Porcine pulmonary surfactant preparations contain the antibacterial peptide prophenin and a C-terminal 18-residue fragment thereof

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Abstract Surfactant preparations obtained from porcine lungs by extraction with chloroform/methanol followed by chromatography over Lipidex-5000 are used for treatment of respiratory distress syndrome in preterm infants. These preparations contain about 98% phospholipids and 1–2% of the hydrophobic pulmonary surfactant-associated proteins B and C (SP-B and SP-C). Separation of the proteins in the surfactant preparation by reversed-phase high performance liquid chromatography revealed, in addition to SP-B and SP-C, the presence of three peptides derived from the cathelicidin family of antibacterial peptides. The 79-residue proline-rich peptide prophenin (identical to that isolated from leukocytes), an 80-residue prophenin with an N-terminal pyroglutamic acid residue, and a C-terminal 18-residue fragment of prophenin were found in approximate molar ratios of 1:20:5. A synthetic version of the C-terminal 18-residue peptide exhibits salt-dependent antibacterial activity (higher activity in the absence of salt) against the Gram-positive bacterium *Bacillus megaterium* Bm11 and, to a lesser extent, against Gram-negative *Escherichia coli* D21 cells. It appears possible that the presence of prophenin peptides may contribute to the antibacterial properties of surfactant preparations.

Key words: Pulmonary surfactant; Lipid-associated peptide; Peptide isolation; Antibacterial peptide; Cathelicidin; Mass spectrometry

1. Introduction

Pulmonary surfactant is a heterogeneous mixture of lipids, mainly phospholipids, and proteins. Surfactant is essential because it reduces the surface tension of the alveolar liquid lining, thus preventing lung collapse at end of expiration. Insufficient amounts of surfactant in preterm neonates give rise to respiratory distress syndrome (RDS), a potentially lethal disease. In addition, surfactant contributes to the lung host defence. Four surfactant proteins have been described to date [1]. Surfactant proteins A and D (SP-A and SP-D) are collagenous lectins (collectins) which are thought to participate primarily in the lung host defence. SP-A knock-out mice have essentially normal respiratory function but are more susceptible to pulmonary infections than wild-type mice [2,3]. SP-B and SP-C on the other hand, are probably primarily involved in the reduction of alveolar surface tension. Children with genetic deficiency of SP-B and SP-B knock-out mice develop severe respiratory distress at birth [4,5]. SP-B and SP-C are structurally unrelated but are both strictly hydrophobic and associated with surfactant phospholipids. Surfactant preparations obtained from animal lungs by extraction and chromatography in organic solvents contain phospholipids, SP-B and SP-C, but not SP-A and SP-D, and are used in the treatment of RDS in premature infants. Instillation of such surfactant preparations give bacteriostatic effects in an animal model of pulmonary infection [6].

Improved methods for the isolation and analysis of pulmonary surfactant constituents have recently been introduced. High resolution separation of SP-B, SP-C and isoforms thereof is achieved with reversed-phase high performance liquid chromatography (RP-HPLC) employing gradient elution with aqueous ethanol or methanol and isopropanol [7]. Lipid-associated proteins and phospholipids can be largely separated by chromatography over Lipidex-5000 in ethylene chloride/methanol 1:4 (v/v) [8]. We have now employed these methods to resolve the proteins present in organic extracts of porcine lung homogenates and find that the antibacterial protein prophenin and a fragment thereof are present in such extracts. Prophenin is likewise present in clinically used surfactant preparations obtained from porcine lungs.

2. Materials and methods

2.1. Organic extraction of lung tissue

Two alternative ways were employed for tissue homogenization. In the first method 100 g of minced lung tissue was homogenized in 400 ml of chloroform/methanol 1:1 (v/v), whereafter 100 ml of water was added. After filtration and addition of 50 ml of chloroform, the homogenate was phase separated and the lower organic phase was collected and evaporated to dryness. In the second method [9] minced lung tissue was initially washed with saline and the mixture was filtered. Cells and debris were removed by centrifugation at $1000\times g$ for 15 min at 20°C, and the supernatant was subsequently centrifuged at $3000\times g$ for 2 h at 4°C. The pellet was then extracted with chloroform/methanol 2:1 (v/v) [9].

2.2. Purification of proteins present in organic lung extracts

The dried organic extracts were resolubilized in ethylene chloride/methanol 1:4 (v/v) and separated by reversed-phase chromatography on a 40×6.5 cm Lipidex-5000 column in the same solvent system. The protein and phospholipid contents of the eluate were monitored by phenylthiocarbamyl amino acid analysis and phosphorus determination, respectively. The protein fractions elute between 35 and 50% of one column volume, while phospholipids start eluting at about 45% [8]. Protein-containing fractions were collected, pooled, and further resolved by gel-filtration over Sephadex LH-60 in chloroform/metha-

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Abbreviations: CID, collision induced dissociation; ES, electrospray; HPLC, high performance liquid chromatography; LB, Luria-Bertani; MALDI-TOF, matrix-assisted laser desorption/ionization time-of-flight; OATOF, orthogonal acceleration time-of-flight; OATOFFPD, orthogonal acceleration time-of-flight focal plane detector; RDS, respiratory distress syndrome; RP, reversed phase; SP, surfactant protein; TFA, trifluoroacetic acid

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nol/0.1 M HCl, 19:19:2 (by vol), or in chloroform/methanol/water, 19:19:2 (by vol). Proteins eluting between 15 and 60% of one column volume (corresponding to the elution positions of SP-B and SP-C) were collected.

Samples of Curosurf (which are obtained by Lipidex-5000 chromatography of organic extracts of minced and saline-washed porcine lungs) were dissolved in chloroform/methanol 1:1 (v/v) and protein-containing fractions were isolated by Sephadex LH-60 chromatography as above.

Sephadex LH-60 fractions were evaporated to dryness, redissolved in ethanol and analyzed by RP-HPLC using a C_{18} column (Vydac, 250×4.6 mm), with a flow of 0.7 ml/min, 60% methanol (aq)/0.1% trifluoroacetic acid (TFA) as the initial solvent and elution with a linear gradient of 2-propanol/0.1% TFA [7].

2.3. Protein analysis

For tryptic digestion, 100 μg of prophenin isolated from lung tissue as above was dissolved in 0.1 M ammonium bicarbonate, pH 8.0, 1 μg of trypsin was added, and the mixture was incubated for 4 h at 37°C, whereafter proteolysis was halted by addition of acetic acid to 1% (v/v).

For matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry, peptides (20–50 ng) were dried on a stainless-steel grid together with approximately 5 μg α-cyano-4-hydroxycinnamic acid. Molecular masses were determined with a Finnigan MAT Lasermat 2000 instrument (Hemelhensted, England), operated in the positive ion mode. Calibration was performed with glucagon (average mass $[M+H]^+ = 3482.8$) and LL-37 (average mass $[M+H]^+ = 4493.3$).

Nano-electrospray (nano-ES) mass spectra were recorded on an AutoSpec orthogonal acceleration time-of-flight focal plane detector (OATOFFPD) (Micromass, Manchester, England) high resolution tandem mass spectrometer [10]. This instrument consists of a double focusing arrangement of electric (E) and magnetic (B) sectors as mass spectrometer 1 (MS1) and an orthogonal acceleration time-of-flight (OATOF) mass analyzer as MS2. Samples were introduced by nano-ES from gold coated borosilicate capillaries. The ion source was operated at a potential of 4 kV in the positive ion mode. For the recording of conventional mass spectra the instrument was tuned to a resolution of approximately 3000 (10% valley definition) and the magnet scanned at a rate of 15 s/decade over m/z ranges 100 (or 400) to 3000. High resolution (10 000, 10% valley definition) spectra were recorded as voltage scans over m/z ranges just sufficient to bracket sample peaks between calibrant ion peaks. Tandem mass spectra were recorded by selecting the precursor ion of interest using MS1, decelerating the ions to 400 eV and focusing them into a collision cell containing methane collision gas at a pressure sufficient to attenuate the precursor ion beam by about 80%. Undissociated precursor ions and fragment ions formed by collision induced dissociation (CID) were orthogonally accelerated into the time-of-flight mass analyzer.

Amino acid sequence analysis was carried out with an Applied Biosystems 494 Prosice protein sequencer with on-line HPLC separation of released phenylthiohydantoin derivatives.

2.4. Antibacterial activity

An inhibition zone assay in 1 mm agarose plates seeded with *Escherichia coli* D21 or *Bacillus megaterium* Bm11 cells was used [11]. Bacteria were grown overnight on agar plates containing 100 μg/ml streptomycin and were then inoculated in Luria-Bertani (LB) medium. Bacteria were added to 1% (mass/vol) agarose in LB medium, LB medium without NaCl, or LB broth containing medium E (0.8 mM $MgSO_4$, 9.6 mM citric acid, 57.4 mM K_2HPO_4 and 16.7 mM $NaNH_4HPO_4$) to a concentration of 6×10^4 cells/ml just before plating. Small wells (3 mm diameter, 3 μl volume) were punched out of the plates and prophenin, isolated from lung tissue, was added dissolved in 0.1% TFA. After overnight incubation at 30°C, the diameters of the inhibition zones were determined. To study the antibacterial activity of the amidated C-terminal 18-residue fragment encompassing positions 62–79 of prophenin-1, this fragment was synthesized (Interactiva, Germany), since we were unable to separate the naturally occurring fragment from the full-length prophenins (see Section 3). The synthetic peptide was purified by RP-HPLC and analyzed by MALDI-TOF (expected molecular mass 2044.5 Da, calculated molecular mass 2044.4 Da).

3. Results

3.1. Identification of prophenin in organic extracts of porcine lung

Proteins in chloroform/methanol extracts of lung tissue were separated from most of the lipids by Lipidex-5000 chromatography and thereafter size-fractionated by Sephadex LH-60 chromatography. RP-HPLC of the Sephadex LH-60 fraction eluting between 25 and 35% of the column volume, i.e. the elution position of SP-B [9], revealed one peak that elutes at about 35% 2-propanol, a second peak at about 60% 2-propanol and a group of peaks eluting at about 80–90% 2-propanol (Fig. 1). SP-B and SP-C are known to elute at about 60 and 90–100% 2-propanol, respectively, under these conditions [7]. Consistent with this, MALDI-TOF analysis of the peptide eluting at 60% 2-propanol showed peaks corresponding to singly and multiply protonated forms of SP-B. The molecular mass of SP-B determined from these peaks is 17 440 Da, which is in good agreement with the calculated molecular mass of SP-B of 17 446 Da. The small peak at 93% 2-propanol (Fig. 1) corresponds to SP-C, as determined by MALDI-TOF analysis (experimental average mass 4218 Da, calculated average mass of methylated and methionine oxidized SP-C 4217 Da) and amino acid analysis. The other peaks in the group of late eluting compounds (Fig. 1) are non-peptide components as determined by amino acid analysis, and probably represent traces of phospholipids.

MALDI-TOF analysis of the early eluting HPLC peak in Fig. 1 revealed two peptides. One peptide produces $[M+H]^+$ ions at m/z 8746 (i.e. corresponding to a molecular mass of 8745 Da), while the other peptide gives $[M+H]^+$ ions at m/z 2044 (corresponding to a molecular mass of 2043 Da). The relative abundance of the two sets of ions varied between

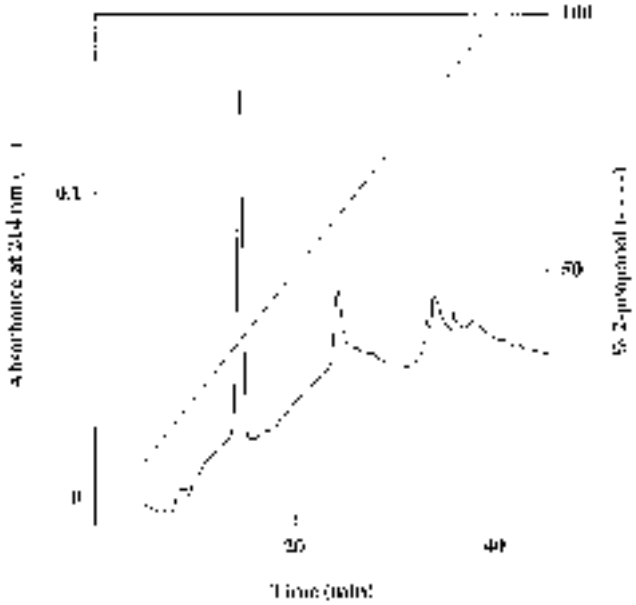

Fig. 1. Reversed-phase HPLC of lipopeptides from porcine lung tissue. HPLC resolution of proteins from organic extracts of porcine lung tissue, purified by Lipidex-5000 chromatography and by gel-filtration over Sephadex LH-60 (elution between 25 and 35% of one column volume). A C_{18} stationary phase and a linear gradient of 2-propanol/0.1% TFA running into 60% methanol (aq.)/0.1% TFA were employed for RP-HPLC.

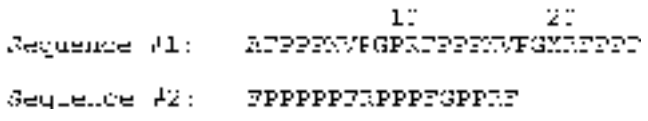

Fig. 2. Identification of prophenin in surfactant preparations. Results of amino acid sequence analysis of proteins eluting under the HPLC peak at 35% 2-propanol in Fig. 1. X denotes a position where no amino acid could be unequivocally assigned.

different experiments, but in most cases both sets could be detected. Attempts to separate the two peptides by HPLC for individual analysis were not successful. Amino acid sequence analysis of the peptides in the early HPLC peak yielded two sequences, which could be individually assigned because of their different amounts (Fig. 2). Sequence #1 in Fig. 2 corresponds exactly to the N-terminal sequence of the antibacterial cathelicidin peptide prophenin, which is a 79-residue polypeptide originally purified from porcine leukocytes [12]. An Val/Phe isomorphism at prophenin position 17 exists [13], but no evidence for the Phe-17 prophenin form was found in the present study. The molecular mass of 8745 Da found by MALDI-TOF analysis is 113 Da higher than expected from the prophenin amino acid sequence containing Val-17, and where the C-terminus is amidated (theoretical average mass 8632 Da). Sequence #2 in Fig. 2 corresponds to prophenin positions 62–78. The calculated average mass of prophenin position 62–79 with a C-terminal amide is 2043 Da, thus in excellent agreement with the experimental molecular mass. Therefore, it is concluded that the peptide isolated corresponds to prophenin position 62–79 with a C-terminal amide, and the C-terminal residue escaped detection by sequence analysis. The molar ratio of 79-residue prophenin and the 18-residue fragment, as estimated from yields of amino acid derivatives during sequence analysis, was about 1:5.

The full-length prophenin and its C-terminal peptide were found in organic extracts of homogenized whole lung, as well as in extracts of saline-washed minced lungs. In the former case the ratio of the areas of HPLC peaks for prophenin and SP-B was about 3:1 (Fig. 1), while in the latter case the ratio was about 1:1. Likewise, analysis of Curosurf preparations revealed the presence of full-length prophenin and the peptide corresponding to prophenin position 62–79, in an approximate ratio of 1:1 compared to SP-B. In contrast, no HPLC peak corresponding to prophenin could be detected in organic extracts of porcine bronchoalveolar lavage fluid.

3.2. *Prophenin-2 is N-terminally elongated with pyroglutamic acid*

In order to resolve the reason for the discrepancy between the molecular mass calculated from the amino acid sequence and that determined by MALDI-TOF mass spectrometry (see above) we analyzed prophenin by nano-ES mass spectrometry. The molecular mass of prophenin was determined to be 8744 Da (Fig. 3), which is 111 Da greater than the theoretical mass of prophenin with Val-17 and a C-terminal carboxylic acid (average mass 8633 Da), or 112 Da greater than the theoretical mass of prophenin with Val-17 and a C-terminal amide (average mass 8632 Da). Small amounts of unmodified prophenin (average mass 8633 Da) were also detected. The molecular masses of prophenin measured by nano-ES above were to an accuracy of ±1 Da. Thus, as it was not known whether the C-terminal is actually amidated or not, the exact magnitude of the modification (111 or 112 Da) was not unequivocally determined. In an attempt to resolve this question,

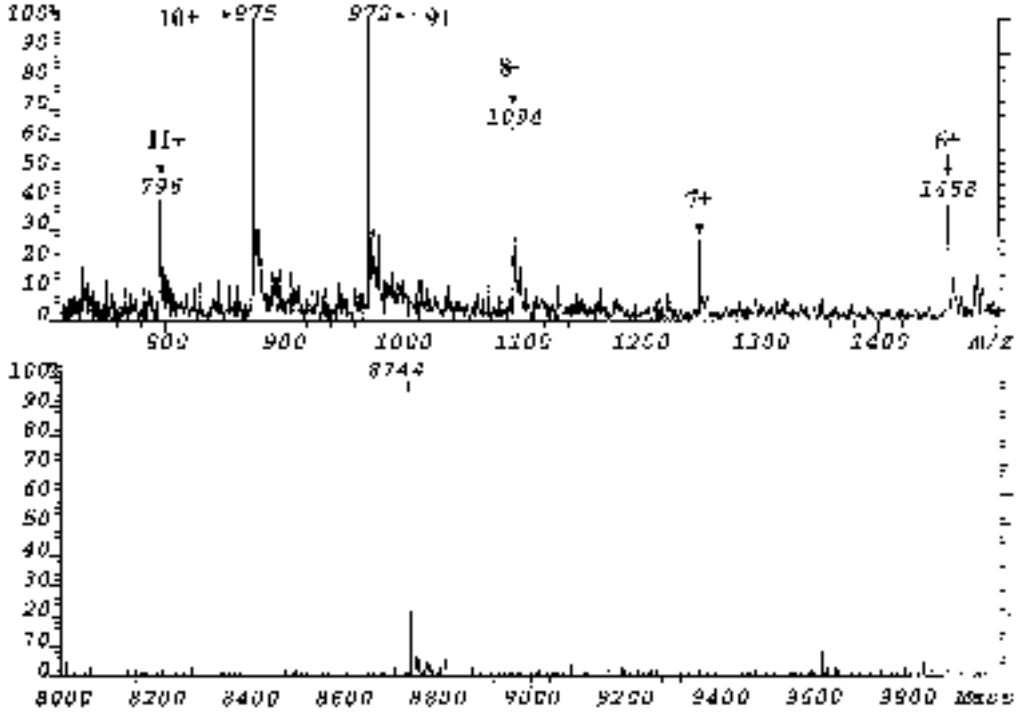

Fig. 3. Nano-ES mass spectrum of prophenin isolated from porcine lung tissue. The top spectrum shows the multiprotonated forms of the protein i.e. $[M+6H]^{6+}$-$[M+11H]^{11+}$. After maximum entropy treatment the lower spectrum of the uncharged protein was attained.

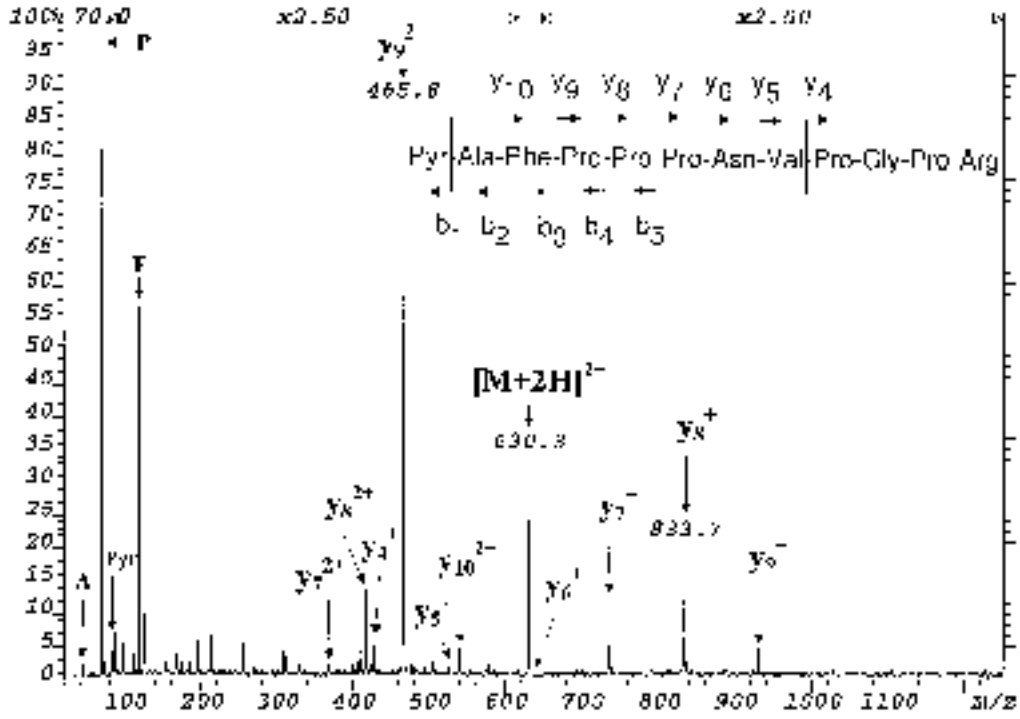

Fig. 4. Identification of pyroglutamic acid in prophenin-2. CID spectrum of the $[M+2H]^{2+}$ ion of m/z 630.3, which corresponds to the doubly protonated T1 fragment modified with pyroGlu. The fragmentation nomenclature is from Roepstorff and Fohlman [24], modified by Biemann [25]. The y-ions are identified in the mass spectrum. The minor b-ions were all detectable at closer inspection of the mass spectrum, but are not identified in the spectrum. Pyr, pyroglutamic acid. A, P, F, and Pyr identify the peaks that represent ammonium ions derived from the respective amino acid.

prophenin was cleaved with trypsin and the tryptic fragments analyzed by high resolution nano-ES mass spectrometry. A tryptic fragment was observed with a monoisotopic mass of 1258.6504 Da which agrees to within 3.6 ppm (0.0046 Da) with the mass of the N-terminal tryptic fragment (T1, cleaved C-terminally of Arg-11, see Fig. 2) modified with a pyroglutamic acid residue (pyroGlu, mass 111.0320 Da). Only a very minor unmodified T1 fragment was observed. The location of this proposed pyroglutamic residue in tryptic fragment T1 was confirmed by tandem mass spectrometry. Shown in Fig. 4 is the CID spectrum of the $[M+2H]^{2+}$ ion of m/z 630.3 which corresponds to the doubly protonated T1 fragment modified with pyroGlu. Series of y_9^+-y_4^+ and y_{10}^{2+}-y_7^{2+} fragment ions locate the modification to the N-terminus. This is confirmed by a minor series of b_1^+-b_5^+ fragment ions. The presence of the pyroGlu modification is further confirmed by the observation of its characteristic ammonium ion at m/z 84 (Fig. 4).

We conclude that prophenin now isolated from lung lipoproteins, as well as prophenin previously isolated from leukocytes [12], consists of a mixture of two full-length polypeptides. One form is the 79-residue polypeptide with Ala as N-terminal residue, previously referred to as prophenin-1 [12] (this form was now detected by sequence analysis; Fig. 2). The second prophenin form has 80 residues with an extra N-terminal pyroGlu compared to prophenin-1 (this form was now observed by mass spectrometry but not by sequence analysis as it is refractory to Edman degradation). The latter polypeptide corresponds to prophenin-2, the covalent structure of which has not been previously resolved [12]. The ratio between prophenin-1 and prophenin-2, estimated from ES mass spectrometry, is approximately 1:20.

3.3. *Antibacterial activity of prophenins isolated from lung tissue by organic extraction and a synthetic 18-residue fragment corresponding to prophenin-1 (positions 62–79)*

The isolated prophenin peptides, which constitute a mixture of prophenin-1 and prophenin-2 and the C-terminal 18-residue fragment in approximate molar ratios of 1:20:5 were

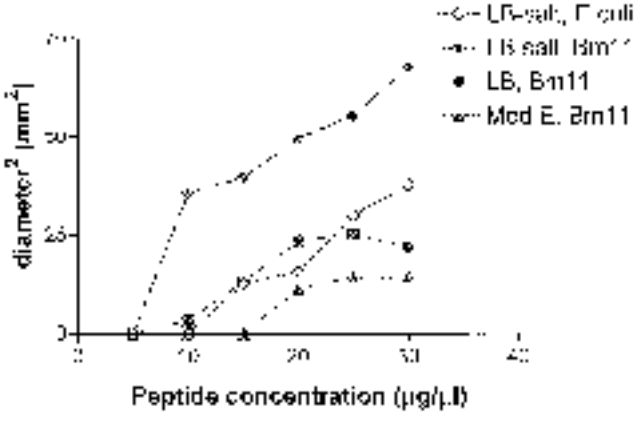

Fig. 5. Antibacterial activity of the prophenin C-terminal 18-residue fragment. Inhibition zone assay of the activity of a synthetic 18-residue peptide corresponding to positions 62–79 of prophenin-1 against *B. megaterium* Bm11 and *E. coli* D21 cells, in the presence of different salt solutions. The diameter^2 of the inhibition zones is plotted against the peptide concentration applied. Filled symbols, activity against *B. megaterium* in LB medium without sodium chloride (diamonds), LB medium (squares) and medium E (triangles). Open symbols, activity against *E. coli* in LB medium without sodium chloride. No activity against *E. coli* was detected in LB medium and in medium E.

tested for antibacterial activity against *E. coli* D21 and *B. megaterium* Bm11 cells using an inhibition zone assay. This showed antibacterial activity against *B. megaterium* grown in salt-free LB medium (6 mm inhibition zones) at a total peptide concentration of 0.5 μg/μl. In the presence of 150 mM NaCl in the growth medium, no activity was detected. The isolated prophenins showed no activity against *E. coli*.

The synthetic C-terminal fragment exhibited dose-dependent antibacterial activity at concentrations > 10 μg/μl. It was more active against *B. megaterium* Bm11 than against *E. coli* D21, and the activity increased with decreasing salt concentration (Fig. 5).

4. Discussion

Organic extracts of porcine lung tissue purified by chromatography over Lipidex-5000 and Sephadex LH-60 contain 79-residue prophenin (previously referred to as prophenin-1), 80-residue pyroGlu-prophenin (previously referred to as prophenin-2) and a C-terminal 18-residue fragment of prophenin. This is an unexpected finding since SP-B and SP-C have been considered to be the exclusive proteins in such preparations. Prophenin has so far only been isolated from porcine leukocytes and in aqueous media [12].

Elastase can liberate the mature antibacterial peptides from the cathelicidin precursors by cleavage C-terminally of a specific Val residue, as shown for bovine proBac5 and proBac7 [14] and porcine proprotegrin [15]. Cleavage after the corresponding Val in the prophenin precursor (Val-128, [16]) would generate a 97-residue long polypeptide, which indicates that the isolated prophenin-1 and -2 are generated either by multiple processing steps or are formed without cleavage after Val-128. The identification of the 80-residue prophenin-2 suggests that proteolytic release from the precursor is mediated by cleavage between the dibasic Arg-Arg pair at positions 144–145 and Gln-146, and the latter subsequently cyclises and forms the N-terminal pyroGlu of prophenin-2. Cleavage after paired basic residues is a common feature in the biosynthesis of peptide hormones [17], although hormones and other bioactive peptides are also generated by cleavages at other sites [18]. The 79-residue prophenin-1 is likely formed from the initially liberated 80-residue prophenin by aminopeptidase activity, similar to the proposed processing of the human cathelicidin antibacterial peptide LL-37 [19].

In addition to full-length prophenins a fragment consisting of prophenin-1 residues 62–79, i.e. covering the C-terminal 18 residues was found in all preparations analyzed. This fragment has not been found previously. Our results also showed that this fragment is active against both *B. megaterium* Bm11 and *E. coli* D21, although only at comparatively high concentrations. Since this fragment was found in organic extracts of homogenized whole lung and saline-extracted minced lungs now prepared, as well as in Curosurf, it appears likely that it constitutes a naturally occurring fragment. Harwig and coworkers found that a fragment of prophenin covering position 42 to nearby the C-terminus, generated by trypsin cleavage, retained partial microbicidal activity against *E. coli*, while fragments covering positions 1–41 were inactive [12]. Together these results suggest that the C-terminal part of prophenin may be of importance for its antibacterial function.

Prophenin copurifies with surfactant phospholipids and the polypeptides SP-B and SP-C during extraction and Lipidex-5000 chromatography in organic solvents. Prophenin and the C-terminal fragment were also detected in Curosurf, a surfactant preparation which is obtained from minced porcine lungs by organic extraction and Lipidex-5000 chromatography [20] and is used for treatment of RDS in premature infants. It is thus conceivable that prophenin is associated with pulmonary surfactant in vivo, but it can not be excluded that prophenin associates with surfactant components after being released from other sources upon tissue homogenization. The latter possibility would be compatible with the fact that prophenin could not be detected in bronchoalveolar lavage surfactant. However, prophenin was detected also when minced lung tissue was extracted with saline and centrifuged in order to remove cells prior to organic extraction, although at a lower yield relative to SP-B compared to after organic extraction of whole tissue homogenates. These results may suggest that prophenin now found is not derived only from leukocytes. Notably, LL-37 is present in bronchoalveolar lavage fluid [21] and lung epithelium [22]. Furthermore, short acidic peptides with antibacterial activity have been found in ovine surfactant [23]. Maybe antibacterial peptides associated with surfactant form a constitutive line of defence against invading pathogens.

Prophenin now isolated in organic solvents exhibits antibacterial activity against the Gram-positive bacterium *B. megaterium*, provided that sodium chloride is omitted from the growth medium. Unlike prophenin isolated from leukocytes by size exclusion chromatography and RP-HPLC in aqueous solvents [12] no activity against *E. coli* was found for prophenin-1 and -2 isolated in organic solvents. Whether this difference is caused by the different bacterial strains used and/or the different isolation procedures cannot be unequivocally determined from the present data. However, also the C-terminal 18-residue fragment, which was purified in aqueous media, is more active against *B. megaterium* than *E. coli*. This argues against the isolation in organic solvents as a primary cause of the observed differences. Pulmonary surfactant preparations from porcine lungs (Curosurf) exhibit bacteriostatic effects against group B streptococci [6]. It is tempting to speculate that this activity, at least in part, is caused by the presence of antibacterially active prophenin and/or fragments thereof in such preparations.

In summary we have found that prophenin, a cathelicidin peptide with antibacterial properties, is present in organic extracts of porcine lung and in a surfactant preparation which is used for treatment of RDS in premature infants. Additionally, a previously unknown 18-residue fragment of prophenin was found in the same preparations. We also explain the existence of two prophenin isoforms as being caused by the presence (prophenin-2) and absence (prophenin-1), respectively, of an N-terminal pyroglutamic acid.

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