Reverse-phase HPLC of the hydrophobic pulmonary surfactant proteins: detection of a surfactant protein C isoform containing N^{ε}-palmitoyl-lysine

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A reverse-phase HPLC protocol for analysis of strictly hydrophobic peptides and proteins was developed. Peptide aggregation is minimized by using only 25–40 % water in methanol or ethanol as initial solvents and subsequent elution with a gradient of propan-2-ol. Analysis of the pulmonary surfactant-associated proteins B (SP-B) and C (SP-C) with this method reveals several features. (1) SP-B and SP-C retain their secondary structures and separate by about 15 min over a 40 min gradient. SP-B is more hydrophilic than SP-C, which in turn behaves chromatographically like palmitoyl-ethyl ester. (2) SP-C exhibits isoforms additional to the major form characterized previously, which contains two thioester-linked palmitoyl groups. The isoforms now observed contain one or three palmitoyl moieties and constitute together 15–20 % of the major form. The tripalmitoylated species contains a palmitoyl group linked to the ϵ-amino group of Lys-11, as concluded from the elution position, MS and amino acid sequence analysis. The tripalmitoylated form increases relative to the dipalmitoylated form on incubation of SP-C in a phospholipid environment. An N^{ϵ}-bound palmitoyl moiety constitutes a third mode of fatty acyl modification of proteins, in addition to the established N^{α}-bound myristoyl groups and S-bound palmitoyl chains. (3) The dimeric structure of SP-B, lacking covalent modifications, is confirmed by MS detection of the dimer. No SP-B isoforms were detected. (4) Denatured, non-helical SP-C can be distinguished chromatographically from the native α-helical peptide. (5) HPLC of SP-C at 60–75 °C reveals an isoform containing an extra 14 Da moiety compared with the main form. This is concluded to arise from inadvertent methyl esterification of the C-terminal carboxy group. In conclusion, this HPLC method affords a sensitive means of assessing modifications and conformations of SP-B or SP-C in different disease states and before functional studies. It might also prove useful for analysis of other strictly hydrophobic polypeptides.

INTRODUCTION

Pulmonary surfactant consists of phospholipids, especially 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, mixed with small amounts of specific proteins, of which surfactant proteins B (SP-B) and C (SP-C) are strictly hydrophobic. The main function of surfactant is to lower the surface tension of the alveolar air/liquid interface and thereby to reduce the otherwise significant tendency of the alveoli to collapse at low lung volumes [1]. A decreased level of pulmonary surfactant in premature babies is associated with respiratory distress syndrome, which can be effectively treated by airway instillation of surfactant preparations containing phospholipids and 1–2 % (w/w) SP-B and SP-C [1]. A vital importance of SP-B is further evident from the findings that hereditary deficiency of proSP-B causes fatal respiratory distress in early infancy [2], and that mice with the proSP-B gene knocked out exhibit lethal respiratory failure at birth [3]. Interestingly, a lack of proSP-B is associated with abnormal processing of proSP-C [3,4]. The functional importance of SP-B and SP-C is thus well documented, but their detailed mechanistic properties are still largely unknown and clear-cut evidence for distinct functions of SP-B and SP-C is missing. This is probably partly due to difficulties in handling these unusually non-polar and complex polypeptides. Both proteins are soluble in organic solvents such as ethanol or chloroform/methanol and most analyses of SP-B and SP-C have been performed on proteins isolated by gel-exclusion chromatography on Sephadex LH-60 in chloroform/methanol/0.1 M HCl [5] as the final purification step.

SP-B is a 79-residue cationic protein with three intrachain disulphide bonds and one interchain disulphide bond forming a homodimer [6]. SP-B is mainly α-helical and the locations of four amphipathic helices of SP-B and homologous proteins have been predicted [7,8]. SP-B seems to be located in the superficial parts of phospholipid bilayers and to lack transmembrane segments [7–9]. Different mechanisms of action of SP-B have been suggested [10–13].

SP-C is a 35-residue lipopeptide with an unusually high content of valine residues, see [6]. Two-thirds of the protein consists of a continuous stretch of hydrophobic residues that form the central part of a transmembrane α-helix 37 Å (3.7 nm) long [14–17]. The hydrophobic character of SP-C is further increased by covalent attachment of palmitoyl chains via *S*-ester bonds to the side chains of Cys-5 and Cys-6 [18–20]. The overall stoichiometry between palmitoyl groups and cysteine residues in pig SP-C was found to be 0.8–0.9 [18]. Canine SP-C contains only one Cys residue and is monopalmitoylated [19]. SP-C is considered to be involved in acceleration of the spread of phospholipids to the air/water interface [6], and molecular models for its action have been put forward [17,21,22].

In general, protein palmitoylation is thought to occur post-translationally on Cys residues, thus forming *S*-esters. Many of the known palmitoylated proteins are integral membrane proteins and do not seem to require the palmitoyl groups for membrane association. The uncommon C_{14} fatty acid myristic acid is co-translationally linked via an amide bond to the α-amino group of specific proteins, many of which are cytosolic without the myristoyl group. The precise functional implications of protein

Abbreviations used: MALDI–TOF, matrix-assisted laser desorption ionization–time-of-flight; SP-B, surfactant protein B; SP-C, surfactant protein C; TFA, trifluoroacetic acid.

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palmitoylation and myristoylation are in several cases not known and the modified proteins exhibit a wide range of functional properties [23]. Both enzymic [24] and non-enzymic [25] addition of the palmitoyl groups to polypeptides have been suggested. Palmitoyl groups have been shown to exhibit a shorter half-life than the polypeptide chain to which they are attached [26], indicating a dynamic turnover of the fatty acyl chains. In SP-C the *S*-ester-linked palmitoyl groups have been proposed to be of functional significance under certain circumstances [21], and of absolute importance for full surface activity under several different conditions [27,28]. Conclusions from studies of depalmitoylated SP-C are complicated by the fact that the peptide exhibits a lower content of helical structure in the absence of *S*-ester-linked palmitoyl groups ([14,27,29] but see also [21]).

SP-B and SP-C and related synthetic peptides are not easily purified by reverse-phase HPLC elution systems containing significant amounts of water, judging from the appearance of broad peaks and correspondingly low resolution [29,30]. This is presumably caused by denaturation and concomitant non-specific aggregation of the non-polar peptides. We now introduce a reverse-phase HPLC separation method, where the water content of the initial solvents, methanol or ethanol, is minimized to just that necessary for retention of the peptides on the column, and a linear gradient of propan-2-ol is used for elution. This method yields rapid and high-resolution separation of SP-B and SP-C, which is conformation-specific for SP-C. In addition, it permits the isolation of an isoform of SP-C, constituting about 4 % of the total SP-C amount. This novel isoform has three palmitoyl groups: in addition to the two *S*-ester-linked palmitoyl groups already known, it contains a third palmitoyl moiety linked via an amide bond to the ϵ-amino group of Lys-11.

EXPERIMENTAL

Isolation of SP-B and SP-C from pulmonary phospholipids

SP-B and SP-C samples used for HPLC analysis were isolated from pig pulmonary phospholipids by chromatography on Sephadex LH-60 in chloroform/methanol (1:1, v/v) containing 5 % 0.1 M HCl [5]. The pulmonary phospholipids were obtained from minced lung tissue or from material collected by bronchoalveolar lavage. The SP-B and SP-C fractions thus obtained are virtually free of contaminating lipids and proteins [5,18] and are commonly used in structure/function analyses [7,13,15,31–33]. All protein samples were stored at −20 °C. Surfactant preparations, containing 1–2 % (w/w) of SP-B/SP-C and about 98 % (w/w) phospholipids, were isolated by chromatography on Lipidex 5000 in ethylene dichloride/methanol (1:4, v/v). The preparations obtained were identical with those used for clinical treatment of respiratory distress syndrome [34], and were, for control experiments, incubated at 4 or 25 °C and at a phospholipid concentration of 80 mg/ml for 12 months, before isolation of SP-B and SP-C by Sephadex LH-60 chromatography and subsequent HPLC analysis.

A non-palmitoylated analogue of SP-C, with the cysteine residues at positions 5 and 6 replaced with serine [SP-C (SS)], was synthesized by using *t*-butoxycarbonyl chemistry and isolated by extraction with formic acid [29].

Reverse-phase HPLC

Reverse-phase HPLC was performed with a Waters instrument and C_{18} (TSK-ODS, LKB, Sweden), C_4 or C_8 (Vydac, Hesperia, CA, U.S.A.) columns, all with 5 μm particle size and dimensions of 4.6 mm × 250 mm. For separations at room temperature the solvent flow was 0.7 ml/min. At elevated temperatures a flow rate of 1.0 ml/min was used. Ethanol (99.5 %, v/v) or methanol with 20–40 % (v/v) water and 0.1 % trifluoroacetic acid (TFA) served as initial mobile phases, and peptides were eluted from the column with a linear gradient of propan-2-ol/0.1 % TFA. Because of the solvent mixing time the actual concentration of propan-2-ol on the column was delayed by approx. 12 min compared with the gradients shown in the figures. The absorbances of the eluate at 214 and 280 nm were monitored continuously and relevant peaks were collected manually. Palmitic acid, palmitoyl-ethyl ester and cholesterol (Sigma, St. Louis, MO, U.S.A.), SP-B and SP-C were dissolved in ethanol before HPLC analysis. SP-B, SP-C and mixtures thereof can be stored in ethanol for at least several weeks without detectable precipitation. Peak areas were estimated by multiplication of the peak height with the width at half peak height.

CD spectroscopy

In the cases where enough material was recovered, CD spectroscopy was performed on peptide fractions from the HPLC without changes in solvent composition or volume. CD analysis of the tripalmitoylated SP-C isoform was performed in 10 mM dodecylphosphocholine/50 mM sodium phosphate buffer (pH 6.0) [29]. The spectra were recorded between 260 and 184 nm at room temperature with a JASCO-720 instrument at a scan speed of 20 nm/min, a response time of 2 s, a band width of 1.0 nm and a resolution of 2 data points/nm. The residual molar ellipticity in degrees·cm^2·dmol^{-1} was calculated after determination of the peptide concentration by amino acid analysis.

MS

Approx. 20–50 ng of SP-C or SP-B from the HPLC fractions were applied to and dried on a stainless-steel grid together with a matrix of approx. 5 μg of α-cyano-4-hydroxycinnamic acid (for analysis of SP-C samples) or sinnapinic acid (for analysis of SP-B samples) dissolved in 70 % acetonitrile. Molecular masses of the proteins were determined with a matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) instrument (Finnigan MAT Lasermat 2000), used in the positive-ion mode. Calibration was performed with pig insulin (m/z 5778.6) or vasoactive intestinal polypeptide (m/z 3326.8).

Amino acid sequence analysis

Sequence analysis was performed with an Applied Biosystems 477A gas-phase sequencer with an on-line 120A HPLC system for detection of phenylthiohydantoin derivatives. Amino acid compositions were determined with an AlphaPlus amino acid analyzer (LKB, Uppsala, Sweden) after hydrolysis of protein samples in 6 M HCl/0.1 % phenol for 24 h at 110 °C in evacuated tubes.

RESULTS

Reverse-phase HPLC separation of SP-B and SP-C

SP-B and SP-C were separated in 40 min on a C_{18} column with a linear gradient of propan-2-ol in 60 % aqueous methanol (Figure 1). For retardation of SP-C on the column, the water content had to be at least 10 %. Separation on C_4 and C_8 columns gave qualitatively similar results provided that the polarity of the initial solvent phase was slightly increased, but the resolution obtained was inferior to that with the C_{18} column. Both the SP-B and SP-C peaks were symmetrical, eluting in approx. 2 and 1.2 ml respectively. Significantly broader peaks

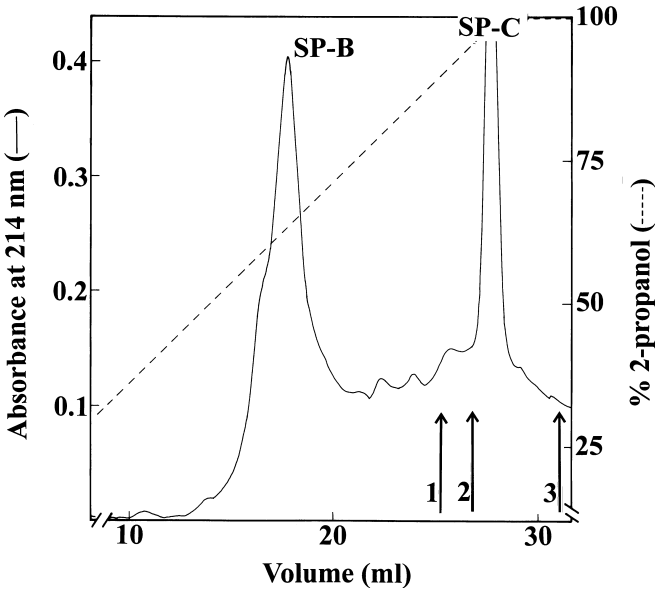

Figure 1 Reverse-phase HPLC of SP-B and SP-C

SP-B (3 nmol of dimer) and SP-C (6 nmol) dissolved and injected in ethanol were separated at room temperature on a C_{18} column (4.6 mm × 250 mm, 5 μm particles) with a linear gradient of 0–100% propan-2-ol/0.1% TFA in 60% aqueous methanol/0.1% TFA over 40 min at a flow rate of 0.7 ml/min. The elution positions of palmitic acid (1) ethyl palmitate (2) and cholesterol (3) under identical conditions are indicated by arrows.

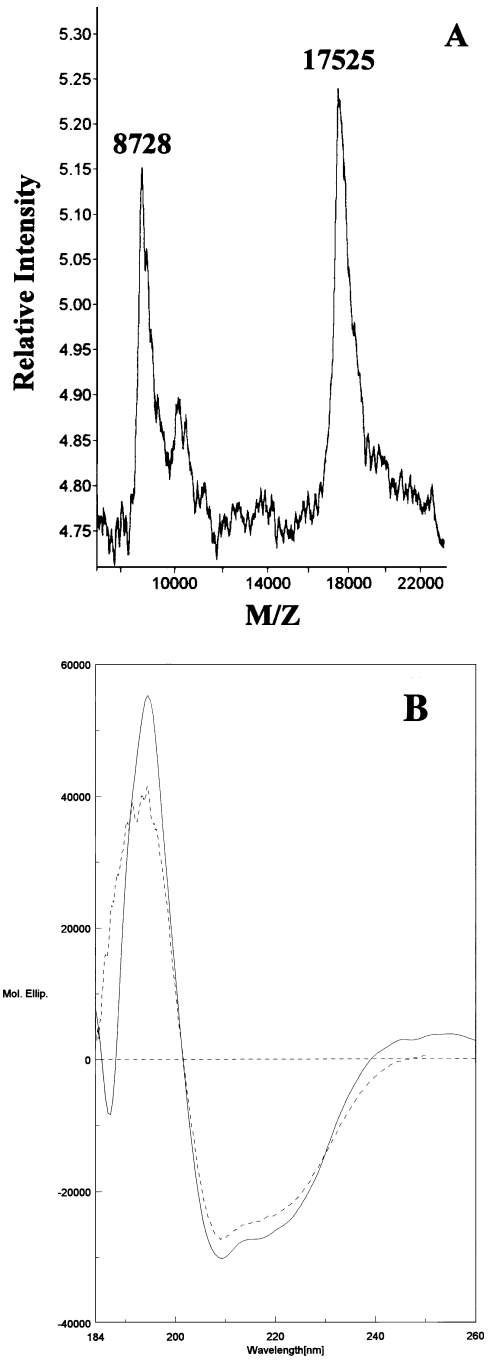

Figure 2 Characterization of SP-B purified by reverse-phase HPLC

(**A**) MALDI–TOF mass spectrum of approx. 20 ng of SP-B isolated as described for Figure 1. (**B**) CD spectrum of SP-B after reverse-phase HPLC recorded in the chromatography solvents (solid line) or after Sephadex LH-60 purification only (dotted line). Abbreviation: mol. ellip., residual molar ellipticity in degrees · cm^2 · $dmol^{-1}$.

were obtained by using more polar initial solvent systems, indicating conformational heterogeneity of the sample (see below), and giving correspondingly low resolution [29,30]. SP-B and SP-C were separated by about 15 min, with SP-B eluting at 62% propan-2-ol and SP-C at 99% propan-2-ol. SP-C is considerably more non-polar than SP-B and eluted between palmitic acid and cholesterol, close to the elution position of ethyl palmitate (Figure 1). The recovery of the peptides was more than 80%.

The SP-B fraction (Figure 1) was analysed by MALDI–TOF MS. This yielded two peaks, one at m/z 8728 and one at m/z 17525 (Figure 2A). Because SP-B on SDS/PAGE under non-reducing conditions exhibits mainly one band at approx. 18 kDa, it is concluded that the m/z 17525 peak represents a singly charged SP-B dimer, whereas the peak at m/z 8728 corresponds to a doubly charged dimer. Coexistence of small amounts of monomeric polypeptides cannot be ruled out. The experimental data give an average molecular mass of the dimer of 17490 Da, which is in reasonable agreement with the mass calculated from the amino acid sequence (17446 Da) considering that the broad MS peaks preclude exact assignments of peak centra and that methylated forms of SP-B might exist (see below). This accuracy of the mass determination of SP-B, including detection of the singly charged dimer, has not been achieved before [18,31]. The data confirm that SP-B lacks covalently linked fatty acyl chains and is a dimeric molecule with two polypeptide chains of 79 residues. CD spectroscopy of SP-B after HPLC gave a spectrum similar to that of SP-B purified by Sephadex LH-60 only [8] (Figure 2B). This confirms that no significant change in SP-B secondary structure occurred during reverse-phase HPLC.

High-resolution separation of SP-C reveals a tripalmitoylated isoform

A few minor peaks were eluted in the vicinity of the main SP-C peak (Figure 1). Isolation of SP-C with 75% ethanol as initial buffer gave a better resolution of these non-polar peptides and disclosed three peaks in addition to the major peak (Figure 3). Two overlapping peaks were eluted at 71–74% propan-2-ol,

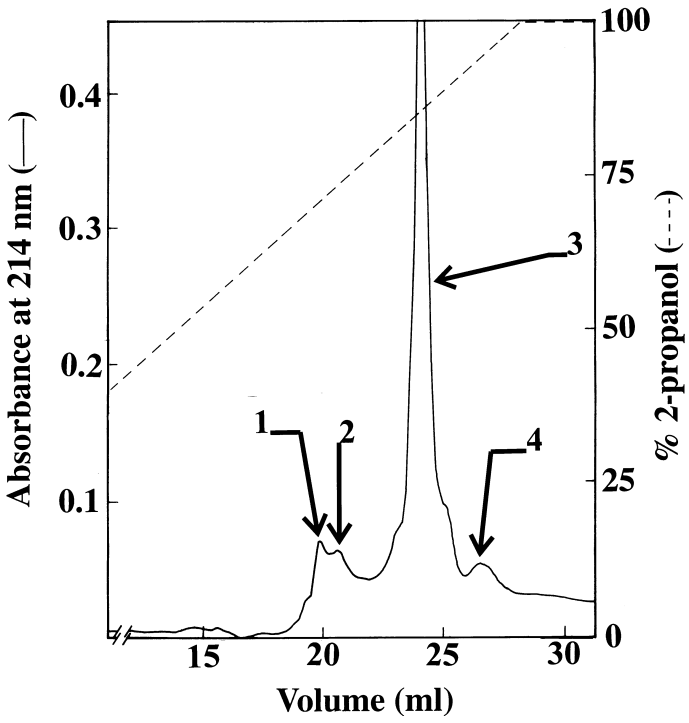

Figure 3 HPLC analysis of SP-C

SP-C isolated by Sephadex LH-60 chromatography was resolved by reverse-phase HPLC. Conditions were as described for Figure 1 except that the initial solvent used was 75% ethanol/0.1% TFA. The peaks were analysed by MS (peaks 1–4, Figure 4), amino acid sequence analysis (peak 4, Table 1) and CD spectroscopy (peak 3, Figure 9).

before the main peak eluting at 86 % propan-2-ol, and a fourth peak was eluted at 95 % propan-2-ol. MS showed that the main peak corresponded to dipalmitoylated SP-C (experimental molecular mass 4217 Da, calculated molecular mass 4186 Da; see below concerning the discrepancy between calculated and experimental molecular masses) and that the two early peaks both contained monopalmitoylated SP-C having lost approx. 240 Da compared with the main species (Figures 4A and 4B). The molecular mass of a palmitoyl moiety is 238 Da. Thus SP-C isolated by Sephadex LH-60 chromatography contains monopalmitoylated peptides at a proportion of approx. 10–15 % of the main dipalmitoylated molecule. No significant amounts of non-palmitoylated peptide (eluting at approx. 50 % propan-2-ol in control experiments with depalmitoylated SP-C) were observed. The two early peaks might correspond to peptides palmitoylated at position 5 or 6. The more polar character of the monopalmitoylated species compared with the dipalmitoylated form is reflected in their earlier elution position. The late peak should then correspond to an unexpected peptide that was even more hydrophobic than dipalmitoylated SP-C. MS of this peak showed an ion at m/z 4453, which is 236 Da higher than the dipalmitoylated peptide (Figure 4C). This suggests that it contains three covalently linked palmitoyl groups.

The amino acid sequence of pig SP-C [19] leaves only two possible sites of covalent attachment of a third palmitoyl moiety, i.e. the α-amino group of Leu-1 or the ϵ-amino group of Lys-11. Edman chemistry-based sequence analysis of the late-eluting peak shows that the α-amino group is not modified to any significant degree, because the N-terminal amino acid was recovered with the expected yield. In contrast, at position 11 only approx. 1 % of the expected amounts of Lys were detected (Table 1). No other residue was detected at this position and amino acid analysis of the tripalmitoylated peptide showed the expected content of Lys. This confirms that the late-eluting peak contains a modified lysine residue at position 11. From the HPLC, MS and sequence analysis we conclude that the peptide that was eluted after the main SP-C peak is a tripalmitoylated SP-C variant with palmitoyl chains covalently linked to the side chains of Cys-5, Cys-6 and Lys-11, i.e. two palmitoyl groups are linked via *S*-esters and one is linked via an amide bond (Figure 5). The amount of the tripalmitoylated species increases slightly relative to the dipalmitoylated form on storage of SP-C in phospholipids. In SP-C freshly purified from pulmonary phospholipids the tripalmitoylated peptide constituted 3.6 ± 2.2 % (means $\pm$ S.D.) ($n = 11$) of the main form, which increased to 6.3 ± 1.6 % ($n = 4$) after 12 months of storage of SP-C in surfactant phospholipids at 4 or 25 °C. In contrast with the effect of incubation in phospholipids, the relative amount of the tripalmitoylated peptide did not change significantly after several weeks of incubation in ethanol of SP-C isolated by Sephadex LH-60 chromatography. Further control experiments showed that incubation of a mixture of 1–2 % (w/w) SP-B and SP-C and 98 % (w/w) phospholipids with Lipidex 5000 resin in ethylene dichloride/methanol (1:4, v/v) at 37 °C for 1 day did not affect the relative amounts of tripalmitoylated SP-C, nor did it cause any detectable formation of SP-B isoforms. Finally, in SP-C isolated from bronchoalveolar lavage the tripalmitoylated form constituted 3.1 % of the main form. It therefore seems unlikely that tripalmitoylated SP-C is formed as a result of blood contamination or during the isolation procedure.

CD spectroscopy of tripalmitoylated SP-C in DPC micelles gave a spectrum with minima at 208 and 222 nm, similar to that of dipalmitoylated SP-C. The secondary structure of tripalmitoylated SP-C is thus not significantly different from that of the dipalmitoylated form.

Analysis of SP-C at elevated temperature reveals a methylated form

Reverse-phase HPLC purification of SP-C at 70 °C split the main peak into two components, whereas the isoforms remained as single peaks (Figure 6). MS of the two major peaks (Figure 7) showed that the early peak exhibited a molecular mass closely corresponding to the calculated mass of dipalmitoylated SP-C (4183 Da determined, compared with 4186 Da calculated). The late peak was composed of two species with m/z 4196 and 4218, most probably representing protonated and sodium ion adducts respectively. The ion at m/z 4196 can be explained by the existence of SP-C peptides carrying a C-terminal methyl ester, which would give a 14 Da increase in molecular mass. Methylated SP-C has been detected before [20]. The existence of methylated peptides, which exhibit better ionization properties because of the loss of a negative charge, and of sodium adducts in mixture with the non-esterified peptide explains why mass values of unresolved SP-C have previously [18,19] been approx. 25–30 mass units above the calculated values.

Conformation-specific separation of SP-C

The polyvalyl α-helix in native SP-C unfolds into a non-helical and aggregated structure under acidic conditions and this seems also to increase in more polar solvents [29,35]. The α-helical secondary structure of SP-C and related synthetic analogues is necessary for optimal functional properties, non-helical SP-C

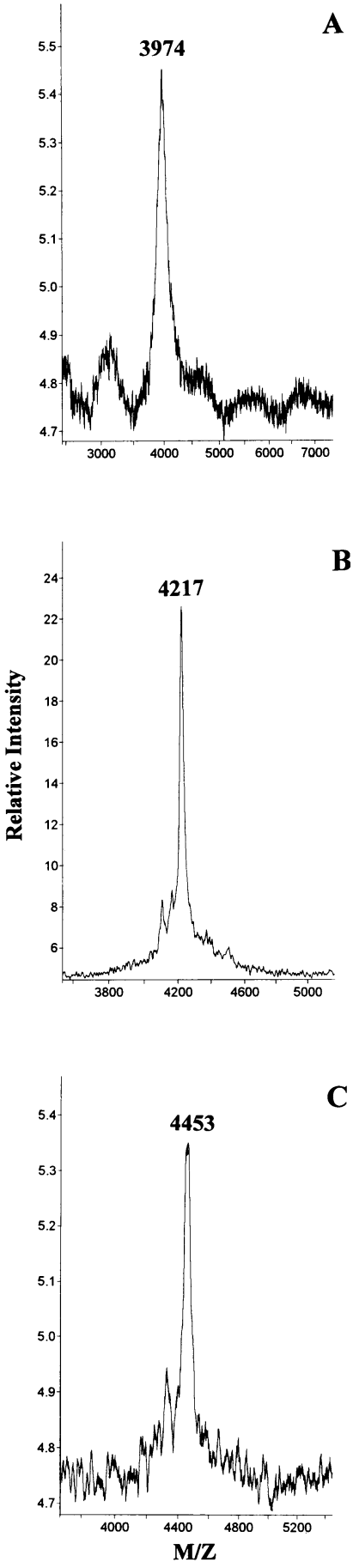

Figure 4 Mass spectra of SP-C isoforms separated by HPLC

MALDI–TOF mass spectra of peptides eluted under the peaks in Figure 3. (**A**) peak 1; (**B**) peak 3; (**C**) peak 4 in Figure 3. MS of peak 2 in Figure 3 gave results essentially identical with those of peak 1.

Table 1 Sequence analysis of tripalmitoylated SP-C

Summary of results from amino acid sequence analysis of the late-eluting peak from HPLC of SP-C (corresponding to peak 4 in Figure 3). The recovery of phenylthiohydantoin (PTH)-Lys in cycle 11 of two different sequencer runs was approx. 1% of that expected. The initial yield was 37%, in the range generally encountered. The recovery of PTH-Arg (positions 2 and 12) is generally low, approx. 10% of the average recovery for most PTH amino acids. The palmitoylated Cys residues at positions 5 and 6 were not detected as PTH derivatives.

Cycle	Amino acid	Average amount of PTH amino acid (pmol)
1	Leu	220
2	Arg	17
3	Ile	223
4	Pro	108
5	Cys	–
6	Cys	–
7	Pro	27
8	Val	26
9	Asn	26
10	Leu	17
11	Lys	0.2
12	Arg	1.4
13	Leu	25
14	Leu	27
15	Val	20

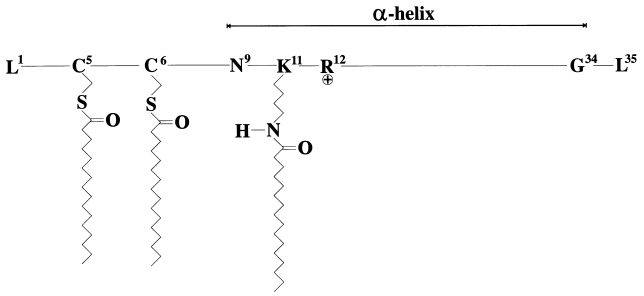

Figure 5 Schematic diagram of tripalmitoylated SP-C

The N- and C-termini, the strictly conserved Arg-12 [19] and the localization of the α-helix of SP-C [16] are indicated. The side chains of Cys-5 and Cys-6 with *S*-ester-linked palmitoyl groups and of Lys-11 with an amide-linked palmitoyl group are shown.

being essentially inactive [29]. Reverse-phase HPLC can be used to differentiate between helical and non-helical SP-C. A synthetic non-palmitoylated analogue of SP-C, which had been treated with formic acid and was non-helical [29], was eluted as a much broader peak than the native peptide (Figure 8). The broad peak is in agreement with a heterogeneous mixture of aggregated peptides. CD spectroscopy of this peptide (Figure 9) confirmed that the broad elution profile corresponds to a β-sheet-like structure, with a CD minimum at approx. 215 nm. In contrast, the sharp main peak of Figure 3 was predominantly helical and exhibited a CD spectrum with minima at 208 and 222 nm (Figure

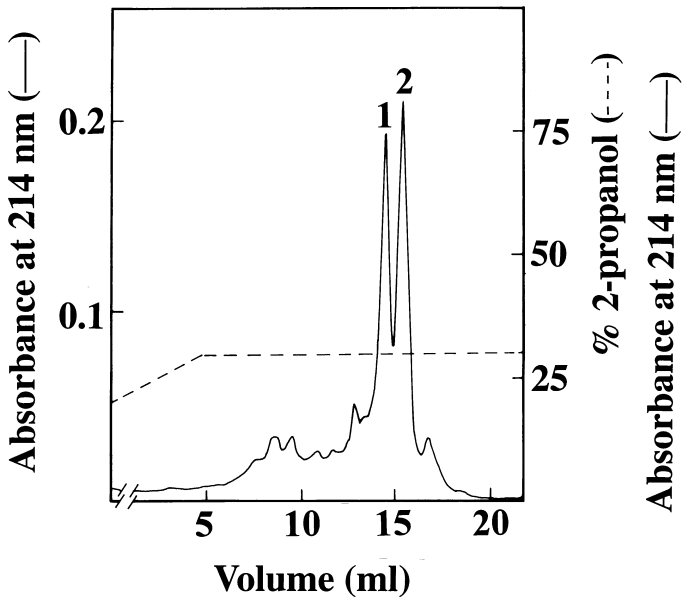

Figure 6 HPLC analysis of SP-C at 70 °C

A linear gradient of 0–30% propan-2-ol/0.1% TFA for 15 min followed by isocratic elution with 30% propan-2-ol/0.1% TFA was used. The flow was 1.0 ml/min and the initial solvent contained 75% ethanol/0.1% TFA.

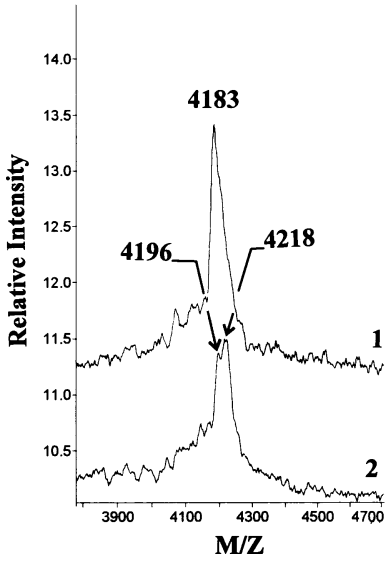

Figure 7 Mass spectra of isoforms of dipalmitoylated SP-C

MALDI–TOF mass spectra of the two major peaks in Figure 6. The upper trace corresponds to the early-eluting peak (labelled 1 in Figure 6) and the lower trace corresponds to the later-eluting peak (2 in Figure 6).

9), which is very similar to the CD spectrum of SP-C purified by Sephadex LH-60 chromatography [17,29]. This shows that the present HPLC method did not affect the α-helical secondary structure of SP-C (or SP-B; see Figure 2B) to any significant degree. HPLC thus affords a way of ensuring the structural integrity of natural or synthetic surfactant peptides before functional studies are undertaken.

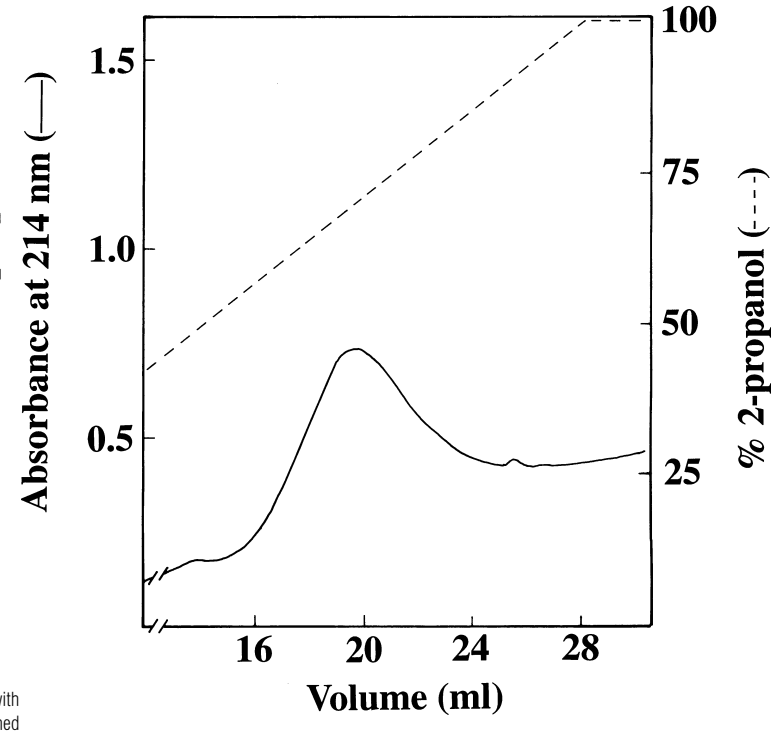

Figure 8 HPLC analysis of a non-helical SP-C analogue

A synthetic non-palmitoylated SP-C analogue, SP-C (SS), which is in a β-sheet-like extended conformation after treatment with formic acid [29], was dissolved and injected in ethanol/formic acid (4:1, v/v). Elution conditions were identical with those used for Figure 3.

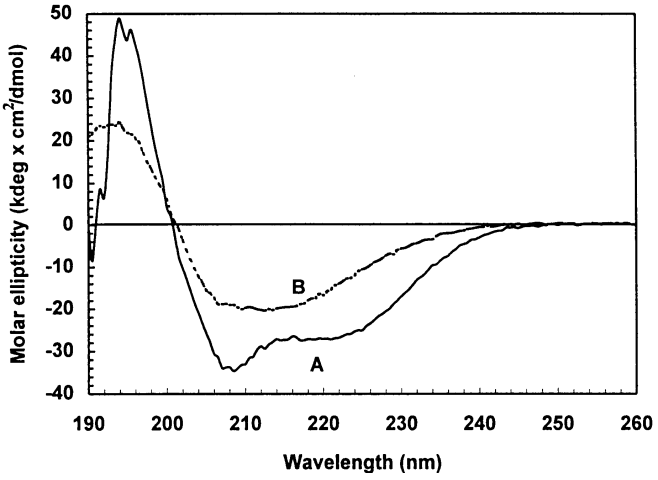

Figure 9 CD spectra of native SP-C and a denatured SP-C analogue after HPLC purification

CD spectra were recorded in the HPLC elution solvent mixture of dipalmitoylated SP-C (trace A) (peak 3 of Figure 3) and denatured synthetic non-palmitoylated SP-C (ss) (trace B) (Figure 8).

DISCUSSION

The present method, using different alcohol/water mixtures to elute hydrophobic polypeptides, resolves isoforms of the lipopeptide SP-C and distinguishes between different peptide conforma-

tions. Previous HPLC methods used to purify SP-B and SP-C [20,29–31,36] exhibit lower resolution. Short-chain alcohols are attractive solvents for reverse-phase HPLC of hydrophobic peptides because their more polar character than that of e.g. terahydrofuran and acetonitrile [37] requires less water for peptide retention on the column. Excessive water contents have an aggregating and denaturing effect on non-polar peptides such as SP-C [35].

Reverse-phase HPLC under the present conditions preserves the secondary structure of SP-B and SP-C and readily separates these non-polar polypeptides in approx. 40 min (Figures 1, 2B and 9). The resolution, time of separation, and peak shapes compare favourably with those of Sephadex LH-60 HPLC for quantification of SP-B and SP-C [38]. Reverse-phase HPLC at 70 °C further increases the resolution, allowing the separation of dipalmitoylated SP-C with or without a methyl ester (Figure 6). N-terminally truncated forms of SP-C [6,18] were not resolved even at high temperature. The apparently better separation of methylated and non-methylated peptides than of truncated SP-C forms is probably due to the difference in charge in the former case. SP-B purified with our system was confirmed by MALDI–TOF analysis (Figure 2) to be homodimeric and to lack covalently linked fatty acyl chains [18,31].

Reverse-phase HPLC analysis of SP-C purified by Sephadex LH-60 chromatography reveals the presence of several isoforms (Figures 3 and 4), in contrast to the situation with SP-B. The existence of monopalmitoylated species can be rationalized from the labile nature of the *S*-ester bonds that link the two palmitoyl moieties to the polypeptide chain in the major form of SP-C [18], resulting in spontaneous depalmitoylation. However, the existence of monopalmitoylated peptides from incomplete palmitoylation of pro-SP-C *in vivo* cannot be excluded. The fact that non-palmitoylated forms were not detected can probably be explained by their expectedly low abundance. Provided that a loss of palmitoyl chains occurs to the same extent in monopalmitoylated SP-C as in the dipalmitoylated peptide, one would expect 1–2% non-palmitoylated peptide. This small amount is likely to escape detection, considering that chemically depalmitoylated SP-C exhibits decreased α-helical content [14,27,29], with concomitant HPLC peak broadening (see Figure 8), and lower yield on Sephadex LH-60 chromatography (T. Curstedt, unpublished work). The palmitoyl/Cys molar ratio of 0.8–0.9 found in SP-C isolated by Sephadex LH-60 chromatography [18] is in perfect agreement with our present results.

The tripalmitoylated isoform now found (Figure 5), constituting approx. 4% of the main form, was unexpected. Peaks eluting later than the main peak have been observed but not further analysed previously [30,31]. It is thus likely that tripalmitoylated SP-C generally occurs, but that relatively low abundance and insufficient chromatographic resolution have previously prevented its detection. Erythrocyte membranes contain a fatty acyl transferase that is capable of transferring palmitoyl groups from palmitoyl-CoA to endogenous erythrocyte membrane proteins and to exogenous acceptor proteins [39]. Inadvertent palmitoylation of SP-C Lys-11 owing to blood contamination during purification is unlikely to occur for three reasons. First, transfer of palmitoyl groups by the erythrocyte fatty acyl transferase is enhanced 10–15-fold by removing *S*-ester-linked fatty acyl chains from the acceptor proteins before incubation with the erythrocytes, indicating that Cys residues are specifically palmitoylated. Secondly, if palmitoylation of SP-C Lys-11 were catalysed by an erythrocyte fatty acyl transferase one would expect to find palmitoylated isoforms of SP-B as well. However, SP-B (with six Lys per dimer, which furthermore are expected to be more solvent-exposed than Lys-11 in SP-C; see further below) apparently does not exhibit palmitoylated isoforms. Thirdly, the same amount of tripalmitoylated SP-C is found in surfactant obtained by bronchoalveolar lavage, where contamination from blood is expected to be minor. Tripalmitoylated SP-C increases relative to the dipalmitoylated form when SP-C is stored in phospholipids, indicating a formation in pulmonary surfactant.

The size of the SP-C α-helix (positions 9–34) and its all-aliphatic part (positions 13–28) is perfectly adapted to interact, in a transmembrane orientation, with a fluid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine bilayer and its fatty acyl chain part respectively [17]. In this orientation Lys-11 is located within the bilayer and in the vicinity of the surrounding phospholipid headgroups [17]. This location, together with the increase in the tripalmitoylated form on storage in phospholipids, supports the notion that that tripalmitoylated SP-C is formed by a non-enzymic O → N-acyl shift of a palmitoyl chain from a phospholipid to the ϵ-amino group of Lys-11. N^{ϵ}-Palmitoyl-lysine in a transmembrane protein can thus be regarded as an 'environmental' adduct analogous to glycation of albumin by blood glucose [40]. The ϵ-amino group of a lysine residue is apparently a preferred site of specific adduct formation both in albumin by glucose [40] and in $NADP^+$-dependent prostaglandin dehydrogenase by pyruvate [41].

Almost exclusively Cys residues have been shown to carry covalently linked palmitoyl chains, whereas amide-bound fatty acyl chains have mainly been detected as myristoyl groups linked to α-amino groups [23,42,43]. To the best of our knowledge adenylate cyclase toxin from *Bordetella pertussis* is, until now, the only known example of a protein containing a palmitoylated Lys, which in that case is formed by a specific enzyme [44]. Lysine residues are generally over-represented in terminal regions of transmembrane helices [45]. For example, proteolipid protein, a hydrophobic integral myelin protein, is proposed to contain at least three lysine residues in transmembrane helices located so that they are situated within or in close vicinity of the phospholipid bilayer [46]. In contrast with SP-C, SP-B, which does not seem to form palmitoyl-lysine-containing isoforms, probably lacks transmembrane segments but contains four amphipathic helices where the non-polar faces interact with the lipid acyl-chains and the polar parts of the helices (including the six Lys residues per dimer) face the extramembraneous environment [8]. Lys residues might thus require a location close to the phospholipid carbonyl groups to be modified via O → N acyl transfer. It seems possible that other integral membrane proteins than SP-C, but less probably membrane proteins lacking transmembrane helices, might contain N^{ϵ}-palmitoyl-lysine. This might have been overlooked because chemical methods used for depalmitoylation, such as treatment with alkaline or reducing agents, cleaves only *O*- and/or *S*-esters, but not amide bonds, whereas fatty acyl-modified proteins are usually detected after brief (hours) exposure to radioactive fatty acids [42,43].

Lys-11 is strictly conserved in all SP-C amino acid sequences determined [19], indicating functional importance, and the palmitoyl-lysine of *Bordetella pertussis* toxin is essential for its biological activities, including haemolytic activity [44]. Neutralization of the positive charges of Lys-11 and Arg-12 in SP-C impaired the binding of phospholipid vesicles to a preformed monolayer, and thereby reduced the insertion of phospholipids into the monolayer [30]. Tripalmitoylated SP-C is likely to exhibit different functional properties from those of the dipalmitoylated form.

The shape of the HPLC peaks permits native α-helical SP-C to be distinguished from denatured and aggregated peptides in β-sheet-like conformation (Figures 3, 8 and 9). This might prove valuable for checking the conformation of SP-C (and SP-B)

before subjecting them to functional studies. The activity of at least SP-C is strictly dependent on an α-helical conformation [29]. In surfactant from alveolar proteinosis patients, non-palmitoylated and monopalmitoylated SP-C variants are increased relative to the dipalmitoylated form [31]. This suggests that the occurrence of isoforms of SP-C might correlate with different lung diseases and might be involved in pathophysiological mechanisms. The resolution of the present HPLC system should facilitate the further screening of hydrophobic pulmonary surfactant proteins in disease states, with respect to the occurrence of both covalent SP-C isoforms and peptides with altered secondary structures.

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