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# Further characterization and mode of action of dactylomelin-P, an antibacterial protein isolated from the ink of the sea hare *Aplysia dactylomela* (Rang, 1828)

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ABSTRACT

Sea hares are well known, nearly shell-less, marine opisthobranchs that use a complex repertoire of chemicals for defense and communication instead of a conventional gastropod shell. The most conspicuous characteristic of these invertebrates is the secretion of ink, which is rich in bioactive proteins. Many of these proteins belong to a family of L-amino acid oxidases (L-AAOs). In the current study, we aimed to determine whether dactylomelin-P, an antibacterial protein isolated from the ink of *Aplysia dactylomela*, could act as an L-AAO. We also investigated its biochemical properties and antibacterial mechanism of action. We found that dactylomelin-P is an acidic protein (pI = 5.0), rich in glutamic acid/glutamine, aspartic acid/asparagine, tyrosine, serine, and proline. It was stable under a broad pH range (3.0–12.0), after heating to 55 °C for 30 min, and after treating with protease. Its N-terminal amino acid sequence was DGVCSNRRQCNKEVCGSSYDVAIVGA and showed high similarity to other sea hare proteins previously identified as L-AAOs. The L-AAO activity was confirmed in an enzymatic assay, which showed that dactylomelin-P could oxidize L-lysine and L-arginine. We also demonstrated that the bacteriostatic activity of dactylomelin-P was mediated by hydrogen peroxide generated in the enzymatic reaction, but it acted as a bactericide in the presence of L-lysine and L-arginine. Transmission electron microscopy analyses showed that dactylomelin-P bound to growth-phase bacteria without causing morphological alterations to the cells. The bactericidal effect seems to involve $H_2O_2$ and other reactive components since it was not counteracted by $H_2O_2$ scavengers. Our findings showed biochemical, functional, and phylogenetic similarities among L-AAOs isolated from sea hares; this offers new insight into the evolution of these proteins and their roles in chemical defense.



## 1. Introduction

There is a long history of success in exploring bioactive molecules from the sea. In this scenario, sea hares have emerged as a rich source of secondary metabolites and proteins of value and interest for pharmaceutical and ecological fields.

Sea hares are nearly shell-less marine opisthobranchs as they do not possess an external protective shell, but only a small, degenerated, and internal one (Derby, 2007; Kamiya et al., 2006), and use a complex repertoire of chemicals for defense and communication (Kicklighter et al., 2005). Species of the genera *Aplysia* and *Dolabella* are well known sources of bioactive proteins with antitumoral, cytotoxic, antiviral, antibacterial, antifungal, and hemagglutinating properties (Butzke et al., 2004; Iijima et al., 1995; Kamiya et al., 1984, 1986, 1989; Kamiya and Shimizu, 1981; Kisugi et al., 1987, 1989, 1992; Melo et al., 1998, 2000; Petzelt et al., 2002; Rajaganapathi et al., 2002; Takamatsu et al., 1995; Yamazaki et al., 1989a,b,c; Yang et al., 2005). Although most of the known bioactive proteins from sea hares have previously been chemically characterized, their primary interactions with target cells are not fully understood. Recent works, however, have shown that some of these proteins act as L-amino acid oxidases. L-AAOs (EC 1.4.3.2) are flavoenzymes, which catalyze the stereo specific deamination of L-amino acids in the presence of oxygen to produce keto-acids, ammonia, and hydrogen peroxide.

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These products are thought to be responsible for the cytotoxic and antibacterial activities of these proteins (Derby, 2007).

Ko et al. (2008) worked with escapin, an L-AAO ink protein from the sea hare *Aplysia californica* (Cooper, 1863), and demonstrated that $H_2O_2$ was the product responsible for most of its bacteriostatic effects. Furthermore, its bactericidal effect was shown to be primarily influenced by the interaction between $H_2O_2$ and α-keto-ε-aminocaproic acid, an intermediate product of L-amino acid oxidation (Kamio et al., 2009). However, a reaction between the initial products of L-amino acid oxidation was shown to lead to the generation of unstable intermediates with more potent bactericidal properties.

Other bioactive proteins isolated from sea hares were also identified as L-AAOs; these included dolabellanin-A (Iijima et al., 2003) and aplysianin-A (Jimbo et al., 2003), found in the albumen gland of *Dolabella auricularia* (Lightfoot, 1786) and *Aplysia kurodai* (Baba, 1937), respectively; and the *Aplysia punctata* (Cuvier, 1803) ink toxin (APIT; Butzke et al., 2005).

Dactylomelin-P, found in the ink of *A. dactylomela* (Rang, 1828) (Melo et al., 2000), is a potent antibacterial protein that shares several features with other ink proteins recognized as LAAOs. Therefore, this study aimed to determine whether dactylomelin-P also acted as an L-AAO and provide a more detailed description of its biochemical properties and antibacterial activity.

## 2. Materials and methods

### 2.1. Dactylomelin-P purification

Dactylomelin-P was purified from sea hare ink with ion exchange and hydrophobic interaction chromatography, according to Melo et al. (2000).

### 2.2. L-AAO activity

L-AAO activity was measured in 0.1 M Tris/phosphate buffer, pH 7.8 at 25 °C in an enzyme-coupled assay (Macheroux et al., 2001). In this assay, $H_2O_2$ generated by the L-AAO enzyme was used by horseradish peroxidase to oxidize *o*-dianisidine to a radical cation, which could be monitored by absorbance at 450 nm. The reaction mixture (total 1 mL) contained 10 µl horseradish peroxidase (1 mg $mL^{-1}$) (horseradish peroxidase type I, Sigma, USA), 50 µl of an *o*-dianisidine solution (8 mM, 20% Triton X-100), and 10 µl of a 100 mM amino acid solution. The reaction was initiated with the addition of 10 µl of L-AAO-solution (1 mg $mL^{-1}$) and it was monitored every 5 min over the 60 min assay. The amino acids L-lysine (L-lys), L-arginine (L-arg), L-histidine (L-his), L-aspartate (L-asp), and L-glutamate (L-glu) were tested as substrates of the reaction. The Michaelis–Menten constant ($K_m$) was determined with the Lineweaver–Burk plot. Each data point represents the mean ± SD of three replicates.

### 2.3. Effects of L-lys, L-arg, and peroxidase on dactylomelin-P activity

We evaluated whether supplementation of the medium with L-lys, L-arg, or peroxidase had an effect on the antibacterial activity of dactylomelin-P. *Staphylococcus aureus* (ATCC 25923) cells were grown to log phase (t = 10 h) in LB broth when 100 µg $mL^{-1}$ of dactylomelin-P or 100 µg $mL^{-1}$ of dactylomelin-P plus 100 µg $mL^{-1}$ peroxidase or 100 µg $mL^{-1}$ of dactylomelin-P plus 100 mM L-arg or 100 mM L-lys were added to the cultures. Bacterial cells grown in LB medium without chemical were used as control. We also tested different concentrations of L-arg and L-lys (50, 25, 12.5, 6.25, and 3.125 mM) to determine the effects of concentration on dactylomelin-P activity and evaluated the result of the concomitant addition of dactylomelin-P, L-arg, or L-lys plus peroxidase on the bacterial growth phase in LB medium. The antibacterial activity was evaluated by measuring turbidity and counting viable cells of three replicates of each treatment. The results were subjected to analysis of variance and the differences among means of three experiments were determined with Bonferroni's test on BioStat software (AnalystSoft Inc., USA). Values of $p<0.05$ were considered significant.

### 2.4. Further biochemical characterization of dactylomelin-P

#### 2.4.1. Isoelectric point

Analytical isoelectric focusing was performed on precast gels in the pH range of 4.0–7.0 (Immobiline IPG strips, GE Healthcare, UK). This was followed by two-dimensional (2D) electrophoresis, based on Görg et al. (2000). Individual 2D gels were then analyzed with ImageMaster 2D Platinum Software (GE Healthcare, UK) in order to determine the isoelectric point of dactylomelin-P.

#### 2.4.2. Amino acid composition

Lyophilized dactylomelin-P (1 mg) was hydrolyzed with 6 M HCl plus 10 g $L^{-1}$ phenol (w/v) in sealed glass tubes under a $N_2$ atmosphere at 110 °C for 22 h. After removal of HCl/phenol by evaporation, the amino acid contents were determined by chromatography on a Biochrom 20 system (GE Healthcare, UK).

#### 2.4.3. N-terminal amino acid sequence

The N-terminal amino acid sequence of dactylomelin-P was determined on a Shimadzu PPSQ-10 Automated Protein Sequencer (Shimadzu, Japan), which used Edman degradation (Edman and Begg, 1967). The sequence was determined from dactylomelin-P that had been isolated by SDS-PAGE and blotted on a polyvinylidene fluoride membrane. Phenylthiohydantoin amino acids were separated on a reversed phase C18 column (4.6 × 2.5 $mm^2$) under isocratic conditions, according to the manufacturer's instructions, and detected at 269 nm. The protein sequence was deposited in the UniProt (Universal Protein Research) database. The sequence was automatically compared to similar proteins by alignment with the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). Additionally, the neighbor-joining method (NJ) in Mega 4 software (Tamura et al., 2007) was used to construct a phylogenetic tree with the N-terminal amino acid sequences of related proteins from the GenBank database. The level of support for the phylogenies derived from NJ analysis was gauged by 1000 bootstrap replicates.

#### 2.4.4. Heat stability

Dactylomelin-P (1 mg $mL^{-1}$) was incubated at different temperatures, ranging from 45 to 60 °C, in 50 mM Tris–HCl, pH 7.0 for 1 h. At intervals of 5 min, aliquots were obtained, immediately cooled, and centrifuged for 5 min at 10,000 × *g*. Then, the stability of dactylomelin-P was tested by assaying the supernatants for antibacterial activity. Antibacterial activity was measured against *S. aureus* (ATCC 25923) cells with the disk-diffusion method on Muller Hinton agar (Bauer et al., 1966).

#### 2.4.5. pH stability

The effect of pH on the antibacterial activity of dactylomelin-P was evaluated by dissolving the protein (1 mg $mL^{-1}$) in the following buffers: 50 mM glycine-HCl (pH 2.0 and 3.0), 50 mM sodium acetate (pH 4.0 and 5.0), 50 mM Bis-Tris (pH 6.0), 50 mM Tris–HCl (pH 7.0 and 8.0), 50 mM glycine-NaOH (pH 9.0 and 10.0), and 50 mM sodium phosphate (pH 11.0 and 12.0). The samples were incubated at 4 °C for 30 min. Afterwards, the samples were dialyzed against 50 mM Tris–HCl pH 7.0, lyophilized, resuspended in 0.15 M NaCl, and tested for antibacterial activity, as previously described (Section 2.4.4).

#### 2.4.6. Resistance to protease

A protease digestion assay was performed with the method originally described by Rajaganapathi et al. (2002). Dactylomelin-P

(1 mg $mL^{-1}$) was incubated with 1 mg of unspecific protease (*Bacillus licheniformis* protease, Sigma, USA) in 50 mM Tris–HCl pH 7.0 at room temperature for 18 h. After incubation, the sample was tested for antibacterial activity, as previously described (Section 2.4.4).

### 2.5. Binding of dactylomelin-P to S. aureus cells

The binding capacity of dactylomelin-P toward *S. aureus* cells was vizualized by Transmission Electron Microscopy (TEM). Initially, the *S. aureus* cells were pre-incubated with 0.5 mg $mL^{-1}$ dactylomelin-P in LB medium, a dose 2500× greater than its minimal inhibitory concentration, for 24 h, at 35 °C, and washed five times with 100 mM sodium-phosphate pH 7.3 buffer by centrifugation at 5000×*g*, 4 °C, 5 min to remove unbound protein. Then, the cells were fixed in 0.1% (v/v) glutaraldehyde plus 2.0% (v/v) paraformaldehyde solution prepared in 100 mM sodium-phosphate buffer pH 7.3, for 2 h at room temperature. Subsequently, the material was rinsed three times with the above buffer and dehydrated through an ethanol series, and embedded with LR Gold resin. Then, the samples were sliced into thin sections. The sections were incubated in phosphate buffered saline (PBS) (10 mM, pH 7.4) and 1% bovine serum albumin (BSA) for 30 min, and then incubated with anti-dactylomelin-P antiserum (diluted 1:100, v/v) for 2 h. After successive washes in distilled water, the sections were incubated with the secondary antibody, rabbit gold-linked anti-IgG (1:50, v/v), for 2 h. This was followed by serial washes and observation by TEM (ZEISS 900). Controls were prepared in the same way, except that the pre-immune serum replaced the anti-dactylomelin-P antibody. The colloidal gold particles density was visualized from random micrographs of cell sections, a minimum of 64 to a maximum of 86 individual cells from each test and control samples. At least six to seven sections were used for analysis in each cell.

## 3. Results

### 3.1. L-AAO activity

Dactylomelin-P was able to catalyze the oxidation of L-lys and L-arg; therefore dactylomelin-P behaved as an L-AAO. The Michaelis–Menten constants ($K_m$) for L-lys and L-arg were 0.22 ± 0.16 mM and 0.015 ± 0.01 mM, respectively (Fig. 1). Dactylomelin-P was not able to oxidize L-his or the acidic amino acids, L-asp and L-glu.

### 3.2. Effects of L-lys, L-arg, and peroxidase on dactylomelin-P activity

In this study, we compared the viable cell number immediately before the addition of dactylomelin-P (t = 10 h) and at the end of the log phase (t = 24 h). We found that the number of bacterial cells was maintained, typical of a bacteriostatic effect. Nevertheless, the treatment with dactylomelin-P supplemented with high L-lys or L-arg led to a significant ($p<0.05$) reduction in the viable cell count at 24 h; this demonstrated a bactericidal mode of action (Fig. 2). The concentration of the amino acids influenced the antibacterial activity of dactylomelin-P. When concentrations of L-lys or L-arg below 25 mM and 50 mM, respectively, were added to LB medium, it resulted in a maximum reduction of 60% in the number of cells, mostly a bacteriostatic effect. On the other hand, higher concentrations of L-lys (25 mM) or L-arg (50 mM) killed about 90% of the cells ($F = 5.75$, $p<0.05$ for L-lys; $F = 3.82$, $p<0.05$ for L-arg).

To demonstrate that the antibacterial activity of dactylomelin-P was mediated by the production of $H_2O_2$, the action of dactylomelin-P against *S. aureus* was evaluated with or without peroxidase. The presence of peroxidase abolished the inhibitory effect of dactylomelin-P; cultures supplemented with dactylomelin-P and peroxidase exhibited growth similar to control; cell growing in LB medium (Fig. 3). When added to cultures supplemented with both dactylomelin-P and L-Lys or

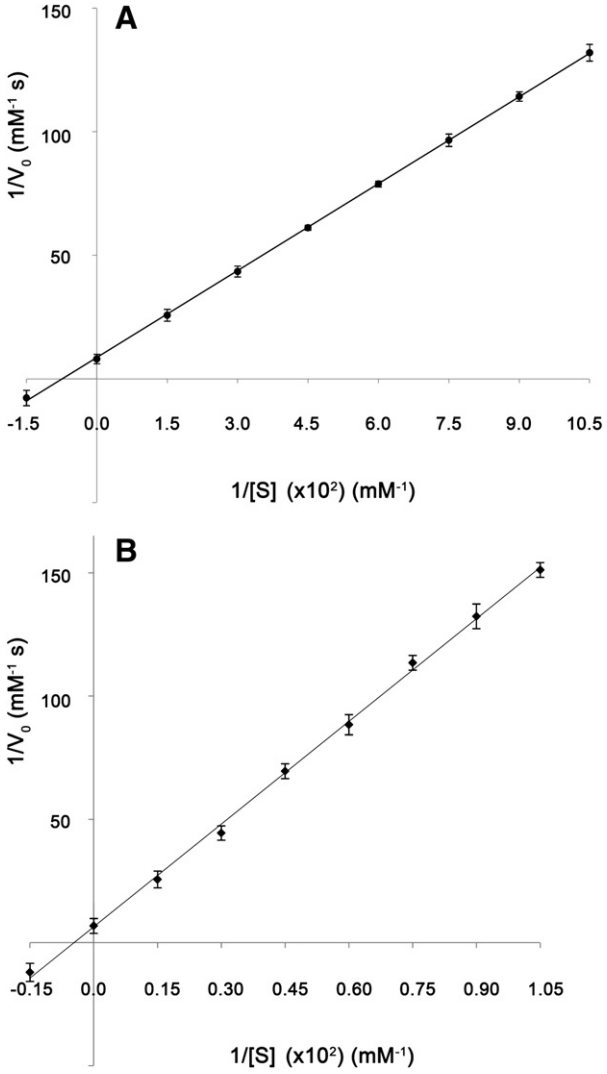


**Fig. 1.** Lineweaver–Burk plot of the L-amino acid oxidase (L-AAO) activity of dactylomelin-P. The reactions were performed at 25 °C for 25 min using 10 μg of dactylomelin-P and L-arginine (A) or L-lysine (B) as substrates. $K_m$ values were 0.015 ± 0.01 mM for L-arg, and 0.22 ± 0.16 mM for L-lys. Each plot represents the mean of three independent experiments each with three replicates.

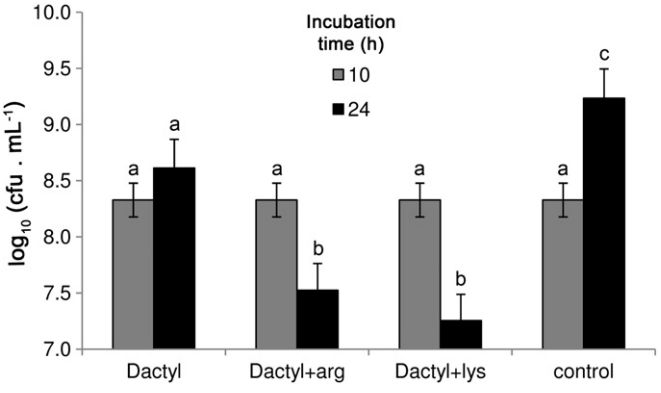


**Fig. 2.** Mode of action of dactylomelin-P in unsupplemented or L-lys and L-arg supplemented LB medium on *Staphylococcus aureus* growth. The antibacterial activity was evaluated using 100 μg $mL^{-1}$ dactylomelin-P (Dactyl) or 100 μg $mL^{-1}$ dactylomelin-P supplemented with 100 mM L-lys (Dactyl + lys) or 100 mM L-arg (Dactyl + arg). Control consisted of bacterial cells growing on LB medium alone. Values represent the means + SD of three independent experiments each with three replicates (ANOVA 1-way for t = 24 h, $F = 17.86$, $p = 0.002$). Bonferroni's test showed significant differences ($p<0.05$) for all treatment comparisons at t = 24 h (a, b, c), except for dactyl + lys and dactyl + arg that exhibited no significant differences (b).

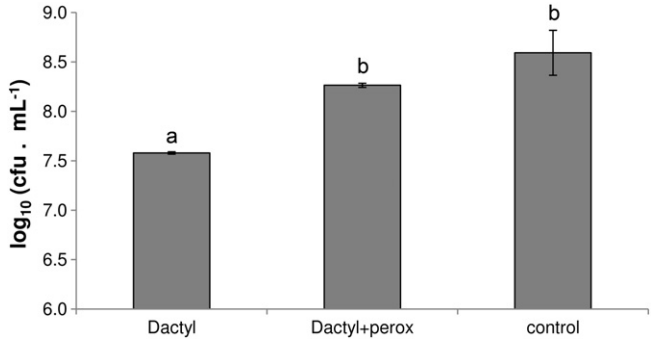


**Fig. 3.** Effect of peroxidase on dactylomelin-P antibacterial activity. *Staphylococcus aureus* was grown on LB medium in the presence of 100 μg $mL^{-1}$ dactylomelin-P (Dactyl) or 100 μg $mL^{-1}$ dactylomelin-P plus 100 μg $mL^{-1}$ peroxidase (Dactyl + perox). Control consisted of bacterial cells grown on LB medium alone. Values are the means + SD of three independent experiments each with three replicates (1-way ANOVA; $F = 182.42$, $p = 0.008$). Bonferroni's test showed significant differences ($p < 0.05$) for comparisons between Dactyl and Dactyl + perox (a, b). Dactyl + perox and control exhibited no significant differences (b).

L-arg, peroxidase did not lead to a recovery in the cell number; the cell counting with or without peroxidase was similar ($F = 1.51$, $p > 0.05$), indicating that the bactericidal effect was not mediated merely by $H_2O_2$.

### 3.3. Further biochemical characterization of dactylomelin-P

#### 3.3.1. Isoelectric point and amino acid composition

Dactylomelin-P is an acidic protein with an isoelectric point of 5.0. Its amino acid composition showed high concentrations of glutamic acid/glutamine, aspartic acid/asparagine, tyrosine, serine, and proline, and small amounts of histidine, methionine and cysteine. The amino acid composition of dactylomelin-P is reported in Table 1 in comparison with two other ink proteins.

#### 3.3.2. N-terminal amino acid sequence

The N-terminal amino acid sequence of dactylomelin-P was DGVCSNRRQCNKEVCGSSYDVAIVGA (Uniprot accession n°**P86163**). A BLAST search showed this sequence shared 96% identity with escapin (an ink protein from *A. californica*, GenBank accession n°**AAT12273**), 80% with APIT (an ink protein from *Aplysia punctata*, GenBank accession n°**AAR14185**), and 40% with aplysianin-A (an albumen gland protein from *A.kurodai*, GenBank accession n°**AAN78211**).

The phylogenetic tree created with the Clustal W program in Mega 4 software (Tamura et al., 2007) divided the sea hare proteins into different groups according to their sources; this suggested that similarities and differences could be related to their origins (Fig. 4). Thus, proteins originated from the ink gland constituted a separate group from proteins that originated from the albumen gland. All were more closely related to each other than to the L-AAO from the terrestrial snail *Achatina fulica* (Férussac 1821) or to L-AAOs from vertebrates.

#### 3.3.3. Biochemical properties of dactylomelin-P

Dactylomelin-P retained its unaltered antibacterial activity after heating at 55 °C for 30 min and after treatments with a protease. The antibacterial activity was retained in pH ranges from 3.0 to 12.0, but was completely abolished at pH 2.0 (Fig. 5).

#### 3.3.4. Transmission Electron Microscopy (TEM)

TEM demonstrated the binding of dactylomelin-P to bacterial cells surface as revealed by an intense labeling on the envelope of cells treated with the protein (Fig. 6A), while no labeling has been seen in the control (Fig. 6B). In addition, no morphological alterations in the shape, size, or surface on the treated cells were observed.

## 4. Discussion

Dactylomelin-P, the most abundant protein in ink released by the sea hare *A. dactylomela*, shares some biological and biochemical characteristics with ink proteins from other sea hares. All known ink proteins have a mass of ~60 kDa, including aplysianin P, isolated from *A. kurodai* (Yamazaki et al., 1989a), APIT from *A. punctata* (Butzke et al., 2005), escapin from *A. californica* (Yang et al., 2005), dolabellanin-P from *Dolabella auricularia* (Yamazaki et al., 1989b), bursatellanin-P from *Bursatella leachii* (de Blainville 1817) (Rajaganapathi et al., 2002), and dactylomelin-P (Melo et al., 2000).

Examination of amino acid composition of dactylomelin-P revealed that, similarly to dollabelanin-P (Yamazaki et al., 1989b), it contains a relatively large amount of the acidic amino acids. These two proteins resemble aplysianin-P (Yamazaki et al., 1989a) in showing low levels of sulfur amino acids. Dactylomelin-P like APIT (pI = 4.59) (Butzke et al., 2005) has an acidic pI (5.0).

**Table 1**
Comparison of amino acid composition of dactylomelin-P, aplysianin-P and dolabellanin-P. Amino acid concentrations are expressed in mol% from lyophilized protein.

| Amino acid | Dactylomelin-P (mol%) | Aplysianin-P[a] (mol%) | Dollabelanin-P[b] (mol%) |
|---|---|---|---|
| Ala | 5.5 | 10.5 | 7.5 |
| Arg | 5.5 | 3.4 | 5.6 |
| Asx | 10.0 | 6.8 | 12.0 |
| Cys | 1.4 | 0.8 | 0 |
| Glx | 10.7 | 7.1 | 10.6 |
| Gly | 6.2 | 11.8 | 7.3 |
| His | 1.6 | 0.6 | 4.8 |
| Ile | 2.5 | 4.0 | 3.3 |
| Leu | 6.1 | 8.2 | 6.0 |
| Lys | 3.3 | 2.6 | 6.1 |
| Met | 1.9 | 2.4 | 2.2 |
| Phe | 5.8 | 3.6 | 4.0 |
| Pro | 8.7 | 12.9 | 5.3 |
| Ser | 8.8 | 5.9 | 9.2 |
| Thr | 6.1 | 10.7 | 8.1 |
| Trp | NT[c] | NT[c] | 0.8 |
| Tyr | 10.0 | 2.2 | 4.1 |
| Val | 5.5 | 6.6 | 4.5 |

[a] Data from Yamazaki et al. (1989a).
[b] Data from Yamazaki et al. (1989b).
[c] NT, not tested.

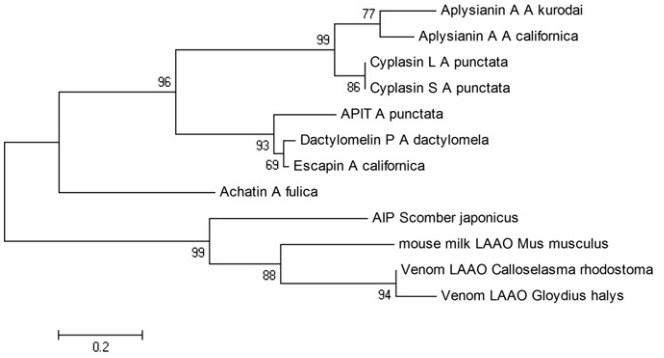


**Fig. 4.** Phylogenetic tree of L-AAOs and related proteins based on N-terminal sequence similarities. Values above the lines represent bootstrap-supported values of nodes from the NJ analysis obtained from 1000 replicates. Data used included: dactylomelin-P (Uniprot accession n°**P86163**), escapin from *A. californica* (GenBank accession n°**AAT12273**, Yang et al., 2005), APIT from *A. punctata* (**AAR14185**, Butzke et al., 2005), aplysianin A from *A. kurodai* (**Q17043**, Jimbo et al., 2003), aplysianin A precursor from *A. californica* (**AAN78211**, Cummins et al., 2004), cyplasin L and S from *A. punctata* (**CAC19362** and **CAC19361**, respectively, Petzelt et al., 2002), achacin from *Achatina fulica* (**CAA45871**, Obara et al., 1992), AIP from the teleostei *Scomber japonicus* (**CAC00499**, Jung et al., 2000), snake venom L-AAOs from the venomous pitviper *Gloydius halys* (**AAR20248**) and from *Calloselasma rhodostoma* (**P81382**, Ponnudurai et al., 1994), and mouse milk L-AAO (**NP_598653**, Sun et al., 2002).

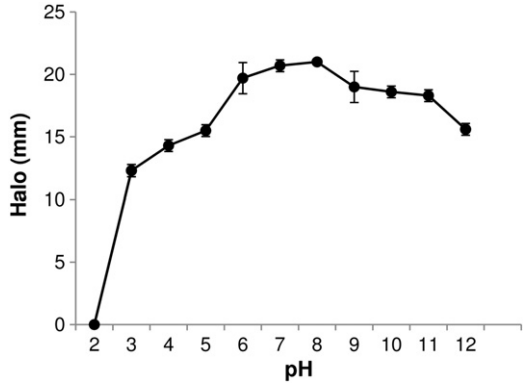


**Fig. 5.** Stability of the antibacterial activity of dactylomelin-P over a pH range. Dactylomelin-P (1 mg $mL^{-1}$) was incubated in buffers with pH values ranging from 2.0 to 12.0, dialyzed, lyophilized, and tested for antibacterial activity against *Staphylococcus aureus* ATCC 25923. Values represent the means + SD of three independent experiments each with three replicates.

The resistance of dactylomelin-P to protease could also be related to its ability to bind to an inhibitor, as reported for aplysianin-P (Yamazaki et al., 1989a) and bursatellanin-P (Rajaganapathi et al., 2002). Butzke et al. (2005), however, attributed the resistance of APIT to trypsin and protease K to the stabilization of its native structure by FAD cofactor.

The thermo stability of dactylomelin-P was comparable to that of bursatellanin-P (Rajaganapathi et al., 2002) and aplysianin-P (Yamazaki et al., 1989a), which were completely inactivated between 55 °C and 60 °C. Dactylomelin-P was very stable with pH variation and retained its antibacterial activity in the pH range of 3.0–12. Its stability under basic pH reinforced a possible function that might be exogenous in the marine environment. Moreover, the stability of dactylomelin-P under acidic pH, similarly to escapin (Shabani et al., 2007), is related to the fact that the pH of inks, in which they act, is naturally acidic. As a matter of fact, both escapin (Ko et al., 2008) and dactylomelin-P possess antimicrobial activity in a broad pH range.

Ink proteins from sea hares show a wide range of biological activities, including antimicrobial and/or cytolytic actions against several strains of bacteria, fungi, and tumors cells. Additionally, dactylomelin-P and bursatellanin-P have hemagglutinating (Melo et al., 2000) and anti-HIV activities (Rajaganapathi et al., 2002), respectively. The similarities among sea hare bioactive proteins are highlighted by comparing their amino acid sequences. The first to be sequenced was aplysianin-A (from the albumen gland; Takamatsu et al., 1995), followed by cyplasin (Petzelt et al., 2002), escapin (Yang et al., 2005), and APIT (Butzke et al., 2005). The similarities among these proteins have been measured, and scientists have speculated about their roles in animal defense and survival. Escapin, for example, shares 93% identity with APIT and 96% identity with dactylomelin-P. Escapin, cyplasin- L, aplysianin-A (from *A. kurodai*), and its counterpart in *A. californica*, share ~60% identity. In addition, the sequences of escapin and the L-AAO from the African snail, *Achatina fulica*, are 48% similar. Yang et al. (2005) reported escapin has also 21% similarity to apoxin-I, an L-AAO isolated from the venom gland of the snake, *Crotalus atrox* (Baird and Girard 1853) (Torii et al., 2000).

The phylogenetic analysis of the sea hare proteins indicated two clades: the first consisted of aplysianin-A from *A. kurodai*, its homologue from *A. californica*, and both cyplasins (L and S) from *A. punctata*; the second comprised APIT, escapin, and dactylomelin-P. As proposed by Kamiya et al. (2006), this pattern suggested separate paralogues of sea hare L-AAOs: one from the albumen gland and the other from the ink gland. Only cyplasins, which were grouped separately in a particular subgroup of the albumen gland cluster, are not known to have L-AAO activity. Moreover, proteins isolated from similar organs of different species (orthologous) appear to be closer than proteins isolated from different organs of the same sea hare species. For example, the APIT (ink protein) produced by *A. punctata* is more closely related to the other ink proteins than to cyplasin (from the mucus of albumen glands) produced by *A. punctata*.

The phylogenetic tree for L-AAOs showed two large clades: one contained invertebrate L-AAOs and the other contained vertebrate L-AAOs. Nevertheless, the invertebrate group contained achacin, a protein from a terrestrial slug, which appeared to be an out-group, separate from the sea hare proteins. This difference could be a reflection of their diverse physiological roles and distinct modes of life; the sea hares are aquatic and the snail is terrestrial (Takamatsu et al., 1995).

The N-terminal amino acid sequence of dactylomelin-P showed similarity to other L-AAOs, and the enzymatic assay confirmed that dactylomelin-P was an L-AAO. The next step was to evaluate the antibacterial mechanism of dactylomelin-P. We previously reported the bacteriostatic effect of dactylomelin-P on *S. aureus* (Melo et al., 2000). In this study we used *S. aureus* as test organism again mostly due to its pathogenicity and to the worldwide emergence of strains resistant to commercial antibiotics (Vandenesch et al., 2003). Furthermore, dactylomelin-P might become an alternative drug to the control of *S. aureus*.

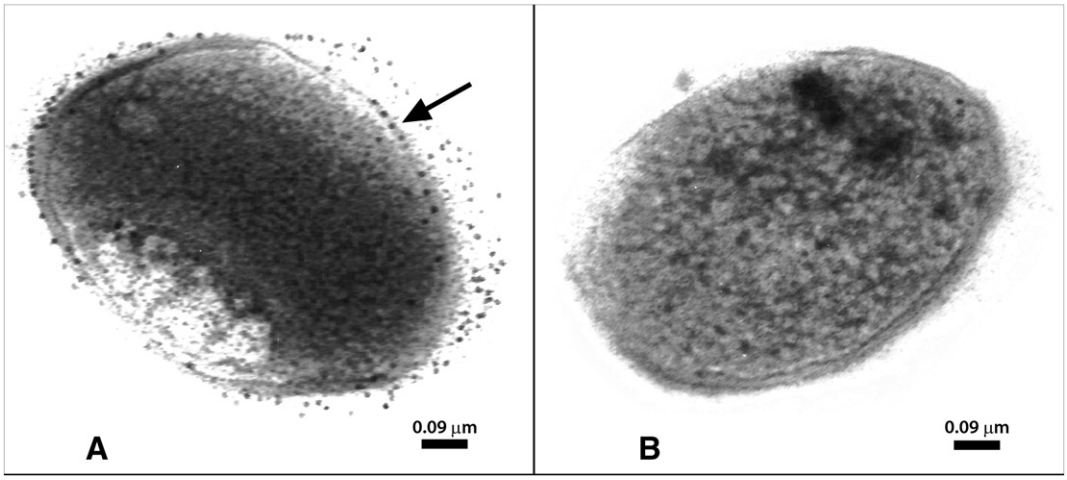


**Fig. 6.** Transmission electron microscopy of *Staphylococcus aureus* ATCC 25923 cells after 24 h of incubation with 0.5 mg $mL^{-1}$ dactylomelin-P in LB medium at 35 °C. The binding to *S. aureus* cells was revealed by incubation of the treated cells with anti-dactylomelin-P antiserum (A) or pre-immune serum (B), followed by incubation of both with rabbit gold-linked anti-IgG secondary antibody. The colloidal gold particles density was visualized from random micrographs of cell sections; a minimum of 64 to a maximum of 86 individual cells from each test and control samples were observed.

The results showed that the bacteriostatic effect of dactylomelin-P was due to the $H_2O_2$, since the cell growth was recovered in the presence of peroxidase. In addition, dactylomelin-P was bactericide in presence of high concentration of L-lys (25 mM) or L-arg (50 mM) leading to 90% of cell death. The bactericidal effect seems to be mediated by $H_2O_2$ and other toxic components resulting from L-AAO reaction, since the death was not avoided by the concomitant treatment of bacterial cells with dactylomelin-P plus L-lys or L-arg and peroxidase. These toxic products might have direct effects on cell growth, with consequences on metabolism; this may lead to growth inhibition and death. Since our main objective was to determine whether dactylomelin-P was an L-AAO and to investigate its antibacterial mode of action, we simply measured the viable cells at the end of the growth curve. Nevertheless, it is known that some L-AAOs show antibacterial activity within few minutes or hours of contact with the growing cells (Ehara et al., 2002; Jimbo et al., 2003; Yang et al., 2005).

Yang et al. (2005) noted the bacteriostatic and bactericidal actions of escapin against *E. coli* and *S. aureus* and attributed these actions to the production of $H_2O_2$ in the culture medium when it was supplemented with L-lys and L-arg. The antifungal action of escapin was also demonstrated to be dependent on the oxidation of L-amino acids; the enrichment of the culture media with large amounts of L-lys increased the activity. According to Ko et al. (2008), escapin products have both bacteriostatic and bactericidal effects. $H_2O_2$ accounts for most of the bacteriostatic action and for a small portion of the bactericide activity. Other compounds appeared to be responsible for the bactericidal effect reported for L-AAOs; the synergistic action of $H_2O_2$ and escapin intermediate products (mainly α-keto-ε-aminocaproic acid) was responsible for most of the bactericidal effect (Ko et al., 2008).

These new data are significant because only the bacteriostatic action of dactylomelin-P had been observed previously (Melo et al., 1998, 2000). Yang et al. (2005) observed that escapin was bacteriostatic in unsupplemented culture medium, had a minor bactericidal effect in arginine supplemented yeast extract, and showed strong bactericidal action in tryptone, peptone, and lysine medium. Dactylomelin-P bactericidal activity significantly decreased ($p<0.05$) with reductions in the L-arg and L-lys concentrations below 50 and 25 mM, respectively. Lower concentrations, like the ones found in unsupplemented LB medium (L-arg, 2.0–2.8 mM; L-lys, 3.8–6.8 mM; Sezonov et al., 2007), would most likely be insufficient to induce a bactericidal effect, resulting in a bacteriostatic activity nearly equal to that previously reported by Melo et al. (2000). Thus, the LB medium concentrations of L-lys and L-arg are insufficient *per se* to assure the bactericidal effect of dactylomelin-P. At the highest amino acid tested concentrations (100 and 50 mM) the effects of dactylomelin-P were about the same, but when we reduced the concentration to 25 mM, only L-lys was able to sustain the bactericidal activity of the enzyme whereas L-arg did not. Escapin was also strongly bactericidal in media with L-lysine at concentrations as low as 3 mM and slightly bactericidal in 50 mM L-arginine (Yang et al., 2005). Thus, although the exact mechanism of bactericidal action of L-AAOs is not known, it probably involves a lysine-dependent mechanism, as shown for escapin (Yang et al., 2005) and dactylomelin-P in the present work.

Our results suggested that the bacteriostatic and bactericidal actions previously reported for proteins in the Aplysianin family (group of bioactive proteins from sea hares) should be revised. The characterization of L-AAO activity has revealed a new perspective, because proteins were found to be bacteriostatic or bactericidal, depending on the amount and nature of the amino acids present in the medium.

In nature, the substrates for the enzymatic action are present in secretions related to the ink. This is the case of opaline secretion, reported to contain lysine and arginine (Derby et al., 2007). This fact allowed the development of a very interesting defense mechanism; the release of ink in combination with opaline, leading to the production of other defensive chemicals, not packaged by the sea hare, after the enzymatic reaction (Johnson et al., 2006).

The bacteriostatic effect of dactylomelin-P is mediated by $H_2O_2$, since when the cultures were incubated with dactylomelin-P and peroxidase the bacterial growth was recovered. Jimbo et al. (2003) and Yang et al. (2005) also reported that the addition of peroxidase to the culture medium containing L-AAO neutralized its biological activity. Ehara et al. (2002) noted the antibacterial activity of achacin and attributed it to the $H_2O_2$ produced by its L-AAO properties. The authors reported that the binding of achacin to the target-cell was essential, because the peroxide could only achieve high concentrations when it was near the target; they described this as site-specific cytotoxicity.

Petzelt et al. (2002) reported that the cytotoxic activity of cyplasin was not associated with any morphological alteration of the treated cells. For those cells, the first signals of toxicity were observed on the external plasma membrane, with unchanged internal morphology. They suggested that cyplasin must bind to the external membrane and initiate a cascade of events that led to cell death. This was consistent with our observations in cells treated with dactylomelin-P. In our study, TEM showed that the bacterial cells preserved their morphology, size, and shape and that dactylomelin-P recognized and bound to growth-phase bacteria. Once this binding was observed, further experiments are required to demonstrate whether like cyplasin dactylomelin-P recognizes and binds to the target cell in order to initiate its biological effects.

Dactylomelin-P was able to oxidize L-lys and L-arg, both basic amino acids. The inability of the protein to oxidize L-his, also a basic amino acid, may have been due to the imidazole radical, an aromatic heterocyclic group present in histidine, which may have been inaccessible to dactylomelin-P. Additionally, dactylomelin-P did not oxidize L-aspartate or L-glutamate, both acidic amino acids. In general, L-AAOs have diverse substrate specificities. Some, like dactylomelin-P, prefer basic amino acids (Butzke et al., 2004, 2005; Jimbo et al., 2003; Yang et al., 2005). Others preferentially oxidize a wider range of substrates, for example, hydrophobic or aromatic amino acids, like L-leucine, L-phenylalanine, L-isoleucine, L-methionine, or L-tyrosine, but show very little affinity for basic amino acids (Du and Clemetson, 2002; Ehara et al., 2002; Macheroux et al., 2001; Torii et al., 2000). L-AAOs from sea hares, however, show a preference for L-lys or L-arg. In addition, L-AAOs isolated from ink of sea hares show low values of $K_m$ for L-lys and L-arg (Butzke et al., 2005; Yang et al., 2005). Nevertheless, the lowest values of $K_m$ reported until now were determined for aplysianin-A, an L-AAO from albumen gland of *A. kurodai* (Jimbo et al., 2003), which are 2.38 μM and 3.34 μM for L-lys and L-arg, respectively.

Interestingly, L-AAOs show different types of activities (Kitane et al., 2008), possibly a reflection of their molecular diversity and the diversity of their oxidation products or intermediates. Although $H_2O_2$ is the main product, other molecules are produced and might be responsible for many biological effects. L-AAOs comprise a large family of molecules that share a common mechanism of action. The distribution of L-AAOs among prokaryotes, invertebrates, and vertebrates suggests that they play an important role. In sea hares, L-AAOs appear to be related to chemical defense against predatory attacks or pathogens. Biochemical, functional, and phylogenetic similarities among L-AAOs of sea hares have revealed the evolutionary history of these proteins and their role in chemical defense.

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