Perturbation of the Wing Color Pattern of a Swallowtail Butterfly, Papilio xuthus, Induced by Acid Carboxypeptidase

Authors: Umebachi, Yoshishige, and Osanai, Minoru

Source: Zoological Science, 20(3) : 325-331

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.20.325

ZOOLOGICAL SCIENCE **20**: 325–331 (2003)

Perturbation of the Wing Color Pattern of a Swallowtail Butterfly, *Papilio xuthus*, Induced by Acid Carboxypeptidase

Yoshishige Umebachi[1†*] and Minoru Osanai[2‡]

Department of Biology, Faculty of Science, Kanazawa University, Kanazawa, Japan

ABSTRACT—Hypodermal injection of Toughmac-E, a digestive mixture composed of nine digestive components, or Molsin induced perturbation of the wing color pattern in 0- to 2-day-old pupae of *Papilio xuthus*, but had no effect on prepupae or 3- to 4-day-old pupae. The effective component in Toughmac-E was identified as Molsin, an acid carboxypeptidase of *Aspergillus saitoi* which specifically liberates tyrosine and phenylalanine from the C-terminal residues of proteins. The pattern perturbation occurred in either side of the fore- and hindwings of both sexes. When this enzyme was administered, stronger melanization than in the normal wings was found in the whole wings of most butterflies, but in other butterflies, the yellow region was enlarged. These findings suggest that the pattern perturbation was caused by changes in the levels of melanin and papiliochromes in scales. Melanin is a black pigment and papiliochromes are yellow pigments; their common precursor is dopamine. The normal pattern is formed by a predetermined balance of melanin and papiliochromes, whereas the deposit of an excess amount of tyrosine and/or phenylalanine disturbs this balance and results in perturbation of the color pattern. Administration of L-dopa or dopamine had no effect on the wing pattern. When the activity of an endogenous acid carboxypeptidase similar to Molsin appears in the early pupa, the summed activities of the endogenous and exogenous acid carboxypeptidases must induce a pattern perturbation. The relations between the endogenous acid carboxypeptidase and its probable substrate, the reserve protein, and the physiological roles of these relations in the regulation, utilization and excretion of amino acids are discussed.

Key words: wing-color-pattern perturbation, melanin, papiliochrome, acid carboxypeptidase, *Papilio xuthus*

INTRODUCTION

Being a matter of great interest from the standpoints of developmental biology, genetics and evolution, the wing color patterns of butterflies have been investigated by many researchers. The patterns have been examined and compared in many kinds of species and also in various kinds of geographical, genetical and seasonal variations. In addition, perturbation experiments such as micro-cautery, micro-excision, and low or high temperature treatments have been made, as reviewed in detail by Nijhout (1991). More recently, new experimental techniques have been used to estimate the enzyme activities in presumptive pattern regions (Koch *et al*., 1998, 2000) and to analyze the appearance of wing patterns using molecular biological methods (Carroll *et al*., 1994; Beldade *et al*., 2002).

Various pigments in the wing of butterflies have also been studied (Needham, 1978; Umebachi, 2000). In this way, melanin, pterins, and ommochromes were found in the wings and identified. Umebachi (1985) isolated papiliochromes, new yellow pigments from the wings of a swallowtail butterfly, *Papilio xuthus*. One of them, papiliochrome II, was characterized and proved to be a combination of N-β-alanyldopamine and L-kynurenine.

The present paper is the first to show that external administration of an acid carboxypeptidase to early pupae produces various blackened or yellow wing-types without any modification to the shape or veins of wings. The occurrence and type of such perturbed wing color patterns depend upon the developmental stage of the treated pupae. These results should thus help to elucidate the mechanism of color pattern formation.

MATERIALS AND METHODS

Animals

Papilio xuthus is a butterfly popular throughout Japan. Eggs or 1st to 5th instar larvae were collected from the field and reared with

* Corresponding author: Tel. +81-76-242-8428;
FAX.+81-76-242-8428.
[1†] Present address: Izumino-machi 2-12-24, Kanazawa, 921-8034, Japan
[2‡] Present address: Sakuradai, 3-35-13-407, Nerima-ku, Tokyo, 176-0002, Japan

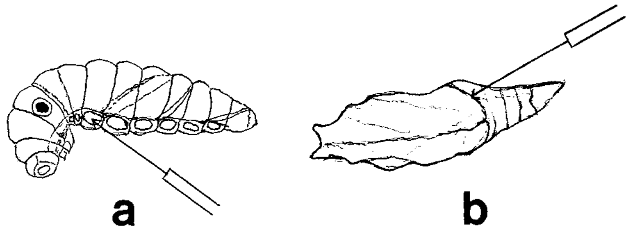

Fig. 1. Injection site of prepupa (a) or pupa (b).

leaves of *Poncirus trifoliata* (Rutaceae) under natural temperature, light and day length from May to September. Under these conditions the prepupal stage was one day long, and the pupal stage was 9–12 days. The emerged adult butterflies were all in the summer form.

Substances administered

Two commercial digestive mixtures of enzymes were used: Toughmac-E (Ono Pharmaceutical Co., Ltd., Osaka, Japan) and Inosea (Sato Pharmaceutical Co., Ltd., Tokyo), and an acid-peptidase of *Aspergillus saitoi*, Molsin (full name, Aspergillopeptidase Molsin) (Sigma Chemical Co., St. Louis, MO). Two substrates for melanin formation, L-dopa (Wako Pure Chemical Industries, Ltd., Tokyo) and dopamine • HCl (Sigma Chemical Co.) were also used.

Injection

After Toughmac-E and Molsin were solubilized to water in concentrations of 4 mg per ml and 1.5-3 mg per ml, respectively, one of them was administered by hypodermal injection to one prepupa or pupa. Likewise, L-dopa solution (1.3 mg per ml) or dopamine solution (3 mg per ml) was used. As to Inosea, only the central part of the tablet was used, and the water solution was prepared in the same way as in Toughmac-E. The injection of 50 μl of each prepared solution was given into the body cavity under the left white patch of the third abdominal segment of the prepupa (Fig. 1a) or under the outer margin of the left wing of the 0- to 4-day-old pupa (Fig. 1b).

RESULTS

I. Administration of Toughmac-E and Molsin by hypodermal injection

A. Administration to prepupa

The greater region of the normal wing pattern of adult *P. xuthus* emerged from untreated prepupae (pharatepupae) and pupae was made up of mainly black and yellow colors (Fig. 2). The yellow tone of the underside was a little stronger than that of the upperside. The male was a paler yellow than the female.

Compared with the normal pattern, there were no variations in color pattern throughout the entire adult wing of individuals administered Toughmac or Molsin as prepupae (data not shown).

B. Administration to 0-day-old pupae

At the time when Toguhmac-E or Molsin was injected into pupae just after pupation, no essential difference of the effects on the wing color pattern was found between these two substances. The upperside of the forewing (forewing recto, f.w.r.) appears normal, but the submarginal, pale yellow band of the underside (forewing verso, f.w.v.) broadens inwards (Fig. 3). A submarginal, pale yellow fleck line on the hindwing upperside (h.w.r.) develops well and distinctly to form a broad band. This tendency is especially clear with a submarginal, yellow band on the hindwing underside (h.w.v.): the inner black band either separates (Fig. 3, a and c) or is substantially reduced (Fig. 3b), causing the appearance of blue flecks and orange areas which were originally covered with the black band in the normal pattern. Such wing color patterns are designated Y (yellow) -type.

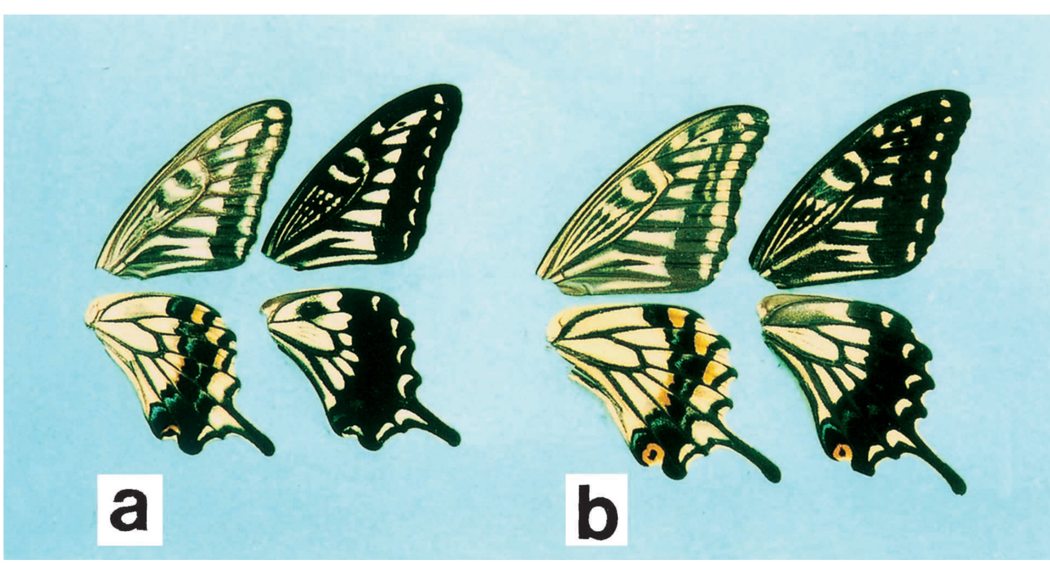

Fig. 2. Normal wing color patterns of *P. xuthus*. Left wings of the (a) male and (b) female show the undersides, and right wings the uppersides. The size of the wings is reduced to 75% of the original.

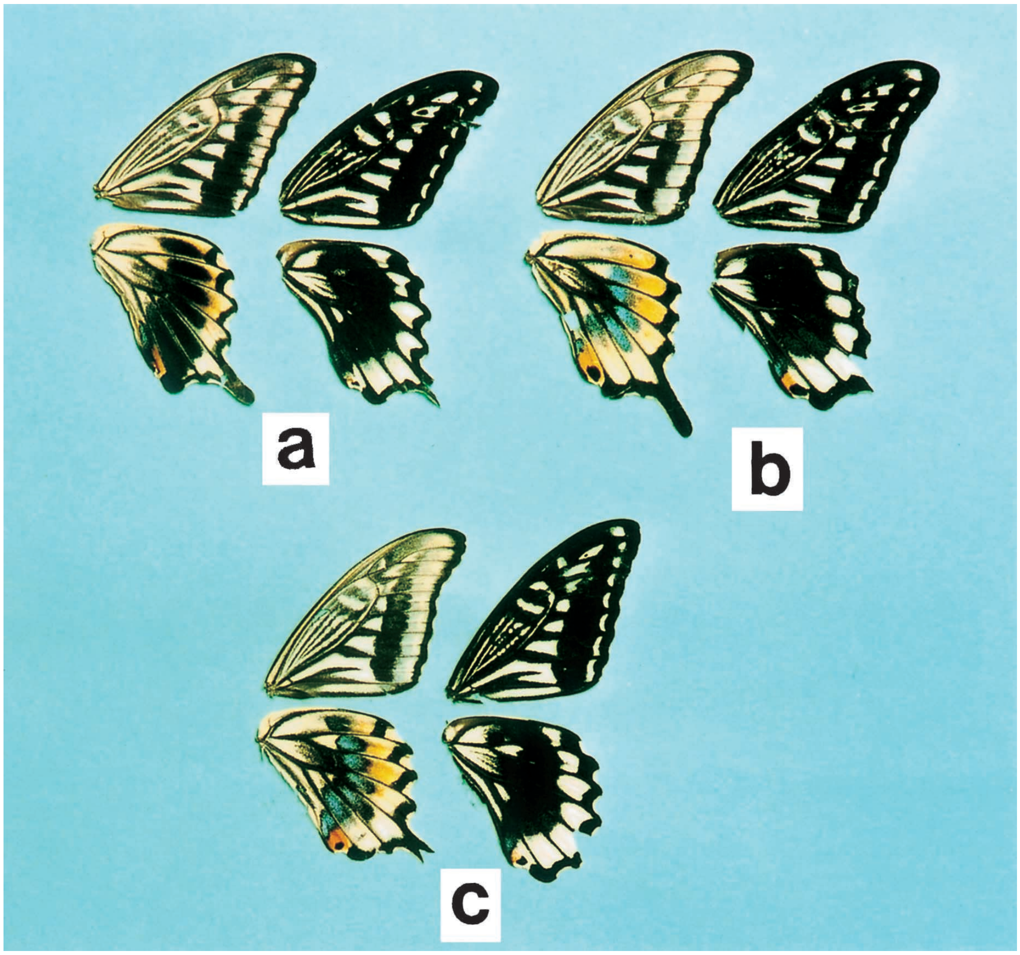

Fig. 3. Yellow-type (Y-type) wings obtained by injection into 0-day-old pupae: (a) male from the Molsin-injected pupa; (b) and (c) males from Toughmac-E-injected pupae. Left wings show the undersides, and right wings the uppersides. The size of the wings is reduced to 75% of the original.

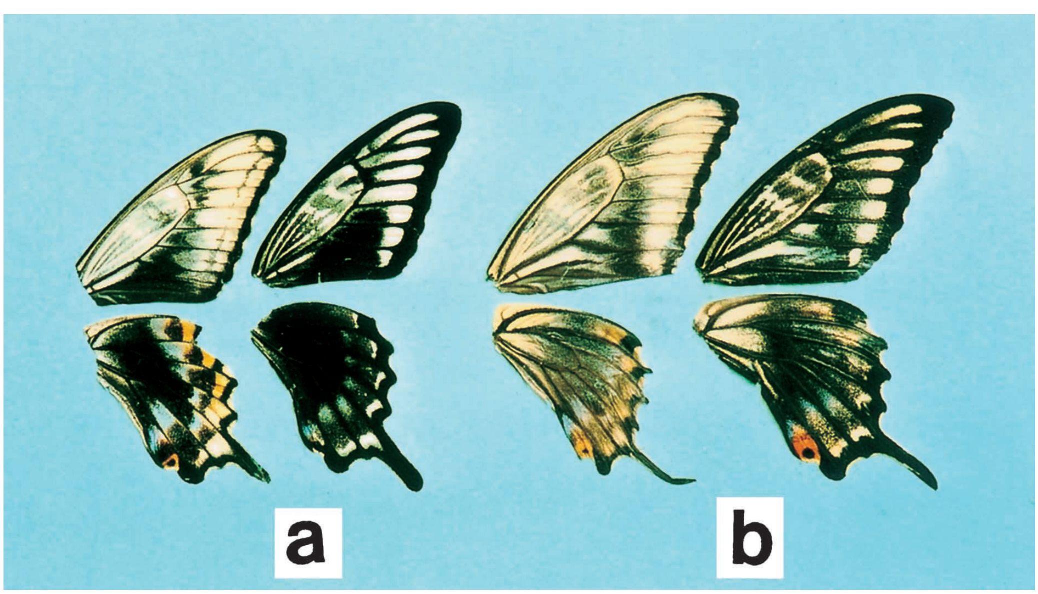

Fig. 4. Black and yellow-type (B-Y type) wings obtained by injection into 1-day-old pupae: (a) Molsin, and (b) Toughmac-E were injected into a male and a female, respectively. Left wings show the undersides, and right wings the uppersides. The size of the wings is reduced to 75% of the original.

C. Administration to 1-day-old pupae

At the time when Toughmac-E or Molsin was injected into 1-day-old pupae, two types of adult butterflies with abnormal wing color patterns, B-Y (black-yellow) -type (Fig. 4) and B (black) -type (Fig. 5b-5d) were obtained.

C-1. Color patterns of B-Y type

On the f.w.r. of both sexes, the pale yellow region was broadly enlarged except for black lines on most veins and around the wing margin, and a large, triangular black marking appears above the inner margin (Fig. 4a and 4b). A similar tendency was also observed in the f.w.v., but the yellow region broadens more strongly than that of the f.w.r., while the triangular black marking is substantially reduced.

On the h.w.r. of the male, the black region is well developed and occupies the majority part (Fig. 4a). In this black region, the submarginal, pale yellow fleck line has a tendency to broaden inwards and separates into two lines: a distinct, original submarginal line and another newly formed, obscure discal line, on which there are faint blue scales. On the female, a perturbation pattern fundamentally similar to that of the male is seen (Fig. 4b), but the enlargement of melanization is much less and its intensity is much weaker. The broadened black region does not reach the prediscal, postbasal or basal parts. On the h.w.v. of the male, not only is the yellow region enlarged, but the black region is more markedly reduced than that of the h.w.r. A postdiscal black band of the normal wing shifts more inwards, and an irregular, discal blue fleck line appears on the black region. The perturbation pattern of the female (Fig. 4b) seems to be similar to that of the male (Fig. 4a), but the yellow region broad-

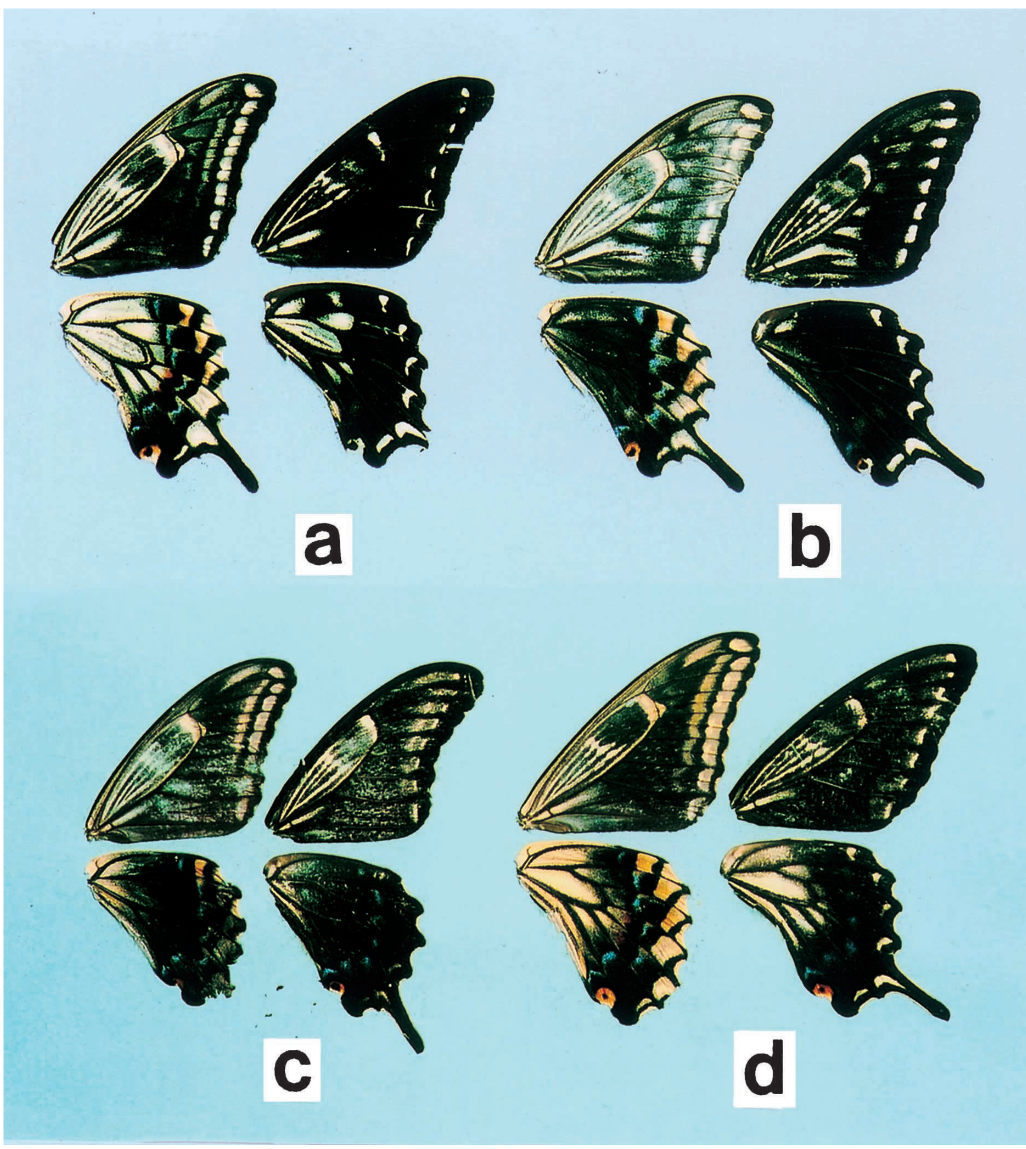

Fig. 5. Blackened wings (B-type) obtained by injection into young pupae. Sex, species of enzyme injected and age are: (a) male, Molsin into 2-day-old pupa; (b) male, Toughmac-E into 1-day-old pupa; (c) female, Molsin into 1-day-old pupa; and (d) female, Toughamac-E into 1-day-old pupa. Left wings show the undersides, and right wings the uppersides. The size of the wings is reduced to 75% of the original.

ens more strongly, whereas the black region has a tendency to be smaller and thinner.

C-2. Color patterns of B-type

Melanization develops markedly in the whole wing, particularly in the f.w.r. (Fig. 5d) and h.w.r. (Fig. 5b and 5c).

D. Administration to 2-day-old pupae

At the time when Molsin was injected into 2-day-old male pupae, the blackend region developed markedly in both sides of the forewing as well as the h.w.r. (Fig. 5a). In contrast, the yellow region developed in the h.w.v., where the pattern was rather similar to that of the normal.

E. Administration to 3- and 4-day-old pupae

Injection of Toughmac-E or Molsin into 3- or 4-day-old pupae did not have any influence on the normal wing color pattern (data not shown). This is the same in injection into prepupae, as shown in experiment A. We therefore concluded] that these substances probably do not perturb the color pattern when injected into pupae of 5-days or older.

II. Examination of the pattern-perturbing component in Toughmac-E

Toughmac-E has an ability to induce perturbation of the wing color pattern of *P. xuthus*, as shown above. This digestive mixture is composed of the following nine digestive enzymes, i.e., hydrolytic enzymes for proteins, carbohydrates and lipids: diastase, pancreatin, Diasmen, polylipase, Molsin, Onotase, Bonlase, Cellulosin AP, and Onoprose A. The former five digestive enzymes were administered to prepupae and pupae. Among them, only Molsin induced a wing-pattern perturbation that was highly similar to that by Toughmac-E (Figs. 2–5), whereas the other four digestive enzymes given had no influence on the wing pattern at all. Diasmen and polylipase were replaced by "Inosea", another commercial digestive, since it contains them as main components. Digestive enzymes 6–9 in Toughmac-E could not be examined, either because they were not obtained or because they have not been characterized. From these results, it was concluded that the perturbation-inducing component in Toughmac-E is Molsin or an acid carboxypeptidase of *Aspergillus saitoi*.

III. Effects of L-dopa and dopamine on the perturbation of the color pattern

L-Dopa and dopamine act as common precursors for formation of the black pigment melanin and the yellow pigments known as papiliochromes in *P. xuthus*. In the present study, however, injection of either of these substances into 0- to 2-day-old pupae did not vary the wing color pattern at all (data not shown).

DISCUSSION

The black and pale yellow markings in the wing of *P. xuthus* result from deposit of the corresponding pigments, melanin and papiliochromes, in scales, whereas the blue is a structural color originating from Thyndall blue in black scales (Nijhout, 1981). Melanin is probably derived from dopamine (Koch *et al.*, 1998; 2000), while papiliochrome II consists of L-kynurenine and N-β-alanyldopamine (Rembold and Umebachi, 1984; Umebachi, 1985, 1993). Therefore, in Papilio butterflies, both black and yellow pigments necessitate dopamine as the common precursor, so that a balance between its supply and utilization in scales must influence the color pattern. L-Kynurenine is derived from tryptophan, and N-β-alanyldopamine is from dopamine and β-alanine formed from uracil (Osanai *et al.*, 1994), respectively.

In addition, various enzymes are included in the formation of pigments and their precursors in scales, as shown in the synthesis of papiliochrome II (Yago, 1989; Soul and Sugumaran, 1991). At the formation of the wing color pattern of another papilionid butterfly, *P. glaucus*, the included enzyme species as well as the timing of the appearance of their activity are different between the presumptive black and yellow regions in wings (Koch *et al.*, 1998, 2000). Even in the same enzyme species, the period of the appearance of the activity varies between these two presumptive regions. Moreover, the growth rate of black scales is also different from that of yellow scales, probably due to the different time program in the presumptive black scales. These internal, physiological conditions of enzymes probably also rule the formation mechanism of the color pattern of *P. xuthus*.

Molsin, an acid carboxypeptidase (EC 3.4.23.18) of *Aspergillus saitoi* has a capacity to liberate tyrosine and phenylalanine at the C-terminal residues of protein and peptide at low pH (Yoshida and Nagasawa, 1956). Therefore, the pattern perturbation induced by Molsin should be attributed not only to the quantitative changes in melanin and papiliochromes but also to the varied degree of formation of colored wing scales. It is possible that a similar, endogenous enzyme, the action of which is the same as that of Molsin, probably another acid carboxypeptidase, functions in *P. xuthus* for supply of dopamine, the precursor indispensable for formation of melanin and papiliochromes. Since the effective period for the pattern perturbation induced by Molsin was strictly limited from day 0 to 2 of pupa, the activity of the endogenous acid carboxypeptidase of this butterfly must appear only during this period. Overhigh activity based on the summation of these endogenous and exogenous acid carboxypeptidases probably causes the abnormal changes in the wing color pattern.

In the Y-type, the pattern of the f.w.r. and f.w.v. obtained from the 0-day-old pupae treated with Molsin showed almost no variation from the normal pattern, such that this exogenous enzyme did not yet sufficiently influence the pattern changes. In contrast, the enhancement of the yellow region of both sides of the hindwing suggests that dopamine derived from tyrosine or phenylalanine freed by the acid carboxypeptidase may be utilized for the synthesis of papiliochrome much more strongly. In the types B-Y (Fig. 4) and B (Fig. 5b–5d) which were obtaind from 1-day-old pupae treated with Molsin, the balance between the formation of the two pigments was not stable: sometimes melanin-rich and papiliochrome-poor pattern occurred, and sometimes the reverse pattern occurred. In the B-type (Fig. 5a) obtained from the 2-day-old pupa treated with Molsin, compared with the B-type from 1-day-old pupae, the pattern perturbation exhibited a tendency to return to normal. Namely, at this stage, the exogenous enzyme did not as strongly affect the pattern perturbation, in particular in the h.w.r. and h.w.v. Probably, the summed overhigh activity was decreased with the reduced activity of endogenous acid carboxypeptidase, because the dose of enzyme exogenously given to pupae was always the same in all the stages used. It is, therefore, concluded that in the normal butterfly of *P. xuthus*, the activity of the endogenous acid carboxypeptidase must appear in the early pupal stage. This activity peaks in the 1-day-old pupa, and is lower at the earlier or later pupal stage. The higher the activity of endogenous acid carboxypeptidase in the pupa is, the greater the quantities of tyrosine and phenylalanine that are liberated.

Unlike vertebrates, insects are unable to easily catabolize aromatic amino acids. For example, histidine is almost not metabolized during the pupal stage of *Bombyx mori* (Osanai and Kikuta, 1981). In contrast, phenylalanine, tyrosine and tryptophan could be metabolized and polymerized to various pigments (Koga and Osanai, 1967; Neehdam, 1978; Kayser, 1985; Umebachi, 2000). Pigments accumulated in the wing scales of butterflies, which are related to the excretion of nitrogenous substances, are regarded as storage excretory materials in "kidneys of accumulation" (Cochran, 1985).

The substrate of the endogenous acid carboxypeptidase is probably the reserve protein synthesized in the larva as a source of amino acids for the de novo syntheses of various enzymes and structural proteins in the pupa and adult (Osanai and Kikuta, 1981). By removal of aromatic amino acids from the C-terminal residues of the original reserve protein in the pupa, this protein is reconstructed to a more utilizable protein. It has been reported that an Arg-C endopeptidase causes the second maturation of spermatozoa of the silkworm, together with the energy-yielding system from arginine (Osanai *et al*., 1987; Osanai, 1992) by cooperation with an Arg-carboxypeptidase (Aigaki *et al*., 1988). Like these proteases, the endogenous acid carboxypeptidase of *P. xuthus* thus plays another important role in the regulation, utilization and excretion of amino acids.

Last but not least, it is interesting that the administration of L-dopa or dopamine did not cause any perturbation of wing color pattern. The difference of action between Molsin and dopa or dopamine is a subject for future research.

ACKNOWLEDGEMENT

The authors are obliged to Prof. Hiroshi Terada, Laboratory of Medical Biochemistry, Faculty of Pharmaceutical Sciences, Tokushima University for his helpful suggestions.

REFERENCES

Aigaki T, Osanai M, Kasuga H (1988) Arginine carboxypeptidase activity in the male reproductive glands of the silkworm, *Bombyx mori*. Insect Biochem 18: 295–298

Beldade P, Brakefield PM, Long AD (2002) Contribution of distalless to quantitative variation in butterfly eyspots. Nature 415: 315–318

Carroll SB, Gates J, Keys DN, Paddock SW, Panganiban GEF, Selegue JE, Williams JA (1994) Pattern formation eyespot determination in butterfly wings. Science 265: 109–114

Cochran DG (1985) Nitrogenous Excretion. In "Comprehensive Insect Physiology Biochemistry and Pharmacology, Vol 4, Regulation: Digestion, Nutrition, Excretion," Ed by KA Kerkut and LI Gilbert, Pergamon Press, Oxford, pp 467–506

Kayser H (1985) Pigments. In "Comprehensive Insect Physiology Biochemistry and Pharmacology, Vol 10, Biochemistry," Ed by GA Kerkut and LI Gilbert, Pergamon Press, Oxford, pp 367–415

Koch PB, Behneck B, Weigmann-Lenz M, Ferench-Constant RH (2000) Insect pigmentation: Activities of β-alanyldopamine synthase in wing color patterns of wild-type and melanic mutant swallowtail butterfly *Papilio glaucus*. Pigment Cell Res 13 (Suppl 8): 54–58

Koch PB, Keys DN, Rocheleau T, Arostein K, Blackburn M, Carroll SB, Ferench-Constant RH (1998) Regulation of dopa decarboxylase expression during color pattern formation in wild-type and melanic tiger swallowtail butterflies. Development 125: 2303–2313

Koga N, Osanai M (1967) Der Gehalt an Tryptophan, Kynurenin, 3-Hydroxykynurenin und Ommochromen bei den ueberwinternden Eiern des Seidenspinners, *Bombyx mori* waehrend der Entwickelung. H-S Z Physiol Chem 348: 979–982

Needham AE (1978) Insect Biochromes: Their Chemistry and Role. In "Biochemistry of Insect", Ed by M Rockstein, Academic Press, New York, pp 233–305

Nijhout HF (1981) The color patterns of butterflies and moths. Sci Amer 245: 104–115

Nijhout HF (1991) The Development and Evolution of Butterfly Wing Patterns. Smithonian Institution Press, London

Osanai M (1992) Motility of apyrene spermatozoa of the silkworm, *Bombyx mori*, induced by initiatorin. In "Comparative Spermatology 20 Years After", Ed by B Baccetti, Raven Press, Basel, pp 521–526

Osanai M, Aigaki T, Kasuga H (1987) Arginine degradation cascade as an energy-yielding system for sperm matulation in the spermatophore of the silkworm, *Bombyx mori*. In "New Horizons in Sperm Cell Research", Ed by H Mohri, Japan Scientific Societies Press, Tokyo, pp 185–195

Osanai M, Kurata N, Umebachi Y (1994) Dynamics of β-alanine associated to degradation of mRNA and sclerotization of the cuticle of the silkworm. Zool Sci 11: 45

Osanai M, Kikuta S (1981) Age-related changes in amino acid pool sizes in the adult silkworm, *Bombyx mori*. Exp Gerontol 16: 445–459

Rembold H, Umebachi Y (1984) The structure of papiliochrome II, the yellow wing pigment of the papilionid butteflies. In "Progress in Tryptophan and Serotonin Research", Ed by HG Schlossberger, W Kochen, B Linzen, H Steinhart, Welter de Gruyter, Berlin, pp 743–746

Soul SJ, Sugumaran M (1991) Quinone methide as a reactive intermediate formed during the biosynthesis of papiliochrome II, a yellow wing pigment of papilionid butterflies. FEBS Lett 279: 145–148

Umebachi Y (1985) Papiliochrome, a new pigment group of butterfly. Zool Sci 2: 163–174

Umebachi Y (1993) The third way of dopamine. Trends Comp Bio-

chem Physiol 1: 709–920
Umebachi Y (2000) The Pigment of Animals (in Japanese), Uchida-Rohkakuho, Tokyo
Yago M. (1989) Enzymic synthesis of papiliochrome II, a yellow pigment in the wings of papilionid butterflies. Insect Biochem 19: 673–678
Yoshida F, Nagasawa M (1956) Studies on the proteolytic enzyme of black Aspergilli. Part III. The specificity of crystalline *Aspergillus saitoi*. Bull Agr Chem Soc Japan 20: 262–266

(Received November 20, 2002 / Accepted January 8, 2003)