Lait 80 (2000) 187–195

Original article

Method for the production of antibacterial peptides from biological fluids at an ionic membrane. Application to the isolation of nisin and caprine lactoferricin

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Abstract — Cationic peptides could be successfully released from a precursor protein bound to a cation-exchange membrane by in-situ enzymatic cleavage with an appropriate enzyme. This procedure allows the washing-off of other hydrolytic fragments from the membrane before the selective removal of the strongly bound target peptide(s) at increased pH or with high ionic strength buffer. Two new applications of this method are presented. The lantibiotic nisin could be released with high efficiency by tryptic hydrolysis of its precursor polypeptide bound to the ionic membrane. Further, the production of a fraction enriched in a novel antibacterial domain from the N-terminal part of caprine lactoferrin is reported. Characterisation of this domain by mass spectrometry and N-terminal sequence analysis revealed that this peptide corresponded to fragment 14–42 of the sequence of mature caprine lactoferrin, here referred to as lactoferricin-C. Thus, the purification procedure shown can be used to isolate cationic peptides initially produced in a longer, inactive form by bacteria (fusion proteins) or naturally occurring antibacterial peptides generated by the digestion of proteins.

antibacterial cationic peptide / membrane cation-exchange chromatography / hydrolysis of membrane-bound protein / nisin / caprine lactoferricin

1. INTRODUCTION

In the past few years, antimicrobial peptides have been isolated from a variety of natural sources [2, 5]. A number of endogenous antimicrobial peptides of plants and animals were found to be cationic, amphiphilic molecules composed of 12 to 45 amino acid residues. In addition to naturally occurring peptides, antimicrobial peptides which arise from the in-vitro digestion of larger proteins have been reported, for

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instance by using bactericidal/permeability-increasing protein [11], or tenecin 1 [9] as starting material.

Several cationic antibacterial peptides have been made by enzymatic hydrolysis of milk proteins, such as the minor whey protein lactoferrin (LF) [1, 4, 6, 12]. The most active LF-derived fragment, named lactoferricin-B [1], has more potent bactericidal effect than undigested LF, suggesting that its much smaller size facilitates access to target sites on the microbial surface [15]. Recently, we have reported two cationic antibacterial peptides prepared from bovine α_{s2}-casein hydrolysed with pepsin [13].

One potential limitation for the useful application of antimicrobial peptides is the cost of production. Peptides derived from longer proteins can be produced in vitro by enzymatic hydrolysis of the precursor protein, but the procedure is expensive and labour-intensive since it comprises, first, the purification of the precursor protein and, in a second step, the isolation of the active peptide from a, generally complex, hydrolysate. For naturally occurring peptides, production from natural sources is, in general, not cost-effective and automated chemical procedures, such as solid-phase synthesis, would also be too expensive, except for very short peptides [2]. An alternative strategy is the production of cationic antibacterial peptides as fusion proteins in bacteria. By this procedure, the cationic peptide is produced in a longer, inactive form (normally with an extra anionic segment) to stabilise the cationic segment and to prevent both antibiotic activity against the host bacterium and proteolysis during recombinant–protein production [5].

Therefore, it is important to develop cost-effective production methods for cationic peptides starting from polypeptides or proteins which may be present in complex mixtures. In a previous paper [12], we have described a method for the production of lactoferricin-B starting from bovine cheese whey. This method includes the concentration of the precursor protein on an ion-exchange chromatographic membrane and the in-situ enzymatic hydrolysis of the membrane-bound protein. This procedure has been also successfully applied to the isolation of an antibacterial domain within the sequence of α_{s2}-casein [13]. In the present paper, we report two new applications. By using this method, the lantibiotic nisin A could be purified from a cell culture of a strain producing nisin precursor. The isolation of an antibacterial domain within the sequence of caprine LF from cheese whey was also achieved with this method.

2. MATERIALS AND METHODS

2.1. Materials

For the production of nisin A and its precursor, *Lactococcus lactis* strains NZ9700 [7] and MG1614 harbouring the plasmid pNZ9111 [14], respectively, were used (NIZO collection). The latter strain produces and secretes nisin exclusively in its precursor form, which is the final intermediate in the biosynthesis of this lantibiotic and does not show antibacterial activity under the conditions used in this study. For nisin A production, *L. lactis* NZ9700 was grown to the stationary phase without aeration at 30 °C in M17 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 0.5 w/v% sucrose (final optical density at 600 nm 1.7–1.8, pH 4.8). For the production of the nisin A precursor, *L. lactis* MG1614 carrying pNZ9111 was cultivated to the early stationary phase in M17 broth supplemented with 0.5 w/v% glucose and 10 $\mu g \cdot mL^{-1}$ erythromycin (EGM17) (final optical density at 600 nm 0.9–1.0, pH 6.2). Cultures were centrifuged for 20 min at 2 500 *g* to remove lactococcal cells. The supernatant was again centrifuged for 30 min at 10.000·*g* and, after dilution with bidistilled water, the pH was adjusted to 4.5–4.8 with acetic acid.

Caprine cheese whey (pH 6.5) was obtained from a local farm. To avoid

membrane clogging, it was microfiltered prior to the membrane ion-exchange procedure.

Porcine pepsin A (E.C. 3.4.23.1., 445 units·mg^{-1} solid) and trypsin from bovine pancreas (E.C. 3.4.21.4, 10 400 units·mg^{-1} solid) were from Sigma Chemical Co. (Saint-Louis, MO, USA).

2.2. Enzymatic hydrolysis of the protein bound to an ion-exchange membrane

Experiments were carried out with a Sartorius Sartobind S cation-exchange membrane in an MA 100 configuration (100 cm^2 adsorption area) (Sartorius, Göttingen, Germany). Binding and recovery of protein material were investigated at room temperature (25 °C) using a flow rate of 20 mL·min^{-1}, which was generated by a peristaltic pump (Verder-Vleuten b.v., Vleuten, The Netherlands). The process was monitored by a UV detector with a 2-mm lightpath flow cuvette (model EM-1 Econo UV Monitor, Bio-Rad Laboratories, Richmond, CA, USA) at 280 nm.

Prior to use, the cation-exchange membrane was equilibrated with a low ionic strength buffer at the pH selected for the protein binding. This buffer pH was chosen to be at least 1 unit below the isoelectric point of the protein. Thus, membrane equilibration was achieved with 10 mmol·L^{-1} sodium phosphate buffer, pH 7.0, for caprine LF and with 10 mmol·L^{-1} ammonium acetate buffer, pH 4.5, for nisin precursor.

The solution containing the precursor protein or peptide (i.e. cheese whey or fermentation medium) was pumped at 20 mL·min^{-1} and at room temperature through the membrane, which was followed by a washing with a low ionic strength buffer at the pH of the desired enzymatic hydrolysis reaction (HCl-acidified water, pH 3.0, before hydrolysis with pepsin or 10 mmol·L^{-1} phosphate buffer, pH 7.0, before hydrolysis with trypsin).

The membrane-bound protein was hydrolysed at 37 °C by recycling at 20 mL·min^{-1} (at reverse flow, to remove air bubbles) a solution containing the appropriate enzyme. An aqueous solution (100 mL, pH 3.0) of porcine pepsin (25 mg·mL^{-1}) was used to hydrolyse caprine LF for a period between 4 h and overnight. Nisin precursor was hydrolysed by using a 50 mL-solution of trypsin (0.1 mg·mL^{-1}) in 10 mmol·L^{-1} sodium phosphate buffer, pH 7.0, for a period between 15 min and 3 h. After hydrolysis, the membrane was washed sequentially with buffers of increasing pH or increasing ionic strength. The remaining, strongly bound peptides were eluted with 1 mol·L^{-1} NaCl or 1 mol·L^{-1} KCl in phosphate buffer pH 7.0. All fractions were collected, tested for activity and analysed by analytical reversed-phase high-performance liquid chromatography (RP-HPLC). Since the antibacterial activity of LF fragments may be diminished in the presence of salts [16], the NaCl fractions from the peptic hydrolysate of caprine LF were desalted by using a Sep-Pak C_{18} cartridge (Waters, Milford, MA, USA) [12].

The cation-exchange membrane was regenerated with 0.2 mol·L^{-1} or 1 mol·L^{-1} NaOH followed by 1 mol·L^{-1} HCl before further use.

2.3. Analytical and preparative RP-HPLC

The RP-HPLC analysis of fractions collected after hydrolysis of caprine LF was carried out by using a 250 × 4.6 mm Widepore C_{18} column (Bio-Rad), as described previously [12]. Fractions of nisin or nisin precursor were analysed by the same procedure, but peptides were eluted with a linear gradient of solvent B in A going from 10% to 25% in 10 min followed by a 50-min linear gradient from 25 to 30% of solvent B in solvent A at a flow rate of 0.8 mL·min^{-1}. Solvent A was a mixture of acetonitrile-water-trifluoroacetic acid (100:900:1, v/v/v) and solvent B contained

the same components (900:100:0.8, v/v/v). For mass spectrometry (MS) analysis (see 2.4), peptides were manually collected from the analytical HPLC system in 1-mL Eppendorf tubes, freeze-dried and kept at –20 °C.

To collect pure material for the antibacterial assay (see 2.5) and the N-terminal sequence analysis, the antibacterial fraction obtained after hydrolysis of bound caprine LF was further purified by preparative RP-HPLC under conditions described previously [12].

2.4. Identification of peptides

Identification of the antibacterial domain of caprine LF was achieved by combining N-terminal sequence and molecular mass data of the peptide, and matching these to the known amino-acid sequence of caprine LF [8]. Nisin A and nisin A precursor were identified by RP-HPLC using reference material prepared at NIZO food research and by the mass spectrometric data of these peptides.

MS spectra were recorded using a Quattro II triple quadrupole mass spectrometer (Micromass, Cheshire, UK) as described previously [12].

The N-terminal sequence of the purified fragment of caprine LF was identified by sequence analysis with a gas-phase sequenator (Model 470A, Applied Biosystems, Foster City, CA, USA) under the conditions described [12].

2.5. Assay for antibacterial activity

Antibacterial activities of the fractions collected from the ion-exchange membrane and of purified peptides were determined by a plate diffusion assay against *Micrococcus flavus* DSM 1790 [12]. Fractions from LF hydrolysates were tested at concentrations ranging from 0.2 to 30 $mg \cdot mL^{-1}$. Nisin and nisin precursor were tested at concentrations lower than 10 $\mu g \cdot mL^{-1}$.

3. RESULTS AND DISCUSSION

3.1. Isolation of nisin produced by *Lactococcus lactis* as nisin precursor

Because proteins and peptides present in a rich medium like M17 may complicate the concentration of nisin precursor on the chromatographic membrane, in a preliminary experiment the breakthrough curve and recovery of nisin were measured using 0, 2, 5 and 10-fold aqueous dilutions of the cell-free extract (CFE) (pH 4.5). Although the adsorption/elution behaviour of nisin and nisin precursor at the membrane may be different, for these experiments a CFE containing nisin was used, because the detection limit of nisin by the bioassay agar activity test is much lower than the detection limit of nisin precursor by RP–HPLC (about 0.1 $\mu g \cdot mL^{-1}$ and 3.2 $\mu g \cdot mL^{-1}$, respectively). The highest recovery of nisin in the 1 $mol \cdot L^{-1}$ NaCl fraction was obtained when a 10-fold diluted CFE was used as feeding solution.

For the binding and recovery study with nisin precursor a 10-fold diluted CFE was therefore used and 1 $mol \cdot L^{-1}$ NaCl or 1 $mol \cdot L^{-1}$ KCl in phosphate buffer (pH 7.0) was used for elution, followed by 0.2 $mol \cdot L^{-1}$ NaOH to regenerate the membrane. After 6 h of growth of a 1% inoculum of precursor–producing *L. lactis* in EGM17, the undiluted CFE contained 7.3 ± 0.5 $\mu g \cdot mL^{-1}$ ($n = 3$) of nisin precursor, as determined by RP-HPLC. To determine the binding and recovery, 100 mL of 10-fold diluted CFE was loaded onto the membrane and the breakthrough effluent, the peaks eluted with 1 $mol \cdot L^{-1}$ KCl or 1 $mol \cdot L^{-1}$ NaCl and the regenerating solution (0.2 $mol \cdot L^{-1}$ NaOH) were analysed by RP-HPLC. The nisin precursor was exclusively recovered in the KCl- or NaCl-containing fraction and equal amounts of nisin precursor were found when using either KCl or NaCl (data not shown). In a separate experiment, three different volumes of 10-fold diluted CFE were loaded onto the membrane and the amount of nisin

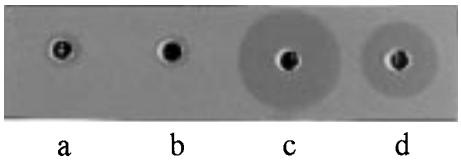

Figure 1. Agar diffusion assay using *M. flavus* as indicator microorganism. Inhibition zones were obtained by spotting the fractions collected from the cation-exchange membrane after hydrolysis of nisin A precursor on the membrane for 15 min with trypsin: (**a**) the 10 mmol·L^{-1} sodium phosphate fraction, (**b**) the solution of trypsin removed immediately after the hydrolysis, (**c**) the 1 mol·L^{-1} KCl fraction, (**d**) the 0.2 mol·L^{-1} NaOH fraction.

precursor in the KCl fraction was quantified by RP-HPLC. A loading volume of 1 L of diluted CFE yielded 362 ± 6 μg of nisin precursor (54% recovery) in the KCl fraction, whereas loading volumes of 2 L and 3 L resulted in 598 ± 6 μg (41%) and 783 ± 6 μg (36%), respectively (n = 3). A similar effect of the loading volume on the recovery of bovine LF from cheese whey was observed earlier using this type of ionic membrane [3].

After passing 3 L of 10-fold diluted CFE, the nisin precursor was at least 540-fold concentrated within the cation-exchange membrane (2 mL of membrane volume) with respect to the concentration of nisin precursor in the feeding solution. After washing the membrane with phosphate buffer to remove unbound material and hydrolysing the nisin precursor bound to the membrane with trypsin at pH 7.0 for different times, all washing solutions, including the 0.2 mol·L^{-1} NaOH solution used for membrane regeneration, were tested for activity against *M. flavus*. No activity was found in the trypsin solution removed after hydrolysis (Fig. 1b) and a negligible halo could be detected in the phosphate buffer fraction used to wash the membrane before elution with KCl (Fig. 1a). Most of the activity was recovered in the KCl-containing fraction after hydrolysis (Fig. 1c), although some activity was also found in the 0.2 mol·L^{-1} NaOH fraction (Fig. 1d). These two active fractions were analysed by RP-HPLC. After 15 min of hydrolysis, the HPLC peak corresponding to nisin precursor was absent in the KCl fraction, but this fraction contained 288 ± 11 μg (n = 2) of nisin as determined by RP-HPLC (Fig. 2c). The solution of NaOH used for regeneration of the membrane contained also a small amount of nisin (57 ± 11 μg) not recovered in the KCl fraction and this explains the antibacterial activity observed in this fraction (Fig. 1d). The small amounts of other components in the KCl fraction (Fig. 2c) precluded further identification of these peptides by MS.

3.2. Isolation of an antibacterial domain of caprine LF from cheese whey

In a preliminary experiment we found that a peptic hydrolysate of caprine LF had antibacterial properties similar to those observed with bovine LF hydrolysed with pepsin. Further, sequence-homology analysis revealed high identity at the amino acid level between caprine and bovine LFs (92%) [8]. In a previous paper [12], we reported the hydrolysis of bovine LF bound to the ion-exchange membrane and the isolation of the bactericidal domain of LF, lactoferricin-B. The same procedure was followed to generate and isolate an antibacterial peptide from caprine LF.

Approximately 1 L of microfiltered caprine cheese whey was passed through the membrane. By using this loading volume, the bound fraction, when eluted with 1 mol·L^{-1} NaCl, consisted of LF and a smaller amount of lactoperoxidase (43% expressed as percentage of the peak area of LF), as determined by RP-HPLC. After prewashing the protein-loaded membrane with acidified water (pH 3.0) to remove unbound material and to bring the membrane to the desired pH for the hydrolysis with pepsin,

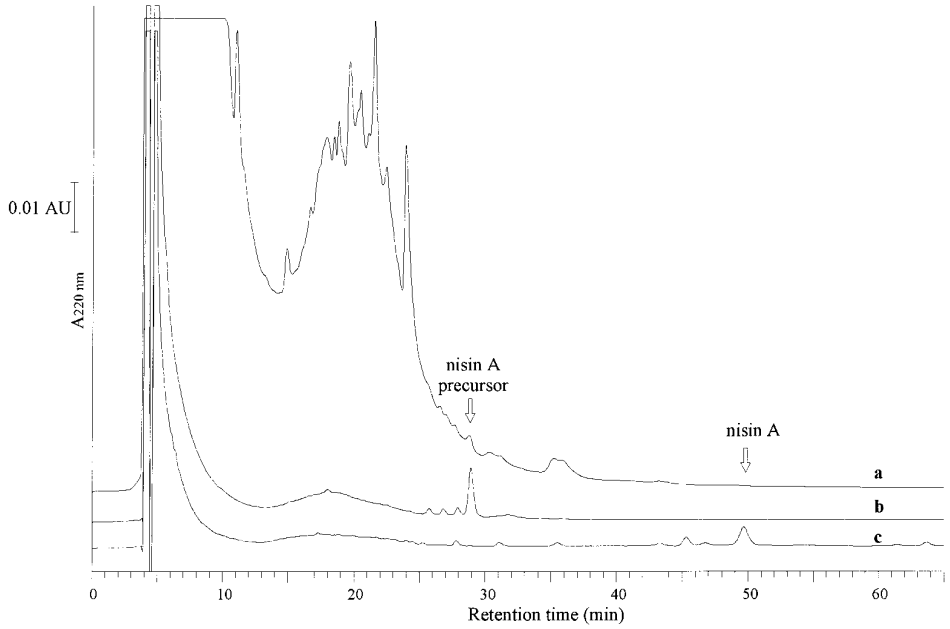

Figure 2. RP-HPLC chromatograms of (**a**) the undiluted cell-free extract (CFE) containing the nisin A precursor, (**b**) the 1 mol·L^{-1} KCl fraction collected from the cation-exchange membrane after passing 3 L of the 10–fold diluted CFE containing the nisin precursor, and (**c**) the 1 mol·L^{-1} KCl fraction collected from the cation-exchange membrane after passing 3 L of 10-fold diluted CFE containing the nisin precursor followed by 15 min hydrolysis with trypsin. For chromatographic conditions, see Section 2.3. Identification of nisin A and nisin A precursor was performed by mass spectrometry and by comparison with the elution behaviour of reference samples.

the bound protein was hydrolysed as described in Section 2.2. The membrane was first washed with 10 mmol·L^{-1} ammonium hydrogen carbonate buffer acidified to pH 7.0 with formic acid, and then with 7 mol·L^{-1} ammonia (pH ca. 12). Finally, it was treated with 1 mol·L^{-1} NaCl in phosphate buffer. Monitoring the hydrolysis as a function of time by RP-HPLC analysis of the NaCl fractions revealed a progressive reduction in the size of the peak corresponding to caprine LF. After 4 h of hydrolysis on the membrane, the major component of the NaCl fraction corresponded to a peptide with molecular mass 22.878, whereas after overnight hydrolysis the caprine LF molecule was completely fragmented into peptides and the major component corresponded to a peptide with mass 3493 (Figs. 3a and 3b). After desalting, the whole fraction shown in Figure 3a had activity against *M. flavus*. The major component of this fraction showed a large inhibition halo when tested by the agar diffusion assay against *M. flavus*. The N-terminal 10-residue sequence of this peptide with mass 3493 was PEWSK?YQWQ. The residue at position 6 could not be identified as it may correspond to the amino acid cysteine. Combining the measured molecular mass with the sequencing data of the peptide and matching these to the known sequence of caprine LF [8] indicated that this peptide corresponded to residues 14 to 42 of the sequence of mature caprine LF (theoretical mass 3494.1). Taken together, these results show that the antibacterial domain of caprine LF produced by peptic hydrolysis of this

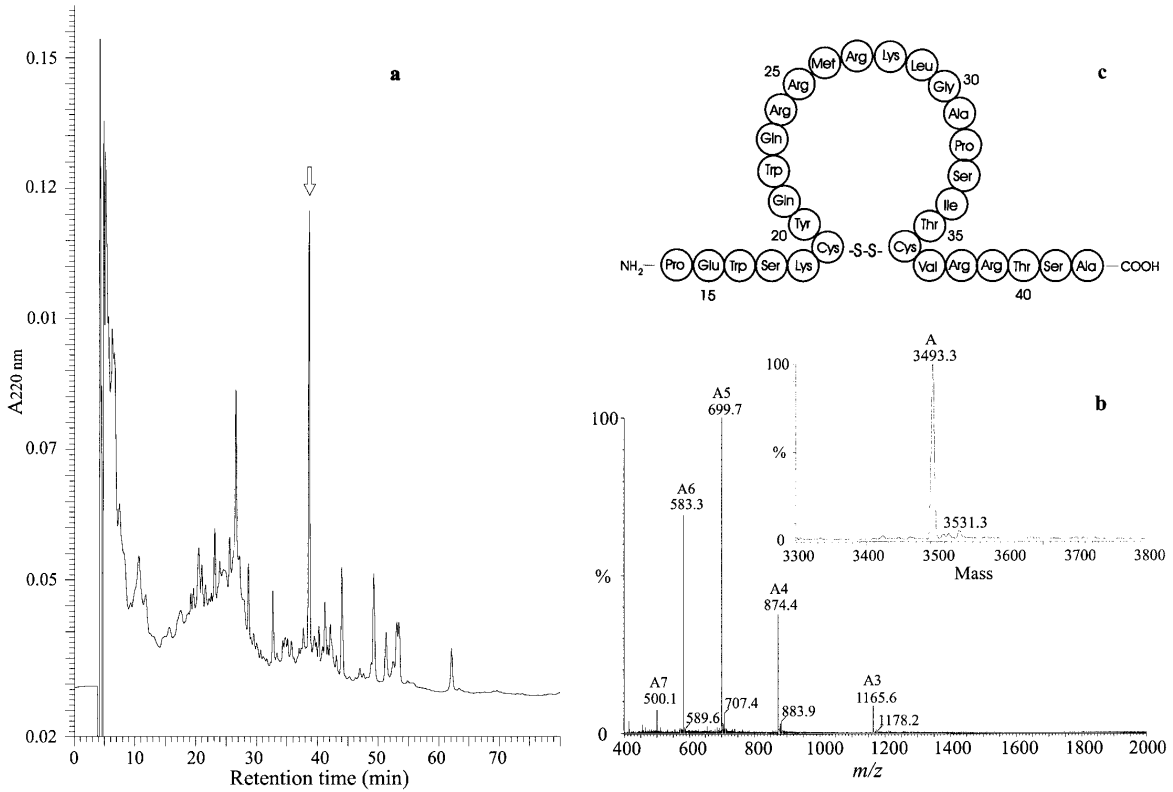

Figure 3. (**a**) RP-HPLC chromatogram of the 1 mol·L^{-1} NaCl fraction collected from the cation-exchange membrane after passing ca. 1 L caprine cheese whey through the membrane followed by overnight hydrolysis of membrane-bound protein with pepsin. The arrow indicates the active component recovered for further purification. (**b**) Electrospray mass spectrum of the component marked with an arrow in Figure 3a. Ax denote components of the main envelope of multiply charged ions of the *m/z* spectrum. The highest peak was set at 100%. The inset represents the deconvoluted spectrum. (**c**) Schematic representation of the primary structure of the identified antibacterial domain of caprine LF. Numbers indicate positions in the sequence of mature caprine LF [8].

protein is a 29-residue peptide (Pro_{14}-Ala_{42}) with 2 cysteines (positions 6 and 23) involved in the formation of an intrachain disulfide bridge (Fig. 3c). This peptide shows a high similarity (72% sequence identity) to the bactericidal peptide isolated from bovine LF, lactoferricin-B, and may therefore be referred to as caprine lactoferricin or lactoferricin-C. Like lactoferricin-B, this peptide is located in the N-terminal part of the LF molecule, is devoid of tyrosine or histidine, which are essential for the metal-chelating functions of this protein, and contains several positively charged residues.

During the binding process, the identified domain of the caprine LF molecule seems to interact with the acidic groups of the cation-exchange membrane and it is retained after hydrolysis of the precursor protein while the other peptides are washed out. Therefore, the antimicrobial fragment of caprine LF might be exposed on the exterior of the folded LF molecule (as is the case in bovine LF [10]) and it seems to be responsible for the binding of the LF molecule to the chromatographic membrane. Further studies are required to determine quantitatively the antibacterial activity of this novel antibacterial peptide in comparison with the bactericidal peptide from bovine LF, lactoferricin-B. In addition, it remains to be determined whether these lactoferricins are produced during gastric digestion of LF in sufficient quantities to have any physiological significance.

4. CONCLUSIONS

Cationic antibacterial peptides are of interest in the food and non-food industry, because of their ability to inhibit pathogens and spoilage microorganisms. For practical use of these peptides as biopreservatives (e.g. in food), a fraction enriched in those peptides may be sufficient. However, a high yield, reproducibility and low cost will determine the choice for a specific purification protocol. This paper reports two new applications of a successful strategy to produce cationic peptides from precursor polypeptides or proteins by in situ enzymatic cleavage of the precursor molecule bound to an ionic membrane. This method presents an obvious economic advantage: the isolation of the precursor is not necessary, since it is concentrated and subsequently hydrolysed within the separation medium. Therefore, the purification protocol, which classically would include three steps, i.e., isolation of the precursor protein, enzymatic hydrolysis of the precursor and isolation of the cationic peptide from the hydrolysate, is reduced to essentially one separation step to obtain a strongly enriched fraction.

ACKNOWLEDGEMENTS

The authors are grateful to C. van Kraaij and M. Beerthuyzen for providing the lactococcal strains and the reference samples of nisin and nisin precursor. The mass spectrometry facility used was funded by PPS MIBITON. I.R. acknowledges the European Commission for a scholarship.

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