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Study on the stability of new promising antimicrobial peptides against protease activities present in infection sites

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Bacterial respiratory infections are one of the leading causes of mortality worldwide, being associated with million deaths annually. Due to the increasing resistance of major respiratory bacteria to available antibiotics, coupled with the depletion of the traditional antibiotic pipeline, treatment options have become severely restricted. The emergence of **antimicrobial resistance** presents a significant threat to global health and highlights the urgent need for novel therapies.

Nature-inspired antimicrobial peptides (**AMPs**) are rapidly gaining attention for clinical translation, as they offer distinct advantages over conventional antibiotics by being molecules with multiple functions with rapid activity against a broad spectrum of microorganisms. AMPs of animal origin are molecules of the innate immunity of eukaryotes which act as a first-line defense against infections (many of them are evolutionarily conserved). They are less likely to elicit resistance than peptides of synthetic origin. Their structure differs from human AMPs, such as LL-37, minimizing the risk of inducing bacterial resistance to our endogenous AMPs or triggering autoimmune responses. Furthermore, AMPs are composed of amino acids: renewable and harmless compounds that do not persist in the environment and also provide harmless degradation products for human health. Therefore, AMPs demonstrate their intrinsic "green" nature through biodegradability and the absence of harmful degradation products. Several naturally occurring peptides with interesting properties were selected, such as (i) short helical AMPs active on the membrane of microorganisms (**MA-AMP**), (ii) or short, proline-rich AMPs that can act at an intracellular level by inhibiting protein synthesis (**PR-AMP**). The combined use of MA- and PR-AMP with different modes of action is expected to increase activity and significantly prevent or slow the incidence of resistance.

The objective of this study is to evaluate the properties of these AMPs for the development of a valid alternative to current therapies for the treatment of lung infections caused by "priority bacterial pathogens" such as *Staphylococcus aureus* and *Pseudomonas aeruginosa*. With this aim, **peptides stability** will be studied in environments where elastases of endogenous or bacterial origin are present. The peptide stability profile will be shown by HPLC and MALDI-TOF analyses. Trials for the development of a new peptide-based drug will continue for the most promising peptides.

STABILITY

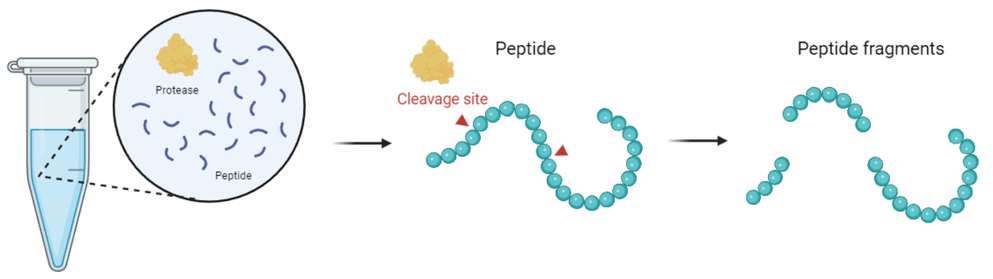

Figure 1. *Enzymatic action inside the test tubes and generation of fragments that will be analyzed by mass spectrometry (MALDI-TOF)*

PEPTIDEs

Peptide A – It is derived from larger peptides (21 residues). They are efficient in countering *P. aeruginosa* lung infection in mice, and they also inhibit *P. aeruginosa* biofilm formation under standard conditions.

Peptide B – It is an unusually short MA-AMPs (13 residues), mainly active against Gram-positive bacteria; can eradicate preformed *S. aureus* biofilms under standard conditions and are also active on intracellular *S. aureus*.

PROTOCOL

Peptide solution: 1 mg/mL
Peptide/enzyme ratio: 120 nmol / 1 unit
Reaction Volume: 130 µL

Incubation at 4 times: T_0, T_1, T_5 and T_{24}

HPLC analyses to verify the formation of fragments
MS analyses to identify the fragments from their weight.

TIME COURSE STABILITY

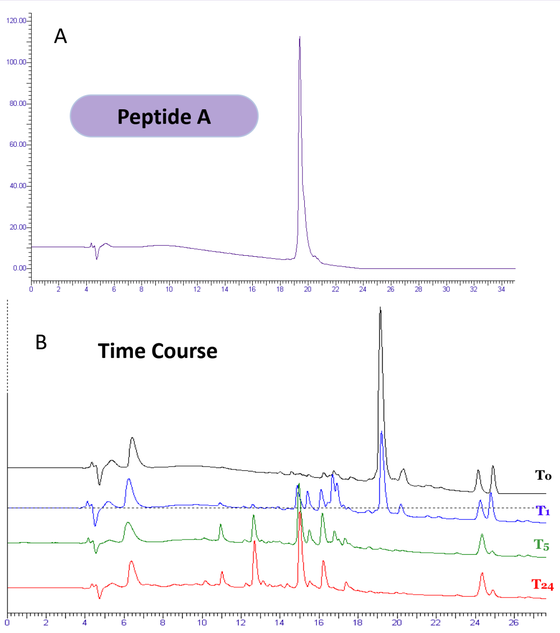

Figure 2. *A) HPLC analysis of Peptide A dissolved in TrisHCl 10 mM. B) HPLC analysis of Peptide A at 4 incubation times (0h, 1h, 5h, 24h) with human elastase in a ratio of 120nmol of peptide for 1unit of enzyme.*

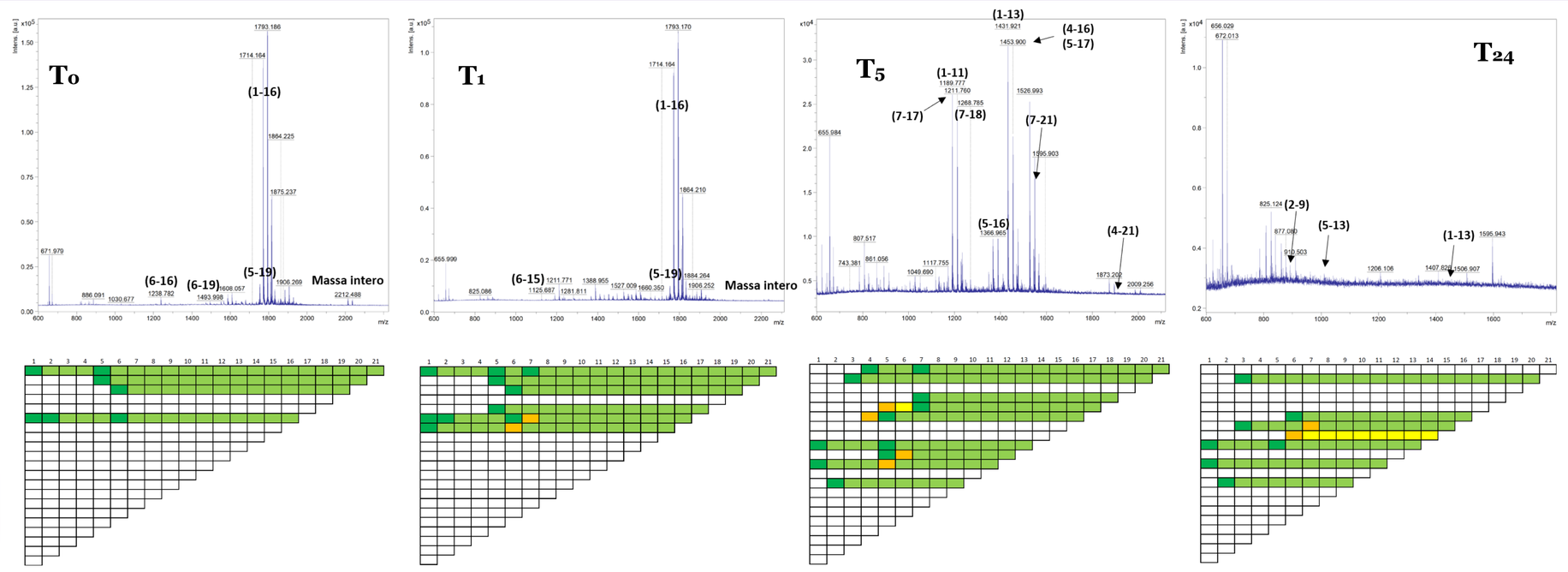

Figure 3. *Above, mass spectrometric analysis of Peptide A at 4 different incubation times. Below, graphic representation of the fragments found in the MS analyses.*

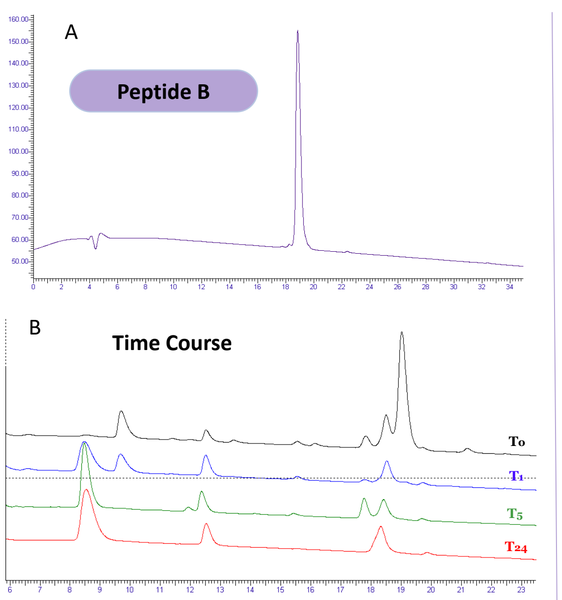

Figure 4. *A) HPLC analysis of Peptide B dissolved in TrisHCl 10 mM. B) HPLC analysis Peptide B at 4 incubation times (0h, 1h, 5h, 24h) with human elastase in a ratio of 120nmol of peptide for 1unit of enzyme.*

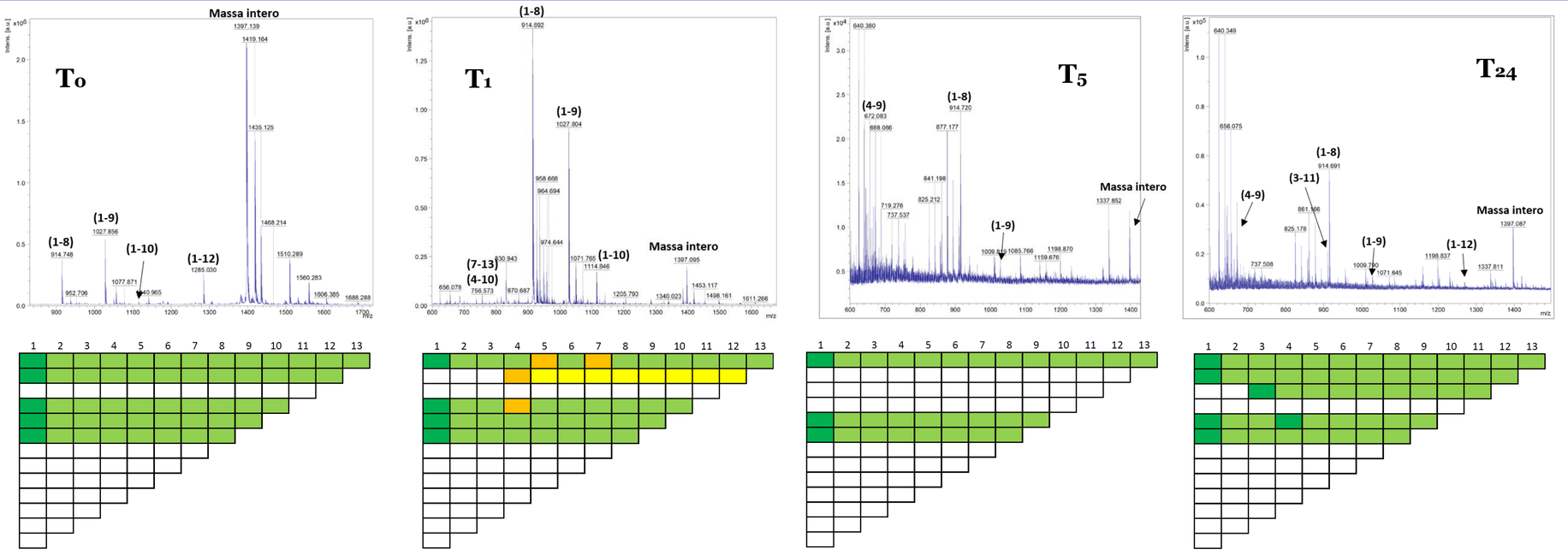

Figure 5. *Above, mass spectrometric analysis of Peptide B at 4 different incubation times. Below, graphic representation of the fragments found in the MS analyses.*

DISCUSSION

One of the main limits in using AMPs for treatment of lung infection is their susceptibility to enzymatic degradation. The lung environment of cystic fibrosis patients is rich in proteases and it is therefore important that any AMPs should not be susceptible to their enzymatic action. To determine whether these proteases affect the antimicrobial activity of peptides, their stability was analysed using purified human neutrophil elastase. AMP were incubated with the enzyme and the resulting solutions were analysed by HPLC and mass spectrometry at defined time points.

Peptide A undergoes complete degradation within 5 hours by human neutrophil elastase as confirmed by both HPLC and mass spectrometry analyses.

In contrast, **Peptide B** showed a faster degradation rate, according to the HPLC analyses, with minimal detectable quantities after 1 hour of incubation. However, mass spectrometry analyses, with a greater sensitivity, detect its presence even after 24 hours of incubation with the enzyme.

CONCLUSIONs

The purpose of this project is to identify the **best therapeutic peptides** for treatment of *P. aeruginosa* and *S. aureus* for lung infection starting from previously tested peptides that have shown promising results.

Among the peptides examined, **Peptide B** appears to have greater resistance to the enzymatic action of human elastase and, given the data previously observed, we expect that this stability will also persist against elastases of bacterial origin.

Other peptides will be tested for their stability. The most valid peptides will also be subjected to **efficacy**, **cytotoxicity**, **synergy** and **resistance** studies. For those peptide that will pass this first screening phase, further studies will be carried out in lung mimicking environments and then and will continue with the *in vivo* testing phase.

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