Pediocin A, a bacteriocin produced by *Pediococcus pentosaceus* FBB61

Andrea Piva¹ and Denis R. Headon²

Author for correspondence: Andrea Piva. Tel: +39 51 792883. Fax: +39 51 792869.

**INTRODUCTION**

An outstanding feature of lactic acid bacteria, accounting for their wide utilization in dairy, meat and vegetable fermentations, is their inhibitory activity against a variety of food-spoilage micro-organisms (Bhunia *et al.*, 1988; Pucci *et al.*, 1988; Spelhaug & Harlander, 1989). Among antimicrobial substances produced by lactic acid bacteria, bacteriocins have gained increasing interest as possible natural food preservatives. Bacteriocins are heterogeneous in terms of spectra of activity, optimal conditions for activity, molecular masses, biochemical characteristics and sequence homologies. Nisin, a bacteriocin produced by certain strains of *Lactococcus lactis*, was approved as a GRAS human food ingredient by the FDA in 1988 (Food and Drug Administration, 1988).

Pediococcal strains are used for meat and vegetable fermentations. Pediocin AcH and pediocin PA-1, produced by different strains of *Pediococcus acidilactici* were purified (Bhunia *et al.*, 1988; Henderson *et al.*, 1992), and later demonstrated to be identical (Motlagh *et al.*, 1992). Pediocins have been successfully used to inhibit *Listeria monocytogenes* in several food systems (Pucci *et al.*, 1988; Yousef *et al.*, 1991). *Pediococcus pentosaceus* FBB61 was originally isolated in 1953 from a cucumber fermentation (Costilow *et al.*, 1956). The inhibitory activity of this strain was observed by Etchells *et al.* (1964) in pure culture fermentations of cucumbers, and further investigated by Fleming *et al.* (1975). Rueckert (1979) characterized the chemical nature of the inhibitory material as proteinaceous and nondialysable across a semi-permeable membrane, and its action as bactericidal.

Daeschel & Klaenhammer (1985) associated the production and immunity phenotype (Bac⁺ Imm⁺) to a 13·6 MDa plasmid. Spelhaug & Harlander (1989) demonstrated that *P. pentosaceus* FBB61 was inhibitory to several strains of *L. monocytogenes*, but had no effect against any Gram-negative bacteria tested.

No additional data are available about the characteristics of pediocin A, its genetic determinants, possible homologies with other bacteriocins and mode of action. This study deals with the purification and partial characterization of pediocin A, produced by *P. pentosaceus* FBB61, with a view to evaluating its possible utilization as a food preservative.

**METHODS**

**Bacterial strains and media.** *P. pentosaceus* FBB61 (ATCC 43200) and *Lactococcus lactis* subsp. *diacetylactis* (*L. lactis* subsp. *lactis*) CH001, used as an indicator strain, were propagated at 30 °C in M17 broth (Sambrook *et al.*, 1989). The medium used for bacteriocin production was M17 + 1·1% (w/v) glucose. Agar media were prepared by adding 1·5% (w/v) granulated agar to the broth media. Soft and overlay agars were prepared with 0·75% agar.

**Bacteriocin production.** *P. pentosaceus* FBB61 was grown in M17 + 1·1% glucose at 30 °C for 24 h. The culture supernatant was then collected, adjusted to pH 6·5, filtered through 0·22 μm pore-size filters, concentrated to 0·1 volume by polyethylene glycol dialysis (Sigma, PEG 20000) and again filter-sterilized. This material was designated crude pediocin A and was frozen at −20 °C when not used immediately.

**Keywords:** pediocin A, bacteriocin, *Pediococcus pentosaceus*, food microbiology
Bacteriocin titration. Serial twofold dilutions of the crude extract and purified protein were made in sterile distilled water. From each dilution 30 μl aliquots were delivered into wells in an M17 agar (1.5% w/v) plate previously seeded with 10 μl of a fresh overnight culture (Lactococcus lactis subsp. lactis CH001) (approx. 10^5 c.f.u.) and incubated at 30 °C. The sample titre was defined as the reciprocal of the highest dilution showing definite inhibition of the indicator lawn and was expressed in activity units (AU) ml⁻¹.

Purification of pediocin A. i) Butanol extraction. Crude pediocin A, prepared from M17 supernatant, was mixed with butanol (2:3 ratio). The mixture was vortexed for 30 s and left for 30 min. The butanol phase was removed and freeze-dried. All manipulations were at 0 °C. The powder was resuspended in ultrapure water in one-fifteenth of the original volume.

(ii) Non-denaturing gels and direct detection of antimicrobial activity. Non-denaturing polyacrylamide gels were prepared with a mixture of 15% (w/v) acrylamide and 0.4% (w/v) bisacrylamide, without SDS. Butanol extract was loaded in two parallel lanes (30 μg per lane). After the electrophoresis, one lane was stained with Coomassie blue R-250 or silver. To detect antimicrobial activity, the other lane was overlaid with 10 μl M17 soft agar seeded with 10 μl of a fresh overnight culture of L. lactis subsp. lactis CH001. Plates were incubated at 30 °C for 20 h. The antimicrobial activity was eluted from these gels as described for helveticin J by Joerger and Klaenhammer (1986).

(iii) Electroendosmotic preparative electrophoresis (EPE). Recovery of the purified protein from the butanol extract was achieved by EPE (Curioni et al., 1988, 1989) using native polyacrylamide gels as described by Reniero et al. (1992). The stacking (T = 3%) and the running gel (T = 10%) were 2 cm and 3 cm in length, respectively. The fraction-collecting device was set to obtain one fraction every 20 drops (400 μl) at a flow rate of 4.8 ml h⁻¹.

SDS-PAGE. SDS-PAGE was carried out with a mixture of 15% (w/v) acrylamide and 0.4% (w/v) bisacrylamide according to Laemmli (1970). After the electrophoresis, the gels were stained with Coomassie blue R-250 or silver.

Sensitivity to heat and proteolytic enzymes. Crude and purified pediocin A was heated in a boiling water bath. Samples were removed at 0, 5, 10, 30 and 60 min, and titres were determined to detect activity. To determine the influence of proteolytic enzymes, the following enzymes (1 mg ml⁻¹) were used: trypsin (EC 3.4.21.4) (110 U mg⁻¹), pronase (7 U mg⁻¹), and proteinase K (EC 3.4.21.64) (20 U mg⁻¹). Pediocin A/enzyme mixtures were incubated for 30 min at 37 °C.

Determination of minimal inhibitory concentration (MIC). One-millilitre vials of M17 were inoculated with approximately 10^9 c.f.u. ml⁻¹ of a fresh overnight culture of the indicator strain. Twofold dilutions of purified bacteriocin were added to each vial, ranging from 1.95 to 1000 AU ml⁻¹. The MIC was defined as the minimal bacteriocin concentration required to inhibit growth after 24 h incubation.

Antimicrobial spectrum. Bacterial strains and growth conditions for activity spectrum determination of purified pediocin A are listed in Table 2. Strain sensitivity to pediocin A was measured by serial twofold dilutions of purified pediocin A (133.3 AU), as described above (bacteriocin titration). Agar plates were seeded with the strains listed in Table 2 and incubated at the growth temperature of the strain. Strain sensitivities were expressed as a percentage of L. lactis subsp. lactis CH001 sensitivity to 133.3 AU of pediocin A.

RESULTS

Production of pediocin A in M17 medium

To maximize the recovery of antimicrobial activity, the relationship of culture growth and production was studied during a 24 h incubation. Titres of activity increased as the growth increased (Fig. 1). There was a linear relation between c.f.u. count and activity that was described by the following linear regression formula:

\[ y = 0.475 x - 3.105 \]  
\[ x = \log (\text{c.f.u. ml}^{-1}); y = \log (\text{AU ml}^{-1}) \]

The antimicrobial protein was constitutively expressed, with no relation to the physiological status of the culture, and it appeared to be stable, with no loss of activity after the exponential phase as described for other bacteriocins.

![Fig. 1. Producer growth and pediocin A production.](attachment:image)

**Table 1. Purification of pediocin A**

<table>
<thead>
<tr>
<th>Purification stage</th>
<th>Vol (ml)</th>
<th>Total activity (AU)</th>
<th>Pediocin A activity (AU ml⁻¹)</th>
<th>Total protein (mg)</th>
<th>Protein concn (mg ml⁻¹)</th>
<th>Sp. act. (AU mg⁻¹)</th>
<th>Activity recovered (%)</th>
<th>Purification (±fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>1000</td>
<td>33000</td>
<td>33</td>
<td>17500</td>
<td>17.5</td>
<td>1.9</td>
<td>100.0</td>
<td>1</td>
</tr>
<tr>
<td>Dialysis against PEG</td>
<td>100</td>
<td>32000</td>
<td>320</td>
<td>13000</td>
<td>130</td>
<td>25</td>
<td>96.9</td>
<td>1.3</td>
</tr>
<tr>
<td>Butanol extraction</td>
<td>10</td>
<td>5333</td>
<td>533.3</td>
<td>6</td>
<td>6</td>
<td>889</td>
<td>16.2</td>
<td>468</td>
</tr>
<tr>
<td>EPE</td>
<td>0.4</td>
<td>1280</td>
<td>3200</td>
<td>0.086</td>
<td>0.215</td>
<td>14884</td>
<td>3.9</td>
<td>7834</td>
</tr>
</tbody>
</table>
Pediocin A

Crude pediocin A preparation (100 ml, 3.2 × 10⁴ AU) was subjected to butanol extraction. Titration of the butanol and aqueous phases for antimicrobial activity indicated that no activity remained in the aqueous phase, whereas the butanol phase, following freeze-drying, resuspension and dialysis against distilled water, was inhibitory against the indicator strain. This indicated the hydrophobic nature of pediocin A. Unfortunately, even if the extraction was carried out at low temperature, pediocin A activity was reduced to 20% by this extraction procedure; nevertheless the enrichment was highly significant (Table 1). Organic solvents can act as denaturing agents, causing considerable loss of activity (Wilson & Goulding, 1986).

Electrophoresis of the butanol extract on non-denaturing polyacrylamide gels resulted in two bands (Fig. 2); direct detection of antimicrobial activity by overlaying the gel with soft agar seeded with the indicator strain revealed that the upper band was active (Fig. 2). This band was eluted and analysed by SDS-PAGE, resulting in a single protein band of approximately 80 kDa, providing direct evidence that this band contained pediocin A.

To scale up the recovery of pure pediocin A, non-denaturing gel elution was replaced by nondenaturing EPE. Butanol extract (5333 AU) was applied to a native polyacrylamide column and the elution profile showed two peaks (data not shown) corresponding to the two bands seen on non-denaturing PAGE (Fig. 2). Each eluted fraction was assayed for antimicrobial activity and was analysed by SDS-PAGE. Only the fraction corresponding to the second peak was highly inhibitory to the indicator strain; on SDS-PAGE it showed only a single band, of approximately 80 kDa (Fig. 3). None of the buffers used in these experiments was inhibitory to L. lactis subsp. lactis CH001. Pediocin A was eluted following electrophoresis for 8.5 h. This protocol allowed 3.9% activity recovery (Table 1).

Enzyme and heat inactivation

Pediocin A activity was completely eliminated upon treatment with trypsin, pronase or proteinase K (data not shown). After incubation of crude or purified pediocin A at 100 °C for 5 min the bacteriocin activity was reduced to 20%, and after 10 min only 5% of the original activity remained. No appreciable difference in inactivation was observed between crude and purified pediocin A. These results do not agree with those of Rueckert (1979): every attempt to detect antimicrobial activity from either crude or purified pediocin A heated at 100 °C for 60 min failed, confirming its heat sensitivity.

MIC

The MIC for L. lactis subsp. lactis CH001 was 31.25 AU ml⁻¹.
Table 2. Activity spectrum of pediocin A

Sensitivity of strains to pediocin A was measured with serial twofold dilutions of purified bacteriocin (133.3 AU). See Methods for details.

<table>
<thead>
<tr>
<th>Target strain</th>
<th>Temp. (°C)</th>
<th>Medium*</th>
<th>Pediocin A sensitivity †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactobacillus acidophilus ATCC 4356</td>
<td>37</td>
<td>MRS</td>
<td>50</td>
</tr>
<tr>
<td>Lactobacillus bulgaricus ATCC 11842</td>
<td>42</td>
<td>MRS</td>
<td>25</td>
</tr>
<tr>
<td>Lactobacillus casei ATCC 334</td>
<td>37</td>
<td>MRS</td>
<td>5</td>
</tr>
<tr>
<td>Lactobacillus curvatus NCFB 2739</td>
<td>30</td>
<td>MRS</td>
<td>5</td>
</tr>
<tr>
<td>Lactobacillus fermentum ATCC 9338</td>
<td>37</td>
<td>MRS</td>
<td>65</td>
</tr>
<tr>
<td>Lactobacillus helveticus ATCC 15009</td>
<td>42</td>
<td>MRS</td>
<td>100</td>
</tr>
<tr>
<td>Lactobacillus plantarum NCDO 1193</td>
<td>37</td>
<td>MRS</td>
<td>5</td>
</tr>
<tr>
<td>Lactobacillus ruminis DMS 20016</td>
<td>37</td>
<td>MRS</td>
<td>5</td>
</tr>
<tr>
<td>Lactobacillus sake NCFB 2747</td>
<td>30</td>
<td>MRS</td>
<td>88</td>
</tr>
<tr>
<td>Lactobacillus salivarius NCFB 2747</td>
<td>37</td>
<td>MRS</td>
<td>10</td>
</tr>
<tr>
<td>Pediococcus pentosaceus FBB 63</td>
<td>30</td>
<td>M17</td>
<td>0</td>
</tr>
<tr>
<td>Pediococcus pentosaceus PC 1</td>
<td>30</td>
<td>M17</td>
<td>38</td>
</tr>
<tr>
<td>Propionibacterium acidipropionici NCDO 563</td>
<td>30</td>
<td>M17</td>
<td>50</td>
</tr>
<tr>
<td>Lactococcus cremoris CNRZ 177</td>
<td>25</td>
<td>M17</td>
<td>50</td>
</tr>
<tr>
<td>Leuconostoc cremoris DB 1275</td>
<td>25</td>
<td>M17</td>
<td>38</td>
</tr>
<tr>
<td>Staphylococcus thermophilus ST 20</td>
<td>42</td>
<td>M17</td>
<td>75</td>
</tr>
<tr>
<td>Staphylococcus thermophilus ST 112</td>
<td>42</td>
<td>M17</td>
<td>75</td>
</tr>
<tr>
<td>Staphylococcus carnosus MC 1</td>
<td>37</td>
<td>BHI</td>
<td>75</td>
</tr>
<tr>
<td>Bacillus cereus ATCC 9139</td>
<td>37</td>
<td>BHI</td>
<td>10</td>
</tr>
<tr>
<td>Listeria innocua BI. 86/26</td>
<td>30</td>
<td>BHI</td>
<td>10</td>
</tr>
<tr>
<td>Enterococcus faecalis EF 1</td>
<td>37</td>
<td>BHI</td>
<td>65</td>
</tr>
<tr>
<td>Clostridium sporogenes C 22/10</td>
<td>37</td>
<td>RCM</td>
<td>65</td>
</tr>
<tr>
<td>Clostridium tyrobutyricus 3.5</td>
<td>30</td>
<td>RCM</td>
<td>50</td>
</tr>
<tr>
<td>Clostridium tyrobutyricus NCDO 1754</td>
<td>30</td>
<td>RCM</td>
<td>75</td>
</tr>
<tr>
<td>Lactococcus lactis CH001</td>
<td>30</td>
<td>M17</td>
<td>100</td>
</tr>
<tr>
<td>Pediococcus pentosaceus FBB61</td>
<td>30</td>
<td>M17</td>
<td>0</td>
</tr>
<tr>
<td>Pediococcus pentosaceus FBB61-2 ‡</td>
<td>30</td>
<td>M17</td>
<td>100</td>
</tr>
</tbody>
</table>

* MRS medium (De Man et al., 1960) was used without Tween 80. BHI, brain-heart infusion (Difco); RCM, reinforced clostridial medium (Oxoid).
† Mean values of at least three experiments, expressed as a percentage of the sensitivity of the standard test strain L. lactis subsp. lactis CH001.
‡ Isogenic mutant that does not produce pediocin A (Daeschel & Klaenhammer, 1985).

Antimicrobial spectrum

P. pentosaceus FBB61 exhibited antimicrobial activity against almost all Gram-positive micro-organisms tested by the agar-spot method (data not shown). The utilization of serial dilutions of purified pediocin A against each strain tested allowed us to measure their relative sensitivities and to verify the broad spectrum of activity of this bacteriocin (Table 2).

DISCUSSION

We have confirmed that the antimicrobial agent produced by P. pentosaceus FBB61, pediocin A, is a bacteriocin. The sensitivity of pediocin A to heat and proteolytic enzymes demonstrated its proteinaceous nature. P. pentosaceus FBB61 and pure pediocin A had an identical broad spectrum of bactericidal activity against almost all Gram-positive strains tested. Purification of pediocin A was achieved by PEG 20000 dialysis, butanol extraction and EPE. The purification resulted in an approximately 7834-fold increase in the specific activity of pediocin A and 3.9% recovery. Electrophoresis experiments showed that a 80 kDa protein could be identified as pediocin A.

Among bacteriocins of the genus Pediococcus that have been purified to date, pediocin A is the largest, at 80 kDa. A wide range of different molecular masses was also observed for Lactobacillus bacteriocins (Joerger & Klaenhammer, 1986; Rammelsberg & Radler, 1990). The heat sensitivity of pediocin A appeared to be consistent with the size and the apparent complexity of this protein compared with other bacteriocins.

Every attempt to detect the antimicrobial activity directly in SDS-PAGE failed, whereas it was successful for pediocin AcH (Bhunia et al., 1987). However, SDS was
highly inhibitory to the indicator strain; it is also possible that SDS inactivated pediocin A by altering its native state.

Pediocin A was also purified by ion-exchange and hydrophobic interaction chromatography (HIC) from a semi-synthetic medium prepared as described by Reniero et al. (1993) (data not shown). The hydrophobic nature of pediocin A was evidenced by the butanol extraction and the HIC elution profile. Chromatography of pediocin A on a C18 reversed-phase column revealed that the bacteriocin was eluted with 32% acetonitrile (data not shown). Hydrophobicity is a common feature of several bacteriocins (Joeger & Klaenhammer, 1986; Muriana & Klaenhammer, 1991; Mortvedt et al., 1991; Piard et al., 1992). This suggests that the cell membrane is a possible target, as demonstrated for lactococcin A (van Belkum et al., 1991).

Because of the potential use of bacteriocins of lactic acid bacteria as food preservatives, it is of great importance to gain insight into their chemical structure and their role as a food preservative.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr M. C. Callegari, Dr R. Reniero and Dr D. McDonagh for their efficient help.

REFERENCES


C. T. Klaenhammer
A. PIVA and D. R. HEADON


Received 3 September 1993; revised 14 October 1993; accepted 22 October 1993.