolymph and integument of fifth instar larvae (Saito, 1998b). The BP molecules from the haemolymph and cuticle are assumed to be monomers, whereas the epidermal BP is a dimer. The amino acid composition and N-terminal amino acid sequences of the BPs from the haemolymph and cuticle (BP-I) are very similar, but the BP from the epidermis (BP-II) is quite different. *R. fugax* BPs consist of two different molecules. The blue colour of BP is due to the presence of bile pigments, which are non-covalently bound to an apoprotein. The blue pigments of BP-I and BP-II are different; BP-I contains a phorcabilin-like pigment, while BP-II contains biliverdin IXα. Furthermore, light stimulates the accumulation of bile pigments in the integument and cocoon, affecting the green colouration (Kato and Miyata, 1994).

In order to obtain information about the possible function of BPs in the larval colouration of *R. fugax*, (1) the localisation of BPs in the integument and the relationship between BPs and larval colouration, and (2) the influence of light intensity on larval colouration, were examined.

2. Materials and methods

2.1. Animals and food plants

Eggs of *R. fugax* were obtained from wild-collected female moths in Fukushima Prefecture. Larvae were reared mainly on fresh oak leaves, *Quercus serrata*, at 25°C under a natural photoperiod. The food plants were branches and leaves harvested from field grown trees. The branches were kept in tightly plugged vials of water. Fifth-instar larvae (5–7 days after last larval ecdysis) were used in the experiments. Animals were selected and their age determined according to live weight.

2.2. Light irradiation

After the last larval ecdysis, some of the larvae were illuminated with light at one of two different intensities [light (1000 lux) and dark (10 lux) conditions] under a photoperiod of 12 h light–12 h dark. White fluorescent tubes (Toshiba, FLR40S•W/M/36) were used as the light source, and light intensity was measured at the centre of the rearing room (2.4×2.4×2.8 m).

2.3. Collection of haemolymph, epidermis and cuticle, and preparation of samples

Haemolymph was collected from fifth-instar larvae from incisions made by cutting off the abdominal pro-

legs. The haemolymph was allowed to drip into ice-cooled plastic micro-centrifuge tubes containing a few crystals of 1-phenyl-2-thiourea, and then centrifuged at 10,000g for 10 min at 4°C to remove the haemocytes. The supernatant was used.

After collecting the haemolymph, the larvae were dissected and the midgut, silk glands, fat bodies, and other undesired tissues were removed with fine forceps. The epidermis was collected with a spatula, and the cuticle was washed with cold distilled water several times. Both tissues were immediately transferred to plastic micro-centrifuge tubes that were chilled on a block of dry ice. The isolated epidermis and cuticle were stored at −20°C until used.

Epidermis or cuticle (50 mg) was homogenised with 0.5 ml of 20 mM Tris–HCl buffer (pH 7.6) containing protease inhibitors (0.1 mM phenylmethyl sulfonyl fluoride and 5 μg/ml soybean trypsin inhibitor) with 0.15 M NaCl. The precipitate was removed after centrifugation at 10,000g for 20 min at 4°C, and the supernatant was used as the sample. After 20 mM Tris–HCl (pH 7.6) extraction, the precipitate was extracted with HCl:MeOH (5:95, v/v) solution.

2.4. Electrophoresis

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed on a 15% polyacrylamide gel containing 0.1% SDS (Laemmli, 1970). Electrophoresis was carried out at 30 mA until the bromophenol blue tracking dye reached the bottom of the gel. The gel was stained with Coomassie Brilliant Blue R-250 (Fluka Chemie AG, Buchs). SDS–PAGE molecular weight standards (Low Range) were purchased from Bio-Rad Laboratories.

2.5. Determination of BP concentration

Each sample was separated on a 15% linear SDS–PAGE. The gel was stained with Coomassie Brilliant Blue G-250 (Fluka Chemie AG, Buchs) and the BP band was scanned. The BP content was determined by NIH Image (ver. 1.61). It was based on densitometry of the BP band and compared to a bovine serum albumin (BSA) band on the same gel. BSA was used as the standard protein.

2.6. Absorbance spectrum

The absorbance spectra of haemolymph and the extracts from the epidermis and cuticle were measured with a spectrophotometer (Beckman DU-650, USA).

2.7. Spectral reflectance

The spectral reflectance of the larval integument of *R. fugax* and *Q. serrata* leaves were measured with a
spectrophotometer (Shimadzu MPC-3100, Kyoto) using barium sulphate (Eastman Kodak Company, Rochester, NY) as a white reflectance standard.

2.8. Statistics

Numerical data were expressed as the mean±SE. For statistical analysis, Student’s t-test was used to compare results for the two light conditions.

3. Results

3.1. Larval colouration of R. fugax

Fifth-instar larvae of *R. fugax* always hang from a branch [Fig. 1(A)]. The larvae are uniquely coloured in two tones separated laterally. The dorsal surface is light yellowish green, whereas the ventral surface is dark green [Fig. 1(B)]. In addition, the body segments closely resemble indented leaf margins. The oak *Q. serrata* is one of the host plants of *R. fugax* [Fig. 1(C)]. The adaxial surface of its leaves are dark green, whereas the abaxial surface are light green [Fig. 1(D)]. Thus, the larvae of *R. fugax* resemble the leaves of the host plant of *Q. serrata* in both colour and shape.

3.2. Spectral reflectance of the larval integument and the leaf of the host plant

Fig. 2 shows the spectral reflectance of the larval integument of *R. fugax* and the leaf of its host plant, *Q. serrata*. In the integument, the reflectance of the ventral surface was lower than that of the dorsal surface [Fig. 2(A)]. Both spectral reflectances had a small peak from 540 to 669 nm. Similarly, in the leaf of the host plant, the reflectance of the adaxial surface was lower than that of the abaxial surface and the spectral reflectance was similar to that of the larval integument [Fig. 2(B)]. These results suggest that the spectral reflectances of the *R. fugax* integument correspond with those of the *Q. serrata* leaf.

3.3. Localisation of BP in the integument

Fig. 3 shows the localisation of BP in the epidermis and cuticle by SDS–PAGE. Large amounts of BPs were detected in the ventral epidermis (BP-II) and cuticle (BP-
I), while BP-I was not found in the dorsal epidermis (lane 4, Fig. 3).

The BP contents of the epidermis and cuticle were compared on the ventral and dorsal surfaces (Fig. 4). The BP-I content of the ventral and dorsal cuticle was 1.40±0.09 and 0.98±0.09 μg/mg tissue weight, respectively. In the epidermis, the BP-II content in the ventral surface was 1.03±0.03 μg/mg tissue weight, whereas little was found on the dorsal surface (0.02±0.02 μg/mg tissue weight). The total amount of BPs (BP-I+II) on the ventral surface was larger than on the ventral surface. Therefore, the difference of larval body colour between the ventral and dorsal surfaces may result from the amount of BP in the integument.

### 3.4. Effects of light intensity on larval body colour

The effects of light intensity on the larval body colour of *R. fugax* were examined under light and dark conditions (Fig. 5). When the larvae were reared under light conditions, the dorsal body was light yellowish-green and the ventral surface was dark green (upper, Fig. 5). In contrast, the dorsal body colour yellowed under dark conditions (lower, Fig. 5).

### 3.5. Effects of light intensity on the quantities of BP and chromophores in the haemolymph

The BP-I content in the haemolymph was compared at two different light intensities [Fig. 6(A)]. The BP-I content under light and dark conditions was 73.4±3.3 and 43.1±1.1 μg/ml, respectively. The amount of blue
Fig. 5. Effects of light intensity on the larval body colour of *R. fugax*. Light (upper) and dark (lower) conditions.

The blue chromophore was measured as the absorbance maximum at 669 nm. The absorbance under light and dark conditions was 1.63±0.05 and 1.04±0.14, respectively [Fig. 6(B)]. For the yellow chromophore, the absorbance spectrum of haemolymph was characterised by three absorbance maxima at 428, 453, and 483 nm. The absorbance of the middle peak (453 nm) under light and dark conditions was 2.64±0.20 and 2.18±0.22, respectively [Fig. 6(C)].

3.6. Effects of light intensity on the quantities of BP and chromophores in the integument

When the larvae were kept in light conditions, the BP-I content in the cuticle was higher in both the ventral and dorsal surfaces than in larvae kept in dark conditions [Fig. 7(A)]. At each light intensity, the amount of BP-I was roughly the same in both surfaces of the cuticle. In the epidermis, BP-II was found mainly in the ventral surface, and the quantity was the same for both light and dark conditions [Fig. 7(A)]. The blue chromophore (absorbance at 669 nm) in the cuticle was soluble in 20 mM Tris–HCl (pH 7.6) buffer, and there was more in light conditions than in dark conditions in both the ventral and dorsal surfaces [Fig. 7(B)]. In the epidermis, the blue chromophore (absorbance at 669 nm) was only detected in the ventral surface, and it was more soluble in HCl:MeOH (5:95, v/v) than in 20 mM Tris–HCl (pH 7.6) buffer [Fig. 7(C)]. The total amount of blue chromophore in both solutions was essentially the same at the two light intensities. These results suggest that light stimulates the accumulation of BP-I and its chromophore in the cuticle, whereas the light intensity does not influence the accumulation of BP-II and its chromophore in the epidermis.

Conversely, in the cuticle, the yellow chromophore (absorbance at 462 nm) was only extracted into 20 mM Tris–HCl (pH 7.6) buffer, and its content was not affected by the light intensity [Fig. 8(A)]. In contrast, in the epidermis, the yellow chromophore (absorbance at 442 nm) was found only in the ventral surface and was extracted by HCl:MeOH (5:95, v/v) alone [Fig. 8(B)]. There was no significant difference between light and dark conditions in the accumulation of yellow chromophore in either tissue.

4. Discussion

4.1. Cryptic colouration

The caterpillar is the eating and growth stage in the life cycle of a moth or butterfly. Survival in an environment filled with predators is difficult for a caterpillar. Predators include social vespid wasps, parasitic wasps, ants, spiders, and birds, and they cause immense damage to caterpillar populations, with larval loss of 70–90%. Birds and parasitic wasps, which are the major predators, can see colour. They have well-developed visual acuity and hunt by sight. As a countermeasure against predators, caterpillars adopt several survival strategies, such as cryptic form and colouration, mimicry, and aposematic colouration (Schmidt, 1990).

The later instar larvae of many groups of Saturniidae are usually reverse countershaded; that is, the dorsal surface, which faces downward in the resting position, is
light than the ventral surface. These larvae closely match the colour of the under surface of their host plant’s leaves. The convex body segments of some genera (e.g. Antheraea and Actias) closely resemble indented leaf margins and compound leaves (Tuskes et al., 1996).

In *R. fugax*, the cryptic colour and shape polymorphism of caterpillars appear to have evolved as strategies to make the caterpillars appear similar to their host plants. The larvae usually hang from a branch. The body segments of the penultimate and final instar larvae closely resemble the indented leaf margins of the host plant. The spectral reflectance of the larval integument of *R. fugax* corresponds with that of the *Q. serrata* leaf (Fig.

4.2. Blue BPs as an effective factor in camouflaging caterpillars

In caterpillars, green pigments are observed in the layer of epidermal cells on the surface of the body, where they play an important role in determining the visibility of caterpillars on their host plants. Blue bile pigments, which give a bright green or blue colour, are frequently found in the haemolymph and integument. The bile pigments are usually associated with proteins such as BPs. It is generally believed that the major function of blue BP is to provide camouflage for protecting the developing larvae (Kawooya et al., 1985; Law and Wells, 1989).

In *R. fugax*, BPs have been efficiently isolated from the haemolymph, epidermis, and cuticle of fifth instar larvae (Saito, 1998b). There are usually two different molecules: BP-I from the haemolymph and cuticle and BP-II from the epidermis. It is very interesting that the phorcabilin-type pigment is distributed only in the haemolymph and cuticle, whereas the biliverdin IXα is found only in the epidermis. BP-I in the haemolymph does not influence the larval body colour, so it probably
does not function as camouflage there. It is likely that BP-I is synthesised in the haemolymph, perhaps in haemocytes, and secreted across the epidermis into the cuticle. It is necessary to determine whether this actually occurs. In Calpodes ethlius, the epidermis is involved with four routing classes of protein (Sass et al., 1993). It secretes peptides apically into the cuticle, basal into the haemolymph, bidirectional, or transported to the cuticle across the epidermis from the haemolymph (Locke et al., 1994; Sass et al., 1993). A 66 kDa peptide is found abundantly in cuticle and haemolymph but is made by spherulocytes and not by the epidermis (Sass et al., 1994).

In this study, the amount of BPs (BP-I+II) in the ventral integument (epidermis and cuticle) was higher than in the dorsal surface (Fig. 4). The quantity of BP-II in the dorsal epidermis was extremely low. The difference in the larval colouration on the two surfaces results from the amount of BPs in each surface of the integument. These results suggest that BPs may play an effective role in the cryptic colouration of R. fugax larvae.

4.3. Effects of light intensity on larval colouration

Three types of bilins (biliverdin IX\(\gamma\), phorcabilin, and sarpedobilin) have been reported in many lepidopteran insects (Barbier, 1981; Kayser, 1985). Phorcabilin and sarpedobilin are obtained by irradiation of biliverdin IX\(\gamma\) with visible light in vitro (Barbier, 1981). However, the process by which biliverdin IX\(\gamma\) is transformed into phorcabilin and sarpedobilin in vivo is unknown. Moreover, the biological significance of the transformation of biliverdin IX\(\gamma\) into phorcabilin and sarpedobilin is still unclear.

Colour changes and colour polymorphism modulated by environmental factors, such as light, temperature, and humidity, have been found in many insects (Fuzeau-Braesch, 1985). In the Japanese oak silkworm, Antheraea yamamai, the larval haemolymph and cocoon are green, due to the presence of blue bilin and yellow pigment. The green colouration of the cocoon is dependent on the intensity of irradiation, whereas the larval haemolymph and integument are green irrespective of the light conditions (Kato et al., 1989). In spectrophotometrical analyses, the bilins of the larval haemolymph and integument differed from those of the cocoon (silk glands) and larval head capsule (Kato and Yamada, 1991). The phototransformation of haemolymph bilin and its incorporation into the silk glands may be involved in the light-stimulated green colouration. Thus, light irradiation of the haemolymph effectively produces green colouration (Kato, 1991). In NMR analyses, the chemical structure of bilin from the cocoon was similar to that of sarpedobilin (Yamada and Kato, 1994), which was isolated from Graphium sarpedon wings (Bois-Choussy and Barbier, 1977). Moreover, similar light-induced green colouration occurs in the larval integument and cocoon of R. fugax (Kato and Miyata, 1994).

Light-induced green colouration was found in the larval haemolymph and integument of R. fugax (Figs. 6, 7). The respective light/dark ratios of the BP-I and blue chromophore (absorbance at 669 nm) contents in the haemolymph were 1.7 and 1.6 [Fig. 6(A, B)]. It seems likely that the amount of blue chromophore in the haemolymph changes in proportion to the change in the BP-I content with light irradiation. This phenomenon is also detected in the cuticle [Fig. 7(A, B)]. The light intensity probably influences the syntheses of BP-I and phorcabilin-type pigment. These results suggest that light stimulates the accumulation of BP-I and its blue chromophore in the haemolymph and cuticle. However, it is an open question whether the light-induced green colouration in this species comes from the synthesis of phorcabilin-type pigment in the haemolymph and cuticle by phototransformation. In contrast to BP-I and its chromophore, BP-II and its blue chromophore (biliverdin IX\(\gamma\)) in the epidermis are not influenced by light intensity. Furthermore, the yellow component of R. fugax integument is a carotenoid (Kato and Miyata, 1994). Light intensity has little effect on the accumulation of carotenoids in the R. fugax integument. Similarly, no change in yellow pigment content was observed in the accumulation of yellow pigment in A. yamamai cocoons, although the yellow pigment is not a carotenoid (Kato et al., 1989). Phytophagous insects generally do not synthesise carotenoids that come from their food plants (Feltwell and Rothschild, 1974; Kayser, 1985). Therefore, it is possible that the accumulation of carotenoids in a tissue is dependent on the food irrespective of the light conditions. Consequently, the phorcabilin-type pigment with its binding protein (BP-I) plays an important role in determining the shade of the green colouration of R. fugax larvae.

The R. fugax larva is a good model for studying the biological significance of bilin transformations. Further experiments will be necessary to elucidate the mechanics of bilin transformations, such as whether light stimulates the synthesis or accumulation of bilins.

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References
