From eggs of the silkworm *Bombyx mori*, we isolated a novel enzyme that is involved in the conversion of physiologically inactive conjugated ecdysteroids, such as ecdysone 22-phosphate and 20-hydroxyecdysone 22-phosphate, to active free ecdysteroids. This enzyme, called ecdysteroid-phosphate phosphatase (EPPase), was located in the cytosol fraction and differed from nonspecific lysosomal acid phosphatases in various enzymatic properties. EPPase was purified about 3,000-fold to homogeneity by seven steps of column chromatography. The cDNA clone encoding EPPase was isolated by reverse transcription polymerase chain reaction using degenerate primers on the basis of the partial amino acid sequence obtained from purified EPPase and by subsequent 3′- and 5′-rapid amplification of cDNA ends. The full-length cDNA of EPPase was found to be composed of 1620 bp with an open reading frame encoding a protein of 331 amino acid residues. A data base search showed that there was no functional protein with the amino acid sequence identical to that of EPPase. Northern blot analysis revealed that EPPase mRNA was expressed predominantly during gastrulation and organogenesis in nondiapause eggs but was not detected in diapause eggs whose development was arrested at the late gastrula stage. In nondiapause eggs, the developmental changes in the expression pattern of EPPase mRNA corresponded closely to changes in the enzyme activity and in the amounts of free ecdysteroids in eggs.

In insects, ecdysteroids, which regulate development and reproduction, are synthesized in prothoracic glands. However, in many insect species, ecdysteroids have been demonstrated to be synthesized in ovaries as well, to accumulate in mature ovaries, and to be transferred to eggs (1–3). Most of these ecdysteroids are physiologically inactive phosphoric esters and synthesized by the enzyme, ATP:ecdysteroid-phosphotransferase (ecdysteroid kinase), which catalyzes phosphorylation of ecdysteroids (Fig. 1) (4, 5). It has been suggested that free ecdysteroids are liberated from maternal ecdysteroid-phosphates during embryonic development and participate in the secretion of serosal cuticle and/or the induction of embryonic cuticleogenesis (embryonic molt) in various insects, such as *Locusta migratoria* (6), *Schistocerca gregaria* (7), *Blaberus craniifer* (8), and *Manduca sexta* (9). Furthermore, the involvement of ecdysteroids in embryonic diapause has also been suggested in *Lepidophyes ulmi* (10), *Bombyx mori* (11), and *L. migratoria* (12).

In *B. mori*, recently, it has been demonstrated that the continuous supply of 20-hydroxyecdysone (20E),1 which has been demonstrated to be an active molecule in *B. mori* eggs (13), is required for embryonic development and that a deficiency of 20E induces embryonic diapause (13); that is, the cessation of embryonic development at the late gastrula stage (14, 15). Furthermore, 20E was demonstrated to be formed by de novo synthesis (16–18) and dephosphorylation of 20-hydroxyecdysone 22-phosphate (20E22P) and ecdysone 22-phosphate (E22P) (in the case of the latter, dephosphorylation was followed by hydroxylation at C-20 position) (18).

Although the potential role of ecdysteroid-phosphates as a "reservoir" of active ecdysteroids has been suggested in various insect eggs as mentioned above, little or no attention has been paid to the enzyme catalyzing the dephosphorylation, ecdysteroid-phosphate phosphohydrolase (ecdysteroid-phosphate phosphatase (EPPase)) (Fig. 1). We now report the purification, characterization, and cDNA cloning of EPPase from *B. mori* eggs. This is the first report of a novel phosphatase that specifically hydrolyzes ecdysteroid-phosphates.2

**EXPERIMENTAL PROCEDURES**

**Animals**—Moths of the silkworm *B. mori* that had been destined to produce diapause eggs, the commercially available bivoltine race (kinyu-showa), were used as the source of eggs. In diapause eggs, embryos ceased development at the late gastrula stage (48–72 h after oviposition) (14, 15). The diapause nature of the eggs was artificially changed to a nondiapause nature by the following treatments: (i) To avoid diapause initiation, prospective diapause eggs were soaked in HCl solution (specific gravity, 1.11 at 15 °C) 20 h after oviposition (15). (ii) To obtain female moths destined to produce nondiapause eggs, the subesophageal ganglion, the source of diapause hormone, was removed soon after larval-pupal ecysis (15). In both cases, the nondiapause eggs obtained continued to develop and larvae hatched 10 days after oviposition. For enzyme purification, HCl-treated 3-day-old nondiapause eggs were used. For RNA extraction, nondiapause eggs laid by the moths with the subesophageal ganglion removed were used, because ommochromes, which disturb first strand cDNA synthesis, are not synthesized in the nondiapause eggs.

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1 The abbreviations used are: 20E, 20-hydroxyecdysone; 20E22P, 20-hydroxyecdysone 22-phosphate; E22P, ecdysone 22-phosphate; EPPase, ecdysteroid-phosphate phosphatase; E, ecdysone; RIA, radioimmunoassay; nNPF, n-nitrogenphylophtalate; PMSP, phenylmethylsulfonyl fluoride; 2DE22P, 2-deoxyecdysone 22-phosphate; 2DE22P, 2-deoxy-20-hydroxyecdysone 3-phosphate; 2DE22P, 2-deoxy-20-hydroxyecdysone; RACE, rapid amplification of cDNA ends; ORF, open reading frame; ConA, concanavalin A.

2 This work was supported in part by Grants-in-aid 11640871 and 13620672 from the Japan Society for the Promotion of Science and by funds from the Hirao Taro Foundation of the Konan University Association for Academic Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AB107356.

3 To whom correspondence should be addressed: Dept. of Biology, Faculty of Science and Engineering, Konan University, Kobe 658-8501, Japan. Tel.: 81-78-435-2512; Fax: 81-78-435-2539; E-mail: sonobe@konan-u.ac.jp.
**Enzyme Assay**—The EPPase activity was measured using E22P as the substrate. E22P was synthesized chemically (19). The amount of ecdysone (E) converted from E22P was quantified by radioimmunoassay (RIA) using antiserum N-6 (provided by Dr. A. Mizuguchi, Nagoya University) generated against 20E-6-carboxy methyleximohapten. This antiserum exhibited over 4,000-fold stronger reactivity against E than against E22P (19). Therefore, the amount of E in the reaction mixture was quantified by RIA without isolating E from E22P. RIA was conducted as described previously (20). For the measurement of EPPase activity, the reaction mixture was made up to a final volume of 100 μl with 200 mM Tris-HCl buffer, pH 7.5, containing 0.1% bovine serum albumin, 100 mM E22P, and 50 μl of the enzyme solution. The reaction was initiated by adding the enzyme solution, and the reaction mixture was incubated at 37 °C for 30 min. The reaction was stopped by the addition of methanol, and the mixture was centrifuged at 3,500 rpm for 30 min to precipitate the protein. Aliquots of the supernatant were dried using a centrifugal evaporator. The residue was dissolved in 50 mM borate buffer, pH 8.4, for use in RIA.

**Acid phosphatase activity** was measured using p-nitrophenylphosphate (pNPP), which is generally used as the substrate of phosphatase (21–24). The assay mixture containing 10 mM pNPP and 200 mM acetate buffer, pH 4.0, was incubated with 50 μl of the enzyme solution at 37 °C for 15 min. After the reaction was stopped by the addition of 0.1 M NaOH, the absorbance of p-nitrophenol (pNP) formed was measured at 405 nm.

**Protein Assay**—The protein concentration was determined by the method of Bradford (25) using the Bio-Rad protein assay reagent according to the manufacturer’s protocol with bovine serum albumin as a standard.

**Preparation of Subcellular Fraction**—All of the procedures were carried out at 4 °C. Subcellular fractionation was performed by differential centrifugation according to previous studies (17, 26) with some modifications. Three-day-old nondiapause eggs (1.0 g) were homogenized in 50 mM Tris-HCl buffer (2 ml), pH 7.5, containing 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM PMSF, 10 μM pepstatin A, and 10 μM leupeptin. The homogenate was centrifuged at 1,000 × g for 10 min to remove the chorion. The supernatant was centrifuged at 10,000 × g for 30 min. The resulting supernatant was further centrifuged at 100,000 × g for 60 min. A lipid layer obtained from centrifugation at 1,000 × g was collected and homogenized to an emulsion. Each pellet (1,000 × g pellet, 10,000 × g pellet, and 100,000 × g pellet), the lipid layer, and the final supernatant were used for the enzyme assay.

**Purification of EPPase**—All of the purification procedures were carried out at 4 °C. Three-day-old nondiapause eggs (350 g) were used for enzyme purification. The eggs were homogenized in 2 volumes of 50 mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM PMSF, 10 μM pepstatin A, and 10 μM leupeptin/g of eggs. An aliquot of 10,000 × g supernatant was loaded onto a Q Sepharose HP column (Amersham Biosciences) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5. Elution was performed using a linear gradient of 0 to 400 mM NaCl in 50 mM Tris-HCl buffer, pH 7.5, for 200 min at a flow rate of 6.5 ml/min. The active fraction was applied to a Chelating Sepharose FF column (Amersham Biosciences), which was previously charged with Zn²⁺ by passing 0.1 M ZnCl₂ through the column and then equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 500 mM NaCl. Stepwise elution was performed using 50 mM EDTA in 50 mM Tris-HCl buffer, pH 7.5, containing 500 mM NaCl at a flow rate of 2.5 ml/min. The active fractions were collected, and NaCl was added to obtain a final concentration of 1.5 M for the following purification step. This enzyme solution was applied to a Phenyl Sepharose HP column (Amersham Biosciences) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl. Elution was performed using decreasing NaCl concentration at a flow rate of 2.0 ml/min. The active fraction was concentrated to about 10-fold, and the buffer was changed to 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM MnCl₂, 1 mM MgCl₂, and 1 mM CaCl₂ using Centriplus 30 (Millipore). The enzyme solution was applied to a HiTrap ConA HP column (Amersham Biosciences) pre-equilibrated with 400 mM borate buffer, pH 8.4, for use in RIA.
(Amersham Biosciences) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5. Elution was performed using a linear gradient of 0–500 mM NaCl in 50 mM Tris-HCl buffer, pH 7.5, for 120 min at a flow rate of 1.0 ml/min. The active fraction was dialyzed against 50 mM Tris-HCl buffer, pH 7.5, and reapplied to the Mono Q column. The enzyme was eluted by a linear gradient of 0–500 mM NaCl for 240 min. The active fraction was concentrated and loaded onto a Superdex 200HR column (Amersham Biosciences). The enzyme was eluted with 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl at a flow rate of 0.25 ml/min.

**Kinetic Analysis**—Besides E22P, other ecdysteroid-phosphates, 20E22P, 2-deoxyecdysone 22-phosphate (2dE22P) and 22-deoxy-20-hydroxyecdysone 3-phosphate (22d20E3P), which were isolated from mature ovaries of *B. mori* (27, 28), were also used as substrates for the kinetic study of EPPase. Substrate concentration was varied from 0.4 to 204.8 μM. Amounts of the products 20E and 2-deoxyecdysone formed in the reaction mixture were determined by RIA using antiserum N-6 as described in the previous section; this antiserum scarcely cross-reacted to their phosphoric esters (0.058% in 20E22P and 0.019% in 2dE22P). The amount of 22-deoxy-20-hydroxyecdysone (22d20E) converted from 22d20E3P was determined by RIA using antiserum H-22 (provided by Prof. L. I. Gilbert, University of North Carolina at Chapel Hill) without isolating 22d20E from 22d20E3P; cross-reactivity of 22d20E3P to this antiserum was below 0.5% of that of 22d20E. Michaelis-Menten constants (Kₘ and Vₘₐₓ) were graphically determined for each substrate from the Lineweaver-Burk plots. The turnover number a Estimated by A₂₈₀ with BSA as a standard.

### Table I

| Step                  | Protein (mg) | Total activity (pmol/min) | Specific activity (pmol/min/mg protein) | Yield (%) | Purification -fold |
|-----------------------|--------------|---------------------------|----------------------------------------|-----------|-------------------|
| 10,000 × g supernatant | 996.15       | 14048                     | 0.14                                   | 100       | 1                 |
| Q Sepharose           | 1426.0       | 9630                      | 6.75                                   | 69.0      | 48                |
| Chelating Sepharose   | 147.0        | 6870                      | 46.71                                  | 49.0      | 334               |
| Phenyl Sepharose      | 34.5         | 5700                      | 165.2                                  | 40.0      | 1180              |
| ConA Sepharose        | 11.4         | 3280                      | 287.7                                  | 23.0      | 2055              |
| Mono Q (first)        | 1.29         | 590                       | 457.4                                  | 4.2       | 3267              |
| Mono Q (second)       | 0.53a        | 220                       | 415.1                                  | 1.6       | 2965              |
| Superdex 200 HR       | 0.42a        | 180                       | 428.6                                  | 1.3       | 3061              |

*a R. Yamada and H. Sonobe, unpublished data.*

**Fig. 3. Purification of EPPase.** *A,* anion-exchange chromatography on Q Sepharose HP. *B,* chelation affinity chromatography on Chelating Sepharose FF. *C,* hydrophobic interaction chromatography on Phenyl Sepharose HP. *D,* first Mono Q HR column chromatography. *E,* second Mono Q HR column chromatography. *F,* gel filtration chromatography on Superdex 200HR. The effluent was monitored at 280 nm (solid lines). The inset in (F) shows SDS-PAGE analysis of the active fraction obtained from the Superdex 200HR column chromatography. The proteins were visualized by silver staining. The positions of the protein markers are shown on the right.
Baculovirus Expression—Recombinant baculovirus harboring the EPPase-cDNA was constructed using the BAC-TO-BAC baculovirus expression system (Invitrogen) according to the manufacturer’s protocol. The cDNA containing the whole open reading frame was cut with BamHI and HindIII from the plasmid as described above and ligated into the pFASTBAC plasmid. The recombinant plasmid was transformed into DH10BAC cells. The colonies containing recombinant bacmid DNA were selected by disruption of the lacZ gene and confirmed by colony PCR using a M13/pUC amplification primer (the forward primer was 5′-CCC AGT CAC GAC GTT GTA AAA CGG, and the reverse primer was 5′-AGC GGA TAA CAA TAC CAC AGG). Spodoptera frugiperda cells (SF9) were cultured in S9005SFm (Invitrogen). The isolated recombinant bacmid DNA (1 µg) was transfected to 1 × 10⁶ cells using a Cell-Fection reagent (Invitrogen). The recombinant virus was harvested from the cell culture medium 72 h after transfection. For protein expression, the recombinant virus was infected to SF9 cells at multiplicity of infection of 10. The cells were collected every 24 h after infection and homogenized in 2 volumes of 50 mM Tris–HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM PMSF, 10 µM pepstatin A, and 10 µM leupeptin/g of cells. The homogenate was used for assaying EPPase activity.

Purification of Recombinant EPPase—Cells infected with recombinant baculovirus at multiplicity of infection of 10 were collected at 72 h by centrifugation (500 × g, 5 min) and homogenized in 2 volumes of 50 mM Tris–HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM PMSF, 10 µM pepstatin A, and 10 µM leupeptin/g of cells. The homogenate was used for assaying EPPase activity.

**TABLE II**

| Substrates | $V_{max}$ | $k_{cat}$ | $K_m$ | $k_{cat}/K_m$ |
|------------|-----------|----------|------|--------------|
| E22P       | 1.25      | 0.94     | 5.88 | 1.61 × 10⁴   |
| 20E22P     | 19.23     | 14.57    | 19.23| 7.64 × 10⁴   |
| 26E22P     | 4.67      | 3.57     | 14.93| 2.32 × 10⁴   |
| 22429E3P   | 0.19      | 0.13     | 163.70| 0.83 × 10⁶   |
| pNPP       | 180.0     | 135.0    | 13145.0| 1.03 × 10⁴   |

$V_{max}$ and $k_{cat}$ were graphically determined for each substrate from the Lineweaver-Burk plots. $k_{cat}/K_m$ was calculated on the basis of molecular mass estimated by SDS-PAGE analysis.
mM Tris-HCl buffer, pH 7.5, containing 250 mM sucrose, 1 mM dithiothreitol, 0.5 mM PMSF, 10 μM pepstatin A, and 10 μM leupeptin/g of cells. The homogenate was centrifuged at 10,000 × g for 60 min at 4 °C. The supernatant was loaded onto a HiTrap Q HP column (Amersham Biosciences) pre-equilibrated with 50 mM Tris-HCl buffer, pH 7.5. Elution was performed by a linear gradient of 0–500 mM NaCl in 50 mM Tris-HCl buffer, pH 7.5, for 90 min at a flow rate of 1.0 ml/min. The active fraction was dialyzed against 50 mM Tris-HCl buffer, pH 7.5, and applied to a Mono Q HR column pre-equilibrated with the same buffer. Elution was performed using a linear gradient of 0–500 mM NaCl in 50 mM Tris-HCl buffer, pH 7.5, for 120 min at a flow rate of 1.0 ml/min. The active fraction was concentrated and loaded onto a Superdex 200HR column. EPPase was eluted with 50 mM Tris-HCl buffer, pH 7.5, containing 150 mM NaCl at a flow rate of 0.25 ml/min. The purity was analyzed by SDS-PAGE (10% gel). The effluent was monitored at 280 nm.

Western Blot Analysis—Polyclonal antibodies against EPPase were raised in rabbits using the purified recombinant EPPase as an antigen. Antiserum from an immunized rabbit was purified using a HiTrap Protein A column (Amersham BioSciences) according to the manufacturer’s protocol.

Recombinant EPPase (1 μg) and native EPPase (1 μg) were subjected to SDS-PAGE (10% gel) and transferred to a polyvinylidene difluoride membrane. The membrane was preincubated for 30 min in 10 mM Tris-HCl buffer saline containing 0.1% casein and then incubated with anti-EPPase antibodies (1:5000 dilution) for 1 h at 25 °C. Bound antibodies were detected with goat anti-rabbit peroxidase-conjugated IgG (1:5000 dilution; Wako Chemical), and the peroxidase activity was visualized using 0.5% of 3,3′-diaminobenzidine tetrahydrochloride in 10 mM Tris-HCl buffer containing 0.02% H2O2.

Northern Blot Analysis—Total RNA from various tissues was isolated using Isogen. Total RNA (20 μg) was separated on a 1.0% formaldehyde-agarose gel, and the separated products were transferred to a Hybond-N’ nylon membrane (Amersham Biosciences) and hybridized with a probe corresponding to the whole open reading frame of the EPPase. The probe was labeled with alkaline phosphatase using the

FIG. 5. Nucleotide and deduced amino acid sequence of EPPase. The nucleotide numbers are indicated on the right, and the amino acid numbers are indicated on the left. The amino acid sequences obtained from six tryptic peptides of purified EPPase are underlined. The termination codon is indicated by an asterisk. The positions and direction of primers are indicated by arrows above the nucleotide sequence.

FIG. 6. The EPPase activity in Sf9 cells infected with recombinant baculovirus. Sf9 cells transfected with recombinant baculovirus at multiplicities of infection of 10 were incubated for 96 h. The open circles indicate the EPPase activity in the infected Sf9 cells. The closed circles indicate the EPPase activity in the uninfected Sf9 cells.
RESULTS

Biochemical Characterization of EPPase—To elucidate the subcellular localization of EPPase, the homogenate prepared from 3-day-old nondiapause eggs was fractionated by differential centrifugation. As shown in Fig. 2A, most of the EPPase activity was localized in the 100,000 × g supernatant, suggesting that EPPase is a soluble enzyme. Acid phosphatase, a typical marker enzyme of lysosomes, was predominantly distributed in the 10,000 × g pellet. The optimal pH levels of EPPase and acid phosphatase were 7.5 and 4.0, respectively (Fig. 2B). Fluoride and L-tartarate, which are strong inhibitors of lysosomal acid phosphatase (29–31), scarcely affected the EPPase activity, although these chemicals also inhibited acid phosphatase in B. mori eggs (Fig. 2C). All of the results obtained indicate that EPPase apparently differs from lysosomal acid phosphatase.

Purification of EPPase—The 10,000 × g supernatant prepared from 3-day-old nondiapause eggs was applied to a Q Sepharose HP column. Two peaks of the EPPase activity were eluted by a linear gradient of NaCl (Fig. 3A). The major peak, eluted within the concentration range from 170 to 200 mM (fractions 146–158), was subjected to zinc-charged Chelating Sepharose column chromatography. Most of the EPPase activity was eluted when 50 mM EDTA was added to the equilibration buffer (Fig. 3B). The active fractions (fractions 44–49) were pooled and applied to a Phenyl Sepharose column. Most of the EPPase activity was eluted with decreasing NaCl concentration (Fig. 3C). The active fractions (fractions 46–52) were pooled and applied to a HiTrap ConA column. EPPase was not bound to the matrix (elution profile not shown), but about 67% of the contaminating proteins were removed at this step (Table I). The active fraction from the HiTrap ConA column was subjected to Mono Q column chromatography twice. In the first Mono Q column chromatography, the EPPase activity with a small shoulder peak was eluted by a linear gradient of NaCl. The major active fractions (fractions 34–37) were pooled and resubjected to Mono Q column chromatography (Fig. 3E). The active fractions from the second Mono Q column chromatography (fractions 67–70) were pooled and subjected to gel filtration chromatography using a Superdex 200HR column, the EPPase activity was eluted as a single and symmetrical peak, and the peak corresponded to that monitored at 280 nm (Fig. 3F). SDS-PAGE analysis revealed that the protein obtained is a single protein with a molecular mass of 42 kDa (Fig. 3G).

The results of purification at each step are summarized in Table I. After seven steps of column chromatography, 0.42 mg of EPPase was obtained from 350 g of 3-day-old nondiapause eggs with 1.3% yield, and the specific activity increased to 3,061-fold (Table I).

Kinetic Analysis of EPPase—To examine the substrate specificity of EPPase, first it was studied whether or not EPPase activity was inhibited by pNPP, which is generally used as a substrate of phosphatase (21–24). As shown in Fig. 4, the EPPase activity was competitively inhibited by pNPP, indicat-
ing that pNPP binds to the same catalytic site in EPPase as in the case of E22P. However, the specific constant \((k_{\text{cat}}/K_m)\) of EPPase was 16-fold lower for pNPP than for E22P (Table II). This result indicates that EPPase has greater specificity for E22P. Next, kinetic properties of the enzyme were analyzed for different ecdysteroid-phosphates in *B. mori* eggs. The \(k_{\text{cat}}/K_m\) value of the enzyme varied according to the substrate used (Table II). The \(k_{\text{cat}}/K_m\) values for 2dE22P and 20E22P were 1.4- and 4.7-fold higher, respectively, than that for E22P. However, the \(k_{\text{cat}}/K_m\) value for 22d20E3P was about 200-fold lower than that for E22P. These results suggest that EPPase has greater specificity for ecdysteroid-phosphates that have a phosphate group at the C-22 position rather than at the C-3 position.

**Molecular Cloning of EPPase**—The amino acid sequences of six peptides obtained from purified EPPase were determined: peptide 1, SDSILITQGGLPMNWELSK; peptide 2, VDLTYGPWVPHCFCNDYVR; peptide 3, LGWFQAAQLVGEGR; peptide 4, GIDFMTPIELXK (where \(X\) represents a gap); peptide 5, AGLNVDTPKYVEMDASAETMDEF; and peptide 6, RGEVAMQAVNDTEDGQGNNFGHA.

Two degenerate primers, EPP 1F and EPP 5R, were designed based on parts of amino acid sequences of peptides 1 and 5. These primers were used to amplify the EPPase-cDNA fragment (567 bp) by reverse transcription PCR using total RNA isolated from 3-day-old nondiapause eggs (Fig. 5). Gene-specific primers derived from this sequence were synthesized and used for 3'- and 5'-RACE. The 3'-RACE product (793 bp) contains a stop codon and an 18-bp poly(A) sequence, and the 5'-RACE product (414 bp) contains a putative translation start site (Fig. 5). Finally, a pair of gene-specific primers, ORF F and ORF R, were synthesized and used to amplify a cDNA fragment (996 bp) including the whole open reading frame of EPPase (Fig. 5).

The nucleotide sequence of this PCR product completely corresponded to that determined by 3'- and 5'-RACE.

The deduced amino acid sequence of the isolated cDNA contained each of the amino acid sequences of six peptides obtained by tryptic digestion of purified EPPase (Fig. 5). The full-length cDNA was composed of 1620 bp with an open reading frame encoding a protein of 331 amino acid residues (Fig. 5).

**Expression of EPPase by Baculovirus Expression System**—To confirm that the isolated cDNA encodes the functional EPPase, the recombinant EPPase was expressed using the baculovirus expression system, and its properties were compared with those of native EPPase. Sf9 cells were infected with recombinant baculovirus harboring the whole open reading frame of EPPase, and the cells were collected every 24 h after infection. The EPPase activity increased rapidly from 24 h after infection and reached a steady state 72 h after infection (Fig. 6). The EPPase activity was not detected in uninfected cells (Fig. 6).

To confirm that the isolated cDNA encodes the functional EPPase, the recombinant EPPase was expressed using the baculovirus expression system, and its properties were compared with those of native EPPase. Sf9 cells were infected with recombinant baculovirus harboring the whole open reading frame of EPPase, and the cells were collected every 24 h after infection. The EPPase activity increased rapidly from 24 h after infection and reached a steady state 72 h after infection (Fig. 6). The EPPase activity was not detected in uninfected cells (Fig. 6). The recombinant EPPase was extracted from the cells infected at multiplicity of infection of 10 and purified according to the methods described under “Experimental Procedures.” By Hi-Trap Q column chromatography, the recombinant EPPase was eluted within the NaCl concentration range from 170 to 210 mM (elution profile not shown). The active fraction was dialyzed and then subjected to Mono Q column chromatography. The recombinant EPPase was eluted within the NaCl concentration range from 150 to 170 mM (elution profile not shown). The active fraction was finally purified using a Superdex 200HR column (Fig. 7A). Western blot analysis of the recombinant and native EPPase using antibodies against recombinant EPPase showed that both proteins have the same antigenicity (Fig. 7B) and the same molecular mass (Figs. 3F and 7B). Furthermore,
V}_{\text{max}} and K_{m} values for E22P and pNPP of the recombinant EPPase (V_{\text{max}}, 1.13 \mu \text{mol/min/mg protein and } K_{m}, 6.12 \mu \text{M}) for E22P; V_{\text{max}}, 120.1 \mu \text{mol/min/mg protein and } K_{m}, 10613.0 \mu \text{M} for pNPP) were almost the same as those of the native EPPase (Table II). These results indicate that the isolated cDNA encodes a functional EPPase.

**Tissue Distribution and Expression Pattern of EPPase during Embryonic Development**—Using a cDNA probe containing the whole open reading frame of EPPase, a transcript of ~1.7 kb was detected in 3-day-old nondiapause eggs but was not detected in diapause eggs (Fig. 8). Furthermore, the transcript was not detected in the mature ovary and testis at the pupal stage and Malpighian tubules, fat body, and midgut at the fifth instar of larvae.

The fluctuations of the amounts of free ecldysteroids and the EPPase activity during embryonic development are shown in Fig. 9A. In nondiapause eggs, the amounts of free ecldysteroids increased from 2 days after oviposition (late gastrula stage) and reached a peak 5 days after oviposition (blastokinesis stage). Thereafter, it decreased gradually as embryonic development proceeded. The EPPase activity increased slightly 1 day after oviposition (early gastrula stage) and reached a peak 3–4 days after oviposition (organogenesis stage). However, the activity decreased gradually to the basal level as embryonic development proceeded. In diapause eggs, the free ecldysteroids were detected only in trace amounts throughout the embryonic development. The EPPase activity was also not detected in diapause eggs. Northern blot analysis of the transcript during embryonic development is shown in Fig. 9B. In nondiapause eggs, the transcript was detected weakly 1 day after oviposition, and its signal intensified between days 2 and 5. However, after that it was scarcely detected. In diapause eggs, the transcript was not detectable throughout the embryonic development.

**DISCUSSION**

In eggs of various insect species, it has been suggested that maternal ecldysteroids are involved in cuticle formation during embryonic development (6–9). However, evidence for this notion is based on temporal correlations rather than direct evidence. On the other hand, it was demonstrated that 20E is required for continuing to embryonic development in B. mori (11, 13). Furthermore, 20E was demonstrated to be produced de novo (11, 13). In nondiapause embryos, it was shown that maternal ecdysteroids are involved in cuticle formation during embryonic development. The EPPase activity was also not detected in diapause eggs. Northern blot analysis of the transcript during embryonic development is shown in Fig. 9B. In nondiapause eggs, the transcript was detected weakly 1 day after oviposition, and its signal intensified between days 2 and 5. However, after that it was scarcely detected. In diapause eggs, the transcript was not detectable throughout the embryonic development.

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