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Method for Rapid Purification of Class IIa Bacteriocins and Comparison of Their Activities

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A three-step method was developed for the purification of mesentericin Y105 (60% yield) from the culture supernatant of *Leuconostoc mesenteroides* Y105. The same procedure was successfully applied to the purification of five other anti-*Listeria* bacteriocins identified by mass spectrometry. Specific activities of the purified bacteriocins were compared.

The increasing numbers of food contaminations by *Listeria* spp. have greatly stimulated research on class IIa bacteriocins produced by lactic acid bacteria. The number of such peptidic compounds may be limited since many of the newly detected strains express previously described bacteriocins. Consequently, the detection and characterization of still-unknown antagonistic peptides is becoming difficult. One of the more time-consuming steps in such studies is the purification of antagonistic compounds. Because bacteriocins are secreted into the culture medium, most strategies start with a step to concentrate bacteriocins from the culture supernatant, such as pH-dependent adsorption of bacteriocins on heat-killed producer bacteria (7), diatomite calcium silicate (Micro-Cel) (9), or rice hull ash or precipitation with silicic acid (19), ammonium sulfate (7), or ethanol (31). Although these procedures are used principally to reduce the working volume, they typically do not provide a high degree of purity. Therefore, subsequent steps of preparative isoelectric focusing (31) and/or multiple chromatographic separations, including cation exchange (7, 10, 22), gel filtration (12, 22), hydrophobic interaction (7, 10, 22), and reverse-phase liquid chromatography (6, 10, 22, 24), are necessary to achieve significant purification of the bacteriocins. Usually, but not always, the protein yields obtained are low (for a review, see reference 7). This is probably due to the high number of steps in the protocol, leading to time-consuming processes and subsequently low yields.

The primary structures of anti-*Listeria* bacteriocins are presented in Fig. 1. All of these peptides are characterized by the consensus sequence YGNGV(n)C(n)$_4$C(n)V(n)$_4$A (where n is any amino acid). More generally, the first 20 residues are well conserved, while a hydrophobic C-terminal half moiety is characterized by a large structural diversity, suggesting its implication in host specificity (11). We hypothesize that these variable C-terminal parts of the peptides result in differences in their specific anti-*Listeria* activities. Indeed, the sensitivity of a particular target strain depends on the bacteriocin concentration of the solution or culture supernatant used for the activity assay. Therefore, the host specificity described for a bacteriocin could depend on host sensitivity. Moreover, comparison of the bacteriocin specific activities described in the literature remains difficult because the methods and the target strains used are heterogeneous.

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Mesentericin Y105, a 37-amino-acid-long peptide produced by *Leuconostoc mesenteroides* Y105 was first characterized by Héchard et al. in 1992 (17). The method used to purify mesentericin Y105 in this initial study consisted of affinity chromatography on blue agarose followed by an ultrafiltration prior to reverse-phase high-pressure liquid chromatography (HPLC). The yield of recovered activity (0.7%) appeared unsatisfactory for any future extensive study on the mode of action of this anti-*Listeria* peptide. Consequently, we sought a simple purification method based on the main physicochemical features of this peptide, namely its net positive charge and the hydrophobicity of its C-terminal region. An efficient purification method involving an ammonium sulfate precipitation was previously described (6). However, this precipitation step appeared unsatisfactory due to the variable composition of its complex culture medium and the difficulties encountered in its pellet dispersion. Indeed, it appeared that the extent and the temperature of sterilization of the culture medium are critical for the amount of hydrophobic, colored contaminants still remaining. The best results were obtained with a 12-min sterilization at 110°C. Consequently, the ammonium sulfate precipitation was replaced by cation exchange chromatography. An overnight culture supernatant of *L. mesenteroides* Y105, propagated at 30°C in MRS broth (DIFCO Laboratories), was half diluted with water and then applied to a carboxy-methyl-cellulose (Cellufine C-200; Amicon)-filled column (2.5 by 18 cm). Activity was found to be bound to the solid phase. After washing successively with water (100 ml) and 0.1 M NaCl (150 ml), mesentericin Y105 was eluted with 0.5 M NaCl (200 ml). After this step, most of the colored contaminant compounds were eliminated. The next two steps were essentially identical to those previously described (6). Briefly, the active fraction (0.5 M NaCl) was applied directly to a C_{18} cartridge (Sep-pak plus; Waters), and mesentericin Y105 was eluted in the 80% acetonitrile fraction. After concentration, the active sample was injected on a C_8 Kromasil analytical HPLC column (5-μm particle size, 100 Å, 4.6 by 250 mm, A.I.T.) and was eluted by using a 40-min acetonitrile-water gradient. HPLC was conducted on a Perkin-Elmer series 200 liquid chromatography pump fitted with a Perkin-Elmer 785A detector, and separation was carried out by using a water-acetonitrile-trifluoroacetic acid 0.1% (vol/vol) solvent system. The elution profile, recorded at 220 nm, is presented in Fig. 2A. Mass spectrometry analysis showed that the 22-min peak corresponds to mesentericin Y105 (molecular weight = 3868.3), and the 26-min peak (molecular weight = 3445.2) corresponds to mesentericin B105 (16). The latter appears to be an anti-*Leuconostoc* bac-

	1 10 20 30 40	Ref.
Mesentericin Y105	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	(13)
Leucocin A	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	(15)
Sakacin P	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK	(29)
Mundticin	KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWSK	(3)
Sakacin A	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	(1)
Piscicocin V1a	KYYGNGVSCNKNGCTVDWSKAIGIIGNNAAANLTTGGAAGWNKG	(5)
Carnobacteriocin BM1	AISYGNGVYCNKEKCWVNKAENKQAITGIVIGGWASSLAGMGH	(26)
Carnobacteriocin B2	VNYGNGVSCSKTKCSVNWGQAFQERYTAGINSFVSGVASGAGSIGRRP	(26)
Bavaricin MN	TKYYGNGVYCNSKKCWVDWGQAAGGIGQTVVxGWLGGAIPGK	(20)
Bacteriocin 31	ATYYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR	(30)
Enterocin P	ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH	(8)
Bifidocin B	KYYGNGVTCGLHDCRVDRGKATCGIINNGGMWGDIG	(32)
Pediocin PA-1	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	(23)
Enterocin A	TTHSGKYYGNGVYCTKNKCTVDWAKATTCIAGMSIGGFLGGAIPGKC	(2)
Divercin V41	TKYYGNGVYCNSKKCWVDWGQASGCIGQTVVGGWLGGAIPGKC	(24)
Consensus	YYGNGV C C V WG A I	

FIG. 1. Sequence alignment of class IIa bacteriocins. Residue numbering is according to the sequence of mesentericin Y105. Piscicocin V1a is identical to piscicolin 126 (18). Sakacin A is identical to curvacin A (28). Carnobacteriocin BM1 is probably identical to piscicocin V1b (5). Sakacin P is probably identical to bavaracin A (21). The consensus sequence includes residues conserved by at least at 75%. The residues conserved by more than 90% are underlined.

teriocin similar to mesenterocin 52B described by Revol-Junelles et al. (27). The mass spectra of the purified bacteriocin samples were obtained from positive electrospray analysis on a Perkin-Elmer Sciex API 165 mass spectrometer equipped with an ion spray source.

The amount of purified mesentericin Y105 obtained from 100 ml of culture supernatant was 120 μg, which appeared significantly better than the final yield of 17 μg per liter obtained for the same molecule by Fleury et al. (12). Protein concentrations of purified fractions were determined by using the bicinchoninic acid protein assay kit (Sigma); synthetic mesentericin Y105 and bovine serum albumin were used as standards with no significant difference.

This quantity corresponds to a yield of 60% of recovered activity, indicating an initial amount of 2 mg of bacteriocin per liter in the culture medium. Bacteriocin assays were performed by the previously described antagonism well diffusion method (12). However, this yield must be considered as indicative. Indeed, several authors (9, 20, 22, 24, 31) reported intermediate or final yields of recovered activity above 100%. In a recent study (14), Ganzle et al. demonstrated that ecological factors, such as protein content, pH, salinity, or/and salt composition of the medium, modulate the activity of bacteriocins (nisin, sakacin A, and sakacin P); for example, divalent cations inhibited bacteriocins, whereas acidic pH and NaCl increased their activity. Moreover, the surfactant Tween 80 may form micelles with the proteins in the growth medium and was described to interfere with bacteriocins, extending or diminishing their apparent activities according to the peptide tested (7). Therefore, this compound, being present in the culture supernatant, must still be present in subsequent fractions tested in order to minimize the error in activity determination. Thus, all the critical dilution assays were made by using a solution of 0.01% Tween 80 as the diluting solvent.

The experiment was repeated more than twenty times and, considering HPLC profiles and purification yields, appeared to be very reproducible. Moreover, the major advantage of this new purification method lies in the duration of the different steps. In a standard experiment, only 6 to 8 h is necessary to purify mesentericin Y105 from 100 ml of an overnight culture of the producer strain.

All described anti-*Listeria* bacteriocins differ essentially in their C-terminal halves (Fig. 1). However, similarly to mesentericin Y105, they all bear a hydrophobic C-terminal side and present positive charges, essentially located within the conserved N terminus. The goal of this study was to demonstrate that our method was easily applicable to the purification of all class IIa bacteriocins. Consequently, the same protocol was applied to culture supernatants of several anti-*Listeria* active strains (Table 1). Bacterial cultures were performed as simply as possible in commercial MRS medium at 30°C, except for *Carnobacterium divergens* V41 cultivated at 20°C (6, 10, 22, 24). As indicated in Table 1, four strains provided by RHODIA-FOOD and Y. Héchard were found to be active against *Listeria*, but the identity of the bacteriocin produced was not known at that stage. The bacteriocins were purified and then analyzed

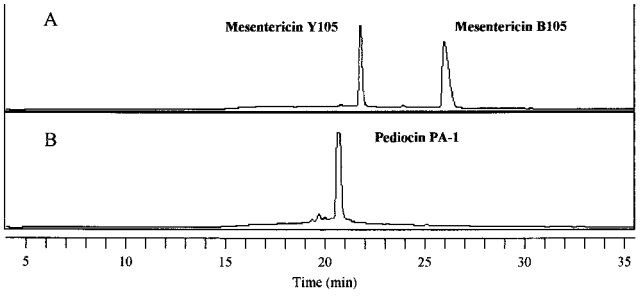

FIG. 2. Reverse-phase 220-nm elution profiles of active fractions obtained from cation exchange chromatography and solid-phase extraction from culture supernatants of *L. mesenteroides* Y105 (A) and *P. acidilactici* 1521 (B).

TABLE 1. Bacteriocin-producing strains used in this study[a]

Strain	Source or reference
L. mesenteroides Y105	17
L. sakei 2675	RHODIA-FOOD
L. sakei 2525	RHODIA-FOOD
P. acidilactici 1521	RHODIA-FOOD
E. faecalis 336	Y. Héchard
C. divergens V41	25

[a] All strains were wild type.

TABLE 2. Purification and identification of class IIa bacteriocins

Bacteriocin-producing strain	Major HPLC peak retention time (min)	Purification yield (%)[a]	Amt of purified bacteriocin (μg)[b]	Bacteriocin initial concn (mg/liter)[c]	MIC (μg/ml)[d]	Mol wt[e]	Identified bacteriocin[f]	Reference
L. mesenteroides Y105	22	60	120	2	33×10^{-3}	3868.3	Mesentericin Y105	17
L. sakei 2675	29	10	35	3.5	65×10^{-3}	4306.1	Sakacin A	This work
L. sakei 2525	20.3	50	50	1	65×10^{-3}	4434.8	Sakacin P	This work
E. faecalis 336	26	66	50	0.75	10×10^{-3}	4828.9	Enterocin A	This work
P. acidilactici 1521	21	25	140	5.6	4×10^{-3}	4624.0	Pediocin PA-1	This work
C. divergens V41	24	10	160	16	1.4×10^{-3}	4509.3	Divercin V41	24

[a] Purification yields correspond to the ratio between total activities of purified bacteriocin solution and culture supernatant.
[b] Amount of bacteriocin purified from 100 ml of culture supernatant.
[c] Concentration is calculated according to the purification yield.
[d] MIC corresponds to the lower concentration of a bacteriocin solution inducing a detectable zone of inhibition.
[e] Molecular weight corresponds to the major peak isolated from HPLC.
[f] Bacteriocins were identified by comparison of their molecular weight with those of known class IIa bacteriocins.

by ion spray mass spectrometry. Identification of bacteriocins was achieved by comparing their molecular weights with those of known class IIa bacteriocins (Table 2). For example, an HPLC profile obtained from the purification of the bacteriocin produced by *Pediococcus acidilactici* 1521 is presented in Fig. 2B. Therefore, according to its molecular weight, a major peak corresponds to pediocin PA-1 (Table 2).

Our results demonstrate the applicability of the method described for the rapid identification of anti-*Listeria* compounds produced by newly detected active strains. Moreover, in all cases, molecular weight shows that the cysteine residues of purified bacteriocins form disulfide bonds between C9 and C14 (according to the numbering in Fig. 1) and also between C24 and C42 for enterocin A and divercin V41 and between C24 and C44 for enterocin A and pediocin PA-1.

In this study, HPLC retention times of purified bacteriocins (Table 2) were found to be very reproducible and sufficiently different from each other to allow their fractionation in cases of mixture production, as seen for mesentericins Y105 and B105.

Purification yields vary from 10 to 66% (Table 2). The highest yield was obtained for enterocin A. It appears that *Enterococcus faecalis* 336 produces a low amount of this bacteriocin (0.75 mg per liter) (Table 2). In contrast, the bacteriocins produced to a larger extent than mesentericin Y105, divercin V41 and pediocin PA-1, are recovered with lower efficiencies (10 and 25% yield, respectively [Table 2]). Such a result is probably due to a saturation of the cation exchange chromatography. Moreover, the 10% yield observed with sakacin A purification, which was lower than the yield of pediocin PA-1, might be explained by the high hydrophobicity of this peptide, as shown by its high retention time of 29 min. In this case, the bacteriocin could be lost during the solid-phase extraction by absorption on the reverse phase.

According to the results of HPLC and mass spectrometry analyses, the purified bacteriocins listed in Table 2 appeared to be at least 95% pure. Antagonistic MICs were calculated by using the well diffusion method (12) and correspond to the lower concentration of bacteriocin solution inducing a zone of inhibition. All the purified molecules were tested simultaneously toward the same indicator, *Listeria ivanovi* BUG 496, in order to compare the specific activities of the six peptides. It appears that the bacteriocins tested can be distributed into two distinct groups according to their MICs (Table 2). The first group, composed of mesentericin Y105, sakacin A, and sakacin P, have MICs above 10^{-2} μg/ml, whereas the second group, composed of the pediocin-like bacteriocins pediocin PA-1, enterocin A, and divercin V41, display MICs lower than 10^{-2} μg/ml. Within the two groups, the measured activities are quite homogeneous, except for that of divercin V41, which was significantly lower than other pediocin-like bacteriocins. This result could be related to the anti-*Listeria* activity detected for the 18 to 43 peptides corresponding to the C-terminal part of divercin V41 (4). Such antimicrobial activity exhibited by a portion of a bacteriocin has never been described for other class IIa bacteriocins and may be related to the higher activity of the intact peptide. Indeed, the C-terminal peptide could act by forming pores in the target membrane according to a nonspecific mechanism, in addition to the specifically directed activity of the entire molecule.

In a previous work (10), pediocin-like bacteriocins pediocin PA-1 and enterocin A were found to be more active than sakacins A and P toward lactic acid bacteria. However, in contradiction with our results (Table 2), sakacin P appeared to be as effective as enterocin A and pediocin PA-1 against *Listeria* species. Such differences can arise from the target strains used, from an erroneous determination of bacteriocin concentration, or from the bacteriocin assay itself. The activities of the purified bacteriocins toward various indicator strains are shown in Table 3. Solutions of bacteriocins were adjusted in

TABLE 3. Comparative inhibition spectra of six purified anti-*Listeria* bacteriocins

Indicator strain	Bacteriocin[a]					
	Mes.	Sak. A	Sak. P	Ped.	Ent. A	Div.
Lactococcus lactis ATCC11454	−	−	−	−	−	−
Leuconostoc paramesenteroides DSM 20288	−	−	−	−	−	−
L. mesenteroides DSM 20484	+	−	−	+	−	−
L. mesenteroides DSM 20240	+	−	−	+	−	−
Lactobacillus delbrueckii DSM 20081	−	−	−	−	−	−
Lactobacillus plantarum DSM 20174	−	−	−	−	−	−
Lactobacillus brevis DSM 20054	−	−	−	−	−	−
Lactobacillus casei DSM 20011	−	−	+	+	+	+
L. sakei 2515	+	+	−	+	+	+
P. acidilactici ENSAIA 583	+	+	+	+	+	+
P. cerevisiae IP 5492	+	−	+	−	+	+
E. faecium ENSAIA 631	−	−	−	+	+	+
E. faecalis IP 5430	+	+	+	+	+	+
E. faecalis ENSAIA 636	+	+	+	+	+	+
E. durans ENSAIA 630	+	+	+	+	+	+
Listeria innocua 8811	+	+	+	+	+	+
Listeria monocytogenes L028	+	+	+	+	+	+
L. ivanovi BUG 496	+	+	+	+	+	+

[a] Mes., mesentericin Y105; Sak. A, sakacin A; Sak. P, sakacin P; Ped., pediocin PA-1; Ent. A, enterocin A; and Div., divercin V41.

order to obtain zones of inhibition with diameters ranging from 1 to 2 cm against *L. ivanovi* BUG496, our most sensitive strain. The results showed that, in agreement with the literature, all the tested *Listeria* strains are sensitive to the six bacteriocins used in this study. Similarly, *Enterococcus durans* and *E. faecalis* are sensitive to all the bacteriocins tested, but *Enterococcus faecium* is inhibited only by pediocin-like bacteriocins. This universal toxicity of class IIa bacteriocins toward *Listeria* spp. and *E. faecalis* and *E. durans* could be due to an identical mechanism of action against these bacteria. For example, we can hypothesize the existence of a common target molecule presented at the surface of the two species. In contrast, the sensitivity of lactic acid bacteria depends upon the purified bacteriocins tested. This result indicates the existence of either a different mode of action toward these bacteria compared to *Listeria* (for example, a different receptor) or a variable accessibility of the bacteriocin to its receptor.

Considering the single-disulfide-bond-containing bacteriocins, mesentericin Y105, which displayed activity against two *L. mesenteroides* strains, *Pediococcus* species, and *Lactobacillus sakei* 2515 (Table 3), has a broader inhibitory spectrum than sakacins A and P. Surprisingly, sakacin P, produced by *L. sakei* 2525, was inactive against *L. sakei* 2515 (nonproducing strain). Unless an immunity protein to sakacin P is still present in the latter strain, this result is in opposition to the widespread idea that the activity of bacteriocins against species is closely related to their producer strains. According to their inhibitory spectra, pediocin-like bacteriocins can be separated in two subclasses. On the one hand, pediocin PA-1 is active toward *Leuconostoc* species but is inactive toward *Pediococcus cerevisiae* IP 5492. On the other hand, enterocin A and divercin V41 present the opposite spectrum. The C-terminal sequences of divercin V41 and enterocin A are quite close, especially for the last twelve residues, which are highly conserved. However, divercin V41 does not present more N-terminal-sequence homologies with enterocin A than with pediocin PA-1. Consequently, as previously mentioned (10, 11), these results show that the specific inhibitory spectra of class IIa bacteriocins depend solely on the sequence of the C-terminal half of the molecule.

With the method described in the present work, very pure preparations of mesentericin Y105 were obtained repeatedly with high yields. This method appeared more simple and rapid than other purification protocols described so far.

This three-step strategy was successfully applied to all class IIa bacteriocin producer strains tested and is probably useful for the purification of any anti-*Listeria* bacteriocin. It also may be efficient for the purification of some other class II bacteriocins, as exemplified by mesentericin B105. Considering the speed of our method, it might be dedicated to the rapid identification of bacteriocins produced by anti-*Listeria* active isolates. Due to the universality of this method, we have been able to obtain pure preparations of six bacteriocins, whose specific activities were then compared. The direct comparison of the specific activities of the bacteriocins showed that the pediocin-like peptides are significantly more active than those bearing a single disulfide bridge.

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