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Purification and Characterization of Vitellin from the Red Flour Beetle, *Tribolium castaneum* Herbst

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The vitellin of the red flour beetle, *Tribolium castaneum* Herbst was purified and characterized. The vitellin of *T. castaneum* was purified by the FPLC techniques, anion exchange chromatography and gel permeation chromatography. In native-polyacrylamide gel electrophoresis, vitellin of *T. castaneum* was detected as a single band. This native vitellin has molecular weight of 440 kDa. The vitellin of *T. castaneum* is composed of three polypeptides, designated Vn1 (178 kDa), Vn2 (168 kDa) and Vn3 (52 kDa) in SDS-polyacrylamide gel electrophoresis. Three subunits of vitellin were presented in the female adult hemolymph and egg extracts, but not observed in the male. These three polypeptides gradually decreased during embryogenesis. Polyclonal antiserum raised against purified vitellin reacted with the three polypeptides, Vn1, Vn2 and Vn3. Antisera raised against Vn1 and Vn2 cross-reacted with the two large subunits, Vn1 and Vn2, respectively. Another subunit, Vn3, however, was not cross-reacted with these two antisera. Also, antiserum raised against Vn3 did not cross-react with the Vn1 and Vn2.

Key words : Vitellin, Red flour beetle, *Tribolium castaneum* Herbst, Embryogenesis

Introduction

In insects, vitellins are the major yolk proteins, which are served as the nutritive source for the developing embryo during embryogenesis. Their precursors, vitellogenins, are synthesized extraovarially under the regulation of specific hormone in the female fat body (Socha *et al.*, 1991; Wyatt, 1991; Venugopal and Kumar, 2000) and subsequently secreted into the hemolymph. These precursors are taken up by the maturing oocytes via receptor-mediated endocytosis (Telfer *et al.*, 1982; Raikhel and Dhadialla, 1992) and deposited as vitellins (Giorgi *et al.*, 1999).

Insect vitellogenins are large oligomeric phosphoglycolipoproteins with the molecular weight ranging from 210 to 652 kDa (Raikhel and Dhadialla, 1992) and containing subunits of the molecular weight ranging from 50 to 180 kDa (Giorgi *et al.*, 1999). However, vitellins of the higher dipterans are known to compose of oligomers of a single polypeptide subunit (Bownes, 1986; White and Bownes, 1997; Kim *et al.*, 2000a), a homologue of vertebrate lipases (Bownes, 1992).

The vitellogenin and vitellin have been purification and characterized in diverse insects (Wheeler and Kawooya, 1990; Hiremath and Eshita, 1992; Venugopal and Kumar, 1999), including coleopterans (Trewitt *et al.*, 1992; Kim *et al.*, 2000b). In the red flour beetle, an economically important stored-product pest, however, the physiology of vitellogenesis is not reported yet.

Therefore, this study was carried out to obtain physiological information of the red flour beetle, *Tribolium castaneum* Herbst. This knowledge would enable developing effective control strategies against this pest. In the present report, we have described the characteristics of vitellin in the red flour beetle, *T. castaneum*.

Material and Methods

Insects

The red flour beetle, *Tribolium castaneum* Herbst, adults

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were collected at the Cheiljedang Co. Ltd., Pusan in Korea, April 2000. Insects were reared in a growth chamber at 30 ± 1°C with 60 ± 5% of relative humidity under a photoperiod of 12L: 12D. The wheat flour was supplied as a diet.

Preparation of hemolymph and egg extracts

Hemolymph samples of *T. castaneum* were collected from the pterothorax as described (Kim *et al.*, 2000b). The hemolymph was subsequently diluted into phosphate-buffered saline (PBS; 120 mM NaCl, 2 mM KCl, 4.5 mM Na_2HPO_4, 1 mM KH_2PO_4, PH 7.4) containing 5 mM EDTA and 1 mM PMSF. The samples were centrifuged at 13,000 rpm for 20 min at 4°C to remove hemocytes and cell debris. The supernatant was stored -70°C until use. Eggs of *T. castaneum* were homogenized in PBS containing 5 mM EDTA and 1 mM PMSF. The mixture was then centrifuged at 13,000 rpm for 20 min at 4°C and the supernatant was stored at -70 until use.

Staged eggs

To examine development of the embryos, female adults were allowed to fertilize and oviposit in the moisten petri dish, and eggs were incubated at 30 ± 1°C with 60 ± 5% of relative humidity. Five eggs of *T. castaneum* were collected from the dish each day over 5 days post oviposition and were punctured by a fine needle in 40 μl PBS containing 5 mM EDTA and 1 mM PMSF to obtain egg component. After the centrifugation of the component at 13,000 rpm for 20 min at 4°C, the supernatant was used for the SDS-PAGE analysis.

Purification of vitellin

Vitellin (Vn) was purified from the oviposited eggs of *T. castaneum* using FPLC (Pharmacia LKB) techniques. Egg extract was subjected to anion exchange chromatography on a Mono Q HR 5/5 column (Pharmacia LKB) in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF with a flow rate of 1 ml/min. Proteins were eluted in a linear segment sodium chloride gradient (0-0.6 M). The Vn-enriched fractions, identified by SDS-PAGE were subjected to gel permeation chromatography on a Superdex 200 HR 10/30 column (Pharmacia LKB) in 20 mM Tris-HCl buffer (pH 8.0) containing 0.1 mM PMSF with a flow rate of 0.5 ml/min. Fractions containing purified Vn were identified by SDS- and native-PAGE analyses.

Electrophoresis

Native-PAGE was performed in 7.5% gel at 4°C according to the method of Davis (1964). SDS-PAGE was conducted on 10% gel at room temperature according to the method of Laemmli (1970). After electrophoresis, gels were fixed and stained with 0.1% Coomassie brilliant blue R-250. Molecular weight markers [myosin (200,000), β-galactosidase (116,000), phosphorylase b (97,000), bovine serum albumin (66,000), egg albumin (45,000) and carbonic anhydrase (31,000); Bio-Rad] were used as standards.

Preparation of polyclonal antibody

The purified vitellin of *T. castaneum* was separated on the SDS-PAGE gel. After staining the gel lightly with Coomassie blue, three vitellin bands were cut off, and each was homogenized, mixed with equal volume of Freunds complete adjuvant (a total of 200 μl), and injected into Balb/c mice. Three successive injections were performed with one-week interval beginning a week after the first injection with antigens mixed with equal volume of Freunds incomplete adjuvant (a total of 200 μl). Bloods were collected 3 days after the last injection and centrifuged at 13,000 rpm for 5 min. The supernatant antisera were stored at -70 until use.

Western blot analysis

For Western blot analysis, SDS-PAGE was carried out as described above. Proteins were blotted to a sheet of nitrocellulose membrane (Sigma, 0.45 μm of pore size) (Towbin *et al.*, 1979). The blotting was performed in transfer buffer (25 mM Tris and 192 mM glycine in 20% methanol) at 30 volt overnight at 4°C. After blotting, the membrane was blocked by incubation in 1% BSA solution for 2 hr at room temperature. The blocked membrane was incubated with Vn antiserum solution (1:1,000 v/v) for 1 hr at room temperature and washed in TBST (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.05% Tween 20). Subsequently, the membrane was incubated with goat anti-mouse IgG alkaline phosphatase conjugate (1:10,000 v/v, Sigma) for 30 min at room temperature. After repeated washing, substrate solution (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM $MgCl_2$) containing NBT (nitro-blue tetrazolium) and BCIP (5-bromo-4-chloroindolyl phosphate) was added. The reaction was quenched with distilled water.

Determination of Molecular weight

The native molecular weight of vitellin was determined by gel filtration chromatography as described (Venugopal and Kumar, 1999). Standard proteins [thyroglobulin (669,000), ferritin (440,000), catalase (232,000), aldolase (158,000) bovine serum albumin (67,000), ovalbumin (43,000), chymototypsinogen A (25,000); Pharmacia LKB] were separately layered onto a Superdex 200 HR 10/30 column (Pharmacia LKB) and eluted in 20 mM Tris-HCl buffer (pH 8.0) with a flow rate of 0.5 ml/min.

Results and Discussion

The red flour beetle, *T. castaneum* Herbst, vitellin was characterized by electrophoresis and FPLC. The egg extract and adult hemolymphs were subjected to native-PAGE (Fig. 1A). Major protein, a single band, was detected in the female hemolymph and the egg extract, but not observed in the male (Fig. 1A). This protein band was probably considered as vitellin in eggs, because vitellin is present as a single phospholipoglycoprotein in most insect eggs (Oliveira *et al.*, 1989; Postlethwait and Giorgi, 1985; Zhu *et al.*, 1986). Also, in the previous paper (Kim *et al.*, 2000b), we reported the firefly vitellins were detected in eggs with a single band on the native gel. In the SDS-PAGE analysis, three major protein bands were detected in egg and were also detected in the female adult hemolymph with a same electrophoretic mobility, but not observed in the male (Fig. 1B). These three protein bands were regarded as subunits of vitellin. Therefore, the vitellin of *T. castaneum* is composed of three subunits, designated Vn1 (178 kDa), Vn2 (168 kDa) and Vn3 (52 kDa) in SDS-PAGE (Fig. 1B).

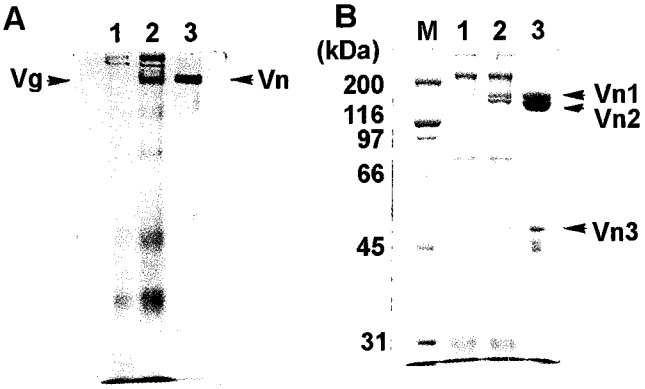

Fig. 1. Native- (A) and SDS-PAGE (B) analyses of proteins from adult male hemolymph (lane 1), adult female hemolymph (lane 2) and egg extracts (lane 3) of *T. castaneum*. Subunits (Vn1 and Vn2 for a large subunits, and Vn3 for a small subunit) of vitellin (Vn) are represented on the right of each panel. Molecular weight markers (M) are indicated.

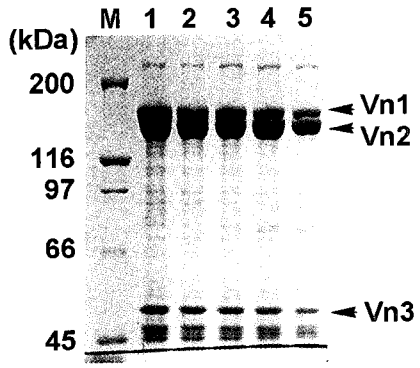

Fig. 2. SDS-PAGE analysis of the staged eggs of *T. castaneum*. Lane 1, Oviposited egg; Lane 2, 1 day-old egg; Lane 3, 2 day-old egg; Lane 4, 3 day-old egg; Lane 5, 4 day-old egg. Subunits (Vn1 and Vn2 for a large subunits, and Vn3 for a small subunit) of vitellin are represented on the right of panel. Molecular weight markers (M) are indicated.

To confirm the vitellin of *T. castaneum*, the staged eggs were analyzed by SDS-PAGE (Fig. 2). Three subunits of Vn bands gradually decreased during embryogenesis. This result is similar to that of many insects (Oliveira *et al.*, 1989, Kim *et al.*, 2000a,b,). Therefore, it is confirmed that these three proteins clearly represent subunits of vitellin.

The vitellins from various insects have been purified by using ultracentrifugation, gel-filtration, hydroxyl apatite chromatography and ion-exchange chromatography (Hiremath and Eshita, 1992; Venugopal and Kumar, 1999; Wheeler and Kawooya, 1990). In this study, vitellin of *T. castaneum* was purified using FPLC techniques (Fig. 3).

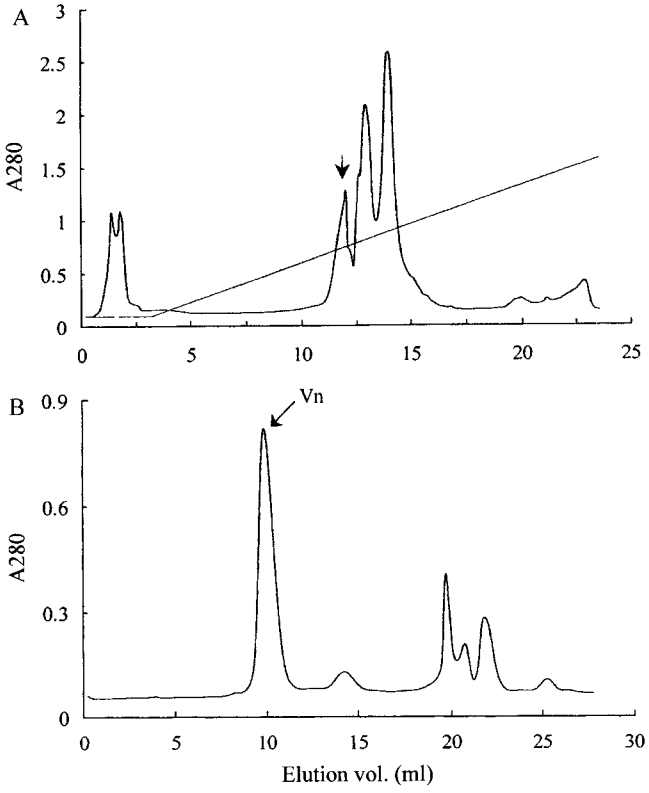

Fig. 3. Purification of vitellin from the *T. castaneum* using FPLC techniques. Egg extract of *T. castaneum* was applied to Mono Q HR anion-exchange chromatography column (A). Arrow indicates vitellin (Vn)-enriched peak. The Vn-enriched fraction was applied to Superdex 200 HR gel permeation chromatography column (B).

The egg extract was applied to Mono Q HR anion-exchange chromatography column (Fig. 3A). The Vn-enriched peak, identified by SDS-PAGE (data not shown), was eluted at 0.31 M NaCl. This Peak fraction was then applied to Superdex 200 HR column gel permeation chromatography (Fig. 3B). The remaining other proteins were separated during gel permeation chromatography and vitellin was highly purified by this procedure in a short time.

The purified vitellin was detected as a single band in native-PAGE analysis (Fig. 4A). In SDS-PAGE analysis, this purified vitellin was confirmed that it consists of three subunits with the molecular weights of about 178 kDa, 168 kDa and 52 kDa (Fig. 4B). The molecular weight of native vitellin of *T. castaneum* was estimated to approximately 440 kDa by gel permeation chromatography (Fig. 5). This method has been reported for determine molecular weights of native vitellogenin in other insects (Osir *et al.*, 1986; Venugopal and Kumar, 1999).

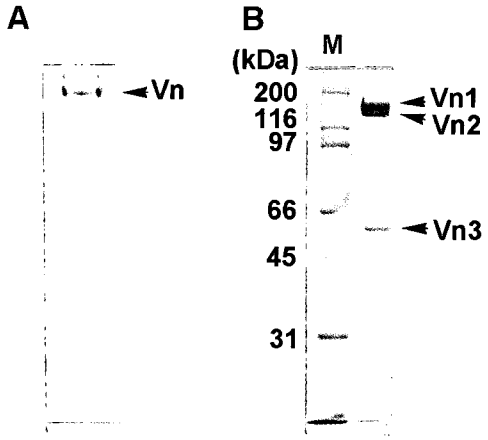

Fig. 4. Molecular weight determination of vitellin by Superdex 200 HR gel permeation chromatography column. Ve, elution volume of protein; Vo, void volume of column by blue dextran. Molecular weight standards: 1, thyroglobulin (669,000); 2, ferritin (440,000); 3, catalase (232,000); 4, aldolase (158,000); 5, bovine serum albumin (67,000); 6, ovalbumin (43,000); 7, chymototypsinogen A (25,000).

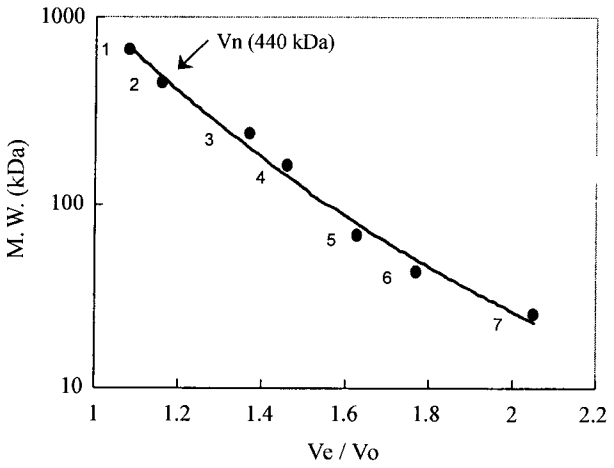

Fig. 5. Native- (A) and SDS-PAGE (B) analyses of purified vitellin from the eggs of *T. castaneum*. Subunits (Vn1 and Vn2 for a large subunits, and Vn3 for a small subunit) of vitellin (Vn) are represented on the right of each panel. Molecular weight markers (M) are indicated.

Western blot analysis using the polyclonal antiserum against purified vitellin showed that three subunits of vitellin bands reacted in the female adult hemolymph and egg extract, respectively (Fig. 6A). However, this reactivity was not observed in the male adult hemolymph. Also, Western blot analysis using each of antiserum against Vn1 and Vn2 indicated that the two large subunits, Vn1 and Vn2, are immunologically related to each other (Fig. 6B). The anti-Vn1 and anti-Vn2 antisera strongly cross-reacted with the Vn1 and Vn2 subunits, respectively. However, The small subunit, Vn3, was not cross-reacted with these two antisera. This is similar to the vitellogenin subunits in *Lymantria dispar*, where two antisera against the large subunits do not recognize the small subunit (Hiremath and Eshita, 1992). Also, anti-Vn3 antiserum did not cross-react with Vn1 and Vn2 subunits (Fig 6B).

In conclusion, these results have shown the vitellin profiles in *T. castaneum*. More detailed studies are needed to determine the gene structure of vitellogenin.

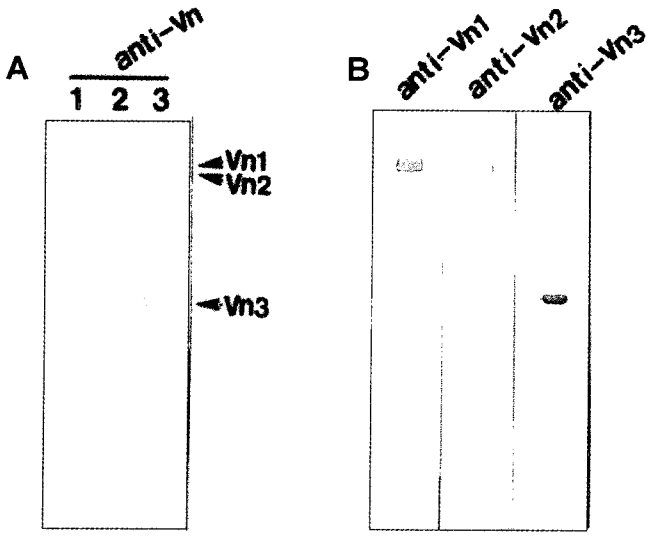

Fig. 6. Western blot analysis from adult hemolymphs and egg extract in T. castaneum. Western blot analysis was used antiserum against purified native vitellin (Vn), Vn1, Vn2 and Vn3, respectively. Panel A, Lane 1, adult male hemolymph; Lane 2, adult female hemolymph; Lane 3, egg extract. Panel B, egg extracts. Subunits (Vn1 and Vn2 for a large subunits, and Vn3 for a small subunit) of vitellin are represented on the right of panel.

Acknowledgments

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