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# Purification and Characterization of Vitellin from the Firefly, *Pyrocoelia rufa*

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**The vitellin of firefly, *Pyrocoelia rufa*, is composed of three polypeptides, designated Vn1 (175 kDa), Vn2 (160 kDa) and Vn3 (45 kDa) in SDS-polyacrylamide gel electrophoresis. Three subunits of vitellin were presented in the female adult hemolymph, ovary and egg extracts, but not observed in the male. This vitellin was purified from the eggs of *P. rufa* by the FPLC techniques, anion exchange chromatography and gel permeation chromatography. In nature, vitellin of *P. rufa* has molecular weight of 400 kDa. Western blot analysis using polyclonal antiserum against purified vitellin showed that the antiserum was reacted with the three polypeptides, Vn1, Vn2 and Vn3 from the female adult hemolymph, ovary and egg extracts. Amino acid residues at N-terminus of three subunits were sequenced. The N-terminal sequences of large subunits, Vn1 and Vn2, were similar to each other. But, the N-terminal sequences of small subunit, Vn3, did not have any significant homology with large subunits.**

**Key words** : Vitellin, Firefly, *Pyrocoelia rufa*, FPLC, N-terminal amino acid sequence

## Introduction

In general, most insect eggs contain a number of diverse proteins. In the silkworm *Bombyx mori*, egg contain three different proteins, a tetrameric vitellin of about 420 kDa, a trimeric egg-specific protein (ESP) of about 225 kDa and a mixture of three monomeric polypetides of about 30 kDa (Zhu *et al.*, 1986). Especially, vitellins are characterized by the major yolk proteins, which are served as the nutritive source for developing embryo during embryogenesis (Kunkel *et al.*, 1985). These insect vitellins are synthesized as single or multiple precursors, vitellogenins, under regulation of specific hormone in the female fat body and secreted into the hemolymph (Socha *et al.*, 1991; Wyatt, 1991; Venugopal and Kumar, 2000). Following secretion into the hemolymph, vitellogenins are sequestrated by the developing oocytes via the receptor-mediated endocytosis (Telfer *et al.*, 1982; Raikhel and Dhadialla, 1992) and deposited as vitellins in the ovary.

The major source of nutrients in the eggs, vitellins, and their precursors, vitellogenins, have been investigated extensively in relation to physiology, endocrinology, biochemistry and molecular biology from various insect species (Bownes, 1986; Giorgi *et al.*, 1999; Kim *et al.*, 2000a; Okuno *et al.*, 2000) and coleopteran insects are no exception (Trewitt *et al.*, 1992; Kim *et al.*, 2001). Also, we have studied previously the characterization of vitellin from the firefly species, *Luciola unmunsana* and *L. lateralis*, which belong to the order coleoptera (Kim *et al.*, 2000b).

*P. rufa* is an abundant firefly species in Korea and is also found in China and only at Tsushima in case of Japan (Suzuki, 2001). The nucleotide sequence of a cDNA encoding the luciferase of *P. rufa* have been reported (Lee *et al.*, 2001). However, the physiological foundation of *P. rufa* is not available currently. In the present study, we have described the purification and characteristics of vitellin in firefly, *Pyrocoelia rufa*.

## Material and Methods

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**Insects**

The firefly, *Pyrocoelia rufa*, adults were collected at Miryang, Kyungsangnam Province in Korea, September 2000. For the oviposition, the firefly was reared under the conditions described previously (Kim *et al.*, 2000b).

**Preparation of hemolymph, ovary, fat body and egg extracts**

Hemolymph was collected by puncturing pterothorax of adults with a fine needle. This 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 solution containing hemolymph was then 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. Ovary and fat body of adults were dissected in the PBS. These samples were rinsed three times with PBS and then homogenized in PBS containing 5 mM EDTA and 1 mM PMSF. The mixtures were then centrifuged at 13,000 rpm for 20 min at 4°C and the supernatants were stored at -70°C until use. Eggs 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.

**Electrophoresis**

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.

**Purification of vitellin**

Vitellin (Vn) was purified from the oviposited eggs of *P. rufa* 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-PAGE analysis.

**Preparation of polyclonal antibody**

The purified vitellin of *P. rufa* was 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 5min. 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 hrs 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.

**N-terminal amino acid sequencing**

The purified vitellin of *P. rufa* was subjected to 10% SDS-PAGE. For N-terminal amino acid sequencing, the protein was electroblotted onto PVDF (polyvinylidene difluoride) membrane in 10 mM CAPS 3-(cyclohexylamino)-1-propanesulfonic acid) buffer and 10% methanol at 50 volt for 45 min at room temperature. Protein samples on PVDF membrane were detected in amido black staining solution (0.1% amido black 10B in 1% acetic acid/40% methanol). The subunits of Vn (S-Vn) were excised from the membrane and then analyzed by protein sequencer (PE

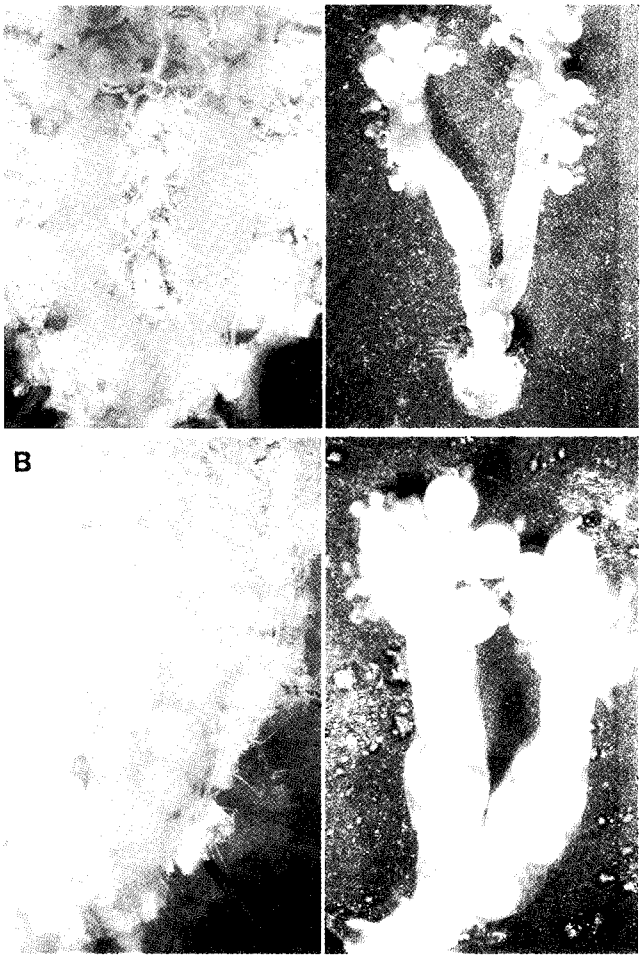


**Fig. 1.** Ovarian development of the firefly, *P. rufa*. A, 2 day-old adult ovary; B, 15 day-old adult ovary.

Applied Biosystems).

## Results

The ovaries of firefly, *P. rufa*, were observed at adult stages (Fig. 1). The ovary of 2 day-old adult was not yet matured (Fig. 1A), but the ovary was matured completely at the 15 day-old adult (Fig. 1B).

For identification of vitellin in *P. rufa* , the fat bodies, hemolymphs of the female and male adult, ovary extracts and egg extracts were analyzed by SDS-PAGE (Fig. 2). In the ovary extracts and egg extracts, three major protein bands with the molecular weights of about 175 kDa, 160 kDa and 45 kDa were detected. These protein bands were also detected in the female adult hemolymph with a same electrophoretic mobility, but not observed in the male. This result showed that the vitellin of *P. rufa* is composed of three subunits, designated Vn1 (175 kDa), Vn2 (160 kDa) and Vn3 (45 kDa).

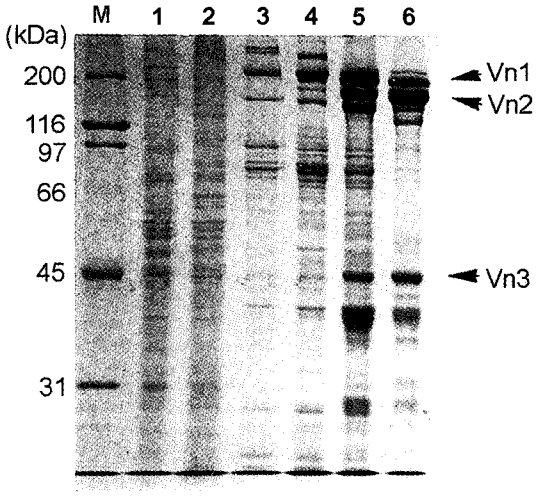


**Fig. 2.** Identification of *P. rufa* vitellin (Vn) using 10% SDS-PAGE. Lane 1, male adult fat body; lane 2, female adult fat body; lane 3, male adult hemolymph; lane 4, female adult hemolymph; lane 5, ovary extract; lane 6, egg extract. M indicates molecular weight markers.

Vitellin of *P. rufa* was purified using FPLC techniques, anion exchage chromatography and gel permeation chromatography (Fig. 3). The Mono Q HR column chromatography separated vitellin from the egg extract of *P. rufa* (Fig. 3A). Vitellin was eluted as a single peak at 0.42 M NaCl. This vitellin-enriched peak fraction, identified by SDS-PAGE (data not shown), was then applied to Superdex 200 HR column chromatography for highly purification (Fig. 3B). Highly purified vitellin was identified that consisted of three subunits with the molecular weights of about 175 kDa, 165 kDa and 45 kDa in SDS-PAGE (Fig. 4). The molecular weight of native vitellin of *P. rufa* was estimated to approximately 400 kDa by gel permeation chromatography (Fig. 5).

In Western blot analysis, the polyclonal antiserum against purified vitellin strongly reacted with three subunits of vitellin in the female adult hemolymph, ovary and egg extracts, respectively (Fig. 6). However, this reactivity was not observed in the male adult hemolymph.

Amino acid residues at N-terminus of three subunits, Vn1, Vn2 and Vn3, were sequenced after blotting on a PVDF membrane, respectively (Table 1). The N-terminal sequences of large subunits, Vn1 and Vn2, were similar to each other. But, the N-terminal sequences of small subunit, Vn3, did not have any significant homology with large subunits.

## Discussion

We have identified and characterized the vitellin of the

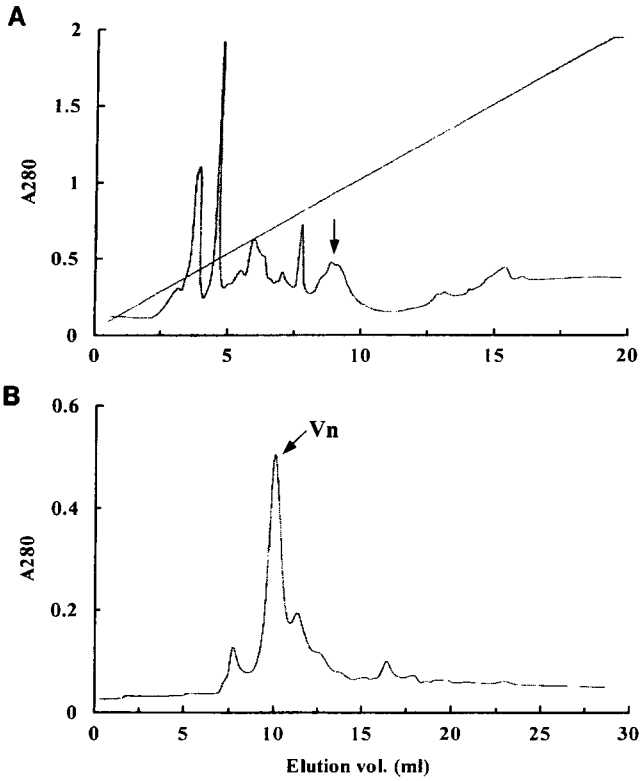


**Fig. 3.** Purification of vitellin from the *P. rufa* using FPLC techniques. Egg extract of *P. rufa* was applied to Mono Q HR anion-exchange chromatography column (A). Arrow indicates vitellin (Vn)-enriched peak. The Vn-enriched fraction was applied to Superdex 200 HR gel permeation chromatography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

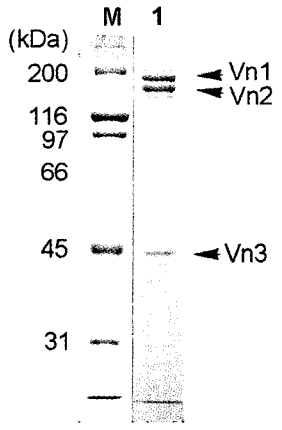


**Fig. 4.** Identification of purified vitellin (Vn) from the egg extract of *P. rufa* using 10% SDS-PAGE. Lane 1, purified vitellin. M indicates molecular weight markers.

firefly, *P. rufa*. *P. rufa* vitellin is composed of three subunits, designated Vn1 (175 kDa), Vn2 (160 kDa) and Vn3 (45 kDa), and these vitellin subunits were detected in egg and female adult hemolymph with a same electrophoretic

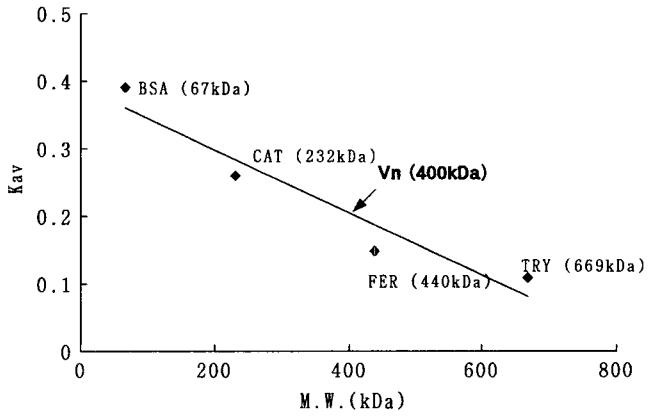


**Fig. 5.** Molecular weight determination of vitellin by Superdex 200 HR gel permeation chromatography column. Molecular weight standards: TRY, thyroglobulin (669,000); FER, ferritin (440,000); CAT, catalase (232,000); BSA, bovine serum albumin (67,000).

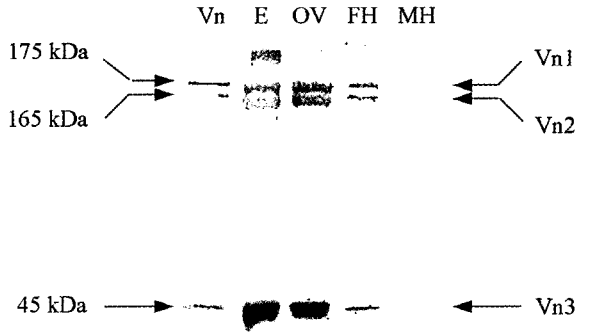


**Fig. 6.** Western blot analysis using antiserum against purified vitellin of *P. rufa*. Vn, purified vitellin; E, egg extract; OV, ovary extract; FH, female adult hemolymph; MH, male adult hemolymph.

**Table 1.** N-terminal amino acid sequences of vitellin subunits in the firefly, *P. rufa*

| Protein | N-terminal sequence |
|---|---|
| Vn1 (175 kDa) | SYNNTNPAWKQ |
| Vn2 (160 kDa) | SYNNTNPAWKQ |
| Vn3 (45 kDa) | YQQQGYPQQYY |

mobility.

Insect vitellins are generally classified into three groups based on their subunit size (Harnish and White, 1982). The first group has both large subunits (100-180 kDa) and small subunits (47-80 kDa), group-2 has only large subunits (170-190 kDa) and group-3 has only small subunits (50 kDa). Accordingly, *P. rufa* vitellin belongs to the first group as it consists of both large and small polypeptides. Several vitellin subunits have been identified in coleopetran insects. Vitellin of the boll weevil, *Anthonomus grandis*, has large

subunits of 160 kDa and small subunit of 47 kDa (Trewitt *et al.*, 1992). The red flour beetle, *Tribolium castaneum*, vitellin has a native molecular weight of 440 kDa with subunits of 178 kDa, 168 kDa and 52 kDa (Kim *et al.*, 2001). Molecular weight and subunit composition of *P. rufa* vitellin are similar to those of red flour beetle, *T. castaneum*, vitellin. In the previous papar (Kim *et al.*, 2000), however, two other species of the firefly, *Luciola unmunsana* and *L. lateralis* vitellins has only large subunits with molecular weight of 195 kDa and 185 kDa, and 195 kDa and 180 kDa, respectively. Although *P. rufa* be- longs to the firefly with *L. unmunsana and L. lateralis*, molecular weight and subunit composition of *P. rufa* vitellin is dissimilar to those of *L. unmunsana* and *L. lateralis*.

Until now, vitellins have been purified from various insects by using ultracentrifugation, affinity chromatography, gel filtration chromatography, hydroxyl apatite chromatography and ion exchange chromatography (Hiremath and Eshita, 1992; Kim *et al*, 2001; Okuno *et al*, 2000; Venugopal and Kumar, 1999; Wheeler and Kawooya, 1990). In this report, vitellin of *P. rufa* was purified using FPLC techniques by two-step purification, gel filtration chromatography and ion exchange chromatography. In this purification, we obtained the molecular weight of native vitellin was approximately 400 kDa by gel filtration chromatography. This method has been reported for determine molecular weights of native vitellogenin and vitellin in other insects (Kim *et al.*, 2001; Osir *et al.*, 1986; Venugopal and Kumar, 1999).

In the present experiment, the N-terminal amino acid sequences of the vitellin subunits in *P. rufa* demonstrated that Vn1 (175 kDa) and Vn2 (160 kDa) shared the same N-terminal sequence. This is similar to the N-terminal sequences of vitellogenin subunits in *Lymantria dispar* (Hiremath and eshita, 1992). The N-terminal sequences provide that Vn2 may be derived from Vn1. Therefore, we assumed that the vitellin of *P. rufa* is a heterogeneous mixture of oligomeric proteins, some consisting of Vn1 (175 kDa) and Vn3 (45 kDa), and some consisting of Vn2 (160 kDa) and Vn3 (45 kDa). More detailed studies are needed to determine the structure of native vitellin in the firefly, *P. rufa*.

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