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Authors: Watanabe, Toshiki, and Kono, Michiko

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Isolation of a cDNA Encoding a Chitinase Family Protein from Cuticular Tissues of the Kuruma Prawn *Penaeus japonicus*

Toshiki Watanabe[1]* and Michiko Kono[2]

[1]*Laboratory of Molecular Biology of Marine Organisms, Ocean Research Institute, The University of Tokyo, 1-15-1 Minamidai, Nakano, Tokyo 164, Japan*
[2]*Fisheries Research Laboratory, Faculty of Agriculture, The University of Tokyo, Maisaka, Shizuoka 431-02, Japan*

ABSTRACT—To identify and characterize a chitinase related to molting in the Kuruma prawn *Penaeus japonicus*, we searched for chitinase-encoding cDNAs expressed in cuticular tissues. Using two degenerate oligonucleotide primers derived from the two conserved regions of the chitinase protein family, a RT(reverse transcription)-PCR product was obtained. This product was used as a probe to screen a cDNA library from a mixture of the tail fan and blade — two tissues which consist mainly of chitinous exoskeleton and underlying epidermis. A positive cDNA clone was analyzed for the sequence. This clone contains an open reading frame for a protein (named Pjchi-2) of 527 amino acids which exhibits sequence similarity to known chitinases. A typical signal sequence could not be found in the Pjchi-2 sequence. Significant accumulation of *Pjchi-2* mRNA was detected in the mixture of the tail fan and blade prior to molting, whereas the transcript level was much lower during the intermolt stage. This observation suggests that Pjchi-2 plays a role in molting. The mRNA was not detected in the hepatopancreas. This expression pattern of *Pjchi-2* makes a contrast to that of *Pjchi-1* which encodes another chitinase family protein in *P. japonicus*, and is expressed in the hepatopancreas but not in the tail fan or blade.

INTRODUCTION

In crustaceans including the Kuruma prawn *Penaeus japonicus*, endo-type chitinolytic enzyme (chitinase) activities have been detected in the hepatopancreas and integument (Buchholz, 1989; Spindler-Barth *et al.*, 1990; Kono *et al.*, 1995). In the integument, the chitinase activity is induced prior to molting, presumably to degrade chitin in the old skeleton which is to be shed off. In contrast, the level of chitinase activity in the hepatopancreas does not vary significantly during the molt cycle. Chitinase expressed in the hepatopancreas is thought to be used to digest chitin-containing food.

We have been trying to isolate and characterize cDNAs encoding chitinases in the hepatopancreas and integument to address the following two questions: (i) Is the chitinase expressed in the integument the same as in the hepatopancreas, or are different chitinase isoforms expressed in different tissues? (ii) How is expression of chitinase differentially regulated in the integument and hepatopancreas? We have reported isolation of a cDNA encoding the Pjchi-1 protein from the hepatopancreas of *P. japonicus* (Watanabe *et al.*, 1996). Pjchi-1 exhibits significant sequence similarities to known chitinases, especially to a chitinase in the tobacco hornworm *Manduca sexta* (Kramer *et al.*, 1993). The *Pjchi-1* mRNA was detected in the hepatopancreas both during the intermolt and premolt stages, but not two cuticular tissues (the tail fan and blade). This result suggests that a different chitinase is expressed prior to molting in cuticular tissues. Here we report isolation of a cDNA encoding a chitinase-like protein from cuticular tissues.

* Corresponding author: Tel. +81-3-5351-6534;
FAX. +81-3-5351-6488.

MATERIALS AND METHODS

Isolation of total RNA and RT-PCR

Total RNA was isolated from a mixture of the tail fan and blade, and subjected to RT-PCR as described in Watanabe *et al.* (1996). Degenerate oligonucleotide primers CHIF (5'-ATGGTNKCNGT-NGGNGGNTGG-3') and CHIR (5'-NGGRTAYTCCCARTCNANRTC-3') were designed based on the amino acid sequences of the two conserved domains of the chitinases (Watanabe *et al.*, 1996).

Construction and screening of a cDNA library from the tail fan/blade

Poly(A)$^+$ RNA was prepared using Oligotex™-dT30 super (Roche Japan). A cDNA library was constructed from the poly(A)$^+$ RNA as described in Watanabe *et al.* (1996) except that λZAPII (stratagene) was used instead of λgt11 as the cloning vector. A product of the RT-PCR reaction was cloned into the pCR™II vector (Invitrogen). The *Eco*RI fragment of this plasmid was self-ligated using T4 DNA ligase to generate longer fragments, radio-labeled with Multiprime™ DNA labeling system (Amersham), and used as a probe to screen the cDNA

library. Conditions for hybridization and subsequent washing were described in Watanabe *et al.* (1996).

Nucleotide sequence analysis

Recombinant pBluescript SK⁻ phagemids were rescued from positive λ phage clones by *in vivo* excision according to the instruction from the manufacturer (stratagene). Insert DNA (3,357 bp) was sequenced as described in Watanabe *et al.* (1996). The nucleotide sequence of 1,229 bp in the 5' untranslated region was determined only on the sense strand, and 2,128 bp on the 3' side (containing the entire open reading frame; see Fig. 2) was sequenced on both strands. Homology search in the PIR and SWISS-PROT databases was carried out using the FASTA E-mailing service by DNA Data Bank of Japan (National Institute of Genetics, Mishima, Japan).

Northern hybridization

Poly(A)⁺ RNA (1 μg per lane) from a mixture of tail fan and blade, and hepatopancreas was run on 1% formaldehyde-agarose gel, transferred to NYTRAN membrane (Schleicher & Schuell), and probed with a cDNA fragment (corresponding to nucleotide position 1,362-2,972 in Fig. 2) that was radio-labeled with [α-^{32}P]dATP. The concentration of the probe in hybridization buffer was 5×10^6 cpm/ml. The final wash of the membrane was done in 0.1 × SSPE and 0.1% SDS at 65°C. RNA ladder (0.24 – 9.5 kb; GIBCO BRL) was used as molecular weight markers.

RESULTS

Preparation of RNA from the tail fan and blade at late premolt stages and RT-PCR

Prawns at late premolt stages (D3 and D4) were identified according to Hong (1977), and total RNA was prepared from a mixture of the tail fan and blade which consist mainly of chitinous exoskeleton and underlying epidermis.

To isolate a cDNA fragment containing a coding sequence of a chitinase, the RNA sample was subjected to RT-PCR using two degenerate oligonucleotide primers CHIF and CHIR which were designed based on the amino acid sequences of the two conserved regions of the chitinase protein family (Watanabe *et al.*, 1996). A RT-PCR product was cloned and sequenced (Fig. 1). A 111 base pair (bp) region which was bounded by the primer sequences was conceptually translated to 37 amino acids, and the sequence was compared to those of the *M. sexta* chitinase (Kramer *et al.*, 1993) and Pjchi-1, a chitinase homologue in *P. japonicus* (Watanabe *et al.*, 1996). In the 37 amino acid region, identity was found at 14 and 13 residues, respectively (data not shown), suggesting that the RT-PCR product contains a coding sequence of a chitinase homologue.

Isolation and sequence analysis of a cDNA clone

This product was used as a probe to screen a cDNA library of the tail fan/blade at the late premolt stage. Six positive clones were isolated, and a clone containing the longest insert was selected for sequence analysis. Within this cDNA clone, an open reading frame (ORF) of 1,581 bp was found (Fig. 2). The ORF was conceptually translated to 527 amino acids, and this putative protein was named Pjchi-2 (*Penaeus japonicus* chitinase homologue-2) due to its sequence similarity to known chitinases (see below).

The first methionine in the ORF (amino acid position 1 in Fig. 2) is shortly preceded by a stop codon in all three reading frames (data not shown). The second and third methionines are found at amino acid positions 8 and 339, respectively (Fig. 2). Assuming that the first methionine is the translation start site, the predicted molecular weight of Pjchi-2 is 59.2 kDa and isoelectric point 8.19. A potential N-glycosylation site was found at amino acid position 230. We failed to find a typical signal peptide (von Heijne, 1986) at the N-terminus of Pjchi-2, as no noticeable stretch of hydrophobic amino acids was found following the first or second methionine.

Using the FASTA program (Pearson and Lipman, 1988), we searched in the PIR and SWISS-PROT databases for proteins which exhibit sequence similarities to Pjchi-2. Proteins with the five highest similarity scores were: (1) a chitinase precursor in a braconid wasp (optimized score 838; Krishnan *et al.*, 1994), (2) a chitinase precursor in the tobacco hornworm *Manduca sexta* (775; Kramer *et al.*, 1993), (3) the human cartilage glycoprotein gp39 precursor (762; Hakala *et al.*, 1993), (4) a swine heparin-binding protein (757; Shackelton *et al.*, 1995), and (5) a bovine oviduct-specific glycoprotein precursor (728; Sendai *et al.*, 1994). The similarity score between Pjchi-2 and Pjchi-1 (Watanabe *et al.*, 1996) was 695. In the optimized alignment between Pjchi-2 and each of the above six structurally related proteins, conserved amino acids were distributed more or less uniformly over a region of about 400 amino acids, and the identity level in the overlap was 35-40% (data not shown). Sequence similarity among Pjchi-2, Pjchi-1 and the two insect chitinases was particularly high in conserved region II (Fig. 3), the putative catalytic domain of the chitinases (Watanabe *et al.*, 1993). Reasonable sequence similarity among those proteins was also seen in conserved region I (Fig. 3).

```
ATGGTCGCTGTTGGTGGATGGAACGACTCAGCCGGTGACAAGTACAGTCGCCTGGTGAAC
        CHIF          N  D  S  A  G  D  K  Y  S  R  L  V  N

AACCCCGAAGCTCGAAGGAAGTTCAACGAGCATGTTATTGAGTTCATCAAGAGGCATAAC
N  P  E  A  R  R  K  F  N  E  H  V  I  E  F  I  K  R  H  N

TTTGATGGTCTCGACATCGACTGGGAATACCCT
F  D  G  L            CHIR
```

Fig. 1. Isolation and sequence analysis of a RT-PCR product. Total RNA from a mixture of the tail fan and blade was subjected to RT-PCR using two degenerate oligonucleotide primers CHIF and CHIR, and PCR products were cloned in pCR™II. Nucleotide sequence of a clone is shown here. Sequences corresponding to the primers are underlined, and the inferred amino acid sequence is shown below corresponding codons.

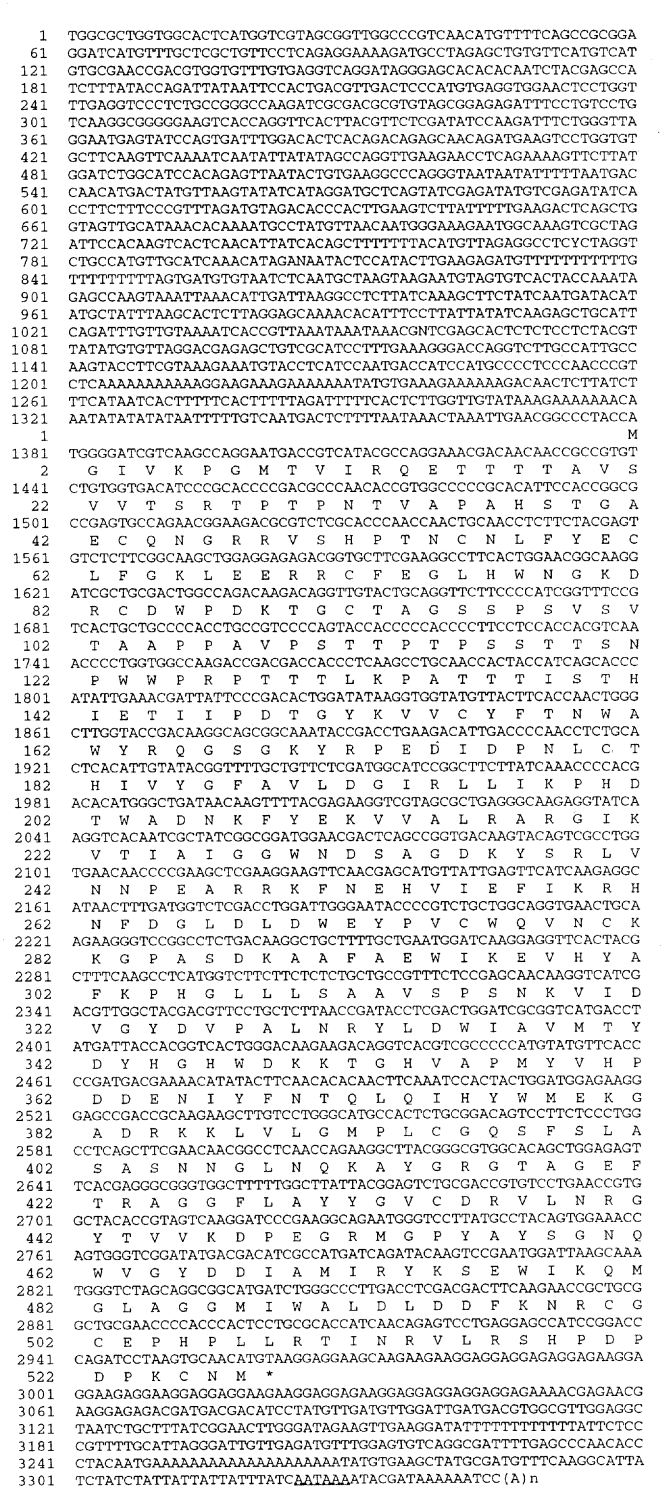

Fig. 2. Nucleotide sequence of the *Pjchi-2* cDNA. Nucleotide sequence of a cDNA clone of 3,357 bp and conceptual translation of an open reading frame of 1,581 nucleotides (nucleotide position 1320-2960) are presented. The amino acid sequence is shown in the one-letter representation below the respective codons. The positions of the nucleotides and amino acids are indicated on the left of the sequences. A typical polyadenylation signal (AATAAA) was found 22 bases upstream from the poly(A) tail (underlined). An asterisk shows the termination codon.

Expression of the Pjchi-2 *mRNA*

Expression of the *Pjchi-2* mRNA was examined, using Northern analysis, in the tail fan/blade and hepatopancreas (Fig. 4). Significant accumulation of transcripts was detected in the tail fan/blade during the premolt stage, whereas the expression level was much lower during the intermolt stage. The top of a smeary band was observed at approximately 3.2 kb (arrow in Fig. 4). The size of this band was in fair agreement with that of the cDNA clone (3,356 bp). The *Pjchi-2* mRNA was not detected in the hepatopancreas.

DISCUSSION

A cDNA encoding Pjchi-2, a new member of the chitinase family, was isolated in cuticular tissues of *P. japonicus*. *Pjchi-2* transcription was seen to be induced prior to molting. In contrast to Pjchi-1 which is expressed in the hepatopancreas but not tail fan/blade (Watanabe *et al.*, 1996), *Pjchi-2* transcription was not detected in the hepatopancreas. The Pjchi-2 sequence shows significant similarity to known chitinases, especially to the two insect chitinases, and the primary structure was particularly well conserved in region II, the putative catalytic domain of the chitinase. These results suggest that Pjchi-2 is a chitinase which digests chitinous exoskeleton prior to molting in *P. japonicus*.

The sequence homology, however, may not be sufficient to assure that Pjchi-2 has chitinase activity. The human glycoprotein gp39 does not exhibit chitinase activity, though it is a member of the chitinase family and possesses conserved region II (Hakala *et al.*, 1993). The level of sequence similarity between Pjchi-2 and gp39 was almost equal to those between Pjchi-2 and the two insect chitinases (see Results). To address this issue, we shall generate Pjchi-2 using a baculovirus system and test its chitinase activity.

A typical signal sequence was not found at the N-terminus of the Pjchi-2 sequence. Though the apparent absence of a signal peptide is unusual among chitinase family proteins, Pjchi-2 is not the first case. A chitinase isolated in the Gram-negative bacterium *Serratia marcescens* lacks a typical signal sequence (Brurberg *et al.*, 1995). This chitinase is exported to the periplasm, *via* an unknown mechanism, without being processed at the N-terminus. It remains to be solved whether Pjchi-2 is cytoplasmic or secreted *via* a novel mechanism to the extracellular region where old exoskeleton is digested prior to molting. We shall address this question by generating antibodies against Pjchi-2 and examining its localization using immunohistochemical techniques.

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The cDNA sequence in Fig. 2 has been deposited in the DDBJ/EMBL/GenBank databases (accession no. D89751).

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                    Region I                                    Region II
Pjchi-2         LRARGIKVTIAIGGWNDSAGDKYSRLVNNPEARRKFNEHVIEFIKRHNFDGLDLDWEYPVC
Chelonus sp.    KKNPSV.IMV.V....AG-SVPF.QMASDQAT.EA.AQN.VK.LQQYQ...F.I.....AQ
M. sexta        SSHPSV.FMV.V...AEG-SS...HM.AQKST.MS.IRS.VS.L.KYD...........GA
Pjchi-1         EKYPDM.TN..V...AEG-.R...QM.MVA.R.AS.IRS.VQLLTDYG...........GA
                      *   * ***         *         *  *   *          *** * *****
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Fig. 3. Alignment of the Pjchi-2 sequence with two insect chitinases and Pjchi-1. The Pjchi-2 sequence in the vicinity of the two conserved regions of the chitinase family (Leu^{215}-Cys^{275}) was aligned using the CLUSTAL V program (Higgins and Sharp, 1989) with a chitinase in a braconid wasp *Chelonus* sp. (Lys^{90}-Gln^{159}; Krishnan *et al.*, 1994), a chitinase in the tobacco hornworm *M. sexta* (Ser^{91}-Ala^{150}; Kramer *et al.*, 1993), and Pjchi-1, a chitinase homologue in *P. japonicus* (Glu^{110}-Ala^{169}; Watanabe *et al.*, 1996). Positions of the two conserved regions are indicated above the sequences. Identity of amino acids between Pjchi-2 and the other proteins is indicated by dots, and a gap by a hyphen. The amino acids which are conserved in all of the proteins are indicated with asterisks.

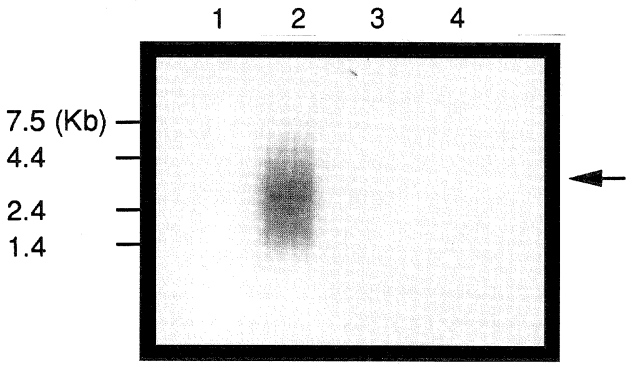

Fig. 4. Northern analysis of Pjchi-2-encoding mRNA in cuticular tissues and the hepatopancreas. A Northern blot of poly(A)$^+$ RNA (1 μg per lane) from a mixture of tail fan and blade (lanes 1 and 2), and the hepatopancreas (lanes 3 and 4) was hybridized with a radio-labeled cDNA probe which corresponds to nucleotide position 1,362-2,972 in Fig. 2. Samples in lanes 1 and 3 are from the intermolt stage (stage C according to Hong, 1977), and lanes 2 and 4 from late premolt stages (stages D3 and D4). A band was recognized at approximately 3.2 kb in lane 2 (arrow); this band was also seen in lane 1 after a long exposure. Positions of four molecular weight markers (7.5, 4.4, 2.4, and 1.4 kb, from top to bottom) are indicated.

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