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# Evidence for the main foot protein gene in *Perna perna* (Mollusca, Mytilidae)

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**ABSTRACT.** Here, we investigated the gene that encodes the main protein component of the byssus in *Perna perna* (*Ppfp*) using a consensus fluorescent probe for the *Mytilus* group and by polymerase chain reaction amplification using specific and random primers. A 524-bp fragment resulting from polymerase chain reaction amplification was found to be homologous to the *fp*-1 gene of *Mytilus coruscus*. This fragment was identified as the 3' end of the *Ppfp*-1 gene, which included the heptapeptide coding sequence for Lys-Pro-Ser-Tyr-Pro-Pro-Ter (probably the incomplete last tandem repeat unit of the giant exon), the stop codon and the polyadenylation signal.

**Key words:** *Perna perna*; Byssus; Foot proteins; *fp*-1 gene; Polyphenolic protein

 

## INTRODUCTION

Proteins that compose the byssus in Mytilidae have high biotechnological value due to their unique properties of elasticity, resistance, and adhesion underwater. Currently, four polyphenolic proteins have been identified in Mytilidae. These polyphenolic proteins are secreted and stored by glands present in the foot of the mussels, and they contain some DOPA residues (Waite, 1997). The main component of the byssus is the fp-1 protein (110-130 kDa), which contains 70 to 80 tandem repeats in the last giant exon. These tandem repeats encode for decapeptides rich in hydroxylated tyrosine (DOPA: 3,4 hydroxyphenyl-L-alanine) and proline (Waite, 1983, 1997; Floriolli et al., 2000) residues. Most marine mussels contain the consensus decapeptide Ala-Lys-Pro*-Ser-Tyr*-Pro**-Pro*-Tyr*(DOPA)-Lys, where P* represents 20% of trans-4-hydroxy-L-proline and P** represents 10% of trans-2,3-cis-3,4-dihydroxyproline. DOPA, which is commonly represented twice in each repeated unit, corresponds to the redox functional group 3,4-dihydroxyphenyl-L-alanine. The catechol functionality of this group is thought to be responsible for adhesion and cross-linking of the proteins, in which the mechanisms are unknown (Yu et al., 1999). Therefore, fp-1 protein constitutes an important model for synthetic polymers, especially in the medical material industry (Warner and Waite, 1999). The sequence of the *fp*-1 gene in *Mytilus edulis* is known and has been used for a variety of biotechnological purposes. No studies have investigated these proteins and their respective genes in *Perna perna*, the most abundant Mytilidae of the Brazilian coast. Therefore, the aim of this study was to find the sequence of the *fp*-1 gene (*Ppfp*-1) in *Perna perna*.

## MATERIAL AND METHODS

The following two strategies were used for the search of the main foot protein gene (*fp*-1) in *P. perna*: 1) Southern blotting and hybridization using a fluorescent degenerate consensus probe (5'-ATA(T/A)GTTGGAGGATAA(G/C)TTGGCTT-3') specific for the *Mytilus* group designed from known sequences of the *M. galloprovincialis fp*-1 gene (Inoue and Odo, 1994), and 2) Specific primers previously described for the polymerase chain reaction (PCR) amplification of the *Mytilus* group *fp*-1 gene as well as random primers.

Twenty samples of *P. perna* were collected from the rocky shores of Praia Vermelha (26° 48'S; 48° 35'W), Santa Catarina, in the south of Brazil. DNA was extracted from the adductor muscle tissue following a modified phenol/chloroform extraction protocol (Hoelzel, 1998). At the most, 100 ng of high quality DNA was extracted.

To have sufficient DNA for Southern blotting and hybridization (50 μg), five independent isolations of DNA were carried out with each sample. Next, DNA was cut with *Hin*dIII and *Alu*I restriction enzymes according to manufacturer instructions. Cleaved DNA was subjected to unidirectional capillary Southern blotting using a *Hybond-N*$^{+}$ (Amersham Biosciences) membrane at neutral pH, as described previously (Darling and Brickell, 1994). Hybridization was performed using a fluorescein-labeled probe, and the membrane was treated according to the protocol published by Sambrook and Russel (2001). Non-radioactive detection of hybridization was performed by the Gene Images ECL detection kit (Amersham Biosciences), which utilizes a chromogenic substrate. The membrane was incubated with anti-fluorescein antibodies conjugated to alkaline phosphatase. Two different substrates were used: naphthol AS phosphate/fast blue BB and BCIP/NTB (5-bromo-4-chloro-3-indolyl-phosphate/nitroblue-tetrazolium), according to previously published reports (Anderson, 1999). X-ray film was then exposed to the membrane for 1 h in the dark. Next, the film

 

was developed for 1 min in developing solution, then bathed with 7% acetic acid, and finally subjected to a fixing solution for 2 min. Lastly, the film was assessed for the presence of hybrids.

Specific primers tested in *P. perna* correspond to those described for different species of the *Mytilus* group (Table 1). PCR amplification was performed in a total reaction volume of 25 µL containing: 1X PCR buffer, 3.75 µL BSA (10 mg/mL), 2.0 µL $MgCl_2$ (50 mM), 0.24 µL dNTPs (100 mM), 0.1 µL each primer (100 µM), 0.3 µL Taq polymerase (10 U/µL), and 10-40 ng of DNA template. The reaction was then subjected to an initial 3-min cycle at 95°C, followed by 44 cycles of 1 min at 94°C, 1 min at the minimum annealing temperature for the combination of primers listed in Table 1, and 1 min at 72°C, followed by a final 10-min elongation step at 72°C. All possible combinations of specific primers from Table 1, and all the combinations with the random primers of 10 nt (P1 to P6 from Amersham Pharmacia and OPA1 to OPA5, OPA7 to OPA9, OPA12, and OPA14 from the Operon Life Technologies) were made. PCR conditions were held constant with the exception of the annealing temperature, which was set at 40°C when amplification was performed with random primers. PCR amplicons obtained in high concentration and persisting after increasing the annealing temperature, as well as those present after combinations of specific-random primers but absent when amplification was performed with only random primers, were selected for sequence analysis (Macrogen, Korea) following purification (Ultrafree-DA, Millipore).

**Table 1.** Specific primer sequences of the *fp*-1 gene from various species of the *Mytilus* group.

| Primer name | Sequence 5'-3' | Primer annealing region at the *fp*-1 | Reference |
|---|---|---|---|
| Forward | | | |
| F1 | cctttcggtttatatggtgccggat<br>AT = 66ºC | Non-identified region at the 5' end of the coding region of the *fp*-1 gene of *Mytilus galloprovincialis* | (1) |
| P1 | gcaaagccaacttataaa<br>AT = 43ºC | Consensus hexapeptide coding repeat unit of the giant exon of the *fp*-1 gene in the *M. edulis* | (2) |
| P2 | atcctccaacttataaa<br>AT = 42ºC | Distal portion of the consensus decapeptide coding repeat unit of the giant exon of the *fp*-1 gene in the *M. edulis* | (2) |
| JH5 | gtaggaacaaagcatgaacca<br>AT = 52ºC | Region at the 5' end of the 2.5-kb giant exon of the *fp*-1 gene from *M. edulis* and *M. galloprovincialis* | (3) |
| PR8 | aagccaagttatcctccaac<br>AT = 53ºC | Decapeptide coding repeat unit number 77 of the giant exon of the *fp*-1 gene of *M. edulis* and the corresponding repeat in *M. galloprovincialis* | (3) |
| Me-13 | ccacttgcaaagaagctgtcatct<br>AT = 56°C | Conserved region of the giant exon of the *fp*-1 gene | (4) |
| Me-15 | ccagtatacaaacctgtgaaga<br>AT = 56°C | Conserved region of the giant exon of the *fp*-1 gene | (4) |
| Reverse | | | |
| R1 | atgcttggatccgtatggatgtgaacc<br>AT = 70ºC | Non-identified region at the 3' end of the *fp*-1 gene of *M. galloprovincialis* | (1) |
| JH4 | ctttcaaatgttcatctgttcctc<br>AT = 58ºC | The 3' untranslated end of the *fp*-1 gene from *M. edulis* and *M. galloprovincialis* | (3) |
| JH54 | ggggggataagttttcttagg<br>AT = 56ºC | The 3' end of coding region of the giant exon of the *fp*-1 gene from *M. edulis* and *M. galloprovincialis* | (3) |
| Me-14 | acaaacgttaaaatgtgtagtacagta<br>AT = 56°C | 3' end of the repetitive region of the giant exon of the *fp*-1 gene | (4) |
| Me-16 | tgttgtcttattaggtttgtaaga<br>AT = 56°C | 3' end of the conserved region of the giant exon of the *fp*-1 gene | (4) |

(1) Inoue et al., 1996; (2) Filpula et al., 1990; (3) Rawson et al., 1996; (4) Inoue et al., 1995.
AT = annealing temperature.

## RESULTS AND DISCUSSION

No hybrids were obtained following Southern blotting and hybridization. The transfer of DNA to the membrane was successful, but no hybrids were obtained, suggesting that the probe was not complementary to the region of the *fp*-1 in *P. perna*.

Combinations of the specific primers Me-13/Me-16, Me-13/Me14, Me-15/Me16, and Me-15/Me14 did not amplify any fragments. Other combinations showed fragments, from which 40 were sent for sequencing, although only one showed similarity with the *fp*-1 of *M. coruscus*. This 524-bp fragment (GenBank Accession Number EU563923) was amplified using the forward PR8 primer (Filpula et al., 1990), which was designed from the 77th repetition unit of the giant exon of the *fp*-1 of *M. edulis*, and the reverse primer (R1) described by Inoue et al. (1996) designed from the *fp*-1 gene of *M. galloprovincialis* (Table 1). The amplicon was successfully aligned with the *M. coruscus fp*-1 gene (GenBank Accession Number D63777) (Inoue et al., 1996) at many places due to the similarity of the 5' end of the fragment found in *P. perna* with the *M. coruscus* 4th, 16th, 18th, 21st, 25th, 28th, 34th, 36th, 39th, 43rd, 49th, 53rd, 63rd, and 72nd repetition units. Nonetheless, the presence of a stop codon, followed by a possible polyadenylation site suggests that the fragment corresponds to the last portion of the gene (3' end). The final, but probably incomplete, repetition unit encodes for a heptapeptide repeat unit of the giant exon of the *fp*-1 gene in *P. perna*. The probable polyadenylation signal is consistent with the position of this signal in *M. coruscus* when the alignment begins at the 72nd repetition unit of *M. coruscus* (Figure 1). Although the possible polyadenylation signal has been shown to be an unconventional hexamer (AAUUAA), Beaudoing et al. (2000) demonstrated that polyadenylation signals are not strictly conserved when they observed a high proportion of alternative hexamers.

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P.perna         AAGCCAAGTTATCCTCCAAC-TGTGTGTAGTACA-CCCTTAGGTTTCCCCGT-TGACCCA
M.coruscus      AAGATAAGTTATCCGCCTACATATAAACCGAAAATAACTTACCCTCCTACATATAAACCA
                ***  ********* ** ** * *     * * *   ****   * *  * * * * ***

P.perna         CTGAAACACGGAAATCAAATTTCAATTAGAACGAAATTCCTTAACATACCTGTACATGTA
M.coruscus      AAGAT-CA-GC-TATCCTCCAGCATATA-AGCCAAA--GATTAG-CTATCCATCACAATA
                  **  ** *   ***      **  ** * * ***    *** * ** *  *     **

P.perna         TTAATA-TAATA-CAT-TATATTCTCTCAACAAAATTTTTTAAACTGCAT--GTTT--GA
M.coruscus      TTAAAAGTAATAACATAAATATACACATTACTGCACTTTACATATTTCTTACGTTTTTGT
                **** * ***** ***  **** * * *  *   * ***  * * * * *  ****  *

P.perna         TGWAGTTG--CATTTTTATTTCATGGAAAAC-TGTTGTTGATATGATAGGAAA-GGAATT
M.coruscus      TGATGTGGAACAGCTTAAGAT-GTAAAAGTAATGTTTAACCGGTTA-AGGATTTGTAAT-
                *** ** *  **  ** *  *  *  **    ****       * * ****   * ***

P.perna         TTTCA-TGATTCTGCTCA-G-GTTCATTAAAGG----CA----TT-TTATAATTAATCGA
M.coruscus      ATTCAATCTTTATGTTTGTGAATTGGTTAT-GTTCTTCAAGTATTGTTTAAAATAAAC-A
                **** *  ** ** *   *  **  ***  *     **    ** **  ** *** * **

P.perna         TTTTTTTTCTCCCTAATTTGGTGGCCTGTACTAAGATAAGATGTAGGTATTCAGAACAGG
M.coruscus      TTTGTTCTCTT
                *** ** ***

P.perna         GAACATCTTATTCCAGTACTAGGATAATGTATTTTACAACATTGTTCATTTTTCTATATA
M.coruscus

P.perna         GACACACAGTAAAAAGGTATACGTGTAGCCATGTATTAATCTTAAATATTATACATACCT
M.coruscus

P.perna         GGTATACAACATGAATAGGGGGTCCTCTGCATCCATCCACTCAGGTTCACATCCATACGG
M.coruscus

P.perna         ATCCAAGCAT
M.coruscus
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**Figure 1.** Alignment of the *Perna perna* sequence obtained with the terminal portion of the *fp*-1 gene of *Mytilus coruscus*. The last repetition unit found in *P. perna* (bold), the stop codon (bold and double underlined), and the most probable polyadenylation signal in *P. perna*, consistent with the position of this signal in *M. coruscus* (bold and single underlined) are shown.

The translated portion (KP*SY*P*P*T) at the 5' end of the amplicon fragment in *P. perna* (Figure 2) is, in fact, part of the consensus repeat unit of most Mytilidae, where P* corresponds to trans-4-hydroxy-L-proline, P** represents trans-2,3-cis-3,4-dihydroxyproline, and Y* (DOPA) represents the redox functional group 3,4-dihydroxyphenyl-L-alanine (Waite, 1983, 1997), which are all hydroxylated residues.


**Figure 2.** Full sequence found in *Perna perna*, showing the last incomplete repeat unit (bold) of the *Ppfp-1* last giant exon, the translated region in the top, the heptapeptide KPSYPPT (bold and underlined), where asterisks represent residues that are normally hydroxylated in the foot proteins, the stop codon (bold and double underlined), and the polyadenylation signal (bold and underlined).

No similarity was found with the repeat units of *P. viridis* (Ohkawa et al., 2004) and *P. canaliculus* (Zhao and Waite, 2005), which demonstrated significant differences with the *Mytilus* group and also between each other. Our results show that *P. perna* is more similar to the repetition units of the *Mytilus* group than to its congenerics. Although the last repeat unit found in *P. perna* may be an incomplete unit, we cannot rule out the possibility of the heptapeptide rule in *P. perna*. Other species have shown smaller repetition units than the characteristic decapeptide observed in the *Mytilus* group. Examples include the heptapeptide in *Aulacomya ater* (Burzio et al., 2000) and the tetrapeptide in *P. canaliculus* (Zhao and Waite, 2005).

After checking similarities in GenBank, we observed that part of the fragment found is very similar to a small region of the acetyl-CoA gene of many species. Nonetheless, this similarity was observed in the anti-sense strand, and our fragment is divided into two by another sequence in the acetyl-CoA gene, suggesting that there may be only an evolutionary relationship between the two genes.

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