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Molecular cloning and identification of the hatching enzyme gene in *Rondotia menciana*

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ABSTRACT: *Rondotia menciana* Moore (Lepidoptera: Bombycidae) is a major pest of mulberry. We cloned the full-length cDNA of the hatching enzyme from *R. menciana* eggs (*RmHE*) using the RACE-PCR and SMART cDNA synthesis technique. The cDNA is 1325 bp long and contains an ORF of 885 bp. It encodes 294 amino acid residues, including a putative signal peptide of 16 amino acid residues. Two conserved signature sequences of insect hatching enzyme (HE) harbour in the RmHE. The deduced amino acid sequence of RmHE has 30–84% identity to HE sequences in other insect species, and RmHE is most similar to that of wild silkworm HE. *RmHE* transcripts could be detected in *R. menciana* eggs at different developmental stages. Levels remained low during the early stages, increased greatly in 7-d eggs, and reached a maximum in 9-d eggs. Changes in the levels of *RmHE* transcripts occurred in accordance with the process of embryo development and eclosion. This indicated that *RmHE* plays an important role in these processes. *RmHE* transcripts were also detected in larval midguts, suggesting that RmHE is involved in food digestion. As far as we know, this is the first report on an *HE* gene in Lepidopteran pests and will offer some basic information for developing an efficient control method during the hatching process for agricultural Lepidopteran pests.

KEYWORDS: expression pattern, Lepidopteran pests

INTRODUCTION

The hatching phenomenon exists in almost all animals during the late development stage of the embryo. Hatching occurs when individuals transform from capsules to a free-living state, and it is significant for postembryonic development. During eclosion, a hatching enzyme (HE) proteinase is secreted by the embryo. The HE digests or softens the eggshell, which facilitates successful eclosion[1,2]. Most HEs belong to the astacin family and are Zn-metalloproteinases characterized by the consensus sequences HExxHxxGFxHExxRxDR (the zinc-binding motif) and SxMHY (the methionine turn)[3,4].

The hatching mechanism has been studied in fish[5–8], crustaceans[9,10], birds[11], amphibians[12–14], echinoderms[15], insects[16–18], and mammals[19,20]. *Rondotia menciana* Moore (Lepidoptera: Bombycidae) is a major pest of mulberry[21]. Control of *R. menciana* is mainly achieved by trapping and insecticide applications[22,23]. However, insecticide use can cause environmental pollution and eventually leads to pesticide resistance. Biotechnology-based pest management could be a better choice[24]. The hatching enzyme gene might provide a target for molecular manipulation and pest control at the embryo stage.

We have characterized HE in several Lepidopterans, including the silkworm, *Bombyx mori*[25,26], Chinese Oak silkworm, *Antheraea pernyi*[27], and Chinese wild silkworm, *B. mandarina*[28]. In the present study, we cloned the full-length cDNA of the *HE* gene from bluish-eggs of *R. menciana*. We characterized the gene using bioinformatics and identified its possible biological function at the mRNA level. To the best of our knowledge, this is the first report of the *HE* gene in Lepidopteran pests and will be useful for providing a molecular basis for understanding

Table 1 Oligonucleotide primers used in the experiments.

Primer name	5′-3′ nucleotide sequence
InsectHE F1	gccsaacracaccgtygtytggga
InsectHE R1	acwccytcgtgytccykcagagc
*RmHE*3′SP1	catcgaaaggcacacatgtgtca
*RmHE*3′SP2	ggatgctatgcccacgtcggcta
3′RACE Outer	Primer taccgtcgttccactagtgattt
3′RACE Inner	Primer cgcggatcctccactagtgatttcactatagg
*RmHE*5′SP1	gtccgttctgtgagaatgcgtga
UPAM†Long	ctaatacgactcactatagggcaagcagtggtatcaacgcagagt
UPAM Short	ctaatacgactcactatagggc
Rmactin3-F	ggatgtccacgtcgcactt
Rmactin3-R	gcgcggctactcgttcact

† UPAM=Universal Primer A Mix.

the complicated mechanism underlying *R. menciana* hatching and for controlling *R. menciana* damage to mulberry. It also brings some clues for developing an efficient control method at hatching for the other agricultural Lepidopteran pests on main crops.

MATERIALS AND METHODS

Materials and reagents

R. menciana were collected from mulberry fields of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences (SRI-CAAS). *Escherichia coli* (Top10) was maintained in our laboratory. RNAiso Plus, restriction enzymes, pMD18-T vectors, T4 DNA ligase, PrimerScript reverse transcriptase, and a 3′RACE Kit were obtained from the Takara Company. An SMARTer 5′RACE Kit was purchased from Clontech. DEPC was purchased from Invitrogen. All PCR primers and DNA sequencing were accomplished by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. All chemicals used were of analytical grade.

Total RNA isolation and synthesis of the first-strand cDNA

Total RNA was isolated from bluish-eggs of *R. menciana* using the RNAiso Plus reagent following manufacturer instructions. The quality of the total RNA was determined by the 260/280 absorbance ratio as well as by electrophoresis[29]. Extracted RNA was stored at −80 °C until subsequent experiments.

The cDNA was synthesized from 1 μg of total RNA by PrimerScript reverse transcriptase with an oligo-dT-adapter primer following the manufacturer's protocol. The cDNA was used as the template for PCR in gene cloning.

Molecular cloning of *RmHE* cDNA

Based on the conserved sequences of *HE* identified in Lepidoptera, the degenerate primers of InsectHE F1, InsectHE R1 (Table 1) were synthesized. Using the first-strand cDNA as the template, PCR was conducted. The cycling protocol for PCR was the following: 1 cycle at 94 °C for 3 min; 5 cycles at 94 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s; then 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s; and a final extension step of 10 min at 72 °C. The PCR products were analysed on 1% agarose gels and purified using the glass-milk method[30]. The purified fragment was then cloned into the pMD18-T vector and sequenced.

To obtain the 3′-end sequence of *RmHE* cDNA, a 3′RACE experiment was carried out according to the Takara 3′RACE Kit User Manual. Outer PCR was performed with the 3′RACE outer primer and the specific primer RmHE3′SP1 (Table 1). Inner PCR was performed with a 3′RACE inner primer and the specific primer RmHE3′SP2 (Table 1). The PCR products were purified, cloned, and sequenced as described above.

The Clontech SMARTer 5′RACE Kit protocol was used for cloning the 5′-end sequence of *RmHE* cDNA. Using the 5′RACE cDNA as a template, PCR reactions were performed with Universal Primer A mix and specific primer RmHE5′SP1. PCR products were purified, cloned, and sequenced as described above.

Bioinformatics analysis

Basic properties, such as the splicing of sequence, searching of the ORF and translation of the nucleotide sequence, as well as isoelectric point prediction and the molecular weight of RmHE were determined using DNAStar software. Signal peptide was identified by the SignalP 4.1 Server (www.cbs.dtu.dk/services/SignalP/). Prediction of the three-dimensional structure of the RmHE protein was accomplished by SWISS-MODEL[31] (swissmodel.expasy.org/), and the Ramachandran plot was analysed by PROCHECK (nihserver.mbi.ucla.edu/SAVS/).

HE cDNA sequences of other species were retrieved from the NCBI database. Multiple sequence alignments were carried out using CLUSTALX. A phylogenetic tree was constructed based on the amino acid sequences by MEGA 4 using the neighbour-joining method with a bootstrap test of 1000 replications.

Semi-quantitative RT-PCR analysis

To study the transcript levels at different embryo development stages of *RmHE*, semi-quantitative RT-PCR was carried out. One microgram of total RNA,

separated from embryos at different development stages, was converted into cDNAs according to manufacturer instructions. The house-keeping *Rmactin 3* gene was used as a reference to minimize differences among samples. PCR reactions were carried out under the following conditions: 1 cycle at 94 °C for 3 min, 25 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, with a final extension step of 10 min at 72 °C. The primers for *RmHE* and *Rmactin 3* were RmHE5′SP1 and RmHE3′SP1, Rmactin3-F and Rmactin3-R (Table 1), respectively. The electrophoresis bands of PCR products were analysed by gel-Pro analyser software.

Expression profile of *RmHE*

We investigated the expression profile of *RmHE* in different tissues of *R. menciana*, using RT-PCR. Total RNA was isolated from the cuticle, posterior silk gland, fat body, midgut, and haemolymph. The PCR conditions were as described above.

RESULTS

Molecular cloning of *RmHE* cDNA sequence

Using the degenerate primers of InsectHE F1, InsectHE R1 (Table 1), a single band of 518 bp was amplified by PCR with first-strand cDNA from *R. menciana*. The deduced amino sequence of the cloned fragment included 2 similar signature sequences of the hatching enzyme, which indicated that *RmHE* mRNA exists in *R. menciana*.

Through the 3′RACE and 5′RACE methods, a full-length *RmHE* cDNA sequence of 1325 bp was obtained (Fig. 1) (GenBank Accession No. KJ642218). This contained a 5′UTR, 3′UTR and an intact ORF of 885 bp. The starting base of the 5′UTR was A, which is consistent with previous results showing that the transcription start site is typically a purine (A or G). In the 3′UTR, there was the polyadenylation signal, AATAAA. The ORF encodes 294 amino acids residues with a molecular weight of 33.41 kDa and an isoelectric point of 5.80. At the N-terminus of *RmHE*, there is a predicted signal peptide of 16 amino acids. The cleavage site is located between Gly16 and Thr17.

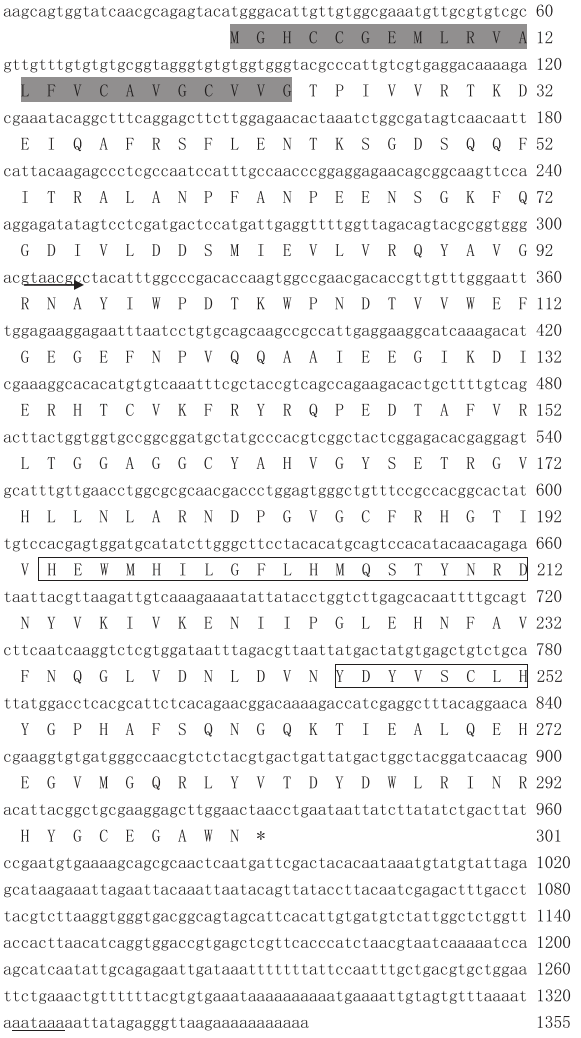

Fig. 1 *RmHE* cDNA sequence and its deduced amino acid residues. Astacin family signature motif sequence was boxed; the spark showed the stop code. The polyadenylation signal sequence (AATAAA) is underlined and highlighted, the signal peptide is shadowed, and the mature enzyme fragment starts from the arrow region.

Homologous alignment and phylogenetic analysis

Homologous analysis results of the deduced protease domain showed that RmHE has identity of 84%, 82%, 80%, 74%, 45%, 42%, 34%, 33%, 33%, 32%, 32%, 32%, 32% and 30% to BmandHE, BmHEL, ApHEL, BmHELII, CuefHCE, DmHEHS, XHE, MLCE, MHCE23, EHE12, ZHCE1, TuHE, QHE and astacin, respectively. Fig. 2a compares the amino acid sequence of RmHE with the sequences of HE of silkworm, wild silkworm, Chinese oak silkworm, fruit flies, mosquito, turtle, quail, fish, *Xenopus*, and astacin, respectively. Two similar signature sequences of HE, HEWMHILGFLHMQSTYNR and YDYVSCLHY, were found in the RmHE. Four cysteine residues were also found which are conserved in all astacin family proteases.

Fig. 2b shows the phylogenetic tree constructed using the neighbour-joining method. HEs were

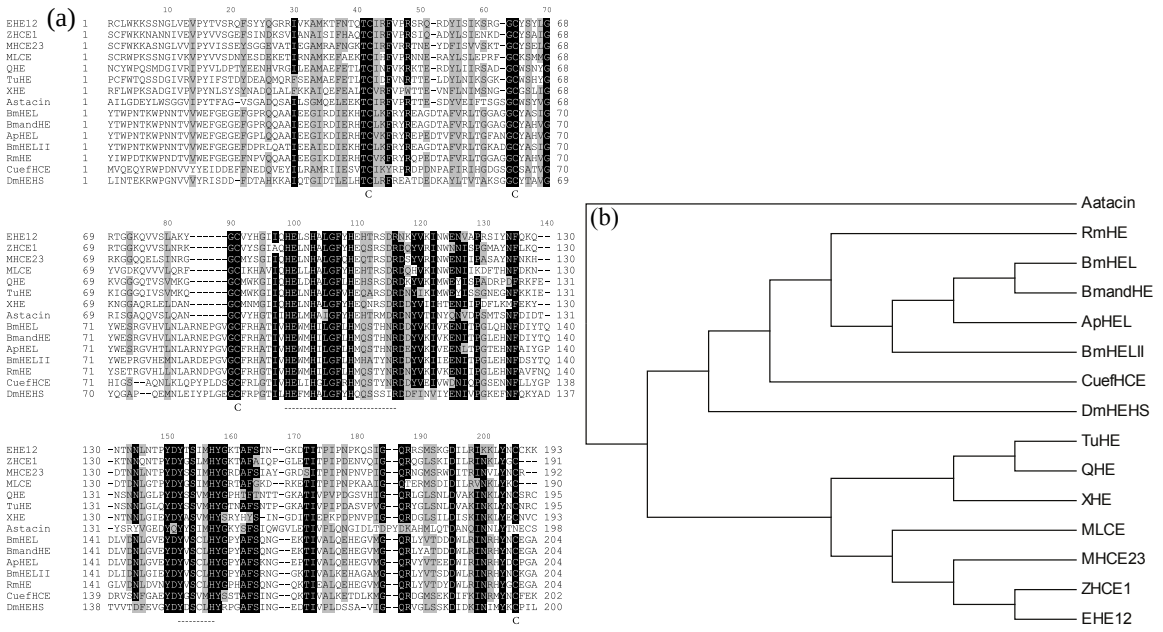

Fig. 2 Comparison of RmHE with other hatching enzymes. (a) Alignment of the amino acid sequences of mature protease domains. (b) A phylogenetic tree based on the protease domains of hatching enzymes. BmHELII *Bombyx mori* hatching enzyme-like II(GenBank accession No. JN627443), BmHEL *B. mori* hatching enzyme-like (FJ147197), BmandHE *B. mandarina* hatching enzyme (JN620366), ApHEL *Antheraea pernyi* hatching enzyme-like (JN205047), CuefHCE house mosquito high choriolytic enzyme 1 (XP_001844559), DmHEHS *Drosophila melanogaster* hatching enzyme homology sequence (NM135911), QHE quail hatching enzyme (BAD95472), TuHE turtle hatching enzyme(BAD95471), XHE Xenopus hatching enzyme (BAA14003), EHE12 eel hatching enzyme (BAB68517), ZHCE1 zebrafish hatching enzyme (NP_998800), MHCE23 medaka high choriolytic enzyme(P31580), MLCE madaka low choriolytic enzyme (NP_001098292), astacin (CAB43519).

clearly classified into two groups. *R. menciana*, silkworm, wild silkworm, Chinese oak silkworm, fruit flies and mosquito were clustered together into the invertebrate group. The vertebrate HEs clustered into a second group. *R. menciana* is most closely related to the wild silkworm and this was consistent with analysis of other genetic markers. *HE* is an appropriate candidate gene for phylogenetic analysis of fish[32] and may also be useful for phylogenetic analysis of insect groups.

The three-dimensional structure of *RmHE*

The amino acid sequence of RmHE was submitted to SWISS-MODEL (swissmodel.expasy.org/), and the three-dimensional structure of the RmHE protein (Fig. 3b) was constructed based on the crystal structure of the zebrafish hatching enzyme 3lqbA[33] (Fig. 3a), which has an identity of 31% to that of RmHE. The protein model was evaluated by PROCHECK (nihserver.mbi.ucla.edu/SAVS/), and the Ramachandran plot provided a visual representation of conformation of amino acid residues, which evaluate the quality of the simulation model. Good quality models typically have over 90% of residues in the allowed regions. The simulated three-dimensional structure of RmHE has 98% in the reasonable area of the Ramachandran, which suggests that the simulated RmHE protein is theoretically reliable (Fig. 3c).

Change of *RmHE* transcript level in embryos

To investigate the relative of *RmHE* transcript in embryos at different development stages, a semi-quantitative PCR technique was employed. The transcript of *RmHE* was maintained at a low level in the early embryo and then dramatically increased to a maximum just prior to eclosion (Fig. 4). The strict temporal pattern of *RmHE* expression was consistent with the possible role of the *HE* gene.

Expression profile of *RmHE* in different tissues

Fig. 5 shows the expression profile of *RmHE* in different tissues of 5th instar *R. menciana* larvae. The expected 394 bp DNA fragment was detected in the

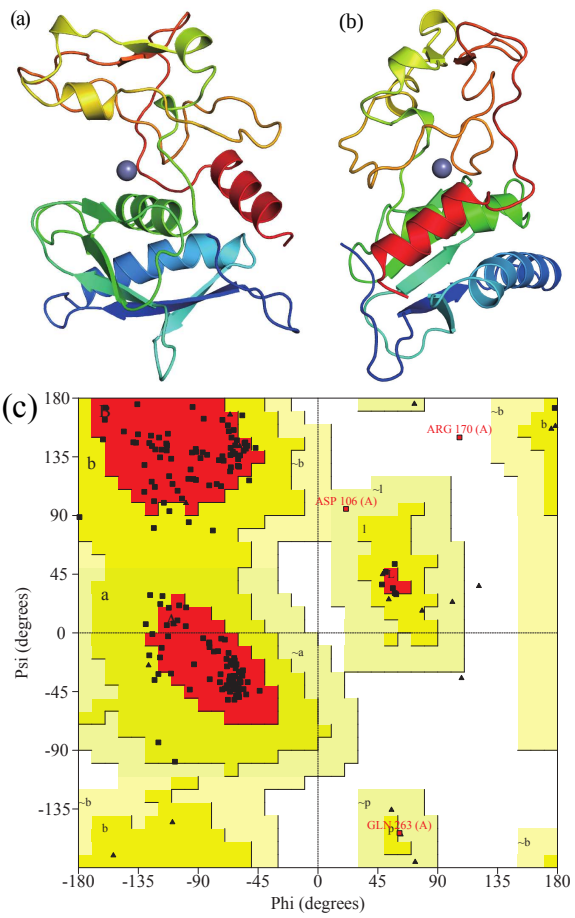

Fig. 3 The prediction of three-dimensional structure of RmHE protein. (a) The three-dimensional structure of 3lqbA protein (template); (b) The three-dimensional structure of RmHE protein; (c) Ramachandran plot, residues in most favoured regions[A,B,L](84%); residues in additional allowed regions[a,b,l,p](14%); residues in generously allowed regions[~a,~b,~l,~p](1%); residues in disallowed regions(0.6%).

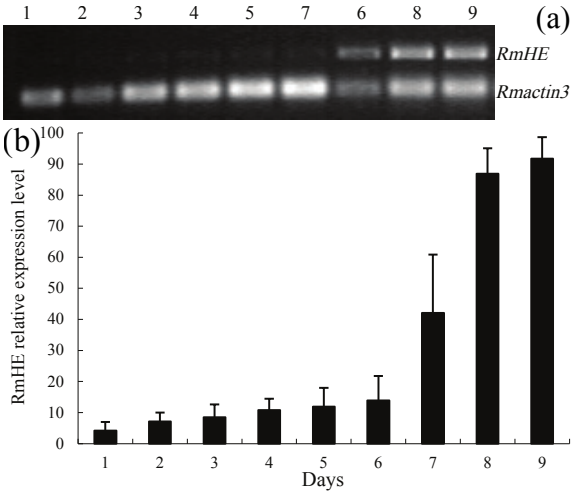

Fig. 4 Change of *RmHE* transcript level in embryo at different development stages. (a) Semi-quantitative RT-PCR products of *RmHE*; (b) *RmHE* relative expression level. (1–9) the days prior to hatching, at 9th day, hatching occurred.

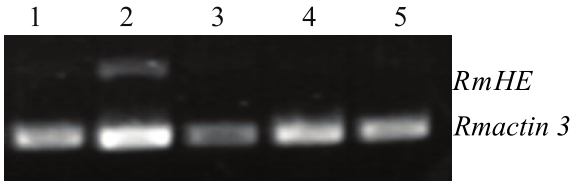

Fig. 5 Expression profile of *RmHE* in different tissues at 5th instar of larval stage. 1% agarose gel analyses of *RmHE* amplifications in comparison with those of controls (*Rmactin 3*), (1) cuticle; (2) midgut; (3) posterior silk gland; (4) fatbody; (5) haemolymph.

midgut, but could not be amplified from the cuticle, posterior silk gland, fat body or haemolymph. These results suggested that *RmHE* might be involved in food digestion.

DISCUSSION

The full-length cDNA of *HE* was cloned successfully from *R. menciana*. Comparison of RmHE to HEs from other insects showed that these proteins share two signature sequences: a zinc-binding motif HExxHxxGFxHxxxxxxR and a methionine turn SxxHY. In the astacin family, these were HExxHxxGFxHExxRxDR and SxMHY. The subtle differences may have resulted from gene mutation, selection and species adaptation to unique environments over evolutionary time[32].

Expression of *RmHE* was low in early embryos and then increased and reached a maximum just prior to eclosion. The spatiotemporal expression pattern of the *RmHE* gene resembles that of the hatching enzyme gene found in other species[6]. Expression of *RmHE* was observed in the midgut of 5th instar larvae but was not detected in other tissues. Two kinds of protein, embryonic astacin[34] and digestive enzyme astacin[35], were found in embryos and adults of the crayfish *Astacus astacus*. We found that the *RmHE* cDNA in the midgut was the same as that in the embryo, which implied that *RmHE* might have a digestive function. Similar results were found in the silkworm and Chinese oak

silkworm[25,27]. The small amounts of testis tissue in *R. menciana* larvae makes it difficult to collect. It therefore remains unclear if the second type of HE exists in the testis of *R. menciana* as it does in the silkworm[26].

Based on the presence of metalloproteases at the time of egg eclosion and the inhibition of egg eclosion in *Pediculus humanus* by metalloprotease inhibitors, therapeutic agents against some parasites could be developed from metalloprotease inhibitors[16]. For *Lucilia cuprina*, the use of rational drug design may lead to a shift away from the use of toxic chemicals towards the use of highly specific inhibitors[36]. This study is the first report on the *HE* gene in a Lepidopteran pest and provides basic information that may aid in the discovery and development of a method to disrupt the egg eclosion of *R. menciana* and other pest Lepidopterans.

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