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Synthesis and Fate of Yolk Protein-3 in *Hyphantria cunea* D.

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Yolk protein-3 (YP3) was purified from the ovary of *Hyphantria cunea* D. and the synthesis and fate during embryogenesis of YP3 were investigated by electrophoresis and fluorography. YP3 purified through gel slice and electrophoretic elution was determined to have M. W. of 18 Kd and consist of one subunit. Haemolymph and fat body of male and female were electrophoresed during vitellogenic stages to indentify the vitellogenin in female. The result showed that there was no distinct difference in electrophoretic patterns between male and female. However, tissue culture of fat body and maturing ovary indicated that YP3 was synthesized by fat body. Also, YP3 in laid eggs was maintained constant until day 6 after oviposition and then decreased, indicating that YP3 was drastically used during late embryogenesis. However, a part of YP3 was present even in newly hatched first instar larvae.

KEY WORDS: *Hyphantria cunea*, Yolk protein, Embryogenesis, Electrophoresis, Fluorography

Yolk proteins (vitellin) used as nutrient sources during embryogenesis are mostly synthesized by fat body and released into the haemolymph and then absorbed by maturing ovary (Wyatt and Pan, 1978; Hagedorn and Kunkel, 1979). In some insects, however, yolk proteins (paravitellin or egg specific protein) are synthesized by ovary (Telfer *et al.*, 1980; Irie and Yamashita, 1983; Chen *et al.*, 1987). Synthetic site of yolk protein was detected by electrophoretic and immunological methods and autoradiography. Generally, synthetic site of vitellogenin, precursor of vitellin can be traced with the difference of electrophoretic pattern between male and female due to female specificity and more clearly with autoradiography (Fourney *et al.*, 1982; Pereira and Bianchi, 1983; Borovsky and Whitney, 1987).

Yolk proteins are degraded to be used as nutrient of embryo and for organogenesis during embryongenesis (Indrasith *et al.*, 1987; Masetti and Giorgi, 1989; Nordin *et al.*, 1990). Also, the time course and rate of the degradation is known to be different with species and kind of yolk proteins within the same species (Masetti and Giorgi, 1989). This process seems to be controlled in order and preceded by trimming and dephosphorylation (Purcell *et al.*, 1988; Oliveira *et al.*, 1989).

Hyphantria cunea Drury was reported to have three kinds of major yolk proteins (YP1, YP2, YP3), and the synthesis and characterization of YP1 and YP2 were already reported (Han and Kim, 1986; Lee *et al.*, 1988). However, the synthetic site of YP3 was not confirmed because of some disadvantages due to small molecular weight. The present work reports on the purification, synthetic site and the fate of yolk protein-3 in *Hyphantria cunea* Drury.

Materials and Methods

Insects

Hyphantria cunea Drury were reared on artificial diet (Dongbang Co.) under the condition of 27 ± 1°C, 75 ± 5% RH and 16L : 8D photoperiod.

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Chemicals

Standard molecular markers were purchased from BRL. [^{3}H]-leucine (250 μCi/mM) was from New England Nuclear. Ampholines (pH 3-10 and 5-7) were from Bio-Rad. Electrophoresis reagents were from Sigma. All other reagents used in the present study were analytical grade.

Collection of Haemolymph

Haemolymph was collected by puncturing the pupal head with a sharp needle and small amounts of phenylthiourea (PTU) added to prevent melanization. Haemolymph was centrifuged at 8,000 g at 4°C for 15 min to remove hemocytes and tissue debris and then stored at −70°C until use.

Preparation of Ovary, Fat body and Oviposited Egg Extracts

Ovaries were dissected out from female adult and rinsed in Ringer solution (128 mM NaCl, 1.8 mM $CaCl_2$, 1.3 mM KCl; pH 7.4) and homogenized in sample buffer (50 mM Tris-HCl buffer, pH 6.8). The homogenate was centrifuged at 8,000 g at 4°C for 30 min and then the overlying lipid layer was carefully removed. The supernatant was used as the sample (10 mg protein/ml). Fat body was extracted from female and male pupae and treated as above. Oviposited eggs were homogenized in sample buffer (0.5 g/ml) and centrifuged at 10,000 g at 4°C for 30 min and the supernatant was used as the sample.

Electrophoresis

Non-SDS polyacrylamide gel electrophoresis (PAGE) was carried out on 7.5% polyacrylamide gel at the constant current of 3 mA per gel as described by Davis (1964). SDS-PAGE was conducted on 8-12% linear gradient polyacrylamide gel at the current of 30 mA according to Laemmli (1970). Two dimensional gel electrophoresis was carried out as described by O'Farrell (1975). First electrophoresis was conducted on 4% polyacrylamide gel including 2% ampholytes (pH 3-10, 0.4%; pH 5-7, 1.6%) at 350 V for 18 hrs for isoelectric focusing and then runned again at 800 V for additional 1 hr. Second electrophoresis was runned on 10-16% concave exponential gradient gel according to the procedure described in SDS PAGE. After electrophoresis, gel was stained in 0.25% Coomassie brilliant blue R 250 and then destained in 50% methanol.

Purification of Yolk Protein-3

Ovary extracts were electrophoresed on 7.5% non-SDS gel and the band equivalent to YP3 was sliced. The sliced bands were put into dialysis sac and electrophoresed at 200 V at 4°C for 2 hrs for electrophoretic elution.

Determination of Molecular Weight

The molecular weight of YP3 subunit was determined on 8-12% linear gradient SDS PAGE as described by Lambin *et al.* (1976). Standard molecular weight markers used was lysozyme (14,300), β-lactoglobulin (18,400), α-chymotrypsinogen (25,700), ovalbumin (43,000), bovine serum albumin (68,000) and phosphorylase b (97,400).

Tissue Culture and Fluorography

Fat bodies and ovaries were dissected out from 8 day old female pupae and incubated in eppendorf tube containing 100 μl of Grace's insect medium and 25 μCi of [^{3}H]-leucine at 30°C for 4 hrs. After incubation, the fat bodies and ovaries were homogenized in sample buffer (62 mM Tris-HCl, pH 6.8) and then the proteins were precipitated by addition of 80% acetone. The precipitates were dissolved in the sample buffer and centrifuged at 5,000 g for 20 min. The supernatant was used for two dimensional electrophoresis. After destaining in 50% methanol, gel was placed in 1 M sodium salicylic acid for 30 min and dried. The gel was exposed to X-ray film at −70°C for 15 days.

Results

Identification and Synthesis of Yolk Protein-3 (YP3)

Paupal haemolymph of male and female was electrophoresed on 7.5% non-SDS polyacrylamide gel, but the result showed that there was no distinct vitellogenin in female haemolymph in any stages (Fig. 1). SDS-PAGE also showed that there

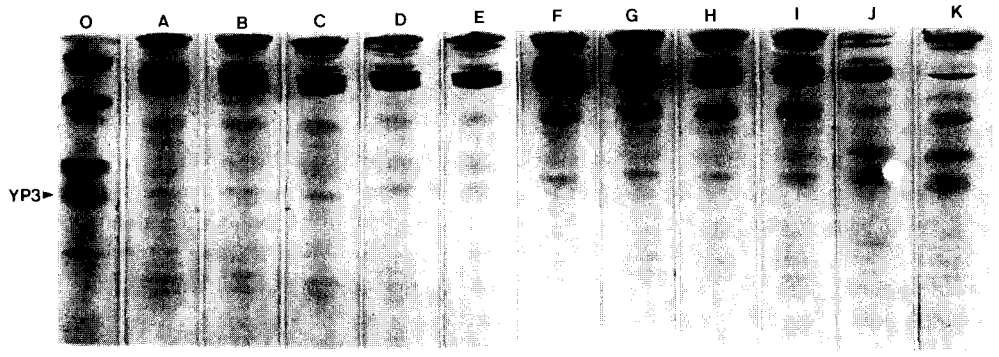

Fig. 1. Non SDS PAGE of haemolymph and ovary extracts of *Hyphantria cunea* D. O, ovary extracts (15 μl); A-E, male haemolymph of 0,2,4,6,8-day old pupae, respectively; F-J, female haemolymph of 0,2,4,6,8-day old pupae, respectively; K, female haemolymph of adults; Seven μl of haemolymph was applied on each well. The arrow indicates Yolk protein-3.

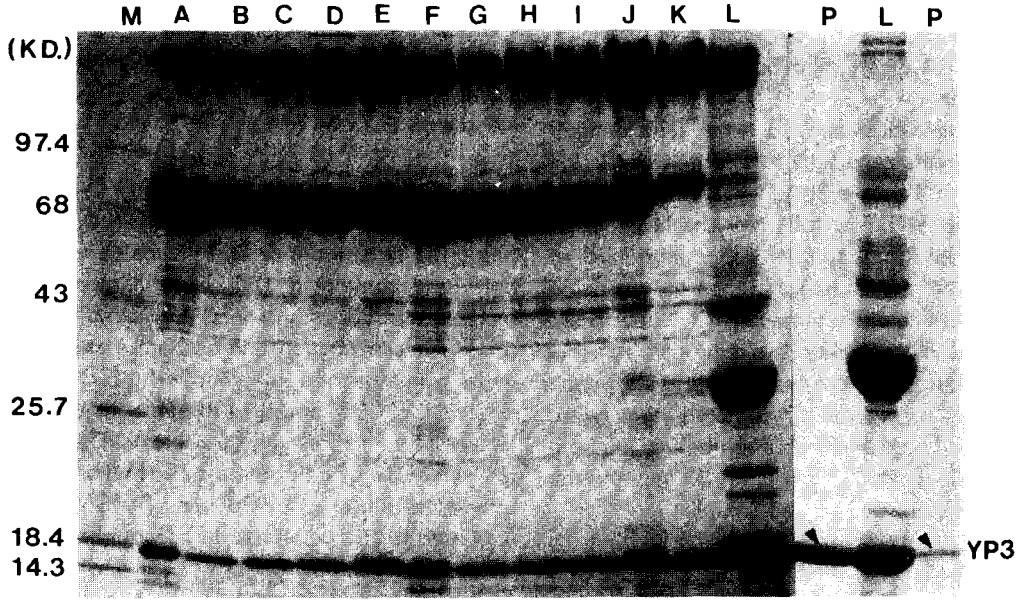

Fig. 2. SDS PAGE of haemolymph and ovary extracts of *H. cunea* D. A-E, male haemolymph of 0,2,4,6,8-day old pupae, respectively; F-J, female haemolymph of 0,2,4,6,8-old pupae, respectively; K, female haemolymph of adults; L, ovary extracts (5 μl); P, purified YP3; M, marker proteins (lysozyme, 14,300; β-lactoglobulin, 18,400; α-chymotrypsinogen, 25,700; ovalbumin, 43,000; bovine serum albumin, 68,000; phosphorylase b, 97,000); Two μl of haemolymph was applied on each well. The arrow indicates YP3.

were no female specific bands in female haemolymph during the whole pupal stages (Fig. 2). Fat body showed similar results by SDS-PAGE (Fig. 3). Thus, vitellogenin in female haemolymph could not be clearly identified by traditional electrophoresis. Therefore, the presence of YP3 vitellogenin in female haemolymph was further investigated by other method. YP3 purified by gel slice and electrophoretic elution was electroporesed on 8-12% SDS gel with standard molecular markers. Molecular weight of YP3 subunit was estimated to be 18 Kd (Fig. 2). Purified YP3 was used for two dimensional PAGE to locate the spot for YP3. At least, one spot was present at lower part on the left side (Fig. 4B). Same spot was obtained from ovary extract by two dimensional electrophoresis

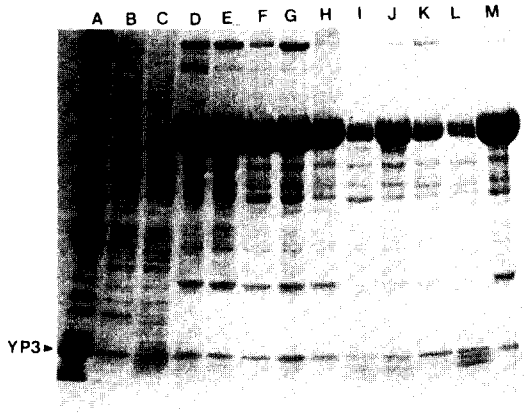

Fig. 3. SDS PAGE of fat bodies and ovary extracts of *H. cunea* D. A, ovary extracts; B-C, ovary extracts of 6,8-day old pupae, respectively; D-H, female fat bodies of 0,2,4,6,8-day old pupae, respectively; I-M, male fat bodies of 0,2,4,6,8-day old pupae, respectively.

(Fig. 4A). Slab gels for fat bodies and ovaries of 8 day old female pupae were exposed to X-ray film, showing that spot equivalent to YP3 was present in fat body but not in ovary (Fig. 5). This result indicates that fat body is the synthetic site for YP3 and also YP3 is present in small amounts in female haemolymph because YP3 should pass through haemolymph to reach the ovary.

Fate of YP3

Newly oviposited egg extracts showed the electrophoretic pattern similar to the one of ovary extract (Figure not show..). YP3 was constantly maintained during the period of days 1-6 after oviposition. Other proteins also showed constant pattern and new proteins did not appear during this period. However, YP3 drastically decreased during the period of days 6-8 after oviposition, indicating that YP3 was drastically degraded during this period (Figs. 6, 7). However, YP3 was present even in small amounts until newly hatched first instar larvae.

Discussion

Yolk proteins are synthesized in most insects by fat body (Wyatt and Pan, 1978; Englemann, 1979; Hagedorn and kunkel, 1979) but in some insects by ovary (Brennan *et al.*, 1982; Kambysellis *et al.*, 1986; Chen *et al.*, 1987). Generally, vitellogenin synthesized by fat body is released into haemolymph to be absorbed by ovary. Therefore, vitellogenin can be identified in female haemolymph by electrophoresis (Gavin and Williamson, 1976; Izumi and Tomino, 1980; Bianchi *et al.*, 1985). In the present work with *Hyphantria cunea,* vitellogenin in female haemolymph could not be identified by electrophoresis (Fig. 1). Therefore, tissue culture of fat body and ovary was conducted to locate synthetic site of yolk protein. Unexpectedly, yolk protein-3 proved to be synthesized by fat body. In spite of that, why vitellogenin is not detected in female haemolymph is quite interesting. Possible interpretation is that vitellogenin is released into haemolymph in extremely small amounts or discontinuously. Also, molecular weight of YP3 subunit was determined to be approximately 18 Kd. This value is quite smaller than that of Lepidoptera including *Hyalophora cecropia* (43 Kd), *Philosamia synthia* (50 Kd), *Manduca sexta* (45 Kd), and *Bombyx mori* (43 Kd) (Hagedorn and Kunkel, 1979; Izumi and Tomino, 1983; Osir *et al.*, 1986). Yolk proteins of maturing egg are used as storage protein for new organs or tissues during embryogenesis (Kunkel and Nordin, 1985). The fate of yolk proteins was investigated at the viewpoint of electrophoretic pattern, immunological identity and degradation pattern. Degradation patterns of yolk protein can be divided into two groups. One group is that specific proteolytic cleavage process is followed by degradation as in *Leucophaea maderae, Periplaneta americana* and *Blattella germanica* (Storella and Kunkel, 1979; Masler and Offengand, 1982; Storella *et al.*, 1985; Wojchowski *et al.*, 1986; Purcell *et al.*, 1988) while the other group is that degradation is processed without specific proteolytic cleavage as in *Rhodnius prolixus* and *Drosophila melanogaster* (Bownes and Hames, 1977; Oliveira *et al.*, 1989). The period of oviposition to hatching of *Hyphantria cunea* is estimated to be 8 days. YP3 maintained constant level until day 6 after oviposition and new polypeptides did not appear during this period. However, YP3 drastically decreased on day 8 after oviposition, indicating that degradation took place during the period of days 6-8 after

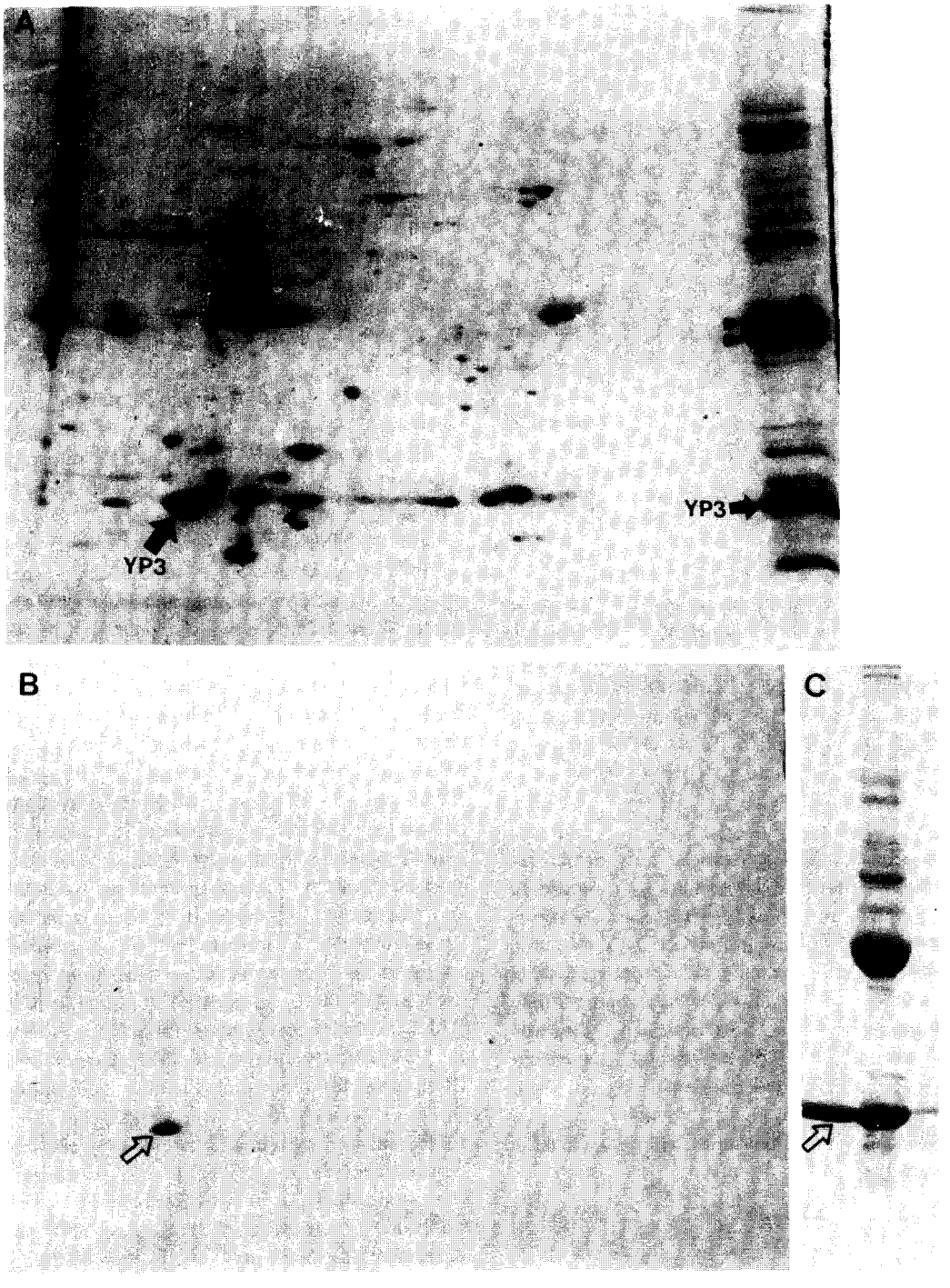

Fig. 4. Two dimensional electrophoresis of ovary extracts and purified YP3 by the method of O'Farrell (1975). A, ovary extracts (50 μl); B, two di. PAGE of purified YP3; C, SDS PAGE of ovary extracts and purified YP3. The arrow indicates purified YP3.

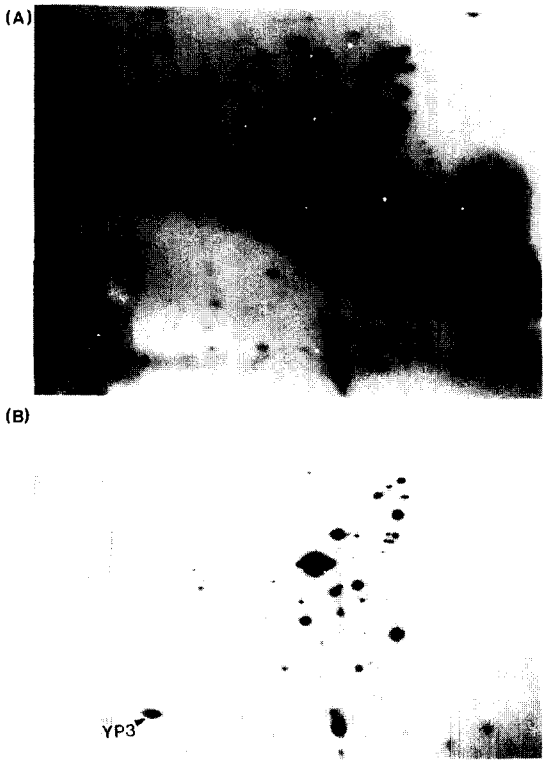

Fig. 5. Fluorography of two dimensional electrophoresis showing proteins synthesized *in vitro* by fat body and ovary during the 4 hr culturing period in insect Grace's medium containing [^{3}H]-leucine as indicated in materials and methods. A, ovaries of 8-day old pupae; B, female fat bodies of 8-day old pupae.

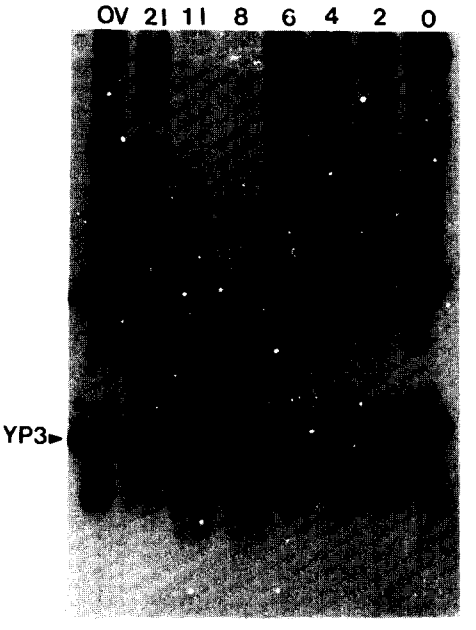

Fig. 6. SDS PAGE of eggs after oviposition. OV, ovary extracts (5 μl); 2I-1I, crude extracts of the 2nd and 1st instar larvae, respectively; 0-8, egg extracts (5 μl) of days 0,2,4,6,8 after oviposition, respectively.

oviposition. Drastic degradation of yolk protein during late embryogenesis was reported in *Bombyx mori* (Indrashith *et al.*, 1987). In *Bombyx mori*, specific cleavage is followed by the occurrence of new polypeptide, but *Hyphantria cunea* had no such new polypeptides. Whether the specific cleavage is taking place or not is dependent on molecular weight of yolk protein. Nordin *et al.* (1990) suggested that yolk proteins of 120 to 180 Kd follow this cleavage. Also, part of YP3 in *Hyphantria cunea* was remained even in newly hatched larvae. Fifty percent of yolk protein was remained in newly hatched larvae of *Rhodnius prolixus* (Oliveira *et al.*, 1989). It is likely that yolk protein is used as food to maintain life of newly hatched larvae. For more concrete data, immunological methods might be required using antibody against YP3.

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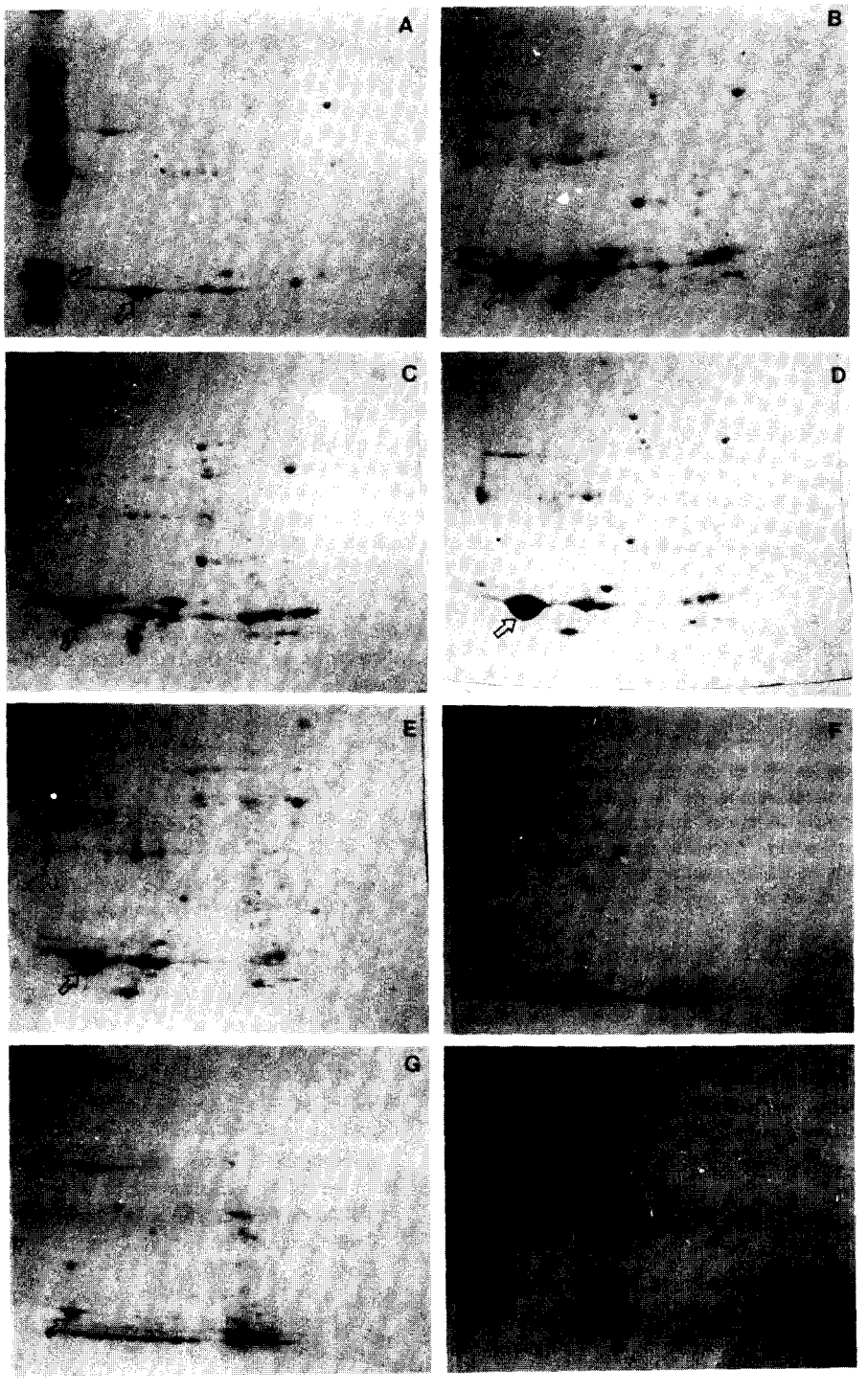

Fig. 7. Two dimensional gel electrophoresis of eggs after oviposition and curde extracts of larvae. A, ovary extracts (50 μl), B-F, egg extracts (50 μl) of days 0,2,4,6,8 after oviposition, respectively; G, crude extracts of the first instar larvae (just hatched); H, curde extracts of the second instar larvae.

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미국흰불나방(*Hyphantria cunea* D.)의 난황단백질-3의 합성 및 이용

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미국흰불나방(*Hyphantria cunea* D.)의 난소에서 yolk protein-3(YP3)를 정제하였으며 이의 합성 및 embryogenesis 기간의 fate를 fluorography와 전기영동적 방법으로 조사하였다. Gel slice에 이은 electrophoretic elution에 의해 정제된 YP3 분자량은 약 18 Kd로서 하나의 구성소단위로 이루어져 있었다. 암 · 수의 haemolymph 및 fat body를 stage별로 non-SDS PAGE와 SDS PAGE한 결과 female specific protein인 vitellogenin을 구별하기 어려웠으나 fat body와 성숙중인 난소를 조직배양시킨 결과 YP3는 fat body에서 합성됨이 밝혀졌다. 또한, 산란된 egg 내에서의 YP3는 산란후 6일까지 유지되다가 그 이후 감소되어 late embryogenesis기간에 급격히 사용되어짐을 알 수 있었다. 그러나, 갓 부화된 1령 유충에서도 YP3는 그 일부가 존재하였다.