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Rapid and Efficient Purification Method for Small, Hydrophobic, Cationic Bacteriocins: Purification of Lactococcin B and Pediocin PA-1

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The bacteriocins lactococcin B and pediocin PA-1 were purified by ethanol precipitation, preparative isoelectric focusing, and ultrafiltration. The procedure reproducibly leads to high final yields in comparison to the generally low yields obtained by column chromatography. Specifically, during isoelectric focusing no loss of activity occurs. The method, in general, should be applicable to small, hydrophobic, cationic bacteriocins.

The well-documented ability of lactic acid bacteria (LAB) to inhibit the growth of other bacteria (14) is of special interest to the food and feed industry, since LAB bacteriocins could potentially be used as food preservatives (2, 12, 16). Lactococcins and pediocins are bacteriocins produced by *Lactococcus* and *Pediococcus* spp., respectively.

Because bacteriocins are secreted into the growth medium, most approaches for purification start with a concentration step from the culture supernatant, such as salt precipitation (8, 9), acid precipitation (5), vacuum concentration, or extraction with organic solvents (18). Although these procedures are necessary for reduction of the working volume, they typically do not provide a high degree of purity. Therefore, several chromatographic steps (including separation by size exclusion, adsorption, and/or hydrophobic interaction) are necessary to achieve significant purification of the bacteriocins. Usually, but not always, the loss of bacteriocin activity is very high, and low protein yields are obtained. For a review on biochemical methods for bacteriocin purification, see reference, 11.

In order to be able to study their mode of action, we developed a purification procedure for lactococcin B and pediocin PA-1 that should, in principle, be applicable to many small, hydrophobic, cationic bacteriocins. It allowed the high-yield purification of lactococcin B, a bacteriocin that proved refractive to purification by column chromatography (including reverse-phase high-pressure liquid chromatography; less than 0.1% of the activity was recovered, but no protein was detectable [8a]).

Growth of *Lactococcus lactis* IL1403 (pMB580) (19) and production of lactococcin B in various media were studied. Figure 1A shows that production was highest in G2M17 (double concentration of normal M17 [GM17]) (17). The bacteriocin was produced during logarithmic growth, and the highest activity was found in the early stationary phase (Fig. 1B). After that, activity declined, possibly by readsorption of lactococcin B to the producer cells or by inactivation of the bacteriocin by the proteolytic-peptidolytic system of the producer. Contrary to what was reported by Geis et al. (4), production in Elliker lactic broth (3) was lower than that on GM17, even with the original strain *Lactococcus lactis* subsp. *cremoris* 9B4. An explanation might be that the media used in these two studies came from different suppliers. Medium-dependent bacteriocin production has been observed before (e.g., see reference 13). No production was observed on a chemically defined medium (CDM) (21) containing glucose (Fig. 1A). Because proteins and peptides present in a rich medium like GM17 complicate purification of bacteriocins, production was also tested in a GM17 dialysate. A 10-times-concentrated M17 solution was dialyzed in a dialysis bag with a molecular mass cutoff of 12 to 14 kDa [the resulting medium was designated GM17d (12–14)] or of 3 kDa [medium GM17d (3)]. To the dialysate, glucose was added to 0.5%, and the lactococcin B producer was inoculated at 1%. Although both dialysates supported good growth of the producer, only 10% of the bacteriocin activity produced on GM17 was obtained (Fig. 1A), indicating that a factor(s) larger than 14 kDa is required for optimal bacteriocin production. For initial purification of lactococcin B, therefore, G2M17 was used.

After 5.5 h of growth of a 1% inoculum of *L. lactis* IL1403 (pMB580) in G2M17, the cell-free supernatant contained 1.0×10^4 arbitrary units [AU] of activity per liter, with *L. lactis* IL1403 as the indicator (see Table 1). After ethanol precipitation, 3.0×10^4 AU/liter was present in the precipitate. This increase in activity is indicative of multimer formation, which has been reported for other bacteriocins (11). The ethanol precipitate was dissolved in demineralized water and subjected to isoelectric focusing after addition of ampholytes (Bio-Rad, Richmond, Calif.), Tween 20, and glycine to final concentrations of 1, 0.1, and 5%, respectively. After 5.5 h at a constant power of 12 W, separation was complete. The pH of each fraction was adjusted to 7 (± 0.1). Bacteriocin activity, present in fractions 14 to 20, was highest in fraction 18 (Fig. 2A). The pI of the bacteriocin was estimated to be approximately 8.5. Fractions 14 to 20 were pooled and ultrafiltered through a membrane with a 3-kDa molecular mass cutoff in a Spin-x UF concentrator (Corning Costar Corporation, Cambridge,

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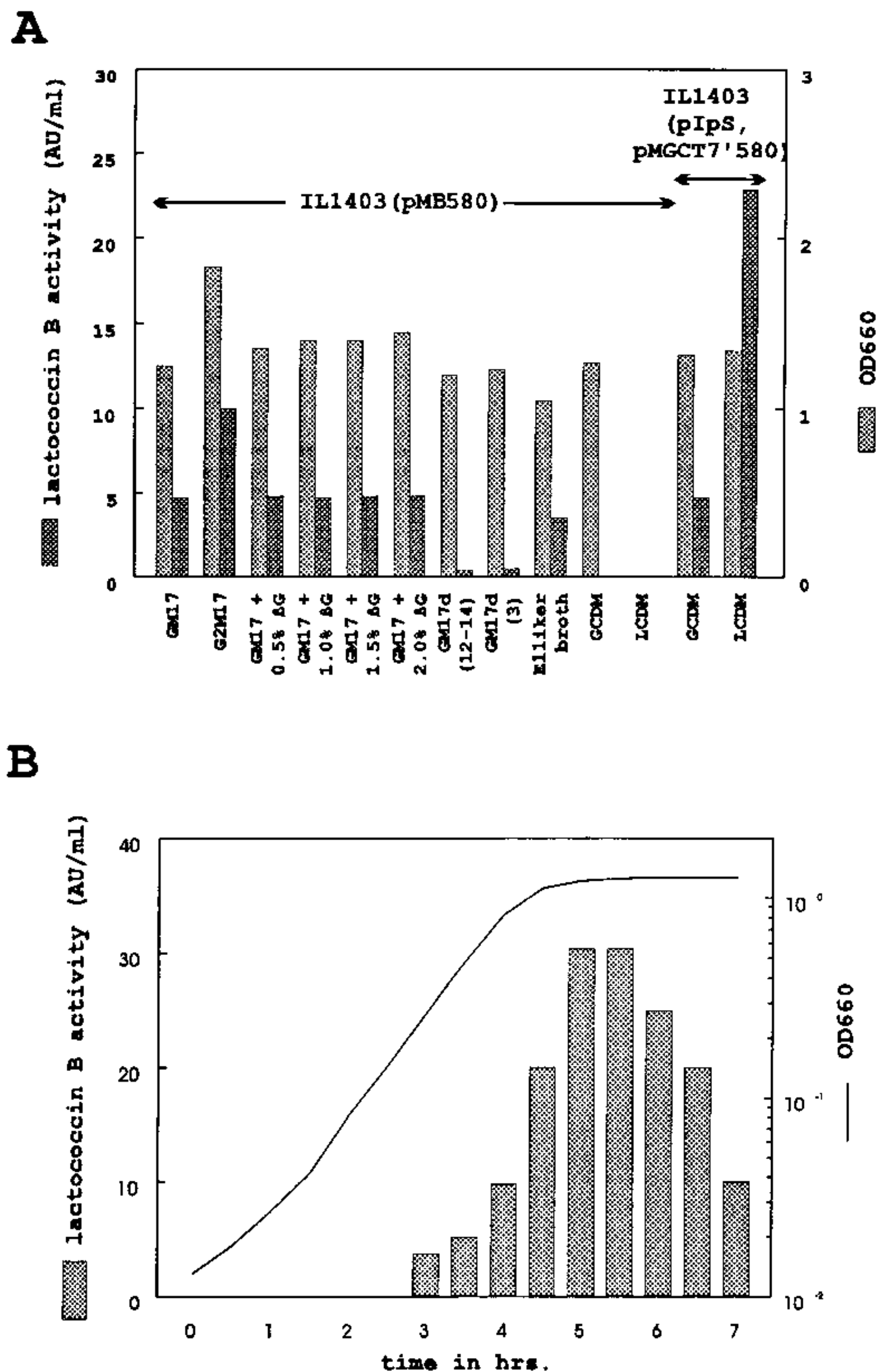

FIG. 1. (A) Lactococcin B production in various media. A 1% inoculum of *L. lactis* IL1403 (pMB580) was grown at 30°C until early stationary phase in the indicated media. Samples were removed, and the optical density at 660 nm (OD660) and the amount of bacteriocin (by using the agar spot test [21] with *L. lactis* IL1403 [1] as the indicator strain) were determined. βG, β-glycerophosphate. (B) Lactococcin B production during growth of *L. lactis* IL1403 (pMB580) in G2M17 at 30°C. The optical density at 660 nm and the amount of bacteriocin (by the agar spot test) were determined at 30-min intervals.

Mass.). The bacteriocin solution was devoid of ampholytes as shown by Tricine-sodium dodecyl sulfate-polyacrylamide gel-electrophoresis (Tricine-SDS-PAGE) (15) and subsequent silver staining (Fig. 3, lane 2). During isoelectric focusing, hardly any loss of activity was observed, but, in contrast to pediocin PA-1 (see below), losses were considerable after ultrafiltration (Table 1).

The amount of lactococcin B purified was insufficient to determine an N-terminal amino acid sequence. Since the initial amount of activity was already low in supernatants of IL1403 (pMB580), lactococcin B was overexpressed by using the bacteriophage T7 RNA polymerase expression system essentially as described by Wells et al. (22). The system was adapted as follows. In plasmid pILpol (22), containing the T7 RNA polymerase gene under the control of the lactose-inducible lactococcal *lac* promoter, the macrolide-lincosamide-streptogramin

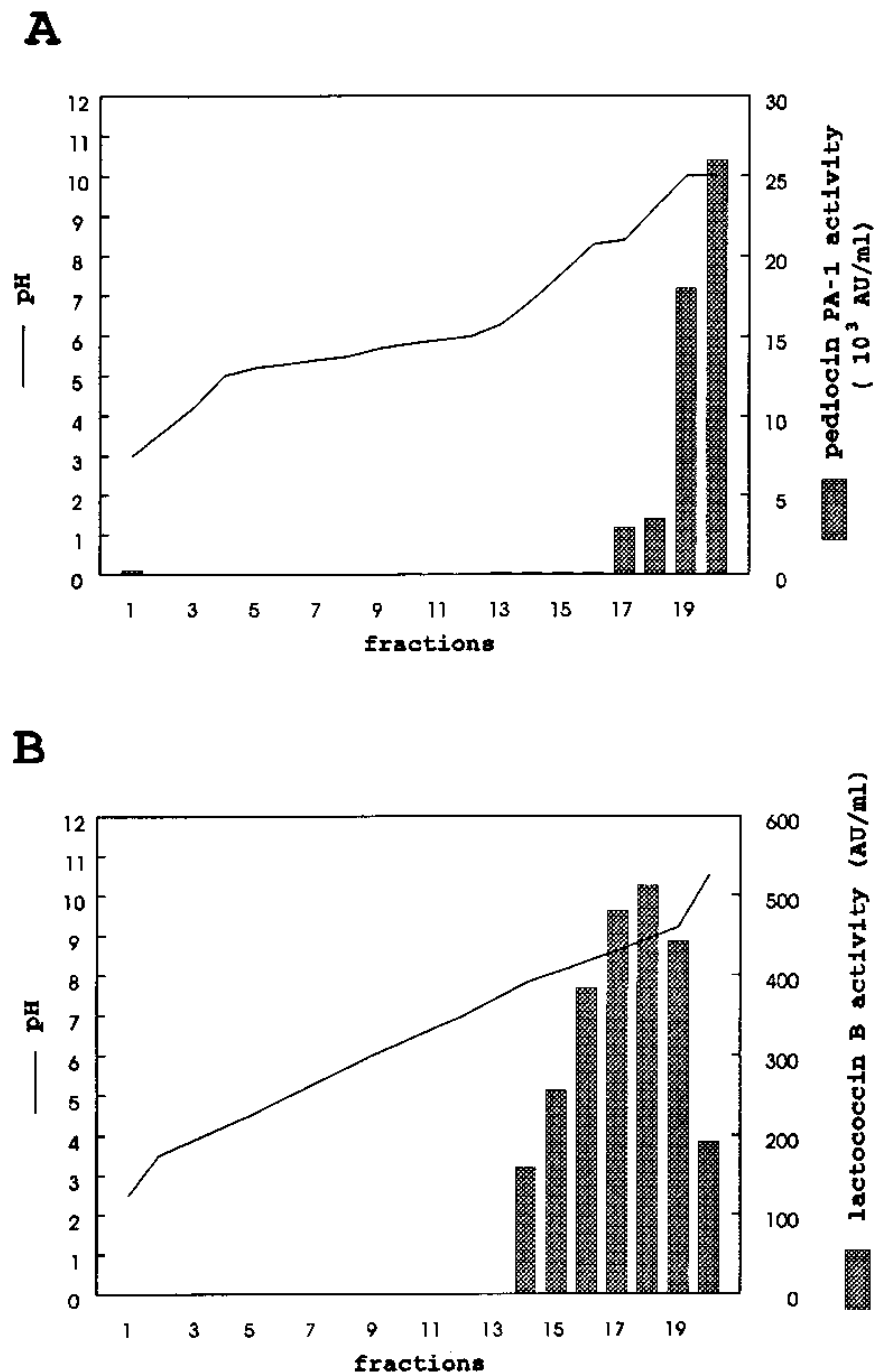

FIG. 2. Activity distribution of pediocin PA-1 (A) and lactococcin B (B) after preparative isoelectric focusing. The amount of bacteriocin in every fraction was determined by the agar spot test using *L. lactis* IL1403 (1) and *Pediococcus pentosaceus* PPE1.2 (7) as the indicator strains for lactococcin B and pediocin PA-1, respectively.

B resistance marker was replaced by a *Cla*I-*Nde*I fragment from pDL55 containing the spectinomycin resistance marker AAD(9) (10), resulting in plasmid pIpS. Instead of pMG280 (22) as the carrier of the *lac* operon, strain IL1414 (1) (a derivative of IL1403 containing a lactose plasmid) was used. The T7 expression vector, containing *lcnB* and *lciB* under control of the T7 promoter, was constructed by cloning the *Hin*dIII-*Xba*I fragment from pT7′580 (19) in pMG36CT (21) cut with the same restriction endonucleases, resulting in pMGCT7′580. IL1414(pIpS, pMGCT7′580) was grown on media containing lactose, spectinomycin, and chloramphenicol. In addition to the strong T7 promoter, plasmid pMGCT7′580 contains the normal lactococcin B promoter. Therefore, lactococcin B production in GM17 and lactose M17 (LM17) could be compared. On GM17 plates lactococcin B production by IL1414(pIpS, pMGCT7′580) was similar to that of IL1403 (pMB580), as judged by the size of the zone of inhibition (halo) around a producing colony. On LM17, production of

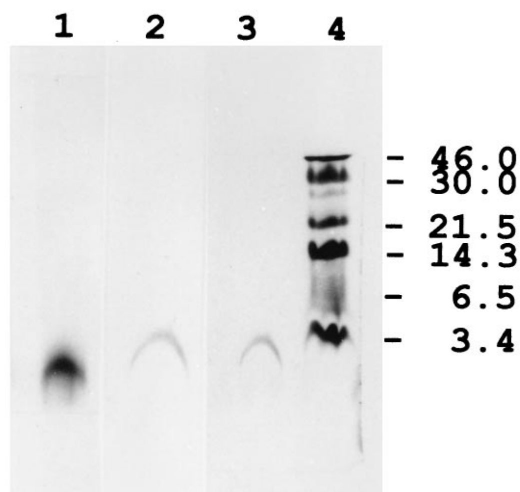

FIG. 3. Silver staining of a 16% Tricine-SDS-polyacrylamide gel containing purified samples of pediocin PA-1 (lane 1) and lactococcin B (lanes 2 and 3, purified from G2M17 and CDM, respectively). Lane 4 contains molecular weight markers (Bio-Rad); sizes (in thousands) are on the right.

lactococcin B by IL1414(pIpS, pMGCT7′580) was similar to that on GM17 by the same strain; no overexpression was obtained. As documented above, production under control of the lactococcin B promoter was observed only in rich media, not in CDM. In contrast, production was observed in glucose CDM (GCDM) by IL1414(pIpS, pMGCT7′580). Since CDM does not contain any constituents with a molecular mass higher than approximately 1.2 kDa, and lactococcin B has a mass of approximately 5 kDa, the bacteriocin was purified from the supernatant of IL1414(pIpS, pMGCT7′580) grown in lactose CDM (LCDM) as follows. The supernatant was freeze-dried (Lyph-lock 4.5 freeze-dry apparatus; Labconco, Kansas City, Mo.) and ultrafiltered after being redissolved in water to 2% of the original volume. The purified sample of lactococcin B (yield, 95% [Table 1]) was subjected to tricine-SDS-PAGE and silver staining and shown to be pure (Fig. 3, lane 3).

Purified lactococcin B was subjected to N-terminal amino acid sequencing by means of Edman degradation. The first 21 N-terminal amino acids were Ser-Leu-Gln-Tyr-Val-Met-Ser-Ala-Gly-Pro-Tyr-Thr-Trp-Tyr-Lys-Asp-Thr-Arg-Thr-Gly-Lys. Comparing this sequence with the sequence deduced from the *lcnB* gene revealed that prelactococcin B, like many other bacteriocins (6), is processed behind a glycine doublet. In addition, it proved that the lactococcin B sample obtained was free from other peptides.

After overnight growth of *Pediococcus acidilactici* PAC 1.0 (7), the filter-sterilized (0.45-μm-pore-size filter; Schleicher and Schuell, Inc., Keene, N.H.) supernatant contained 1.6×10^6 AU of pediocin PA-1 activity (Table 1). About 32% could be precipitated with cold ethanol (Table 1). The precipitate was dissolved in demineralized water and, after addition of carrier ampholytes, Tween 80, and glycine (to final concentrations of 1, 0.1, and 5%, respectively), subjected to preparative isoelectric focusing. After 5.5 h, spot testing indicated that bacteriocin activity was present in fractions 17 to 20, with the highest activity in fraction 20 (Fig. 2B). The pI of pediocin PA-1 was estimated to be around 9. The active fractions were pooled and ultrafiltered. During isoelectric focusing almost no activity was lost, and after ultrafiltration all activity remained. After precipitation, preparative isoelectric focusing, and ultrafiltration, the bacteriocin was pure as judged by tricine-SDS-PAGE and silver staining (Fig. 3, lane 1).

Pediocin PA-1 has been purified previously by column chromatography (7). The yield in the procedure used by Henderson et al. was only 0.6%. With our method we repeatedly obtained very pure bacteriocin preparations, with yields between 30 and 40% for lactococcin B and pediocin PA-1. Usually, yields are between 0.5 and 1% with other purification protocols. The great advantage of the present procedure is the omission of columns. With preparative isoelectric focusing, losses are minimal (less than 1% for lactococcin B and 3% for pediocin PA-1). Usually, the hydrophobic bacteriocins stick to the matrix of columns, causing losses of 40 to 80% on cation- and/or anion-exchange columns (11).

We believe that the procedure described here could also be used for many other (small) hydrophobic, positively charged bacteriocins. A trial with lactococcin A (previously purified by Holo et al. [8] with a yield of 16%) showed that it could be purified with a yield over 30% (data not shown). Also, mutant

TABLE 1. Purification of lactococcin B and pediocin PA-1

Sample and fraction	Vol (ml)	OD_{280}[a]	Total activity (AU)[b]	Sp act (AU/ml × OD_{280})	Purification (fold)	Yield (%)
Lactococcin B (G2M17)						
Culture supernatant	1,000	35	1.0×10^4	0.3×10^0	1	100
Ethanol precipitate	50	50	3.0×10^4	1.2×10^1	40	300
Rotofor samples	20	3.1	2.9×10^4	4.8×10^2	1,600	299
Retentate	2.5	2.7	4.2×10^3	6.1×10^2	2,033	41
Lactococcin B (CDM)						
Culture supernatant	1,000	6.0	5.0×10^4	8.3×10^1	1	100
Lyophilisate	50	5.5	7.3×10^4	6.6×10^2	8	146
Retentate	2.5	2.5	6.9×10^4	5.6×10^4	675	138
Pediocin PA-1						
Culture supernatant	1,000	3.3	1.6×10^6	4.8×10^2	1	100
Ethanol precipitate	40	0.7	5.1×10^5	1.8×10^4	37	32
Rotofor samples	11.4	0.7	4.7×10^5	6.3×10^4	131	29
Retentate	11.4	0.5	4.6×10^5	8.1×10^4	169	29

[a] OD_{280}, optical density at 280 nm.
[b] Determined in an agar spot test using *L. lactis* IL1403 (for lactococcin B) (1) or *P. pentosaceus* PPE1.2 (for pediocin PA-1) (7) as the indicator strain.

lactococcin B molecules have been purified by this method (20).

Bacteriocins are of potential interest to the food industry because of their ability to inhibit food-related pathogens and spoilage organisms. Some applications (e.g., mode-of-action studies) would require highly purified bacteriocin preparations, whereas in other applications (e.g., use as a biopreservative) a less purified sample may be sufficient. In both cases a high final yield is desirable. Cost and reproducibility will also influence the industries' choice for a specific purification protocol. The method described here reproducibly provides high yields of purified bacteriocin and could be easily scaled up for industrial purposes.

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