Engineering Increased Stability in the Antimicrobial Peptide Pediocin PA-1

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Pediocin PA-1 is a food grade antimicrobial peptide that has been used as a food preservative. Upon storage at 4°C or room temperature, pediocin PA-1 looses activity, and there is a concomitant 16-Da increase in the molecular mass. It is shown that the loss of activity follows first-order kinetics and that the instability can be prevented by replacing the single methionine residue (Met31) in pediocin PA-1. Replacing Met by Ala, Ile, or Leu protected the peptide from oxidation and had only minor effects on bacteriocin activity (for most indicator strains 100% activity was maintained). Replacement of Met by Asp was highly deleterious for bacteriocin activity.

Bacteria produce ribosomally synthesized antimicrobial polypeptides termed bacteriocins. Bacteriocins produced by gram-positive bacteria are usually membrane-permeabilizing cationic peptides with less than 50 amino acid residues (19, 20, 23, 25). These bacteriocins may be divided into two classes; class I contains bacteriocins (often referred to as lantibiotics) with modified residues, and class II contains bacteriocins without modified residues. Within class II, the so-called pediocin-like bacteriocins produced by a variety of lactic acid bacteria constitute a dominant group. At least 14 different bacteriocins belonging to this group are presently known, and pediocin PA-1 (4, 15, 17, 22), leucocin A UAL-187 (13), mesentericin Y105 (14), sakacin P (28), and curvacin A (identical to sakacin A) (16, 28) were the first of these to be identified.

The pediocin-like bacteriocins are characterized by a YNGGV motif and a disulfide bridge in a highly conserved N-terminal region, by high antiinisterial activity, and by their membrane-permeabilizing mode of action (6, 7, 20). Some of the pediocin-like bacteriocins (such as pediocin PA-1) also contain a disulfide bridge in the C-terminal region, whereas others (such as sakacin P) do not. The highly conserved N-terminal region is hydrophilic and cationic, and it has been proposed that this region mediates the initial binding of these bacteriocins to target cells through electrostatic interactions (5). The somewhat less conserved C-terminal half is hydrophobic and/or amphiphilic and is thought to penetrate into the hydrophobic part of the target cell membrane, thereby mediating membrane leakage (10, 18). Structural analysis indicates that a 15- to 20-residue stretch from the middle toward the C-terminal end forms an amphiphilic α-helix upon interaction with membrane-like structures and that the remaining C-terminal residues are relatively unstructured (12, 30).

Much of the interest in pediocin-like bacteriocins is due to their antiinisterial activity and thus to their potential for use as antimicrobial additives in food. Their use as additives requires that they be sufficiently stable and consequently devoid of residues that are prone to potentially damaging chemical modifications. However, several of the pediocin-like bacteriocins contain methionine residues whose sulfur atom may be oxidized, which results in destabilization of the bacteriocin. In this study, we focused on the methionine residue present in the C-terminal half of pediocin PA-1, which presently is perhaps the pediocin-like bacteriocin that is most promising for use as an antimicrobial additive. Pediocin PA-1 variants were constructed in which Met31 was replaced by Ala, Leu, and Asp, and the effects of these mutations on bacteriocin stability, activity, and target cell specificity were determined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Wild-type pediocin PA-1 was produced by and purified from Pediococcus acidilactici LMG2351, which was isolated from commercial starter cultures obtained from Christian Hansen Laboratories, Copenhagen, Denmark (22). Mutant pediocin PA-1 and wild-type sakacin P were produced by and purified from a two-plasmid bacteriocin expression system developed recently (3, 9). This system is based on the use of pSAK20 and either pSP2 (for production of wild-type sakacin P) or pPED2 (for production of mutant pediocin PA-1) introduced into the bacteriocin-deficient strain Lactobacillus sake Lb790. pSAK20 and pSP2/pPED2 confer resistance to chloramphenicol and erythromycin, respectively. Mutant sakacin P and pediocin PA-1 variants were produced by and purified from L. sake Lb706 and L. sake Lb790, respectively. The EFSAKRTE operon contains genes encoding proteins necessary for activation of the bacteriocin-specific promoters and for processing and secretion of the prebacteriocins (2, 3, 21).

Epicurian Coli XL-1-Blue supercompetent cells (Stratagene) were used for cloning all of the mutated pPDE2 plasmids, and plasmids with desired mutations were transformed into L. sake Lb790/pSAK20.

E. coli was grown at 37°C in Luria-Bertani medium (Difco) with vigorous agitation, whereas the lactic acid bacteria were grown at 30°C without agitation. The indicator strains used in the bacteriocin assays were L. sake NCDO 2714 (type strain), Lactobacillus casei subsp. torquens NCDO 2740, Enterococcus faecalis NCDO 581, P. acidilactici NCDO 1859, Pediococcus pentosaceus FBI83B, Leuconostoc mesenteroides subsp. dextranicum NCDO 529, and Carnobacterium piscicola UI49 (27). C. piscicola UI49 was grown in M17 medium (Oxoid) supplemented with glucose and Tween 80 at final concentrations of 0.4% (wt/vol) and 0.1% (vol/vol), respectively. The other lactic acid bacteria were grown in MRS broth (Oxoid). For agar plates, the media were solidified by adding 1.5% (wt/vol) agar. The selective antibiotic concentrations used were 150 μg of erythromycin per ml for E. coli, 10 μg of erythromycin per ml and 10 μg of chloramphenicol per ml for normal growth of plasmid-containing L. sake LB790, and 2 μg of erythromycin per ml and 5 μg of chloramphenicol per ml for initial selection of L. sake LB790/pSAK20 transformed with pPDE2 variants.

Purification of sakacin P, pediocin PA-1, and mutant pediocin PA-1. Wild-type and mutant bacteriocins were purified to homogeneity from 400-ml cultures of Lb706 (2, 3). The operon contains genes encoding proteins necessary for activation of the bacteriocin-specific promoters and for processing and secretion of the prebacteriocins (2, 3, 21).

E. coli was grown at 37°C in Luria-Bertani medium (Difco) with vigorous agitation, whereas the lactic acid bacteria were grown at 30°C without agitation. The indicator strains used in the bacteriocin assays were L. sake NCDO 2714 (type strain), Lactobacillus casei subsp. torquens NCDO 2740, Enterococcus faecalis NCDO 581, P. acidilactici NCDO 1859, Pediococcus pentosaceus FBI83B, Leuconostoc mesenteroides subsp. dextranicum NCDO 529, and Carnobacterium piscicola UI49 (27). C. piscicola UI49 was grown in M17 medium (Oxoid) supplemented with glucose and Tween 80 at final concentrations of 0.4% (wt/vol) and 0.1% (vol/vol), respectively. The other lactic acid bacteria were grown in MRS broth (Oxoid). For agar plates, the media were solidified by adding 1.5% (wt/vol) agar. The selective antibiotic concentrations used were 150 μg of erythromycin per ml for E. coli, 10 μg of erythromycin per ml and 10 μg of chloramphenicol per ml for normal growth of plasmid-containing L. sake LB790, and 2 μg of erythromycin per ml and 5 μg of chloramphenicol per ml for initial selection of L. sake LB790/pSAK20 transformed with pPDE2 variants.

Purification of sakacin P, pediocin PA-1, and mutant pediocin PA-1. Wild-type and mutant bacteriocins were purified to homogeneity from 400-ml cultures by ammonium sulfate precipitation followed by cation-exchange chromatography, hydrophobic interaction chromatography, and reverse-phase chromatography as described previously (22). The primary structures of the compounds were confirmed by determining the molecular masses with a Voyager-DE RP matrix-assisted laser desorption ionization–time of flight mass spectrometer (Perseptive Biosystems); α-cyano-4-hydroxycinnamic acid was used as the matrix. Typically, the errors in the masses which were determined were ±1 Da. The purities of the bacteriocins were verified to be greater than 90% by analytical reverse-phase chromatography.

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chromatography by using a µRPC SC 2.1/10 C18 C18 column (Pharmacia Biotech) in the SMART chromatography system (Pharmacia Biotech).

The concentrations of purified bacteriocins were determined by measuring UV absorption at 280 nm, and the values were converted to protein concentrations by using molecular extinction coefficients calculated from the contributions of individual amino acid residues.

**Bacteriocin assay**. Bacteriocin activity was measured by using a microtiter plate assay system, essentially as described previously (24). The wells of a microtiter plate contained 200 µl of culture medium with bacteriocin fractions at twofold dilutions and an indicator strain at an optical density at 610 nm of about 0.01. The microtiter plate cultures were incubated overnight (12 to 16 h) at 30°C, after which growth of the indicator strain was measured spectrophotometrically at 610 nm with a microtiter plate reader. The MIC was defined as the concentration of bacteriocin that inhibited growth of the indicator strain by 50%.

**Plasmid isolation and transformation**. Plasmids were isolated from E. coli and L. sake by using the Wizard Plus SV Miniprep DNA purification system (Promega). To ensure lysis of L. sake, lysozyme and mutanolysin were added to the cell resuspension solution included in the Wizard Plus SV Miniprep kit to final concentrations of 5 mg/ml and 15 U/ml, respectively.

Chemosensitive *Epicurian Coli* XLI-Blue supercompetent cells were transformed by using the protocol provided with a Quick Change site-directed mutagenesis kit (Stratagene). The PCR reactions were performed in a GeneAmp 2400 PCR system (Perkin-Elmer) by using Pfu DNA polymerase (Stratagene). The 30-µl reaction mixtures each contained about 40 ng of plasmid template, 125 ng of each oligonucleotide primer (Eurogentec), each deoxynucleoside triphosphate (Stratagene) at a final concentration of 0.05 mM, and 2.5 U of Pfu DNA polymerase. After a 1-min hot start at 95°C, 16 cycles of the following program were run: denaturation for 30 s at 95°C, primer annealing for 1 min at 50°C, and polymerization for 12 min at 68°C. The PCR product was digested for 1 h at 37°C with restriction enzyme DpnI (Stratagene) to eliminate the original template and thereby increase mutation efficiency. The DNA sequencing profiles were run in the Wizard Plus SV Miniprep kit to final concentrations of 5 mg/ml and 15 U/ml, respectively.

**Site-directed mutagenesis and DNA sequencing**. Mutations in the bacteriocin PA-1- and sakacin P-encoding genes cloned in pPED2 were obtained by using a Quick Change site-directed mutagenesis kit (Stratagene). The PCR reactions were performed with a GeneAmp 2400 PCR system (Perkin-Elmer) by using Pfu DNA polymerase (Stratagene). The 30-µl reaction mixtures each contained about 40 ng of plasmid template, 125 ng of each oligonucleotide primer (Eurogentec), each deoxynucleoside triphosphate (Stratagene) at a final concentration of 0.05 mM, and 2.5 U of Pfu DNA polymerase. After a 1-min hot start at 95°C, 16 cycles of the following program were run: denaturation for 30 s at 95°C, primer annealing for 1 min at 50°C, and polymerization for 12 min at 68°C. The PCR product was digested for 1 h at 37°C with restriction enzyme DpnI (Stratagene) to eliminate the original template and thereby increase mutation efficiency. The DNA sequencing profiles were run in the Wizard Plus SV Miniprep kit to final concentrations of 5 mg/ml and 15 U/ml, respectively.

**RESULTS**

**Production and purification of sakacin P, pediocin PA-1, and mutant pediocin PA-1 molecules**. The methionine residue in pediocin PA-1 (position 31) (Fig. 1) was in all mutant molecules replaced by another residue, either alanine, isoleucine, leucine, or aspartate (designated ped[M31A], ped[M31I], ped[M31L], and ped[M31D], respectively). The mutants were all produced by using the L. sake Lb790/pSAK20/pPED2 two-plasmid expression system, whereas wild-type pediocin PA-1 was produced by using the natural pediocin PA-1 producer (*P. acidilactici* LMG2351), as it yielded about five times more bacteriocin than the two-plasmid expression system. Wild-type sakacin P was produced by using the L. sake Lb790/pSAK20/pSPP2 expression system, since it yielded about five times more than the natural sakacin P producer (L. sake LTH673). More-over, sakacin P produced by the expression system was stable for months, whereas sakacin P produced by the natural producer lost activity during purification and storage as a result of degradation by contaminating extracellular proteases produced by *L. sake* LTH673 (10).

**Oxidation and partial inactivation of pediocin PA-1 during storage**. During storage pediocin-like bacteriocins that contain a methionine residue change to a less active form (10, 26; see below), apparently due to oxidation of the methionine sulfur atom to sulfoxide. This oxidation increases the molecular mass by 16 Da. For pediocin PA-1, the kinetics of this conversion was determined by separating the unoxidized and oxidized forms by reverse-phase chromatography after the bacteriocin was stored for various lengths of time under various conditions (Fig. 2). The relative amounts of the two forms were determined from the reverse-phase chromatography absorbance profiles. The first of the two peptide forms to elute from the reverse-phase column had the molecular mass (as determined by mass spectrometry) expected for pediocin PA-1 containing an oxidized methionine (4,640 Da), whereas the second form to elute had the molecular mass expected for the active unoxidized form of pediocin PA-1 (4,624 Da). The specific activity of the latter was about 100-fold greater than that of the former.

The conversion of active unoxidized pediocin PA-1 to the less active oxidized form followed first-order kinetics with half-

**TABLE 1. Theoretical and experimental molecular masses of pediocin PA-1 and mutant bacteriocin molecules**

<table>
<thead>
<tr>
<th>Bacteriocin</th>
<th>Theoretical mass (Da)</th>
<th>Observed mass (Da)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pediocin PA-1</td>
<td>4,624.3</td>
<td>4,624.1 ± 0.1</td>
</tr>
<tr>
<td>ped[M31A]</td>
<td>4,564.2</td>
<td>4,563.9 ± 0.1</td>
</tr>
<tr>
<td>ped[M31D]</td>
<td>4,608.2</td>
<td>4,608.0 ± 0.1</td>
</tr>
<tr>
<td>ped[M31L]</td>
<td>4,606.3</td>
<td>4,606.3 ± 0.1</td>
</tr>
<tr>
<td>ped[M31D]</td>
<td>4,606.3</td>
<td>4,606.4 ± 0.2</td>
</tr>
</tbody>
</table>

* The theoretical molecular masses were calculated from the amino acid sequences, assuming that the cysteine residues form disulfide bridges and that methionines (only present in wild-type pediocin PA-1) are not oxidized.

* The observed masses are means based on three parallel measurements obtained by mass spectrometry.
lives of 15, 27, 42, 65, and 100 days when preparations were stored at room temperature in 100, 50, 25, 10, and 0% propa- nol respectively, containing 0.1% (vol/vol) trifluoracetic acid (TFA) (Fig. 3). Freezing the bacteriocin protected it from oxidation. After 55 days (in 20 mM phosphate buffer, pH 7), no oxidation was detected when preparations were stored at 2°C, in contrast to the 20 to 30% oxidation that occurred when preparations were stored at either 4°C or room temper- ature.

Mutational effects on bacteriocin activity. The susceptibility of pediocin PA-1 to inactivation, apparently because of oxidation of its methionine residue, prompted us to construct methionine-free mutant pediocin PA-1 molecules. Seven indicator strains were used to test the effect that the mutations had on potency and target cell specificity. Four of these strains (L. sake NCDO 2714, L. coryneformis subsp. torquens NCDO 2740, E. faecalis NCDO 581, and C. piscicola UI49) were sensitive to both pediocin PA-1 and sakacin P, whereas three of the strains (P. pentosaceus FBB63B, P. acidilactici NCDO 1859, and L. mesenteroides subsp. dextranicum NCDO 529) were about 100 to 1,000 times more sensitive to pediocin PA-1 than to sakacin P (Table 2). The latter three strains were, consequently, useful for analyzing whether replacement of the methionine residue (which is absent in sakacin P [Fig. 1]) significantly alters the target cell specificity of pediocin PA-1.

The mutant pediocin PA-1 molecules in which the methio- nine residue was replaced by a hydrophobic amino acid residue (alanine, isoleucine, or leucine) were as active as pediocin PA-1 and sakacin P against the four strains that were sensitive to both pediocin PA-1 and sakacin P (Table 2). These mutant molecules were only slightly less potent (two to five times less potent) than pediocin PA-1 against the three strains that were sensitive to pediocin PA-1 but relatively resistant to sakacin P, but they were more potent than sakacin P (Table 2). Re- placing the methionine residue with a negatively charged hy- drophilic amino acid residue (aspartate) rather than with a hydrophobic residue resulted in a 100-fold reduction in the bacteriocin activity (Table 2).

A more stable variant of pediocin PA-1 was clearly obtained by replacing the methionine residue with either alanine, leu- cine, or isoleucine. In contrast to pediocin PA-1, the methi- onine-free mutant pediocin PA-1 molecules retained activity, and no oxidation was detected even after 4 weeks of storage at room temperature in the presence of 80% propanol (Fig. 4). Methionine-free mutant molecules in 25% propanol–0.1% TFA in fact remained active without detectable oxidation for more than 7 months at 4°C or for 70 days at room tem- perature.

DISCUSSION

Pediocin PA-1 readily changed to a less active form, which had a molecular mass that was 16 Da greater and which was somewhat more polar (as judged by reverse-phase chromatography), as one would expect upon oxidation of the methionine sulfur atom to sulfoxide. The change followed first-order kinetics, with the half-life varying between a few weeks and several months, apparently depending on the amount oxygen present. Oxygen, a nonpolar molecule, is expected to have higher solubility in organic solvents with low polarities than in water, and this might be the reason why the conversion rate in water-propanol mixtures increased with increasing propanol concentration. Pediocin PA-1 was clearly much more stable when the methionine residue was replaced by either an ala- nine, isoleucine, or leucine residue, indicating that methionine was indeed the destabilizing residue in pediocin PA-1.

When a bacteriocin contained four cysteine residues, as is the case in pediocin PA-1, our expression system produced about equal amounts of three variants, each displaying one of the three possible patterns of disulfide formation (9). In con- trast, the wild-type producer of pediocin PA-1 yielded basically...
one variant with correct disulfide bridges (9). A protein present in the wild-type producer of pediocin PA-1, but not in our expression system, thus apparently helps generate the correct disulfide bridges (9). The formation of incorrect disulfide bridges may explain why more complex absorbance profiles were obtained in the last reverse-phase chromatography step when we purified the mutant bacteriocins and pediocin PA-1 produced by our expression system, compared to the simple absorbance peak which was obtained when we purified pediocin PA-1 produced by the wild-type producer.

Despite similarities in their primary structures, the pediocin-like bacteriocins have different target cell specificities (8). Their hydrophobic-amphiphilic C-terminal halves appear to be important in determining their specificities, since hybrid bacteriocins containing N- and C-terminal regions from different pediocin-like bacteriocins have antimicrobial spectra similar to those of the bacteriocins from which the C-terminal halves are derived (10). The fact that 15-mer fragments from the C-terminal half of pediocin PA-1, but not fragments from the N-terminal half, inhibit pediocin PA-1 to a greater extent than they inhibit other closely related pediocin-like bacteriocins also suggests that the C-terminal half contains important specificity determinants (11). The disulfide bridge in the C-terminal half of some pediocin-like bacteriocins is clearly one such specificity determinant. Introducing this bridge in sakacin P (which naturally lacks the bridge) by inserting two cysteine residues made the specificity of sakacin P more similar to that of pediocin PA-1 (which naturally contains the bridge), whereas removing the bridge in pediocin PA-1 by replacing cysteine with serine residues made the specificity of pediocin PA-1 more similar to that of sakacin P (9). Other residues in the C-terminal half may also influence the target cell specificity. Replacement of the methionine residue in the C-terminal half of pediocin PA-1 appeared, however, to have only a minor effect on the target cell specificity, since ped [M31A], ped[M31L], and ped[M31I] were as potent as pediocin PA-1 against the four strains that were sensitive to both pediocin PA-1 and sakacin P and were only slightly less potent than pediocin PA-1, but much more potent than sakacin P, against the three strains that were sensitive to pediocin PA-1 but relatively resistant to sakacin P. Replacing the methionine residue with a hydrophilic negatively charged residue (aspartate) instead of a hydrophobic residue resulted in a marked decrease in potency against all strains tested, which is consistent with the proposal that the region interacts with the hydrophobic part of target cell membranes (10, 18). Similarly, replacement of methionine with a hydrophilic but uncharged threonine residue reduces the activity (18). Among the pediocin-like bacteriocins, pediocin PA-1 is perhaps the molecule which is considered to be the most promising antimicrobial additive. Making pediocin PA-1 more stable by replacing the methionine with another hydrophobic residue...
and retaining the bacteriocin activity is an important step in developing pediocin PA-1 into a useful antimicrobial additive.

ACKNOWLEDGMENT

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REFERENCES