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Insecticyanin of *Agrius convolvuli*: Purification and Characterization of the Biliverdin-Binding Protein from the Larval Hemolymph

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ABSTRACT—Blue biliprotein, insecticyanin (INS), has been purified from the hemolymph of the sweet potato hornworm, *Agrius convolvuli*. The protein was efficiently isolated from the hemolymph of fifth instar larvae using three successive column chromatographic techniques: hydrophobic interaction chromatography, ion exchange chromatography and gel-filtration. The purified INS showed a native molecular weight of approximately 59,000 by gel-filtration. SDS-PAGE revealed a single band with Mr of approximately 26,000. Moreover, the molecular mass of INS was 21, 213 by MALDI-TOF/MS. Thus, the native *A. convolvuli* INS molecule was assumed to be a trimer in solution. The blue coloration of *A. convolvuli* INS from the hemolymph was attributed to the presence of biliverdin IXγ, due to the absorbance maxima at 360 and 695 nm, which was non-covalently bound with the apoprotein. Amino acid composition and N-terminal sequence of *A. convolvuli* INS is similar to *M. sexta* INS. *A. convolvuli* INS represents one of the biliverdin-binding proteins in lepidopteran insects.

INTRODUCTION

Caterpillars are often green in body color and this coloration presumably serves as a protective camouflage on the host plants. A green coloration also has been observed in the hemolymph and integument of various insects. The green color results from a combination of a blue pigment, biliverdin, and yellow pigments, carotenoids. These pigments are known to be intimately associated with proteins (Kawooya *et al.*, 1985; Kayser, 1985; Law and Wells, 1989). Blue biliproteins have been isolated from the hemolymph of several lepidopteran insects. In *Manduca sexta*, insecticyanin (INS) has been purified and characterized from the hemolymph (Cherbas, 1973; Riley *et al.*, 1984; Goodman *et al.*,1985; Holden *et al.*, 1986, 1987). *M. sexta* INS is synthesized and stored in pigment granules in the epidermis and secreted into both the hemolymph and the cuticle (Riddiford, 1982; Riddiford *et al.*, 1990). The amino acid sequence (Riley *et al.*, 1984) and cDNA sequence (Li and Riddiford, 1992) of INS have been determined. In another lepidopteran insect, *Pieris brassicae*, the bilin binding protein has been crystallized and its crystal structure was determined (Huber *et al.*, 1987a, b). Moreover, the complete amino acid sequence (Suter *et al.*, 1988) and cDNA sequence (Schmidt and Skerra, 1994) of the bilin binding protein have been determined. Blue chromoproteins have also been isolated and characterized from the larval hemolymph of *Heliothis zea* (Haunerland and Bowers, 1986) and *Trichoplusia ni* (Jones *et al.*, 1988). In the Eri-silkworm, *Samia cynthia ricini*, the biliverdin-binding protein (BBP) found in the molting fluid during the larval-pupal ecdysis, has been purified and characterized (Saito, 1993). The N-terminal amino acid sequence of *S. cynthia ricini* BBP has also been determined (Saito, 1994).

The rearing system of the sweet potato hornworm, *A. convolvuli*, as an experimental insect was developed in our laboratory (Kiguchi and Shimoda, 1994). *A. convolvuli* belongs to Sphingidae, and is colsely related to *M. sexta*. *A. convolvuli* shows a population density-dependent polychromatism in the larval body color. This various phase-related phenomenon cannot be found in *M. sexta* larvae. In this study, as a first step toward understanding the physiological function of *A. convolvuli* INS in the larval coloration, we have isolated INS from the larval hemolymph and examined its biochemical properties. We have also compared the N-terminal amino acid sequence of *A. convolvuli* INS with that of the biliverdin-binding proteins from three other species of Lepidoptera.

MATERIALS AND METHODS

Animals

Larvae of the sweet potato hornworm, *Agrius convolvuli*, were reared on an artificial diet at 25 ± 1°C and 70-80% RH under a long day photoperiod of 16 hr light-8 hr dark, as described in Kiguchi and Shimoda (1994).

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Chemicals

Phenyl-Sepharose HP, Q-Sepharose HP, HiLoad Superdex 200 pg were purchased from Pharmacia Biotech (Uppsala, Sweden). TSKgel G2000SWXL was purchased from Tosoh Corporation (Tokyo, Japan). Biliverdin IX dimethyl ester was obtained from PORPHYRIN PRODUCTS INC. (Logan, Utah, USA). All other chemicals obtained were of the highest purity commercially available.

Collection of hemolymph

Hemolymph (approximately 60 ml) was collected from the fifth instar larvae (day 3, 50 animals) from an incision made by cutting off the horn. Hemolymph dropped into saturated ammonium sulfate solution and was centrifuged at 10,000 × g for 20 min at 4°C. The precipitate was dissolved in 60 ml of buffer A [50 mM Tris-HCl buffer (pH 7.8)/0.1 mM PMSF (phenylmethylsulfonyl-fluoride)], containing ammonium sulfate at 40% saturation. The precipitate was removed after a centrifugation at 10,000 × g for 20 min at 4°C and the supernatant was used as the crude extract for insecticyanin (INS) purification.

Purification of insecticyanin (INS)

The crude extract (62 ml) was applied to a Phenyl-Sepharose HP (1.5 × 20 cm) column, which had been equilibrated with buffer A containing 40% saturated ammonium sulfate. The column was eluted with a linear gradient of ammonium sulfate 40 →0% saturation in buffer A at a flow rate of 2 ml/min. INS fractions were pooled and dialyzed against buffer A. The Phenyl-Sepharose fraction (78 ml) was then applied to a Q-Sepharose HP (1.5 × 15 cm) column equilibrated with buffer A. The proteins were eluted with a linear gradient of 0 to 0.4 M NaCl in buffer A at a flow rate of 2 ml/min. INS fractions (38 ml) were pooled and concentrated to 3.2 ml using a Minimodule NM-3 fiber filter (Asahikasei, Tokyo). The Q-Sepharose fraction was further purified through a gel-filtration column (1.6 × 60 cm) of Superdex 200 pg equilibrated with buffer A containing 0.15 M NaCl at a flow rate of 1 ml/min. The blue peak fraction was collected and used to determine the biochemical properties of INS. For all chromatographic steps, INS eluted fractions were monitored for absorbance at 674 nm using a spectrophotometer (Beckman DU-650, USA). All purification procedures were carried out at 4°C or in ice, unless otherwise stated.

M. sexta INS was isolated from the hemolymph (a gift from Dr. Kiyoko Taniai, NISES) using the same column chromatographic techniques.

Determination of the protein concentration

The protein concentration in the fractions was determined by the Bio-Rad protein assay kit with bovine serum albumin (BSA) as a standard protein (Bio-Rad Laboratories, Richmond, CA, USA).

Electrophoresis

Sodium dodecyl sulfate-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 bromphenol 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 were purchased from Bio-Rad Laboratories (Richmond, CA, USA).

Molecular weight determination

The molecular weight of native INS was determined by comparing retention times with those of marker proteins using gel-filtration (HPLC). Purified INS was applied to a column (0.78 × 30 cm) of TSKgel G2000SWXL equilibrated with 50 mM sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl at a flow rate of 1 ml/min. The molecular weight of subunits was determined by SDS-PAGE as described above from the comparison of mobilities with those of marker proteins. Moreover, the molecular mass of INS was determined by MALDI-TOF/MS (matrix-assisted laser desorption ionization-time of flight/mass spectrometry) using Voyager™ RP Biospectrometry Systems (PerSeptive Biosystems, USA).

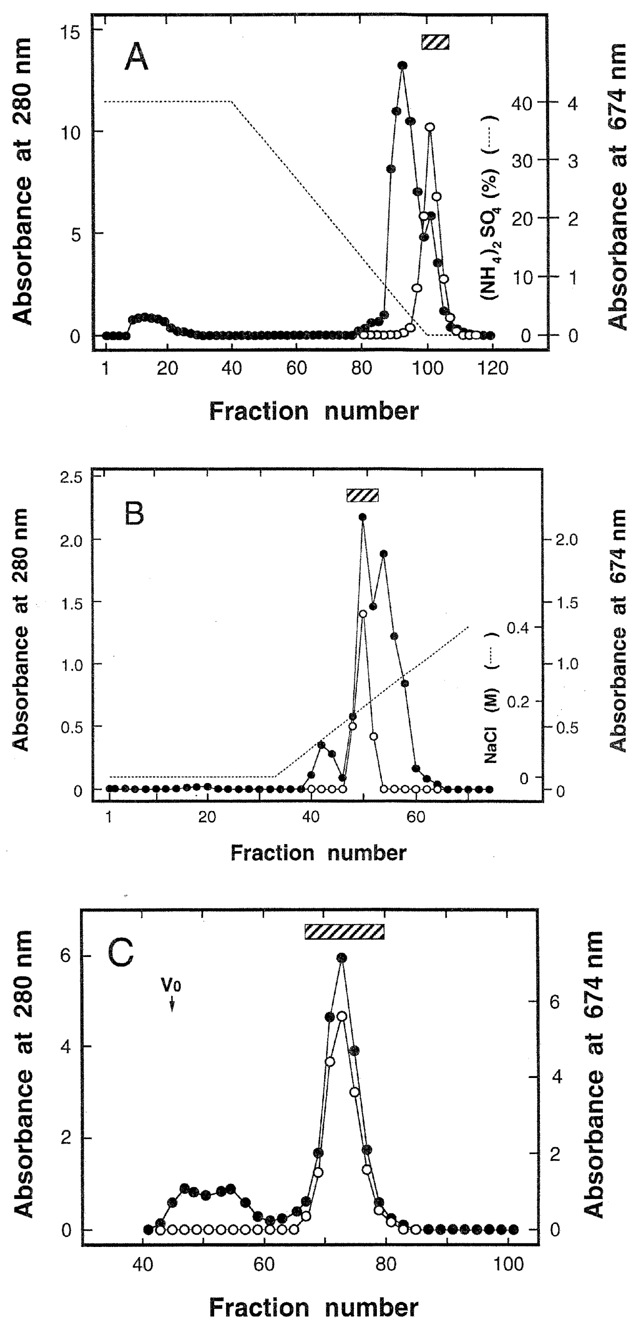

Fig. 1. Purification of *A. convolvuli* INS from the larval hemolymph. (**A**) Hydrophobic interaction chromatography on a Phenyl-Sepharose HP column. (**B**) Q-Sepharose HP column chromatography. (**C**) Gel-filtration on Superdex 200 pg. Protein and INS were monitored by absorbance at 280 nm (●) and 674 nm (○), respectively. Hatched bars indicate the pooled fractions.

Amino acid analysis

The purified INS (5 μg) was hydrolyzed with 6N HCl containing 4% mercapto acetic acid for 21 hr at 110°C. After hydrolysis, the amino acids of hydrolysate was analyzed on an automatic amino acid analyzer (Hitachi, Model L-8500, Tokyo).

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Table 1. Purification of insecticyanin from *Agrius convolvuli* hemolymph

Purification step	Total protein (mg)	Total units*	units/mg	Yield (%)
1. Hemolymph	481.1	67.5	0.14	100
2. 40% $(NH_4)_2SO_4$	356.5	55.1	0.15	82
3. Phenyl-Sepharose HP	72.2	46.3	0.64	69
4. Q-Sepharose HP	43.7	39.4	0.90	58
5. Superdex 200 pg	22.4	35.6	1.59	53

*One unit represented 1.0 absorbance at 674 nm.

N-terminal sequencing

The N-terminal sequence of purified INS was determined with a gas phase protein sequencer (Beckman, Model LF-3400 DT, USA). Phenylthiohydantoin (PTH) derivatives of individual amino acids were identified by reverse-phase high performance liqid chromatography (HPLC).

Analysis of chromophore

Purifid INS was incubated with an equal volume of ice-cold methanol for 30 min. The sample was centrifuged, and the supernatant was pooled and evaporated. The chromophore of INS was resuspended in methanol:HCl (95:5, v/v). The absorbance spectra of the chromophore of INS and of biliverdin IX dimethyl ester used as a standard, were measured for comparison of their absorbance maxima using a spectrophotometer (Beckman DU-650, USA).

RESULTS

Purification of INS from the larval hemolymph

INS from the larval hemolymph was purified using three steps of column chromatography. The first step was a hydrophobic interaction chromatography on a Phenyl-Sepharose HP. The elution profile showed two peaks of protein (Fig. 1A). INS was eluted in the fractions 99-107, which corresponded to the second peak. Next, the INS fractions were pooled and applied to a column of Q-Sepharose HP. The elution profile of the Q-Sepharose HP column is shown in Fig. 1B. INS (fractions 47-53) was detected at 0.15 M NaCl. In a further purification step, the INS fractions (3.2 ml) were applied to a Superdex 200 pg gel-filtration column. INS was detected in the fractions 67-80 as a major peak (Fig. 1C) and highly purified at this step.

The purification process of INS from the hemolymph is summarized in Table 1. This purification procedure allowed us to obtain about 22.4 mg of purified INS from 481.1 mg of hemolymph protein, increasing the units/mg about 11.4-fold with 53% recovery.

Determination of the molecular weight of INS

The molecular weight of native *A. convolvuli* INS was determined by gel-filtration with TSKgel G2000SWxL column (Fig. 2). INS had an apparent molecular weight of approximately 59,000 by comparing retention times with those of marker proteins. The INS obtained was also analyzed by SDS-PAGE and was found to be a single band (lane 6, Fig. 3A) with an apoprotein Mr of approximately 26,000 (Fig. 3B). The molecular mass of *Agrius* INS and *M. sexta* INS were 21,213

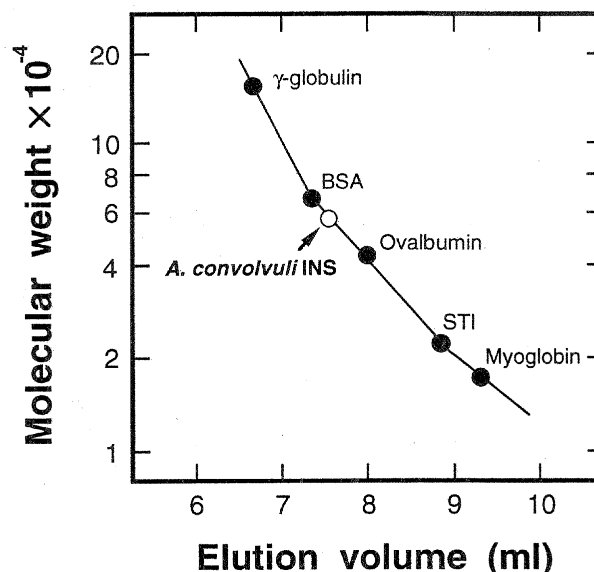

Fig. 2. Molecular weight determination of native *A. convolvuli* INS by gel-filtration. Purified INS and marker proteins were applied to TSKgel G2000SWxL column (0.78 × 30 cm) and eluted with 50 mM sodium phosphate buffer (pH 7.0) containing 0.3 M NaCl. Marker proteins were γ-globulin (160,000), BSA (66,200), ovalbumin (45,000), STI (21,500) and myoglobin (17,000).

and 21,504, respectively. Moreover, the subunit size of *A. convolvuli* INS and *M. sexta* INS are quite similar by SDS-PAGE (Fig. 4). Therefore, the *A. convolvuli* INS molecule was probably assumed to be a trimer in solution.

Amino acid composition of INS

The amino acid composition of *A. convolvuli* INS was determined and compared with other lepidopteran biliverdin-binding proteins (Table 2). The amino acid composition of *A. convolvuli* INS was essentially the same as *M. sexta* INS. However, we also found that there were some differences in amino acid composition between the two INSs, such as the mol % of Asp, Ser, Pro and Cys residues.

N-terminal sequence of INS

N-terminal amino acid sequence of the purified *A. convolvuli* INS was analyzed and compared with those of *M. sexta* INS, *P. brassicae* BBP and *S. cynthia ricini* BBP. *A. convolvuli* INS and *M. sexta* INS showed 97% identity in the first 48

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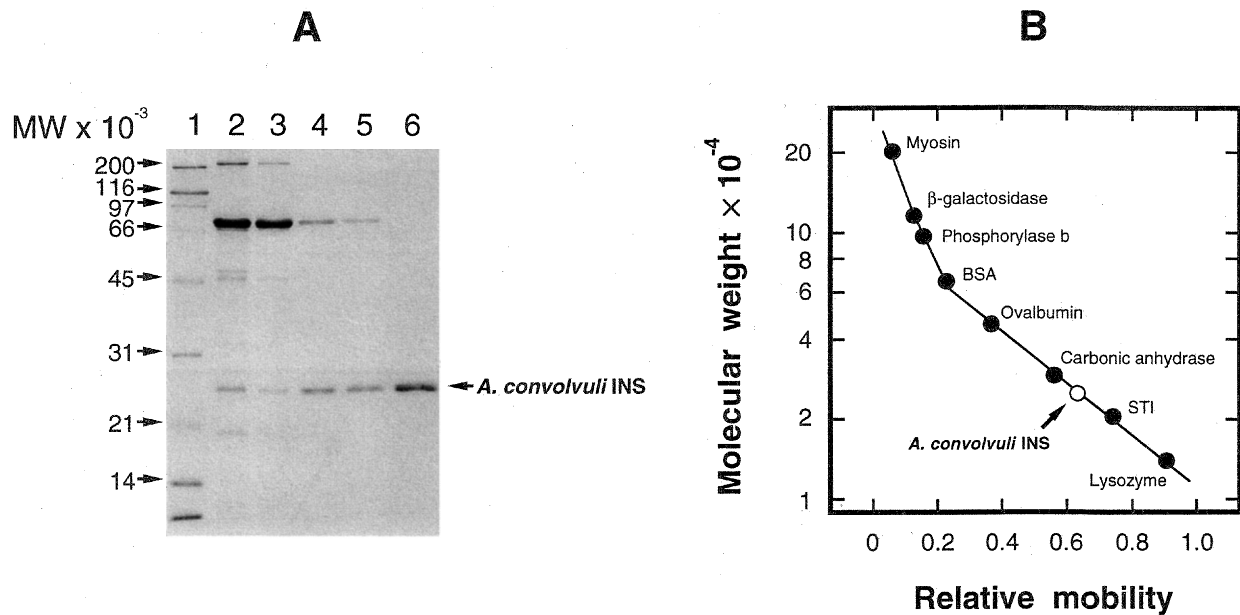

Fig. 3. (**A**) SDS-PAGE of the purification steps of *A. convolvuli* INS from the larval hemolymph. Lane 1, molecular weight markers; lane 2, larval hemolymph (fifth instar, day 3); lane 3, ammonium sulfate precipitate of the hemolymph; lane 4, Phenyl-Sepharose fraction; lane 5, Q-Sepharose fraction; lane 6, Superdex 200 fraction (purified *A. convolvuli* INS). (**B**) Molecular weight determination by SDS-PAGE. Molecular weight marker proteins are myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,400), BSA (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), STI (21,500) and lysozyme (14,400). Open circle indicates the relative mobility value of INS subunit.

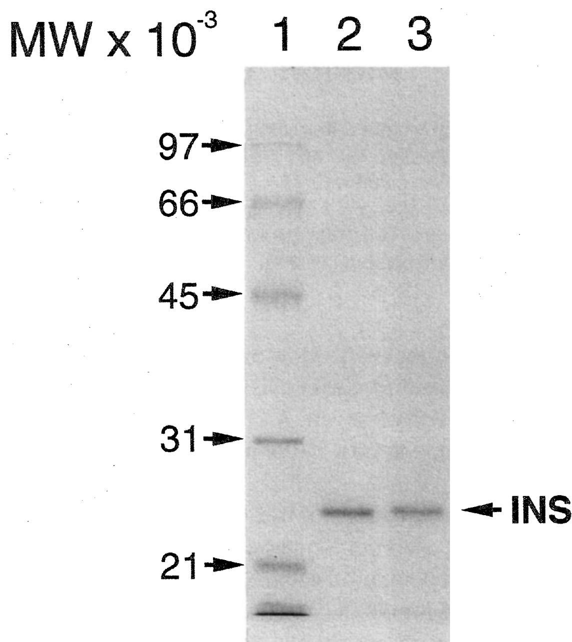

Fig. 4. The comparison of subunit size of *A. convolvuli* INS and *M. sexta* INS by SDS-PAGE. Lane 1, molecular weight markers; lane 2, *A. convolvuli* INS; lane 3, *M. sexta* INS.

residues, with only two amino acid residue differences at 26th [T (threonine)-A (alanine)] and 40th [I (isoleucine)-G (glutamine)] (Fig. 5). *A. convolvuli* INS showed a 46% identity with *P. brassicae* BBP and a 42% identity with *S. cynthia ricini* BBP.

Absorbance maxima of INS and the blue pigments

The absorbance maxima of *A. convolvuli* INS and *M. sexta* INS, the extracted blue pigments from purified INSs, and a standard biliverdin IX dimethyl ester are shown in Table 3. In *M. sexta*, the blue chromophore of INS is the biliverdin IXγ isomer (Goodman *et al.*, 1985). *A. convolvuli* INS had three major absorbance peaks at 277, 377 and 674 nm in buffer A. The blue chromophore was easily extracted with methanol:HCl (95:5 v/v) from the purified INS, indicating that the chromophore is associated non-covalently with the apoprotein. The absorbance maxima of extracted blue chromophore from *A. convolvuli* INS were at 360 and 695 nm, which is consistent with those of biliverdin IXγ from the larval hemolymph of *M. sexta.* Moreover, the absorbance ratio ($\lambda_{max1}/\lambda_{max2}$) of the chromophore from *A. convolvuli* INS (1.60) and biliverdin IXγ from *M. sexta* (1.62) are quite similar (Table 3). These results suggest that the blue chromophore of *A. convolvuli* INS is biliverdin IXγ.

The molar ratio of biliverdin to apoprotein of INS was determined by comparison of the absorbance peak at 674 nm with the absorbance peak of standard biliverdin. This analysis indicated that 1 mg of INS contains 33.3 μg of biliverdin, these amounts are equivalent to 169.5×10^{-7} mmol of INS (Mr=59,000) and 571.2×10^{-7} mmol of biliverdin (Mr=583).

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Table 2. Amino acid composition of the biliverdin-binding proteins (mol %)

Amino acid	*A. convolvuli*	*M. sexta* [1]	*P. brassicae* [2]	*S. cynthia ricini* [3]
Asp	15.9	13.5	5.8	11.3
Glu	7.4	7.1	6.9	6.7
Ser	2.8	4.3	6.9	8.1
Gly	7.5	6.5	8.7	8.3
His	2.8	3.1	3.5	3.5
Arg	1.3	1.0	0.6	4.4
Thr	5.0	4.0	4.0	6.0
Ala	8.7	7.8	4.6	6.7
Pro	5.9	3.7	3.5	3.3
Tyr	7.0	7.0	9.2	4.7
Val	8.9	7.7	11.6	10.9
Met	1.8	0.8	0.0	0.5
Cys	0.1	1.7	2.3	1.3
Ile	4.0	4.2	4.0	4.8
Leu	6.8	5.8	3.5	6.1
Phe	4.4	4.4	2.9	6.2
Lys	9.6	9.4	11.6	6.7

Tryptophan was not determined.
[1]Riley *et al.* (1984).
[2]Suter *et al.* (1988).
[3]Saito (1993).

	1 5 10 15 20 25 30 35 40 45
A. convolvuli **INS**	GDIFYPGYCPDVKPVNDFDLSAFAGTWHEIAKLPLENENIGKCTVAEY
M. sexta **INS**	GDIFYPGYCPDVKPVNDFDLSAFAGAWHEIAKLPLENENEGKCTVAEY
P. brassicae **BBP**	NVYHDGACPEVKPVDNFDWSNYHGKWWEVAKYPNSVEKYGKGGWAEY
S. cynthia ricini **BBP**	DVILDGPCPNFKGVSNFDMKAYQGAWYQITKL

Fig. 5. N-terminal amino acid sequence alignments of *A. convolvuli* INS, *M. sexta* INS (Riley *et al.*, 1984), *P. brassicae* BBP (Huber *et al.*, 1987) and *S. cynthia ricini* BBP (Saito, 1994). Identical residues are boxed.

Table 3. Absorption characteristics of biliverdin IX and of blue chromophore from INS

Compound	Solvent	$\lambda_{max\ 1}$	$\lambda_{max\ 2}$	Ratio[1]
Biliverdin IX dimethyl ester	MeOH:HCl (95:5, v/v)	374 nm (A=1.693)	691 nm (A=0.791)	2.14
M. sexta INS	20 mM Tris-HCl (pH 7.8)	377 nm (A=0.488)	674 nm (A=0.405)	1.20
Biliverdin IXγ from *M. sexta* INS	MeOH:HCl (95:5, v/v)	359 nm (A=0.583)	696 nm (A=0.360)	1.62
A. convolvuli INS	20 mM Tris-HCl (pH 7.8)	377 nm (A=0.305)	674 nm (A=0.256)	1.19
Chromophore from *A. convolvuli* INS	MeOH:HCl (95:5, v/v)	360 nm (A=0.173)	695 nm (A=0.108)	1.60

[1]A at $\lambda_{max\ 1}$/A at $\lambda_{max\ 2}$

Thus, *A. convolvuli* INS molecule has three molecules of biliverdin associated with it, suggesting that one molecule of biliverdin associates with each subunit.

DISCUSSION

Biliverdin-binding proteins have been found in the eggs, hemolymph and integument of several insects. These proteins can be classified into three groups according to their molecular properties. The first group is the insecticyanin (INS) type of proteins, which are composed of a low molecular weight subunits (range from 20,000 to 27,000). This group contains INS of *M. sexta* (Cherbas, 1973; Riley *et al.*, 1984; Goodman *et al.*, 1985), bilin-binding protein of *P. brassicae* (Huber *et al.*, 1987a, b; Suter *et al.*, 1988), the blue chromoprotein of *Antheraea yamamai* (Yamada and Kato, 1989, 1991) and

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biliverdin-binding protein of *S. cynthia ricini* (Saito, 1993, 1994). These proteins are associated non-covalently with biliverdin and are not glycosylated. The second group contains the cyanoproteins (CPs) of hemimetabolous insects, *Locusta migratoria* (Chino *et al.*, 1983; deBruijn *et al.*, 1986; Koopmanschap and deKort, 1988) and *Riptortus clavatus* (Chinzei *et al.*, 1990; Miura *et al.*, 1994), which are composed of subunits of Mr 83,000 and 76,000, respectively. The third group contains the blue chromoproteins from three species of Noctuidae, *H. zea* (Haunerland and Bowers, 1986), *T. ni* (Jones *et al.*, 1988) and *Spodoptera litura* (Yoshiga and Tojo, 1995). The chromoproteins of *H. zea* and *T. ni* have molecular masses of 560,000 and 320,000, respectively, with 150,000 subunits. In *S. litura*, the four biliverdin-binding proteins (BPs) have a native molecular weights of 390,000, and are composed of 150,000 subunits. These proteins differ in several physicochemical properties. Thus, three apparently distinct types of biliverdin-binding proteins have been described in the hemolymph of insects (Kanost *et al.*, 1990).

In many insect species, the hemolymph has a green or bluish-green color throughout their larval life. The green coloration of caterpillar is a result of biliverdin present in the epidermis and cuticle. It is generally believed that the major function of the blue biliprotein is to provide camouflage for protection of the developing larvae (Kawooya *et al.*, 1985; Law and Wells, 1989). *M. sexta* INS is synthesized and stored in the pigment granules in the epidermis and secreted into both the hemolymph and cuticle (Riddiford, 1982; Riddiford *et al.*, 1990). Thus, we presume that INS type of proteins may play an important role in the body coloration of caterpillar as a camouflage. In contrast, the second and third group of proteins frequently contain lipids or carbohydrates and their physiological role is believed to function as storage proteins.

In *M. sexta*, INS has been isolated from the larval hemolymph and epidermis, and this protein is a trimer in solution (Cherbas, 1973; Petratos *et al.*, 1986). The protein (Mr=71,600) is composed of three subunits (Mr=23,000) (Goodman *et al.*, 1985). The amino acid sequence of *M. sexta* INS was determined and its subunit contains 189 amino acid residues of Mr 21,378, and then cross-linking experiments suggest that the protein was a tetramer of four identical subunits (Riley *et al.*, 1984). An X-ray crystallographic study of *M. sexta* INS suggests that it is believed to be a tetramer in solution; There are two subunits per asymmetric unit (Holden *et al.*, 1986, 1987). There is some discrepancy about the subunit composition of *M. sexta* INS. Thus, the molecular structure of INS, *in vivo*, is still open to question.

In the present study, we have purified the biliverdin-binding protein from the larval hemolymph of the sweet potato hornworm, *A. convolvuli*. The molecular weight of native *A. convolvuli* INS was estimated to be 59,000, which was close to that of *Manduca* INS (Mr=60,000), by gel-filtration with TSKgel G2000SWXL column (Fig. 2). Moreover, both INSs are similar in the molecular mass [*A. convolvuli* INS (21,213) and *M. sexta* INS (21,504)] determined by MALDI-TOF/MS. The amino acid composition of INSs show high similarity (Table 2), and the N-terminal sequence of both INSs indicate a 97% identity (Fig. 5). It is concluded from these data that *A. convolvuli* INS is a trimer in solution and an INS-type protein. In addition, it seems likely that *A. convolvuli* INS and *M. sexta* INS have a similar function.

The hemolymph of *A. convolvuli* and *M. sexta* have a green or bluish-green color throughout the larval, pupal and adult stages, and INS is also found in the hemolymph throughout the same stages. Interestingly, the penultimate and last instar larvae of *A. convolvuli* show a population density-dependent changes in body color as a phase polychromatism. The larval body color of the isolated larvae (solitary form) is green, whereas crowded ones (gregarious form) show a dark melanization resulted from a non-transparent black cuticle. This phenomenon cannot be found in the larvae of *M. sexta*. However, *A. convolvuli* INS is present in the hemolymph of both form larvae. Althought the physiological function of *A. convolvuli* INS remains unclear, it seems as if *A. convolvuli* INS in the hemolymph does not function as a camouflage. It will be interesting to study the relation between blue biliprotein of *A. convolvuli* INS and larval color changes. Further structural analysis of *A. convolvuli* INS should give a clue to the understanding of the functionary and evolutionary relationship between *A. convolvuli* INS and the biliproteins from other insects. Determination of the complete amino acid sequence of *A. convolvuli* INS is currently in progress.

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