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A Molecular Biomarker for Disruption of Crustacean Molting: The *N*-acetyl-*β*-glucosaminidase mRNA in the Epidermis of the Fiddler Crab

Yanling Meng · Enmin Zou

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Abstract Several environmentally persistent chemicals have been found to be capable of disrupting crustacean molting. Considering the importance of molting in the life of crustaceans, there is a need to develop a molecular biomarker that can reflect the disrupting effects of contaminants on ecdysteroid signaling in crustaceans. *N*-acetyl-*β*-glucosaminidase (NAG) is a chitinolytic enzyme found in crustacean epidermis. The results of the present investigation show that the transcription of NAG gene in the epidermis of the fiddler crab, *Uca pugilator*, is inducible by the molting hormone 20-hydroxyecdysone, which validates the use of NAG mRNA as a biomarker for molt-disrupting effects of xenobiotics.

Keywords Endocrine disruption · Biomarker · *N*-acetyl-*β*-glucosaminidase · *Uca pugilator*

Aquatic environments are increasingly contaminated with various persistent chemicals, and because of the generally high lipophilicity of these chemicals, most can readily accumulate in fatty tissues of crustaceans. For instance, Mattig et al. (1997) reported the accumulation of organochlorine compounds, including polychlorinated biphenyls (PCBs), dichlorodiphenyltrichloroethanes (DDTs), hexachlorocyclohexanes (HCHs), and hexachlorobenzene (HCB), in the shore crab, *Carcinus maenas*, and the sand shrimp, *Crangon crangon*. Heptachlor epoxide, dieldrin, endosulfan, chlordane, DDT and metabolites, and HCHs were found to accumulate in the burrowing crab, *Chasmagnathus granulata* (Menone et al. 2000). Several of these chemicals, such as kepone (Schimmel et al. 1979), certain PCBs (Zou and Fingerman 1997a), methoxychlor (Baer and Owens 1999), and endosulfan (Zou and Fingerman 1997b; Montagna and Collins 2007), are capable of inhibiting crustacean molting. Stimulatory effects of xenobiotics on crustacean molting have also been reported. For instance, the nonsteroidal ecdysone mimic RH 5849 (Clare et al. 1992) and the pesticide emamectin benzoate (Waddy et al. 2002) have been found to be capable of stimulating crustacean molting. Since the disruption of crustacean molting is not readily seen in the field, this type of endocrine disruption has been called the invisible endocrine disruption (Zou 2005). Considering the importance of molting in the life of crustaceans, it is necessary to develop a sensitive molecular biomarker for disrupting effects of environmental chemicals on crustacean molting. In vertebrates the induction of transcription of estrogen-sensitive genes, such as vitellogenin and choriogenin genes (Lee et al. 2002; Prakash et al. 2007), have been used as biomarkers for estrogenic effects of environmental contaminants. However, to our knowledge, no such molecular biomarker has been reported yet for disrupting effects of environmental chemicals on crustacean molting. We describe herein a molecular biomarker, the mRNA of the chitinolytic enzyme *N*-acetyl-*β*-glucosaminidase (NAG) in the epidermis of the fiddler crab, *Uca pugilator*, that is inducible by the molting hormone, and thus can be used to assess the disrupting effects of xenobiotics on crustacean molting.

Materials and Methods

Female fiddler crabs were purchased from the Gulf Specimen Marine Laboratories (Panacea, FL, USA). In the

Y. Meng · E. Zou (✉)
Department of Biological Sciences, Nicholls State University, Thibodaux, LA 70310, USA
e-mail: em.zou@nicholls.edu

laboratory the crabs were maintained in tanks containing artificial seawater, salinity 12–14‰, made with Instant Ocean synthetic sea salt (Aquarium Systems, Mentor, OH, USA). The animals were maintained under the natural light regime of ~14 h light/10 h dark at a temperature of 19–21°C. The animals were allowed to acclimate to laboratory conditions at least 4 days before use in an experiment. Only intermolt crabs, selected using the setogenic technique (Vigh and Fingerman 1985), were used.

The molting hormone 20-hydroxyecdysone (20-HE) (Sigma, St Louis, MO, USA) was first dissolved in absolute ethanol, and then one aliquot of this solution was mixed with nine aliquots of saline to produce the final solution for injection. For the time response experiment, 40 crabs (wet weight 1.675 ± 0.342 g) were injected with 20-HE at a dose of 1 μg/g wet weight. Another 40 crabs were injected with 10% ethanol solution as control group. Epidermal tissues were harvested at 0.25, 0.75, 1.5, and 3 h. For each time point, 5–7 crabs from each group were sacrificed, and epidermal tissues taken from beneath the carapace were pooled to make the total weight ~600 mg. For dose–response experiment, 4 groups of 10 crabs each were injected with 10% ethanol and 20-HE at 0.25, 1 and 16 μg/g wet weight, respectively. One and half hours after injection, epidermal tissues were harvested from 5 to 7 crabs of each group.

Since the blood volume of various decapods varies between 10 and 50% of the wet weight and the density of the blood in various decapods ranges from 1.025 to 1.052 (Maynard 1960), a mean blood volume/wet weight ratio of 30% and a blood density of 1.0 can be used to estimate the total blood volume of *U. pugilator*. For instance, a crab of 1.6 g wet weight is estimated to have a blood volume of 0.48 mL. An injection dose at 1 μg/g wet weight would give rise to a 20-HE concentration of about 2.08 μg/mL hemolymph. The peak ecdysteroid concentration in *U. pugilator* during the molting cycle is known to be around 0.15 μg/mL hemolymph (Hopkins 1983). Considering the possible loss of injected hormone from excretion and through injection wound, the 20-HE doses used in the present study were greater than the physiological hormone level.

Total RNA was isolated from epidermal tissues using the RNeasy mini kit (Qiagen, Valencia, CA, USA). RNA concentration was determined by absorption spectrophotometry at a wavelength of 260 nm. Quality of isolated RNA was determined by the ratio of absorbance at 260–280 nm, which varied, in the present study, from 1.6 to 2.0.

Semiquantitative reverse transcription polymerase chain reaction (RT-PCR) was used to quantify NAG mRNA level. RT-PCR was carried out according to the protocol of the AMV Transcriptase System (Promega, Madison, WI, USA). The total RNA used for different experiments ranged from 0.5 to 1.0 μg. Primers (forward primer: 5′-CACTGGCACATCACCGACTCC-3′; reverse primer: 5′-GTAGGGGCTGCACCAGTTGTT-3′) were designed on the basis of the conservative regions of NAG cDNA sequences. A PCR product of ~750 bp was obtained with this primer pair. This PCR band was cut out from a 2% agarose gel and purified using Promega Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). After DNA sequencing (ACGT, Inc., Wheeling, IL, USA), a pair of more specific primers (forward primer: 5′-TCGTGGCATGGATGATTG-3′; reverse primer: 5′-GCGTCGTGGTTACTGAAAA-3′) were designed based on the sequencing result of this PCR product (Fig. 1). This more specific primer pair was used in the subsequent semiquantitative RT-PCR experiments with18S rRNA used as internal control. Primers designed for *Callinectes sapidus* 18S rRNA sequence were used (Zheng et al. 2006). PCR reaction mixture was prepared using Promega PCR Master Mix. NAG mRNA and 18S rRNA primers were added into the same reaction system. PCR conditions included first denaturing at 94°C for 5 min, followed by 35 cycles of amplification (94°C for 30 s, 58°C for 30 s and 72°C for 45 s) and the final extension at 72°C for 5 min. After agarose gel electrophoresis of the PCR products, DNA bands were documented and quantified with the FluorChem FC2 imaging system (Alpha Innotech), and densitometric analyses were performed using the accompanying software (AlphaEaseFC).

Results and Discussion

The use of a primer pair based on the conserved regions of NAG cDNA sequences yielded a PCR product of ~750 bp. DNA sequencing results revealed a 749 bp cDNA with an open reading frame of 245 amino acid residues (Fig. 1). ClustalW analyses of sequence similarities identified by tBlastx searches show that the predicted sequence of this cDNA fragment is highly similar (47–65% identical) to deduced amino acid sequences from NAG cDNAs of other arthropods (Fig. 2). Apparently, this acquired fragment represents the partial sequence of *U. pugilator* NAG cDNA. A pair of more specific primers, bracketing a 266 bp sequence, were subsequently used for semiquantitative RT-PCR experiments to determine the relative abundance of NAG mRNA level in epidermal tissues from *U. pugilator*. The results of further DNA sequencing verified that the RT-PCR product, ~270 bp in length, obtained with the more specific primer pair, is indeed a 266 bp segment of the *U. pugilator* cDNA sequence shown in Fig. 1.

Time–response results show that injection of the exogenous 20-HE at a dose of 1 μg/g wet weight resulted

Fig. 1 Partial sequence of *Uca pugilator* NAG cDNA and deduced amino acid sequence

```
tctggactctctgcccaacatggccttctacggcgcctactcccccaggcaggtgtacacg
  L  D  S  L  P  N  M  A  F  Y  G  A  Y  S  P  R  Q  V  Y  T
cctgcaatggtgcgcagcttggtgcagtatgcgcgcgtacgtggcgtgaggctgctgccc
 P  A  M  V  R  S  L  V  Q  Y  A  R  V  R  G  V  R  L  L  P
gaattcgacgcgccagcgcatgtcggcaacggttggcagtggggcgagaaggaagggctg
 E  F  D  A  P  A  H  V  G  N  G  W  Q  W  G  E  K  E  G  L
gggaaactggctgtctgtgttaaccaggaaccatggcagcagttttgcgtggagcctcca
 G  K  L  A  V  C  V  N  Q  E  P  W  Q  Q  F  C  V  E  P  P
tgcggtcagctcaacattgctaaccccgccatatataacgttttggggcagatctatcac
 C  G  Q  L  N  I  A  N  P  A  I  Y  N  V  L  G  Q  I  Y  H
gaaatgcttgacatcttcgccccagttgatctgtttcatttcggcggtgacgaggtcaac
 E  M  L  D  I  F  A  P  V  D  L  F  H  F  G  G  D  E  V  N
                                Forward primer
cttaattgctggaacacaacggaggagatcgtggcatggatgattgccaacggaggtggt
 L  N  C  W  N  T  T  E  E  I  V  A  W  M  I  A  N  G  G  G
cttgacgcagatgcttactacacgcaatggggagtgttccaggagaaggcatggcagctg
 L  D  A  D  A  Y  Y  T  Q  W  G  V  F  Q  E  K  A  W  Q  L
ctacaggcggctgcaggggttggacaaggagaggaggaggtgaagggaatactgtggacc
 L  Q  A  A  A  G  V  G  Q  G  E  E  E  V  K  G  I  L  W  T
tcagaactcacggatccggctcgtcttgatgtcttcctgaactcctcccagtacatcata
 S  E  L  T  D  P  A  R  L  D  V  F  L  N  S  S  Q  Y  I  I
caaatttggagcgctcgcgatgaccccgccattcaggaggctttaactaaaggctacaga
 Q  I  W  S  A  R  D  D  P  A  I  Q  E  A  L  T  K  G  Y  R
     Reverse primer
gtaattttcagtaaccacgacggcatggtacttggactgtggtgccggggcatgggtgggc
 V  I  F  S  N  H  D  A  W  Y  L  D  C  G  A  G  A  W  V  G
gagggcaacaactggtgagcccccctaca
 E  G  N  N  W
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Fig. 2 Multiple sequence alignment (ClustalW) of the deduced amino acid sequence of NAG mRNA from *Uca pugilator* with that of other arthropods, including *Fenneropenaeus chinensis*, *Manduca sexta* (GenBank accession no. AY368703), *Bombyx mori* (GenBank accession no. NM001044001), *Bombyx mandarina* (GenBank accession no. AF326597), *Ostrinia furnacalis* (GenBank accession no. DQ887769) and *Choristoneura fumiferana* (GenBank accession no. DQ005717)

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B.mandarina  IKRTIDAMAAVKLNTFHWHITDSQSFPLVLQKSPNFSKLGAYSPTKVYTKQDIREVVEYG
B.mori       IKRTIDAMAAVKLNTFHWHITDSQSFPLVLQKRPNLSKLGAYSPTKVYTKQDIREVVEYG
M.sexta      IKKTIDAMASAKLNTFHWHITDSQSFPFVMDKRPNLVKYGAYSPSKVYTKKAIREVVEYA
O.Fumacalis  IKRTIEAMAAVKLNTFHWHITDSQSFPFVTTKRPNLYKFGALSPQKVYTKAAIREVVRFG
C.fumiferana IKKTIDAMAAVKLNTFHWHITDSQSFPFVSERRPNLSKYGAYTPAKIYTKAAIRDVVQFG
U.pugilator  ----------------------------LDSLPNMAFYGAYSPRQVYTPAMVRSLVQYA
F.chinensis  IERTLDAMAANKLNTFHWHITDSHFPFMQLETLPNMAYYGAYGSRFIYSTADIRNLVEYG

B.mandarina  LERGVRVLPEFDAPAHVGEGWQDTG------LTVCFKAEPWTKFCVEPPCGQLNPTKEEH
B.mori       LERGVRVLPEFDAPAHVGEGWQDTG------LTVCFKAEPWTKFCVEPPCGQLNPTKEEL
M.sexta      LERGVRCLPEFDAPAHVGEGWQESD------LTVCFKAEPWAKYCVEPPCGQLNPIKDEL
O.Fumacalis  LERGVRVLPEFDAPAHVGEGWQDTD------LTVCFKAEPWKSYCVEPPCGQLNPTKDEL
C.fumiferana LERGVRVLPEFDAPAHVGEGWQDTG------LTVCFKAEPWASYCVEPPCGQLNPTKDEL
U.pugilator  RVRGVRLLPEFDAPAHVGNGWQWGEKEGLGKLAVCVNQEPWQQFCVEPPCGQLNIANPAI
F.chinensis  RIRGIRVLAEFDAPAHVGNGWRWGEGQGLGKLAVCVNREPWQSYCVEPPCGQLNLANPNM

B.mandarina  YDYLVDIYVEMAEAFESTDMFHMGGDEVSERCWNSSEEIQNFMIQNRWNLDKSSFLKLWN
B.mori       YDYLEDIYVEMAEAFESTDMFHMGGDEVSERCWNSSEEIQNFMIQNRWNLDKSSFLKLWN
M.sexta      YDVLEDIYVEMAEAFHSTDMFHMGGDEVSDACWNSSEEIQQFMIQNRWDLDKSSFLKLWN
O.Fumacalis  YQYLEDIYSDMAEVFDTTDIFHMGGDEVSEACWNSSDSIQNFMMQNRWDLDKESFLKLWN
C.fumiferana YDVLEDIYTDMAEVFK-PDIFHMGGDEVSERCWNASDDIQQFMMQHRWDLDKSSFLNLWD
U.pugilator  YNVLGQIYHEMLDIFAPVDLFHFGGDEVNLNCWNTTEEIVAWMIANGGGLDADAYYTQWG
F.chinensis  YDVLGQIYNEMVELFSPIDLFHFYGGDEVNLNCWNTTDEITSWMDENNFGRDDDAYYNQWS

B.mandarina  YFQKNAQDRAYKAFG-----KRLPLILWTSRLTDYTHVEKFLDKDEYIIQVWTTGADPQI
B.mori       YFQKNAQDRAYKAFG-----KRLPLILWTSTLTDYTHVEKFLDKDEYIIQVWTTGADPQI
M.sexta      YFQTKAEDRAYKAFG-----KNIPLVMWTSTLTDYTHVDKFLDKEKYIIQVWTTGVDPQI
O.Fumacalis  YFQQKAQDKAYKAFG-----KKLPLILWTSTLTNYKHIDDYLNKDDYIIQVWTTGVDPQI
C.fumiferana YFQRKAQEKVYKAFG-----KKVPIILWTSTLTDHVYVDKYLNKDDYIIQVWTTGVDPQI
U.pugilator  VFQEKAWQLLQAAAGVGQGEEEVKGILWTSELTDPARLDVFLNSSQYIIQIWSARDDPAI
F.chinensis  IFQEKSRQLPTTANG----GNEVPGILWTSHLTEEGRADQYLDPTKYIIQIWTTGTDKLI

B.mandarina  QGLLQKGYRLIMSNYDALYFDCGFGAWVGSGNNWCSPYIGGQKVYGNSPAVMAL-----S
B.mori       QGLLQKGYRLIMSNYDALYFDCGFGAWVGSGNNWCSPYIGGQKVYGNSPAVMAL-----S
M.sexta      QGLLQKGYKLIISNYDALYFDCGFGAWVGSGNNWCSPYIGWQKVYDNSPAVMAL-----S
O.Fumacalis  KGLLEKGYRLIMSNYDALYFDCGYGAWVGAGNNWCSPYIGWQKVYDNSPAVIAL-----E
C.fumiferana LGLLQKGYRLIMSNYDALYLDCGFGAWVGSGNNWCSPYIGWQKVYDNSPAVMAL-----D
U.pugilator  QEALTKGYRVIFSNHDAWYLDCGAGAWVGEGNNW-------------------------
F.chinensis  GELLEKNFRVIFSNYDHWYLDCGFGAWVGEGNNWCSPYKGWQAVYDNSPLDIATDLTGSA
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in upregulation of NAG mRNA level in the epidermis of *U. pugilator* at 1.5 h after injection, but interestingly downregulation of epidermal NAG gene expression was observed 3.0 h after injection (Fig. 3a, b). Since an increase in NAG mRNA was apparent at the 1.5 h mark, epidermal tissues were taken 1.5 h after hormone

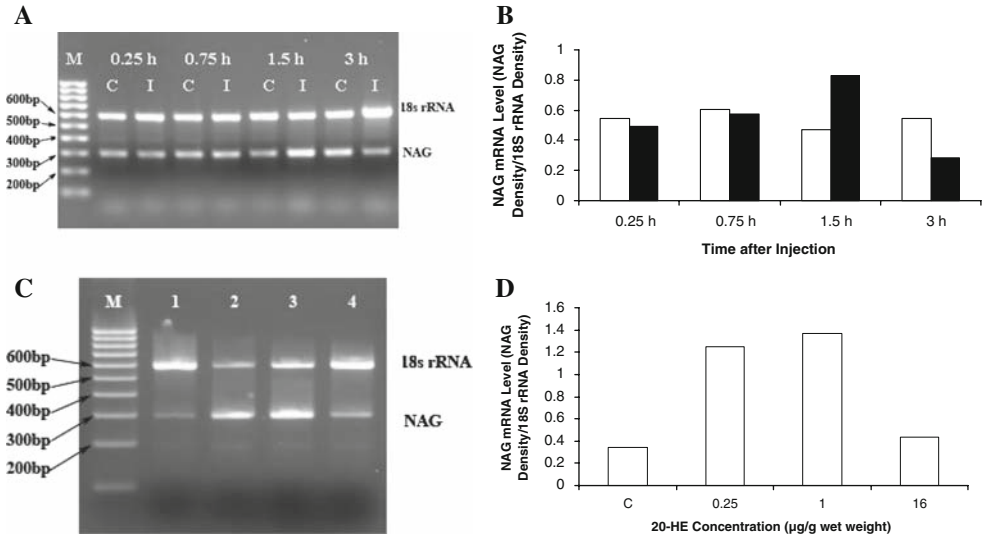

Fig. 3 Induction of *N*-acetyl-*β*-glucosaminidase (*NAG*) mRNA in the epidermis of *Uca pugilator* by 20-hydroxyecdysone (20-HE). **a** Time course of NAG mRNA induction by 20-HE at 1 µg/g wet weight [(*C*) Ethanol control, (*I*) 20-HE-injected group]. **b** Densitometric analysis for time course results (*White bars*, control; *solid bars*, 20-HE-treated); **c** Induction of NAG mRNA at different dose of 20-HE [(*M*) Marker, (*1*) control, (*2*) 20-HE at 0.25 µg/g wet weight, (*3*) 20-HE at 1 µg/g wet weight, (*4*) 20-HE at 16 µg/g wet weight]; **d** densitometric analysis of dose–response results. *Vertical axis* shows the ratio between densities of NAG mRNA and18S rRNA bands

treatment in the subsequent dose–response experiment. Injection of 20-HE at 0.25–1 µg/g wet weight upregulated the level of epidermal NAG mRNA (Fig. 3c, d). But such induction of epidermal NAG mRNA was absent when 20-HE was administered at 16 µg/g wet weight. This non-response at a high dose of 20-HE could be the result of a negative feedback mechanism, with an excess of newly synthesized NAG leading to the turnoff of transcription machinery. At the level of enzymatic activity, significant increases in activity of chitinolytic enzymes in the epidermis of *U. pugilator* are still apparent 2 days after second injection of 20-HE at 25 µg/g wet weight (Zou and Fingerman 1999a; Zou and Bonvillain 2004). Perhaps, it only needs a brief period of upregulation of NAG gene expression in the epidermis to produce a sufficient amount of NAG for degradation of cuticular chitin following an injection of a high dose of 20-HE. This newly synthesized NAG is presumably compartmentalized intracellularly and released to the extracellular environment when needed.

The induction of NAG mRNA in the epidermis by the molting hormone has never been studied before in any crustaceans. Using a staining method specific for NAG Zou and Fingerman (1999a) found that there is only one NAG isoenzyme with a molecular mass of 89 KDa in the epidermis of *U. pugilator*. High similarity of the predicted amino acid sequence from the 749 bp RT-PCR product to that of other arthropod species as shown by the ClustalW results indicates that this RT-PCR product indeed corresponds to a fragment of NAG mRNA found in epidermal tissues of *U. pugilator*. The results of time–response and dose–response experiments, obtained with a pair of more specific primers based on this partial sequence of NAG cDNA, clearly show that epidermal NAG mRNA in *U. pugilator* is inducible by 20-HE. This is further evidence that NAG gene expression in the epidermis of *U. pugilator* is under control of the molting hormone.

Molting in crustaceans is regulated by a multi-hormonal system, but is under immediate control of the steroid hormones called ecdysteroids (Chang et al. 1993). When molt cycle proceeds to premolt stage, ecdysteroid synthesis in the Y-organ increases due to the lift of inhibition by the molt-inhibiting hormone. Ecdysteroid titer in the hemolymph is, as a result, elevated. In the epidermis ecdysteroids regulate gene activities at the transcriptional level through interaction with the ecdysteroid receptor (EcR), which then forms a heterodimer with crustacean retinoid X receptor (RXR). This EcR/crustacean RXR dimer binds to the DNA response elements of the genes regulated by the molting hormones. Among the products of the genes regulated by the molting hormones are enzymes responsible for degradation of the old exoskeleton such as NAG. A molt-disrupting xenobiotic could render its effects through interfering with ecdysteroidogenesis and/or perturbing intracellular ecdysteroid signaling. The fact that the expression of NAG gene represents the terminal event in the ecdysteroid signaling and that the response of NAG mRNA to 20-HE is rapid makes epidermal NAG mRNA an effective molecular biomarker for molt-interfering effects of environmental chemicals.

In vertebrates the disruption of sexual development by environmental contaminants usually involves the abnormal expression or suppression of the genes regulated by sex steroids. The expression in males of vitellogenin and choriogenin genes, which normally express only in females, is indicative of an estrogenic effect. This is why vitellogenin and choriogenin mRNAs have been used as molecular biomarkers for feminizing effects of environmental chemicals in vertebrates (Lee et al. 2002; Prakash et al. 2007). However, the disruption of molting in Crustacea by environmental contaminants is not sex-specific; or in other words, a chemical capable of disrupting molting of males can also perturb molting of females. Therefore, when it comes to using NAG mRNA as a biomarker for molt-disrupting effect, a control group, whether it be crustaceans from a clean site for field assessment or cultured epidermal tissues for in vitro assay, must be used. Additionally, since NAG activity varies during the molting cycle (Zou and Fingerman 1999b), crustaceans in the same molt stage, preferably, intermolt stage, must be used.

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