Different strategies for purification of antimicrobial peptides from Lactic Acid Bacteria (LAB)

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Bacteriocins are natural peptides secreted by several bacteria that exert bactericidal activity against other bacterial species. Research in this topic is promoted not only by the need to develop alternatives to antibiotics and drugs that have shown non-desirable effects, but also to the capacity of some bacteriocin to inhibit saprophytic and food-borne pathogens in food stuffs. Many LAB are bacteriogenics, and the purification of the bacteriocins produced is helpful for the knowledge of the mechanism of action, structure, and other characteristics, which helps to isolate the bacteriocin biosynthetic genes. In this chapter, different techniques applied to purify some bacteriocins from LAB are described. The most frequently applied techniques involve salt precipitation followed by various combinations of ion-exchange (IEC) and reverse phase C18 solid phase extraction, absorption-desorption (AD), reverse-phase high-performance liquid chromatography (RP-HPLC) and Sodium Dodecyl Sulphate Polyacrilamide Gel Electrophoresis (SDS PAGE). The results obtained through the purification of different LAB bacteriocins (enterocin and salivaricin) carried out in our laboratory are included to discuss the different purification strategies applied.

Keywords: bacteriocin, purification, Lactic acid bacteria, Lactobacillus, Enterococcus

1. Introduction

Microbes compete for the limited space and nutrients present in natural ecological niches, therefore they have developped several strategies in order to survive: production of antimicrobial agents such as bacteriocins is one of them. Gram-positive bacteria, and mainly lactic acid bacteria (LAB), are now being increasingly studied for their production of bacteriocin-like substances [1].

Bacteriocins are ribosomally synthesized antimicrobial peptides widely distributed in nature. This peptide biodiversity is supported by several differences in their structures. All constitutively synthesized peptides, regardless of sub-classification, share a net positive charge which causes them to fold into an amphiphilic conformation upon interaction with bacterial membranes [2]. Most characterized bacteriocins are heat-stable, nontoxic, and susceptible to degradation by proteolytic enzymes present in the gastrointestinal tract [3].

The increased consumption of foods containing additives formulated with chemical preservatives and consumer concerns have created a higher demand for more natural and minimally processed foods, therefore, there is a high interest in naturally produced antimicrobial agents that do not produce adverse effects. This interest and also the potential applications in health care sectors have attracted the interest of academia and industry resulting in increased numbers of published research on bacteriocin production, purification, genetics, and applications.

Adequate purification of bacteriocins is necessary for their characterization. The elucidation of their biochemical structure requires a pure, homogeneous peptide as well as an adequate yield of protein. A

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rapid, reliable, quantitative and sensitive method for detection of bacteriocins became essential in the search for new alternatives with potential industrial applications.

Based on the heterogenous nature of bacteriocins a general method or protocol is not available [1], and a wide range of methodologies for the purification Lactic Acid Bacteria (LAB) and related microorganisms bacteriocins have been described, as resumed in Table 1.

Table 1. Bacteriocins from LAB and related microorganisms. Some aspects of their purification

Bacteriocin	Purification	Steps	Yield	Ref
Plantaricin ST31	Ammonium sulfate precipitation - Sep-pack C18 cartridge – RP-HPLC	3	0.8%	[4]
	Cation exchange Chromatography	1	5.9%	
Nisin Z	Expanded bed, Ion-exchange chromatography (EB-IEC)	1	90%	[5]
Enterocin AS48	Cation exchange – Reverse Phase Chromatography	2	24.3%	[3]
Plantaricin C19	Adsorption/release – RP-HPLC	2	15%	[6]
Pediocin ACM	Adsorption-desorption – RP-HPLC	2	40,4%	[7]
Salivaricin	Ammonium sulfate precipitation – C_{18}	2	7,3%	This work
Unnamed bacteriocins	Ammonium sulfate – Tricholoroacetic acid Precipitation - Ultrafiltration	3	1.0 - 1.2%	[8]
Pediocin PA-1	Etanol precipitation - Isoelectric focusing - Ultrafiltration	3	29	[9]
Lactococcin B		3	41	
Mesentericin Y105	Cation exchange chromatography – C_{18} – HPLC	3	60%	[10]
Sakacin A		3	10%	
Sakacin P		3	10%	
Enterocin A		3	66%	
Pediocin A-1		3	25%	
Divercin V41		3	10%	
Acidocin D20079	Ammonium sulphate precipitation - Cation exchange chromatography - Octyl Sepharose column.	3	16%	[11]
Lacticin Q	Acetone precipitation - Cation exchange chromatography – RP-HPLC	3	64%	[12]
Lactobin A	Ammonium sulfate precipitation - Chloroform-methanol extraction - RP-FPLC	3	0,07%	[13]
Divergicin M35	Cation exchange chromatography - Sep-Pack C_{18} - RP-HPLC	3	10%	[14]
Enterocin CRL35	Ammonium sulfate precipitation - Molecular filtration Biogel P-6 - Cation exchange chromatography - HPLC	4	2%	[15]
Bozacin 14	Ammonium sulfate precipitation – Sep-Pack C_{18} – RP-HPLC	4	4.4%	[16]
Mutacin B-Ny266	C_{18} column - HPLC (x3)	4	1.0 %	[17]
Abp118	Ammonium sulfate precipitation - Hydrophobic interaction - Cation exchange chromatography – C_8/C_{18} RP-FPLC.	4	6,4%	[18]
Macedocin	Ammonium sulfate precipitation - Anion exchange chromatography - Cation exchange chromatography - Reverse-phase chromatography - Gel filtration	5	1,6%	[19]

Michiganin A	Ammonium sulfate precipitation - Cation exchange chromatography - RPC column - pepRPC HR 5/5 column - μRPC C_2/C_{18} column.	5	3%	[20]
Piscicocin CS526	Ammonium sulfate precipitation - gel-filtration - cation exchange chromatography - C_{18} cartridge - RP-HPLC.	5	7%	[21]
Propionicin F	Ammonium sulphate precipitate - Anion-exchange chromatography - Reverse-phase chromatography (x3)	5	0,5%	[22]
Pediocin PD-1	Ammonium sulphate precipitation – dialyses – lyophilization – methanol – chloroform extraction – cation exchange.	6	34%	[23]

In this chapter, some strategies implemented in our laboratory for the purification of bacteriocins are described using two different antimicrobial compounds as models: salivaricin CRL1328, and enterocin CRL35.

Lactobacillus (*Lb.*) *salivarius* CRL1328 is a probiotic microorganism isolated from the vagina of a healthy woman [24]. Ths strain produces a bacteriocin, named salivaricin CRL1328 which was characterized preliminarily, and was the first bacteriocin described as being produced by a vaginal isolate [24]. Recently, the salivaricin biosynthesis genes have been characterized (data not shown, Genbank accesion number AF592482). This bacteriocin resulted to be a two component bacteriocin (class IIb) identical to the bacteriocin described by Flynn et al. [18] produced by *Lb. salivarius* UCC118 of intestinal origin. On the other hand, enterocin CRL35 is produced by *Enterococcus* (*E.*) *mundtii* CRL35, isolated from an artisanal cheese [15]. Enterocin is a class IIa bacteriocin [15],[25].

2. Protocols for bacteriocins purification

2.1. Culture media.

The composition of the growth medium greatly affects the production of individual bacteriocins [26]. Several media have been evaluated by numerous authors to improve bacteriocin synthesis [27], because these peptides are not always produced in standard or enriched culture media. Lactic acid bacteria are fastidious microorganisms that require rich media containing milk, whey ultrafiltrate, or complex synthetic media such as MRS [28], M17 [29] or LAPTg [30] for growth. Therefore, the isolation of a peptide(s) in rich-medium supernatant is an additional problem, making the purification of the bacteriocin a relatively complicated protocol. Furthermore, many bacteriocin molecules tend to associate with other substances present in the culture medium, where they can display a high degree of hydrophobicity and form protein aggregates. In this sense, a chemically defined medium (CDM) which allows the growth and bacteriocin production by LAB would be be very useful for determining the influence of different nutrients on bacteriocin production and for an easy and efficient downstream processing. However, CDMs for LAB are very complex and time consuming, reasons for which they are not used routinely. Their use is limited to very specific studies, and traditional methods are normally used for screening strategies. In our laboratory, the use of LAPTg (15g/L peptone, 10g/L tryptone, 10g/L yeast extract, 10g/L glucose, and 1 mL/L Tween-80, final pH 6.5) medium have been appropriate for some bacteriocinogenic strains of lactobacilli and enterococci. In the specific studies of salivaricin CRL 1328, even though it is produced in MRS broth, the highest activity was detected in a less enriched broth, such as LAPTg [27].

2.2. Screening of inhibitory activity.

There are several screening methods available to detect antimicrobial activity from supernatants of hypothetical producer strains: a) spot-on-lawn assay [31], b) disc diffusion [32], c) microtiter plate assay [33], d) agar well diffusion assay [34], e) multi-well plate assay [35], among others. Even though there are several methods applied by the scientist with this purpose, the most frequently used are described below. It is very important to be able to screen many strains in an easy, fast and reliable way.

In our laboratory, a few indicators organisms were selected to be used in routine antimicrobial test: *Listeria innocua* 7 (INRA collection), *E. faecalis* CRL341 and *Lb. plantarum* CRL691. This last strain has resulted to be sensitive to a wide range of bacteriocins: class I (lantibiotics), IIa (pedicocin-like anti-Listeria), and IIb (two component bacteriocins).

When performing a screening of potential bacteriocin producer strains, a parameter to take into account is the culture media used to grow the sensitive strain. Recent results have shown that when a sensitive strain is grown in a medium that contain enzymatic casein hydrolisate smaller inhibition haloes are formed than when grown in a medium with acid hydrolysate of casein [36]. Probably the higher content of medium size peptides in the enzymatic hydrolysate may interfere in some way with the bacteriocin activity.

2.2.1. Spot-on-lawn method.

In the protocol used, usually 10 μl of an overnight culture of the sensitive strain was inoculated in 7 ml of the appropriate soft-agar media (0,7% agar) and this mixture (top agar) transferred to plates prepoured with agar base (1,5% agar). 5 μl of each supernatant (or extract) of 16 h cultures of LAB were spotted onto this lawn and plates incubated in upright position. The inhibition zone was evaluated after overnight incubation.

2.2.2. Well diffusion assay method.

The soft-agar culture media was inoculated with the sensitive strain (top agar) and plated over the agar base. Wells were performed with a sterile plastic straw and inoculated either with the cell supernatants or purified extracts.

The inhibitory activity detected as described previously in paragraphs 2.2.1 or 2.2.2 was expressed quantitatively as arbitrary units per milliliter (AU/mL) by testing serial two-fold dilutions. The arbitrary unit (AU) was defined as the reciprocal of the highest dilution able to produce a clear zone of growth inhibition of the indicator strain.

2.2.3 Microtiter plate assay.

The microtiter plate assay is an alternative technique useful to screen several supernatants or extracts for inhibitory activity. Although we have not used this methods for preliminary screenings of supernatant, it has been widely applied for the analysis of the antimicrobial activity of the "peaks" collected through the different purification and chromatographic steps, for the comparative analysis of the Minimal Inhibitory Concentration (MIC), and to study of the mechanism of action of the antimicrobial compounds. For routine analysis using microtiter plates, a microplate reader with temperature regulation and agitation capacity is recommened, so that the plate can be shaken before every absorbance determination. The Versamax Microplate Reader (Molecular Device) associated with its Softmax pro software has been shown to be very useful for these purposes.

In our laboratory, different bacteriocins were detected and studied, by the application of the methodologies described above. The sensitivity patterns of salivaricin [24] and enterocin [15] have been partially published before, and are shown in Table 2

Table 2. Sensitivity patterns of Salivaricin CRL 1328 and Enterocin CRL 35.

Indicator strain	Salivaricin CRL1328	Indicator strain	Enterocin CRL35
Enterococcus faecium ATCC 19434	+	*Lb. plantarum* CRL691	+
E. faecalis	+	*Listeria monocytogenes* FBUNT	+
E. faecalis ATCC 19433	+	*Listeria innocua* 7	+
E. faecalis CRL 318	+	*Lb. sakei* DSM20017	+
E. faecalis CRL 341	+	*Lb. delbruekii subsp lactis* LKT	-
N. gonorrhoeae	+	*Lb. helveticus* CRL1176	-
L. paracasei subsp. *paracasei* CRL 1289	+	*Lb. reuteri* CRL1098	-
Candida sp.	-	*Lb. johnsonii* NCK65	-
Streptococcus agalactiae ATCC 27956	-		
Gardnerella vaginalis	-		
Streptococcus group B spp.	-		

2.3. Concentration of the bacteriocin-containing supernatant.

To begin the characterization of a new antimicrobial peptide is important to decide and to implement a purification strategy. Many LAB bacteriocins are not produced in high amounts by the producer strain, therefore it is very important to concentrate the supernatant that contains the antimicrobial substance at the very initial steps. For this purpose, many protocols have been implemented: ammonium sulfate concentration,, absortion-desortion, and organic solvent extraction.

2.3.1. Ammonium sulfate precipitation.

Due to their proteinaceus nature, bacteriocins can be concentrated through the application of salting-out methods, being ammonium sulfate the most frequently used. In this procedure the solid salt is added to the sample slowly until the desired saturation percentage of ammonium sulphate is reached.

A 18-h-old culture of the bacteriocinogenic LAB strain was centrifuged (9000×g, 10 min, 4ºC) and the peptidic fraction precipitated from the cell-free supernatant with 70% saturated ammonium sulphate [37]. Some bacteriocins can precipitate at lower ammonium sulphate concentrations, or even in a small range of saturation, then is important to assay which is the concentration of salt that precipitates the peptide of interest. The suspension was incubated overnight at 4°C and agitated with a magnetic stirrer. Salted-out proteins were precipitated by centrifugation (10000×g for 20 min) and dissolved in a small volume of 10 mM phosphate buffer (pH 7.0) or distilled water. The suspension can be desalted by dialysis with phosphate buffer at 4°C during 12h by using benzoylated membranes (molecular weight cut off 1200; Sigma-Aldrich) or with dialysis cassettes with cut-off of 2000 to 3500 (Pierce Biotechnology, Inc). Since most bacteriocins have a size smaller than 10000 Da, the use of regular dialysis bags with cut-off of 10000 -12000 Da is inappropriate for this procedure.

The concentration of salivaricin CRL 1328 by the application of this methodology is showed in Table 4.

2. 3.2. Adsorption-desorption method.

This protocol developed by Yang [38] relies on the property of several bacteriocins to adsorb to the producer cells at neutral pH, and their release after being treated with a low pH (between pH 2–2.5). In the protocol used for salivaricin, the bacterial strain was grown overnight in LAPTG broth and heated at 70ºC for 25 minutes to inactivate the microorganisms. The pH of the culture was adjusted to pH 7.0 with 5M NaOH to allow bacteriocin adsorption to the bacterial cells. The cells were collected by centrifugation, washed twice with 5mM sodium phosphate buffer (pH 7), resuspended in 0,1M NaCl solution (pH 2.0, adjusted with HCl) to 40 times the original volume of the culture, and agitated at 4°C

for 1 h to release the bacteriocin molecules from the cell surface. The cells were separated by centrifugation (14000 x g 30min at 4ºC) and the supernatant subjected to different studies. This extract was rather clear and can be loaded onto a SPE C_{18} cartridge as described below. In Table 4 an example of the adsorption-desorption assay with salivaricin CRL1328 is shown. The recovery of salivaricin by this methodology is very low, probably because is a complex two-components bacteriocin. One of the advantages is that a lower number of contaminant proteins is obtained compared with the ammonium sulphate precipitation described before.

Table 3. Absorption-desorption method applied to the purification of salivaricin CRL132

Fraction	Volume ml	Activity UA/ml	Protein ug prot/ml	Spec. Act. UA/ug prot	Total Act. UA	Total Prot mg	Yield %
Supernatant	1000	400	223.0	1.79	400000	223000.0	100.0
1st wash	10	0	151.0	0.00	0	1510.0	0.0
2nd wash	10	0	55.0	0.00	0	550.0	0.0
Desorbed	50	200	43.0	4.65	10000	2150.0	2.5

2.3.3. Organic solvent (ethanol or acetone) extraction.

This protocol has been used successfully for the concentration of some bacteriocins including Lactococcin B, Pediocin PA1, and recently Lacticin Q with acetone [12]. In our laboratory, to concentrate the bacteriocin, the free-cell supernatant was treated with three volumes of cold acetone, stored a -30°C overnight and later centrifuged to recover the peptidic fraction in the pellet. The suspension was then subjected to Cation Exchange Chromatography.

2.4. Purification of bacteriocins.

The methodology described above produces a partially purified extract of bacteriocins. These extracts must be subjected to other purification step, that includes the use of different strategies. The most frequenly used techniques are C18 Solid Phase exctraction (SPE) or Ion Exchange Chromatography (IEC). The selection of one or other possibility (or both) will depend on the knowledge of the peptide characteristics.

2.4.1.Ion Exchange Chromatography.

The IEC chromatography could be used either with cation or anion exchange resins, that are able to separate the peptides by their electric charge at definited pH. Since most bacteriocins have positive charges at pH near neutrality, the use of cation exchange resins is appropiate for their purifications. Numerous type of cationic resins have been employed, from simple Carboxy-methyl cellulose to a more "sophisticated" strong cation exchanger like SP Fast Flow Sepharose (Table 1). In a standard protocol the bacteriocin extract is passed through the cation-exchange column, whashed with a phosphate buffer containing NaCl at concentration of 50 to 100 mM (this step will remove the anionic contaminants), and the bacteriocin eluted with a gradient of NaCl from 100 to 1000 mM. A convenient alternative to conventional column is the use of cartridges or SPE columns that are packaged with a strong-cation exchange resin (Mega-BE SCX, Varian).

2.4.2. Reverse Phase SP Extraction columns

The salted-out proteins or the dialysate (without salt) containing the bacteriocin activity are loaded onto a C_{18} cartridges/columns previously equilibrated with the adequate buffer. The column was washed with the same buffer, followed by a gradient of acetonitrile or 2-propanol (10-80% in 20 mM ammonium acetate), collecting all the fractions and selecting the active fractions for the next steps. Depending on the initial amount of protein in the sample one or more C_{18} columns can be used. In addition, cartridges of different sizes and resin contents are available in the market. For large protein contents the high capacity Mega Bond Elut C_{18} columns are suitable (Varian Inc.). The bacteriocins crude extract was eluted directly with a gradient of 20-80% (v/v) 2-propanol in distilled water. The flow rate was 0.5 ml/min. One ml fractions were collected, concentrated with nitrogen flux or vacuum and tested for antibacterial activity by the spot-on-lawn or well diffusion methods by using the appropriate sensitive strain. The extract are stored at -20 ºC. One example of the results obtained by the application of this methodology to salivaricin is shown in Table 4.

Table 4: Ammonium sulfate precipitation and solid phase extraction

Fraction	Volume ml	Activity UA/ml	Protein mg prot/ml	Spec. Act UA/mg prot	Total Act. UA	Total Prot mg	Yield %
Supernatant	130	800	187.5	4.27	104000	24377.3	100.0
A.sulfate	1.5	12800	6102.9	2.10	19200	9154.4	21.8
C_{18}	4	1600	625.3	2.56	6400	2501.1	7.3

2.4.3. Reverse-phase (RP) HPLC.

This analytical technique has been shown to be extremely valuable for the analysis of these antimicrobial peptides, since bacteriocins are generally resistant to different organic solvents used as mobile phases and the high pressures employed trough the chromatographic process.

In the HPLC protocol, after the sample has been separated by cation exchange or SPE chromatography, the bacteriocin extract is loaded onto the HPLC column. In the purification protocols described by the authors listed in the references of this chapter, several types of RP columns bas been used, being the silica-based C4 to 18 ODS the most frequently employed (Table 1). Although for peptide separation a 300 Å pore column is sometimes recommended, the frequent experimentation shows that 100 A pore columns are also effective for bacteriocin purification. In the protocols used in our laboratory, purifications were performed by the frequent use of a 300 Å C8 Vydac and a Tosoh TSK gel ODS120T columns. In figure 1, an example of the HPLC chromatogram generated from the purification of salivarin CRL1328, a complex bacteriocin, is shown. Since this bacteriocin belong to class IIb type bacteriocin, two peptides are required for antimicrobial activity, so an even more complicated chromatographic separation is expected. In the figure, the elution of the peptidic components of the bacteriocin is indicated with arrows. The inhibitory activity is found in fractions collected in between the retention time of the peptides (hatched area). The mobile phase consisted of (i) acetonitrile and (ii) HPLC-grade water containing 0.1% trifluoroacetic acid (TFA). The sample was loaded on the C_{18} column (TSKgel ODS120T, TOSOH Bioscience) and separated by a linear biphasic gradient of 20 to 80% acetonitrile over 30 min at a flow rate of 0.5 ml/min.

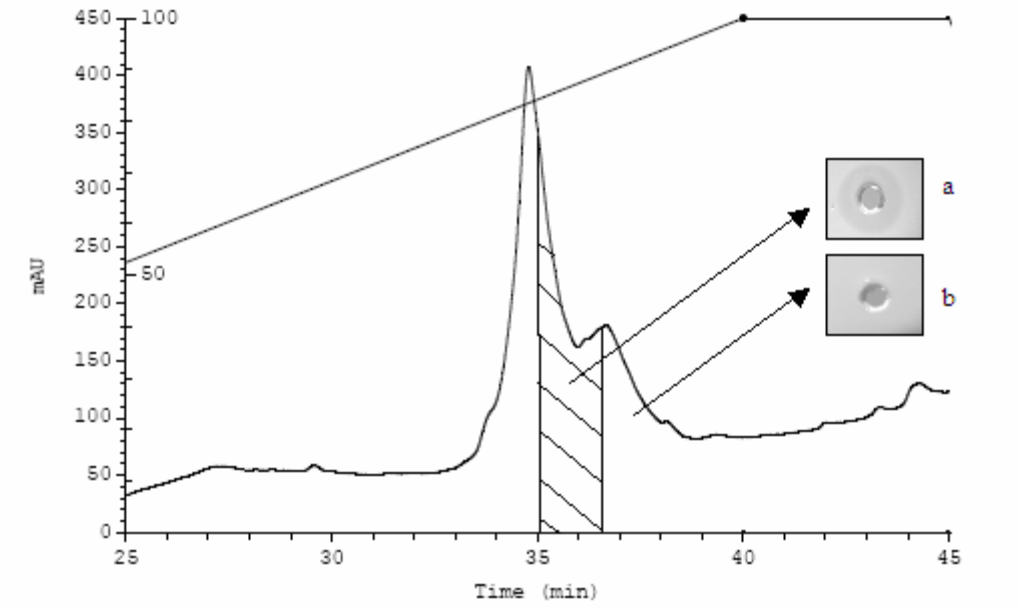

Figure 1. RP-HPLC chromatogram of a salivaricin CRL1328 purified extract. The HPLC fractions corresponding to two peaks with retention times (RT) of 34,5 and 37 min correspond to the peptides of this two component bacteriocin. These fractions did not shown activity (inset c), but the mixture of the fractions collected at RT 34 and 37 min also showed a strong inhibitory activity against *E. faecalis (inset a)*. The inhibitory activity (inset b) was detected in the sample collected in minutes 35 and 36.

3. A comment of different strategies applied for the purification of Enterocin CRL 35

In the first protocol of purification described by Farias et al, [15] a five steps procedure was applied for enterocin CRL35. Besides ammonium sulfate precipitation and a Cation Exchange Chromatography, a molecular filtration step was introduced by using Biogel P6. Later, the protocol was modified, eliminating the cation exchange and molecular filtration, and including a larger capacity reverse phase SPE chromatography that allowed a better recovery of the bacteriocin. In this last step the active fractions were eluted by using a gradient of different solvents such as acetonitrile, isopropanol or methanol. In our experiments, better recoveries of enterocin CRL35 were obtained with 2-propanol, but acetonitrile is more adequate for other bacteriocins (data not shown). The pooled active fractions obtained from SPE can be concentrated by a nitrogen flux or by vaccum, reloaded on a new C_{18} cartridges, or processed directly by RP-HPLC.

Samples from the C_{18} SPE column were concentrated and loaded onto a C_{18} (TSKgel Tosho Bioscience ODS120T) column. For HPLC chromatography, a KNAUER Smartline model coupled with a DAD array like detector (Knauer UV 2600), particularly useful for detecting the peptides at different wavelengths (220, 254, 280nm), was used. The mobile phase was (i) 100% 2-propanol and (ii) HPLC-grade water containing 0.1% trifluoroacetic acid (TFA). A 100-µl aliquot of samples was loaded and chromatografied on the C_{18} column by a linear biphasic gradient of 20 to 80% isopropanol over 40 min at a flow rate of 1 ml/min. Elution was monitored at different wavelengths: 220, 254 and 280 nm, and fractions were collected manually, concentrated by a nitrogen flux, and tested for antimicrobial activity by using one of the methods described previously. Peaks with antimicrobial activity were repooled and rechromatographied. The samples were controlled for the degree of purification by SDS-PAGE as described below, or even used for amino acids analysis, MS spectrometry or NH_2 sequencing.

4. Electrophoresis of Enterocin CRL35 by SDS-PAGE

4.1 Estimation of the bacteriocin MW using SDS-PAGE.

In addition to RP-HPLC, it is possible to analyze the partial or purified bacteriocin extracts by SDS-PAGE. For this technique, active extracts were electrophoresed by using the Laemmli protocol modified by Schäffer and von Jagow [39], that utilizes the "discontinuous" tricine buffer which is appropriate for the separation of low MW peptides. After electrophoresis, the peptides were stained with Coomasie Blue, silver-staining or with the luminescent stain SYPRO-Ruby (Molecular Probes-Invitrogen). This last dye stains proteins at concentration of less than 2 ng for band, and is compatible with downstream analysis as mass spectrometry and sequencing. Therefore, the peptide band can be cut out and electroeluted for further processing. Recently, another fluorescent dye, Lumitein, was introduced to the market (www.biotium.com). The manufacturer indicates that Lumitein is as sensitive as silver, then it could be a good alternative for bacteriocin staining, as unlike silver staining, this fluorescent dye is also compatible with downstream processing. In Fig. 2a, a purified extract of enterocin CRL35 stained with SYPRO-Ruby is shown. A parallel gel stained with Coomasie Blue did not show a similar band (data not shown).

4.2 Bioassay.

When the peptide bands do not stain adequately with the previous protocols, or just to confirm the identity of a bacteriocin, the application of bioassay is particularly useful to detect the antimicrobial compound in the gel. In this protocol, the SDS-PAGE gel is placed over a lawn of a sensitive strain. A clear zone of inhibition is observed at the level of the migration site of bacteriocin after the appropriate incubation. In this procedure is important to wash the gel previously with sterile water to reduce the SDS content, in order to avoid the inhibition of the sensitive strain by the detergent. If the sample is electrophoforesed in duplicate, is possible to process one strip by the bioassay and then to compare the migration of the inhibitory compound in the second strip, cutting the band for downstream processing.

This procedure has been particularly useful for the characterization of lactocin 705 [40]. Fig. 2b presents a bioassay performed with the purified enterocin CRL35, showing the inhibition of the sensitive strain (*Listeria innocua*) in the position corresponding to the migration of inhibitory peptide.

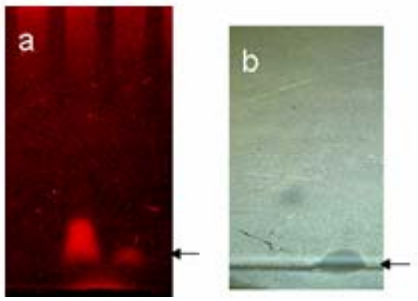

Fig. 2 a) Detection of enterocin CRL35 in PAGE using the sensitive luminescent stain SYPRO-Ruby (b) Bioassay of purified enterocin CRL35 after SDS-PAGE using *L. innocua* as sensitive strain. Arrows indicate the location of the peptide (MW 4.3).

5. Advanced strategies.

Note to the researcher or student that plan to characterize a "new" bacteriocin or BLIS (Bacteriocin-like inhibitory substance). Since two decades up to the present, the microbial journals have shown an explosion in the publication of papers describing the characterization of new bacteriocins from LAB or related Gram positive microorganisms. Then, is possible that the antimicrobial peptide that it is intended to be characterized and purified had already been described previously. Therefore, before the design of a purification strategy for the "new" bacteriocin, a good approach is the screening of the producer strain by PCR with a set of "bacteriocin-specific" primers that are available in the literature or can be designed

based on the sequences available in GenBank (http://www.ncbi.nlm.nih.gov/Genbank/). For example, if a strain is suspected to produce a lantibiotic-type bacteriocin, is possible to perform a PCR reaction with primers directed to the maturation genes (lanBC) present in all lantibiotic biosynthetic clusters [1]. On the other hand, numerous pediocin-like bacteriocin has been described. Primers to amplify their genes can also be designed to verify if the bacteriocin that is being characterized belongs to the class IIa antimicrobial peptides.

6. Final considerations.

As a review, the different techniques that can be applied for the purification of bacteriocins, or small inhibitory peptides are shown in Figure 3. This figure represents the basic strategies that can be applied for a standard purification process. Before deciding which one should be applied, is important to know some specific characteristics of the bacteriocin.

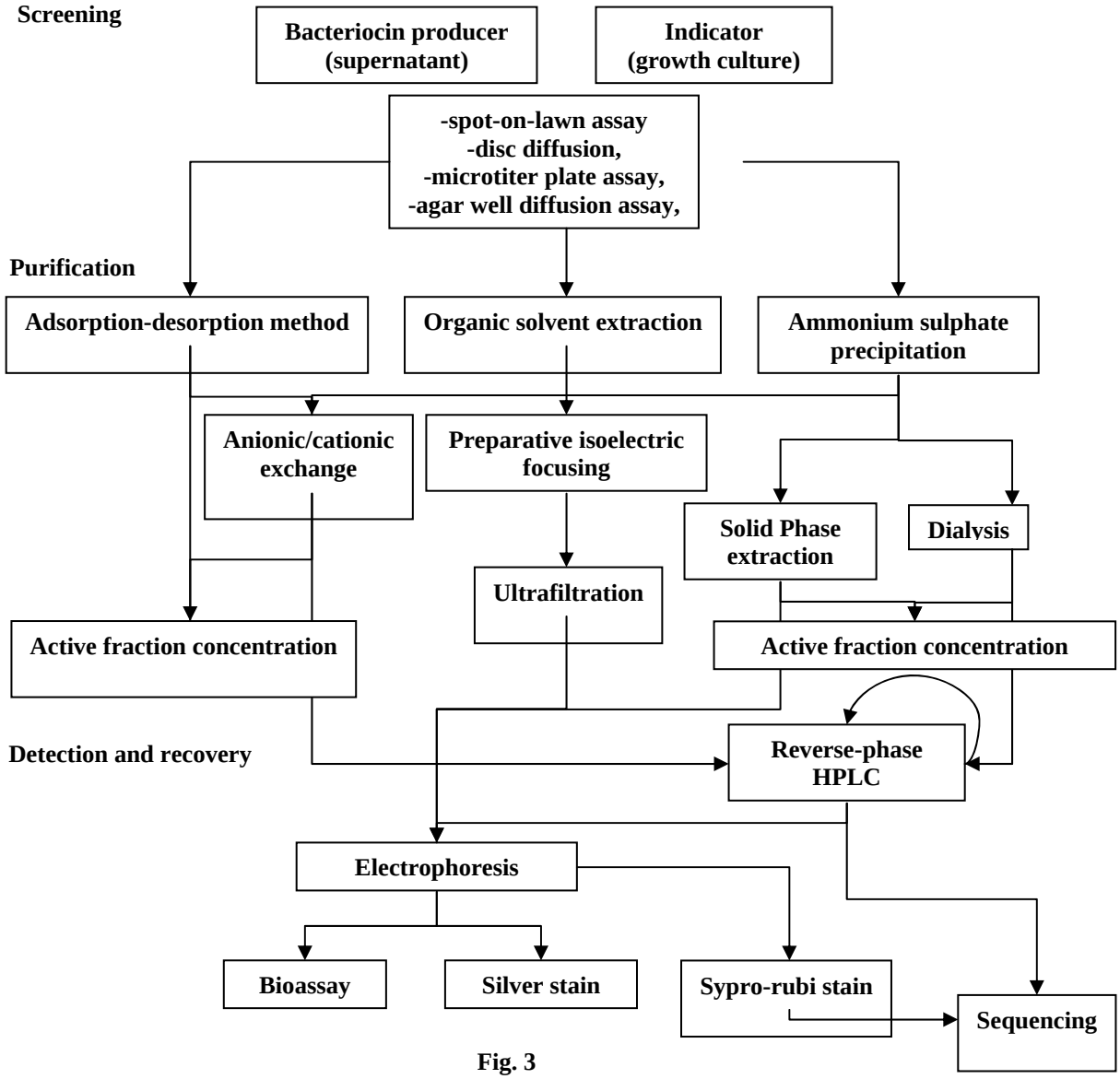

Fig. 3

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