An 86 kDa diapause protein 1-like protein is a component of early-staged encapsulation-relating proteins in coleopteran insect, *Tenebrio molitor* larvae[1]

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Abstract Recently, we reported two novel early-staged encapsulation-relating proteins (56 kDa and 48 kDa ERPs) isolated from the hemolymph of coleopteran insect, *Tenebrio molitor* larvae [Cho et al. (1999) Eur. J. Biochem. (in press)]. Here, a cDNA clone for another early-staged encapsulation-relating protein (86 kDa) was isolated. We found that the 86 kDa protein shows high homology with insect diapause protein 1. The 86 kDa protein was localized in the fat body and hemolymph, but not hemocyte lysate. A significant level of 86 kDa protein was detected in pre-pupae stage, but it decreased rapidly at late larvae and pupae, and no protein was found in embryo, early larvae and adult stages. This diapause protein 1-like protein is likely to be a component of early-staged encapsulation-relating proteins in the insect cellular defense reaction.

Key words: Diapause protein I; Defense protein; Encapsulation; Parasitoid; Insect

1. Introduction

Insects utilize two broad categories of defense responses against invading parasites and pathogens: humoral and cellular responses. The well-known humoral responses involve the synthesis of antimicrobial proteins [1–3] and the pro-phenoloxidase (pro-PO) activating system [4,5]. Previously, we reported the antimicrobial proteins and pro-phenoloxidase in the larvae of the coleopteran insects, *Tenebrio molitor* and *Holotrichia diomphalia* [6–10]. The cellular defense reactions include phagocytosis, nodule formation and encapsulation. Recently, we reported that the activated phenoloxidase of *T. molitor* larvae was involved in cell clump/cell adhesion [11]. Encapsulation and nodule formation in insects are major cellular defense reactions against foreign biotic or abiotic materials that are too large to be phagocytosed by individual hemocytes [12,13]; however, the biochemical process of encapsulation is still obscure.

Previously, to isolate and characterize the early-staged encapsulation-relating proteins (ERPs), we injected three different kinds of material: positively charged DEAE-Sepharose beads, negatively charged CM-Sepharose beads, non-charged gel filtration beads, or inserted pieces of surgical suture into the abdomen of *T. molitor* larvae [14]. The beads or sutures were recovered within 10 min after their injection or insertion, and proteins enriched on their surface were analyzed on SDS-PAGE under reducing conditions. Four different ERPs (86 kDa, 78 kDa, 56 kDa, and 48 kDa) were commonly enriched from the crude hemolymph regardless of provocateurs. Among them, we previously reported the primary structures and functional properties of the 56 kDa and 48 kDa proteins [14].

This paper describes the purification and molecular cloning of the 86 kDa ERP. The overall structure of the 86 kDa ERP was very similar to that of diapause protein 1 that is utilized during post-diapause development, but the biological function of diapause protein 1 is still unknown. Our results suggest that the 86 kDa ERP (diapause protein 1-like protein) is crucial in encapsulation of cellular defense reaction in insects. This is the first demonstration that a diapause protein 1-like protein participates in the defense mechanism of insect.

2. Materials and methods

2.1. Animals, collection of hemolymph and hemocytes

T. molitor larvae (mealworms) were maintained on a laboratory bench in terraria containing wheat bran. Vegetables were placed on top of the bran to provide water. Hemolymph and hemocytes were collected as previously described [14].

2.2. Purification and molecular cloning of 86 kDa early-staged ERP

Triggering an early-staged encapsulation response and recovering of the early-staged ERPs from larvae were performed essentially as previously described [14]. Briefly, the swollen beads (DEAE-Sepharose CL-6B, 1 g) with insect saline were suspended in 1 ml of insect saline (130 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, pH 6.0). 20 μl of suspended solution was injected into each third instar larva anesthetized on ice. After the injection of suspended beads (20 μl), the puncture was immediately sealed with liquid paraffin. To harvest the hemolymph after 10 min, larvae were injected with 50 μl of modified anti-coagulation buffer (30 mM trisodium citrate, 26 mM citric acid, 20 mM EDTA, and 15 mM sodium chloride, pH 5.5) using a 25 G needle. The tail of each larva was cut off using fine scissors and the hemolymph was placed in a test tube that was kept on ice. The beads were collected from the hemolymph by centrifugation (1000×*g*, 10 s), and washed twice with an anti-coagulation buffer. The materials coated on the beads were recovered by using 6 M guanidine-HCl in 50 mM Tris-HCl containing 1 mM EDTA solution (pH 7.0). The proteins were precipitated with trichloroacetic acid (TCA) and analyzed on 12% SDS-PAGE under reducing conditions.

The 86 kDa protein was purified to homogeneity from polyacrylamide gel using the Micro-Electroeluter (Centrilutor, Amicon) accord-

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the accession number AB021700.

Abbreviations: ERP, encapsulation-relating protein; TCA, trichloroacetic acid; PVDF, polyvinylidene difluoride

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ing to the manufacturer's instructions. The purified 86 kDa protein was reduced and *S*-pyridylated according to a previously published method [10]. The *S*-pyridylated 86 kDa protein was digested with trypsin, and the resulting peptides were separated by HPLC on a C_{18} reverse phase column (Gilson). To determine the amino-terminal sequences of the 86 kDa protein, the purified 86 kDa protein was subjected to SDS-PAGE under reducing conditions. The band of the 86 kDa protein was blotted onto a polyvinylidene difluoride filter (PVDF filter, Millipore), cut out from the filter, and subjected to automated amino acid sequence analysis [15]. A cDNA library of *T. molitor* larvae was constructed according to a previously published method [6]. To screen the 86 kDa protein clones, we performed immunoscreening using an affinity-purified antibody against the 86 kDa protein. Following isopropyl-β-D-thiogalactoside (IPTG) induction, 5×10^4 plaques were screened with an affinity-purified antibody against the purified 86 kDa protein. A secondary antibody (alkaline phosphatase-conjugated anti-rabbit IgG, Bio-Rad) was used at a dilution of 1:1000. Phage DNA was isolated from phage lysates using the lambda DNA preparation kit according to the manufacturer's instructions. We analyzed all of the plaques showing positive signals on immunoscreening. We sequenced the clones according to the dideoxy chain termination method of Sanger et al. [16]. The amino acid sequences of the 86 kDa protein were compared with the protein sequence database of the National Center for Biotechnology Information (NCBI) using the Genetyx system (Software Development Co., Ltd., Tokyo). The protein concentrations were determined by the method of Lowry et al. [17] using bovine serum albumin as the standard.

2.3. Antibody and immunoblotting

Antibody against the 86 kDa protein was raised by injecting 20 μg of the purified protein into a male albino rabbit with complete Freund's adjuvant and giving a booster injection of the same amount of protein 14 days later [18]. The resulting antibodies were affinity-purified as previously described [10]. For immunoblotting, the proteins separated on the gel by electrophoresis were transferred electrophoretically to a PVDF filter and the filter was immersed in 5% skim milk solution containing 1% horse serum for 12 h, after which they were transferred to rinse solution I (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 0.1% Tween 20 and 2.5% skim milk) containing affinity-purified antibody against 86 kDa protein (50 ng/ml), and kept at 4°C for 2 h. The bound antibodies were identified using the ECL Western blotting reagent kit (Amersham Life Science kit).

3. Results

3.1. Purification of 86 kDa protein

To isolate early-staged ERPs from *T. molitor* larvae, we injected DEAE-Sepharose beads as the provocateur and recovered the injected beads after 10 min. As shown in Fig. 1, four different proteins (86 kDa, 78 kDa, 56 kDa and 48 kDa) were enriched from the crude hemolymph on the bead surface (lane 2), as we previously reported [14]. Among them, we purified the 86 kDa protein to homogeneity from the crude encapsulated materials (lane 3 in Fig. 1). The yield of purified 86 kDa ERP was 60 μg from 10 mg of the crude retrieved proteins. Then, we determined the amino-terminal sequence of the 86 kDa protein from the blotted filter as VSVQNEPVTNPQR. We also determined three partial amino acid sequences as GELFYYMY, DPAFYYQLFK, and TSYYEVYQK.

3.2. cDNA cloning of 86 kDa protein

To determine the whole amino acid sequence of the 86 kDa protein, we screened the cDNA library of *T. molitor* larvae with an affinity-purified antibody against the 86 kDa protein by an immunoscreening method. We obtained 15 positive clones for the 86 kDa protein and sequenced one of them, which contained the largest insert. This cDNA contained an

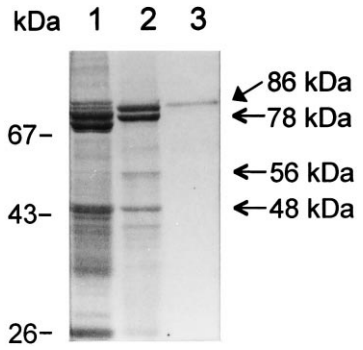

Fig. 1. 12% SDS-PAGE analysis of the purified 86 kDa ERP from injected DEAE-Sepharose beads. The early-staged ERPs from beads were recovered with 6 M guanidine-HCl in 50 mM Tris-HCl containing 1 mM EDTA solution (pH 7.0). The proteins were precipitated with TCA and analyzed on 12% SDS-PAGE under reducing conditions. Lane 1, 30 μg of the crude hemolymph; lane 2, the early-staged ERPs from DEAE-Sepharose beads (10 μg) after 10 min; lane 3, the purified 86 kDa ERP (0.5 μg).

open reading frame of 2265 nucleotides corresponding to 755 amino acid residues (Fig. 2A). The chemically determined 13 amino acid residues of the amino-terminal of the 86 kDa protein were present in this sequence starting from Val at position 17 (indicated as an arrowhead in Fig. 2A). Moreover, three partial amino acid sequences of the 86 kDa protein were included in the deduced amino acid sequence in this open reading frame (indicated by underlines). Therefore, we concluded that this is a cDNA for the 86 kDa protein. Using a method predicting a signal peptide cleavage site [19], we assigned the most likely cleavage site by a signal peptidase as between Ala^{15} and Ser^{16}. There were three potential *N*-glycosylation sites (Asn-Xaa-Ser/Thr) (indicated as closed diamonds). We searched for sequence homology between the sequence of the 86 kDa ERP and those in the NCBI database, and found that the deduced amino acid sequence of the 86 kDa protein was in good coincidence with that of the well-known diapause protein 1 [20]. As shown in Fig. 2B, the sequence identity between the 86 kDa protein and diapause protein 1 was 43.3% [20], suggesting that the 86 kDa protein is a diapause protein 1-like protein. This result indicated that diapause protein 1-like protein is a component of early-staged ERPs in *T. molitor* larvae. This is the first time that the biological function of diapause protein 1-like protein has been demonstrated in insect cellular defense response.

3.3. Immunoblotting analysis

To determine whether the purified 86 kDa protein was a component of the early-staged encapsulation response, we performed immunoblotting experiments. For this, we raised polyclonal antibody against the purified 86 kDa protein. The proteins recovered from the beads were subjected to SDS-PAGE, blotted onto a PVDF filter, and probed with affinity-purified antibody against the purified 86 kDa protein. As is evident from Fig. 3A, the recovered proteins were found to contain the 86 kDa protein (lane 3 in Fig. 3A). The positions of these signals coincided with that of the purified 86 kDa protein (lane 1 in Fig. 3A).

Next, we examined the localization of the 86 kDa protein. For this, we prepared the hemocyte lysate and plasma solution from the hemolymph of *T. molitor* larvae. We also prepared fat bodies from *T. molitor* larvae. As shown in Fig. 3B,

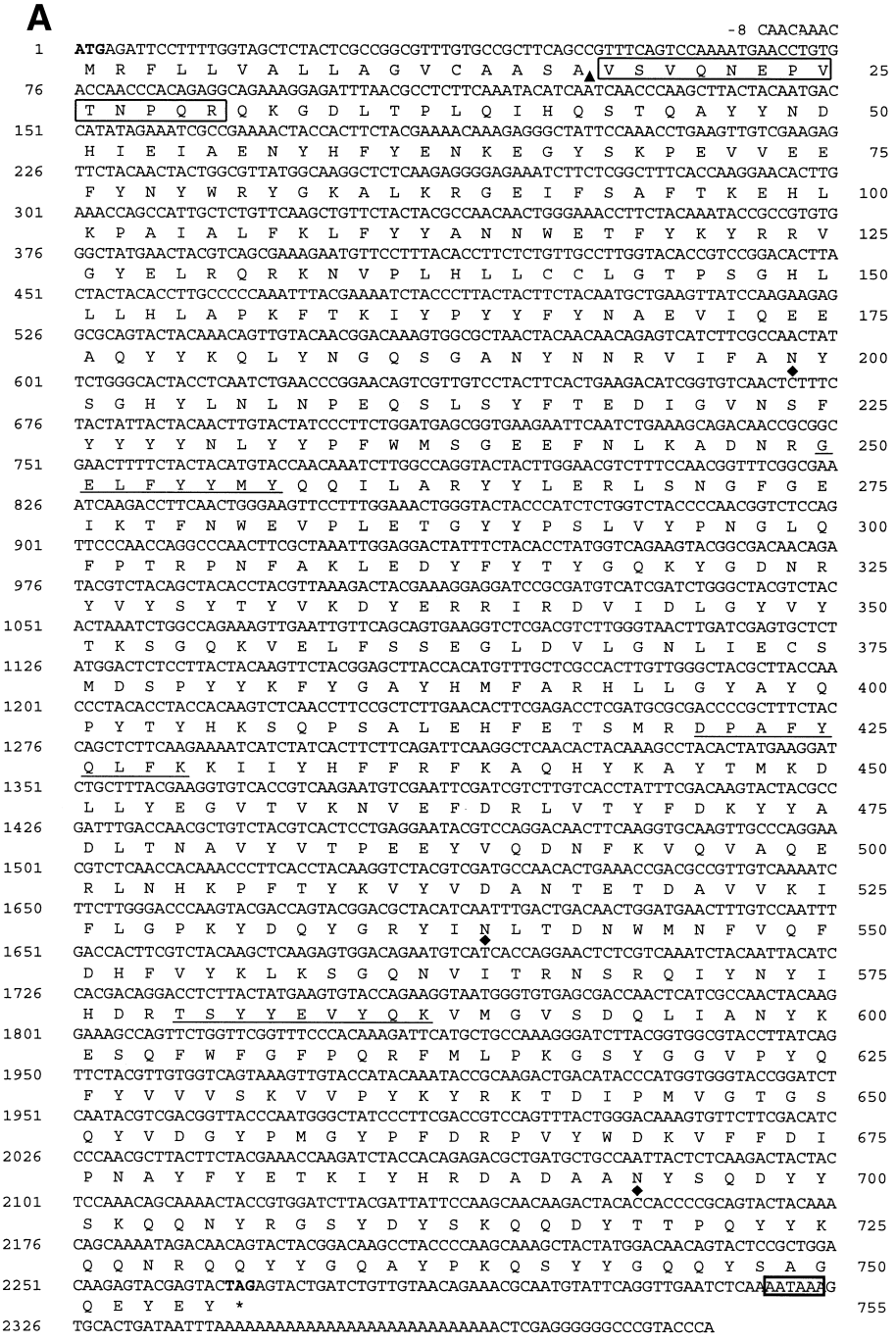

Fig. 2. Nucleotide and deduced amino acid sequence of cloned cDNA encoding the 86 kDa ERP (A) and amino acid similarity between the 86 kDa ERP and insect diapause protein 1 (B). A: Numbers of nucleotides starting from the first base at the 5′-end are shown on the left of each line; the deduced amino acid sequence is numbered from the initiating Met residue on the right of each line. The chemically determined partial amino acid sequences of the 86 kDa ERP are underlined. The chemically determined amino-terminal sequence of the 86 kDa ERP is boxed. The potential attachment sites for *N*-linked carbohydrate chain are indicated by closed diamonds. The amino-terminal residue of 86 kDa is shown by the arrowhead. A poly(A) additional signal is bold-boxed. B: Numbers refer to the predicted protein sequence. Gaps (-) were inserted to maximize the sequence alignment. Identical residues are boxed.

appreciable 86 kDa protein was not detected in the hemocyte lysate (lane 5 in Fig. 3B). However, a significant amount of proteins was detected in the hemolymph, plasma and fat body (lanes 3, 4 and 6 in Fig. 3B, respectively), indicating that the 86 kDa protein was synthesized in the fat body and secreted in the hemolymph.

We analyzed the expression of the 86 kDa protein at various developmental stages of *T. molitor*. The 86 kDa protein was detected in the late larvae, pre-pupae, and pupae (lanes 4, 5 and 6 in Fig. 4, respectively). Its content reached a maximum at the pre-pupal stage (lane 5). No appreciable 86 kDa protein was detected in the embryos, early larvae, and adults (lane 2, 3 and 7 in Fig. 4). These results indicated that the amount of the 86 kDa protein is several times higher in dia-

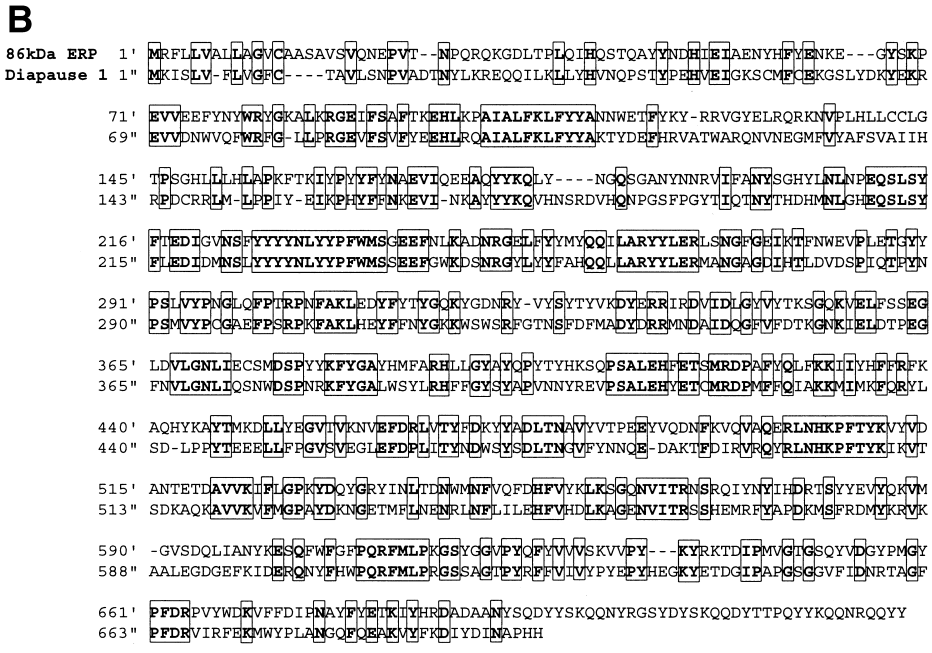

Fig. 2 (continued).

pausing periods than in non-diapausing periods during metamorphosis. Other groups have suggested that diapause protein accumulated in the hemolymph during the last larval stage and in adults reared under short-day (diapause-inducing) condition [20].

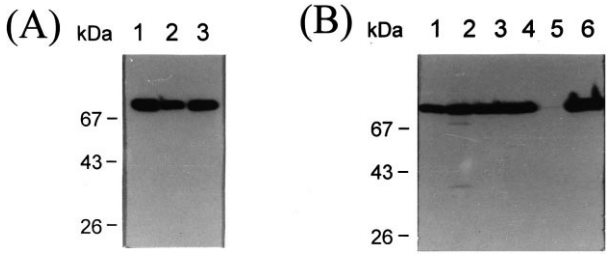

Fig. 3. Immunoblotting of the 86 kDa ERP against the recovered early-staged ERPs from beads (A) and determination of localization of the 86 kDa ERP (B). A: The purified 86 kDa ERP (lane 1, 0.5 μg), the crude hemolymph (lane 2, 30 μg), and the early-staged ERPs from DEAE-Sepharose beads (lane 3, 10 μg) were separated onto the gel by electrophoresis and were immunoblotted with the affinity-purified antibodies against the 86 kDa ERP. B: The hemolymph was obtained from 150 larvae and centrifuged at 3000 rpm at 4°C for 10 min. Precipitated hemocytes were washed with 500 μl of anti-coagulation buffer (pH 5.5) and suspended again with 500 μl of anti-coagulation buffer (pH 5.5). 100 μl of suspended solution was sonicated for 15 s at 4°C and centrifuged at 15000 rpm (22000×*g*) at 4°C for 10 min. The supernatant was used as hemocyte lysate. The fat body was also obtained from 150 larvae and centrifuged at 3000 rpm at 4°C for 10 min. The protein from the fat body was extracted with 100 μl of 6 M guanidine-HCl in 50 mM Tris-HCl containing 1 mM EDTA solution (pH 7.0). Proteins were precipitated with TCA and subjected to SDS-PAGE and then immunoblotting was performed with an affinity-purified antibody against the 86 kDa ERP. Lane 1, 0.5 μg of the purified 86 kDa ERP; lane 2, 10 μg of the early-staged ERPs from DEAE-Sepharose beads; lane 3, 30 μg of the crude hemolymph; lane 4, 30 μg of plasma solution; lane 5, 30 μg of hemocyte lysate; lane 6, 30 μg of the soluble fat body protein.

4. Discussion

In this study, we found that the deduced amino acid sequence of the 86 kDa protein was in good coincidence with known diapause protein 1. Since diapause protein 1 belongs to the family of insect arylphorin-type storage proteins, it has been suggested to be implicated in cuticle formation during molting and in the formation of adult proteins during metamorphosis. However, the exact biological function of diapause protein 1 is still obscure. Diapause is a genetically controlled period of developmental arrest that enables insects to survive under adverse environmental conditions and synchronize the active stage of their life cycle with available food sources [21,22]. In the biological points, encapsulation response of cellular defense reactions against foreign non-self materials may be similar to diapause-inducing conditions in insects.

When foreign biotic or abiotic materials are introduced into the insect body, it is suggested that insects react quickly and

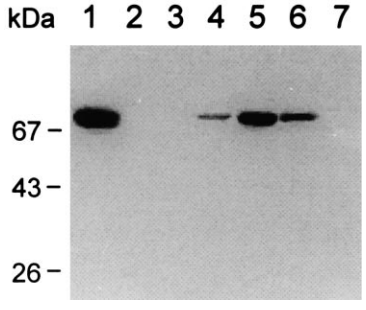

Fig. 4. Immunoblotting analysis of the 85 kDa ERP at various developmental stages. Samples were prepared from animals at various developmental stages and subjected to immunoblotting analysis with an affinity-purified antibody against the 86 kDa ERP. Lane 1, 0.5 μg of the purified 86 kDa ERP; lane 2, 20 μg of embryo protein; lane 3, 20 μg of early larval protein; lane 4, 20 μg of late larval protein; lane 5, 20 μg of pre-pupal protein; lane 6, 20 μg of pupal protein; lane 7, 20 μg of adult protein.

form capsules that include hemocytes and extracellular hemolymph components. Previously, we demonstrated that the 56 kDa and 48 kDa ERPs are novel glutamine-rich proteins, which are exclusively detected in the membrane fraction of hemocytes [14]. However, in the present study, the 86 kDa protein was localized in the fat body and plasma, indicating that the 86 kDa protein is an extracellular component.

Another band (78 kDa protein) was also enriched on the early-staged encapsulation induced by beads. Further studies are necessary to purify this protein to homogeneity and determine its primary structures. We hope that future studies will elucidate the molecular mechanism of the early-staged encapsulation response, and identify which protein accumulates first to non-self foreign objects as well as which proteins are engaged in coating of non-self foreign materials.

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