Purification, Kinetic Characterization, and Molecular Cloning of a Novel Enzyme, Ecdysteroid 22-Kinase*

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This is the first report succeeding in the isolation and characterization of an enzyme and its gene involved in the phosphorylation of a steroid hormone. It has been demonstrated that ecdysteroid 22-phosphates in insect ovaries, which are physiologically inactive, serve as a “reservoir” that supplies active free ecdysteroids during early embryonic development and that their dephosphorylation is catalyzed by a specific enzyme, ecdysteroid-phosphate phosphatase (Yamada, R., and Sonobe, H. (2003), J. Biol. Chem. 278, 26365–26373). In this study, ecdysteroid 22-kinase (EcKinase) was purified from the cytosol of the silkworm Bombyx mori ovaries to about 1,800-fold homogeneity in six steps of column chromatography and biochemically characterized. Results obtained indicated that the reciprocal conversion of free ecdysteroids and ecdysteroid 22-phosphates by two enzymes, EcKinase and ecdysteroid-phosphate phosphatase, plays an important role in ecdysteroid economy of the ovary-egg system of B. mori. On the basis of the partial amino acid sequence obtained from purified EcKinase, the nucleotide sequence of the cDNA encoding EcKinase was determined. The full-length cDNA of EcKinase was composed of 1,850 bp with an open reading frame encoding a protein of 386 amino acid residues. The cloned cDNA was confirmed to encode the functional EcKinase using the transformant harboring the open reading frame of EcKinase. A data base search showed that EcKinase has an amino acid sequence characteristic of phosphotransferases, in that it harbors Brenner’s motif and putative ATP binding sites, but there are no functional proteins that share high identity with the amino acid sequence of EcKinase.

In steroid metabolism, it is generally accepted that cytochrome P450-mediated reaction, referred to as phase I metabolism, converts hydrophobic compounds into more polar metabolites, whereas phase II metabolism involves an adduct formation via a conjugation reaction. Phase II metabolism, such as sulfation, glucuronidation, and fatty acyl esterification, leads to a change of polarity or to a charge modification of the given metabolite, thereby modulating biological activity or facilitating elimination. In mammals, the sulfation pathway can be thought of as a reversible process, comprising two enzyme systems: the sulfotransferases, which catalyze the sulfation reaction, and the sulfatases, which catalyze the hydrolysis of sulfate esters formed by the action of sulfotransferases. Much is now known concerning the function of these enzyme systems as well as the molecular structure of the relevant cDNA and genes comprising these systems (1–4). However, to our knowledge, there exists very little information regarding the phosphorylation of steroid hormones in mammals.

Ecdysteroids, which include the arthropod molting hormone, mediate a wide variety of developmental and reproductive events in insects (5). During the larval and pupal stages, ecdysteroids are synthesized in steroidogenic glands, known as the prothoracic glands (6). The detailed ecdysteroid biosynthetic pathway constituted by phase I metabolism is not fully clarified, but cytochrome P450 enzymes catalyzing the last four steps of the biosynthesis of 20-hydroxyecdysone (the principal molting hormone of arthropods) (i.e. CYP306a1 (25-hydroxylase), CYP302a1 (22-hydroxylase), CYP315a1 (2-hydroxylase), and CYP314a1 (20-hydroxylase), have been identified and characterized in Drosophila melanogaster, Bombyx mori, and Aedes aegypti (7–13).

Although glucosides (14), fatty acyl esters (15), and sulfate esters (16) of ecdysteroids are detected as minor products of phase II reaction in several insect species, the major products of the phase II reaction in most insect species are phosphate esters (17–20). The ovaries of most insect species have the capacity to accumulate ecdysteroid phosphates, which are physiologically inactive (21), in addition to the capacity to synthesize free ecdysteroids de novo (22, 23). It has been suggested that the ecdysteroid phosphates that are accumulated in the ovaries are transferred to the eggs and function as a source of active free ecdysteroid before the prothoracic glands differentiate during embryonic development (24–26). Recently, in B. mori eggs, a novel enzyme ecdysteroid-phosphate phosphatase (EPPase) (Fig. 1), which is specifically involved in dephosphorylation of ecdysteroid phos-

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3 The abbreviations used are: EPPase, ecdysteroid-phosphate phosphatase; EcKinase, ecdysteroid 22-kinase; HPLC, high performance liquid chromatography; RT, reverse transcription; RACE, rapid amplification of cDNA ends; EST, expressed sequence tag.
Phosphorylation of Steroid Hormones

![Diagram showing the conversion of ecdysone to ecdysone 22-phosphate](image)

**FIGURE 1. Reciprocal conversion of free ecdysone and ecdysone 22-phosphate by EcKinase and EPPase.**

The phosphorylation of steroid hormones is a critical process in the development of insects. The enzyme EcKinase, which is responsible for phosphorylation at the C-22 position of ecdysteroids, was isolated, characterized, and revealed to be a vital enzyme that may control the “on/off switch” of embryonic development (27–29).

The discovery of EPPase in *B. mori* eggs emphasizes the physiological significance of phase II phosphorylation reaction in the ovary-egg system of insects. However, detailed characterization of enzymes involved in the formation of ecdysteroid phosphates has not been carried out, although the occurrence of enzyme activity has been reported in the ovaries of *Schistocerca gregaria* (30) and *B. mori* (31). We now report the purification, kinetic characterization, and cDNA cloning of an enzyme responsible for phosphorylation at the C-22 position of ecdysteroids in the mature oocytes of *B. mori*, named as ecdysteroid 22-kinase (EcKinase) (Fig. 1), and show that EcKinase belongs to a novel kinase family.

**EXPERIMENTAL PROCEDURES**

**Experimental Animals**—The pupae of a hybrid race (kinshu x showa) of the silkworm *B. mori*, which have been programmed to lay diapause eggs (diapause egg-producing pupae), were mainly employed. In order to obtain pupae programmed to lay nondiapause eggs (nondiapause egg-producing pupae), the subesophageal ganglion, the source of diapause hormone, was removed soon after larval-pupal ecdysis (32, 33). Pupal-adult ecdysis in both types of pupae took place 12–13 days after the larval-pupal ecdysis at 23–24 °C. There was no appreciable difference in the growth rate of ovaries between both types of pupae.

**Chemicals**—[23,24,3H]Ecdysone (2,112 GBq/mmol) was obtained from PerkinElmer Life Sciences. Ecdysone and 20-hydroxyecdysone were purchased from Sigma. Ecdysone 22-phosphate was synthesized chemically (34). Other ecdysteroids, 2-deoxyecdysone, 20-hydroxyecdysone, 22-deoxy-20-hydroxyecdysone, and 2,22-dideoxy-20-hydroxyecdysone, were extracted from *B. mori* ovaries and purified by high performance liquid chromatography (HPLC), as described previously (35–37).

**Preparation of Enzyme Solution**—All operations were carried out at ~4 °C. For subcellular fractionation, mature ovaries were excised from adult moths in insect Ringer’s solution, and their chorions were removed using tweezers in a small volume of homogenization buffer: 10 mM Hepes-NaOH buffer, pH 7.5, containing 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 mM pepstatin A, and 10 mM leupeptin. The ooplasm obtained did not contain follicle cells and nurse cells as observed by light microscopy. The ooplasm was centrifuged at 1,000 × g for 5 min. The resultant pellet (yolk granule fraction) was washed again with the same buffer solution and centrifuged, and the pellet was used as the yolk granule fraction. The supernatant was combined, homogenized, and used for differential centrifugation, the procedure of which is essentially the same as that described in our previous paper (38). The homogenate was first centrifuged at 1,000 × g for 30 min, and the resultant supernatant was further centrifuged at 10,000 × g for 30 min. The next resultant supernatant was further centrifuged at 150,000 × g for 60 min. The yolk granule fraction, 1,000 × g pellet, 10,000 × g pellet, 150,000 × g g pellet, and the lipid layer obtained from centrifugation at 10,000 × g were sonicated in a small volume of homogenization buffer to an emulsion state. These emulsions and the final 150,000 × g supernatant (cytosol) were used for the enzyme assay.

To determine changes in enzyme activity during ovarian development, ovaries during pupal-adult development were dissected and homogenized with ~5 volumes of the homogenization buffer. The homogenate was centrifuged immediately at 150,000 × g for 60 min. The supernatant was used as the enzyme source.

For enzyme purification, mature ovaries (450 g) from diapause egg-producing pupae were employed. The ovaries were ground using an agate mortar in ~2 volumes of homogenization buffer, and the homogenate was filtered through gauze to remove chorions. The filtrate was further homogenized with a glass-Teflon homogenizer, and the homogenate was centrifuged at 150,000 × g for 60 min. The resultant supernatant was applied to column chromatography (see below).

**Enzyme Assay**—The activity of ecdysone 22-kinase, which was subsequently named EcKinase (see “Results”), was measured using [3H]ecdysone as the substrate. The standard assay system for ecdysone 22-kinase activity contains the following in 100 μl of 10 mM Hepes-NaOH buffer, pH 7.5, containing 0.1% bovine serum albumin: 2 mM ATP, 10 mM MgCl₂, 740 Bq of [3H]ecdysone, and enzyme solution. The reaction was initiated by adding the enzyme solution, the reaction mixture was incubated at 35 °C for 30 min, and the reaction was stopped by adding 4 volumes of methanol. The mixture was centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was collected and evaporated to dryness under vacuum. The residue was dissolved in 1 ml of distilled water and applied to a C18 cartridge (Varian). After washing with 4 ml of distilled water to remove ATP and any salts, ecdysteroids were eluted with 6 ml of methanol. The ecdysteroid fraction was dried, and the residue was dissolved in 1 ml of 80% (v/v) chloroform/methanol/water. The radioactivity of the [3H]ecdysone 22-phosphate was eluted with 6 ml of 80% (v/v) methanol/water. The radioactivity of the [3H]ecdysone 22-phosphate fraction was measured using a liquid scintillation counter (LSC-5101; Aloka) to quantify ecdysone 22-phosphate formed. The recovery of radioactivity throughout the entire procedure was more than 90%.

For the characterization of substrate specificity of ecdysone 22-kinase, 20-hydroxyecdysone, 2-deoxyecdysone, 2-deoxy-20-hydroxyecdysone, 22-deoxy-20-hydroxyecdysone, and 2,22-dideoxy-20-hydroxyecdysone were used in addition to ecdysone. Enzyme activity was assayed using the standard assay system for ecdysone 22-kinase activity by substituting the above mentioned ecdysteroids (20 μM) for the usual substrate, [3H]ecdys-
one. Following HPLC (see below), the reaction products were monitored at 254 nm and quantified using an on-line integrator D-2500 (Hitachi). The relative activity of the enzyme was expressed by defining the amount of ec dysone 22-phosphate produced in the control assay as 100%. For the identification of the products of enzyme reactions, the ec dysosteroid phosphate fraction was divided into two portions. One portion was used to compare retention times with those of authentic ec dysosteroid phosphates by HPLC (see below). The remainder was used in EPPase hydrolysis; the retention times of free ec dysosteroids liberated after EPPase hydrolysis were compared with those of authentic free ec dysosteroids.

A competition assay was performed by adding ec dysosteroids at a concentration of 100 µM as a competitor into the standard assay system. The radioactivity of [3H]ec dysone 22-phosphate produced was compared with the control without the competitor. The inhibitory effect was expressed as a percentage of control.

The activity of the recombinant EcKinase was measured using the standard assay system by substituting 20 µM ec dysone for the usual substrate, [3H]ec dysone. The ec dysosteroid fraction in the reaction mixture was applied to HPLC (see below), and the effluent was monitored at 254 nm.

Protein Assay—Protein concentration was determined by the method of Bradford (39), using the Bio-Rad protein assay reagent (Bio-Rad) according to the manufacturer’s protocol with bovine serum albumin as a standard.

HPLC Analysis of Ec dysosteroids—Ec dysosteroid phosphates and free ec dysosteroids liberated by enzymatic hydrolysis (see below) were identified using HPLC (LC-10A system; Shimazu) equipped with a reverse-phase column (4.6 × 250 mm; Wakosil 5C18; Wako, Japan). The column was eluted with a 50- or 60-min linear gradient of methanol in 20 mM potassium phosphate buffer, pH 5.6, changing from 0 to 70% (v/v) at a flow rate of 1 ml/min at 40°C.

Quantification of Ec dysosteroid Phosphates in Ovaries—Ec dysosteroids were extracted from ovarian homogenate by adding 4 volumes of methanol, and ec dysosteroid phosphates were separated from free ec dysosteroids as described previously (37). To enhance the sensitivity to radioimmunoassay using the anti-serum N6 (40), ec dysosteroid phosphates were hydrolyzed to free ec dysosteroids, using a digestive enzyme preparation from Helix pomatia (see below). The radioimmunoassay was conducted as described previously (41). Ec dysosteroid quantity was expressed in terms of ec dysone equivalents.

Enzymatic Hydrolysis of Ec dysosteroid Phosphates—Ec dysosteroid phosphates were hydrolyzed to free ec dysosteroids by recombinant EPPase (27) or the H. pomatia digestive enzyme preparation (Nakarai Tesque). The former specifically catalyzes the dephosphorylation of ec dysosteroid 22-phosphate, whereas the latter contains non-specific phosphatase activity. The procedures of each enzymatic hydrolysis have been described previously (27, 28, 37).

Purification Procedures of Ec dysone 22-Kinase—All of the purification procedures were carried out at ~4°C. An aliquot of the 150,000 × g supernatant prepared from mature ovaries (see “Preparation of Enzyme Solution”) was loaded onto a blue Sepharose 6 FF column (26 × 75 mm; Amersham Biosciences) pre-equilibrated with 10 mM Hepes-NaOH buffer, pH 7.5. Stepwise elution was performed using the same buffer, containing 2 mM KCl at a flow rate of 4 ml/min. The active fraction was applied to a chelating Sepharose FF column (26 × 75 mm; Amersham Biosciences) pre-equilibrated with 10 mM Hepes-NaOH buffer, pH 7.5, containing 2 mM KCl. Stepwise elution was performed using the same buffer, containing 100 mM histidine at a flow rate of 4 ml/min. The active fraction was loaded onto a Sephacryl S-100 HR column (26 × 600 mm; Amersham Biosciences) pre-equilibrated with 10 mM Hepes-NaOH buffer, pH 7.5, containing 150 mM NaCl. The enzyme was eluted with the same buffer at a flow rate of 2 ml/min. The active fraction was concentrated to about 50-fold, and the buffer was changed to 5 mM Tris-HCl buffer, pH 7.5, using an Amicon Ultra-15 centrifugal filter device (Millipore Corp.). The enzyme solution was applied to a Mono Q HR 5/5 column (Amersham Biosciences) pre-equilibrated with 5 mM Tris-HCl buffer, pH 7.5. Elution was performed using a linear gradient of 0–100 mM NaCl in the same buffer for 20 min at a flow rate of 1 ml/min. The active fraction was concentrated to about 100-fold, and the buffer was changed to 10 mM Hepes-NaOH buffer, pH 7.5. The enzyme solution was applied to a ceramic hydroxyapatite column (Type 1, 7 × 52 mm; Bio-Rad) pre-equilibrated with 10 mM Hepes-NaOH buffer, pH 7.5. Elution was performed by changing the elution buffer linearly to 200 mM potassium-phosphate buffer, pH 7.5, for 90 min at a flow rate of 1 ml/min. The active fraction was dialyzed against 5 mM Tris-HCl buffer, pH 7.5, and applied again to the Mono Q HR 5/5 column pre-equilibrated with 5 mM Tris-HCl buffer, pH 7.5. The enzyme was eluted by a linear gradient of 0–100 mM NaCl for 45 min.

The purity was analyzed using HPLC equipped with a reverse-phase column (CAPCELL PACK C18, 2 × 250 mm; Shiseido Fine Chemicals) pre-equilibrated with 0.5% trifluoroacetic acid. Elution was performed using a 30-min linear gradient of 0–80% acetonitrile in 0.5% trifluoroacetic acid at a flow rate of 1 ml/min at 280 nm. The purity was also analyzed by SDS-PAGE, using a 10% gel. Several standard proteins (e.g. myosin (205 kDa), phosphorylase b (97.4 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), and trypsin inhibitor (21.5 kDa)) were used to estimate the molecular mass of purified ec dysone 22-kinase. The proteins were visualized by silver staining.

Amino Acid Sequence Analysis—In order to analyze the internal amino acid sequence of purified ec dysone 22-kinase, 500 pmol of purified enzyme were reduced with dithiothreitol and alkylated with iodoacetamide and digested with lysylendopeptidase. The resultant peptides were applied to reverse-phase HPLC using a TSK gel ODS-80Ts (2.0 × 250 mm; Tosoh) pre-equilibrated with 0.1% trifluoroacetic acid. Elution was performed with a 90-min linear gradient of 0–90% acetonitrile in 0.09% trifluoroacetic acid at a flow rate of 0.4 ml/min. Among the peptides obtained, four peptides, named K54, K71, K75-1, and K75-2, were applied to an amino acid sequencer (see below), and one peptide, named K94, was digested again with trypsin. The digests were separated by reverse-phase HPLC using a Symmetry C18 column (1.0 × 150 mm; Waters) pre-equilibrated with 2% acetonitrile containing 0.1% trifluoroacetic acid. Elution was performed with a 100-
min linear gradient of 2–90% acetonitrile in 0.1% acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.05 ml/min. Two fragments obtained, named K94-T1 and K94-T2, were applied to an amino acid sequencer. All amino acid sequence analyses were performed using a Procise 494 cLC protein sequencing system (Applied Biosystems).

Isolation of EcKinase cDNA—The 12–40-h-old diapause eggs of strain p50T were collected, and total RNA was prepared using TRizol Reagent (Invitrogen). Construction of the full-length cDNA library, named fdpe, was carried out by Hitachi Instruments Service Co., Ltd. (Tokyo, Japan). Sequences from the 5'-end of each cDNA were determined, and the expressed sequence tags (ESTs) were compiled into a local data base. One EST clone, which is expected to encode the amino acid sequences of six peptides obtained from purified EcKinase, was searched by means of standard BLAST analysis, and the full length of its nucleotide sequence was determined.

5'-Rapid Amplification of cDNA Ends (5'-RACE)—Total RNA was prepared from mature ovaries and 72-h-old diapause eggs according to the same methods as described above. Each first-strand cDNA was synthesized with random hexamer using Ready-to-Go RT-PCR Beads (Amersham Biosciences) according to the manufacturer's instructions. The following primers were used: R1 (5'-TCGCTGTCACAGCTGTGATTG-3') and R2 (5'-CATGAGGGTCTGTGTTGATCG-3'), were designed based on the nucleotide sequence of one EST clone, named fdpeP12_F_109. The 5'-end of EcKinase cDNA was amplified by two rounds of PCR. In the first PCR, amplification was performed with R2 and a 5'-adapter primer, UPM (Clontech), using the first-strand cDNA as a template. PCR was carried out as follows: first denaturation at 95 °C for 30 s; five cycles of 95 °C for 10 s, 68 °C for 10 s, 72 °C for 2 min; five cycles of 95 °C for 10 s, 66 °C for 10 s, 72 °C for 2 min; five cycles of 95 °C for 10 s, 68 °C for 10 s, 72 °C for 2 min; five cycles of 95 °C for 10 s, 66 °C for 10 s, 72 °C for 2 min; and final extension at 72 °C for 2 min.

Detection of EcKinase Transcript—Total RNA was prepared from mature ovaries and 72-h-old diapause eggs according to the same methods as described above. Each first-strand cDNA was synthesized with random hexamer using Ready-to-Go RT-PCR Beads (Amersham Biosciences) according to the manufacturer's instructions. The following primers were designed to detect the expression of EcKinase and elongation factor 2 as an internal control: F1 (5'-AAACACTGTTGCTGAGGACCTG-3') and R3 (5'-TCTGGCTCTCTTCTCTACCTACCT-3') for EcKinase, EF2-F (5'-GTCCTGACGGTGAGGCATGTAGCTG-3') and EF2-R (5'-CAATGCTCCTCGGACGACCCTAGC-3') for elongation factor 2. The first strand cDNA was used as templates, and each PCR amplification was conducted by the following program: first denaturation at 94 °C for 30 s; 45 cycles of 94 °C for 20 s, 64 °C for 20 s, 72 °C for 1 min 30 s; and final extension at 72 °C for 1 min 30 s. PCR products were electrophoresed by an 1.5% agarose gel and visualized after staining with ethidium bromide.

Construction of the EcKinase Expression Plasmid—In order to avoid the artificial effects of additional sequences, such as His tag and/or some epitope tags, which are encoded in commercial expression plasmids, on biological activity of the recombinant enzyme, we constructed the expression plasmid to express the whole open reading frame of EcKinase without artificial sequences to avoid the artificial effects of additional sequences, such as His tag and/or some epitope tags, which are encoded in commercial expression plasmids, on biological activity of the recombinant enzyme, we constructed the expression plasmid to express the whole open reading frame of EcKinase without artificial sequences.
sequence) and 23 residues (nucleotide positions 1,170–1,192 in Fig. 10), including the stop codon (boldfaced in the primer sequence). Using these primers, PCR was conducted with the EST clone, fdpeP12_F_I09, as the template. The amplified cDNA insert was subcloned into pCR2.1-TOPO plasmids (Invitrogen), and the sequence fidelity was confirmed. Subsequently, the insert was released from pCR2.1-TOPO by HindIII/EcoRI digestion and then ligated into the HindIII/EcoRI site of the expression plasmid pIB/V5-His (Invitrogen).

Expression of the EcKinase Construct in Spodoptera frugiperda Cells (Sf9 cells)—Sf9 cells were cultured in Sf900IISFM (Invitrogen). Transfection with the expression plasmid was performed using Cellfectin reagent according to the manufacturer’s protocol (Invitrogen). Stable transformant was selected using 40 \( \mu \)g/ml blasticidin S (Invitrogen) and maintained at 10 \( \mu \)g/ml blasticidin in 75-cm\(^2\) bottles at 28 °C. To prepare the recombinant EcKinase, \(4 \times 10^7\) cells transfected with the EcKinase construct were collected at centrifugation (3,500 rpm, 20 min) and homogenized in 500 \( \mu \)l of 50 mM Hepes-NaOH buffer, pH 7.5, containing 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 \( \mu \)M pepstatin A, and 10 \( \mu \)M leupeptin. The homogenate was centrifuged at 150,000 \( g\) for 60 min at 4 °C. The supernatant was dialyzed against 500 ml of 50 mM Hepes-NaOH buffer, pH 7.5, at 4 °C using a Slide-A-Lyzer dialysis cassette (10,000 molecular weight cut-off; Pierce). The dialysate was used for immunoblot analysis (see below) and the measurement of EcKinase activity (see "Enzyme Assay").

Immunoblotting—In order to prepare antibodies against EcKinase, two different EcKinase fragments, EcKinase (amino acids 44–57) and EcKinase (amino acids 356–368) that had been extended on each N terminus with a cysteine residue, were synthesized chemically and were coupled to bovine serum albumin with \( m \)-maleimidobenzoyl-N-hydroxysuccinimide ester. In a data base search, we could not find other proteins that have identity with the amino acid sequences of those fragments. Each conjugated fragment was mixed, emulsified in complete Freund’s adjuvant, and used to immunize rabbits. Antibody activity was examined by enzyme-linked immunosorbent assay using the synthetic EcKinase (amino acids 44–57) and EcKinase (amino acids 356–368) fragments. The serum obtained was positive at a 5,000-fold dilution level of the antiserum.

Recombinant EcKinase and purified native EcKinase were subjected to SDS-PAGE (10% gel) and transferred to a polyvinylidene difluoride membrane. The membrane was preincubated for 1 h with 20 mM...
Tris--HCl buffer saline containing 2% blocking agent (GE Healthcare). Then membranes were incubated with anti-EcKi
nase antiserum (1:2000 dilution) and goat anti-rabbit peroxi
dase-conjugated IgG (1:20,000 dilution; Wako) for 1 h at 25 °C. 
The peroxidase activity was visualized using an ECL-plus detec
tion system (GE Healthcare).

RESULTS

Identification of Reaction Product in the Standard Assay System—Since ecdysone 22-kinase activity in mature oocytes is 
localized in the cytosol (see below), the cytosol was incubated 
with [3H]ecdysone under standard assay conditions, and the 
product was analyzed by HPLC. As shown in Fig. 2, only one 
radioactive product was obtained corresponding to the position 
of elution of authentic ecdysone 22-phosphate, having a retention 
time of 29.3 min. When these radioactive fractions (fractions 
58–60) were pooled and incubated with EPPase, total 
radioactivity recovered corresponded to the position of elution 
of authentic ecdysone, having a retention time of 43.2 min 
(radiochromatogram not shown). These results confirmed the 
identity of the reaction product as ecdysone 22-phosphate, 
indicating that ecdysone 22-kinase exists in the mature oocytes 
of B. mori.

Changes in Ecdysone 22-Kinase Activity during Ovarian Development—Ovarian homogenate (see “Experimental Proce
dures”), which was prepared separately from the ovaries of dia
pause egg-producing pupae and nondiapauser egg-producing 
pupae, was used to measure ecdysone 22-kinase activity and the 
quantity of ecdysteroid phosphates during ovarian develop
ment. Enzyme activity in the ovaries of diapause egg-producing 
pupae was maintained at a low level until the 6th day after 
aviposition. Thereafter, activity increased rapidly until the 10th 
day and then leveled off until emergence (Fig. 3A). The profile 
of enzyme activity in nondiapause egg-producing pupae was 
almost identical to that of diapause egg-producing pupae (Fig. 
3A). The quantity of ecdysteroid phosphates in the ovaries of 
diapause egg-producing pupae increased gradually, corre
sponding to ovarian development, and reached a maximum in 
mature ovaries (Fig. 3B). This result is essentially the same as 
that reported earlier (42). The developmental change in the 
quantity of ecdysteroid phosphates in nondiapause egg-pro
ducing pupae was almost identical to that in diapause egg-pro
ducing pupae (Fig. 3B).

Taken together, it is obvious that quantities of ecdysteroid phosphates during ovarian development increased in parallel 
with the increase in ecdysone 22-kinase activity in the ovaries of 
both diapause egg-producing pupae and nondiapause egg-pro
ducing pupae. This result is consistent with a previous postula
tion that the accumulation of ecdysteroid phosphates in the 
ovaries was brought about by a phosphorylation reaction in the 
ovaries themselves but not by the transport of ecdysteroid phosphates from the hemolymph (31). Two conditions (i.e. that 
high enzyme activity remains in mature ovaries and that dia
pause egg-producing pupae can be obtained from local sericul
turists more readily than nondiapause egg-producing pupae) 
compelled us to use mature ovaries of diapause egg-producing 
pupae in the purification of the kinase and in the following 
investigation of biochemical characterization.

Subcellular Localization of Ecdysone 22-Kinase—The yolk granule fraction and supernatant fraction were prepared from ma
ture ovaries and homogenized for use in the enzyme assay. As 
shown in Fig. 4A, ecdysone 22-kinase activity was detected pre
dominantly in the supernatant fraction, but little or no enzyme 
activity was found in the yolk granule fraction. Subsequently, the 
supernatant fraction was separated into the 1,000 x g pellet, 10,000 x g 
pellet, lipid layer, 150,000 x g pellet, and cytosol by differential centrifuga
tion. Each pellet was sonicated in homogenization buffer and used for measuring ecdysone 22-kinase activ
ity. As shown in Fig. 4B, the cytosol fraction exhibited high enzyme activity.

Purification of Ecdysone 22-

KInase—The cytosol fraction pre
pared from mature ovaries was 
first applied to a blue Sepharose 6 FF col
umn. Enzyme activity was eluted with

![FIGURE 6. Analysis of purity of the finally obtained enzyme fraction. A, reverse-phase HPLC analysis of the 
active fraction obtained from the second Mono Q chromatography. The concentration of acetonitrile is indi
cated by the dashed line. B, SDS-PAGE analysis of the active fraction obtained from the second Mono Q chro
matography. The positions of the standard proteins are shown on the right.](Image 60x223 to 396x347)

| Purification step | Total activity | Protein | Specific activity | Yield | Purification |
|-------------------|----------------|---------|------------------|-------|-------------|
| 150,000 x g supernatant | 644.6 | 14691.6 | 0.044 | 100 | 1 |
| Blue Sepharose 6 FF | 327.2 | 7653.9 | 0.042 | 50.8 | 0.96 |
| Chelating Sepharose FF | 232.8 | 3959.1 | 0.059 | 36.1 | 1.34 |
| Sephacryl S-100 HP | 126.4 | 23.4 | 5.4 | 19.6 | 122.7 |
| Mono Q HP 5/5 (first) | 113.2 | 12.1 | 9.3 | 17.6 | 212.7 |
| Hydroxyapatite Type 1 | 43.6 | 1.35 | 32.3 | 6.8 | 734.1 |
| Mono Q HP 5/5 (second) | 20.9 | 0.26 | 80.4 | 3.2 | 1827.3 |

* Estimated by A 280 with BSA as a standard.
2 mM KCl solution (Fig. 5A). Enzyme activity was not affected by the 2 mM KCl solution used (data not shown). The active fractions (fractions 42–45) were pooled and subjected to chelating Sepharose column chromatography. Enzyme activity was eluted from the second Mono Q column, the active fraction was subjected to reverse-phase HPLC. A single sharp peak of protein was observed in this system (Fig. 6A). SDS-PAGE analysis also revealed that the protein obtained was a single protein having a molecular mass of 44 kDa (Fig. 6C). The active fractions (fractions 23–26) were pooled and subjected to gel filtration chromatography using Sephacryl S-100 column. Enzyme activity was observed in fractions of molecular mass ranging from 30 to 45 kDa (Fig. 5C). The fractions having the high activity (fractions 43–47) were pooled and subjected to Mono Q column chromatography. Enzyme activity was eluted at 35–50 mM NaCl (Fig. 5B). The active fractions (fractions 23–26) were pooled and subjected to gel filtration chromatography using Sephacryl S-100 column. Enzyme activity was observed in fractions of molecular mass ranging from 30 to 45 kDa (Fig. 5C). The fractions having the high activity (fractions 43–47) were pooled and subjected to Mono Q column chromatography. Enzyme activity was eluted at 35–50 mM NaCl (Fig. 5D). No appreciable effect of NaCl on enzyme activity was observed at concentrations lower than 150 mM (data not shown). Fractions 12 and 13 were pooled and subjected to a hydroxypatite column. Enzyme activity was eluted with about 30 mM potassium phosphate buffer, pH 7.5 (Fig. 5E). The active fractions (fractions 23–25) were pooled and subjected to a second Mono Q column chromatography. A large and symmetrical peak, being detectable at 280 nm, was eluted with 50 mM NaCl solution, and the peak corresponded to enzyme activity (Fig. 5F).

In order to analyze the purity of the enzyme fraction obtained from the second Mono Q column, the active fraction was subjected to reverse-phase HPLC. A single sharp peak of protein was observed in this system (Fig. 6A). SDS-PAGE analysis also revealed that the protein obtained was a single protein having a molecular mass of ~44 kDa (Fig. 6B).

The results of purification at each step are summarized in Table 2. The results were highly reproducible.

### Biochemical Characterization of Ecdysone 22-Kinase
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The reaction rate was linear for at least 60 min (Fig. 7A) and at protein concentrations up to 2 μg/100 μl (reaction mixture) (Fig. 7B). The optimal reaction temperature was about 35 °C (Fig. 7C). The optimal pH was about 7.5 (Fig. 7D). Enzyme activity was stable at ~30 °C for at least 6 months. Kinase activity was markedly influenced by Mg²⁺, and maximal activity was obtained in the presence of 10 mM (Fig. 8A). Activity was strongly inhibited in the presence of Ca²⁺ (Fig. 8B), and 50% inhibition (IC₅₀) was obtained at a concentration of ~1 mM Ca²⁺. Activity required the presence of ATP as a phosphate donor, but no other nucleotide triphosphates (namely GTP, CTP, or UTP) were required (Fig. 8C). The Kₘ for ATP was 16.5 μM (Fig. 8D), and the Kₘ for ecdysone was 8.5 μM (Fig. 9).

In B. mori, ovaries have been found to contain four C-22 phosphate esters and three C-3 phosphate esters of ecdysteroids as follows: ecdysone 22-phosphate, 20-hydroxyecdysone 22-phosphate, 2-deoxyecdysone 22-phosphate, 2-deoxy-20-hydroxyecdysone 22-phosphate, 22-deoxy-20-hydroxyecdysone 3-phosphate, 2,22-dideoxy-20-hydroxyecdysone 3-phosphate, and a trace amount of 2,22-dideoxy-23-hydroxyecdysone 3-phosphate (29, 36, 37, 43). Thus, using the free type of these ecdysteroid phosphates, substrate specificity of ecdysone 22-kinase could be analyzed. The retention times of reaction products and free ecdysteroids liberated from reaction products by EPPase hydrolysis are shown in Table 2. The retention times of ecdysteroid phosphates produced in each reaction mixture were identical to those of authentic ecdysone 22-phosphate, 20-hydroxyecdysone 22-phosphate, 2-deoxyecdysone 22-phosphate, and 2-deoxy-20-hydroxyecdysone 22-phosphate. The retention times of free ecdysteroids liberated by EPPase hydrolysis were also identical to...
those of authentic free ecdysteroids: ecdysone, 20-hydroxyecdysone, 2-deoxyecdysone, and 2-deoxy-20-hydroxyecdysone. However, 22-deoxy-20-hydroxyecdysone and 2,22-deoxy-20-hydroxyecdysone, which are phosphorylated at the C-3 position in ovaries, did not serve as phosphate acceptors (Table 2). In a competitive assay, the phosphorylation of ecdysone was remarkably inhibited by 20-hydroxyecdysone, 2-deoxyecdysone, and 2-deoxy-20-hydroxyecdysone but was scarcely inhibited by 22-deoxy-20-hydroxyecdysone and 2,22-deoxy-20-hydroxyecdysone (Table 2). These results suggest that the ecdysone 22-kinase we obtained has a greater specificity for phosphorylation at the C-22 position than at the C-3 position.

Further kinetic analysis demonstrated that ecdysone 22-kinase activity is competitively inhibited by 2-deoxyecdysone (Fig. 9), indicating that 2-deoxyecdysone binds to the same catalytic site in ecdysone 22-kinase as in the case of ecdysone. From the results indicated in Fig. 9 and Table 2, there is no doubt that 20-hydroxyecdysone and 2-deoxy-20-hydroxyecdysone also bind to the enzyme in the same manner as do ecdysone and 2-deoxyecdysone. Therefore, we propose that the ecdysone 22-kinase we obtained should be named hereafter ecdysteroid 22-kinase (EcKinase) as a generic term.

**Molecular Cloning of a cDNA Encoding EcKinase**—Purified EcKinase was N-terminally blocked. Therefore, the enzyme was digested with lysylendopeptidase. The resultant five peptides were named K54, K71, K75-1, K75-2, and K94. Among them, the peptide K94 was digested again with trypsin, and the resultant two peptides were named K94-T1 and K94-T2. The amino acid sequences of six peptides obtained from purified EcKinase were determined: K75-2, MSENVRAHAINSVD (peptide 1 in Fig. 10); K94-T1, FVFPALYGAR (peptide 2 in Fig. 10); K94-T2, LHALGFALQLQAP (peptide 3 in Fig. 10); K54, LPFGGLK (peptide 4 in Fig. 10); K75-1, IAVYTLPVITVEED (peptide 5 in Fig. 10); K71, TSSRFPAIRI (peptide 6 in Fig. 10).

In order to obtain a cDNA clone encoding EcKinase, standard BLAST analysis was carried out on the diapause egg EST data base using amino acid sequences of six peptide fragments obtained from EcKinase. One EST clone, named fdpeP12_F_I09, was found to encode three peptides (peptides 1–3) in its 5’-part (660 bp). Thus, the entire nucleotide sequence of the fdpeP12_F_I09 was determined (Fig. 10); the fdpeP12_F_I09 consisted of 1,850 bp comprising a putative translation start site (ATG), a 5’-untranslated region (31 bp), a stop codon (TGA), a 3’-untranslated region (639 bp), a poly(A) tail (22 bp), and a consensus polyadenylation signal (AATAAA) 14 bp upstream from the poly(A) tail. The molecular mass (44,236 Da) estimated from the open reading frame (1,158 bp) completely corresponded to that estimated by SDS-PAGE (Fig. 6B). The protein contained amino acid sequences of all six peptides obtained by enzymatic hydrolysis of purified EcKinase. Furthermore, a highly conserved cluster, known as Brenner's motif HXDhX3N3...D (where h represents a large hydrophobic amino acid, and X is any amino acid), which is shared by many phosphotransferases (44, 45), was observed in EcKinase (amino acids 236–260 in Fig. 10). The ATP binding sites proposed in choline kinase (46) were also conserved in EcKinase (Asp<sup>238</sup>, Asn<sup>243</sup>, and Asp<sup>260</sup> in Fig. 10).

The above mentioned results were obtained by analyzing the EST clone derived from 12–40-h-old diapause eggs. We next examined whether EcKinase transcript exists in mature ovaries, in which EcKinase activity reached a maximum (Fig. 3A), by RT-PCR using EcKinase gene-specific primers (F1 and R3 in Fig. 10). As shown in Fig. 11, a clear band, expected to have 1,279 bp from the translation start site (ATG), was expressed whether EcKinase transcript exists in mature ovaries, in which EcKinase activity reached a maximum (Fig. 3A), by RT-PCR using EcKinase gene-specific primers (F1 and R3 in Fig. 10). As shown in Fig. 11, a clear band, expected to have 1,279 bp from the
nucleotide sequence of EcKinase cDNA, was detected in mature ovaries, although an unexpected band having 700 bp was detected only in trace amounts, probably due to the degradation of PCR product. Furthermore, it was confirmed by 5′-RACE that the 5′-end of the ovarian clone is the same as that of fdpeP12_F_109 (data not shown). These results indicate that EcKinase transcript exists in ovaries as well. On the other hand, EcKinase transcript was not detected in 72-h-old diapause eggs (late gastrula stage) in which embryos have ceased development (47), although elongation factor 2 used as an internal control was observed in 72-h diapause eggs as well as in mature ovaries (Fig. 11).

Expression of EcKinase in Sf9 Cells—To confirm that the cloned cDNA encodes EcKinase, the extract from the stable transfected harboring the whole open reading frame of EcKinase was used for immunoblot analysis and the measurement of EcKinase activity. Immunoblot analysis using anti-EcKinase antiserum showed that the recombinant EcKinase has the same antigenicity and the same molecular mass as the purified native EcKinase (Fig. 12A, lanes 1 and 3). However, we could not observe any protein bands that corresponded to the molecular mass of EcKinase in the extract from untransfected control Sf9 cells (Fig. 12A, lane 2). These results indicate that the cloned cDNA encodes EcKinase.

FIGURE 11. Agarose gel electrophoresis of products generated from RT-PCR of total RNA from mature ovaries and 72-h-old diapause eggs. RT-PCR was carried out using EcKinase gene-specific primers, F1 and R3 (see Fig. 10), and elongation factor 2 gene-specific primers, EF2-F and EF2-R (see "Experimental Procedures"). Lane 1, RNA markers (phiX 174 HaeIII-digested); lane 2, EcKinase in mature ovaries; lane 3, EcKinase in 72-h-old diapause eggs; lane 4, elongation factor 2 in mature ovaries; lane 5, elongation factor 2 in 72-h-old diapause eggs.

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nucleotide sequence of EcKinase cDNA, was detected in mature ovaries, although an unexpected band having ~700 bp was detected only in trace amounts, probably due to the degradation of PCR product. Furthermore, it was confirmed by 5′-RACE that the 5′-end of the ovarian clone is the same as that of fdpeP12_F_109 (data not shown). These results indicate that EcKinase transcript exists in ovaries as well. On the other hand, EcKinase transcript was not detected in 72-h-old diapause eggs (late gastrula stage) in which embryos have ceased development (47), although elongation factor 2 used as an internal control was observed in 72-h diapause eggs as well as in mature ovaries (Fig. 11).

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**DISCUSSION**

In *D. melanogaster*, metabolic deletions in the pathway of 20-hydroxyecdysone biosynthesis during embryonic development bring about morphological disruptions of the embryo (7–9, 11, 48). In *B. mori* (21, 49), *Lepidosaphes ulmi* (50), and *Locusta migratoria* (51), 20-hydroxyecdysone plays an indispensable role in the developmental difference between diapause eggs and nondiapause eggs. In *B. mori* eggs, 20-hydroxyecdysone has been demonstrated to originate from both *de novo* biosynthesis (23, 38, 52, 53) and dephosphorylation of ovary-derived ecdysone phosphates (27–29, 53). Furthermore, in *L. migratoria* (54), *S. gregaria* (55), *Blaberus craniifer* (56), and *Manduca sexta* (14, 15), free ecdysteroids liberated from ovary-derived ecdysteroid phosphates are suggested to participate in controlling the early events of embryonic development, such as embryonic cuticleogenesis and germ band elongation, which occur before the prothoracic glands differentiate during embryonic development. As mentioned above, the physiological significance of the presence of ecdysteroids during early embryonic development has been emphasized in many insects, but there has been little or no biochemical information concerning the enzyme systems involved in the reciprocal conversion of free ecdysteroids and ecdysteroid phosphates.

Ecdysteroids (*e.g.* 20-hydroxyecdysone) have six hydroxyl groups, three of which (the C-2, C-3, and C-22 positions) are secondary alcohols and thus accessible to esterification. It has been suggested that ecdysteroids phosphorylated at the C-22 position are storage forms from which active ecdysteroids can be released, whereas ecdysteroids phosphorylated at the C-2 position or the C-3 position are end products in ecdysteroid metabolism (18, 19, 29). In *B. mori* ovaries, only C-3 and C-22 phosphate esters have been detected (36, 37, 43, 57–59). We have already demonstrated that a crude preparation of *B. mori* ovaries has the capacity to phosphorylate the hydroxyl groups of the C-22 and C-3 positions using ecdysone, 2-deoxyecdysone, and 2,22-dideoxy-20-hydroxyecdysone as the phosphate acceptors: the former two ecdysteroids are phosphorylated at the C-22 position, and the latter is phosphorylated at the C-3 position (31). However, in our present experiments, purified Ecdysone did not have the capacity to phosphorylate the C-3 position of 22-deoxy-20-hydroxyecdysone and 2,22-dideoxy-20-hydroxyecdysone, which are phosphorylated at the C-3 position in ovaries (Table 2). These results suggest the possibility that two separate enzymes are involved in phosphorylation at the C-3 and C-22 positions. Since Ecdysone has greater specificity for the phosphorylation at the C-22 position rather than at the C-3 position (Table 2), and EPPase has also greater specificity for the ecdysteroid 22-phosphates in comparison with ecdysteroids 3-phosphates (27), Ecdysone and EPPase are indispensable to the reciprocal conversion between free ecdysteroids and ecdysteroid 22-phosphates in the ovary-egg system of *B. mori*.

In addition to Ecdysone isolated from mature ovaries of *B. mori*, it has also been reported that 2-deoxyecdysone 22-kinase occurs in the crude preparation of the follicle cells of *S. gregaria* (30). Ecdysone shares some common biochemical characteristics with 2-deoxyecdysone 22-kinase, such as cytosolic localization, dependence on ATP and Mg2+, inhibition by Ca2+, and similar pH and temperature optima (Figs. 4, 7, and 8). However, since Ecdysone of *B. mori* was isolated from mature ovaries in which follicle cells and nurse cells have degenerated, enzyme localization within ovaries seems to differ between both enzymes. We are now attempting to elucidate the detailed localization of Ecdysone in *B. mori* ovaries by means of immunohistochemistry using anti-Ecdysone antiserum.

In our present study, on the basis of the partial amino acid sequence obtained from ovarian Ecdysone, the nucleotide sequence of the cDNA encoding Ecdysone was determined from an EST clone prepared from *B. mori* eggs, and the presence of its cDNA in ovaries was confirmed by RT-PCR and 5′-RACE. Furthermore, the cloned cDNA was confirmed to encode a functional Ecdysone, using the expression construct for Ecdysone (Fig. 12). The kinase data base (available on the World Wide Web at crf.medic.kumamoto-u.ac.jp/CSP/Kinase.html) has shown that 157 enzymes (or enzyme families) belong to class 2.7.1, which is composed of enzymes that target a hydroxyl group that accepts phosphoryltransfer from ATP. In the data base Pfam (available on the World Wide Web at www.sanger.ac.uk/Software/Pfam/index.shtml), 165 kinases (or kinase families) have been deposited. However, we could not find enzymes involved in the phosphorylation of steroid hormones in these databases. Using the DNA Data Bank of Japan (DDBJ) (available on the World Wide Web at www.ddbj.nig.ac.jp/) with tBLASTn analysis, it was revealed that the amino acid sequence of Ecdysone did not show over 30% identity with any other known sequences. These results
suggest that EcKinase is a novel enzyme that does not belong to an already known kinase family from the viewpoint of amino acid sequence.

According to DDBJ and Protein Data Bank Japan (PDBJ) (available on the World Wide Web at www.pdbj.org/), in which the three-dimensional structural data of proteins are deposited, the deduced three-dimensional structure of EcKinase (amino acids 121–324) is remarkably similar to the three-dimensional structure of choline kinase (amino acids 146–363), despite no significant similarity in amino acid sequences between the two enzymes. Alignment of the protein sequences between the two enzymes showed that EcKinase contains the conserved Brenner’s phosphotransferase motif HXDHX₃NH₃...D (Fig. 10) also observed in choline kinase. Three highly conserved residues from the ATP binding sites proposed in choline kinase (46) were also observed in EcKinase (Fig. 10). Our comparison suggests that EcKinase could utilize a mechanism similar to that of other kinases during catalysis.

In examination of the NCBI conserved domain data base (available on the World Wide Web at www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), EcKinase (amino acids 84–308) was shown to share ~45% identity with a “domain of unknown function (DUF227, amino acids 1–232; see pfam 02958 in the NCBI conserved domain data base).” All known members of this group are proteins from D. melanogaster and Caenorhabditis elegans, which belong phylogenetically to Ecdysozoa, containing molting animals (60). The DUF227 proteins harbored both Brenner’s motif and the proposed ATP binding sites. Therefore, it is conceivable that some protein that includes DUF227 is involved in the phosphorylation of ecdysteroids.

We reported previously that there was no functional protein having an amino acid sequence identical to that of EPPase in the data base Pfam and Swiss-Prot using FASTA analysis (27). However, an amino acid sequence motif RHGXRXP (61–64), which is highly conserved in acid phosphatases, mutases, and bisphosphatases, was present in EPPase (amino acids 79–85). Furthermore, according to DDBJ and PDBJ, the deduced three-dimensional structure of EPPase (amino acids 130–272) shared a common three-dimensional structure with phosphoglycerate mutase (amino acids 36–175) and fructose-2,6-bisphosphatase (amino acids 239–366), although EPPase did not share the same order of amino acid sequence with those two enzymes. These results suggest that EPPase belongs to the histidine phosphatases family, in which the histidine residue included in the RHGXRXP motif is responsible for the formation of a phosphoenzyme transient intermediate in the course of the catalytic process (65, 66). Thus, studies on EcKinase and EPPase not only shed new light on the steroid metabolism but also may provide new information relevant to understanding the molecular mechanisms of the phosphorylation and dephosphorylation of organic molecules.

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