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# Synthesis and Structural Characterization of Human-identical Lung Surfactant SP-C Protein

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Abstract: An efficient synthesis for human-identical lung surfactant protein SP-C is described with a semi-automated solid phase synthesizer using Fmoc chemistry. Double coupling and acetic anhydride capping procedures were employed for synthetic cycles within the highly hydrophobic C-terminal domain of SP-C. Isolation of the protein was performed by mild cleavage and deprotection conditions and subsequent HPLC purification yielding a highly homogeneous protein as established by sequence determination, electrospray, plasma desorption and MALDI mass spectrometry. A general method has been employed for the preparation of Cys-palmitoylated protein by using temporary Cys(tButhio) protection, *in situ* deprotection with $\beta$-mercaptoethanol and selective palmitoylation of resin-bound SP-C. The mild synthesis and isolation conditions provide SP-C with a high $\alpha$-helical content, comparable to that of the natural SP-C, as assessed by CD spectra. Furthermore, first biophysical data indicate a surfactant activity comparable to that of the natural protein. © 1998 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: lung surfactant; human-identical SP-C protein; solid phase synthesis; palmitoylation; structural characterization

## INTRODUCTION

Pulmonary surfactant is a complex mixture of phospholipids and c. 5–8% surfactant-associated proteins (SAP). SAPs are secreted by alveolar pneumocytes and exert important biological functions, notably by reducing the surface tension and controlling the dynamics of the lipid monolayer at the air-water interface [1–3]. Among the surfactant proteins known thus far [2], the hydrophobic SP-C protein has recently found considerable interest as a constituent of surfactant preparations for the therapy of neonatal respiratory distress syndrome (NRDS) [4,5]. The structure of the mature human SP-C, formed by proteolytic processing from a c. 21 kDa precursor protein [2], has been shown to consist of 34/35 amino acid residues with an extremely hydrophobic valine- and leucine-rich C-terminal sequence, and an *N*-terminal part in which two adjacent Cys residues are thioester-palmitoylated [6,7]. Primary structures of SP-C proteins from several mammalian species have been elucidated by a combination of protein-chemical and mass spectrometric methods [6–10]. For all natural proteins, the presence of characteristic Cys-palmitoylated residues and an $\alpha$-helical C-terminal domain has

Abbreviations: SAP, surfactant associated protein; NRDS, neonatal respiratory distress syndrome; PyBOP, 1-benzotriazolyloxytripyrrolidino-phosphoniumhexafluorophosphate; DIPEA, *N*-ethyldiisopropylamine; DMAP, 4-dimethylaminopyridine; PamCl, palmitoyl chloride; PD, $^{252}$Cf-plasma desorption; ESI, electrospray ionisation; MALDI, matrix assisted laser desorption/ionisation; ΔCS, collimator/skimmer potential; $\beta$-MeSH, $\beta$-mercaptoethanol.

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been established [2]; the latter extremely hydrophobic sequence is thought to be responsible for the formation of specific dimeric complexes [10]. The $\alpha$-helical palmitoylated structure appears to be important for both the biological activity and solution stability of SP-C, whereas transition to a $\beta$-sheet conformation has been reported to yield oligomeric aggregates [11,12]. The precise physiological role of the palmitoylation has not yet been elucidated, but partial or complete lack of palmitate residues have been observed in pathophysiologically altered SP-C proteins [9].

Recombinant human and bovine SP-C polypeptides have been successfully expressed in *E. coli* from which the palmitoylated protein was obtained by subsequent Cys-S-palmitoylation with suitably activated palmitoyl-derivatives [11,13]. By contrast, chemical syntheses using standard SPPS procedures have met with considerable difficulties due to the extreme hydrophobicity of the C-terminal sequence. Syntheses of non-palmitoylated SP-C and several sequence analogues have been reported with relatively high purity, i.e. sequence homogeneity using standard *t*-butyloxycarbonyl (t-BOC) and Fmoc protection and deprotection procedures [14–16]. However, in a previous study the non-palmitoylated protein revealed a considerably lower α-helix content compared to natural SP-C which has been ascribed to the lack of palmitate residues and corresponding lower stability in solution [15]. Furthermore, a correlation between the decreased $\alpha$-helical content and diminished surfactant activity has been reported [3,15].

In the present study, we describe the synthesis of human-identical SP-C with a semi-automated peptide synthesizer which provided convenient features for double coupling and capping procedures to eliminate truncated sequences. Using mild procedures for Fmoc/trifluoracetic acid protection/deprotection and final purification, SP-C with high primary structure homogeneity and an $\alpha$-helicity comparable to that of the natural protein was obtained, as assessed by sequence determination, mass spectrometry and circular dichroism (CD) data. The complete, palmitoylated SP-C protein was prepared by integration of Fmoc-Cys(t-Buthio)-OH in the SPPS protocol using reductive cleavage and palmitoylation of the resin-bound protein [13]. As shown by structural and first biophysical studies, this procedure provided human-identical SP-C of high purity as a useful model protein suitable for detailed biochemical pharmacological studies.

## MATERIALS AND METHODS

### Synthesis and Purification Procedure

All syntheses were carried out with an ABIMED EPS 221 semi-automated peptide synthesizer with the Fmoc protection strategy [13]. Carboxy-terminal $N_\alpha$-protected leucine was linked with 4-hydroxymethylphenoxy acetic acid to a PEG-graft polymer (Nova Syn® TGA resin, Novabiochem). Other $N_\alpha$-Fmoc-protected amino acids (*S*-trityl-Cys, *N*-trityl-His, $\varepsilon$-*t*-Boc-Lys, 2,2,5,7,8-pentamethylchroman-6-sulphonyl-Arg (Pmc-Arg) and PyBOP as activator were obtained from Novabiochem (Bad Soden, Germany). Reagents and solvents were obtained at highest available purity from Aldrich (Steinheim, Germany) (*N*-methylmorpholine; *N*-ethyl-diisopropylamine; piperidine; triethylsilane). DMF was obtained from Merck (Darmstadt, Germany) and trifluoracetic acid (TFA) and acetic anhydride were purchased from Riedel de Haen (Seelze, Germany).

Chain elongation on the Fmoc-Leu-pre-loaded resin (0.1 mmol) was performed for 24 cycles of the 34 amino acid SP-C sequence according to a protocol, which included Fmoc-deprotection by treatment with 20% piperidine in DMF (5 min), followed by double coupling (20 min) with a $2 \times 5$ M excess of PyBOP-activated Fmoc-amino acid. Subsequent capping of unreacted $N_\alpha$-amino groups was carried out by addition of a 10 M excess of acetic anhydride. The further chain elongation (residues (1–10) of the complete SP-C sequence) was performed with a 0.025 mmol aliquot of resin-bound polypeptide, using the same protocol but only a single PyBOP-activated coupling step for 45 min. All Fmoc-deprotection steps were monitored with a JASCO UV/DEC- 100-II spectrophotometer. After the final Fmoc-deprotection cycle, simultaneous cleavage of the polypeptide from the resin and side chain deprotection was carried out by treatment with TFA for 120 min at 20°C, using triethylsilane as scavenger. A half-aliquot of the peptide was precipitated with *t*-butyl-methylether (8 ml) according to the manufacturer's standard protocol. The remaining TFA solution was extracted with 5 ml chloroform: methanol: 0.1 M HCl (47.5:47.5:5), and the organic phase separated at $-20$°C.

### Synthesis of Palmitoylated SP-C

Palmitoylation of resin-bound SP-C was carried out with 10 μmol of the Cys(tButhio)-protected polypeptide resin which was reduced by adding 10 ml of a

$\beta$-mercaptoethanol:DMF mixture (1:1), and reaction of the resulting suspension for 24 h at 20°C under an $N_2$ stream. The suspension was then filtered, the resin washed with 10 ml each of dichloromethane, ethanol and ether and used directly for palmitoylation.

Palmitoylation was carried out with the resin-bound protein (10 μmol) by addition of a solution of 1 mg dimethylaminopyridine (DMAP) in 0.5 ml absolute pyridine, followed by addition of 120 μl palmitoyl chloride (PamCl) in 0.5 ml absolute THF. The resulting suspension was reacted under gentle shaking for 1 h at 20°C, while developing a slight orange colouring. The reaction mixture was then filtered, the resin washed with 20 ml dichloromethane and 10 ml each of ethanol and ether, and the palmitoylated protein cleaved with a solution of TFA:triethylsilane:water (90:5:5) as described above. Depalmitoylation of the palmitoylated SP-C was carried out as previously described [9,17] by reacting an SP-C solution in 2-propanol:$H_2O$ (4:1) with a 1 M solution of DTT in 1 M $NH_4HCO_3$ (pH 8). The reaction product was purified by HPLC on a C4-column (see below) and characterized by MALDI-MS [13].

### HPLC Characterization

The ether-precipitated crude product was redissolved in $CHCl_3$:MeOH:0.1 M HCl (47.5:47.5:5), centrifuged at 13000 rpm and the clear supernatant analyzed on a Waters-Millipore (Milford, USA) HPLC instrument equipped with a 6000A/M-45 gradient pump system, a 490E multi-wavelength detector and an 820-maxima data transfer interface for data acquisition and evaluation. Separation was performed on a Grom (Herrenberg, Germany) protein-C4 column (250 × 4 mm; 7 μM) with 0.1% TFA in water (solvent A) and 0.1% TFA in 2-propanol (solvent B), using a linear gradient 40% B to 98% B in 40 min at a flow rate of 0.8 ml/min. The column was thermostated at 40°C.

### Primary Structure Analysis

For amino acid determination the ether-precipitated SP-C was dissolved in 2-propanol: 1% HAc (4:1) and hydrolyzed for 24 h at 150°C in TFA: 12 M HCl (1:2) containing 1% phenol. HPLC analysis of OPA-pre-column derivatized amino acids was performed on a Merck-Hitachi work station with an F1050 fluorescence detector. N-terminal Edman sequence analysis was performed with a Knauer (Berlin, Germany) model 477 gas phase sequencer.

### Mass Spectrometry

$^{252}$Cf-PD mass spectra were obtained on a BIO-ION 20K time-of- flight spectrometer (BIO-ION™, Uppsala, Sweden) as described previously [9,10]. Samples of approximately 2 μg dissolved in $CHCl_3$: MeOH: 0.1 M HCl (47.5:47.5:5) were adsorbed on a nitro-cellulose sample target [18] and spectra recorded for $5 \times 10^5$ counts at 16 kV acceleration voltage and evaluated for fragment ions. MALDI-MS analysis was performed on a Bruker-Biflex (Bruker-Franzen, Bremen, Germany) linear TOF mass spectrometer equipped with a UV-nitrogen laser (337 nm) and a sun-spark-1 workstation and Bruker-XMass software for data acquisition. A modified thin layer method [19] was used for sample preparation, by first crystallization of a saturated solution of 4-hydroxy-$\alpha$-cyanocinnamic acid (HCCA) as matrix in acetonitrile: 0.1% TFA (2:1) on the sample target; the protein solution was then added on top of the crystal layer resulting in eventual co-crystallization of sample and matrix.

Electrospray-MS (ESI-MS) analyses were performed with a Vestec-201A quadrupole mass spectrometer (Vestec, Houston, Texas), equipped with a previously described ESI interface without employing a countercurrent gas [20]. The temperature at the ionization region was kept at approximately 42°C for all measurements. The voltage difference between collimator and skimmer (declustering potential, ΔCS) was varied from 20 V at incomplete 'desolvation conditions' for analysis of non-covalent complexes and solvent adducts, to 200 V which induces covalent fragmentation [20]; other instrumental conditions were as previously described [21]. Protein solutions of 0.1 μg/μl (approx. 25 μM) were prepared in 2-propanol: 1% HAc (4:1). Sample delivery to the needle tip was performed with a Harvard-44 (Franklin, MA, USA) microinfusion pump through a 50 μm (i.d.) fused silica capillary, at a flow rate of 2–3 μl/min.

### Circular Dichroism Spectroscopy

CD spectra were recorded on a JASCO J-500 C spectropolarimeter at 20°C with a scan rate of 1 nm/min, using cuvettes of 0.05 cm path length, with sample solutions of 50 μM in 2-propanol/1% HAc (4:1).

### SP-C Proteins

Recombinant SP-C protein sequences (non-palmitoylated, **2**) were prepared as fusion products with

chloramphenicol-acetyltransferase in *E. coli* and release of human-identical SP-C by hydroxylamine cleavage using an Asn-Gly linkage [10]. Selective Cys-palmitoylation of **2** to human-identical palmitoylated SP-C (**6**) was performed as previously described [10,17].

## RESULTS AND DISCUSSION

### Solid Phase Synthesis of Human-Identical SP-C

All reactions for the synthesis of human-identical SP-C were performed with a semi-automated peptide synthesizer using the Fmoc strategy for $N_{\alpha}$-protection. An optimized cycle protocol was developed by initial synthesis of the hydrophobic C-terminal sequence (10–34), and the single steps were carefully controlled by MALDI-MS analysis. The mass spectrometric characterization revealed the formation of significant amounts of truncated sequences especially during the C-terminal synthetic cycles, so that a modified protocol with acetic anhydride capping and double coupling was employed for chain prolongation. Furthermore, mild conditions for the deprotection and cleavage from the resin were used with regard to the palmitoylation of SP-C (see below). The ether precipitated crude product was used for final purification, since MS analyses showed the presence of some truncated products in the extraction phase.

The same general SPPS protocol was used for the synthesis of palmitoylated human-identical SP-C. For the palmitoylation Fmoc-Cys(tButhio)-OH was employed instead of the Trt-protected cysteine. The protecting group was removed from the resin-bound polypeptide by treatment with $\beta$-mercaptoethanol and Boc-Gly-OH was coupled as N-terminal amino acid in order to protect the N-terminus during the palmitoylation. With this procedure the Cys residues could be directly palmitoylated by palmitoyl chloride. Other protecting groups used were Pmc for Arg, Boc for Lys, and Trt for His.

The MALDI-MS analysis of the crude non-palmitoylated SP-C **1** from the TFA cleavage solution (Figure 1b) revealed a most abundant molecular ion $[M + H]^+$ together with some by-product ions. HPLC purification yielded a single homogeneous peak with a retention time identical to that of recombinant (non-palmitoylated) human SP-C as shown in Figure 1b. This protein showed high purity as ascertained by $^{252}$Cf-plasma desorption (PD-MS) and electrospray mass spectrometry (ESI-MS) which yielded complementary sequence determinations (Table 1 and Figure 3).

### Primary Structure and Homogeneity of SP-C

The primary structure and homogeneity of the HPLC-purified SP-C was ascertained by molecular weight determinations using MALDI-MS and PD-MS, partial mass spectrometric and Edman sequence analysis and amino acid determinations. The results are consistent with previous primary structure determinations of natural and recombinant SP-C proteins and confirm the high purity of the synthetic SP-C (Table 1) [10,11]. The PD mass spectrum provided partial sequence determinations of the hydrophobic C-terminal domain (residues (9–34)) by sequence-specific fragmentation as previously described for natural SP-C [6]. In addition, partial Edman sequence determination and fragment ions by ESI-MS provided complementary data

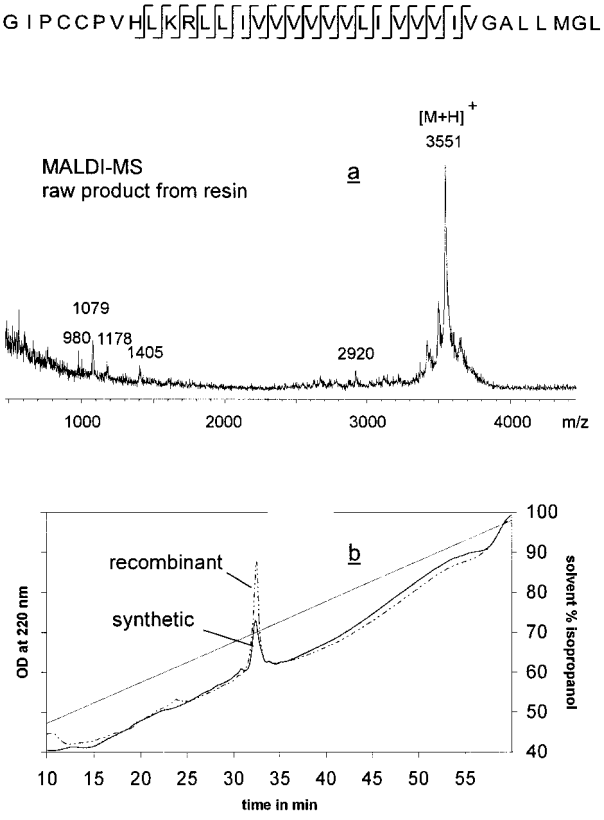


Figure 1 Purification and MALDI-MS characterization of human-identical non-palmitoylated SP-C. (a) MALDI-MS analysis of the raw product **1** from the TFA cleavage solution; (b), HPLC purification of synthetic SP-C **1** (solid line; ether precipitate) and recombinant non-palmitoylated SP-C **2**.

Table 1 Primary structure characterization of synthetic human SP-C by Edman sequencing, plasma desorption mass spectrometry (PD-MS), electrospray ionization mass spectrometry (ESI-MS) and amino acid analysis

| | SP-C sequence | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | | | | 5 | | | | | 10 | | | | | 15 | | | | | 20 | | | | | 25 | | | | | 30 | | | | | 34 | |
| | G | I | P | C | C | P | V | H | L | K | R | L | L | I | V | V | V | V | V | V | L | I | V | V | V | I | V | G | A | L | L | M | G | L |
| Edman sequencing | G | I | P | – | – | P | V | H | L | K | R | L | L | I | V | | | | | | | | | | | | | | | | | | | |
| PD-MS (fragmentation)[a] | | | | | | | | | L | K | R | L | L | I | V | V | V | V | V | V | L | I | V | V | V | I | | | | | | | | |
| ESI-MS (fragmentation)[b] | | | | | | | | | | | | | | | | V | V | V | V | L | I | V | V | V | I | V | G | A | L | L | M | G | L |
| amino acid analysis[c] | G (3.2/3) I (4.1/4) P(–) C (2.2/2) V (10.5/11) H (1.4/1) K (1.1/1) R (0.9/1) L (7.0/7) M (0.6/1) | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | | |

[a] $a_n$ – fragment ions for sequence position (8–26); Reference [9].
[b] $b_n^{2+}$ – fragment ions for sequence position (17–34); Figure 3b.
[c] Quantitation for amino acid residues (measured/calculated) in SP-C. No determination was made for proline residues.'

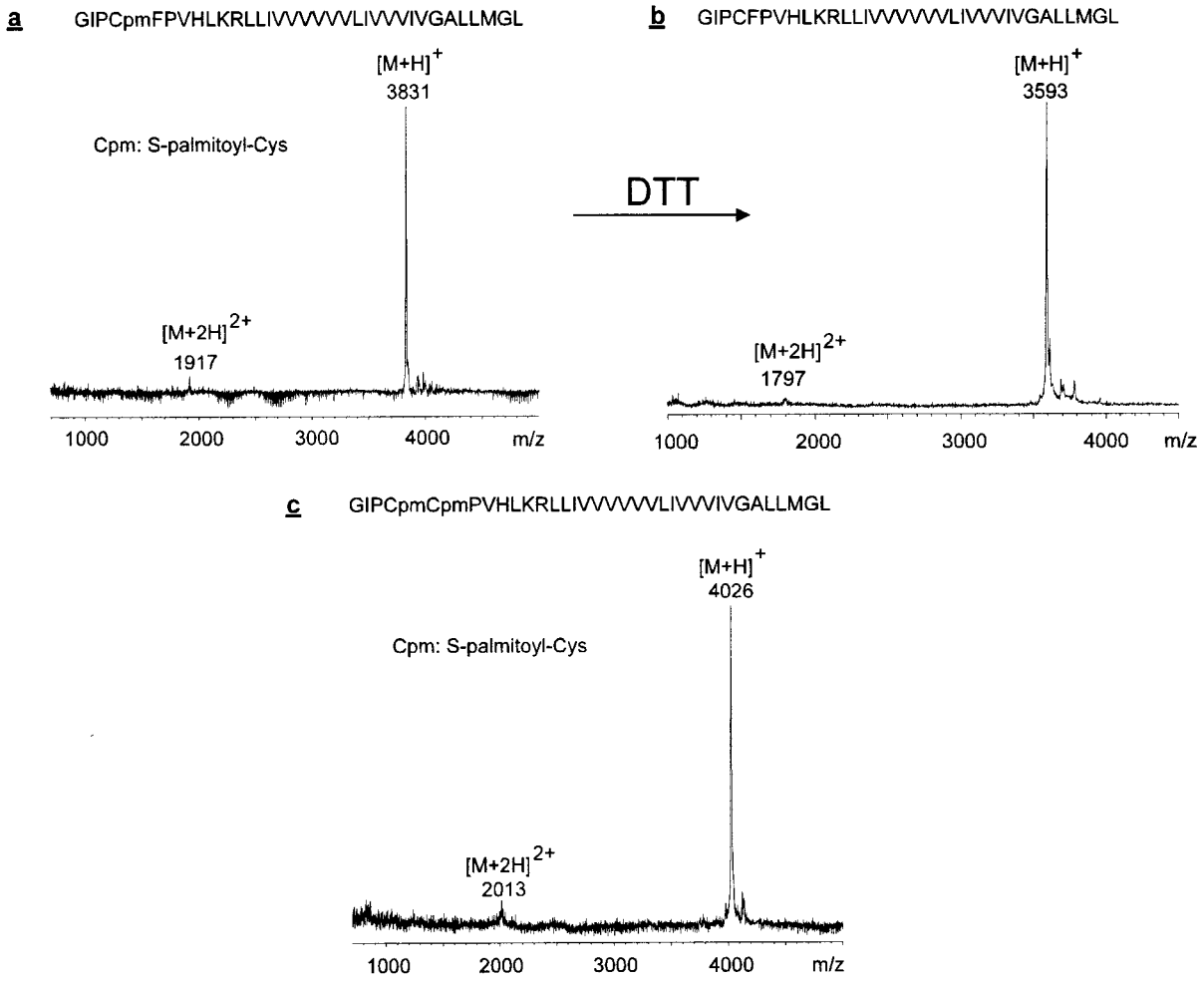


Figure 2 MALDI Mass spectra of (a), palmitoylated Cys[4](Pam) Phe[5]-SP-C (**4**); (b), depalmitoylated SP-C (**5**) obtained by DTT cleavage of **4**; (c), synthetic human-identical Cys[4,5]-palmitoylated SP-C 3.

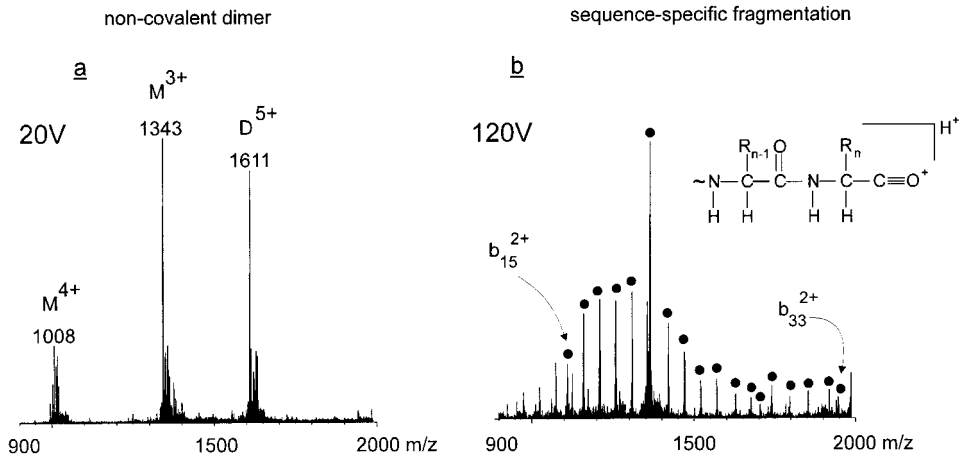


Figure 3 ESI Mass spectra of synthetic palmitoylated SP-C (3) at a counter electrode-skimmer potential ΔCS of 20 V (a), and ΔCS of 120 V (b).D, M in (a) denote protonated dimer and monomer ions with the charge states indicated. Other ions of low abundance are due to solvent adducts. Sequence-specific fragment ions $b_n$ in (b) are indicated by dots.

for the complete primary structure characterization. The amino acid determinations yielded close to theoretical values. Furthermore, the high optical purity of SP-C is indicated by the nearly identical secondary structure data with natural SP-C from CD (Figure 4) and 2D-NMR analyses, as previously reported by Wüthrich *et al.* [12]. Amino acid determinations by HPLC upon hydrolysis of a synthetic N-terminal peptide SP-C (1–14) prepared under identical conditions also showed an essentially racemization free peptide.

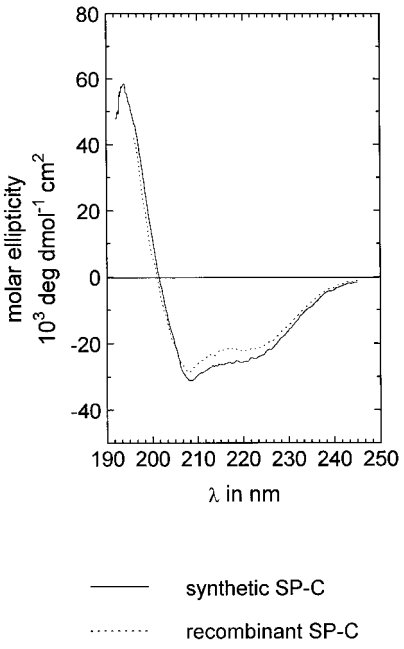


Figure 4 CD-spectra of synthetic non-palmitoylated SP-C **1** (—) and recombinant SP-C **2** (···).

## Cys-Palmitoylation of Resin-Bound SP-C

The palmitoylation of the complete, protected SP-C on the resin by reductive removal of the Cys-(tButhio) group was found to be an efficient procedure for the preparation of the human-identical protein [13]. In contrast previous chemical syntheses of SP-C proteins have been carried out in non-palmitoylated form [14–16,23], and the palmitoylation of recombinant SP-C required highly selective *S*-palmitoylation conditions to prevent concomitant N-terminal acylation [9,17]. Mass spectrometric studies showed that complete Cys-palmitoylation was obtained with PamCl in DMF/NMM. However, at acidic reaction conditions the His-Trt group was found unstable resulting in partial His-palmitoylation. In order to provide slightly basic conditions (pH 7–8) comparable to Fmoc protection/deprotection, a reaction scheme was developed by palmitoylation in THF/pyridine with addition of 4-dimethylaminopyridine which has been successfully employed for acylation reactions of oligopeptides [22]. Using this procedure, selective and nearly quantitative Cys-*S*-palmitoylation was obtained with a 20-fold molar excess of PamCl (see Materials and Methods). The correct primary structures and *S*-palmitoylation were ascertained by mass spectrometric analysis of the cleavage products with DTT, as previously employed in the identification of Cys-palmitoylated natural SP-C proteins [3,6,9]. Corresponding MALDI-MS analyses of the

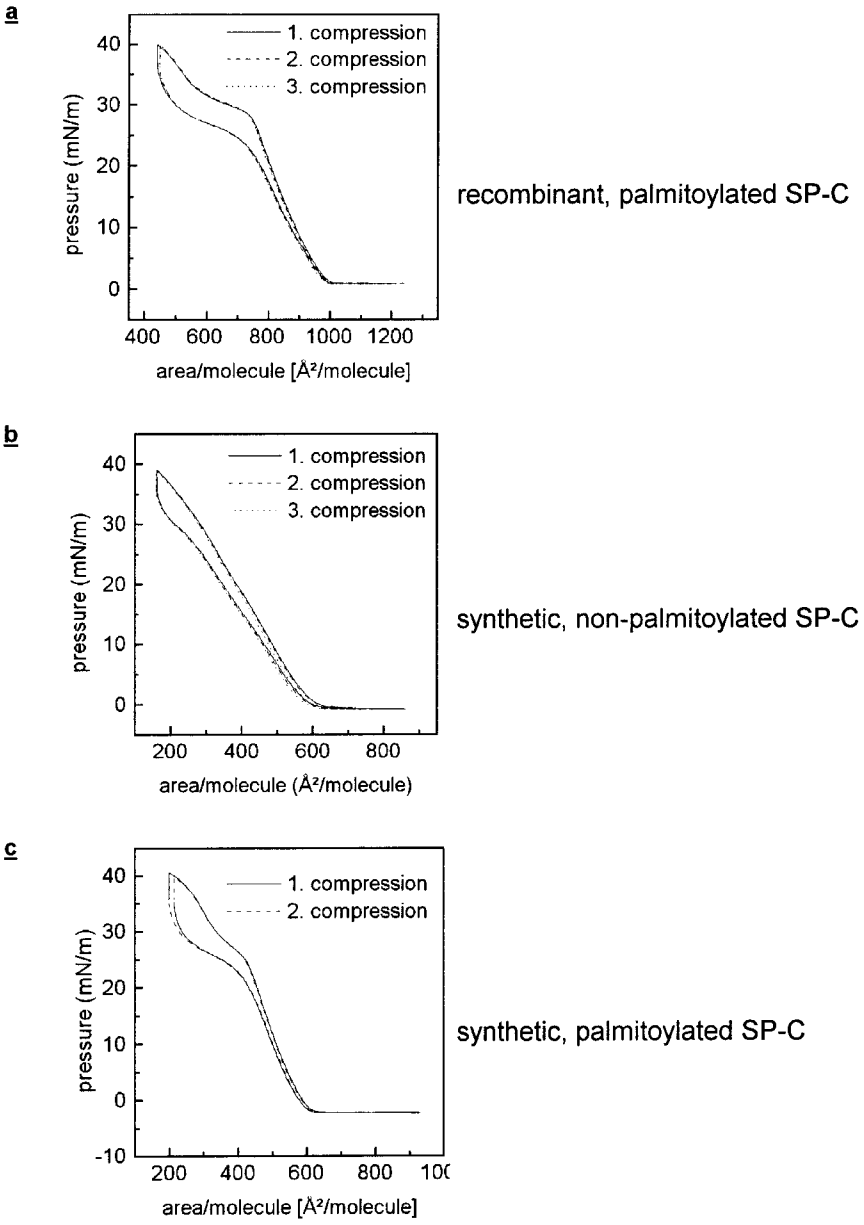


Figure 5 Surface area-pressure isotherms of (a) recombinant palmitoylated SP-C **6**; (b), synthetic non-palmitoylated SP-C **1**; (c), synthetic palmitoylated Cys[4](Pam)Phe[5]-SP-C **4**.

human-identical Cys[4], Cys[5]-palmitoylated SP-C **3** and the monopalmitoylated Cys[4], Phe[5]-SP-C **4** are illustrated in Figure 2. The reductive cleavage with DTT at slightly basic conditions (pH 8) resulted in the selective removal of the Pam group and corresponding mass shift of the molecular ion; by contrast the Cys-Pam group is stable at slightly acidic conditions (pH 5-7) [9,10].

Furthermore, the homogeneity of the palmitoylated synthetic SP-C proteins was confirmed by ESI-MS and H/D exchange experiments yielding identical results with the natural and recombinant palmitoylated proteins. The ESI mass spectrum of the palmitoylated SP-C **3** at mild desolvation conditions (ΔCS, 20 V) showed the presence of the specific non-covalent dimeric complex by the 5+ charged ion at $m/z$ 1611, as identified previously for natural SP-C proteins (Figure 3a) [10]. The identification of the SP-C dimer is in agreement with a native-like conformation and high $\alpha$-helix content as determined by CD spectra (see below). In contrast, at high repeller potential (ΔCS $\sim$ 120 V) the non-covalent dimer is

 

completely dissociated; at these harsh desolvation conditions covalent fragmentation provide sequence-specific fragment ions from which the partial sequence (15–33) was determined (Figure 3b).

### Secondary Structure Characterization and Surfactant Activity of Synthetic SP-C

The native-like solution conformation of the synthetic SP-C protein was ascertained by CD-spectra which showed a high extent of $\alpha$-helical conformation. CD-spectra of synthetic SP-C **1** and the recombinant SP-C **2** are compared in Figure 4 and revealed $\alpha$-helical contents of 66 and 59%, respectively. By contrast, a substantially lower $\alpha$-helical content has been previously reported for non-palmitoylated SP-C prepared by chemical synthesis when compared to the natural protein [15]. The high $\alpha$-helix content is also consistent with 2D-NMR data for natural SP-C [12] in comparison with synthetic SP-C (unpublished data), indicating a high optical purity. Furthermore, first biophysical studies for the synthetic and recombinant SP-C indicate comparable surfactant activities as shown by the surface area pressure diagrams (Figure 5). These results are also consistent with a native-like helical conformation which has been suggested previously as a pre-condition for the surfactant activity of SP-C [3,15]. In monolayer preparations, the synthetic and recombinant proteins showed a qualitatively comparable behaviour with reversible compression and expansion cycles [11]. However, it is interesting to note the differences between the palmitoylated and non-palmitoylated SP-C (Figure 5a, b). These differences and the role of the palmitate residues for the surfactant activity of SP-C are presently the subject of detailed studies.

## CONCLUSIONS

In the present study a highly efficient procedure has been developed for the solid phase synthesis of the complete human-identical lung surfactant SP-C protein. This approach employs a double coupling and capping strategy during the chain prolongation cycles in the critical, highly hydrophobic C-terminal domain of the SP-C sequence, thereby eliminating any significant formation of truncated sequences as observed in previous syntheses. The careful analytical control by mass spectrometry greatly facilitated a synthesis protocol for obtaining highly homogeneous material. The high purity and sequence homogeneity was confirmed by mass spectrometric analysis, sequence data and amino acid determinations. Key steps in the present synthesis are mild conditions of protection, deprotection, and the subsequent purification providing a fully soluble protein. The solution conformation was found comparable to both natural and recombinant SP-C by spectroscopic data. Moreover, the present method incorporates the direct on-resin palmitoylation of the cysteine residues using the temporary Cys(t-Buthio) protection. This procedure clearly provides advantages to the previously used palmitoylation of deprotected SP-C thus avoiding problem of concomitant *N*-acylation by activated Pam derivatives [11,23]. Hence, the present procedure provides authentic SP-C of high purity, a useful model for both biochemical and biophysical activity studies.

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