Nisin Z as Potential Alternative to Traditional Antibiotics: Purification by Two Multi-chromatographic Procedures

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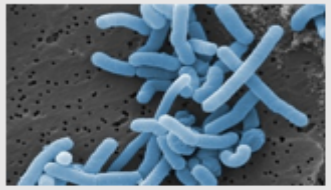
Introduction

The intensification of the aquaculture sector has led to the emergence and occurrence of infectious diseases in fish farming. Bacterial disease outbreaks, the erroneous use and overuse of antibiotics, and the emergence of antibiotic-resistant bacteria urge the development of alternative antimicrobial strategies to the conventional antibiotherapy, both in human and veterinary medicine. In this scenario, Lactic Acid Bacteria (LAB) are receiving a great interest as probiotics in aquaculture.



LAB exert antimicrobial activity through several mechanisms, such as production of organic acids, D-amino acids, ethanol, reuterin, and ribosomally-synthetized peptides, with or without post-translational modifications (*i.e.*, bacteriocins). Nisin A (NisA), a 34-residue long lantibiotic produced by several *Lactococcus lactis* strains, is the most well-characterized bacteriocin, and exerts a broad antimicrobial spectrum against several bacterial ichthyopathogens. To date, several NisA variants have been discovered, with nisin Z (NisZ) being the most commonly found in nature.

As bacteriocins are proteinaceous compounds secreted to the extracellular medium, their purification is regarded as crucial for their application as alternative to antibiotics. In this context, the aim of this work was to compare two distinct multi-chromatographic procedures for purification of NisZ from cell-free culture supernatants (CFS) from *L. lactis* RBT18, a bacteriocinogenic strain previously isolated by our group from cultured rainbow trout (*Oncorhynchus mykiss*, Walbaum).

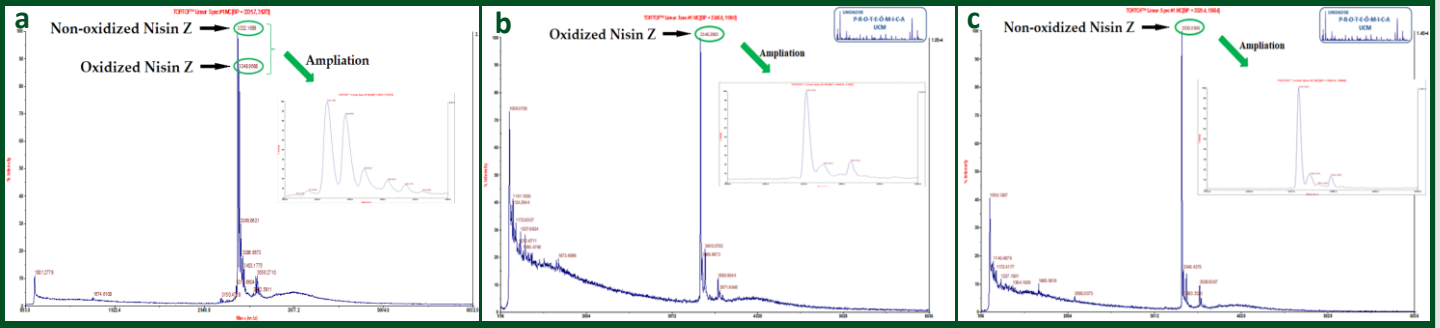


Figure 1. MALDI/TOF-TOF mass spectrometry analyses of purified NisZ from *L. lactis* RBT18: a) oxidized and non-oxidized forms of NisZ after Purification Procedure I; b) oxidized form of NisZ after Purification Procedure II, and c) non-oxidized form of NisZ after Purification Procedure II.

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Materials and Methods

Table I. Summary of the two multi-chromatographic purification procedures *.

Steps	Purification Procedure I Modification of the procedure described by Cintas *et al.* (1995)	Purification Procedure II Based on the procedure described by Field *et al.* (2012)
1	*L. lactis* RBT18 grown overnight in MRS broth (30 ºC)	*L. lactis* RBT18 grown overnight in GM17 (30 ºC)
2	Centrifugation and obtention of a CFS	Cultured *L. lactis* RBT18 inoculated into TY broth and re-cultured overnight
3	Concentration step with ammonium-sulphate	Centrifugation and obtention of a CFS
4	Gel filtration with PD-10 columns	Elution at a column packed with Amberlite XAD-16 beads, with isopropanol 70% supplemented with 0.1% TFA
5	Cation exchange chromatography with a SP Sepharose Fast Flow resin	Isopropanol evaporation through use of a rotary evaporator
6	Hydrophobic interaction with Octyl Sepharose CL-4B resin	Elution through a Mega BE-C18 column lined to a vacuum pulling system
7		Isopropanol re-evaporation through use of a rotary evaporator
8	**Reverse-phase Fast Protein Liquid Chromatography (RP-FPLC)**	

* Antimicrobial activity of fractions obtained during bacteriocin purification was quantified by a microtiter plate assay (MPA) using *Lactococcus garvieae* CF00021 as indicator microorganism.

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MALDI/TOF-TOF MS

Results and Discussion

Table II. Final RP-FPLC of bacteriocins from *L. lactis* RBT18 by using the two multi-chromatographic purification procedures.

		Volume (ml)	Total A_{280} [a]	Total activity (10^3 BU) [b]	Specific activity [c]	Increase in specific activity [d]	Yield (%) [e]
	Procedure I						
a	**Reversed-phase chromatography**	1.5	0.031	983	31,700,000	348,000	77
	Procedure II						
b	**Oxidized NisZ**	0.5	0.010	10	975,000	2,700	0.4
c	**Non-oxidized NisZ**	0.6	0.007	98	12,600,000	35,000	3.8
	Oxidized NisZ + Non-oxidized NisZ	1.1	0.017	108	6,300,000	16,400	4.2

[a] Absorbance at 280 nm (A_{280}) multiplied by the volume (mL). [b] Antimicrobial activity in bacteriocin units per milliliter (BU/mL) and multiplied by the total volume (mL). [c] Specific antimicrobial activity expressed as the total antimicrobial activity (BU) divided by total A_{280}. [d] Specific antimicrobial activity of a fraction (BU/A_{280}) divided by the specific antimicrobial activity of the first supernatant (BU/A_{280}). [e] Yield expressed as the total antimicrobial activity (BU) of a fraction multiplied by 100 and divided by the total antimicrobial activity (BU) of the CFS.

The first purification procedure resulted in an absorbance peak (eluted at 58% elution buffer, v/v) showing antimicrobial activity, with a yield of 77% and a 348,000-fold increase in the specific antimicrobial activity compared to that of the cell-free supernatant. The second purification procedure resulted in two antimicrobial active absorbance peaks (55 and 58% elution buffer, v/v, respectively), with a combined yield of 4% and a 16,000-fold-increase in the initial specific antimicrobial activity. In both cases, MALDI-TOF MS analyses confirmed the presence of purified NisZ (3,330 Da) and its oxidized-form (3,346 Da), both showing antimicrobial activity.

Concluding Remarks

In summary, our results show that while both procedures are suitable for NisZ purification, the first one is more appropriate and effective, since the antimicrobial activity yield and the increase in specific antimicrobial activity were 18- and 21-times higher, respectively. Nevertheless, further research is necessary to optimize bacteriocin production, and purification and to reduce bacteriocin oxidation in order to obtain economically viable yields of highly active NisZ that allow its use as an effective alternative to antibiotics in aquaculture.