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Performance and Applications of Polyclonal Antipeptide Antibodies Specific for the Enterococcal Bacteriocin Enterocin P

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Polyclonal antibodies with specificity for enterocin P (EntP) have been generated by immunization of rabbits with two chemically synthesized N-terminal peptides (P1 and P2) and a C-terminal peptide (P3) of this bacteriocin conjugated to the carrier protein KLH. The sensitivity and specificity of the peptide−KLH-generated antibodies were evaluated by a noncompetitive indirect enzyme-linked immunosorbent assay (NCI-ELISA) and a competitive indirect (CI)-ELISA. The NCI-ELISA but not the CI-ELISA was valuable for detecting the existence of EntP specific antibodies in the sera of the P2−KLH and P3−KLH immunized animals and to detect and quantify the EntP in the supernatant of producer strains. The anti-P2−KLH sera cross-reacted with the supernatant of a strain producer of sakacin A, a bacteriocin closely related to EntP. Immunoaffinity chromatography columns with anti-P2−KLH or anti-P3−KLH immunoglobulins retained the EntP from the supernatant of the producer strain. Western blotting of EntP with the anti-P2−KLH-generated antibodies suggests that purified EntP tends to the formation of aggregates with no antimicrobial activity. Monitoring the purification of EntP with antipeptide antibodies suggests that while the performance of the evaluated purification procedures would be reasonably acceptable in terms of recovery of the antimicrobial activity of the bacteriocin, their yield is far from attractive in terms of recovery of the initial concentration of enterocin P.

KEYWORDS: Enterococci; bacteriocins; enterocin P; antipeptide antibodies; ELISA

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides produced by different bacterial species including many members of the lactic acid bacteria (LAB) and are attracting a considerable interest for their potential use as natural and nontoxic food preservatives (*1*, *2*). The LAB bacteriocins are often cationic, amphiphilic, membrane-permeabilizing peptides classified into three main groups: class I consists of the modified bacteriocins, the lantibiotics; class II comprises the nonmodified heat stable bacteriocins including the subclass IIa (pediocin-like bacteriocins), the subclass IIb (two peptide bacteriocins), and the subclass IIc (other peptide bacteriocins); and finally, class III consists of the protein bacteriocins (*3*−*8*). The subclass IIa bacteriocins are characterized by the occurrence of a hydrophilic conserved YGNGVxCxxxxCxV sequence motif in their N-terminal half including two cysteines that form a disulfide bridge and a hydrophobic variable C-terminal domain of equivalent size; another characteristic of these bacteriocins is their strong inhibitory effect on *Listeria* (*8*). It has been determined that enterocin P (EntP) produced by *Enterococcus faecium* is synthesized as a prepeptide that after cleavage produces the mature bacteriocin of 44 amino acids; it seems to be processed and secreted by the *sec*-dependent pathway and exhibits a broader spectrum of antimicrobial activity when compared to other subclass IIa bacteriocins (*9*−*11*). The EntP has also been shown to deplete the intracellular level of ATP dissipatting the membrane potential but not the transmembrane pH gradient of energized cells and to form specific, potassium ion-conducting pores in the cytoplasmic membrane of target cells (*12*, *13*). However, it is still not known how the peptide inserts into the membrane to form a structured pore.

Because of the potential use of bacteriocins as food preservatives, a myriad of applications is envisaged for them. Bacteriocins, either alone or in combination with other antimicrobial barriers, may be useful tools to reduce the load of foodborne pathogens and spoilage bacteria. Also, because some bacteriocins such as the enterocins are produced by microorganisms coding for potential virulence factors (*14*, *15*) and many industrial strains with interest as protective or starter cultures

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do not produce such antagonistic peptides, interest in the heterologous expression or coexpression of class II bacteriocins is growing rapidly (*16*−*18*). The generation of antibodies against bacteriocins may provide sensitive and specific methods for identification and detection of different bacteriocins and their producing strains and for their evaluation in different substrates by the use of immunochemical assays (*19*−*21*). Antibodies also offer potential alternative methods for the purification of bacteriocins by the use of immunoaffinity chromatography strategies (*22*−*25*).

Reports on the generation of antibodies against bacteriocins have been scarce. Initial attempts to generate antibodies against bacteriocins relied on the use of whole bacteriocin molecules, either alone or conjugated to carriers, as the immunogen. However, while this approach would be valid for bacteriocins such as the lantibiotics nisin A and Z (*20*, *21*, *26*, *27*) and the subclass IIc bacteriocins enterocin B (*24*) and propionicin PLG-1 (*28*), antibodies generated against bacteriocins of the subclass IIa with strong consensus similarities may show a lack of specificity although changes in single amino acid residues drastically affect protein recognition (*29*, *37*). We report in this work the generation of specific rabbit polyclonal antibodies against chemically synthesized N-terminal and C-terminal fragments of the bacteriocin EntP and the development of sensitive immunoassays for EntP analysis. The antibodies were further evaluated for the sensitive and specific detection and quantification of EntP by enzyme-linked immunosorbent assay (ELISA) and Western blotting, for purification of EntP by immunoaffinity chromatography, and for evaluation of the recovery of such bacteriocin during its purification using two multistep chromatographic procedures.

MATERIALS AND METHODS

Materials. The amino acid sequences of the N-terminal fragments of EntP used in this work were NH_2-ATRSYGN-COOH (peptide P1) and NH_2-ATRSYGNGVYC-COOH (peptide 2), while the amino acid sequence of the C-terminal fragment of EntP was NH_2-ASGLAGMGH-COOH (peptide P3). All peptides were synthesized by 9-fluorenylmethoxycarbonyl (Fmoc) chemistry with an Applied Biosystems 413A automated solid phase peptide synthesizer in the Protein Chemistry Facility at the Centro de Biología Molecular Severo Ochoa (Madrid, Spain). Purity of the peptides was monitored by HPLC, being higher than 95%, and the peptide identity was confirmed by mass spectrometry (MS). Ovalbumin (OA) (grade III and fraction VII), Tween 20, glutaraldehyde, ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid], and Freund's adjuvants were obtained from Sigma Chemical Co. (St. Louis, MO). The Imject Maleimide Activated mcKLH kit and the Imject Immunogen EDC kit with mcKLH were obtained from Perbio Science UK Ltd. (Cheshire, U.K.). The Immunopure Protein A gel, the immunopure rProtein A IgG Plus Orientation kit, the disposable columns sample kit, and the immunopure binding/elution buffer system were also obtained from Perbio Science. Goat anti-rabbit immunoglobulin G (IgG) conjugated to HRP was obtained from Cappel Laboratories (West Chester, PA). The Novex 16% tricine gels, the tricine sodium dodecyl sulfate (SDS) buffer kit, and the XCell SureLock mini-Cell chamber were obtained from Invitrogen S. A. (Barcelona, Spain). The Amberlite XAD-16 hydrophobic polyaromatic resin, the molecular weight marker ultralow range for SDS−polyacrylamide gel electrophoresis (PAGE) (1060−26 600), and the color marker for SDS−PAGE and protein transfer ultralow range (1060−26 600) were also from Sigma. The econo-column chromatography support (5.0 cm × 30 cm) and the Silver Stain Plus reagent were obtained from Bio-Rad laboratories (Madrid, Spain). The Hybond ECL membrane, the ECL Plus Western blotting detection kit, the Hyperfilm ECL MP, the dessalting and buffer exchange PD-10 columns, the cation exchanger SP Sepharose Fast Flow gel, the hydrophobic interaction Octyl Sepharose CL-4B gel, the ion exchange Resource S 1 mL column, and the reversed phase PepRPC HR 5/5 chromatography column, as well as the automated fast-performance liquid chromatography (FPLC) system were all from Amersham Biosciences Europe GmbH (Cerdanyola, Spain). The 0.25 μm pore size filters (PES 25 mm GD/X, sterile syringe filters) and the Whatman 3M cellulose membranes were from Whatman Int. Ltd. (Maidstone, U.K.). The LAB strains were grown in the MRS broth (Oxoid Ltd., Basingstoke, U.K.). Pediocin PA-1 produced by *P. acidilactici* 347 was purified to homogeneity as described previously (*19*). Pure enterocin Q was chemically synthesized at the Molecular Biology Unit (University of Newcastle Upon Tyne, U.K.) with a peptide purity >95% by HPLC. Pure nisin A (30 000 IU/mg) was purchased from NBS Biologicals (Hartfield, U.K.). Rabbits (New Zealand White females) were purchased from a local supplier (Navarra, Spain).

Preparation of Immunoconjugates and Immunization. Peptides P1 and P3 were conjugated to the carrier protein keyhole limpet haemocyanin (KLH) (P1−KLH and P3−KLH, 1:2 w/w) by EDC coupling using the components of the Imject Immunogen EDC kit for use as the immunogens. Peptide P2 was conjugated to maleimide-activated KLH (P2−KLH, 1:2 w/w) using the components of the Imject Maleimide activated KLH kit, also for use as the immunogen. The chemically synthesized peptides P1, P2, and P3 were also conjugated to OA (peptide−OA, 11:1 mol/mol) by the glutaraldehyde method (*30*) for use as the solid phase antigens. The immunogenic potential of the peptide fragments according to their hydrophilicity and antigenic index was determined by the use of a sequence analysis software package (*31*). Rabbits were immunized with the immunogens (P1−KLH, P2−KLH, and P3−KLH), according to a previously described scheme (*19*). Rabbits were bled via marginal ear veins on days 28 and 61, and a final bled was performed on day 63 by cardiac puncture.

ELISAs. Most of the procedures were performed as previously described (*29*) with modifications. Briefly, for antisera titration, flat-bottom polysterene microtiter plates (Maxisorp, Nunc, Roskilde, Denmark) were coated overnight (4 °C) with 100 μL of peptide P (either P1, P2, or P3) conjugated to OA (5 μg/mL) in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.6 (coating buffer, CB). Plates were washed three times with washing solution (0.05% Tween 20 in phosphate-buffered saline 0.01 M, pH 7.4, PBS). Wells were blocked for 1 h at 37 °C with 300 μL of 1% (w/v) OA (grade III) in PBS (OA−PBS) and then washed six times. Next, 50 μL of serially diluted serum was added to each well and incubated for 1 h at 37 °C. Unbound antibody was removed by washing four times, and 100 μL of goat anti-rabbit IgG peroxidase conjugate (diluted 1:500 in OA−PBS) was added to each well. Plates were incubated for 30 min at 37 °C and washed eight times, and bound peroxidase was determined with ABTS as the substrate by measuring the absorbance of the wells at 405 nm with a Labsystems iEMS reader (Helsinki, Finland) with a built-in software package for data analysis. The titer of each serum sample was arbitrarily set as the maximum dilution that yielded at least twice the absorbance of the same dilution of nonimmune control serum.

For determination of antiserum specificity and sensitivity to EntP, two types of ELISA were designed. In the noncompetitive indirect (NCI)-ELISA, wells of microtiter plates were coated with 100 μL of different concentrations of pure EntP, pure pediocin PA-1, pure nisin A, pure OA, or neutralized and filter-sterilized supernatants from a number of LAB strains in CB. The plates were maintained for 16 h at 4 °C and then blocked and washed as described for the antiserum titration procedure. Next, 50 μL of antiserum (diluted 1:100 in PBS) was added, and the plates were incubated for 1 h at 37 °C. After a washing step and the addition of goat anti-rabbit IgG−peroxidase conjugate (diluted 1:500 in OA−PBS), the amount of bound peroxidase was determined with the ABTS substrate as previously described. The increase in the absorbance was proportional to the amount of specific antigen in the samples. In the competitive indirect (CI)-ELISA, microtiter plates were coated with 100 μL of EntP (0.5 μg/mL) in CB and then blocked and washed as described for the antiserum titration procedure. Next, 50 μL of the analytes was simultaneously incubated with 50 μL of antiserum (diluted 1:100 in PBS) for 1 h at 37 °C. After the washing step and addition of the goat anti-rabbit IgG peroxidase, the bound peroxidase was determined with the ABTS substrate as described previously. Relative antibody affinity was arbitrarily desig-

nated as the bacteriocin concentration required to inhibit antibody binding by 50%. For both immunoassays, the concentrations of antibodies, hapten conjugates, or enzyme tracers were optimized by checkerboard titration. Competition curves were obtained by plotting absorbance against the logarithm of the analyte concentration. Sigmoid curves were fitted to a four parameter logistic equation by use of the Labsystems software package (Genesis version 1.60).

Construction of the Immunoaffinity Columns and Immunopurification of EntP. For recovery of total IgGs from the immunosera of interest, 5 mL of a protein A gel slurry was packed into a 5 mL disposable polysterene column. After the column was equilibrated with binding buffer (10 mM Tris, pH 8), 1 mL aliquots of the anti-P3−KLH serum diluted in binding buffer (1:7, v/v) were applied to the column. The column was then washed with 40 mL of binding buffer, and the retained IgG fraction was eluted with 10 mL of elution buffer (0.1 M glycine buffer, pH 2.8). The eluted fractions (2 mL each) were neutralized by the addition of 100 μL of binding buffer, and those showing higher absorbances at 280 nm were desalted using PD-10 columns (Sephadex G-25M) and further lyophilized. This purification procedure was repeated three times, and 17.64 mg of IgGs was obtained by measuring absorbance of the fractions at 280 nm and the calculated extinction coefficient for total IgGs (an A_{280} of 1.35 corresponds to 1 mg/mL). This fraction was named fraction A. The same procedure was followed for the recovery of total IgGs from the anti-P2−KLH serum. A total of 14.75 mg of such IgGs was obtained and named fraction B.

The fractions A and B were further used for construction of immunocolumn A (anti-C-terminal EntP) and immunocolum B (anti-N-terminal EntP). For construction of immunocolum A, 16 mg of IgGs from fraction A was dissolved in 2 mL of antibody binding/wash buffer (50 mM sodium borate, pH 8.2) and deposited in a 2 mL immunopure rProtein A Plus Orientation column, and bound antibodies were cross-linked to the matrix with disuccinimidyl suberate (DSS). For construction of immunocolum B, 14.75 mg of IgGs from fraction B was cross-linked to another 2 mL Immunopure rProtein A column as previously described. The performance of immunocolumns A and B on the immunopurification of bacteriocins was evaluated with culture supernatants of *E. faecium* P13, an EntP producer strain (9), and with *Lactobacillus sakei* 706, a sakacin A producer (*32*). Microorganisms were grown in MRS broth at 32 °C for 16 h, and the supernatants were obtained by centrifugation of the cultures at 12000*g* for 10 min at 4 °C, adjusted to pH 6.2 with 1 N NaOH, and filtered through 0.2 μm pore size filters. Then, supernatants diluted in binding buffer (1:1, v/v) were applied to the immunocolumns. The columns were washed with 10 mL of binding buffer, and bacteriocins were eluted with 10 mL of elution buffer. The eluted fractions were neutralized by addition of 100 μL of binding buffer and further desalted by passage through gel filtration PD-10 columns. For both immunoaffinity columns, the elution of bound bacteriocins was monitored by the anti-P2−KLH and anti-P3−KLH antibodies and the NCI-ELISA, as previously described.

Protein Electrophoresis, Western Hybridization, and Overlay Assay. Aliquots of immunopurified EntP and sakacin A were subjected to Tricine-SDS−PAGE as described by Shägger and Von Jagow (*33*). Protein electrophoresis was performed on preformed Novex 16% Tricine gels in a XCell SureLock mini-Cell, at 80 V constant current. Gels were silver-stained with the Silver Stain Plus reagent or blotted onto a Hybond ECL membrane (pore size, 0.2 μm). The electrophoresed gel was first immersed in buffer K (0.025 M Tris, 0.04 M 6-amino-*n*-hexanoic, and 20% methanol [v/v]) during 10 min before contact with the nitrocellulose membrane. Above the gel and toward the cathode, six membranes of Whatman 3M paper embedded in buffer K were placed, while under the nitrocellulose membrane three membranes of Whatman 3M paper embedded in buffer A2 (0.025 M Tris with 20% methanol [v/v]) were allocated, followed by another set of Whatman 3M paper embedded in buffer A1 (0.03 M Tris with 20% methanol [v/v]). After allocation of a 2 kg weight tare over the cathode, blotting was performed by application of an electrical current of 0.08 A for 1 h. The transfer of peptides from the gel to the membrane was monitored by the use of a color marker. Further treatment of the blotted membrane included blocking in 50 mL of deffated milk powder (5% deffated milk in PBS-T) at 37 °C for 1 h and incubation with 30 mL of the anti-P3−KLH serum (diluted 1:100 in PBS) for 1 h at 37 °C. Incubation of the membrane with 30 mL of the goat anti-rabbit IgG peroxidase conjugate (diluted 1:5000 in defatted milk powder) permitted visualization of the expected antigen−antibody interactions by chemiluminiscence with the ECL Plus Western blotting detection kit. The light emission was detected by a short exposure of the membrane to a blue light sensitive Hyperfilm ECL MP autoradiography film. To determine the antimicrobial activity of pure bacteriocins, an overlay assay was performed (*34*). Briefly, the electrophoresed gel was fixed in a solution of 20% 2-propanol and 10% acetic acid in distilled water (v/v) and further washed during 1, 16, and 1 h in distilled water. The first 1 h wash was performed at room temperature with agitation, while the other two washings were carried without agitation at 4 °C. After the gel was fixed, washed, and drained, it was overlaid with the indicator strain *Lb. sakei* NCFB 2714 (National Collection of Food Bacteria, Shinfield, Reading, U.K.) at 1×10^5 cfu/mL in soft 0.8% MRS agar and incubated overnight at 32 °C.

Table 1. Reactivities of Anti-P2−KLH and Anti-P3−KLH Serum Polyclonal Antibodies against Culture Supernatants of LAB As Determined by a NCI-ELISA[a]

strain (bacteriocin produced)	source[b]	cross-reactivity (%)[c] anti-P2	anti-P3
E. faecium P13 (EntP)	LAB 1153	90	93
E. faecium AA13 (EntP)	LAB 1507	93	92
E. faecium G16 (EntP)	LAB 1505	100	100
E. faecium LA5 (enterocins L50A, L50B, and P)	FVM	80	82
E. faecium L50 (enterocins L50A, L50B, P, and Q)	FVM	59	66
E. faecium T136 (enterocins A and B)	LAB 1152	NR	NR
E. faecium P21 (enterocins A and B)	LAB 1508	NR	1
E. faecalis INIA-4 (enterocin AS-48)	INIA	NR	1
Lb. sakei 706 (sakacin A)	NHL	100	NR
Lb. sakei LTH673 (sakacin P)	NHL	NR	NR
L. lactis BB24 (nisin A)	LAB 1154	NR	1
L. lactis IL1403 (nonbacteriocin producer)	IFR	NR	NR
P. acidilactici 347 (pediocin PA-1)	LAB 1497	2	3
P. pentosaceous FBB61 (pediocin A)	TNO	3	3

[a] NR, no reactivity. [b] Source: LAB is the dessignation for strains of our collection at the BCCM/LMG, Universiteit Gent, Gent, Belgium; FVM, Facultad de Veterinaria, Universidad Complutense de Madrid, Madrid, Spain; INIA, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain; NHL, Laboratory of Microbial Gene Technology, Agricultural University of Norway, Ås, Norway; IFR, Institute of Food Research, Norwich Laboratory, Norwich, U.K.; TNO, Nutrition and Food Research, Zeist, The Netherlands. [c] Cross-reactivity defined as (absorbance reading produced by a culture supernatant above the absorbance reading produced by MRS/absorbance reading produced by supernatant of *E. faecium* P13 above the absorbance reading produced by MRS) × 100.

Microorganisms, Media, and Bacteriocin Assays. The LAB strains tested for antibody cross-reactivity and EntP production are listed in **Table 1**. All microorganisms were propagated in MRS broth at 32 °C, and the supernatants were obtained by centrifugation at 12000*g* for 10 min at 4 °C, adjusted to pH 6.2 with 1 M NaOH, filtered through 0.2 μm pore size filters, and stored at −20 °C until use. The antimicrobial activity of the supernatants against the most appropriate indicator strains was evaluated by an agar diffusion test and, when stated, by a microtiter plate assay (MPA), performed as previously described by Cintas et al. (*11*).

Purification of EntP. The antimicrobial activity of *E. faecium* P13 (*9*), used as the source of EntP, was purified to homogeneity using two different procedures. In the first procedure, performed as described by Casaus et al. (*35*) with modifications, the bacteriocin was purified from a 1 L *E. faecium* P13 culture grown in MRS at 32 °C until the late logarithmic phase (A_{620} = 0.8). The cells were removed by centrifugation at 10000*g* for 10 min at 4 °C, and 40 g of Amberlite XAD-16 was added to the supernatant. The supernatant with the hydrophobic resin was maintained for 2 h at 4 °C with stirring and further loaded into an econo-column chromatography support. After elution of the supernatant, the resin was washed with 100 mL of distilled water in the first step and with 75 mL of 40% ethanol in water (v/v) later, to remove weak or nonhydrophobic compounds. The adsorbed bacteriocin was eluted with 200 mL of 85% 2-propanol, pH 2.0, in water (v/v) and after addition of 0.1% trifluoroacetic acid (TFA) was

subjected to cation exchange chromatography in a Resource S column, integrated in an automated FPLC system. The column was washed with 200 mM sodium phosphate buffer in 50% metanol/water (v/v), pH 5, plus 0.1% TFA (buffer A), and the bacteriocin was eluted with a gradient of buffer A with 1 M NaCl. The eluted fractions with antimicrobial activity were further subjected to reverse phase chromatography in a C_2 to C_{18} column (PepRPC HR 5/5) integrated in a FPLC system (RP-FPLC). The bacteriocin was eluted from the column with a 55 min linear gradient of 20−35% 2-propanol in aquoeus 0.1% TFA at a flow rate of 0.5 mL/min. Fractions with high bacteriocin activity were mixed and rechromatographed on the same RP column to obtain chromatographically pure bacteriocin. During purification, bacteriocin activity was calculated by a MPA with *Lb. sakei* NCFB 2714 as the indicator microorganism. Growth inhibition was measured spectrophotometrically at 620 nm with a microtiter plate reader after 14 h of incubation at 32 °C. One bacteriocin unit (BU) was defined as the reciprocal of the highest dilution of the bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin).

In the second purification procedure, EntP was purified as previously described (*9*−*11*). Briefly, supernatants from early stationary phase 1 L *E. faecium* P13 cultures grown in MRS broth at 32 °C were subjected to precipitation with ammonium sulfate (50%, w/v). The sample was kept at 4 °C with stirring for 3 h. After centrifugation at 12000*g* for 30 min at 4 °C, the pellet and floating materials were mixed and solubilized in 100 mL of 20 mM sodium phosphate buffer, pH 6. The fraction was applied to gel filtration PD-10 columns and further subjected to cation exchange (SP Sepharose Fast Flow) and hydrophobic interaction (Octyl Sepharose CL-4B) chromatographies, followed by RP chromatography in a C_2 to C_{18} column (PepRPC HR 5/5) integrated in a FPLC system. Fractions with high bacteriocin activity were mixed and rechromatographed on the RP column to obtain chromatographically pure bacteriocin. Bacteriocin activity was determined as described above.

RESULTS

Sensitivity of Rabbit Antipeptide Antibodies for EntP. Three regions within the EntP amino acid sequence were selected for production of chemically synthesized peptides. Synthetic peptides P1 (amino acid residues 1−7) and P3 (amino acid residues 34−42) were conjugated to KLH by EDC coupling, while synthetic peptide P2 (amino acid residues 1−11) was conjugated to maleimide activated KLH and used to immunize rabbits. On day 63 of the immunization process after six doses of the immunogens were administered, all animals had apparent titers in serum ranging from 1/3200 to 1/102 400. The sensitivity of the anti-P1−KLH, anti-P2−KLH, and anti-P3−KLH antibodies for EntP was determined by a NCI-ELISA. **Figure 1** shows the results obtained with the most sensitive sera for detection of pure bacteriocins in either CB or MRS broth. The anti-P1−KLH evaluated sera were not able to detect EntP in this assay (results not shown), while the anti-P2−KLH and anti-P3−KLH sera recognized the EntP present in the wells of the microtiter plates, although recognition of EntP was higher in CB than in MRS broth. The detection limits for EntP when using the anti-P2 antibodies were 0.05 μg/mL in CB and 0.25 μg/mL in MRS broth, while those when using the anti-P3 antibodies were 0.01 μg/mL in CB and 0.1 μg/mL in MRS broth. Such antibodies could not detect the presence of equivalent concentrations of pure pediocin PA-1 and nisin A in the wells of the microtiter plates. The sensitivity for EntP of the peptide-generated antibodies was also determined by a CI-ELISA. In this assay, the plates were coated with pure EntP. However, when using this assay (results not shown), the average detection limits for EntP by the anti-P2 or anti-P3 antibodies were not improved as compared to those obtained with the NCI-ELISA, while the amount of free EntP required for 50% binding inhibition was higher than 10 μg/mL.

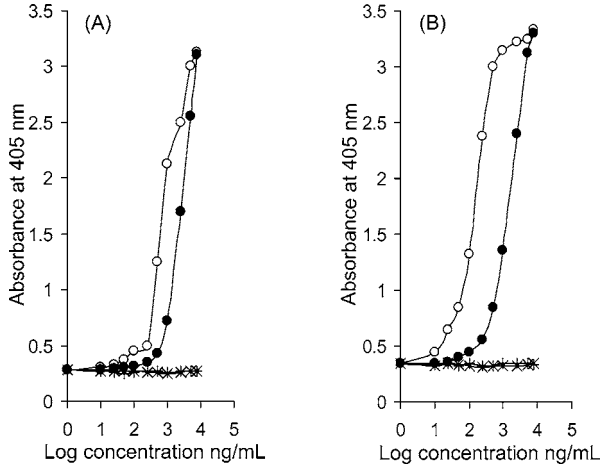

Figure 1. NCI-ELISA detection of EntP in CB (○) or MRS broth (●), pediocin PA-1 in CB (×), and nisin A in CB (+), using the anti-P2–KLH (**A**) or anti-P3–KLH (**B**) antibodies.

Table 2. EntP Concentration in the Culture Supernatants of *E. faecium* Producers As Determined by a NCI-ELISA

	EntP concentration (ng/mL)	
strains	anti-P2–KLH	anti-P3–KLH
E. faecium P13	7300	7250
E. faecium AA13	7500	7200
E. faecium G16	7900	7900
E. faecium LA5	6500	6300
E. faecium L50	4700	5060

Immunoreactivity of the Antipeptide Antibodies to Different Bacteriocins. The specificities of serum polyclonal anti-P2−KLH and anti-P3−KLH antibodies in neutralized and filter-sterilized supernatants of 16 h cultures of representative LAB strains were evaluated by NCI-ELISA (**Table 1**). The anti-P2−KLH and anti-P3−KLH antibodies showed a high cross-reactivity with the supernatants of the *E. faecium* strains P13, AA13, G16, LA5, and L50, producers of EntP, with negligible to no reaction against supernatants of strains producing bacteriocins such as enterocin A, sakacin A, sakacin P, and pediocin PA-1, four subclass IIa bacteriocins with the N-terminal amino acid motif KYYGNGVxC (residues 1−9). However, it should be noticed that the anti-P2−KLH antibodies displayed a high cross-reactivity (100%) against the supernatant of *Lb. sakei* 706, producer of sakacin A, a bacteriocin of the subclass IIa sharing with EntP nine identical amino acids RSYGNGVYC (residues 2−10) at the N-terminal end. The concentration of EntP in the supernatants of *E. faecium* strains previously isolated from Spanish fermented sausages and producing EntP was also determined (**Table 2**). While the *E. faecium* strains P13, AA13, and G16 displayed a similar EntP production (91−100%), this was slightly lower (59−82%) in *E. faecium* LA5 and *E. faecium* L50, both strains producing more than one bacteriocin.

Immunoaffinity Columns and Immunopurification of EntP. The antipeptide anti-P2−KLH and anti-P3−KLH IgGs were recovered from the immunosera of interest by adsorption and elution from a Protein A gel. A total of 17.64 mg of IgGs (fraction A) was obtained from the anti-P3−KLH serum, while 14.75 mg of such IgGs (fraction B) was also obtained from the corresponding anti-P2−KLH serum. The fractions A and B were further used to construct immunocolumn A (recognizes the C-terminal fragment of EntP) and immunocolumn B (recognizes the N-terminal fragment of EntP) by the cross-linking with DSS of the purified IgGs to 2 mL of Immunopure rProtein A Plus

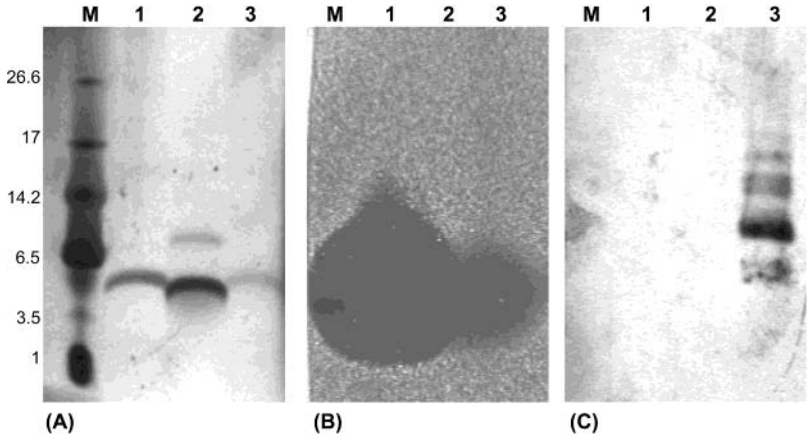

Figure 2. Tricine-SDS–PAGE of different bacteriocins after silver staining (**A**), antimicrobial activity after overlay with the indicator strain *Lb. sakei* NCFB 2714 (**B**), and Western blotting with the anti-P2–KLH antibodies (**C**). M, molecular weight marker with sizes indicated in the left margin. Lane 1, 1 μg of pure pediocin PA-1; lane 2, 1 μg of pure enterocin Q; and lane 3, 2 μg of pure EntP.

Table 3. Immunoaffinity Chromatography Recovery of EntP from the Supernatants of *E. faecium* P13 As Determined by a NCI-ELISA

	immunocolum A (C-terminal)		immunocolum B (N-terminal)	
EntP incorporated (ng)	EntP eluted (ng)	recovery (%)	EntP eluted (ng)	recovery (%)
1000	1076	100	1102	100
1500	1405	94	1520	100
2500	1590	64	1509	60
5000	2366	47	1690	34
10 000	2556	26	1635	16
20 000	1964	10	3062	15
30 000	2668	9	4125	14
40 000	4135	10	5925	15

Orientation matrix. The performance of immunocolumns A and B on the immunopurification of bacteriocins was evaluated with culture supernatants of *E. faecium* P13 and *Lb. sakei* 706. The results obtained (**Table 3**) indicate that EntP from the supernatant of the producer *E. faecium* P13 is retained with maximum efficiency (94−100%) on immunocolumns A and B until the EntP incorporated in columns is 1500 ng but with a lower recovery (10−64%) when the EntP incorporated on the columns was progressively higher (2500−40 000 ng). Although the saturation level for recovery of EntP by both columns seems to be low, the immunopurification of EntP in a single step gives a reasonably high yield as compared to that obtained by the use of multistep chromatographic techniques, as evaluated below. The results obtained indicate that 0.15−4.5 mL of neutralized and filter-sterilized supernatants of *E. faecium* P13 grown in MRS permit the recovery of 1.0−4.1 μg of EntP by immunocolumn A and 1.1−5.9 μg of EntP by immunocolumn B. Supernatants (2 mL) of *Lb. sakei* 706 producing sakacin A were also subjected to immunopurification of this bacteriocin on immunocolumn B. The anti-P2−KLH antibodies recognized the presence of sakacin A on the eluted fractions, although a precise quantification of the immunopurified bacteriocin was not pursued.

The purity of EntP and sakacin A eluted from the immunoaffinity columns was also evaluated. Eluted fractions containing the bacteriocins were desalted and lyophilized. Aliquots of the samples were sent for amino acid sequencing at the University of Oslo (Oslo, Norway) and also subjected to protein electrophoresis by Tricine-SDS−PAGE on preformed 16% tricine gels. Both the immunopurified EntP and the sakacin A samples gave multiple amino acid sequences indicating that they were not purified to homogeneity, while the electrophoretic pattern of the same samples on Tricine-SDS−PAGE gels showed an intense protein smearing of the gel slots containing the immunopurified EntP and sakacin A (results not shown).

Western Blotting of Purified EntP. The anti-P2−KLH antibodies with specificity for the N-terminal end of EntP were used for characterization of this bacteriocin purified by a multiple step chromatographic procedure comprising an initial ammonium sulfate precipitation step, followed by gel filtration, cation exchange, hydrophobic interaction, and RP chromatographies. As shown in **Figure 2A**, visualization of purified pediocin PA-1, enterocin Q, and EntP by silver staining indicates that the chemically synthesized enterocin Q tends to the formation of aggregates and that all bacteriocins show a strong antimicrobial activity in the overlay assay (**Figure 2B**), with the highest activity being displayed by pediocin PA-1. However, after Western blotting (**Figure 2C**), the anti-P2−KLH antibodies only recognized reactive antigenic bands in the lane corresponding to the purified EntP. These results suggest that EntP has a strong tendency to the formation of aggregates not visualized by silver staining and not displaying antimicrobial activity.

Use of the Anti-P3−KLH Antibodies To Monitor Purification of EntP. Bacteriocins have been mostly subjected to purification procedures based on measurement of their antimicrobial activity after each purification step. Accordingly, the performance of most of the purification procedures relies on the evaluation of the antimicrobial activity and not on the specific detection and quantification of the bacteriocin being subjected to purification. In this work, the antimicrobial activity of *E. faecium* P13, used as the source of EntP, was purified using two different multistep chromatographic procedures. In the first procedure, EntP was purified by adsorption onto a XAD-16 polymeric resin followed by cation exchange and RP chromatographies. Results shown in **Table 4** indicate that although the antimicrobial activity of the fraction subjected to adsorption onto a XAD-16 polymeric resin remains the same (100%) as in the supernatant, the presence of EntP is reduced to a 0.63% of the initial concentration. When the resulting fraction was subjected to cation exchange chromatography, the antimicrobial activity of the resulting fraction was reduced to a 2.4%, while the recovery of EntP was 0.46%. After purification of the sample by RP-FPLC, two chromatographic fractions with antimicrobial activity were obtained. In fraction A, the antimicrobial activity was 0.9% of the original and the recovery of EntP was 0.004%, while the antimicrobial activity of fraction

Table 4. Purification of EntP from *E. faecium* P13 Using the First Purification Procedure

purification stage	volume (mL)	total A_{254}[a]	total activity[b] ($10^3 \times$ BU)	total activity (%)	enterocin P[c] (μg)	enterocin P (%)
culture supernatant	1000	28 500	1550	100	8840	100
adsorption to XAD-16 polymeric resin	200	888	1560	100	56.4	0.63
cation exchange chromatography	50	22.75	37.01	2.4	41.5	0.46
reverse phase chromatography						
fraction A	0.400	0.077	14.96	0.9	0.41	0.004
fraction B	0.675	0.082	38.01	2.4	0.54	0.006

[a] A_{254} multiplied by the volume in milliliters. [b] Antimicrobial activity in BU. [c] EntP concentration as determined by ELISA.

Table 5. Purification of EntP from *E. faecium* P13 Using the Second Purification Procedure

purification stage	volume (mL)	total A_{254}[a]	total activity[b] ($10^3 \times$ BU)	total activity (%)	enterocin P[c] (μg)	enterocin P (%)
culture supernatant	1000	28 500	1550	100	8840	100
ammonium sulfate precipitation	100	3670	1509	97	2656	30
gel filtration chromatography	200	1620	1478	95	2450	28
cation exchange chromatography	50	17	764	49	160	1.8
hydrophobic interaction chromatography	10	4.04	1196	77	22.60	0.2
reverse phase chromatography						
fraction A	0.675	0.05	49.93	3	0.50	0.006
fraction B	0.700	0.04	443.12	28	1.53	0.017

[a] A_{254} multiplied by the volume in milliliters. [b] Antimicrobial activity in BU. [c] EntP concentration as determined by ELISA.

B was 2.4% of the original in the supernatant with a recovery of 0.006% of the initial concentration of EntP.

In the second purification procedure, EntP from *E. faecium* P13 was purified by precipitation with ammonium sulfate, followed by gel filtration, cation exchange, hydrophobic interaction, and RP chromatographies. The results shown in **Table 5** suggest that although after the ammonium sulfate and gel filtration steps the purified sample shows a 95% of the antimicrobial activity of the supernatant, detection and quantitation of EntP by the anti-P3−KLH antibodies and the NCI-ELISA indicate that only a 28% of the initial bacteriocin remains. When the resulting fraction was subjected to cation exchange chromatography, 49% of the original antimicrobial activity remains with a recovery of a 1.8% of the initial EntP concentration. The hydrophobic interaction step further improves the antimicrobial activity (77%) of the bacteriocin, although only a 0.2% of the initial EntP is present in the purified sample. Finally, after the RP-FPLC, two chromatographic fractions with antimicrobial activity were resolved. In fraction A, the antimicrobial activity was 3% of the original while the recovery of EntP was 0.005%, while the antimicrobial activity of fraction B was 28% of the original with a recovery of 0.017% of the initial concentration of EntP.

DISCUSSION

Although during the past decade a large effort has been carried out on the biochemical and genetic characterization of bacteriocins produced by LAB, their detection and quantification have relied mostly on the use of bioassay-based and other biochemical tests in which nonspecificity is a major drawback. Moreover, although highly specific immunochemistry-based methods have been developed and routinely used as analytical tools in many areas of research, surprisingly, the impact of these techniques in the bacteriocin research field has been marginal. Furthermore, the lack of commercially pure bacteriocins makes difficult their availability while their low molecular masses (<5000 Da) make them poorly immunogenic or nonimmunogenic. In addition, peculiar characteristics of the bacteriocin molecules (*6*, *8*) such as the occurrence of hydrophilic conserved sequence motifs, their hydrophobicity, the formation of intrachain disulfide rings, and, in the case of lantibiotics, the presence of modified amino acids might also interfere with the sensitivity and specificity of the antibodies and with the development of immunoassay formats. However, antibodies generated against a short chemically synthesized fragment of a bacteriocin of interest might be useful to generate antibodies of predetermined specificity (*18*, *19*, *29*).

Of great interest is the generation of antibodies and the development of sensitive immunoassays for detection and quantification of EntP. This bacteriocin may be employed as a mixture with other bacteriocins to increase the antimicrobial activity of the resulting pool and to reduce the presence of resistant microorganisms, while it also shows potential for its production in heterologous hosts that may produce the bacteriocin in larger quantities, may facilitate its purification, or may be considered as safer producers of EntP for their direct use in foods. However, because EntP is a subclass IIa bacteriocin, it exhibits a strong amino acid sequence homology with other bacteriocins at the N terminus. Accordingly, for generation of antibodies specific for EntP, the peptide fragments P1 and P2 (N-terminal residues) were designed to generate antibodies, which could be either specific or show cross-reactivity against related bacteriocins, while peptide fragment P3 (C-terminal residues) might display a high specificity for EntP. All peptide fragments were potentially highly immunogenic according to its hydrophilicity and antigenic index.

The sensitivity of the P1−KLH-, P2−KLH-, and P3−KLH-generated polyclonal antibodies for EntP was evaluated by a NCI-ELISA. The anti-P1−KLH sera were not able to detect EntP probably because the epitope was too short to induce an antibody response (*36*), while the anti-P2−KLH and anti-P3−KLH sera recognized the EntP (**Figure 1**) with a higher sensitivity of the anti-P2 as compared to the anti-P3 antibodies and with a larger recognition in CB than in MRS broth. The effect of CB to enhance the detection of EntP was similar to that observed for detection of enterocin A (*18*) and pediocin PA-1 (*29*). However, the results obtained with the CI-ELISA suggest that contrary to results obtained for detection of pediocin

PA-1 (*29*) and enterocin B (*24*), but similarly to those obtained for enterocin A (*18*), this immunochemical format significantly reduces the detection of free EntP. These results heighten the importance of the development of proper immunoassay formats for detection of each bacteriocin. The limit of detection and sensitivity of the NCI-ELISA developed for EntP were in the range of values previously reported for nisin A (*22*, *26*), nisin Z (*25*, *27*), pediocin PA-1 (*19*, *29*), enterocin A (*18*), and enterocin B (*24*).

Both the anti-P2−KLH- and the anti-P3−KLH-generated antibodies showed a high affinity for EntP in the supernatants of *E. faecium* P13, AA13, G16, LA5, and L50 grown in MRS broth with a nonsignificant cross-reactivity with cell culture supernatants from enterocin A, enterocin B, enterocin AS-48, sakacin P, nisin A, pediocin PA-1, and pediocin A producer strains (**Table 1**), some of which share the shorter (YGNCVxC) consensus amino acid motif with EntP at their N-terminal end. This absence of cross-reactivity is not surprising since it has been reported that closely related proteins have been distinguished by the use of antisera as a probe for a specific sequence within the protein molecule (*29*, *36*) and that changes in a single amino acid residue drastically affect protein recognition (*37*). However, the anti-P2 (N-terminal) but not the anti-P3 (C-terminal) antibodies showed a high affinity for supernatants of *Lb. sakei* 706 producer of sakacin A (*32*), a bacteriocin identical to curvacin A (*8*) that shares with EntP the long (RSYGNGVYC) N-terminal consensus amino acid motif, permitting the simultaneous recognition by these antibodies of the two distinct bacteriocins, EntP and sakacin A. Thus, the specificity of any antipeptide antibody should be fully addressed considering the potential cross-reactivity of such antibodies against closely related bacteriocins. The EntP produced by a number of *E. faecium* strains was also quantified by a NCI-ELISA (**Table 2**). While the use of both sera gave comparable results on the production of EntP by all *E. faecium* strains, the lower production of EntP by the *E. faecium* strains LA5 and L50 may be reasonably adscribed to their multiple bacteriocin production (*11*). EntP production by *E. faecium* P13 displays primary metabolite kinetics (*9*, *38*), and its production as well as that of other bacteriocins may also depend on strains, pH, and other environmental factors (*39*). With the results obtained, it is hypothesized that the use of the P3−KLH-generated antibodies would constitute a valuable tool for detection of EntP in the supernatant of LAB isolates producing still unknown antagonistic peptides and for quantification of the EntP produced by LAB isolates in which the existence of the structural gene for production of this bacteriocin has been genetically determined. Such antibodies might also be useful for quantification of EntP in foods after fermentation with an EntP-producer strain and to evaluate the production of EntP by genetically modified hosts producers or coproducers of EntP and other bacteriocins, as it has been already shown during coproduction of pediocin PA-1 and nisin A (*17*), and pediocin PA-1 and enterocin A (*18*), by *Lactococcus lactis*.

Immunoaffinity chromatography columns containing immunoglobulins with specificity for EntP retained the EntP from supernatants of *E. faecium* P13 grown in MRS (**Table 3**), with an efficiency of 9−100% for column A and 15−100% for column B, depending on the initial content of EntP incorporated into the columns. Although the recovery of EntP, as measured by the NCI-ELISA, from both column A (1.0−4.1 μg) and column B (1.1−5.9 μg) seems to be low, the immunopurification of EntP in a single step and from very low volumes of supernatants (0.15−4.5 mL) gives a reasonably high yield as compared to those obtained by the use of multistep chromatographic techniques. The column B also retained the sakacin A produced by *Lb. sakei* 706. However, the eluted fractions containing either EntP or sakacin A were not purified sufficiently to give a unique sequence, while the electrophoretic pattern of the same samples on Tricine-SDS−PAGE showed an intense protein smearing (results not shown), suggesting that for these bacteriocins the possibility exists for formation of aggregates and/or protein complexes with proteins and peptides from the cytoplasm of dead cells or from those present in the MRS broth. An immunoaffinity chromatography column with polyclonal antibodies has also been developed for purification of enterocin B in a single step (*24*), but higher amounts and better yields of pure nisin A (*22*) and nisin Z (*20*, *23*) have been attained by the use of immunoaffinity matrixes coated with monoclonal antibodies with specificty for nisin A or nisin Z. Thus, the construction of immunoaffinity columns with a higher amount of IgGs or monoclonal antibodies with specificity for EntP would further improve the recovery of this bacteriocin in a single step.

The anti-P2−KLH antibodies have been also useful to identify a relevant feature of EntP. Western blotting of pure pediocin PA-1, enterocin Q, and EntP with the anti-P2−KLH antibodies has shown that these antibodies only recognize reactive antigenic bands in the slot containing purified EntP (**Figure 2**). These results further strenghten the recognition that these antibodies do not cross-react with closely related bacteriocins of the subclass IIa and confirm previous hypotheses suggesting that EntP is a hydrophobic bacteriocin with a strong tendency to the formation of aggregates (*9*). The results obtained may also help to explain the formation of an intense smearing after Tricine-SDS−PAGE of EntP and sakacin A retained by immunoaffinity chromatography. Furthermore, although the molecular structure of such aggregates is not yet known, their formation in the purified EntP significantly reduces its biological activity (i.e., antimicrobial activity on sensitive microorganisms). Clearly, the impact of this behavior for the biotechnological potential of EntP as a natural food presevative in the food industry should be evaluated further.

For the first time in the bacteriocin research field, antipeptide (anti-P3−KLH) antibodies have been also used to monitor the purification of EntP by two different multistep chromatographic procedures. When EntP produced by *E. faecium* P13 was purified by adsorption onto a XAD-16 polymeric resin followed by cation exchange and RP chromatographies, the results obtained (**Table 4**) show the existence of two chromatographic fractions for purification of EntP. The existence of multiple chromatographic peaks after purification to homogeneity of EntP (*10*) and other bacteriocins such as nisin Z (*40*), pediocin PA-1 (*41*, *42*), and carnobacteriocin BM1 (*42*) has been adscribed to the coexistance after RP-FPLC of two peptide forms, oxidized and nonoxidized, of the same bacteriocin. The results obtained (**Table 4**) also suggest that while the purification procedure for EntP would be reasonably acceptable in terms of final recovery of the initial antimicrobial activity (3.3%), its final yield is far from attractive (0.95 μg EntP) in terms of recovery of the initial concentration of the bacteriocin (0.010%). Furthermore, while it is recognized that the apparent increase in the antimicrobial activity of bacteriocins during its purification may be the result of removal of antimicrobial inhibitors, disaggregation of the bacteriocin or conformational change to a more active form of the bacteriocin in the hydrophobic solvent (*9*, *11*, *19*), the results obtained in this work suggest that reduced adsorption/desorption of EntP to the XAD-16 polymeric resin and to the RP

chromatographic column are the rate-limiting steps for purification of larger quantities of EntP. When EntP was purified by a second purification procedure (**Table 5**), the results obtained indicate that while this purification procedure for EntP would also be acceptable in terms of recovery of the initial antimicrobial activity (31%), its final yield is again far from attractive (2.03 μg of EntP) in terms of recovery of the initial concentration of this bacteriocin (0.023%). The high losses of EntP after ammonium sulfate precipitation, cation exchange, hydrophobic interaction, and RP-FPLC suggest that these are also rate-limiting steps for purification of larger quantities of EntP using this purification procedure.

In most efforts toward development of improved methods to increase the yield of purified bacteriocins (*44*−*46*), the presence of bacteriocins in the purified fractions has been evaluated by measurement of their antimicrobial activity, by determination of protein concentrations by colorimetric assays, or by measuring UV absorption at 280 nm, which is converted to protein concentration using molecular extinction coefficients, calculated from the contribution of individual amino acid residues. However, all of these methods used to determine bacteriocin concentrations suffer from lack of specificity, and thus, bacteriocin recovery yields are difficult to ascertain. The use of specific antipeptide antibodies to monitor purification of EntP suggests that while both of the evaluated purification protocols are useful for purification of EntP to homogeneity (*9*−*11*), however, the yield of pure EntP is far from attractive. If the food industry demands larger quantities of EntP for their use as a natural food preservative, the use of antibodies of predetermined specificity for EntP would be of help on the development and optimization of more efficient purification protocols of this bacteriocin. For the food industry, the current application of LAB bacteriocins other than nisin relies mainly on bacteriocin production **in situ** by starter or protective cultures. Such applications would greatly benefit from sensitive and specific methods for the quantification of bacteriocins in foodstuffs, and the antibodies described here may prove to be instrumental in developing such methods. Their suitability for food analysis, however, remains to be established.

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