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Note

Purification and Characterization of Biliverdin-binding Protein from Larval Hemolymph of the Swallowtail Butterfly, *Papilio xuthus* L.

Akira YAMANAKA,† Takamasa ITO, Daizo KOGA,* Toshiyuki SATO,**
Masanori OCHIAI,*** and Katsuhiko ENDO

Department of Physics, Biology and Informatics, Faculty of Science, Yamaguchi University, Yamaguchi 753-8512, Japan
**Department of Environmental and Biological Science, Faculty of Agriculture, Yamaguchi University, Yamaguchi 753-0841, Japan*
***Department of Biology, Faculty of Science, Shinshu University, Matsumoto 390-0802, Japan*
****Biochemical Laboratory, The Institute of Low Temperature Science, Hokkaido University, Sapporo 060-0819, Japan*

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The biliverdin-binding protein from the larval hemolymph of the swallowtail butterfly, *Papilio xuthus* L., was purified and characterized. The crude biliverdin-binding protein, obtained by ammonium sulfate fractionation, was purified in two steps, the first one by gel filtration chromatography and the second one by ion-exchange chromatography. The molecular mass of the purified protein was analyzed by SDS-polyacrylamide gel electrophoresis and estimated to be 21 kDa. The N-amino terminal sequence of *P. xuthus* biliverdin-binding protein analyzed up to the 19th residue showed that 42% of the amino acid sequence are sequence similarity to the bilin-binding protein from *Pieris brassicae*. These results suggest that the *P. xuthus* biliverdin-binding protein belongs to the insecticyanin-type.

Key words: biliverdin-binding protein; *Papilio xuthus*; butterfly; insecticyanin

Camouflage of the host plant are the typical morphological adaptations of insects in the larval stages. It is known that the expression of the larval green coloration is based on a relationship between a yellow pigment, carotenoid, and a blue pigment, biliverdin, in hemolymph; the blue biliprotein, which binds blue bile pigment, also plays an important role in coloration.[1] In butterflies and moths, it is known that their blue pigment is biliverdin IX_γ.[2] Blue biliproteins that are known by different names such as biliverdin-binding protein (BP), bilin-binding protein, insecticyanin, insect cyanoprotein, etc., have been widely found in various insect orders (Orthoptera, Heteroptera, and Lepidoptera), and these proteins have been purified and characterized by their hemolymph in a number of species, such as *Riptortus clavatus*,[3] *Locusta migratoria*,[4] *Manduca sexta*,[5] and *Agrius convolvuli*.[6] However, there has been no report from other butterfly species in spite of many reports concerning bilin-binding protein of the large white butterfly, *Pieris brassicae*.[7–9] To date, little is known about the physiological and biochemical mechanism of larval color adaptation of *P. xuthus*. In this paper, we report on the purification and characterization of the BP in larval hemolymph of *P. xuthus*.

P. xuthus, which had been collected in the towns of Yamaguchi and Hofu Cities, was used. Larvae of *P. xuthus* were reared on leaves of *Fagara ailanthoides* at 25°C with a photoperiod of 16 hr light and 8 hr dark.[10] One unit of BP represented 1.0 absorbance at 648 nm. The protein was measured by the method of Smith *et al.* using BSA as a standard.[11] SDS gel electrophoresis was done on 12.5% polyacrylamide slab gels as described by Laemmli.[12] Proteins were stained with Coomassie Brilliant Blue R-250. All other chemicals were of analytical grade.

Larval hemolymph of *P. xuthus* was collected from 20 last instar larvae by a "flushing out" method; 0.1 M phosphate buffer containing 150 mM KCl and 10 mM EDTA, pH 6.0, was injected into each haemocoel[13] and the effluent was centrifuged at $500 \times g$ for 5 min to remove hemocytes. The resulting supernatant was used as the crude extract for BP purification. Ammonium sulfate was added to the crude extract up to 65% saturation, and the precipitate was removed by centrifugation at 12,100 $\times g$ for 10 min. Additional ammonium sulfate was added to the supernatant up to 95% saturation. The

† To whom correspondence should be addressed. Fax: 81-83-933-5720; E-mail: yamanaka@po.cc.yamaguchi-u.ac.jp
Abbreviation: BP; biliverdin-binding protein

precipitate collected by centrifugation was dissolved in 300 μl of 20 mM Tris-HCl buffer (pH 7.8), containing 0.1 M NaCl. The solution obtained was put on a Superose 12 column (1.0×30 cm; Pharmacia, Uppsala, Sweden) equilibrated with the same buffer as above. Protein was eluted with the same buffer at a flow rate of 0.2 ml/min. The eluted BP fraction (1.2 ml) was concentrated by an UltrafreeC3LGC (Milipore, Tokyo) to 100 μl. The resulting solution was diluted with 1.0 ml of 20 mM Tris-HCl buffer (pH 7.8) and put on a Sep-Pak plus QMA-CM cartridge column (Waters, Massachusetts, U.S.A.) equilibrated with 20 mM Tris-HCl buffer (pH 7.8). The column was, then, washed with the same buffer. The nonabsorbed solution (referred to as purified BP) was collected and concentrated by ultrafiltration to 100 μl.

Table 1 summarizes typical results of the step purification. In this preparation, the BP was thought to be purified approximately 4.6-fold over the larval hemolymph of *P. xuthus* by the absorbance at 648 nm and recovered in 1.2% yield. However, the BP purification was estimated to reach about 21-fold on computerized image analysis of the BP band of a CBB stained gel using RFLP Scan Plus software (Scanalytics, Bsllerica, MA) (data not shown). Although the BP was rapidly purified with ammonium sulfate fractionation and two step chromatographies, the yield was low. The final protein preparation was electrophoretically homogeneous, and the molecular mass of the protein was found to be 21 kDa by SDS-polyacrylamide gel electrophoresis (Fig. 1). In addition, the molecular mass of the purified BP was estimated to be 24 kDa from gel filtration with Superose 12. A calibration curve was made with the following proteins: bovine serum albumin (66,000), carbonic anhydrase (29,000), cytochrome *c* (12,400), and aprotinin (6.500) (Sigma, U.S.A.) (data not shown). These results suggest that *P. xuthus* BP is a monomer.

Furthermore, the optical absorption spectra of BP and blue pigment were measured with a Hitachi U-2000A. The two absorption maxima of the purified native BP in 20 mM Tris-HCl (pH 7.8) were at 378 nm ($A_{378}=0.342$) and 648 nm ($A_{648}=0.175$); the A_{648}/A_{378} ratio was 0.51. The two absorption maxima of the blue pigment extracted from the purified BP with methanol:HCl (95:5, v/v), as described in Sato,[14] were 359 nm ($A_{359}=0.246$) and 695 nm ($A_{695}=0.124$); the A_{695}/A_{359} ratio was 0.50. The two absorption maxima of the biliverdin IX dimethyl ester (Porphyrin Products INC, U.S.A) as a standard were 374 nm ($A_{374}=0.338$) and 691 nm ($A_{691}=0.158$); the A_{691}/A_{374} ratio was 0.47. These results suggest that the pigment of *P. xuthus* BP is biliverdin IX.

For analysis of the amino acid composition of the purified BP, The protein was hydrolyzed in 6 N HCl at 110°C for 21 h, and amino acids were analyzed by the system of Pico-Tag. Amino acid compositions of

Table 1. Summary of Purification of Biliverdin-binding Protein from *P. xuthus*

Purification step	Total protein (mg)	Total units	units/mg	Yield (%)
Crude extract	119.68	14.43	0.12	100.0
65%-95% $(NH_4)_2SO_4$	3.00	1.16	0.39	8.0
Superose 12	0.96	0.58	0.60	4.0
QMA-CM cartridge	0.31	0.17	0.55	1.2

One unit represents 1.0 absorbance at 648 nm.

Table 2. Amino Acid Composition of BP (mol%) from *P. xuthus*

Amino acid	*P. xuthus*	*P. brassicae*[9]	*M. sexta*[5]
Asx	15.6	5.8	13.5
Glx	7.9	6.9	7.1
Ser	7.1	6.9	4.3
Gly	7.4	8.7	6.5
His	0.5	3.5	3.1
Arg	1.9	0.6	1.0
Thr	7.1	4.0	4.0
Ala	6.1	4.6	7.8
Pro	3.8	3.5	3.7
Tyr	4.8	9.2	7.0
Val	8.3	11.6	7.7
Met	0.4	0.0	0.8
Cys	0.2	2.3	1.7
ILe	5.8	4.0	4.2
Leu	2.0	3.5	5.8
Phe	7.0	2.9	4.4
Lys	14.2	11.6	9.4

Tryptophan was not determined.

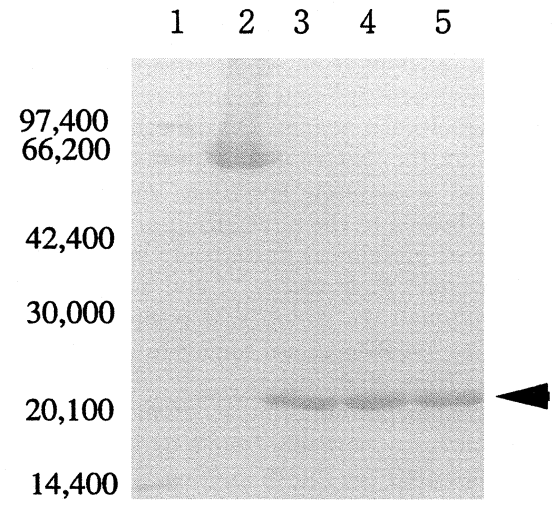

Fig. 1. SDS-PAGE of BP during Purification Steps and Estimation of the Molecular Weight.

Lane 1, molecular weight standards. The numbers in the left-side show molecular size markers; Lane 2, crude extract; Lane 3, 65-95% ammonium sulfate fraction; Lane 4, fraction from Superose 12 column; Lane 5, fraction from QMA-CM cartridge column. The arrow indicates the position of BP.

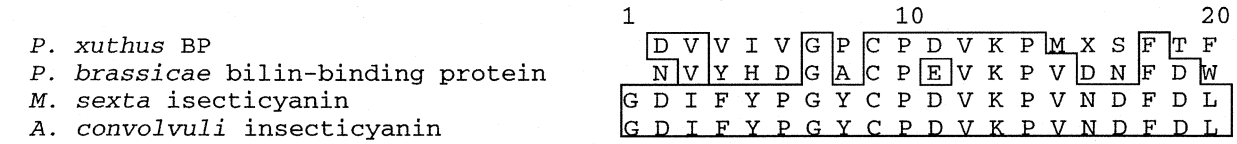

Fig. 2. N-terminal Amino Acid Sequence Alignments of *P. xuthus* BP with Other Blue Biliproteins.
a) bilin-binding protein from *P. brassicae*;[9] b) insecticyanin from *M. sexta*;[5] c) insecticyanin from *A. convolvuli*.[6] The boxes indicate identical amino acids.

the protein were found to be rich in Asx and Lys. The amino acid composition of *P. xuthus* BP was compared to that of *P. brassicae* bilin-binding protein[6] and *M. sexta* insecticyanin.[5] The composition profiles of *P. xuthus* BP was not similar to those of bilin-binding protein or insecticyanin (Table 2). It is unclear whether the difference in the composition profiles depended on the species, or the primary structure themselves.

Since the deduced amino acid sequence of *P. brassicae* bilin-binding protein has only been analyzed in butterflies,[8] we next examined whether the N-terminal amino acid sequence of *P. xuthus* BP has similarity to that from *P. brassicae*. The purified BP was blotted onto a polyvinylidene difluoride membrane (Milipore Lit. Tokyo). The N-terminal amino acid sequence of BP was analyzed by Edman degradation with a Shimadzu protein sequencer PPSQ-10 (Shimadzu, Kyoto). The N-terminal sequence of the purified BP was analyzed for 19 residues, and the *P. xuthus* BP showed the N-terminal sequence similarity with bilin-binding protein[9] of *P. brassicae* (42%), the moth insecticyanin of *M. sexta*[5] (47%), and *A. convolvuli*[6] (47%) (Fig. 2).

The biliverdin-binding protein purified in this study were different from *R. clavatus* biliverdin associated cyanoprotein[3] and *L. migratoria* BP[4] with respect to molecular weight. On the other hand, the similarity of the N-terminal sequence, molecular weight, and absorption spectrum imply that *P. xuthus* BP belong to the same group of the insecticyanin-type of proteins as has already been reported from *M. sexta*,[5] *P. brassicae*,[7-9] and *Samia cynthia ricini*,[14] although the amino acid composition profiles of *P. xuthus* BP was not similar to those of blue biliproteins from other insects.

In the swallowtail butterfly, *P. xuthus*, the larval cuticle color turns markedly from a dark-brown into a green color after the fourth molting. Studies of the relationship between BP metabolism and biliverdin IX biosynthesis may shed light on the physiological regulation of the morphological color adaptation in *P. xuthus* larvae. We are currently studying the structure and gene cloning of BP.

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References

1) Kanost, M. R., Kawooya, J. K., Law, J. H., Ryan, R. O., Heusden, M. C. V., and Ziegler, R., Insect hemolymph proteins. *Adv. Insect Physiol.*, **22**, 299–396 (1990).
2) Kayser, H., Pigments. In "Comprehensive Insect Physiology, Biochemistry and Pharmacology", eds. Kerkut, G. A., and Gilbert, L. I., Vol. 10, Pergamon Press, Oxford, pp. 367–415 (1985).
3) Chinzei, Y., Haruna, T., Miura, K., Numata, H., and Nakayama, S., Purification and characterization of biliverdin-associated cyanoprotein from eggs and hemolymph of the bean bug, *Riptortus clavatus* (Heteroptera: Alydidae). *Insect Biochem.*, **20**, 545–555 (1990).
4) Chino, H., Abe, Y., and Takahashi, K., Purification and characterization of a biliverdin-binding cyanoprotein from locust haemolymph. *Biochim. Biophys. Acta*, **748**, 109–115 (1983).
5) Riley, C. T., Barbeau, B. K., Keim, P. S., Kezdy, F. J., Heinrikson, R. L., and Law, J. H., The covalent protein structure of insecticyanin, a blue biliprotein from the hemolymph of the tobacco hornworm, *Manduca sexta* L. *J. Biol. Chem.*, **259**, 13159–13165 (1984).
6) Saito, H. and Shimoda, M., Insecticyanin of *Agrius convolvuli*: purification and characterization of biliverdin-binding protein from the larval hemolymph. *Zool. Sci.*, **14**, 777–783 (1997).
7) Huber, R., Schneider, M., Epp, O., Mayr, I., Messershmidt, A., Pflugrath, J., and Kayser, H., Crystallization, crystal structure analysis and preliminary molecular model of the bilin binding protein from the insect *Pieris brassicae*. *J. Mol. Biol.*, **195**, 423–434 (1987).
8) Schmidt, F. A. and Skerra, A., The bilin-binding protein of *Pieris brassicae* cDNA sequence and regulation of expression reveal distinct features of this insect pigment protein, *Eur. J. Biochem.*, **219**, 855–863 (1994).
9) Suter, F., Kayser, H., and Zuber, H., The complete amino-acid sequence of the bilin-binding protein from *Pieris brassicae* and its similarity to a family of serum transport proteins to be the retinol-binding proteins, *Biol. Chem. Hoppe-Seyler*, **369**, 497–505 (1988).

10) Yamanaka, A., Endo, K., Nishida, H., Kawamura, N., Hatase, Y., Kong, W., Kataoka, H., and Suzuki, A., Extraction and partial characterization of pupal-cuticle-melanizing-hormone (PCMH) in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera, Papilionidae). *Zool. Sci.*, **16**, 261–268 (1999).
11) Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C., Measurement of protein using bicinchoninic acid. *Anal. Biochem.*, **150**, 76–85 (1985).
12) Laemmli, U. K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685 (1970).
13) Hayakawa, Y., Jahagirdar, A. P., Yaguchi, M., and Downer, R. G. H., Purification and characterization of trehalase inhibitor from hemolymph of the American cockroach *Periplaneta americana*. *J. Biol. Chem.*, **264**, 16165–16169 (1989).
14) Saito, H., Purification and characterization of two insecticyanin-type proteins from the larval hemolymph of the Eri-silkworm, *Samia synthia ricini*. *Biocim. Biophys. Acta*, **1380**, 141–150 (1998).