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Abstract : *Pediococcus pentosaceus* Pep1 was isolated from vacuum-packed sausages, and it was shown to produce a potentially novel antimicrobial agent active against several species of *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Staphylococcus, Enterococcus, Bacillus* and *Listeria*, many of which are associated with food spoilage and health hazards of food origin. Gram-negative organisms such as *Yersinia enterocolitica* and *Escherichia coli* were not inhibited by this antimicrobial agent. Since the antimicrobial agent was found to be sensitive to proteolytic enzymes, resistant to heat and organic solvents and active over a wide range of pH values, between 3 and 8, apparently most stable in the lower part of that range, it was identified as a bacteriocin and was designated as pediocin P.

Key Words: Vacuum-packed sausages, *Pediococcus pentosaceus, pediocins, bacteriocins,* characterization

Vakum ile Paketlenmiş Sosis Örneklerinden Pediosin Üreten *Pediococcus pentosaceus* Pep1 Suşunun İzolasyonu ve Karekterizasyonu

Özet : Vakum ile paketlenmiş sosis örneklerinden izole edilen *Pediococcus pentosaceus* Pep1 suşunun *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Staphylococcus, Enterococcus, Bacillus* ve *Listeria* gibi birçok türü gıda bozulmalarına ve gıda kökenli hastalıklara sebebiyet veren bakterilere karşı potansiyel bakımdan yeni olarak tanımlanabilecek antimikrobiyal bir madde ürettiği tespit edilmiştir. Gram-negatif organizmalardan *Yersinia enterocolitica* ve *Escherichia coli*'nin bu antimikrobiyal maddeden etkilenmedikleri görülmüştür. Proteolitik enzimlere karşı hassas, ısıya ve organik çözücülere karşı dayanıklı ve 3 ile 8 arasında geniş bir pH skalasında, özellikle bu skalanın alt kısımlarında, aktif olan bu antimikrobiyal madde bakteriyosin olarak tanımlanmış ve pediosin P olarak isimlendirilmiştir.

Anahtar Sözcükler: Vakum paket sosis, *Pediococcus pentosaceus, pediosin, bacteriyosin*, karekterizasyon

Introduction

Many lactic acid bacteria produce bactericidal proteins or bacteriocins that have potential for use as food biopreservatives. Several species and strains of pediococci which are used as starter cultures in fermentation of meat (1, 2), sausage products (3), vegetables (4) and cheddar cheese (5) have also been the subject of much recent investigation with regard to their bacteriocin-producing ability. Bacteriocins have been characterized from *Pediococcus acidilactici* (6-9) and *Pediococcus pentosaceus* (10, 11) for effective use in foods. Pediocin AcH and pediocin PA-1 produced by different strains of *Pediococcus acidilactici* were purified (6, 12), and later demonstrated to be identical (13). Characteristics common to these bacteriocins are that their genetic determinants appear to be plasmid-borne and the bacteriocins are active against a broad spectrum of Gram-positive bacteria, many of which are associated with food spoilage and food-related health hazards. The ability of these bacteriocins to inhibit many foodborne pathogens, including *Listeria monocytogenes* (8, 14), make them attractive as potential food preservation agents.

Pediococcus pentosaceus FBB61 was originally isolated in 1953 from a cucumber fermentation (15). The inhibitory activity of this strain was observed by Etchells et al. (16) in pure culture fermentations of cucumbers, and further investigated by Fleming et al. (17). Rueckert (18) characterized the chemical nature of the inhibitory material as proteinaceous, and its action as bactericidal. Daeschel and Klaenhammer (10) linked the bacteriocin production and immunity phenotype (Bac^+ Imm^+) to a 13.6 MDa plasmid. Spelhaug and Harlender (19) demonstrated that *P. pentosaceus* FBB61 was inhibitory to several strains of *Listeria monocytogenes*, but had no effect against any Gram-negative bacteria tested. Piva and Headon (20) confirmed that the antimicrobial agent produced by *Pediococcus pentosaceus* FBB61 is a heat-stable bacteriocin, designated pediocin A, with a molecular weight of 80 kDa as determined by SDS-PAGE and active against many species of *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Staphylococcus, Enterococcus, Listeria* and *Clostridium*.

However, no additional data are available on the characteristics of pediocin produced by different strains of *P. pentosaceus*, its genetic determinants, possible homologies with other bacteriocins and mode of action. This study deals with the purification and characterization of the potentially novel bacteriocin of the pediocin P (anti-*Listeria*) family of peptides from *Pediococcus pentosaceus* Pep1 isolated from vacuum-packed sausages.

Materials and Methods

Bacterial cultures and media. A *Pediococcus pentosaceus* strain was used for production of a potentially novel bacteriocin of the pediocin (anti-Listeria) family of peptides. The strain was grown in TGE (trypticase glucose yeast extract) medium (21) at 35°C unless stated otherwise. As necessary, 1.5% agar was added to TGE broth to make a solid medium. The soft

agar, used for overlaying TGE agar in some studies, was prepared by adding 0.75% agar to the broth. *Lactobacillus plantarum* NCDO 955, *Lactococcus lactis* subsp. lactis OZ1, *Enterococcus faecalis* MB1, *Pediococcus acidilactici* Ped L, *Leuconostoc mesenteroides* Ly, *Listeria monocytogenes* NCTC 5105, *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis* were used as Gram-positive indicator organisms while *Escherichia coli* and *Yersinia enterocolitica* were used as Gram-negative indicators to check their sensitivity and resistance to pediocin P. All lactic acid bacteria were propagated in a TGE medium while tryptic soy broth supplemented with 0.5% yeast extract was used for growth of non-lactic acid bacteria. Cultures were maintained at -70°C as frozen stock cultures in equal volumes of 10% non-fat dry milk and 25% glycerol. *Lactobacillus plantarum* NCDO 955 strain was used as an indicator organism to assay pediocin P production.

Isolation of pediocin producing Pediococcus. Four different commercially produced vacuum-packed sausages were obtained from local supermarkets in Ankara, Turkey. Each bag was opened, and 1 ml of the liquid in the packages was collected and serially diluted (22). Samples from 3 to 4 consecutive dilutions were pour plated in sterile TGE agar. The plates were incubated at 35°C overnight. Plates containing 50 to 100 colonies were overlaid with 5ml TGE soft agar seeded with *L. plantarum* NCDO, a sensitive test organism. The plates were further incubated at 35°C overnight and examined for zone of growth inhibition of *L. plantarum* NCDO 955 around a colony. Representative colonies which produced a clear, defined zone of inhibition were considered pediocin producers and were purified and examined for morphology using a phase-contrast microscope and Gram-stain characteristics, and if found to be pediococci microscopically (4) were identified by the standard carbohydrate fermentation tests to determine their genera and species.

Biochemical tests for characterization of the isolated strains. The isolates were examined for carbohydrate fermentation patterns and other characteristics to determine their genera and species. Carbohydrate fermentation tests were done in TGE agar (TGE broth plus 1.5% agar) plates containing 0.004% chlorophenol red as an indicator. Membrane-filtered carbohydrates at 1% level were supplemented in place of glucose in TGE agar. The cells were harvested and washed twice with sterile 5 mM phosphate buffer, pH 7.0. The cells were resuspended in the same buffer to original volume and 5µl of each strain was inoculated by stabbing in the agar media in the plates. The plates were then incubated at 35°C overnight and examined for change in the color of the indicator. Each test was done at least twice, and the species of each isolate was determined by comparing the reactions with the table in Bergey's Manual (23). Further confirmation of the genera and species of isolates was also carried out by use of the API-20C identification test kit.

Antimicrobial activity assay. An aliquot of a test culture broth was heated in boiling water for 15 min and serially diluted (1:10 to 1:250) with sterile deionized water. A 5-µl portion, in duplicate, from each dilution was spotted directly over a lawn of appropriate indicator bacteria

over a TGE agar plate. The plates were incubated overnight and examined for the presence of 2 mm or larger clear zones of inhibition. The highest dilution that produced a distinct inhibition zone of 2mm or more was multiplied by 200 (AU/ml = dilution factor X 1ml/5µl) (21).

Partial purification of pediocin P by adsorption onto producer cells. *Pediococcus pentosaceus* Pep1 was grown to early stationary phase in TGE broth. Broth culture was heated at 72°C for 30 min to kill the cells after its pH was adjusted to 6.5. The cells were harvested by centrifugation at 15000 rpm for 15 min. After the cells had been washed with 5mM sodium phosphate (pH 6.5), they were resuspended in dH2O, and 0.1 M NaCl was added and the pH of the mixture was adjusted to 2.0. The mixture was kept at 4°C for 2 h by mixing with stirrer. Cell suspensions were then centrifuged at 20000 rpm for 20 min, and the supernatants containing free pediocin P were filter sterilized (0.22-µm-pore-size filter), dialyzed against deionized water in a 1000 molecular-weight-cutoff (Spectra/pore) dialysis bag at 4°C for 24 h, and freeze-dried (24).

The nature of antimicrobial materials. Following exposure to several pH values between 3 and 12, heat, organic solvents and enzymes, the presence or loss of antibacterial activity of the freeze-dried pediocin preparations from *Pediococcus pentosaceus* Pep1 were examined. The freeze-dried partially purified pediocin P preparation was dissolved in deionized water at a concentration of 50 mg/ml. Samples from this preparation were adjusted with sterile 5 mM NaOH or 5 mM HCl to different pH levels between 3 and 12 and the final pediocin P concentration was adjusted to yield 10 mg/ml. Samples were then kept as follows: (i) 2 h at 25°C, (ii) 24 h at 25°C, and (iii) 15 min at 121°C. Each sample was adjusted to pH 7.0 with sterile 5 mM phosphate buffer and the residual pediocin activities were determined by the agar-spot method (17). The freeze-dried partially purified pediocin P preparations were dissolved in organic solvents such as acetone, acetonitrile, chloroform, ethanol, formaldehyde and isopropanol at a concentration of 10 mg/ml. The samples were incubated at 25°C for 1 h. Solvents were then evaporated. Dried samples were reconstituted with sterile deionized water to a concentration of 10 mg/ml and assayed for antimicrobial activity (17). The sensitivity of the partially purified pediocin P to proteolytic enzymes such as protease K, protease IV, protease XIV, protease XXIV, chymotrypsin, trypsin, papain and ficin, and to other enzymes such as catalase, DNase, ribonuclease A, lipase, and lysozyme was also tested. The enzymes were dissolved in sterile 4 mM phosphate buffer, pH 7.0, at a concentration of 200 µg/ml. The freeze-dried pediocin P preparations were added to the enzyme solutions at a concentration of 10 mg/ml. Control samples contained only buffer solution. The samples were incubated at 37°C for 1 h and assayed as above for activity.

Antimicrobial spectrum of partially purified pediocin P and *Pediococcus pentosaceus* Pep1. Selected strains of Gram-positive and Gram-negative bacteria, many of which are associated with food-spoilage and food-related health hazards were used as indicators to determine their sensitivity or resistance to *P. pentosaceus* Pep1 and its partially purified pediocin P prepa-

ration. The spot-on-lawn method using 5 µl heated broth per spot, the disc-assay-method using 25 µl of heated broth per disc or the well-diffusion method using 50 µl of heated broth per well, in duplicates, was used over a lawn of each indicator strain on agar plates for testing the antimicrobial activity spectrum of *P. pentosaceus* Pep1, while the disc assay method was used for testing that of partially purified pediocin P. The plates were incubated for 24 h at 35°C and examined for the presence or absence of zones of growth inhibition.

Results

Selection of isolates with inhibitory properties. A liquid from vacuum-packed sausages was pour plated in TGE agar. The plates were incubated for colonies to develop, and overlaid with soft agar containing 1 of the 5 lactic acid indicator bacteria. After inoculation, the plates were examined for zone of growth inhibition. The colonies of isolates producing zones against at least 5 indicators were purified and designated on the basis of sources and sample numbers. Only 3 of 10 isolates were capable of producing zone of growth inhibition against all 5 indicators from 5 genera. The remaining 7 isolates produced zones against either 1 or 2 indicators.

Biochemical and physiological profiles of the isolates. The isolate had cocci cells arranged in tetrad formation occurring due to the division of cells in 2 perpendicular directions in a single plane. This feature allows the rapid presumptive identification with microscopic observation. It was nonmotile, nonsporulating, and Gram-positive. *P. pentosaceus* Pep1 showed some differences in its ability to ferment carbohydrates (Table 1). The isolate was defined as homofermentative because it did not produce gas (CO_2) from glucose. A comparison of the biochemical reaction profiles and growth temperature with the published data and API-20C test kit suggested that 1 of the 3 producers of the 10 isolates was *Pediococcus pentosaceus* and 2 of the 3 producers of the 10 was *Pediococcus acidilactici*. *Pediococcus pentosaceus* was designated as Pep1 on the basis of genus, species and sample number, and used throughout this study.

Nature of the antimicrobial compounds. To determine if the antimicrobial substances produced by *P. pentosaceus* Pep1 were bacteriocins or not, retention of the activity of the freeze-dried partially purified pediocin following several treatments was tested. The bactericidal property of pediocin P was found to be most effective at a pH between 3 and 8, and, except for incubation at a pH below 3 and above 8.0, the antimicrobial activity of the materials was retained. The inhibitory effect of the antimicrobial peptide from *P. pentosaceus* Pep1 against *Lactobacillus plantarum* NCDO 955 is presented in Figure 1. The pediocin P molecule is quite heat stable and retains fairly high bactericidal activity even after exposure to 121°C for 15 min. However, the activity level dropped (disc no: 2 in Figure 2). The bactericidal property of pediocin P is completely lost following incubation with proteolytic enzymes, such as proteases IV, XIV, XXIV, trypsin, chymotrypsin and ficin. The activity of the pediocin P was not affected by DNase, ribonuclease A, lipase, lysozyme or catalase. Inactivation of antimicrobial activity by

Table 1. Biochemical and physiological profiles of *Pediococcus pentosaceus* Pep1.

Characteristics	profile of isolate[a]
Growth at	
35°C	+
40°C	+
50°C	-
Growth in	
4% NaCl	+
6.5% NaCl	+
18% NaCl	-
Production of "catalase"	+
Hydrolysis of arginine	+
Fermentation of	
Arabinose	+
Maltose	+
Melezitose	-
Mannitol	-
Trehalose	+
Rhamnose	-
Raffinose	+
Sucrose	-
Xylose	+
Glycerol	-
Sorbitol	-
Ribose	+
Genus and species[b]	Pp

[a] +, able to or -, unable to perform the functions. All tests were done at least twice
[b] *Pediococcus pentosaceus*, Pp

proteases suggested that the substances could be antimicrobial peptides or bacteriocins. Treatment of pediocin P with organic solvents such as acetone, acetonitrile, chloroform, ethanol, formaldehyde and isopropanol did not cause the loss of its antibacterial activity.

Antimicrobial activity of bacteriocin. Both *P. pentosaceus* Pep1 and partially purified pediocin P were found to be inhibitory against cells of Gram-positive bacteria from many genera (Table 2). These include nonpathogenic species and strains of *Lactobacillus, Pediococcus, Enterococcus, Lactococcus, Leuconostoc, Staphylococcus* and *Bacillus*. Many of these nonpathogenic strains were associated with the spoilage of meat and dairy products, such as *Leuconostoc mesenteroides* and *Enterococcus faecalis*. Among the pathogens, species and strains that were found to be sensitive include *Listeria monocytogenes*, *Bacillus cereus*, and *Staphylococcus aureus*.

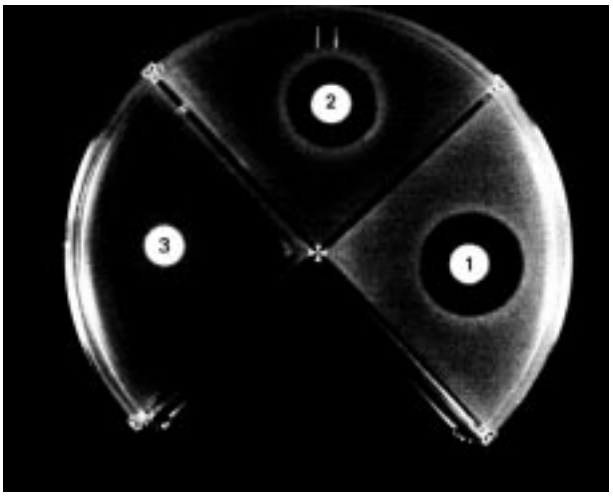

Figure 1. Pediocin P of *Pediococcus pentosaceus* Pep1 colonies produced a zone of growth inhibition on a lawn of *Lactobacillus plantarum* NCDO 955 used as indicator.

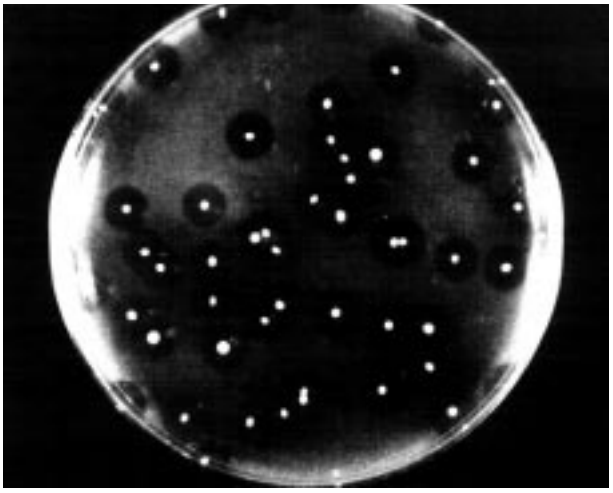

Figure 2. Disc assay of antimicrobial activity of antimicrobial peptide from *Pediococcus pentosaceus* Pep1 against *Lactobacillus plantarum* NCDO 955 (1) untreated, (2) pediocin P after heating at 121°C for 15 min, (3) pediocin P after treatment with trypsin.

Gram-negative bacteria including *Escherichia coli* and *Yersinia enterocolitica* were found not to be susceptible to the pediocin P of *P. pentosaceus* Pep1 and *P. pentosaceus* Pep1 itself. Activities of the pediocin P in the cell-free culture supernatant fluids were found to be 30000 AU/ml.

Discussion

A strain of *Pediococcus pentosaceus* was isolated from vacuum-packed sausages and designated Pep1. It was found to produce an extracellular antimicrobial peptide. The inhibitory effect

Table 2. Antimicrobial spectrum of partially purified pediocin P and *Pediococcus pentosaceus* Pep1.

Bacterial species[a]	Antimicrobial activity of pediocin P and Pep1[b]
Lactobacillus plantarum NCDO 955	+
Pediococcus acidilactici Ped L	+
Enterococcus faecalis MB1	+
Leuconostoc mesenteroides Ly	+
Lactococcus lactis subsp. lactis OZ1 (our food isolate)	+
Leuconostoc isolates from spoiled meats	+
Lactobacilli isolates from spoiled meats	+
Listeria monocytogenes NCTC 5105	+
Staphylococcus aureus (our food isolate)	+
Bacillus subtilis (our food isolate)	+
Bacillus cereus (our food isolate)	+
Yersinia enterocolitica (our food isolate)	-
Escherichia coli (our food isolate)	-

[a] *Lactobacillus plantarum* NCDO 955, *Leuconostoc mesenteroides* Ly, *Enterococcus faecalis* MB1 and *Pediococcus acidilactici* Ped L were kindly provided by Dr. Bibek Ray of the University of Wyoming

[b] *P. pentosaceus* Pep1 and pediocin P showing presence (+) or absence (-) of zone of growth inhibition around the colonies against indicator bacteria.

of the antimicrobial peptide from *P. pentosaceus* Pep1 against *Lactobacillus plantarum* NCDO 955 is presented in Figure 1. The loss of antimicrobial activity following treatment with proteolytic enzymes implied the presence of an essential protein or peptide moiety and indicated that the active component secreted extracellularly by *P. pentosaceus* Pep1 was proteinaceous. Since the factors including possible inhibitory effects of organic acids, hydrogen peroxides and bacteriophages were respectively eliminated by using pH-neutralized, catalase-treated and heated-culture supernatants of *P. pentosaceus* Pep1, the antimicrobial activity of it reported here confirmed it to be a bacteriocin. The wide spectrum of antimicrobial activity and the proteinaceous nature of the substance further indicated that it was a bacteriocin, and it was given the name pediocin P. Treatment with lipase and organic solvents did not cause any loss of activity, probably because of the absence of lipid moieties in the molecule. Retention of antimicrobial activity following treatment with lipase, ribonuclease, lysozyme and organic solvents also indicated that it was a pure protein, rather than a conjugated one. The peptide remained active after high temperature treatment (121°C, 15 min) and at a relatively low pH. The ability to retain its antimicrobial property after treatment with formaldehyde or high temperature also indicated that the molecule may be fairly small. Since the antimicrobial agent was found to show activity between the pH values of 3 and 8, it seems that it is relatively more stable in acidic con-

ditions than in basic ones. The loss of activity at a higher pH could be due to degradation of the molecule (6, 7). This is similar to the properties reported for bacteriocins produced by other lactic acid bacteria such as pediocin AcH (6), pediocin PA-1 (7), pediocin A (10), lactacin (25), lactocin 27 (26), and nisin (27). This stability, over a wide range of pH values, could be useful when this antimicrobial agent is used in food products as a biopreservative. The loss of activity subsequent to exposure to alkaline pH of 9 and above suggests that the molecules have some type of secondary structure, that is important for its antimicrobial activity; alkaline treatment destroys that structure resulting in loss of activity.

Some bacteriocins of Gram-positive bacteria, in contrast to Gram-negative bacteria, have broad activity spectra against other Gram-positive bacteria (28). This appears to be especially true of pediocin P, to which every Gram-positive bacterium tested in this study proved to be sensitive. Both *P. pentosaceus* Pep1 and its partially purified pediocin P were found not to be effective against the Gram-negative organisms tested, but were shown to be effective against many Gram-positive organisms, many of which are associated with food spoilage, but more importantly it was also effective against foodborne pathogenic bacteria such as *Listeria monocytogenes*. There is increasing concern regarding *L. monocytogenes* in milk and meat products, and the use of this bacteriocin may be a way of controlling the growth of this pathogen in milk, meat and other food items. Fermented meat products are often involved in staphylococcal food poisoning outbreaks and leuconostoc spoilage (14). Since bacteriocins from lactic acid producing bacterial starter cultures are present in some naturally fermented food products, their addition to foods in a purified form should pose no risk to consumers. Starter cultures of *Pedioccoccus* spp. which possess pediocin P activity may be useful in controlling *Listeria, Leuconostoc* and *Staphylococcus* contamination in fermented meats. Their active proteins may also have potential as biopreservatives in a variety of perishable foods.

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