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Two ion-exchange chromatographic methods for the isolation of antibacterial peptides from lactoferrin In situ enzymatic hydrolysis on an ion-exchange membrane

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Abstract

Two ion-exchange chromatographic methods are reported for the rapid isolation of antibacterial peptides from lactoferrin (LF). Using the first method, a pepsin hydrolysate of LF was fractionated by bead-based cation-exchange chromatography. After removal of weakly bound material by washing with ammonia, highly purified lactoferricin-B (LFcin-B) was obtained in a single step by elution with 2 *M* NaCl. Some other cationic peptides, copurified as minor components, were also characterised by N-terminal sequencing, mass spectrometry and antibacterial activity determination. With the second method, cheese whey was filtered through a cation-exchange membrane, and the selectively bound LF was directly hydrolysed in situ with pepsin. Inactive LF fragments were washed off the membrane with ammonia, and a fraction enriched in LFcin-B was obtained by further elution with 2 *M* NaCl. The membrane method is more rapid and offers several economic advantages.

Keywords: Whey; Peptides; Antibacterial agents; Lactoferrin

1. Introduction

Lactoferrin (LF) is a bilobate iron-binding glycoprotein and exists in most biological fluids of mammals, such as saliva, tears and mucous secretions, as well as in milk [1]. This protein is active against a wide range of pathogenic bacteria [2–5] and it is thought to contribute to the protection of infants from infectious disease [6]. During milk processing (e.g. cheesemaking), most of the LF is retained in the cheese whey fraction, i.e., about 30–100 mg/l [1,7–9]. At pH 6.5, LF and lactoperoxidase (LP) are positively charged, whereas the main part of the whey proteins is negatively charged. Therefore, LF and LP may be purified from whey using ion exchangers. Separation of LF and LP from cheese whey by using ion-exchange beds [8,10–13] or ion-exchange membranes [9,14] has been demonstrated.

Some bioactive peptides derived from bovine LF have been reported. Bellamy et al. [15] found that a pepsin-generated hydrolysate of LF displayed more potent bactericidal properties than undigested LF. These investigators isolated a potent antibacterial cationic peptide named lactoferricin-B (LFcin-B), which was identified as fragment 17–41 of bovine LF. Other, less active peptides, also from the N-

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terminal region of LF, have recently been reported [16,17]. The isolation of these antibacterial peptides was carried out by starting from pepsin or chymosin LF hydrolysates, using two steps of reversed-phase high-performance liquid chromatography (RP-HPLC) [15] or by ion-exchange chromatography (IEC) followed by RP-HPLC [16,17]. In each case, pure LF was used as the starting material. Those experiments imply, first, the isolation of LF from milk, colostrum or whey and, then, the purification of LFcin-B from the enzymatic LF hydrolysate.

We have developed two ion-exchange chromatographic methods. Hereby, a traditional bead-based system and a microporous membrane-based system were used to isolate the antibacterial peptide LFcin-B. In the first method, isolated LF was digested with pepsin and the hydrolysed material was separated, in one step, on an ion-exchange bed. In the second method, LFcin-B was obtained directly from cheese whey, without the isolation of LF (alternatively, isolated LF was used as the starting material). Gouda cheese whey (or a solution of LF) was separated with an ion-exchanger membrane, after which the bound LF was hydrolysed in situ with pepsin and the resulting LFcin-B was released by selective elution.

2. Experimental

2.1. Digestion of LF with pepsin in solution

Bovine LF was kindly donated by Domo Food Ingredients (Beilen, The Netherlands). A 5% (m/v) aqueous solution of LF was adjusted to pH 3.0 with 1 *M* HCl and digested with 3% (m/m of substrate) porcine pepsin A (EC 3.4.23.1, 445 U/mg solid, from Sigma, St. Louis, MO, USA) for 4 h at 37°C, although different periods, between 2 and 21 h, and different pepsin concentrations were tested in preliminary experiments. The reaction was terminated by heating at 80°C for 15 min and the pH was adjusted to 7.0 by the addition of 1 *M* NaOH. The digest was centrifuged at 16 000 *g* for 15 min and the supernatant was retained and freeze-dried.

2.2. Purification and characterization of peptides

2.2.1. Ion-exchange chromatography

The LF hydrolysate, prepared as described above, was dissolved in bidistilled water (5%) and 2 ml were injected onto a column (150×26 mm I.D.) of SP-Sepharose Fast Flow resin (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated at 4°C with 10 m*M* ammonium hydrogen carbonate buffer acidified to pH 7.0 with formic acid. Peptides were eluted at a flow-rate of 5 ml/min with a 0 to 100% gradient in 70 min of 3, 5 or 7 *M* ammonia solution. Finally, after 5–15 runs, the column was eluted with 2 *M* NaCl. The fractions were tested for activity and further analysed by analytical RP-HPLC.

Prior to the activity test, the 2 *M* NaCl fraction was desalted using a Sep-Pak C_{18} cartridge (part no. WAT023635; Waters, Milford, MA, USA) with a linear 0 to 100% gradient in 5 min of acetonitrile in water.

2.2.2. Analytical and preparative RP-HPLC

Analytical RP-HPLC was carried out by using two M 6000A pumps in combination with a high-sensitivity accessory block (Waters), an ISS-100 injector (Perkin-Elmer, Überlingen, Germany), a Waters Model 680 gradient controller and a Kratos 783 detector (Kratos Analytical, Ramsey, NJ, USA). A 250×4.6 mm Widepore C_{18} column (Bio-Rad Laboratories, Richmond, CA, USA) was used with a C_{18} cartridge (Bio-Rad) as a guard column. The equipment was linked to a data acquisition and processing system (Turbochrom, Perkin-Elmer). Solvent A was a mixture of acetonitrile–water–trifluoroacetic acid (TFA) (100:900:1, v/v/v) and solvent B contained the same components (900:100:0.8, v/v/v). Peptides were eluted with a linear gradient of solvent B in A going from 0 to 50% in 70 min at a flow-rate of 0.8 ml/min. The absorbance of the eluent was monitored at 220 nm. Sample concentrations were approx. 1 mg/ml and injection volumes were 50 μl.

The equipment used for preparative RP-HPLC consisted of two pumps (Model 510, Waters), an automatic sample injector (Model 231, Gilson Medical Electronics, Villiers le Bel, France), a UV detector (Model 759A, Applied Biosystems, Foster City, CA, USA) and a Waters Model 680 gradient controller. A Widepore C_{18} 250×20 mm column (Bio-Rad) was used with a C_{18} cartridge (Bio-Rad) as a guard column. A linear gradient of solvent B in A from 10 to 33% in 55 min at a flow-rate of 6.6 ml/min was used. The injection volume was 1–2 ml of the 2 *M* NaCl IEC fraction (see Section 2.2.1).

2.2.3. Mass spectrometry

The mass of each purified peptide was measured by mass spectrometry with a Quattro II triple quadrupole instrument (Micromass, Cheshire, UK). Samples were dissolved to a concentration of 10 μg/ml in 50% (v/v) aqueous acetonitrile, 0.3% (v/v) in formic acid, and analysed by infusion of the sample solution (20 μl) with a syringe pump type 22 (Harvard Apparatus, South Natick, MA, USA) using a flow of 50% (v/v) aqueous acetonitrile at 4 μl/min through the electrospray interface. Nitrogen was used as a nebulizing and drying gas. The capillary was held at 3.9 kV; the cone voltage was 40 V. Mass calibration over the mass/charge (m/z) range from 200 to 2000 was performed with a NaI–CsI mixture. About 10–15 spectra were averaged to obtain an adequate signal-to-noise ratio of about 1500. By using Masslynx software (version 2.2, Micromass), on a Windows NT workstation, the m/z spectral data were processed and transformed to spectra representing mass values.

2.2.4. Amino acid analysis

Amino acid analysis was carried out with an amino acid analyser (type 4151, LKB Biochrom, Cambridge, UK). L-Norleucine was used as an internal standard. Hydrolysates were made with 6 *M* HCl in evacuated tubes at 110°C for 24 h.

2.2.5. N-terminal sequence analysis

The N-terminal sequence of the purified peptides was identified by sequence analysis with a gas-phase sequenator (Model 470A, Applied Biosystems). Phenylthiohydantoin (PTH) amino acids were analysed on-line by RP-HPLC with a PTH analyser (Model 120A, Applied Biosystems). For the separation, a PTH C_{18} column (220×2.1 mm) (Applied Biosystems) was applied.

2.2.6. Peptide identification

Peptide identification was achieved by combining N-terminal sequence and molecular mass data of the peptide, and matching these to the known amino acid sequence of LF.

2.2.7. Assay for antibacterial activity

Antibacterial activities of LFcin-B and other peptides from the LF hydrolysate were determined by a plate diffusion assay. *Micrococcus flavus* DSM 1790 was used as an indicator organism. *M. flavus* was grown in MF broth (0.1% sucrose, 1% peptone, 0.3% meat extract, 0.2% NaCl, 0.15% yeast extract, pH 7.0). Purified fractions from LF hydrolysates were tested at concentrations ranging from 0.2 to 50 mg/ml in bidistilled water.

2.3. Pepsin digestion of LF in situ on an ion-exchange membrane and isolation of lactoferricin

Protein separation was 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 determined at room temperature (25°C) at a flow-rate of 9.9 ml/min, which was generated by a 6000 A pump (Waters). The process was monitored by a UV detector with a 2-mm lightpath flow cuvette (model EM-1 Econo UV Monitor, Bio-Rad) at 280 nm. Prior to use, the ion-exchange membrane was pre-equilibrated with 10 m*M* sodium phosphate buffer (pH 7.0).

The maximum binding capacity for LF was measured by passing a solution containing 0.15 mg/ml of LF ($2 \cdot 10^{-6}$ *M*) in 10 m*M* sodium phosphate buffer through the ion-exchange membrane. To ensure that the membrane was fully loaded, the application of sample was continued until the maximum LF breakthrough was obtained. The recovery of LF was also measured after passing through the membrane 1800 ml of microfiltered Gouda cheese whey (pH 6.5) or Gouda cheese whey to which NaCl had been added in solid form to final concentrations of 0.05, 0.1 and 0.2 *M* added salt.

To carry out the hydrolysis of membrane-bound LF, ca. 1450 ml of a 0.15 mg/ml solution of LF ($2 \cdot 10^{-6}$ *M*) in phosphate buffer (sufficient to ensure membrane saturation) or 1800 ml of microfiltered Gouda cheese whey were pumped through the membrane followed by a washing with acidified water (pH 3.0) to remove unbound material. The LF bound to the membrane was hydrolysed overnight at 37°C by recycling (at reverse flow, to remove air bubbles) 100 ml of an aqueous solution (pH 3.0) of porcine pepsin (25 mg/ml) at 3.0 ml/min. The membrane was washed with a 7-*M* ammonia solution until baseline level of the elution pattern was achieved. Finally, the strongly bound peptides were eluted with 2 *M* NaCl. The 2 *M* NaCl fraction

obtained was adjusted to pH 7.0 with HCl and heated at 85°C for 15 min. It was desalted by using a Sep-Pak C_{18} cartridge, as described in Section 2.2.1.

The concentrations of LF and LFcin-B in the samples obtained from the ion-exchange membrane were determined by analytical RP-HPLC (see Section 2.2.2); but, for the samples containing intact LF, the gradient of solvent B was from 15 to 60% in 30 min. In experiments starting with pure LF, concentrations of retained LF were calculated from absorbance values measured at 280 nm. An absorption coefficient of $A^{1\%}_{280,1\ cm}$ 12.3 was determined for LF, the estimation being based on the dry weight of the pure protein. An absorption coefficient of $A^{1\%}_{280,1\ cm}$ 12.7 was previously reported in the literature [18].

A flow chart of the experimental steps for the isolation of antibacterial peptides from LF, according to Section 2, is outlined in Fig. 1.

3. Results and discussion

3.1. Ion-exchange chromatography of LF hydrolysate

Using an SP-Sepharose Fast Flow column for the separation of the LF hydrolysate, the active peptides remained attached to the column even when it was washed with 5 *M* ammonia solution. When using the gradient containing 7 *M* ammonia, some activity was found in the fraction collected with 100% 7 *M* ammonia. In all cases, the main activity was found in the fraction obtained after subsequent elution of the column with 2 *M* NaCl. During this elution, two peaks were obtained (results not shown), which were collected in two separate fractions and were analyzed by RP-HPLC. The first (minor) IEC fraction was resolved by RP-HPLC into several components; the composition of the entire fraction varied with the concentration of ammonia used in the washing step (Fig. 2A). However, the second (major) fraction from the SP–Sepharose column contained essentially one prominent peak in the RP-HPLC chromatogram, regardless of the concentration of ammonia used (Fig. 2B). After desalting, the minor and the major IEC fraction both showed activity against *M. flavus*.

The desalted second IEC fraction, containing essentially one UV-absorbing HPLC peak (Fig. 2B), was subjected as such to mass spectrometry and to amino acid and sequence analysis. The mass spectrum of this fraction showed, besides some mass components of relatively low intensity (molecular masses 3154, 3211 and 3308), two major components of molecular mass 3194 and 3123 (Fig. 3a), which corresponded to residues 17 to 42 and 17 to 41, respectively, of the mature sequence of bovine LF (theoretical values, 3194.9 and 3123.8) [19]. The results of amino acid analysis were consistent with the sequence data. The isolated main component of Fig. 2B was also subjected to mass spectrometry (Fig. 3b). Mass components of 3194 and 3123 were still present, indicating that, under our chromatographic conditions, these two peptides could not be resolved. However, the minor mass components of 3154 and 3308 were no longer present, indicating that these could correspond to the minor peaks observed in the RP-HPLC chromatogram of Fig. 2B. The latter mass of 3308 probably corresponds to residues 17 to 43 of bovine LF (theoretical value, 3308.1). The minor component of mass 3211, which is present in both spectra, could correspond to the oxidised form of the fragment 17–42 (Met→MetO).

The two main peptides (masses 3194 and 3123) have recently been found in a ratio of approximately 1:1 in a chymosin hydrolysate of LF [17]. We found that, by increasing the concentration of pepsin or the hydrolysis time, the ratio of the peptides was shifted towards the 17–41 sequence (results not shown). This is the sequence of the bactericidal peptide, LFcin-B, as described by Bellamy et al. [15]. However, Jones et al. [20] analysed a LFcin-B sample obtained from the group of Bellamy and found an N-terminal sequence that matched the sequence reported by Bellamy et al. [15], but two distinct peptides were shown by electrospray mass spectrometry. The two peptides shown by mass spectrometry differed by only one amino acid residue at the carboxyl terminus and corresponded to the two peptides described above, f(17–41) and f(17–42). They suggested that both peptides exhibited antimicrobial activity because the molecular configuration would be little affected by this single deletion at the carboxyl terminus. It is probable that the 16–17 linkage in the mature sequence of LF is rapidly split by the action of pepsin, but different ‘LFcin-like

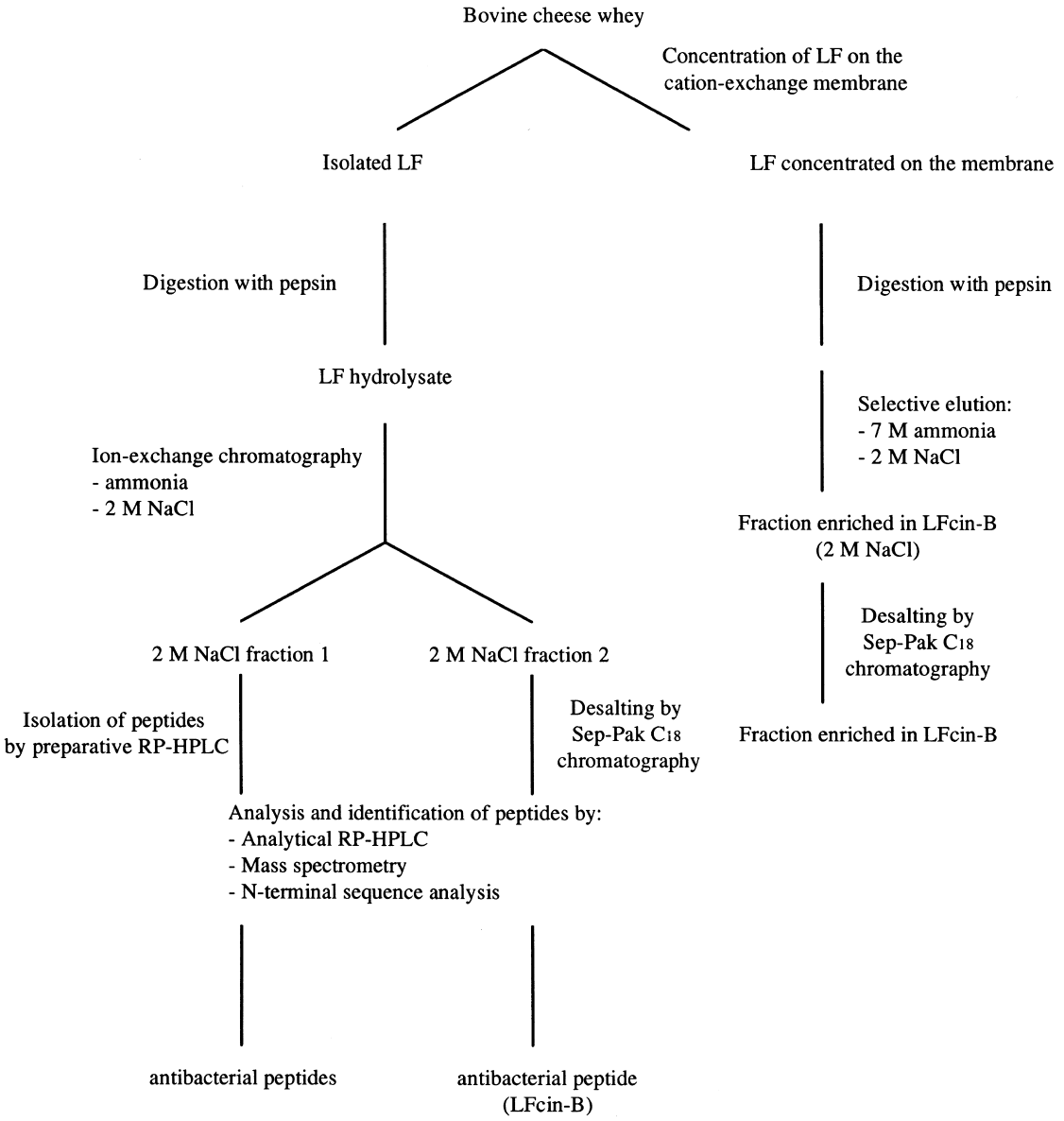

Fig. 1. Flow chart of the experimental steps used for the isolation of antibacterial peptides of lactoferrin from cheese whey. LF, lactoferrin; LFcin-B, lactoferricin-B. For details, see Section 2.

peptides' can be formed by pepsin digestion. These peptides differ in one, two or three amino acid residues at the carboxyl terminus.

Peptides 1–5 in the first eluting (minor) fraction obtained with 2 *M* NaCl from the SP–Sepharose column after using 3 *M* ammonia (Fig. 2A, trace c) were further purified by preparative RP-HPLC and tested for their activity against *M. flavus*. Purified peptides were subjected to mass spectrometry and N-terminal sequence analysis in order to determine their location in the native LF sequence (Fig. 4).

The combination of mass spectrometric data (experimental mass, 1439) and the N-terminal sequence data (KFGKNK) revealed that peptide 1 corres-

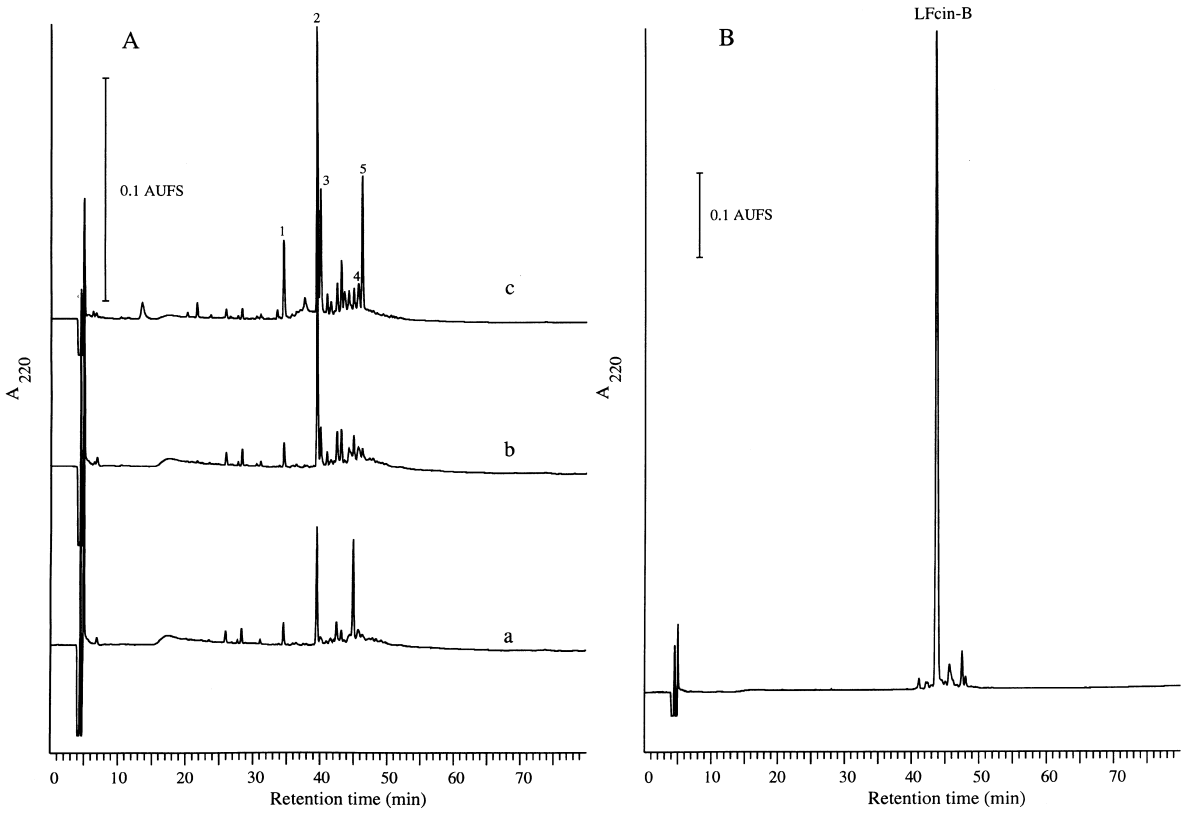

Fig. 2. (A) RP-HPLC chromatograms of the first eluting (minor) peak obtained during the elution of the SP–Sepharose Fast Flow column with 2 *M* NaCl after prior washing with a gradient using (a) 7 *M* ammonia solution, (b) 5 *M* ammonia solution and (c) 3 *M* ammonia solution. (B) RP-HPLC chromatogram of the second eluting peak during the elution of the SP–Sepharose Fast Flow column with 2 *M* NaCl. Chromatographic conditions are detailed in Section 2. LFcin-B, lactoferricin-B. Peak components 1–5 in A were purified and characterized (see text and Fig. 4).

ponded to the fragment 277–288 of the mature sequence of LF [19,21]. This peptide (theoretical mass value, 1439.7) was not located in the N-terminal part of the LF sequence, like LFcin-B, and did not contain any iron-binding site. Although the peptide is positively charged (four of the 12 residues are basic amino acids), it did not show antibacterial activity against *M. flavus* under the conditions tested. However, it has been reported [22] that, besides electropositivity, other factors may determine the antibacterial character of a protein or peptide.

Peptides 2 and 3 were collected together as a single fraction after preparative RP-HPLC and the entire fraction showed activity against *M. flavus*. However, the activity of this fraction should be attributed only to peptide 2, because peptide 3 was also present as the major peak of an inactive fraction collected in a separate experiment. The fraction was further purified by RP-HPLC on an analytical scale, and the constituent peptides were identified according to Section 2.2.6. Peptide 2 consisted of two fragments connected by a disulphide bond (a heterodimer), and showed a total molecular mass of 2430. The amino acid sequences at the two N-termini of peptide 2 were established as APRKNV and CIRA, and the full sequence is presented in Fig. 4 (theoretical mass, 2430.8). This sequence of the heterodimer [f(1–16)–f(45–48)] flanked that of the active peptides f(17–41), f(17–42), f(17–43) and f(17–44) (the latter not found in this study). This is consistent with the existence of various splitting sites at the carboxyl terminus of the ‘lactoferricin-like peptides’.

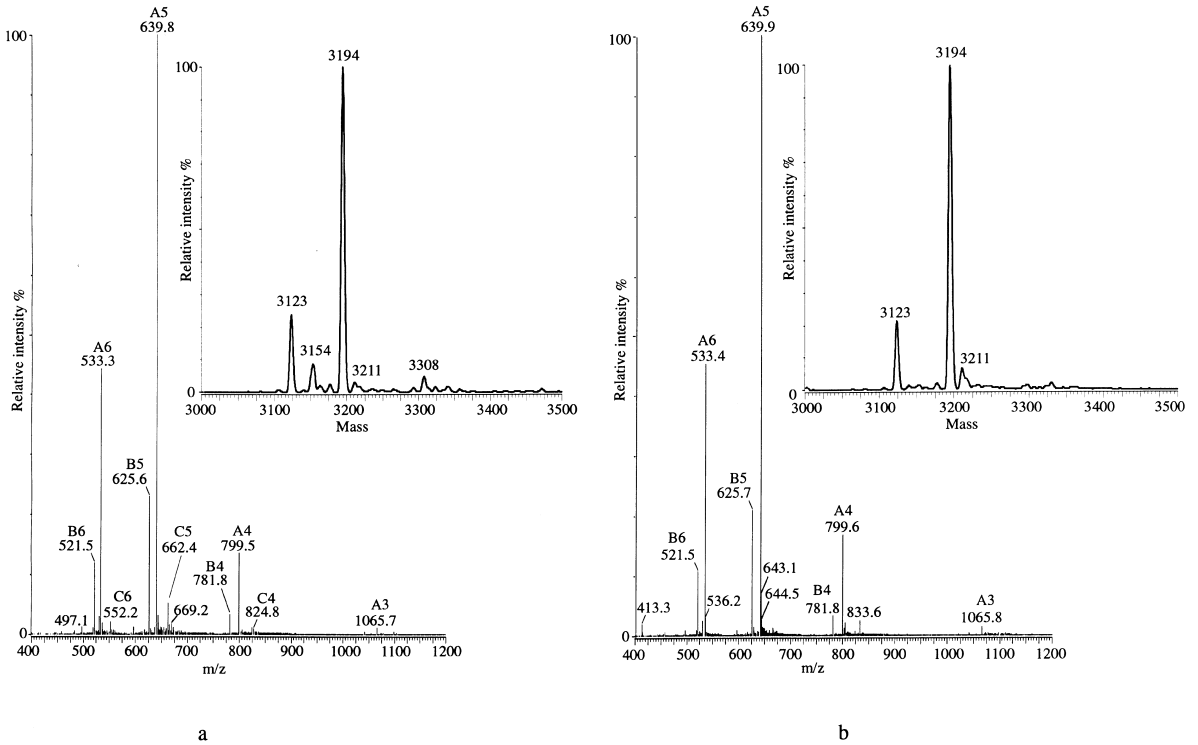

Fig. 3. Electrospray mass spectra of (a) the total fraction shown in Fig. 2B and (b) the main component (LFcin-B) shown in the same figure. Ax, Bx and Cx denote components of main envelopes of multiply charged ions of the m/z spectra. The insets represent the deconvoluted spectra.

Peptide 2 is similar to an active peptide that was described recently [16] for which the sequence f(1–16)–f(43–48) was reported. Peptide 3 was a linear peptide with an N-terminal sequence of IWKLLS and a molecular mass of 2248, which corresponds to the fragment f(267–285) of the mature chain of LF (theoretical mass, 2248.7). This fragment has the residues 277 to 285 in common with peptide 1 and, like peptide 1, it did not show antibacterial activity against *M. flavus*, in spite of its electropositive character (six of the 19 residues are basic amino acids).

Peptides 4 and 5 were also collected as a combined fraction after preparative RP-HPLC. Mass spectrometric analysis of this fraction revealed the presence of two peptides with masses of 2637 (major component) and 5153 (very minor component). Analytical RP-HPLC analysis of this fraction showed a major peak accompanied by a shoulder. After treatment with dithiothreitol, the shoulder disappeared, giving two new minor 'non-shoulder' components, whereas the position of the major peak was not affected by reduction. The sequencing data for this fraction confirmed the presence of a single peptide and a heterodimer at a lower concentration. From these data, it could be derived that the sequence of the major component, i.e., single peptide 5 (Fig. 2A), comprised residues 267 to 288 (theoretical mass, 2637.1), and that the sequence of the minor component, i.e. heterodimer peptide 4 (Fig. 2A), corresponded to residues 1–11 connected to 17–47 by a disulphide bridge at residues 9 and 45 (theoretical mass, 5151.4). The entire fraction (consisting of peptides 4 and 5) showed activity against *M. flavus*, although the antibacterial activity is probably due to the heterodimer, because this contained the most active antibacterial region of the LF molecule. Hoek et al. [17] have recently described a similar heterodimer isolated from a chymosin LF hydrolysate [f(1–16)–f(17–48)], which displayed antibacterial activity

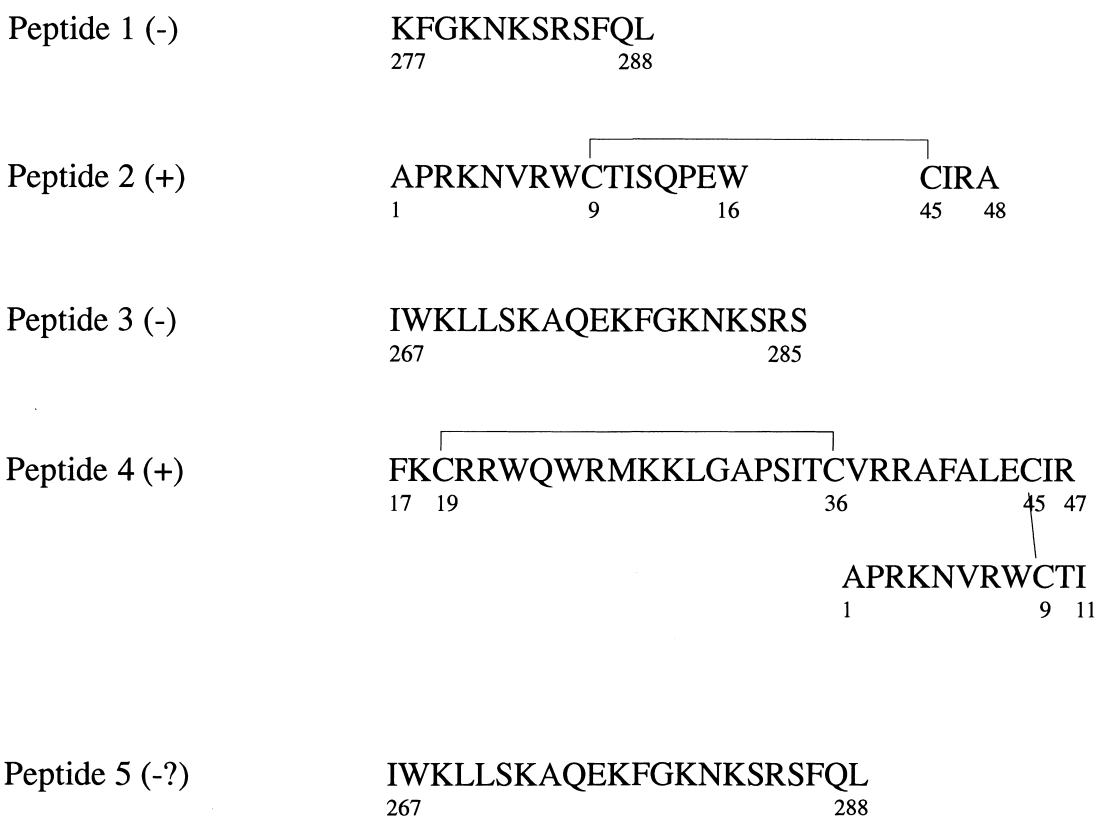

Fig. 4. Primary structures of peptides generated by pepsin digestion of bovine lactoferrin (LF). Peptide numbers correlate to the HPLC fraction numbers given in Fig. 2A. Residue numbers indicate the sequence position in bovine LF [19,21] and disulphide bonds are shown as lines. (+) indicates antibacterial activity against *M. flavus*, (−) indicates no antibacterial activity.

towards *Escherichia coli*. The small amount of the heterodimer in the IEC fraction or in the total LF hydrolysate precluded further isolation and antibacterial testing. This longer peptide may be further hydrolysed by pepsin, giving different active fragments, i.e., 17–41, 17–42 or 17–43 and a part of peptide 2 (Fig. 4).

The advantage of the present ion-exchange method, which uses ammonia to elute inactive LF fragments, is that a fraction containing antibacterial, cationic fragments of LF is retained by the column and that a concentrated fraction of LFcin-B with at least 86% purity (on the basis of peak areas in Fig. 2B) can be obtained in a single elution step with NaCl. Desalting of the LFcin-B-containing fraction may be performed by reversed-phase chromatography, but also by other means such as ultrafiltration or electrodialysis because of the high purity of the fraction obtained in the single ion-exchange chromatographic step.

3.2. Pepsin digestion of LF on an ion-exchange membrane

To measure the maximum binding capacity of LF to the membrane, a solution of pure LF (0.15 mg/ml in phosphate buffer) was pumped through a Sartobind S strong cation-exchange membrane. At a flow-rate of 9.9 ml/min and 25°C, approximately 137 mg/100 cm^2 ($2 \cdot 10^{-8}$ mol/cm^2) were bound to the membrane. Under similar conditions, Mitchell et al. [23] found a comparable binding capacity of the same membrane type for LF.

The association of LF with the whey proteins

β-lactoglobulin and bovine serum albumin reported [24] takes place at low ionic strength of the solution. In order to determine the optimal ionic strength for binding LF to the ion-exchange membrane in the isolation of LF from cheese whey, different amounts of NaCl were added to the whey. A concentration of 0.05 *M* NaCl resulted in a recovery of LF in the bound fraction that did not differ notably from the amount obtained without NaCl addition. From 1800 ml of cheese whey, approx. 28 mg of LF/100 cm^2 ($4 \cdot 10^{-9}$ mol/cm^2) was obtained. Higher NaCl concentrations, 0.1 and 0.2 *M* NaCl, decreased the recovery of LF in the bound fraction to 26 and 14 mg/100 cm^2, respectively. However, the addition of small amounts of NaCl had a marked effect on the binding of LP to the ion-exchange membrane. By adding 0.05 *M* NaCl, the area under the curve of the LP peak, obtained by RP-HPLC, in the bound fraction decreased by about 90%, and LP was not detected at all when 0.1 *M* NaCl was added to the whey. These results are in agreement with those of Dionysius et al. [25], who studied the effect of the electrical conductivity of whey on the binding of LF and LP to a weak cation-exchange resin; those experiments confirm the greater affinity of LF for cation-exchange media at higher conductivities. The differential binding capacity of the two proteins could, therefore, be used to separate them and, for example, the use of whey with 0.05 *M* NaCl added may yield a product that consists of LF with negligible LP contamination.

After passing a solution of LF in phosphate buffer (0.15 mg/ml) through a membrane until saturation of the membrane, or 1800 ml of Gouda cheese whey without and with 0.05 *M* NaCl, the LF bound to the membrane was hydrolysed overnight (see Section 2.3). After washing the membrane with a 7-*M* ammonia solution, the membrane was treated with 2 *M* NaCl to produce a fraction enriched in LFcin-B. Fig. 5 shows the RP-HPLC chromatograms of the 2 *M* NaCl fractions obtained after hydrolysis on the membrane, starting from a solution of LF in phosphate buffer (Fig. 5a), Gouda cheese whey (Fig. 5b) and Gouda cheese whey with 0.05 *M* NaCl added (Fig. 5c). Mass spectrometric analysis (not shown) of the RP-HPLC LFcin-B peak in these 2 *M* NaCl fractions confirmed the presence of the two 'LFcin-like peptides' with masses of 3123 and 3194 [fragments f(17–41) and f(17–42)]. The minor peak, marked with an asterisk in Fig. 5, corresponded to the fragment 1–8 of bovine LF, as was determined by mass spectrometric analysis (found, 1026; expected, 1026.2) in combination with the amino acid sequence data of the N-terminal sequence (APRKNV).

The yield of one cycle (LF binding, LF hydrolysis on the membrane and LFcin-B elution) was estimated by quantifying the amount of LFcin-B [f(17–41) plus f(17–42)] obtained in the 2 *M* NaCl fraction by analytical RP-HPLC, using an external calibration curve. Based on the maximum values found in the LF binding experiments, the yields of LFcin-B were ca. 50% when starting from cheese whey (0.52 ± 0.04 mg LFcin-B/100 cm^2, $2 \cdot 10^{-9}$ mol/cm^2; $n=2$) or cheese whey with NaCl added (0.51 ± 0.07 mg LFcin-B/100 cm^2, $2 \cdot 10^{-9}$ mol/cm^2; $n=2$) and 65% starting from pure LF (3.66 ± 0.16 mg LFcin-B/100 cm^2, $14 \cdot 10^{-9}$ mol/cm^2; $n=2$). A similar yield (50%) was achieved by Hoek et al. [17] when isolating LFcin-B from a LF hydrolysate by cation-exchange chromatography followed by a final purification by RP-HPLC. It may be noted that in the fractionation of whey by IEC–membrane technology, Chiu and Etzel [14] observed an optimum in the recovery of LF as a function of the loading volume of whey. Deviation from such an optimum may also have influenced our final yield of LFcin-B.

Compared to the methods already described for the isolation of LFcin-B [15–17,26,27], the present membrane-based method, which includes the hydrolysis of LF on the ion-exchange membrane, presents several economic advantages. The isolation of a fraction enriched in LFcin-B can be achieved directly from cheese whey, without the need for the intermediate isolation of LF. This procedure can be applied not only by using an IEC membrane, but also with other IEC material (either on a column or batchwise). However, membrane-based processes are more rapid by allowing a higher flow-rate (ca. 20-fold) compared with bead-based systems [14]. In addition, these processes can be easily scaled up to gram or even kilogram quantities. Similarities between ion-exchange and ultrafiltration membrane equipment may allow whey protein concentrate manufacturers to easily convert to the production of antimicrobial peptides, like LFcin-B, without invest-

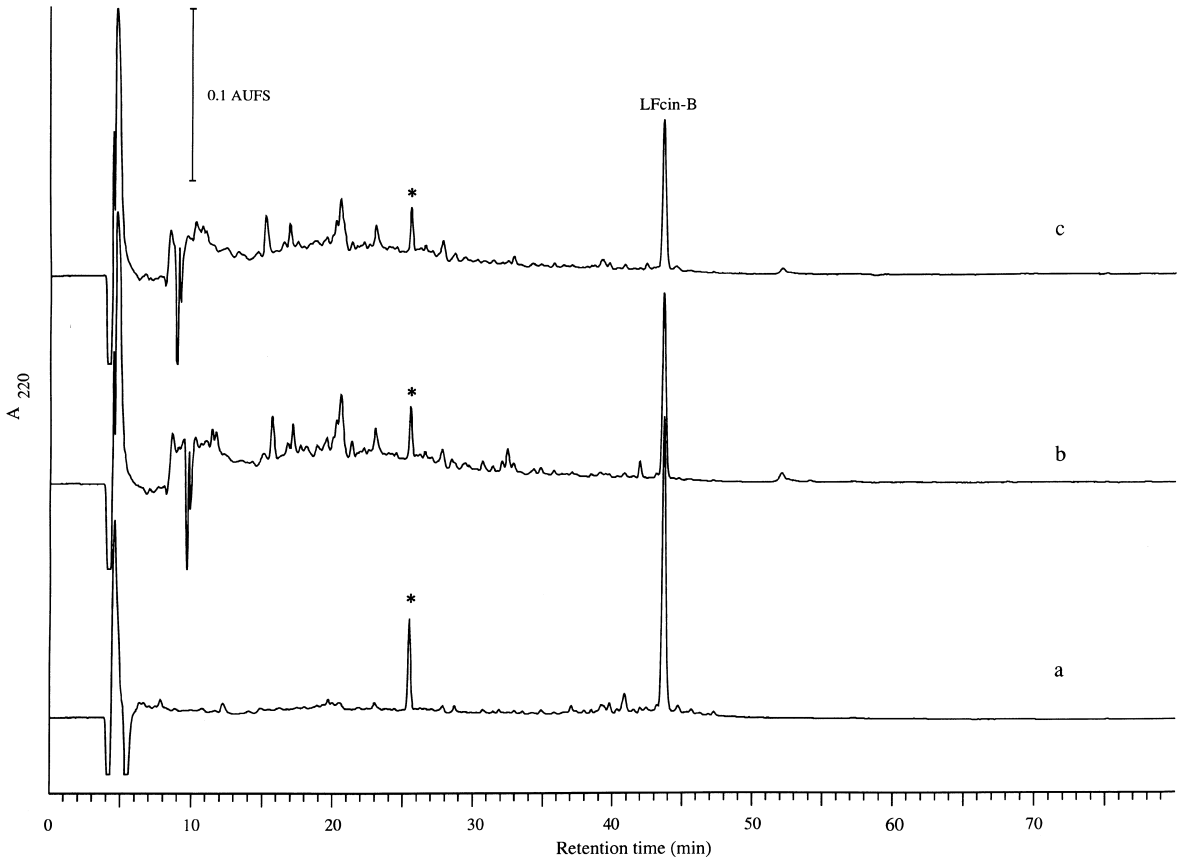

Fig. 5. RP-HPLC chromatograms of the 2 *M* NaCl fraction obtained after hydrolysis on the ion-exchange membrane starting from (a) 0.15 mg/ml LF solution in phosphate buffer, (b) microfiltered Gouda cheese whey and (c) microfiltered Gouda cheese whey with 0.05 *M* NaCl added. The sample in chromatogram (a) was diluted 1:1 with RP-HPLC solvent A. For chromatographic conditions, see Section 2. LFcin-B, lactoferricin-B; * corresponds to fragment 1–8 of bovine LF.

ing in a new plant. Moreover, because the amount of LF in whey is only a minor portion of the total protein content, the whey devoid of LF may still be used for further processing.

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