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Short communication

Molecular characterization and protein localization of the antimicrobial peptide big defensin from the scallop *Argopecten purpuratus* after *Vibrio splendidus* challenge

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ABSTRACT

Big defensins are antimicrobial peptides (AMPs) that are proposed as important effectors of the immune response in mollusks, chelicerates and chordates. At present, only two members of the big defensin family have been identified in scallop. In the present work, a cDNA sequence encoding a new big defensin homologue was characterized from the scallop *Argopecten purpuratus*, namely *ApBD1*. *ApBD1* cDNA sequence comprised 585 nucleotides, with an open reading frame of 375 bp and 5'- and 3′-UTRs of 41 and 167 bp, respectively. The deduced protein sequence contains 124 amino acids with a molecular weight of 13.5 kDa, showing characteristic motifs of the big defensin family and presenting 76% identity with the big defensin from the scallop *A. irradians*. Phylogenetic analysis revealed that ApBD1 is included into the cluster of big defensins from mollusks. Tissue-specific transcript expression analysis by RT-qPCR showed that *ApBD1* was present in all tissues tested from non-immune challenged scallops but it was most strongly expressed in the mantle. The transcript levels of *ApBD1* were significantly up-regulated in gills at 24 and 48 h post-injection with the heat-attenuated bacteria *Vibrio splendidus*. Additionally, immunofluorescence analysis using a polyclonal anti-ApBD1 antibody showed that this protein was abundantly located in epithelial linings of gills and mantle; and also in digestive gland showing ApBD1-infiltrating hemocytes from immune challenged scallops. This is the first time that a big defensin is detected and located at the protein level in a mollusk. These results suggest an important role of ApBD1 in the mucosal immune response of *A. purpuratus*.

1. Introduction

Antimicrobial peptides (AMPs) are key effectors of humoral innate immunity present in all organisms, and are represented by highly diverse families [1]. AMPs generally are composed by 12–100 residues, are positively charged and display an amphiphilic structure [2]. These characteristics allow a specific interaction of AMPs with microbial membranes, whereby they can destroy the microorganism, either by causing a change in the membrane permeability, forming pores in the membrane with the subsequent leakage of cellular content, or by entering into the microorganism and killing it by several non-lytic mechanisms [3].

Within AMPs, the big defensin family is found in mollusks, chelicerates and chordates [4]. This family comprises large peptides (of ~9–13 kD; i.e., twice the size of classic defensins) composed of an N-terminal hydrophobic region and a C-terminal cationic region, containing 6 conserved cysteine residues bound by three internal disulfide bridges [5]. Big defensins have a broad spectrum of microbicidal activity against Gram negative and Gram positive bacteria, and fungi [6]. Big defensins could play a crucial role in the

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immune response of mollusks, as have been proposed for the oyster *Crassostrea gigas* [5,7], the clam *Venerupis phillippinarum* [8], the mussel *Mytilus galloprovinciallis* [9] and the scallops *Argopecten irradians* [6] and *Chlamys nobilis* [10], in which cDNA sequence encoding big defensin homologues have been described. Still, these studies in mollusks have characterized the expression of this AMP only at the transcript level.

The majority of studies on the immune response of invertebrates are based on hemocyte response to infection, although the specific response driven by the circulating hemocytes and effector molecules in plasma does not reflect the systemic mechanisms in response to pathogen invasion [11]. Indeed, the first pathogen - host interaction occurs in the epithelia [11], thus it is very important to know how immune molecules such as big defensins participate in the defense mechanisms in this kind of tissue at protein level [11,12].

The scallop *Argopecten purpuratus* is a species of great commercial interest, being placed among the most produced native species in Latin America (FAO, 2014). However, scallop production has gradually declined in the last decade due to episodes of massive mortalities whose causes are unknown. The mortality of *A. purpuratus* larvae has been associated with the presence of the Gram-negative bacteria *Vibrio splendidus* [13]. Despite its commercial importance and the potential impact of pathogens in disease outbreaks, the knowledge of the immune response of *A. purpuratus* is limited [14–17]. Indeed, no AMPs have been characterized in this species until now. The characterization of AMPs and their systemic role on immune defense in *A. purpuratus* is fundamental to understand its mechanisms of defense, and to generate management strategies that contribute to improve their resistance to potential pathogens. For this reason, in this study we aimed to identify and characterize a big defensin AMP from *A. purpuratus* and to determine the expression of *ApBD1*, at both the transcript and protein level, in scallop tissues after an immune challenge with *V. splendidus*.

2. Material and methods

2.1. Animals, bacterial challenge and tissue collection

Animal care was carried out in strict accordance with the recommendations in the CCAC guidelines (http://www.ccac.ca/Documents/Standards/Guidelines). Adult *Argopecten purpuratus* scallops (70–80 mm shell height; n = 200) were transferred from Tongoy bay to the laboratory at the Universidad Católica del Norte, Coquimbo, Chile (30°16′ S, 71° 35′W); and acclimatized for 1 week in 1000 L tanks supplied with filtered, aerated, running seawater (~16 °C), and fed with a diet of 50% *Isochrysis galbana* and 50% *Nannochloris* sp (6×10^6 cells/mL/day). Samples from muscle, gill, mantle, digestive gland, gonad and hemocyte tissues were extracted from 5 non immune-challenged scallops. Hemolymph was collected from the pericardial cavity and hemocytes were isolated by centrifugation to discard plasma (600 × *g* for 5 min at 4 °C); and all tissues were kept in RNA*later* Stabilization Reagent (Ambion Inc., Austin, Texas, USA) at −80 °C until total RNA extraction.

For the experimental immune challenge, 100 μL of a mix of 3 strains (VPAP16, VPAP18 and VPAP23) of heat-attenuated virulent *V. splendidus* in sterile sea water (1×10^6 cells/scallop) [13] or 100 μL of sterile sea water (SSW), as injury control, were injected in the scallop adductor muscle. Hemolymph from scallops was collected from the pericardial cavity at 6, 12, 24, 48 and 72 h post-injection. Hemocytes were isolated as described above. Samples from gill and mantle tissues were harvested by dissection and kept in RNA*later* at −80 °C until total RNA extraction. Four groups of four scallops were considered in each condition and no mortality was observed during the experimental challenge.

2.2. Total RNA extraction, reverse transcription and molecular cloning of ApBD1 cDNA

Total RNA from each tissue was extracted and treated with RNase-free DNase with an AxyPrep Multisource total RNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA). RNA from hemocytes was extracted and treated with RNase-free DNase with the SV Total RNA Isolation System (Promega, Madison, WI, USA) according to the manufacturer's protocol. Quantification and quality of total RNA were determined with an Epoch spectrophotometer (BioTek, Winooski, VT, USA) and formaldehyde/agarose gel electrophoresis, respectively. Reverse transcription (RT) of RNA was carried out with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Santa Clara, CA, USA) according to the manufacturer's protocol.

In order to isolate a big defensin homologue from *A. purpuratus*, a pair of primers was designed (Table 1) from the big defensin gene from *A. irradians* (GenBank Acc. No. FJ824733). A 353-bp PCR product from hemocyte cDNA was amplified, purified and sequenced. In order to obtain the 5′- and 3′-untranslated regions (UTRs), specific primers were designed (Table 1) and 5′- and 3′-RACE (rapid amplification of cDNA ends)-Ready cDNAs were obtained with a SMARTer™ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Thermal cycling parameters were set following manufacturer's instructions. The amplification products were visualized on agarose gels, purified, and ligated into pGEM-T Easy vector (Promega, Madison, WI, USA). Subsequently, competent cells of *E. coli* JM-109 (Promega, Madison, WI, USA) were transformed with the plasmid by heat shock and then cultured in agar plates LB/Amp/IPTG/X-gal overnight at 37 °C. Plasmids containing the corresponding insert were purified with an UltraClean®15 DNA Purification Kit (Mo Bio Laboratories, Carlsbad, CA, USA). The resulting cDNAs were sequenced and aligned at the overlapping regions to produce a full-length cDNA sequence.

2.3. Sequence analyses

The search of nucleotide sequences was performed using the BLAST algorithm at the National Center for Biotechnology Information. Nucleotide sequences were translated to predicted protein sequences using the *Expert Protein Analysis System* at the Expasy web server (http://www.expasy.org/). Signal peptide prediction was performed by SignalP [18] and the domain analysis was performed with SMART (Simple Modular Architecture Research Tool) [19]. Homology modeling of the protein was performed using Phyre^2 server [20]. The multiple alignments were generated using the Clustal Omega program (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic analysis was performed using MEGA v6.0 (Molecular Evolutionary Genetic Analisis) [21], using the Neighbor-Joining method [22], with bootstrap values calculated from 1000 pseudoreplicates.

2.4. Real-time quantitative PCR (RT-qPCR) analysis of gene transcription

Primers for RT-qPCR reaction were designed (Table 1). The amplified region included a partial 3′-UTR sequence in order to amplify specifically the gene under study. *β-actin* was used as endogenous control in order to normalize experimental results. *β-actin* was previously validated as housekeeping gene for *A. purpuratus* [17,23]. RT-qPCR assays were performed in triplicate on an Eco™ Real-Time PCR System (Illumina, San Diego, CA, USA) using Maxima® SYBR Green/ROX qPCR Master Mix (Thermo Scientific, Rockford, IL, USA). By serial dilution of cDNA, RT-qPCR

Table 1
Primers used for the molecular characterization and recombinant production of a big defensin from the scallop *Argopecten purpuratus* (ApBD1).

Primer name	Forward primer (5′-3′)	Reverse primer (5′-3′)
PCR		
ApBD1	CTCGTCCCTCCCTAGTAAGATG	GCACTTGTAACTTCCACAAACG
RACE		
rApBD1	GGAGCGTCGTAGCACGACATGTTTTCA	CTCTCGTATGAGCGGCATGCACTTCG
RT-qPCR		
qApBD1	TGCCGTGTTCCAGATGA	TCGTACAAGATTTGAGAAAACGAAA
qActin	CACTGCTCTTGCTCCACAAAC	GAAGGTGGACAGAGATGCCAA
Recombinant		
recApBD1	TACATATGGCCCTACCCCTTGCTTATG	GCTAGCTCATCATCGTGGAACACA

efficiency was set to be between 95 and 110%. Relative expression was calculated using the standard curve-based method [24]. Statistical differences among *ApBD1* transcriptional levels were estimated with one-way analysis of variance (ANOVA) to compare tissues; and two-way ANOVA to compare among injection treatment and time post-injection. ANOVAs were followed by Tukey test to establish the difference between specific conditions.

2.5. Recombinant expression of ApBD1, anti-ApBD1 polyclonal antibody production, ELISA and Western Blotting

The coding sequence for the mature peptide of ApBD1 was cloned in-frame with the N-terminal His6 in the *Nhe*I/*Nde*I sites of the pET-28a expression vector (Novagen, Madison, WS, USA). Directed mutation at the VI cysteine residue of ApBD1 was introduced by PCR as described previously [25], in order to maximize its antigenic properties. The recombinant ApBD1 protein was expressed in *E. coli* Rosetta (DE3) and produced as previously described for other antimicrobial peptides [25]. The molecular mass of the purified peptide was determined by SDS-PAGE.

Anti-ApBD1 polyclonal antibody was generated against the recombinant ApBD1 mature peptide in 4-weeks-old CF-1 mice. For antibody production, animals were subcutaneously injected at 1, 14 and 21 days with 30 μg of the recombinant peptide in 1:1 Freund's adjuvant (Thermo Scientific, Waltham, MA, USA). The antiserum was collected on day 28, centrifuged at 700 g for 10 min and the supernatant was stored at −20 °C. Antibody efficiency was determined by indirect ELISA and antibody specificity was determined by Western blot as described before [26] (Fig. S1).

2.6. Histological analysis and immunofluorescence

Scallop tissues mantle, gill and digestive gland were dissected under sterile conditions and fixed in Bouin's solution (0.9% picric acid, 9% formaldehyde, 5% acetic acid). Fixed samples were dehydrated through an ascending ethanol series, embedded in Histosec (Merck, Darmstadt, Germany), and mounted on glass slides. Paraffin sections (5 μm) were cleared in Neoclear (Merck, Darmstadt, Germany) and hydrated in a descending ethanol series.

Immunofluorescence analysis was carried out as described previously [27]. Briefly, paraffin sections were incubated with 50 mM NH_4Cl for the quenching of the autofluorescence, incubated overnight at 4 °C with anti-ApBD1 (1:100) in 1% BSA and then incubated for 1 h with Goat anti-Mouse Alexa Fluor 568-conjugate (Thermo Scientific, Waltham, MA, USA) (1:200) in 1% BSA. To-Pro Iodide® (Thermo Scientific, Waltham, MA, USA) (1:1000) was used for nuclear staining. Control slides were incubated with the mouse prebleed serum. Slides were analyzed using a Leica TCS SP5 II spectral confocal microscope (Leica Microsystems, Wetzlar, Germany).

3. Results and discussion

3.1. Identification and characterization of ApBD1

The complete cDNA sequence obtained from *Argopecten purpuratus* hemocytes was named *ApBD1* (GenBank Acc. No. KU499992). The *ApBD1* cDNA displays a length of 585 bp, with a 5′-UTR of 41 bp and a 3′-UTR of 167 bp, with a canonical polyadenylation signal sequence (AATAAA) and a polyA tail at 3′-UTR. The open reading frame (ORF) was composed of 375 bp coding for a protein of 124 amino acids of 13.5 kD. The predicted protein comprises a 28-residue signal peptide at the amino-terminal; followed by a putative propeptide region of 10 amino acid residues; and a putative mature peptide of 86 amino acid residues at the carboxy-terminal (Fig. S2). The predicted molecular weight of the mature peptide was 9.18 kDa, with a theoretical isoelectric point of 9.81. The predicted protein displays the motifs and structural characteristics similar to other big defensins such as a helical structure containing disulfide bridges, positive net charge (+13) and a high proportion of hydrophobic residues (36%) [5]. The predicted transmembrane region (composed of 23 amino acid residues) starts at amino acid position 7 and ends at amino acid position 29 of the predicted protein sequence.

Multiple alignment of amino acid sequences revealed a high identity of ApBD1 with other sequences of big defensins from mollusks and arthropods (Fig. S2). The C-terminal region of ApBD1 exhibits six conserved cysteine residues forming the consensus pattern C-X6-C-X3-C-X14-C-X4-C-C that is characteristic of the family [6]. ApBD1 amino acid sequence displays the highest amino acid identity (76%) with *A. irradians* big defensin (GenBank Acc. No. FJ824733). Analyses also indicated that this peptide exhibits the two characteristic domains of the group as the N-terminal hydrophobic domain, and the C-terminal cationic domain, which has been linked to the bactericidal activity of big defensins [5,28]. Thus, these characteristics strongly suggest that ApBD1 is a new member of the family of the big defensins.

The deduced amino acid sequence of ApBD1 was analyzed to obtain a three-dimensional model of its protein tertiary structure. Based on the tertiary structure of the big defensin from the horseshoe crab *Tachypleus tridentatus* (PDB: 2RNG), with which ApBD1 has the closest structural similarity, 90% of the ApBD1 residues were modeled with 100% confidence. The analysis showed that ApBD1 presents 6 predicted β-sheets and 3 predicted α-helices. In ApBD1, the 6 conserved cysteine residues are joined by three disulfide bonds whose predicted connectivity is $^{89}C_1{-}C_5^{120}$, $^{96}C_2{-}C_4^{115}$, and $^{100}C_3{-}C_6^{121}$ (Fig. S3).

Phylogenetic analysis was performed based on the amino acid sequences of different classes of defensins, including α-defensins, β-defensins, CSαβ-defensins and big defensins. Resulted analysis revealed that ApBD1 is included into the clade of big defensins from scallops *Chlamys nobilis* and *Argopecten irradians* (bootstrap values

99 for both). This clade is included into the clade of oyster *Crassostrea gigas* big defensin isoforms; and big defensin of the arthropod *Tachypleus tridentatus*. Big defensins clustered in a separate clade from other defensin families. This clade of big defensins was more related to the β-defensins than to α-defensins or CSαβ defensins (Fig. S4).

3.2. Tissue-specific expression of ApBD1

The relative transcription of *ApBD1* was evaluated in different tissues from unstimulated adult scallops to determine constitutive expression of this gene. *ApBD1* mRNA transcripts were constitutively expressed in all tissues examined including mantle, muscle, gills, digestive gland and gonad (Fig. 1). *ApBD1* transcript expression was significantly ($P < 0.05$) higher in mantle (about 30–45-fold) than in most analyzed tissues. *ApBD1* transcript expression was low in gills, gonads and digestive gland, and almost undetectable in hemocytes; and no significant differences were detected among them. This result contrasts with the results obtained from the oyster *Crassostrea gigas* and the scallop *Argopecten irradians,* where big defensin expression was restricted to hemocytes [5,6]. However, our results agree with the pattern of basal transcription described for the big defensins from the bivalves *Mytilus galloprovinciallis, Chlamys nobilis* and *Hyriopsis cumingii* [9,10,29]. In these species, as observed for *ApBD1*, the expression of big defensins was higher in mantle than in the other analyzed tissues. The mantle is one of the most exposed tissues in bivalves, and a high level of expression of this AMP could indicate an important role of this molecule in localized protection against pathogens [11].

3.3. ApBD1 expression after V. splendidus challenge

To determine whether *ApDB1* expression was induced after bacterial challenge, relative transcript levels of *ApBD1* were assessed in hemocytes, gills and mantle tissues of *A. purpuratus* over a period of 72 h post-immune challenge with *V. splendidus* (Fig. 2). The levels of *ApBD1* transcripts in hemocytes from control and immune challenged scallops were very low and no significant differences were found among them (data not shown). However, *ApBD1* was found to be significantly ($P < 0.05$) up-regulated in gills after 24 h and 48 h of *V. splendidus* challenge (Fig. 2). At these time points, *ApBD1* mRNA level showed an overexpression of 7- and 5-fold change, respectively, compared to the gills from corresponding control scallops injected with sterile sea water. The relative level of *ApBD1* mRNA in mantle gradually increased after 2 h of exposure to the pathogen up to 24 h post-injection; however, no significant differences were observed compared to corresponding control scallops injected with sterile sea water (Fig. 2). The highest expression of *ApBD1* at constitutive levels in mantle may explain this result, and emphasized the role of this tissue as first line of defense. Therefore, a potential role of ApBD1 in the epithelial immune response is suggested, as it has been described for other molluscan big defensin [29]; and also to local tissue damage, as it has been described for other vertebrate AMPs [30,31]. Further research considering different types of immune stimulation will be necessary to determine the potential functions of this peptide.

3.4. Tissue localization of ApBD1 by immunofluorescence

Since increased gene expression of *ApBD1* was observed in gills from immune challenged scallops and mantle was the tissue showing the highest constitutive expression, further investigation was performed to characterize ApBD1 protein localization in these and other epithelial tissues. For this, an anti-ApBD1 polyclonal antibody was raised in mice and immunodetection was performed in mantle, gills and digestive gland from scallops 24 h after injection with *V. splendidus*. ApBD1 protein was detected in mantle, gills and digestive gland tissues of challenged scallops, as seen by immunofluorescence analysis (Fig. 3). Furthermore, hemocytes infiltrating the digestive gland of challenged scallops were positively labelled. Control scallops did not show any positive staining (data not shown).

The observed tissue localization of ApBD1 protein coating several epithelial tissues of *A. purpuratus* after the immune challenge supports the hypothesis that this peptide could play a role in the mucosal immune response. Because the presence of the signal

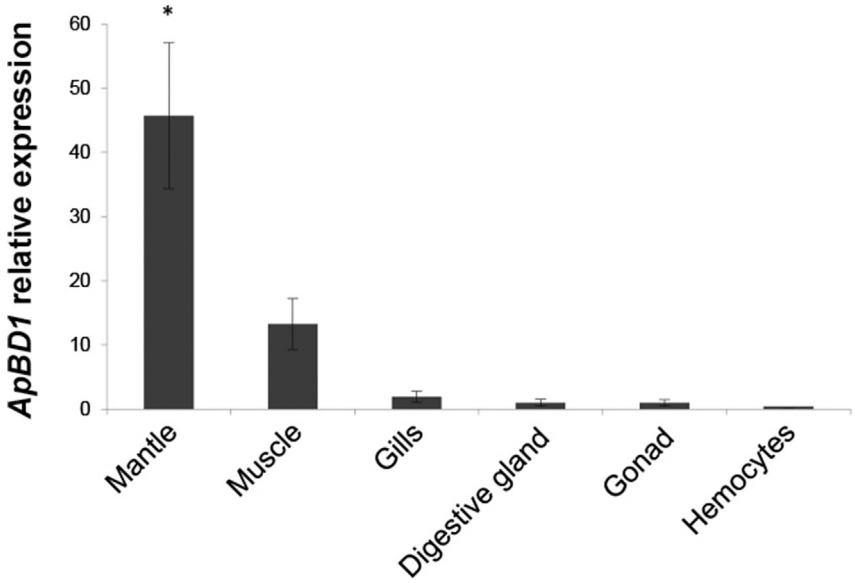

Fig. 1. Relative mRNA basal levels of *ApBD1* in different tissues from *Argopecten purpuratus*. Relative expression levels of *ApBD1* were obtained by RT-qPCR according to the standard curve-based method. *β-actin* was used as housekeeping gene. Gills, mantle, muscle, digestive gland and gonad were collected from 5 individual scallops. Results represent the mean value ± standard error (SE). The asterisk indicates significant difference compared to the other tissues (*$P < 0.05$).

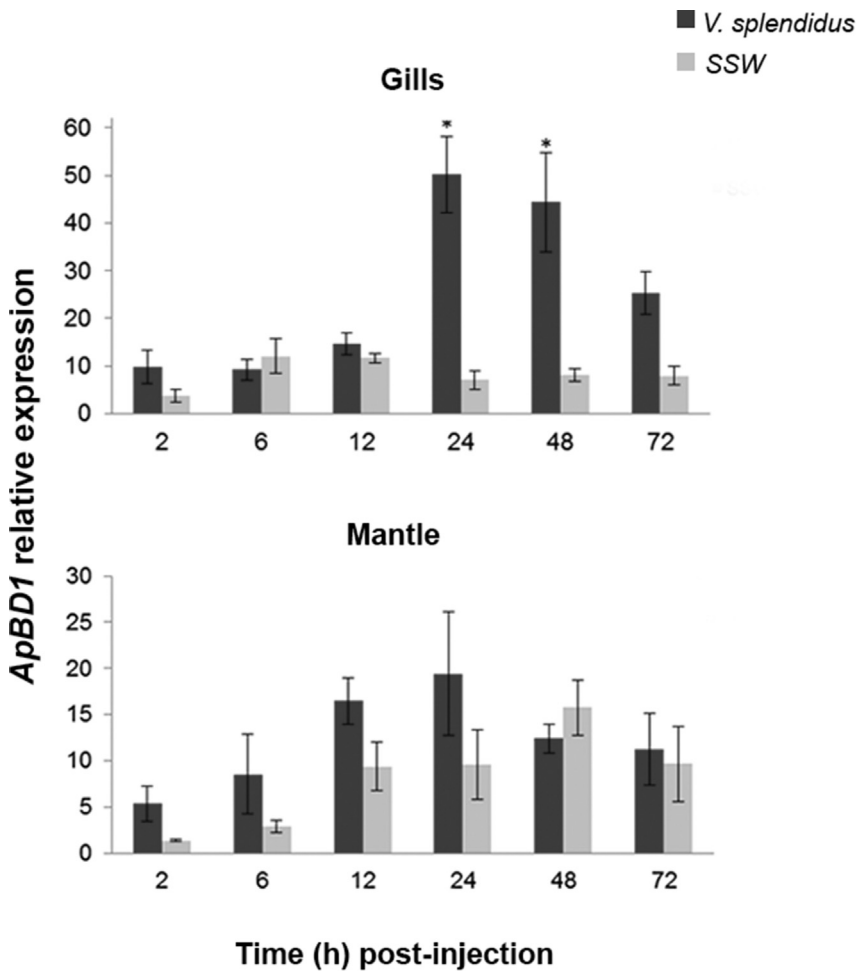

Fig. 2. Temporal changes in mRNA levels of *ApBD1* in gills and mantle from *Argopecten purpuratus* after an immune challenge. Relative expression levels of *ApBD1* were obtained by RT-qPCR according to the standard curve-based method. *β-actin* was used as internal control gene. Gills and mantle tissues were evaluated at different time point after the injection with *Vibrio splendidus* (dark grey bars) or with sterile seawater (SSW, grey bars). Results represent the mean value ± standard error (SE). n = 4 pools of 4 individuals each. Asterisks indicate significant differences from the respective control group (*$P < 0.05$).

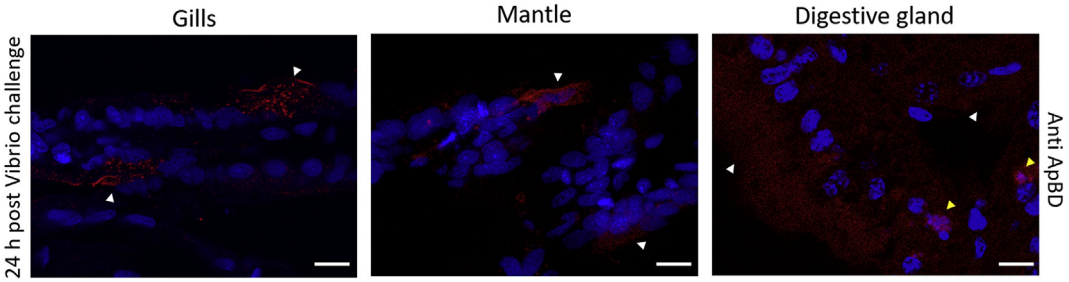

Fig. 3. Detection of ApBD1 protein in tissues from immune challenged *A. purpuratus* by immunofluorescence. Confocal analysis on gills, mantle and digestive gland tissues from immune challenged scallops with *Vibrio splendidus*. Alexa 568 goat anti-mouse antibody was used for ApBD1 detection (in red) and To Pro iodide® was used as the nucleic acid stain (in blue). Immunostained positive tissues (white arrow heads) and infiltrating hemocytes (yellow arrow heads) are indicated. Magnification 400X, scale bar, 25 μm.

peptide, it is expected to observe the extracellular localization of ApBD1 following immunostimulation, as reported for many AMPs [6,32]. Interestingly, transcript expression results from hemocytes disagree with results observed by immunofluorescence, where infiltrating hemocytes positive for ApBD1 were detected in the digestive gland. It has been postulated that hemocytes seem to serve as vehicles for AMPs like Cg-Defhs [33], thus is possible that circulating hemocytes may differ in their *ApBD1* transcriptional activity from infiltrating hemocytes. Also, considering the recognized diversity of this family of AMPs [5], we could alternatively hypothesize the existence of other isoforms of big defensin in *A. purpuratus* which could be detected by gene expression studies and/or by the antibody. In this work, *ApBD1* primers were designed to amplify a region including a partial 3′-UTR sequence, in order to amplify specifically the homologue under study. Indeed, the transcript overexpression of a big defensin from *A. purpuratus* was recently reported in hemocytes using different primers [15], suggesting the existence of several isoforms of big defensin in this

species. The identification and characterization of other isoforms are required to assess the possible differential of expression between them, as observed among big defensins from *C. gigas* [7]. Furthermore, it has been described that some molluscan AMPs are expressed by specific tissues besides hemocytes in different species, such as the mantle defensin from *C. gigas* [34]. The use of techniques such as *in situ* hybridization to localize the *ApBD1* mRNA in tissues and cells from *A. purpuratus* will help to obtain information about specific tissues expressing this AMP.

4. Conclusions

The filtering nature of marine bivalves keeps them constantly exposed to an enormous amount and diversity of microorganisms [35]. Gills, mantle and digestive gland tissues are considered as important mucosal interfaces that act as a powerful barrier against infectious agents. As a result, many of the infections in mollusks start at organs exposed to the external environment, such as the pallial organs in bivalves [11]. The detection of ApBD1 at the protein level in these epithelial tissues illustrates the specific phenotype of immune response, since the correlation between gene transcription and protein expression is not always strong. Regulatory processes such as post-transcriptional, translational and protein degradation processes can determine the abundance and stable state of proteins [12]. To date, gene transcription of big defensins from bivalve mollusks has been evaluated in several species [6,8,10]. However, this is the first time that a big defensin has been detected and located at the protein level in mollusks.

The main importance of characterizing the immune mechanisms at the epithelial level is that many of the infectious processes are neutralized by the immune response at these sites [11]. Therefore, the efforts on the exploration for pathogen resistance by mollusks should be oriented towards the study of the epithelial immune response at the protein level. This will help to understand the factors that affect the resistance of the animal and the true specific response to the infection.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.fsi.2017.07.010.

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