Characterization and Affinity Purification of Juvenile Hormone Esterase from *Bombyx mori*

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Received February 16, 2000; Accepted April 7, 2000

Juvenile hormone esterase (JHE) from hemolymph of the silkworm moth *Bombyx mori* was characterized for substrate specificity and inhibitor sensitivity. *B. mori* JHE hydrolyzed the juvenile hormone surrogate substrate methyl n-heptylthioacetothioate (HEPTAT) more efficiently than n-propylthioacetate and 1-naphthyl acetate substrates widely used to assay total carboxylesterase activity. *B. mori* JHE was sensitive to 3-oc-tylthio-1,1,1-trifluoro-2-propanone (OTFP), which was developed as a selective inhibitor for lepidopteran JHE, and relatively insensitive to disisopropyl fluorophosphate (DFP), an inhibitor of serine esterases but not of all JHes. Affinity purification with a trifluoromethyl ketone ligand was more efficient for purification of *B. mori* JHE than DEAE ion exchange chromatography.

**Key words:** affinity purification; *Bombyx mori*; juvenile hormone esterase; transition-state analog; trifluoromethyl ketone

Juvenile hormone (JH) plays an important role in insect ecdisis and metamorphosis in reproduction in some species. The JH titer is regulated by the rates of degradation and of biosynthesis. JH is degraded by JH epoxide hydrolase, which is intracellular, and by juvenile hormone esterase (JHE), which is primarily secreted into hemolymph. JHE has been purified from several lepidopteran species by affinity chromatography with 3-(4-mercaptopbutylthio)-1,1,1-trifluoro-2-propanone (MBTFP) as a ligand. The amino acid sequence of affinity-purified JHE from *Heliothis virescens* was used to isolate a cDNA clone encoding *H. virescens* JHE. The cDNA encoding the JHE of *Choristoneura fumiferana* and a JHE-related carboxylesterase from *Trichoplusia ni* have also been cloned.

Since JHE has a critical role in insect development, agents that disrupt JHE or JHE gene regulation are potential insecticides. Such agents have high species selectivity and minimal detrimental effect on nontarget organisms and the environment. The silkworm *Bombyx mori* has been used as a model insect for studies of the role of JH in gene regulation during development, including analyses of edysone receptors (EcR), cuticular proteins, and silk proteins, JHE in hemolymph of *B. mori* larvae was purified, but has not been well characterized. We studied the substrate selectivity and inhibitor sensitivity of *B. mori* JHE, and described purification of *B. mori* JHE by affinity- and by ion exchange-chromatography. We found that a surrogate substrate, HEPTAT was useful for the JHE assay with a quantity of samples even of crude hemolymph and that affinity chromatography with MBTFP as a ligand was efficient in purifications yielding active JHE directly from larval hemolymph in a single step.

**Materials and Methods**

**Chemicals.** Specific JHE inhibitors—3-octylthio-1,1,1-trifluoro-2-propanone (OTFP) and 3-butylthio-1,1,1-trifluoro-2-propanone (BTFP)—and a carboxylesterase (CE) substrate—methyl n-heptylthioacetothioate (HEPTAT)—were synthesized by the methods of Hammock et al. and McCutchen et al. HEPTAT is a JH analog developed as a surrogate spectrophotometric substrate for JHE. All other reagents were reagent grade.

**Insect and hemolymph preparation.** The silkworm

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Abbreviations: JH, juvenile hormone; JHE, juvenile hormone esterase; CE, carboxylesterase; HEPTAT, methyl n-heptylthioacetothioate; OTFP, 3-octylthio-1,1,1-trifluoro-2-propanone; BTFP, 3-butylthio-1,1,1-trifluoro-2-propanone; DFP, disisopropyl fluorophosphate; MBTFP, 3-(4-mercaptopbutylthio)-1,1,1-trifluoro-2-propanone; p-NPA, p-nitrophenyl acetate; 1-NA, 1-naphthyl acetate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)
Bombyx mori (C145 × N140) was reared at 25°C on an artificial diet (Silkmate, Nippon-Nosan Kogyo Co., Yokohama) that included mulberry leaves. To collect hemolymph samples, an abdominal leg was cut and hemolymph collected in ice-chilled microtubes containing approx. 0.05% (w/v) phenylthiourea crystal. Samples were stored at −20°C until use.

Ammonium sulfate precipitation was used to fractionate hemolymph samples. Hemolymph was centrifuged at 10,000 × g for 5 min and the supernatant diluted 10-fold in 0.01 M sodium phosphate buffer (pH 7.4). The sample was fractionated with stepwise saturated ammonium sulfate at increasing concentrations.

Enzyme assays. Enzyme assays were done in 0.05 M of sodium phosphate (pH 7.4). This buffer was supplemented with 10% sucrose for all substrates except 1-NA. Esterase activity in hemolymph was measured in a microtiter plate format with three substrates: p-nitrophenyl acetate (p-NPA) and 1-naphthyl acetate (1-NA) were used to assay CE activity as reported by Grant et al., and HEPTAT was used to assay JHE activity as reported by McCutchen et al. Briefly, hemolymph samples (5–10 μl) were diluted in assay buffer to a final volume of 298 μl per well and incubated at 25°C for 5 min. To assay JHE activity using HEPTAT, the buffer was supplemented with 0.025% (w/v) 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). To assay CE activity using 1-NA, the assay buffer was supplemented with 0.05% (w/v) Fast Blue RR salt. Two microliters of substrate solution [p-NPA (75 mM), 1-NA (30 mM), or HEPTAT (30 mM) in EtOH] were then added to the well. Absorbance was monitored at 405 nm for p-NPA and HEPTAT, or at 450 nm for 1-NA, for 2 min period in a microtiter plate reader (SPECTRA-Max, Molecular Devices, Menlo Park, CA, USA). Hydrolysis rates were calculated by the SOFTmax PRO program (Molecular Devices). JHE activity was also assayed using tritiated JH III as a substrate by a partition assay as reported by Hammock and Sparks. For inhibition experiments, enzyme samples were incubated with inhibitors for 10 min before the assay for enzymatic activity.

Protein concentrations of hemolymph samples were measured by the method of Bradford using a BioRad Protein Assay Kit (BioRad) with bovine serum albumin as a standard.

Polyacrylamide gel electrophoresis (PAGE). PAGE was done with 1.0 mm polyacrylamide gels (10%) using a vertical slab instrument (Atto, Tokyo, Japan). Nondenaturing (native) PAGE, was used to detect CE activity in the gel by staining. After electrophoresis, the gel was incubated in 50 ml of filtered 0.05% Fast Blue RR salt in 0.05 M sodium phosphate (pH 7.4), for 5 min at room temperature; 100 μl of 75 mM 1-NA in EtOH was then added and the gel incubated at room temperature until well stained as described by Stauffer et al. Catalytic activities of esterases separated by native PAGE were measured by slicing native gels (5 mm slices), incubating gel slices overnight in 0.05 M sodium phosphate (pH 7.4), and assaying with HEPTAT and p-NPA as described above.

SDS-PAGE was done with 10% acrylamide gel and hemolymph proteins stained with Coomassie Brilliant Blue R-250.

Affinity chromatography. An affinity gel (MBTTP-gel) was prepared and affinity chromatography conducted as described previously. All procedures were done at 4°C in a purification buffer [0.1 M sodium phosphate buffer (pH 7.4) containing 5% sucrose and 0.02% sodium azide]. The gel was packed into a column with a fluoropolymer frit at the base (Econo-Column, Bio-Rad). The column was washed sequentially with EtOH, 50% EtOH, distilled water, and the purification buffer. Hemolymph of larvae at day 5 after the 4th molting (L5DS) diluted 10-fold with the purification buffer (10 ml) was added to the column (0.5 ml bed volume of gel) and the column was shaken gently for 8–16 h. Following settling of the gel, the solution was removed and enzymatic activity measured by a spectrophotometric assay with HEPTAT. After samples were put on, the gel was washed in the same column with 20 ml of purification buffer, and 50 ml of the same buffer containing 1% n-octyl β-d-glucopyranoside (OG). To elute the enzyme from the affinity gel, the gel was shaken gently in 1 ml purification buffer containing 1% OG (w/v) and 1 mM of n-butythiothiolo-1,1,1-trifluoro-2-propanone (BTTFP) for 12 h. Elution was repeated with fresh eluent until the protein concentration of the eluate was less than 5 mg/ml. Eluted samples were combined and concentrated with a microconcentrator (Centricon-30, Amicon). Samples were then dialyzed in the purification buffer to elute BTTFP from the eluted enzyme.

To compare the efficiency of affinity purification procedure with DEAE-ion exchange chromatography, an enzyme sample diluted 4-fold with 10 mM of Tris-HCl buffer (pH 8.5) was put on a DEAE-Sephacel (Sigma) column. JHE was eluted from the column with a 50 to 200 mM linear gradient of NaCl as described previously.

Results

Developmental changes in CE activity of B. mori larval hemolymph

Four substrates were used to measure carboxylesterase (CE) activity in B. mori hemolymph from day 0 of the 4th instar (L4D0) until pupation (day 9
or 10 of the 5th instar: L5D9 or L5D10) (Fig. 1). Two of the substrates, p-NPA and 1-NA, are widely used to assay nonspecific CEs ("general" esterases). The other two substrates, 3H-JH III and HEPTAT, were used to assay JHE.16

During the 4th instar (from 0 to 4 days after the 3rd molting), CE activity with HEPTAT or 3H-JH III was low [Fig. 1(C) and (D)]. Activity with these substrates increased from day 3 of the 5th instar (L5D3). The time course of enzyme activity with HEPTAT is very similar to that with 3H-JH III even though the enzyme sample was crude hemolymph. In contrast, CE activity using p-NPA or 1-NA as substrates increased gradually early in 4th to late 5th instar larvae.

Hemolymph CE activity in 4th and 5th instar larvae was analyzed on native (nondenaturing) PAGE by staining with 1-NA for enzymatic activity. CE-2 was found throughout the 5th instar [from 0 to 8 days after the 4th molting (Fig. 2)], and throughout the 4th instar (data not shown). After L5D3, CE-1 was observed (Fig. 2). CE-1 was not detected in hemolymph from 4th instar larvae. The appearance of CE-1 after L5D3 correlates with the increase in CE activity with substrates HEPTAT and 3H-JH III (Fig. 1(C) and (D)). A third CE ("CE-i" in Fig. 2) was previously shown to be induced by bacterial infection.21

**Substrate selectivity of CEs of B. mori larval hemolymph**

The substrate selectivity of CE-1 and CE-2 detected in native gels was measured by separating the gel into 5 mm slices and assaying enzyme activity with different substrates after eluting them out into an assay buffer. NPA, HEPTAT, and 3H-JH III were used for enzyme assays. The profile of enzyme activities in gel slices assayed with the substrate HEPTAT was similar to that assayed with 3H-JH III (Fig. 3). HEPTAT and JH III activity was thus primarily associated with CE-1, indicating that CE-1 is JHE. In contrast, p-NPA activity was primarily associated with CE-2 (Fig. 3).

**Inhibition of CEs of B. mori larval hemolymph**

The effect of inhibitors on CE activity of L5D5 hemolymph was investigated. Table 1 shows half-inhibitory concentrations (I50) for DFP, known to inhibit CEs and proteases with serine at the catalytic site, and for trifluoromethyl ketones OTFP and BTFP, selective inhibitors of lepidopteran JHE.13 p-NPA hydrolysis was partially inhibited by DFP, but DFP did not inhibit HEPTAT hydrolysis up to 10⁻¹ M. In contrast, OTFP strongly inhibited HEPTAT and 3H-JH III hydrolysis.

**Table 1. Inhibitory Activity of DFP and Trifluoromethyl Ketones OTFP and BTFP on CE Activity of B. mori Larval Hemolymph at L5D5 with Three Substrates.a**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>I50, M</th>
<th>HEPTAT</th>
<th>3H-JH III</th>
</tr>
</thead>
<tbody>
<tr>
<td>DFP</td>
<td>1.84×10⁻⁴</td>
<td>&gt;1.0×10⁻⁴</td>
<td>&gt;1.0×10⁻⁴</td>
</tr>
<tr>
<td>OTFP</td>
<td>1.13×10⁻⁴</td>
<td>9.31×10⁻⁹</td>
<td>2.67×10⁻⁹</td>
</tr>
<tr>
<td>BTFP</td>
<td>6.83×10⁻⁴</td>
<td>2.17×10⁻⁶</td>
<td>8.54×10⁻⁷</td>
</tr>
</tbody>
</table>

a. Substrate concentrations were 75 mM, 30 mM, and 0.5 μM for NPA, HEPTAT, and [3H]-JH III.
b. Inhibition: 10 min.

**Fig. 1. Specific Activity of Esterases in B. mori Hemolymph.**

Assays were done on samples taken on each day from the beginning of the 4th instar until pupation. The molt from the 4th to the 5th instar is indicated by (M). Esterase activities were measured with the substrates (A) p-NPA, (B) 1-NA, (C) HEPTAT, and (D) JH III. Mean values and standard deviations were calculated from 8 insects, 4 female and 4 male.
Separation of CEs of B. mori larval hemolymph

CEs with activity on p-NPA were separated from CEs with activity on HEPTAT by ammonium sulfate fractionation. HEPTAT hydrolysis was found in the 30–45% saturated ammonium sulfate fraction, while p-NPA hydrolysis was detected in the 60–80% fraction [Fig. 4(B)]. Proteins in ammonium sulfate fractions were separated on native-PAGE and detected with staining for CE activity with 1-NA. CE-1 and some CE-2 activity was detected in the 30–45% ammonium sulfate fraction, while CE-2 activity alone was detected in the 60–80% fraction. The observations that CE-1 was inhibited by OTFP and CE-2 by DFP strongly suggest that CE-1 is JHE and CE-2 is a nonspecific CE.

Affinity Chromatography of the B. mori JHE

B. mori JHE was purified from diluted larval hemolymph of L5D5 larvae by affinity chromatography using MBTFP gel and the inhibitor BTFP for elution. BTFP is a less potent inhibitor than OTFP and therefore easier to remove from the purified enzyme by dialysis. A purification factor of 25.3 was reached with 9.0% recovery by affinity chromatography for purification of B. mori JHE (Table 2). In comparison, DEAE-ion exchange chromatography resulted in a purification factor of only 5.3 with a recovery of 12.5% of the enzyme. SDS-PAGE analysis of the affinity purified sample of JHE is shown in Fig. 5.

Discussion

A number of useful biological tools have been developed for studying JHEs from Manduca sexta and Heliothis virescens. These tools include a series of trifluoromethyl ketone inhibitors that specifically inhibit JHE, an affinity gel (MBTFP-gel) for JHE purification, and the colorimetric substrate HEPTAT for rapid analysis of JHE activity in a microtiter format. We used all three of these tools to analyze of JHE from Bombyx mori.

<table>
<thead>
<tr>
<th>Relative activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Activity staining</td>
</tr>
<tr>
<td>0 20 40 60</td>
</tr>
</tbody>
</table>

Fig. 2. Isozyme Patterns of CEs in the Hemolymph of Individual Larvae at the 5th Instar.

Hemolymph proteins were separated by native PAGE (10% gel) and detected by activity staining with 1-NA (0.1 mm) in 0.1 m of sodium phosphate buffer (pH 7.4) with Fast Blue RR salt (0.025%) as a color-developing reagent. The three CEs detected by this technique, CE-1, CE-2 and CE-3, are indicated.

Fig. 3. Substrate Selectivity of CEs in the Hemolymph of Larvae at Day 5 of the 5th Instar (L5D5) Separated by Native PAGE.

(A) CEs detected in a native polyacrylamide gel by activity staining (Fig. 2). Substrate selectivity was measured for CEs eluted from native polyacrylamide gel slices with substrates (B) NPA, (C) HEPTAT, and (D) 3H-JH III.
**Table 2. Purification of JHE from Bombyx mori Hemolymph at L5D5**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Volume ml</th>
<th>Conc. mg/ml</th>
<th>NPA activity</th>
<th>HEPTAT activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Specific µM/min/mg</td>
<td>Total µM/min</td>
</tr>
<tr>
<td>Crude</td>
<td>155.6</td>
<td>40.0</td>
<td>2.02</td>
<td>371.5</td>
</tr>
<tr>
<td>Purified</td>
<td>0.46</td>
<td>3.86</td>
<td>4.11^a</td>
<td>2.71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(11.3)^a</td>
<td></td>
</tr>
<tr>
<td>DEAE-Ion Exchange Chromatography</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude</td>
<td>111.9</td>
<td>40.0</td>
<td>6.81</td>
<td>381.0</td>
</tr>
<tr>
<td>Purified</td>
<td>2.62</td>
<td>3.25</td>
<td>8.18</td>
<td>21.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(1.20)^a</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(12.5%)^c</td>
<td></td>
</tr>
</tbody>
</table>

a. This represents the minimum specific activity because full reactivation may not have occurred and some enzyme activity may have been lost during dialysis.  
b. Purification factor calculated from the specific activity of the purified sample divided by that of the crude preparation.  
c. Recovery of enzyme activity as calculated by total activity of the purified sample divided by that of the crude preparation.

**Fig. 4.** Separation of CE s in the Hemolymph of Larvae at Day 5 of the 5th Instar (L5D5) by Ammonium Sulfate Precipitation.  
(A) Proteins separated by native PAGE and stained with 1-NA (Fig. 2). Lane 1, crude hemolymph; lane 2, 30-45% saturated ammonium sulfate fraction; lane 3, 60-80% saturated ammonium sulfate fraction. The positions of CE-1 and CE-2 are indicated. (B) Substrate selectivity and inhibitor sensitivities of CE in ammonium sulfate fractions. Specific activities were measured with substrates (a) NPA and (b) HEPTAT for controls (uninhibited), and samples inhibited with OTFP or DFP.

**Fig. 5.** Proteins in Crude and Purified Preparations of CEs from *B. mori* Separated by SDS-PAGE and Gel Stained with Coomassie Brilliant Blue.  
Lane 1, molecular weight markers in Da; lane 2, crude hemolymph; and lane 3, CE purified by MBTFP affinity chromatography.

The hydrolytic activity of CE in *B. mori* hemolymph with ^3^H-JH III was sensitive to inhibition by OTFP, insensitive to inhibition by DFP, and moderately sensitive to inhibition by BTFP—features are similar to those of JHEs from other lepidopteran species. Affinity chromatography with MBTFP as an affinity ligand was efficient in purifying of *B. mori* JHE. By using BTFP, which has a shorter carbon chain and is a less potent inhibitor of JHE than OTFP, as an eluting free ligand, we obtained enzymatically active purified *B. mori* JHE. With affinity chromatography, JHE could be purified directly from larval hemolymph in a single step.

A series of spectrophotometric substrates for partially purified JHE of Lepidoptera were developed. Because HEPTAT was the best of the series of substrates developed to analyze JHE of *M. sexta* and *H. virescens*, this substrate was used to analyze the hydrolytic characteristics of *B. mori* JHE. First, CE activity of crude hemolymph was measured with four substrates. CE activity with all four substrates gradually increased during the 5th instar, peaking just before pupation (Fig. 1). The time course of HEPTAT hydrolysis was similar to that for ^3^H-JH III with low
activity in the 4th instar and a drop in activity before pupation. HEPTAT hydrolysis is readily monitored with a microplate reader even with a quantity of samples; in contrast, that of $^3$H-JH III hydrolysis is measured by scintillation counting through extraction with a solvent for each sample. Hemolymph CEs were separated by native PAGE and detected by activity staining with substrate 1-NA. CE-1 hydrolyzed HEPTAT and JH III, while CE-2 hydrolyzed p-NPA, commonly used to detect nonspecific CE. In other work on B. mori hemolymph CEs, it was found that CE-1 had an isoelectric point of 4.8-4.9, and was inhibited by OTFP but not by DFP, while CE-2 had an isoelectric point of 4.9-5.1, and was inhibited by DFP but not by OTFP. Based on these results, we surmise that CE-1 is a JHE and CE-2 is a nonspecific CE. HEPTAT was a good selective substrate for JHE with no hydrolysis by nonspecific CE, and this substrate was applicable for JHE assay of a crude preparation. CE-1 was separable by different ammonium sulfate precipitation from CE-2, which has similar characteristics in molecular weight and pI. Surrogate substrate HEPTAT was useful for assaying JHE in crude B. mori hemolymph without inhibition of nonspecific esterases, because HEPTAT was not hydrolyzed by CE-2. In conclusion, HEPTAT was a useful substrate for the JHE assay with a quantity of crude and purified samples, and MBTFP-afinity chromatography was efficient in rapidly and simply purifying B. mori JHE.

In Lepidoptera, JHE is considered to have an important role in reducing JH titer during the early 5th instar. This reduction in JH titer is required to reprogram tissues for metamorphosis. An imidazole insect growth regulator KK-42, causes precocious metamorphosis of B. mori when applied to early larval stages. The time course of JHE activity in the KK-42-treated 4th instar larvae was similar to that in control 5th instar larvae. The titer of JHE in control larvae remained low throughout the 4th instar, and increased 1 day after molting to the 5th instar. JHE activity increased 1 day after KK-42 treatment of 4th instar larvae. These results support the hypothesis that JHE has an important role in reducing JH titer for metamorphosis.

Induction of JHE synthesis may be the trigger for commitment to metamorphosis in early final instar larvae. Hydrolysis of JH, a methyl ester, by JHE produces JH acid, which may also have a biological function that differs from that of JH. If JH acid has such a biological function, JHE would be the key enzyme for regulating that function. Research is now under way to isolate cDNA and gene encoding B. mori JHE to facilitate further study of JHE biology.

Acknowledgments

This work was supported in part by Enhancement of Center of Excellence, Special Coordination Funds for Promoting Science and Technology, Science and Technology Agency, Japan. Additional support was provided by the USDA (94-37302-0567).

References

Bombyx Juvenile Hormone Esterase


