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BIOCHEMICAL CHARACTERIZATION OF ANTI-METHICILLIN RESISTANT *S. aureus* PROTEIN (P-80) FROM MARINE *Pseudoalteromonas*

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ABSTRACT

The marine *Pseudoalteromonas* are recognized as major active metabolite producers against different biological targets. These compounds include cyclic peptides, toxic proteins or enzymes, among others. In previous studies, we isolated a protein named P-80 from *Pseudoalteromonas* sp with antibiotic activity against *S. aureus*, and proteolytic action. In the present work, we studied properties such as structure and the function of P-80. We performed different assays, including MALDI-TOF mass spectrometry, antibacterial activity by the standard disc diffusion, pH and temperature stability, cytotoxicity *in vitro* and finally the effect of metal ions and protease inhibitors on antimicrobial activity. The purified P-80 showed a molecular mass of 78.5 kDa as determined by MALDI-TOF mass spectrometry. It was highly active over pH range of 7.0 to 9.2, and remained stable over a pH range of 7.0 to 8.0 although its activity was reduced at pH 9.2 (only 55% was retained). This protein showed quite a good antibacterial activity over a broad temperature range (> 65% of activity is retained between -20 and 70 ºC). The inhibitor tested such as EDTA, EGTA, PMSF, DTT and TLCK (at 5 mM) had no effect on the antibacterial activity. Whereas Mn^{2+} ions enhanced the antibiotic activity but Zn^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Cu^{2+} and Co^{2+} ions had no effect. In conclusion, the P-80 protein remained 55% and 65 % of its antimicrobial activity against *Staphylococcus aureus* at pH 9.0 and 70 ºC respectively, suggesting high stability compared to other marine antibacterial proteins indicating intrinsic features that could enable commercial applications

Keywords: Marine bacterium, antimicrobial protein, *Pseudoalteromonas*, biochemical properties

INTRODUCTION

Conventionally after plants, especially terrestrial microorganisms of the *Actinomyces* genus are the most common producers of antimicrobial agents. Although medicinal applications of bioactive substances from marine microorganisms have not traditionally been used, in the last years they have become one of the main topics of study in search of molecules with various pharmacological activities and established a thriving resource for the development of a wide range of novel compounds. These products have shown antimicrobial activities, antiviral, immunosuppressive, enzyme inhibitors, receptor antagonists, etc. (**Reichenbach, 2001**).

The adaptation of bacteria to diverse marine habitat can determine their special skills to produce structurally unique secondary metabolites while others belong to known classes of compounds (**Bernan *et al.*, 1997; Demunshi and Chugh, 2010**). In the marine environment, communities of bacteria associated with abiotic or biotic surfaces often live encapsulated in a self-synthesized polymer matrix where an interactive environment exists (biofilm). As part of this, the complex community formed has the advantage in that there is an inherent increase in the efficiency of nutrient acquisition, enhance tolerance to toxic compounds and physicochemical stress and presumably excellent opportunities for genetic exchange (**Bowman, 2007**). Also, many surfaces attached marine bacteria are likely to produce substances that inhibit growth and survival of competing species (**Egan *et al.*, 2008**) and they can be secondary metabolites and proteins.

Antimicrobial proteins called AMPs can be either low molecular weight peptides or large molecules, such as proteins (**Wilson *et al.*, 2011**). Terrestrial and marine organisms, including invertebrates, vertebrates, plants, mushrooms, fungi and bacteria produce these biomolecules. They have attracted much research interest because of their biochemical diversity, broad specificity on antiviral, antibacterial, antifungal, antiprotozoal parasites, and even antitumoral or wound-healing effect (**Zasloff, 2002**). Antimicrobial proteins and peptides (AMPs) are such alternative class of active antibiotic compounds that offers tremendous potential against multidrug-resistant bacteria to common classes of antibiotics (**Domingues *et al.*, 2014**).

Different marine species of the bacterial genus *Pseudoalteromonas* are recognized as forming biofilm and large producers of a wide range of bioactive metabolites with therapeutic possibilities. *Pseudoalteromonas* species secrete several proteins, enzymes, and other soluble substances exopolymer high molecular weight showing antibacterial activity against pathogenic microorganisms (**Bowman, 2007; López *et al.*, 2012**).

The mechanisms of action of some of the marine antimicrobial proteins have been disclosed, among them are those associated with suppression of ATP synthesis or RNA synthesis, those that interfere with bacterial membrane electrical potential or proteolytic activity related to increasing oxygen transport and accumulation of hydrogen peroxide, etc. However, the mechanisms of many other marine compounds await elucidation and novel mechanisms may be unraveled in future investigations (**Ng *et al.*, 2015**).

In general, proteins are responsible for a huge amount of different and essential functions in the living system. In addition, some of the proteins are important as biomarkers, for example, in the diagnosis of pathologies or targets for different therapies and interventions. Therefore, it is necessary to delve into exploring structure, function, mechanisms, working and other properties related to these biological molecules. Thus, understanding the biochemical properties and the function-structure relationship of new proteins could contribute to the development of novel antibiotics.

An 80-kDa protein (P-80) was isolated from sediment samples of the Gulf of México and showed antimicrobial activity against a clinic isolate methicillin-resistant *S. aureus* (MRSA) (**Cetina *et al.*, 2010**). In previous publications, it was

shown that its antimicrobial activity could be induced under microaerophilic condition (**López *et al.*, 2012**) and inhibited by medium containing a high concentration of amino nitrogen (**López and Monteón, 2013**). Besides, the proteolytic activity was an evident property but different antimicrobial mechanism (**Bautista, *et al.*, 2015**).
The present work focuses on characterization of the biochemical properties of anti-*S. aureus* (MRSA) protein from the marine bacterium *Pseudoalteromonas* sp.

MATERIALS AND METHODS

Bacterial strains and medium

Pseudoalteromonas sp. was originally isolated from sediment samples in the Gulf of Mexico, Campeche (**Cetina *et al.*, 2010**). The strain was conserved in the Biotechnology Laboratory of Biomedical Research Centre. The strain was inoculated in sterile seawater medium (0.9% glucose, 0.1% yeast extract, 0.5% peptone, pH 8.0) and cultivated at 28 °C for 73 h under static conditions.
The human pathogenic *Staphylococcus aureus* (MRSA) was grown in CST broth at 37°C for 24 h and used for evaluating the antibacterial activity. The *Staphylococcus aureus* ATCC 25923 strain was used as a control.

Purification of P-80 protein

The P-80 protein was isolated from biomass of *Pseudoalteromonas* sp. as described by **López *et al.* (2012)**. The exhausted culture medium of *Pseudoalteromonas* sp. was centrifuged at 3500 g for 15 min at 4 ° C. The bacterial pellet was collected and washed with natural sterile seawater twice. Two grams were suspended in 50 mM phosphate buffer at pH 7.4 and sonicated at 93.5 watts. The suspension was centrifuged at 4025 g for 20 min at 4 ° C and the pellet was discarded. Cold 100% ethanol was added to the supernatant solution (9: 1), mixed and kept at least 4 hours at - 20 ºC. The precipitate fraction (crude extract) was recovered and washed with cold 90% ethanol and dried by inversion on tissue paper. The crude extract was suspended in 50 mM phosphate buffer pH 7.4 and filtered through the 30 kDa cut-off membrane. The fraction was washed with 10 volumes of the same buffer to remove proteins with masses below 30 kDa and then filtered through 0.22 µm membrane (Millipore). All the purification steps were carried out at 4 ºC.
Afterwards, this fraction was subjected to gel filtration chromatography in a TSK gel G2000 SW column (7.5 mm × 30 cm × 5 µm, Agilent Technologies, Germany) using a HPLC system (HP Agilent 1100 series, Agilent Technologies, Germany) equilibrated with 100 mM glycine buffer, pH 9.2 containing 200 mM NaCl. Protein fractions were eluted with the same buffer at a flow rate of 0.9 mL min^{-1}, collected and analyzed for antimicrobial activity. The fraction with activity was subjected to anion exchange chromatography in a POROS ^{TM} HQ/H columns (4.6 mm × 100 mm, Life Technologies, USA) equilibrated with 50 mM Tris-HCl buffer, pH 7.4. The protein was eluted with a linear gradient from 0 to 1 M of NaCl at a flow rate of 3 mL min^{-1}. The pure protein was dialyzed against the same buffer. Protein purity was analyzed by SDS–PAGE and protein concentration were determined using the bicinchoninic acid method (BCA) with Pierce^{TM} BCA protein assay kit (Pierce Biotechnology, USA) using bovine serum albumin (BSA) as a standard.

Antimicrobial activity assay

The antibacterial activity of the P-80 was tested by the standard disc diffusion (Kirby-Bauer) method against the Gram-positive bacteria *Staphylococcus aureus* MRSA as the test microorganism and *S. aureus* ATCC 25923 as a control. Paper discs (6 mm) were saturated with 20 µL of purified antimicrobial protein P-80 solution (1.2 mg mL^{-1}) in phosphate buffer (0.1 M, pH 7.5). The discs were placed onto the surface of Muller-Hinton agar (Difco®) containing the test microorganisms. Clear zones of inhibition around the discs indicated antibacterial activity, which was measured after 24 h of incubation at 37°C. Vancomycin and phosphate buffer were used on the plates as the positive or negative control, respectively.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 4% acrylamide in the stacking gel and 12% acrylamide in the separating gel. The running buffer was Tris- glycine and 0.1% SDS, pH 8.3. The protein solution was mixed with a half volume of the tray buffer (Tris-HCl, pH 6.8, 15% glycerol and 0.075% bromophenol blue). Protein band was observed by staining with Coomassie Brilliant Blue.

Mass spectrometry

Matrix-assisted laser desorption/ionization-Time of flight (MALDI-TOF) Mass Spectrometry was used for determining the molecular mass of the antimicrobial protein using a Microflex Bruker spectrometer (Bruker Daltonic, Bremen, Germany). The pure protein was mixed with a saturated solution of 2,5-dihydroxybenzoic acid (DHB) in 30% (v/v) acetonitrile, 70% (v/v) water and 0.1% (v/v) trifluoroacetic acid. The spectrometer was equipped with a 20 Hz nitrogen laser controlled by FLEX-CONTROL software (version 3.0; Bruker Daltonics, Billerica,MA). Mass spectra were collected in the positive linear mode.

Effect of pH and temperature on antimicrobial activity

The pH stability was determined by incubating P-80 protein in a reaction mixture adjusted to the desired value of pH using the following 50 mM buffer; potassium phosphate (pH 6.5-8.0) and glycine-sodium hydroxide (pH 9.2). After incubation, the antimicrobial activity was determined by the Kirby-Bauer method. The thermal stability was determined by incubating the purified antimicrobial protein at different temperatures (from -20 to 70 ºC) for 2 h in phosphate buffer (20 mM, pH 8.0) and then immediately cooled in ice, and the residual activity was tested against *Staphylococcus aureus* MRSA under standard assay conditions by Kirby-Bauer methods. The residual activity (%) of the pretreated sample was done using the standard assay condition and taken as 100%.

Effect of metal ions and inhibitors on antimicrobial activity of P-80

The effect of metal ions on the purified P-80 protein were determined by treating the antibacterial protein with various metal ions, like Zn^{2+} (Zinc sulfate), Fe^{2+} (ferric chloride), Mg^{2+} (magnesium chloride), Ca^{2+} (calcium chloride), Cu^{2+} (copper sulfate), Co^{2+} (cobalt chloride) and Mn^{2+} (manganese chloride), at final concentration of 1 mM and 5 mM for 24 hours at 8 °C (**Matsushima *et al.*, 2010**). The antimicrobial activity assay was subsequently performed as described earlier and buffer pH 7.0 with diluent ions was used as negative control. The residual activity (%) was calculated with reference to the activity of the P-80 protein without these supplements while the inhibitors affect the proteolytic activity of P-80. Thus, to investigate the effect on its antimicrobial activity it was pre-incubated with different inhibitor compounds as EDTA (ethylene diamine tetraacetic acid), PMSF (phenylmethylsulfonyl fluoride), a mix of these EDTA-PMSF, DTT (dithiothreitol), TLCK (Tosyl-L-lysyl-chloromethane hydrochloride), at a concentration of 5 mM in various tubes for 1 h at 37°C. After the pre-incubation, the antimicrobial activity was assayed as described earlier and purified P-80 without inhibitor compounds was used as the control and its activity considered as 100%.

Cytotoxicity assay of P-80 protein

Cell Lines and culture conditions HeLa (cervical adenocarcinoma) and Vero (African green monkey kidney) cell lines were cultured in RPMI medium, supplemented with 10% (v/v) fetal bovine serum (FBS) in 5 cm^2 plastic bottles until 100% confluent cells.
For *in vitro* cytotoxicity assay the old medium was replaced with respective growth medium containing 0, 25, 50, 75, 100 or 500 µL of purified protein P-80 solution (1 mg mL^{-1}). As negative control 500 µl of PBS (diluted sample solvent) was added and DMSO at 0.5% final concentration as positive control, the blank receiving medium only. The cells were incubated for 12, 24 and 48 h at 37 ºC in the above conditions. After incubation, the medium was discharged and the cell was treated with 0.1% trypan blue and incubated for 10 min.
Finally, it was observed under an inverted microscope and the cell viability (%), vacuolization (%) and cellular morphology were determined. Blue stained cells were considered dead. The percentage of mortality was established as follows: (Number of blue cells/number of no blue cells) 100. We counted at least five hundred cells. All experiments were performed in triplicate.

Statistical analysis

In all figures, the data are expressed as a mean standard error of the mean (SEM). Statistical analysis was performed by using the Prism 5 software. The mean and standard deviations of triplicate determinations were computed and reported.

RESULTS

The crude extract of *Pseudoalteromonas* biomass showed anti- *Staphylococcus aureus* MRSA activity and SDS-PAGE revealed one main protein band corresponding to about 80 kDa (data not shown). The ethanol precipitated fraction was submitted to ion-exchange chromatography with a linear gradient of NaCl in phosphate buffer and one peak was obtained (Fig 1). An antibacterial activity was detected in this eluted fraction. The data suggested a high level of purification of the marine protein.

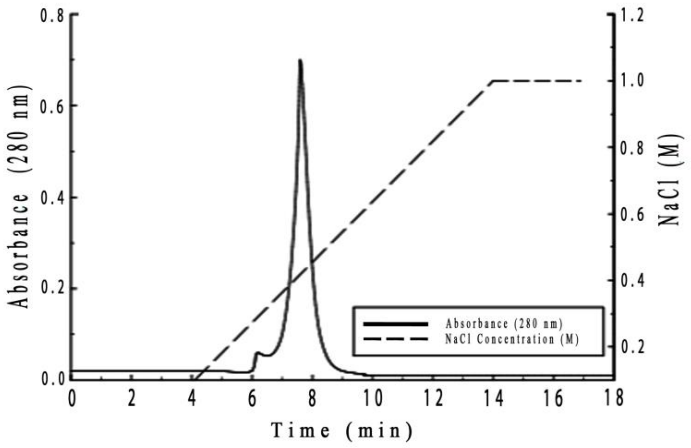

Figure 1 Isolation of the antimicrobial protein by ion-exchange chromatography

Molecular mass determination by MALDI-TOF mass spectrometry

The purified antibacterial protein showed a molecular mass of 78.6 kDa (Fig 2) as determined by MALDI-TOF mass spectrometry that is consistent with SDS-PAGE results. Weak signal of a dimeric form (*m/z* 155,000) was also detected.

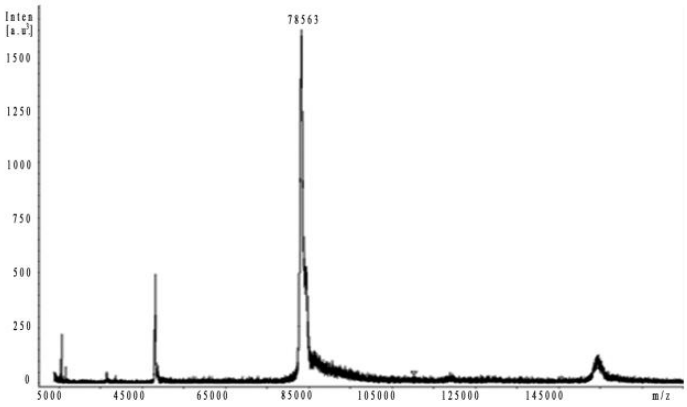

Figure 2 MALDI-TOF analysis to P-80 purified protein. The registered signal corresponds to a molecule with of weight of 78563 Da.

Influence of pH on antimicrobial activity

Figure 3 shows the antibacterial activity of the P-80 protein against *S. aureus* after treatment at pH 7.0 to 9.3 for 2 h. After exposure at pH 8.0, the antimicrobial protein retained its maximum antimicrobial activity. But treatment at pH 7.0 - 7.5, it was observed about 10 % of the antimicrobial activity was lost and remarkably reduced at pH 9.2 (only 55% was retained). Then, the data showed that P80 protein retained 100 % of maximum antimicrobial activity and stability at pH 8.0 under our experimental conditions.

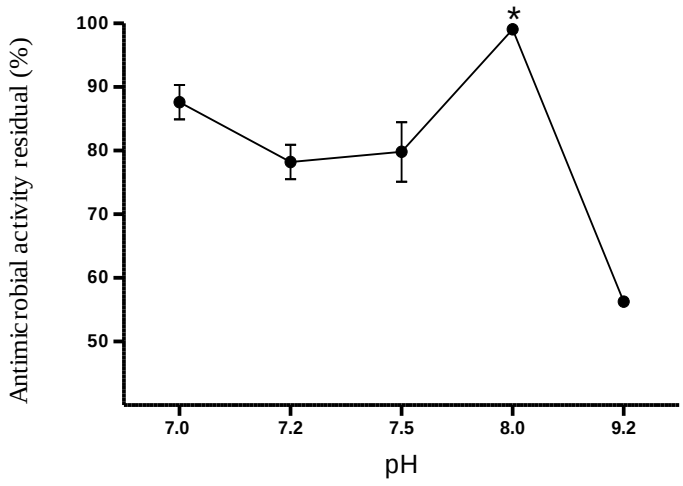

Figure 3 Effect of pH on P-80 antimicrobial activity

Influence of Temperature on antimicrobial activity

The effect of temperature on the antimicrobial activity of the P-80 protein was measured at pH 8.0 by incubating the protein over a temperature range of -20 - 70 ºC (Figure 4). The P-80 protein remained active over a wide range of temperature. Namely, it retained 100% and 65% of its activity when protein was heated to 50 ºC and 70ºC, respectively. Almost no change was observed from -20 to 25 ºC (about 10% activity reduction) and it lost 15% activity at -70ºC.

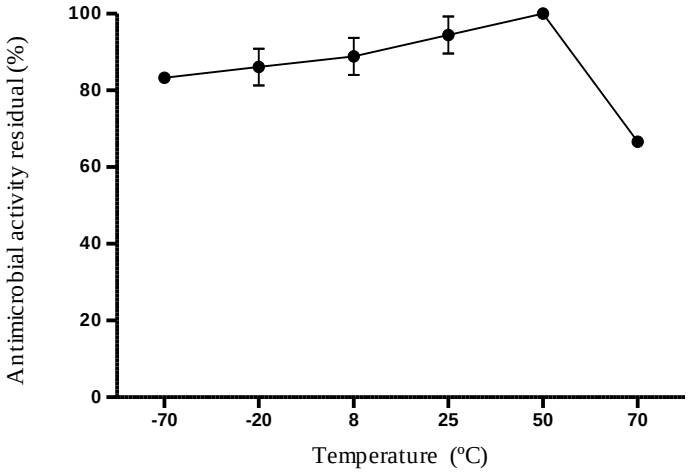

Figure 4 Effect of temperature on P-80 antimicrobial activity

Effect of various inhibitors and metal ions on antimicrobial activity

The effects of various inhibitors on antimicrobial activity were examined in order to characterize the purified protein P-80 against *Staphylococcus aureus* ATCC 25923. Results in Table 1 indicate that none of the inhibitors tested had an impact on the antimicrobial activity against the pathogenic bacteria. On the other hand, in order to investigate the role of metal ions such as Zn^{2+}, Fe^{3+}, Mg^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+} and Mn^{2+}on the antimicrobial activity of the P-80. We observed that antimicrobial activity of P-80 against *S. aureus* was not reduced when it was in the presence of all metal ions. However, Mg^{2+} ions stimulated over 20% its activity. The last data probably suggest that Mg^{2+} ion had effects on the structure of the P-80 protein.

Table 1 Effects of metal ions and inhibitors on P-80 activity in *Pseudoalteromonas* sp.

Metal ions and inhibitors (5 mM)	Relative antimicrobial activity (%)	Induction (%)	Inhibition (%)
EDTA	102	-	-
PMSF	99	-	-
EDTA-PMSF	97	-	-
TLCK	101	-	-
Zn^{2+}	106	-	-
Fe^{3+}	107	-	-
Mg^{2+}	120	120*	-
Ca^{2+}	99	-	-
Cu^{2+}	106	-	-
Co^{2+}	102	-	-
Mn^{2+}	102	-	-

* Statistically significant P< 0.001 Dunnett method

Ultraviolet-visible absorbance spectrum of antimicrobial P-80 protein

In Figure 5, the spectrum of antimicrobial protein in addition to showing the typical absorbance peak near of 280 nm revealed the presence of certain absorption in the UV region of longer wavelength (as bumps near of 363 nm), this features is characteristic of metalloproteins or proteins prone to aggregation.

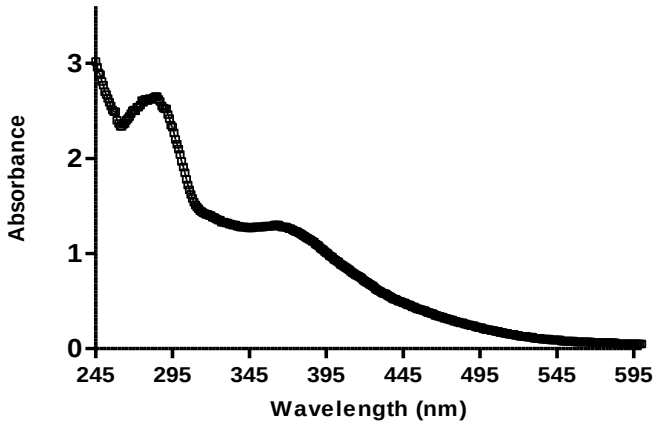

Figure 5 Ultraviolet-visible absorbance spectrum of antimicrobial P-80 protein of marine bacterium *Pseudoalteromonas* sp.

Cytotoxic effect of P-80 protein on cell lines

The antimicrobial protein (P-80) was found to be non-cytotoxic towards HeLa and Vero cells. The 95% of the confluence was observed and showed no alteration in the cellular morphology on cultures of 12, 24 or 48 h of incubation on all experimental conditions and controls, indicating that the P-80 protein showed no cytotoxic or antineoplastic effect (Figure 6)

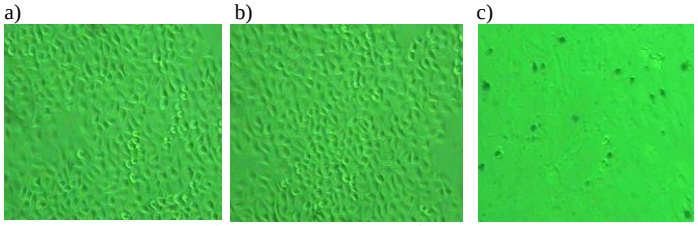

Figure 6 Morphology of HeLa cells 48 hours after incubation with P-80 protein: only one dose is shown since no differences were observed among different doses. A) P-80 dose of 25 mg/ ml, b) untreated control cells and c) positive control showing cells treaed with DMSO (0.5%).

DISCUSSION

An antimicrobial protein from *Pseudoalteromonas* sp. (*Ps*) was purified by ethanol precipitation followed by ion exchange chromatography (Fig 1). The molecular mass of P-80 determined by MALDI-TOF mass spectrometry was 78563 Da (Fig 2). According to previous reports (**López *et al*., 2012; Bautista *et al*., 2015**), they agree with this, antimicrobial proteins of high molecular mass from marine gender *Pseudoalteromonas* have been reported. For example, *Ps. tunicata* produces an antibiotic toxic protein with a molecular weight of 190 kDa or *Ps. piscisida* X153 and NJ6-3-1 produce an unstable tetrameric antimicrobial protein with a molecular weight of 280 kDa (**Chen *et al*., 2010**). In addition, some of them confirmed also the proteolytic capacity (**Mai-Prochnow *et al*., 2006**). The MALDI-TOF spectrum showed also a dimeric form around 155000 m/z suggesting a high tendency of P-80 to aggregation (Fig 2). Protein aggregation of other proteins has been studied and reported that protein aggregates can induce light scattering (**Bloemendal *et al*., 1989; Severinovskaya *et al*., 2014**). It is well known that proteins are only marginally stable in their folded state and the free energy of the system will favor associated state over individual molecules, e.g. depending on the environmental conditions largest proteins are highly prone to aggregation (**Munishkim *et al*., 2004**). **Ramshini *et al*. (2011)** mentioned that aggregation is an intrinsic characteristic of some big size proteins. Along the same line, a given mechanism dictates that initial stage of aggregation is quite specific and occurs when hydrophobic surface elements interact in an intermolecular manner (**Amin *et al*., 2014**), in other words, hydrophobic surface regions of proteins are the preferred sites for ligand binding and protein-protein association.

After exposing the P-80 protein to different pH values, we noted that the protein was stable at pH ranging 7.0 - 8.0 retaining its activity (around 90 % of relative activity). But at pH value above 9.0, it remarkably lost its antimicrobial activity (about 45%) (Fig 3). A similar pattern of pH effect was also reported for the PfaP antibiotic marine protein of *Pseudoalteromonas flavipulchra* JG1 where antibacterial activity against *Vibrio anguillarum* remained unaltered after treatment at pH 5.0-8.0 (**Yu *et al*., 2012**). **Chen *et al*. (2010)** reported an antimicrobial protein against methicillin-resitant *S. aureus* with tested activity at pH 7.8. But in contrast with these results an extracellular serine protease of *Pseudoalteromonas* sp. strain A28 involved in algicidal activities showing optimum at pH 8.8 (**Lee *et al*., 2000**). Our data are consistent with bacterial neutral proteases that show activities in a pH range between 5 and 8. Changes in pH of the environment greatly influence the protein-protein, protein-ligand, protein-membrane and peptide-membrane associations, so it can be said that the pH affects the main properties of these macromolecules, such as activity and stability (**Talley and Alexov, 2010**).

Regarding the temperature, P-80 protein retained its antimicrobial activity throughout the entire range that was tested (> 65% of activity is between -20 and 70 ºC) (Fig 4) Our data are in accordance with the CvL antibacterial protein from marine sponge *Cliona varians*, where activity was reported stable at temperature ranging from 4 to 60 ºC for 60 min, although its activity was rapidly lost at 70ºC and above (**Moura *et al*., 2006**). On the other hand, the antimicrobial activity of P-80 against *Staphylococcus aureus* was highly stable compared to other marine antibacterial proteins. For example, the P3 protein of oyster *Crassostrea belcheri* with antibacterial activity against *Vibrio harveyi, V. parahaemolyticus and V. cholera* was stable up to 45ºC within 30 min. (**Nuchchanart *et al*., 2007***)*, and the antibacterial activity of the protease of marine *Bacillus proteolyticus* was maximum between 40ºC and 50ºC and lost > 40% of its activity around 60ºC (**Bhaskar *et al*., 2007**). However, there are also thermally more stable antimicrobial proteins than the marine P-80 of *Pseudoalteromonas.* For example, the bacteriocin of *Bacillus amyloliquefaciens* that retained its antibacterial activity when exposed to temperatures ranging from 40ºC to 100°C for 30 min. (**Lim, 2016**). The heat- induced effect on the structure of proteins is well documented. However, having the most information about how a protein loses its stability due to the influence of the temperature is essential for almost all thermally related process for handling, storing, transporting or preservation of proteins. Protein thermal stability is an important property considered in medical or industrial applications and several elements contribute to resisting to the irreversibility of chemical or physical changes due to increase in temperature. **Kumar *et al*. (2000)** explored the thermostability with respect to the melting temperatures and found that salt bridges appear to show a correlation with structural stability of proteins. However, this property is recognized as a consequence of the combination of several factors acting in a synergistic manner, for example, the extent of hydrophobicity or increased surface area buries upon oligomerization, salt bridges, among others (**Kumwenda *et al*., 2013**).

It is well known that some metallic ions can participate in the correct folding of macromolecules and they can be responsible for the stabilization of the tertiary structure or to form part of the catalytic site. In this study, Mg^{2+} improved the antimicrobial activity of the p-80 protein by 20% although antimicrobial activity was not inhibited by EDTA. The rest of the ions had no effect on activity as shown in Table 1. Probably magnesium ion may have a role in stabilizing the structure of an antimicrobial protein or the metal was not bound directly to the protein, but was part of a ligand, as a Mg^{2+} ion in bacteriochlorophyll protein (**Rutkowska-Zbik *et al*., 2013**). This result was consistent with the presence of certain absorption as bumps at 363 nm in the UV region of longer wavelength, extending to the visible in the ultraviolet-visible spectrum (Fig 5), it is well known that this feature correspond to metalloproteins or proteins prone to aggregation.

On the other hand, a marine antimicrobial protein of marine *Pseudoalteromonas* sp. showed no cytotoxic effect *in vitro* in cultures of HeLa and Vero cells, even at high concentration (500 $\mu g\text{-}ml^{-1}$). Drug-induced toxicity is of considerable concern in drug discovery and development, placing emphasis on the need for predictive in vitro technologies that identify potential cytotoxic side effects of drugs (**Hughes and Karlen, 2014**). In summary, the above results are important because the molecule may be considered safe with a minimal risk of toxicity. Early toxicological assessment of candidate molecules is increasingly critical in the development of pharmaceutical compounds.

CONCLUSION

In the present study, we focused on structure and function of P-80. The antimicrobial protein showed a molecular mass of 78.5 kDa and no cytotoxic effect *in vitro*. In addition, while inhibitors, compounds have no effect on its antimicrobial activity, magnesium ion seems to have a role in stabilizing its structure by improving it. Likewise, P-80 remained 55 % and 65 % its antimicrobial activity against *Staphylococcus aureus* at pH 9.0 and 70 ºC respectively, suggesting high stability compared to other marine antibacterial proteins. Then P-80 protein has intrinsic features that could enable commercial applications.

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REFERENCES

Amin, S., Barnett, G.V., Pathak, J.A., Roberts, C.J., Sarangapani, P.S. (2014). Protein aggregation, particle formation, characterization and rheology. *Curr Opin Colloid Interface Sci*, 19, 438 – 449. https://doi.org/10.1016/j.cocis.2014.10.002

Cetina, A., Matos, A., Garma, G., Barba, H., Vázquez, R., Zepeda-Rodríguez, A., Jay, D., Monteón, V., López, R. (2010). Antimicrobial activity of marine bacteria

isolated from Gulf of Mexico. *Rev Peru Biol*, 17, 231- 236. http://www.redalyc.org/articulo.oa?id=195016139014
Bautista, J., Hernández, A., Monteón, V., López, R. (2015). Antimicrobial protein of the marine bacterium *Pseudoalteromonas* sp. and its proteolytic property. *JPR:BioMedRx: An Int J*, 9, 457- 462. http://www.jprsolutions.info
Bhaskar, N., Sudeepa, E.S., Rashmi, H.N., Selvi, A.T. (2007). Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. *Biores Technol*, 98, 2758 – 2764. http://dx.doi.org/10.4236/aer.2016.42004
Bernan, V.S., Greenstein, M., Maiese, W.M. (1997). Marine microorganisms as a source of new natural products. *Adv Appl Microbiol*, 43, 57-90. https://doi.org/10.1016/S0065-2164(08)70223-5
Bloemendal, M., van Amerongen, H., Bloemendal, H., van Grondelle, R. (1989). A structural study of bovine lens a-crystallin and its subunits by absortion and linear dichroism spectroscopy. *Eur J Biochem*, 184, 427 – 432. DOI: 10.1111/j.1432-1033.1989.tb15034.x
Bowman, J.P. (2007). Bioactive compound synthetic capacity and Ecological significance of marine bacteria genus Pseudoalteromonas. *Mar Drugs*, 5, 220-241. DOI: 10.3390/md504220
Brown, N.C., Eliasson, R., Reichard. P., Thelander, L. (1969). Spectrum and iron content of protein B2 from ribonucleoside diphosphate reductase. *European J Biochem*, 9, 512- 518. DOI: 10.1111/j.1432-1033.1969.tb00639.x
Chen, W.M., Lin, C.Y., Chen, C.A., Wang, J.T., Sheu, S.Y. (2010). Involvement of an l-amino acid oxidase in the activity of the marine bacterium Pseudoalteromonas flavipulchra against methicillin-resistant *Staphylococcus aureus*. *Enz Microb Technol*, 47, 52 – 58. https://doi.org/10.1016/j.enzmictec.2010.03.008
Demunshi, Y., Chugh, A. (2010). Role of traditional knowledge in marine bioprospecting. *Biodivers Conserv*, 19, 3015-3033. doi:10.1007/s10531-010-9879-9
Dominques, M.M., Silva, P.M., Franquelim, H.G., Carvalho, H.G, Castanho, M.A., Santos, N.C. (2014). Antimicrobial protein rBPI21-induced surface changes on Gram-negative and Gram-positive bacteria. *Nanomedicine*, 10, 543-551. DOI: http://dx.doi.org/10.1016/j.nano.2013.11.002
Egan, S., Thomas, T., Kjelleberg, S. (2008). Unlocking the diversity and biotechnological potential of marine surface associated microbial communities. *Curr Opin Microbiol*, 11, 219 —25. https://doi.org/10.1016/j.mib.2008.04.001
Kumar, S., Tasi, C-J., Nussinov, R. (2000). Factors enhancing protein thermostability. *Protein Eng*, 13, 179- 191. https://doi.org/10.1093/protein/13.3.179
Lee, S.O., Kato, J., Takiguchi, N., Kuroda, A., Ikeda, T., Mitsutani, A., Ohtake, H. (2000). Involvement of an extracellular protease in algicidal activity of the marine bacterium *Pseudoalteromonas* sp. strain A28. *Appl Environ Microbiol*, 66, 4334 – 4339. doi: 10.1128/AEM.66.10.4334-4339.2000
Lim, K.B., Balolong, M.P., Kim, S.H., Oh, J.K., Lee, J.Y., Kang, D-K. (2016). Isolation and characterization of a broad spectrum bacteriocin from *Bacillus amyloliquefaciens* RX7. *BioMed Res Int* V ID 8521476, 7 pages. http://dx.doi.org/10.1155/2016/8521476
López, R., Monteón, V., Chan, E., Montejo, R., Chan, M. (2012). Oxygen limitation favors the production of protein with antimicrobial activity in *Pseudoalteromonas* sp. *Braz J Microbiol*, 43, 1206 – 1212. http://dx.doi.org/10.1590/S1517-83822012000300048
López, R., Monteón, V., González, E., Montejo, R., Monteón, Y., Chan, M. (2012). Isolation and assessment of the crude extract antimicrobial activity of marine bacterium *Pseudoalteromonas* sp. *Rev Mex Cienc Farm*, 43, 38- 46. http://www.redalyc.org/articulo.oa?id=57928311006
López, R., Monteón, V. (2013). Influence of different organic nitrogen sources on antimicrobial activity of *Pseudoalteromonas* sp. *JPR:BioMedRx: An Int J*, 10, 952- 956. www.jpronline.info
Mai-Prochnow, A., Lucas-Elio, P., Egan, S., Thomas, T., Webb, J.S., Sanchez-Amat, A., Kjelleberg, S. (2006). Hydrogen peroxide linked to lysine oxidase activity facilitates biofilm differentiation and dispersal in several Gram negative bacteria. *J Bacteriol*, 190, 5493 – 5501. doi: 10.1128/JB.00549-08
Matsushima, R., Dano, H., Uchida, M., Ishihara, K., Suzuki, T., Kaneniwa, M. (2010). Analysis of extracelular alginate lyase and its gene from a marine bacterial strain, Pseudoalteromonasatlántica AR06. *Appl Microbiol Biotechnol*, 86, 567-576. doi:10.1007/s00253-009-2278-z
Moura, R.M., Queiroz, A.F.S., Fook, J.M.S.L.L., Dias, A.S.F., Monteiro, N.K.V., Ribeiro, J.K.C., Moura, G.E.D.D., Macedo, L.L.P., Santos, E.A., Sales, M.P. (2006). CvL, a lectin from the marine sponge *Cliona varians*: Isolation, characterization and its effects on pathogenic bacteria and Leishmania promastigotes. *Comp Bioc*, A 145, 517-523. https://doi.org/10.1016/j.cbpa.2006.08.028
Munishkim, L.A., Cooper, E.M., Uversky, V.N., Fink, A.L. (2004). The effect of macromolecular crowding on protein aggregation and amyloid fibril formation. *J Mol Recognit*, 17, 456 – 464. DOI: 10.1002/jmr.699
Ng, T.B., Cheung, R.C.F., Wong, J.H., Bekhit, A.A., Bekhit, A.E. (2015). Antibacterial products of marine organisms. *Appl Microbiol Biotechnol*, 99, 4145- 4173. DOI: 10.1007/s00253-015-6553-x
Nuchchanart, W., Poompuang, S., Tunkijanukij, S., Nakasathien, S. (2007). Characterization of antibacterial protein from hemolymph of oyster *Crassostrea belcheri*. *Kasetsart J (Nat Sci)*, 41, 169 – 179. http://kasetsartjournal.ku.ac.th/kuj_files/2008/A080402162915.pdf.
Ramshini, H., Parrini, C., Relini, A., Zampagni, M., Mannini, B., Pesce, A., Saboury, A.A., Nemat-Gorgani, M., Chiti, F. (2011). Large proteins have a great tendency to aggregate but a low propensity to form amyloid fibrils. *PlosOne*, 6, 1–10. https://doi.org/10.1371/journal.pone.0016075
Reichenbach, H. (2001). Myxobacteria producers of novel bioactive substances. *J Ind Microbiol Biotechnol*, 27, 149-156. doi:10.1038/sj.jim.7000025
Rutkowska-Zbik, D., Witko, M., Fiedor, L. (2013). Ligation of wáter to magnesium chelate of biological importance. *J Mol Model*, 19, 4661 – 4667. doi:10.1007/s00894-012-1459-3
Severinovskaya, O.V., Kovalska, C.B., Losytskyy, M.Y., Cherepanov, V., Subramaniam ,V., Yarmoluk, S.M. (2014). Application of MALDI-TOF mass spectrometry for study on fibrillar and oligomeric aggregates of alpha-synuclein. *Biopolym Cell*, 30, 190 – 196. DOI: 10.7124/bc.000895
Talley, K., Alexov, E. (2010). On the pH-optimum of activity and stability of proteins. *Proteins*, 78, 2699 – 2706. DOI: 10.1002/prot.22786
Wilson, G.S., Raftos, D.A., Nair, S.V. (2011). Antimicrobial activity of surface attached marine bacteria in biofilms. *Microbiol Res*, 166, 437- 448. https://doi.org/10.1016/j.micres.2010.08.003
Yu, M., Wang, J., Tang, K., Shi, X., Wang, S., Zhu, W.M., Zhang, X.H. (2012). Purification and characterization of antibacterial compounds of *Pseudoalteromonas flavipulchra* JG1. *Microbiology*, 158, 835 – 842. doi: 10.1099/mic.0.055970-0
Zasloff, M. (2002). Antimicrobial peptides on multicellular organism. *Nature*, 415, 389- 395. doi:10.1038/415389a