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Method of purification affects some interfacial properties of pulmonary surfactant proteins B and C and their mixtures with dipalmitoylphosphatidylcholine

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Abstract

Two methods were employed for preparation of lipid extracts from porcine lung surfactant. Pulmonary surfactant proteins SP-B and SP-C were isolated from the extracts using gel-exclusion chromatography on LH-60 with chloroform:methanol acidified with hydrochloric acid. Monolayers of pure SP-B or SP-C isolated from butanol lipid extracts spread at the air–water interface showed larger molecular areas than those determined in films of SP-B or SP-C isolated from chloroform surfactant extracts. Aqueous dispersions of dipalmitoylphosphatidylcholine (DPPC) supplemented with 2.5 and 5.0 wt% of SP-B or SP-C obtained from butanol extracts adsorbed faster to the air–water interface than their counterparts reconstituted with proteins isolated from chloroform extracts. Surface pressure–area characteristics of spread monolayers of DPPC plus SP-B or SP-C did not depend on the method of isolation of the proteins. The diagrams of the mean molecular areas vs. composition for the monolayers of DPPC plus SP-B or SP-C showed positive deviations from the additivity rule, independently of the procedure used for preparation of lipid extract surfactant. Matrix-assisted laser desorption/ionization spectrometry of the proteins isolated from different extraction solvents was consistent with some differences in the chemical compositions of SP-Bs. Butylation of SP-B during extraction of surfactant pellet with butanol may account for the differences observed in the molecular masses of SP-Bs isolated by the two different extraction protocols. The study suggests that the method of purification of SP-B and SP-C may modify their ability to enhance the

Abbreviations: DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; SP-B(but), pulmonary surfactant protein B isolated from butanol lipid extract of surfactant; SP-B(chl), pulmonary surfactant protein B isolated from chloroform lipid extract of surfactant; SP-C(but), pulmonary surfactant protein C isolated from butanol lipid extract of surfactant; SP-C(chl), pulmonary surfactant protein C isolated from chloroform lipid extract of surfactant; X_r, mole fraction of protein amino acid residues in protein–lipid monolayers determined from the amounts of protein and lipid initially spread in the monolayers. $X_r = N_r/(N_r + N_{DPPC})$, where N_r and N_{DPPC} are the numbers of amino acid residues of protein and molecules of DPPC that were spread initially in each monolayer; MALDI, matrix-assisted laser desorption/ionization; ATR, attenuated total reflectance

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adsorption rates of DPPC/protein mixtures, and this may be relevant to the formulation of protein-supplemented lipids for exogenous treatment of pulmonary surfactant insufficiency. © 1998 Elsevier Science B.V.

Keywords: Lung surfactant protein; Adsorption; Mass spectrometry

1. Introduction

Hydrophobic pulmonary surfactant proteins, SP-B and SP-C, are found in lipid extracts of natural surfactant. SP-B is a disulphide-linked dimer of 79 amino acid residue monomers which contains regions of amphipatic α-helix and has a net positive charge at physiological pH [1]. SP-C is an extremely hydrophobic 35-residue protein which has two palmitoyl groups bound through thiol esters of cysteines at positions 5 and 6 of its sequence. It has a 23-residue C-terminal α-helical portion and also has a net positive charge associated with residues near its N-terminal [2].

In-vitro studies have shown that the two hydrophobic pulmonary surfactant proteins modify interfacial properties of lung surfactant phospholipids, which are essential for the function of lung surfactant in vivo. SP-B and SP-C enhance the rate of adsorption of phospholipids from vesicles in the subphase into the air–water interface [3–5]. The hydrophobic surfactant proteins improve surface tension lowering ability of mixtures with synthetic phospholipids in oscillating bubbles [6,7] and promote the post-collapse respreading of DPPC monolayers [8]. The improvements in these surface behaviors generated by SP-B and SP-C in phospholipid mixtures have suggested that the two hydrophobic proteins have functional roles in the surface phenomena of alveoli during the breathing cycle. Combination of synthetic phospholipids, with SP-B and SP-C, has produced physiologically active surfactant substitutes [7,9].

This study compares the surface active properties of hydrophobic pulmonary surfactant proteins which have been prepared from lipid extracts of surfactant made by different methods. It examines the effect of the preparation method on the biophysical activities of SP-B and SP-C in promoting phospholipid adsorption to the air–water interface. Mass spectrometry evidence is presented about potential chemical modifications of the proteins by the extraction solvents. Inconsistencies in the biophysical activities of SP-B and SP-C isolated by different groups have been discussed [10] and some studies have shown effects of the protein purification method and the resulting extent of delipidation on the dynamic surface tension lowering abilities of the proteins [5,6].

2. Materials and methods

2.1. Materials

DPPC, purchased from Sigma (St. Louis, MO) showed a single spot on thin-layer chromatography on silica gel with a solvent system of chloroform–methanol–water (65 : 25 : 4, by volume). Sodium chloride, chloroform, methanol, 1-butanol, hydrochloric acid were A.C.S. grade or higher and were purchased from Fisher (Ottawa, ON). Sephadex LH-60 was from Pharmacia (Uppsala, Sweden). Water was deionized and doubly-distilled in glass, the second distillation being from a dilute potassium permanganate solution.

2.2. Purification of SP-B and SP-C

Lungs from freshly slaughtered pigs were lavaged two times with cold 0.15 M NaCl, followed by centrifugation at $800 \times g$ for 10 min. The supernatant was centrifuged at $8000 \times g$ for 60 min and the surfactant pellet obtained. Two different protocols were used to prepare lipid extracts of surfactant from surfactant pellets:

(i) According to the method of Haagsman et al. [11], part of the surfactant pellet was resuspended in a small volume of double distilled water and was injected into stirred distilled 1-butanol, and then stirred for 30 min at room temperature. After centrifugation at $3000 \times g$ for 30 min, the butanol phase (supernatant) containing surfactant lipids plus hydrophobic surfactant proteins was dried by rotary evaporation at 50°C and the residue was dissolved in chloroform:methanol (1 : 1, v/v) acidified with 2% by volume of 0.1 M HCl. Proteins

isolated from the lipid extracts of surfactant prepared by this method will be referred to as SP-B(but) and SP-C(but).

(ii) Another part of surfactant pellet was resuspended in double distilled water and was extracted with chloroform:methanol according to the method of Bligh and Dyer [12]. The chloroform layer of the biphasic system, containing the lipids and hydrophobic proteins was reduced to dryness by rotary evaporation at 37°C and the residue was dissolved in chloroform : methanol (1 : 1, v/v) acidified with 2% by volume of 0.1 M HCl. Proteins isolated from the lipid extracts prepared by this method will be called SP-B(chl) and SP-C(chl).

The hydrophobic surfactant proteins were chromatographically separated from the surfactant lipids and each other by gel-exclusion chromatography on Sephadex LH-60 (2.5 × 90 cm) using chloroform:methanol (1 : 1, v/v) acidified with 2% by volume of 0.1 M HCl [7]. The eluted fractions (4 ml) were monitored for protein by SDS-polyacrylamide gel electrophoresis (16% gels) [13] utilizing silver staining (Daiichi, Tokyo). Fractions which contained SP-B showed a single band at ca. 18 kDa under non-reducing conditions. SP-C fractions showed a single band at ca. 5 kDa under non-reducing conditions. The patterns of the gels for SP-B and SP-C were not affected by the method of preparation of the lipid extract.

SP-Bs and SP-Cs isolated by either method were stored in chloroform : methanol (1 : 1, v/v).

2.3. Analytical methods

Concentrations of SP-B and SP-C were determined using the fluorescamine method of Udenfriend et al. [14], a procedure which we have standardized with quantitative amino acid analysis [15]. Analysis of phosphorus [16,17] was performed on aliquots of 50 μg of SP-B or SP-C. DPPC was dissolved in chloroform and the concentrations of the solutions were determined by phosphorus assay [16,17].

2.4. Surface pressure–time measurements on adsorbed films

The adsorption kinetics of aqueous dispersions of DPPC plus either SP-B or SP-C were monitored in a Teflon dish ($r = 1.2$ cm) with a subphase volume of 5 ml. At time zero, desired volumes of lipid-protein dispersions were injected below the surface of the subphase through an injection septum, and surface tension was measured as a function of time using the Wilhelmy plate method and a computer-controlled transducer readout, TSAR 1 (Tech-Ser, CA). The subphase (0.15 M NaCl) was stirred continuously with a Teflon-coated stirring bar and a magnetic stirrer to minimize diffusion resistance. Subphase temperature was 35 ± 1°C.

To prepare the samples, chloroform solutions of DPPC were mixed with solutions of SP-B or SP-C in chloroform : methanol (1 : 1, v/v). Solvents were evaporated under nitrogen at room temperature. The lipid–protein films were hydrated with 0.15 M NaCl for one hour at 50° with intermittent mechanical vortexing. Under these experimental conditions, multilamellar liposomes are formed spontaneously [18].

2.5. Surface pressure–area measurements on spread monolayers

Measurements on spread monolayers of SP-B or SP-C alone or mixed with DPPC were performed in a Langmuir trough which employed a continuous Teflon ribbon barrier (Applied Imaging, Dukesway Team Valley, Gateshead; Tyne and Wear, England). Surface tension was measured by the Wilhelmy plate method with a roughened platinum plate. Lipid–protein mixtures for spreading were prepared by premixing of stock solutions of the components. The subphase was 0.15 M NaCl; the pH was adjusted to 7.0 immediately before each experiment with 0.1 M NaOH and it did not change by more than 1 pH unit during the surface pressure–area measurements. Subphase temperature was 20–22°C. After a 10-minute period allowed for solvent evaporation and film equilibration, monolayers were continuously compressed at a rate of 40 cm^2/min between a maximal area of 500 cm^2 and a final area of 100 cm^2.

The initial compositions of the spread protein–lipid monolayers were given by the weight % protein and by the mole fraction of protein amino acid residues, X_r. The mean area in the spread lipid–protein films, A_{mean} = trough area/($N_r + N_{DPPC}$), was defined as

mean area per ''residue'', where ''residue'' denoted an amino acid residue of the protein or a molecule of DPPC. For all calculations, molecular weights of 17 400 for SP-B (158 amino acid residues, dimeric form) and 4186 for SP-C (35 amino acid residues, dipalmitoylated) were used [2].

2.6. Matrix-assisted laser desorption / ionization mass spectrometry.

MALDI mass spectrometry was performed on a VG TofSpec spectrometer (Manchester, UK). SP-B or SP-C was dissolved in chloroform:methanol (3 : 1, v/v) to a final concentration of 100 pmol/μl. The samples were then mixed in a 1 : 1 ratio with a saturated solution of α-cyano-4-hydroxy cinnamic acid (using the same solvent as sample) and 2 μl of the solution was placed on the stainless steel sample support. External calibration was used with either recombinant Eglin-C or trypsinogen. Typically ca. 20 laser shots were averaged.

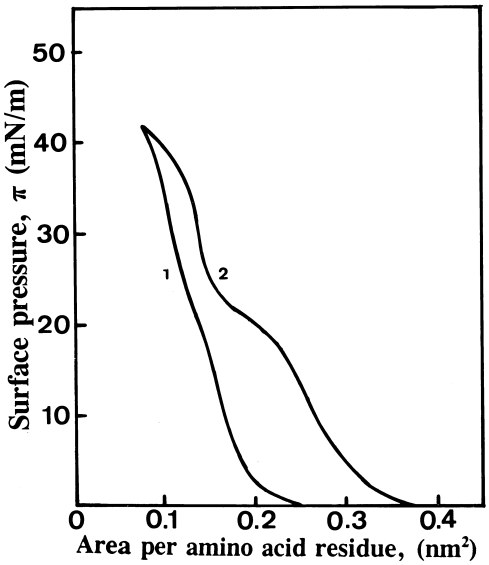


Fig. 1. Surface pressure versus area per amino acid residue for spread monolayers of (1) SP-B(chl) and (2) SP-B(but). The curves are average of measurements on four different protein preparations (S.D. at various points were within $\pm 0.02\,nm^2$/amino acid residue).

3. Results

Fig. 1 shows the compressional isotherms of surface pressure versus area per amino acid residue for monolayers of SP-B(chl) – curve 1, and SP-B(but) – curve 2. Independently of the organic solvent used for extraction of surfactant pellet, each isotherm exhibited an inflection point at ca. 20 mN/m. The origin of this inflection is not known. Spread films of synthetic polypeptides [19,20] have shown an inflection or, in some cases, a flat plateau and this property of the films has been interpreted in terms of a transformation from a monolayer to a bilayer [19], or in terms of changes in the degree of packing density of the α-helices in the monolayer plane [20]. Comparison of the two curves in Fig. 1 shows that, at a given π, SP-B(but) occupied a larger molecular area in the spread monolayers than did SP-B(chl); the difference in the molecular areas being more pronounced at the low pressure, whereas at collapse surface pressure ($\pi \approx 42$ mN/m) the two curves converged. The area at lift-off pressure for monolayers of SP-B(but), $0.38\,nm^2$/amino acid residue, was ca. 60% larger than the corresponding area for SP-B(chol), $\approx 0.24\,nm^2$/amino acid residue. An increase in the molecular areas for monolayers of SP-B isolated from butanol lipid extracts [21], compared to the areas observed for films SP-B isolated from chloroform extracts, has already been reported [22,23]. The differences in the experimental conditions of monolayer measurements in the two studies partly accounted for the observed discrepancy. The present study demonstrates that the method of preparation of lipid extract of surfactant represents an additional factor contributing to the differences in the monolayer areas observed in the two studies [21,23].

Surface pressure–area isotherms were measured for SP-Cs isolated from either chloroform or butanol lipid extracts (Fig. 2). The area at lift-off pressure for SP-C(but) – curve 2, was ca. 60% larger than the corresponding area for SP-C(chl) – curve 1. The two curves in Fig. 2 showed a kink point at a surface pressure of ca. 20 mN/m. An inflection or, a flat plateau, at a similar surface pressure has been observed in spread monolayers of human recombinant SP-C [24,25]. The elipsometric observation of increase in the thickness of the films of SP-C at this characteristic point has been accounted for by the

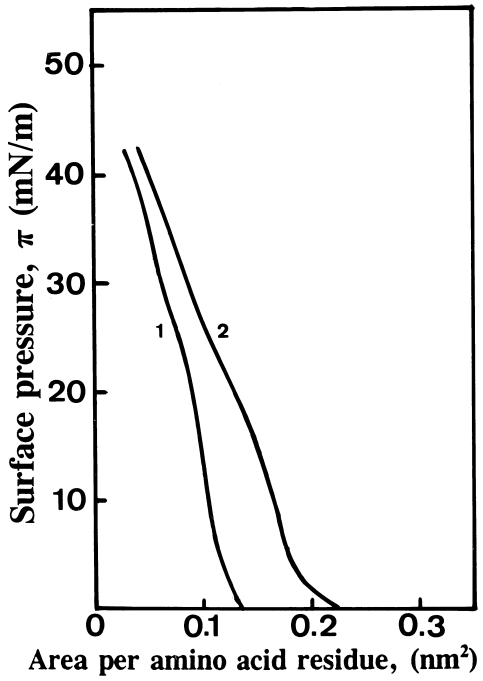


Fig. 2. Isotherms of surface pressure vs. area per amino acid residue for spread monolayers of (1) SP-C(chl) and (2) SP-C(but). The curves are average of measurements on three different preparations (S.D. at various points were within $\pm 0.02\,nm^2$/amino acid residue).

reversible formation of bilayer/multilayer structures at the interface [25].

To study potential effects of the method of isolation of SP-B and SP-C on their ability to enhance the adsorption rates of phospholipids, surface pressure–time measurements were performed during adsorption of aqueous dispersions of DPPC plus SP-B or SP-C that had been isolated by either of the two methods of extraction. Multilamellar liposomes of DPPC at a subphase concentration 60 μg/ml did not adsorb to the air–water interface during the time of observation (curve a in Fig. 3). The rates of adsorption of mixtures of DPPC plus either SP-B(but) (curves d and e in Fig. 3) or SP-B (chl) (curves b and c in Fig. 3) were significantly increased compared to that of DPPC alone. The enhancement of adsorption by either form of the protein was consistent with previous reports on the effects of the hydrophobic pulmonary surfactant proteins on adsorption rates of some phospholipid mixtures [5,21,26]. The data in Fig. 3 also reveal that, at similar concentrations, significantly higher adsorption rates and final surface pressures were detected for protein–lipid dispersions supplemented with SP-B(but) (curves d and e in Fig. 3) than those for SP-B(chl)/DPPC combinations (curves b and c in Fig. 3), i.e. SP-B(but) appeared to be a more potent agent than SP-B(chl) in promoting the adsorption rates of the protein/DPPC dispersions. A similar tendency was observed for aqueous dispersions of DPPC supplemented with 5 wt% SP-C(but) (curve e in Fig. 4) compared to those containing same levels of SP-C(chl) (curve c in Fig. 4). At lower protein concentrations, e.g. 2.5 wt%, SP-C(but) and SP-C(chl) displayed similar rates of adsorption as judged from their $\pi(t)$ dependences (curves d and b in Fig. 4).

To assess effects of the method of isolation of the hydrophobic pulmonary surfactant proteins on their capacity to interact with phospholipids in spread monolayers, compressional isotherms for binary monolayers of DPPC plus either SP-B(but) or SP-C(but) were measured and compared with previously reported data for films of SP-B(chl)/DPPC and SP-C(chl)/DPPC [23,27,28]. The $\pi(A_{mean})$ isotherms for SP-B(but)/DPPC monolayers (data not shown) had interfacial characteristics similar to those observed for SP-B(chl)/DPPC films of comparable initial protein concentrations [23]. For example, the isotherms for SP-B(but)/DPPC films which contained more than 10 wt% exhibited discontinuities at $\pi \approx 45\,mN/m$, associated with partial exclusion of the protein from the surface [23,29]. The presence of up to 17 wt% of SP-B(but) in the mixed lipid–protein monolayers did not interfere with the property of DPPC films to attain very high collapse pressure of ca. 72 mN/m. A similar property was observed in the binary SP-B(chl)/DPPC monolayers [23].

The compressional surface pressure–area curves for monolayers of SP-C(but)/DPPC (data not shown) resembled those obtained for SP-C(chl)/DPPC monolayers [27]. A characteristic plateau-like region at $\pi \approx 51–53\,mN/m$ was seen in the isotherms for SP-C(but)/DPPC monolayers which contained more than 5 wt% protein. As discussed before, at $\pi > 50\,mN/m$ lipid–protein phases were excluded from the SP-C/DPPC monolayers [27,29].

Plots of the mean area per ''residue'' at constant surface pressure in the SP-B(but)/DPPC films as a function of the mole fraction of the amino acid residues of SP-B(but) showed positive deviations from

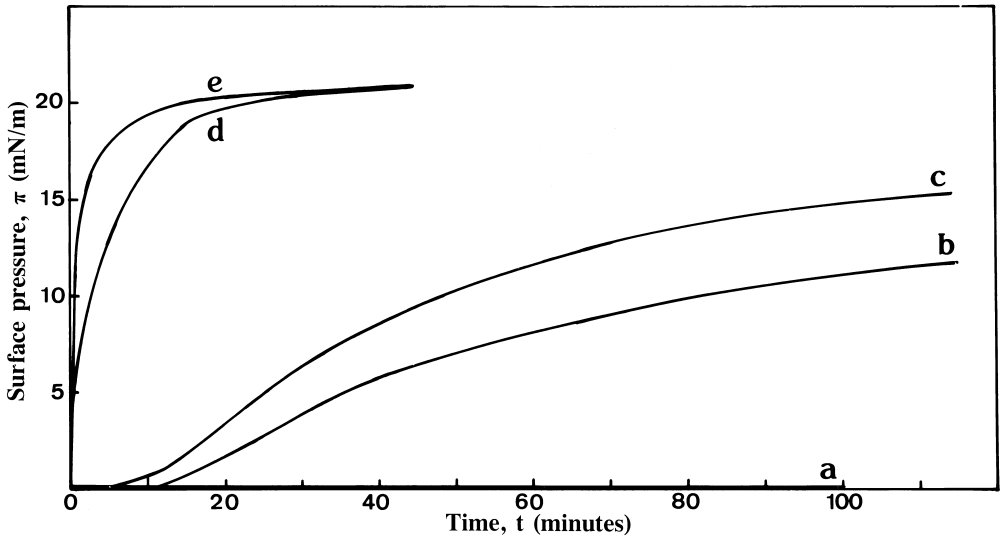


Fig. 3. Time dependence of surface pressure during adsorption of aqueous dispersions of SP-B(chl)/DPPC (curves b and c) and SP-B(but)/DPPC (curves d and e). The concentration of SP-B was (a) 0 wt%, (b and d) 2.5 wt%, (c and e) 5.0 wt%. Final concentration of DPPC in the subphase was 0.06 mg/ml. The curves are averaged from two experiments; the range of values of π at any time point did not exceed 1.5 mN/m.

the additivity rule, consistent with interactions between SP-B(but) and DPPC (data not shown). These properties of the $A_{mean}(X_r)$ diagrams were very similar to those observed in SP-B(chl)/DPPC monolayers [23,28]. The $A_{mean}(X_r)$ plots for the films of SP-C(but)/DPPC (data not shown) resembled those for SP-C(chl)/DPPC monolayers [27]. The additivity of the mean areas seen for films which contained

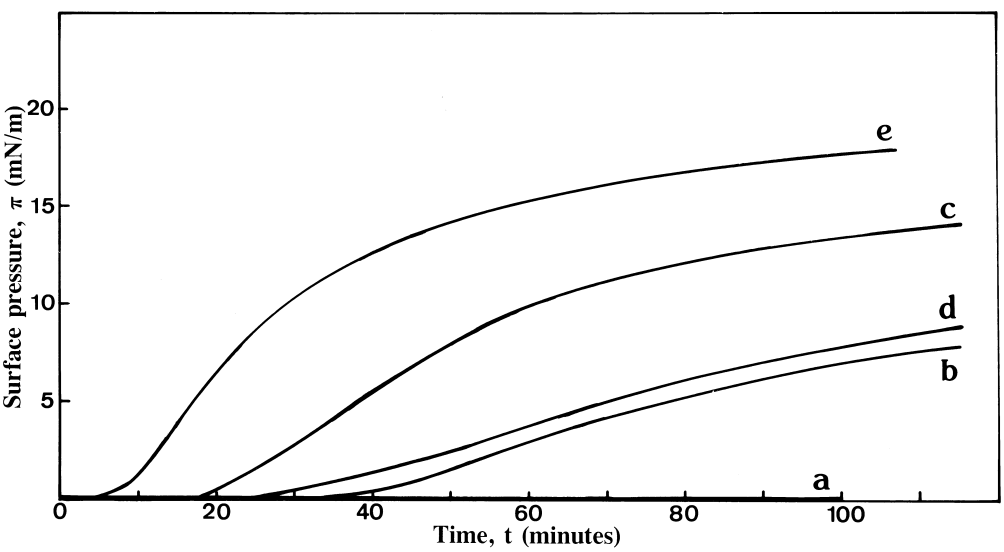


Fig. 4. Surface pressure-time adsorption isotherms for SP-C(chl)/DPPC (curves b and c) and SP-C(but)/DPPC (curves d and e). The concentration of SP-C was (a) 0 wt%, (b and d) 2.5 wt%, (c and e) 5.0 wt%. Final concentration of DPPC in the subphase was 0.06 mg/ml. Curves are averaged of two experiments; the range of π values at any time point did not exceed 1.5 mN/m.

> 30 wt% SP-C(but) was consistent with self-association of SP-C and its aggregation in the lipid monolayer [27,29].

To examine alterations in molecular masses of the proteins due to potential chemical modifications of the proteins during the different isolation procedures,

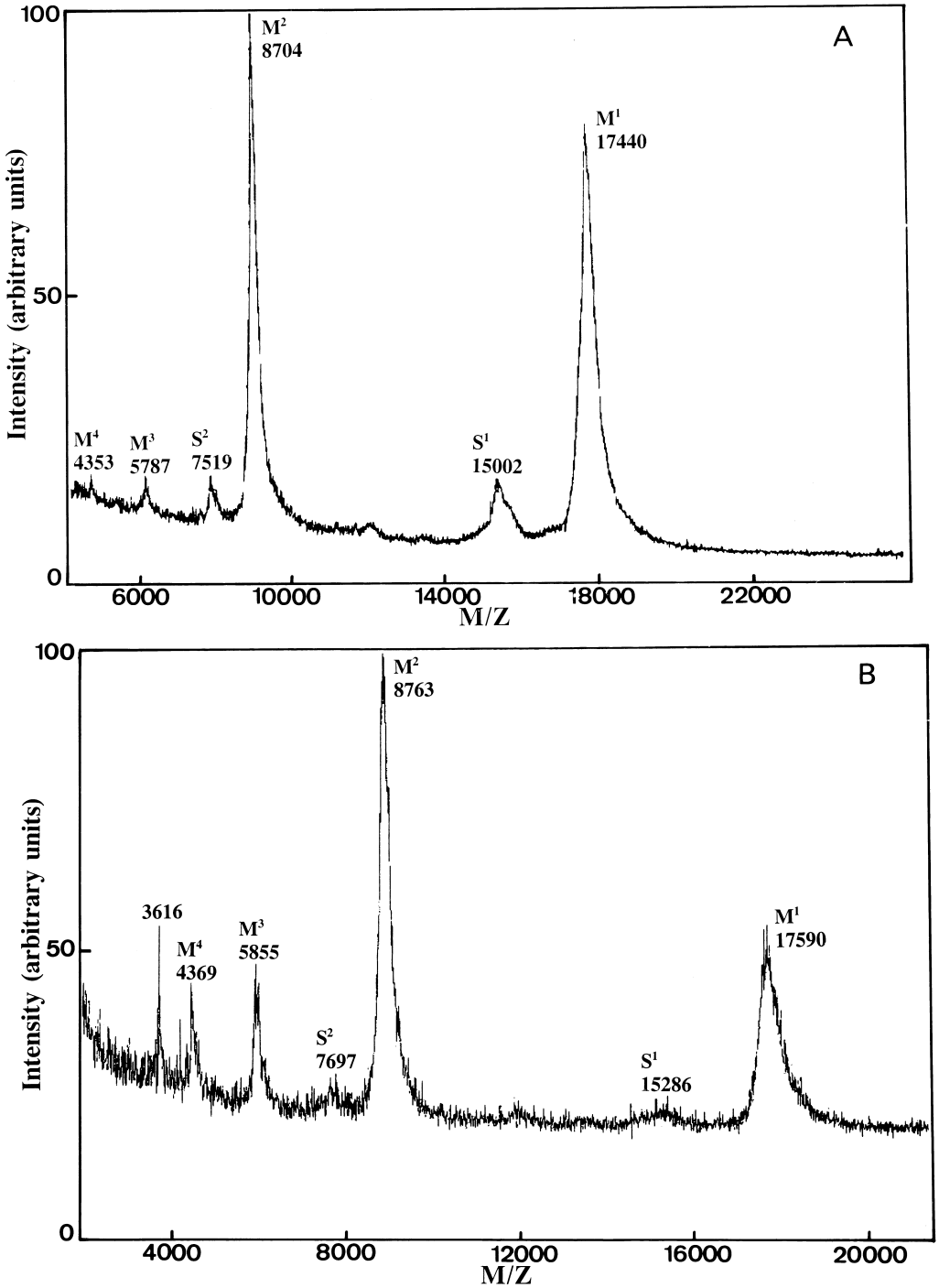


Fig. 5. (A) MALDI mass spectrum of SP-B(chl) isolated from chloroform lipid extract surfactant. (B) MALDI mass spectrum of SP-B(but) isolated from butanol lipid extracts.

MALDI mass spectra of SP-B(but) and SP-C(but) were compared to those measured for SP-B(chl) and SP-C(chl), respectively. The MALDI mass spectrum of non-reduced SP-B(chl), shown in Fig. 5(A), is characterized with signals of multiply charged ions with up to four charges (the number of charges on each ion species is given by the superscript M^n). To determine the molecular mass of a protein, the apparent ion mass M of each of the assigned peaks was multiplied with the corresponding number of charges Z and the average of these values was calculated [30]. A molecular mass of 17405 ± 32 (± S.D.) was determined for SP-B(chl) from the four assigned peaks in Fig. 5(A). This value corresponded well to the mass of porcine SP-B observed by plasma desorption mass spectrometry on nitrocellulose backing by Curstedt et al. [2] who used a similar method of extraction of the hydrophobic surfactant proteins. Mass spectra for different preparations of SP-B(chl) showed little variability, e.g. the average masses determined for two different preparation of SP-B(chl) differed only by 5 Da. The ion peaks at M/Z 15002 and 7519, S^n in Fig. 5(A), probably representing ions with one and two charges, gave an average molecular mass of 15020. This value is considerably lower than the molecular mass of 17405 determined from the M^n ion peaks in Fig. 5(A). The difference in the molecular masses of ca. 2385 Da may be accounted for by the presence of a molecular species which was of ca. 20–22 amino acids shorter than the native dimeric form of SP-B. The origin of this ''short'' form of SP-B is not known. However, both PAGE and mass spectrometry on the individual chromatography fractions of LH-60 column, suggested the presence of a secondary shorter form of SP-B (data not shown). So far we have not been able to separate and better characterize the two possible forms of SP-B.

The MALDI mass spectrum of SP-B(but) prepared from butanol lipid extracts of porcine lung surfactant is shown in Fig. 5(B). Similar to the spectrum of SP-B(chl), shown in Fig. 5(A), the spectrum in Fig. 5(B) was characterized by the presence of multiply charged molecular-ion species. The ion peaks (M^n), corresponding to four different charge states, allowed calculating a molecular mass of 17539 ± 49 (average mass ± S.D) for SP-B(but). The two peaks (S^n) at M/Z 15286 and 7697 which, most likely, represented ions with one and two charges, confirmed the presence of a truncated form of SP-B(but), (molecular mass of 15340), similar to the observation of a

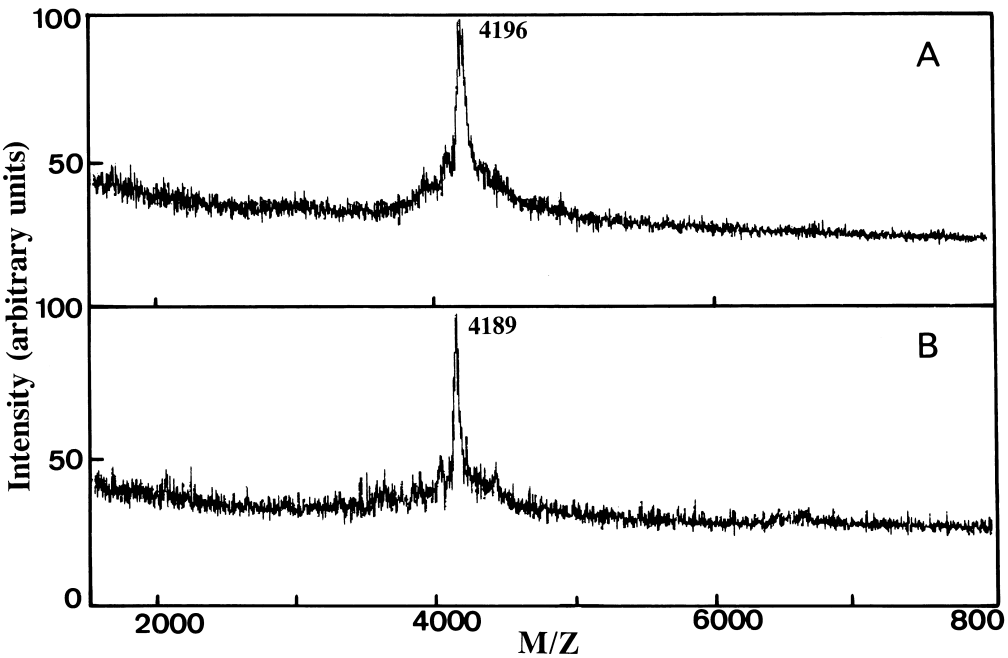


Fig. 6. (A) MALDI mass spectrum of SP-C(chl) prepared from chloroform lipid extract surfactant. (B) MALDI mass spectrum of SP-C(but) isolated from butanol lipid extracts.

short form in the SP-B(chl) sample (Fig. 5(A)). The data in Fig. 5(B) also reveal an ion peak at M/Z 3616 consistent with the presence of some non-palmitoylated N-terminally truncated SP-C [2]. Comparison between the average masses of the major forms of SP-B(chl) (17405) and SP-B(but) (17 539) revealed a difference of 134 Da, consistent with protein modification probably due to the different solvent systems used for the preparation of lipid extract surfactant.

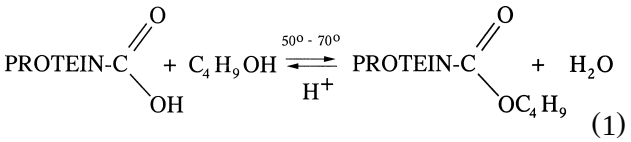
The mass spectrum of SP-C(chl) isolated from chloroform extracts of surfactant showed a molecular-ion peak at M/Z 4196 (Fig. 6(A)), consistent with the theoretical molecular mass (4186) of the major dipalmitoylated non-truncated form of porcine SP-C [2]. A similar molecular mass of 4189 was determined for SP-C(but) from its mass spectrum, shown in Fig. 6(B). The average values determined for different preparation of SP-C(chl) and SP-C(but) were 4217 ± 18 (mean $\pm$ S.D., $n = 3$) and 4204 ± 22 (mean $\pm$ S.D., $n = 4$). The spectra in Fig. 6(A and B) revealed that the two protein preparations were fairly homogeneous and the molecular masses of SP-C(but) and SP-C(chl) appeared to be independent of the method of extraction of surfactant pellet.

4. Discussion

The present study compares properties of hydrophobic pulmonary surfactant proteins SP-B and SP-C isolated from lipid extracts of surfactant obtained by either chloroform : methanol or butanol extraction of surfactant pellet. It is worth noting that the purification protocols for the proteins from porcine surfactant were identical for the two sets of proteins, SP-B(chl) and SP-C(chl) vs. SP-B(but) and SP-C(but), except for the method of preparation of lipid extract as described in Section 2. Since differences in the extent of delipidation of the proteins may alter both, their interfacial properties [23] and their ability to lower the dynamic surface tension of reconstituted lipid–protein mixtures [5,6], the amounts of lipid which remained associated with SP-B and SP-C after their purification were carefully monitored. The results from the phospholipid estimation [16,17] showed that, independently of the method of extraction, SP-B preparations contained < 1 mol of phospholipid per mole (dimer) of protein. SP-C preparations showed 0.5 mol of phospholipid per mole (monomer) of protein prepared by either of the extraction methods. The results suggested that the endogenous lipid which remained associated with the proteins was unlikely to be a variable which could account for the differences observed in the properties of SP-Bs and SP-Cs isolated by the two methods. The levels of phospholipid in the protein preparations were consistent with those already reported for SP-B and SP-C isolated by LH-60 chromatography [21,23,27]. They also indicated that the hydrophobic pulmonary surfactant proteins purified by this technique contained amounts of endogenous phospholipid comparable to the levels reported for the proteins isolated by other methods [5,6].

The analysis of the pressure–area characteristics of the proteins isolated by the two extraction protocols, as well as their ability to facilitate adsorption rates of aqueous dispersions of DPPC suggested that the utilization of two different organic solvents for preparation of lipid extracts of surfactant possibly induced some protein modifications. These can include changes in chemical compositions or in secondary and tertiary structures, or both, of the proteins. The structures of SP-Bs and SP-Cs were studied by circular dichroism (CD) and identical spectra were measured for SP-B(but) and SP-B(chl) in methanol as well as in butanol (data not shown). Likewise, SP-C(but) and SP-C(chl) showed similar spectra in the two organic solvents (data not shown). Though the CD spectra obtained were not used to compute the proportions of secondary structures present, the similarities of the spectra suggested that simple transfer of the proteins from chloroform : methanol to butanol as well as utilization of butanol as an extraction solvent did not cause significant changes in the secondary structures of either SP-Bs or SP-Cs. However, the fluorescence emission spectra, excited at 275 nm, of the two sets of SP-B showed that the fluorescence emission maximum of SP-B(but) at 334 nm was blue-shifted compared to the emission maximum of SP-B(chl) at 339 nm, and the fluorescence emission intensity of SP-B(but) was ca. 60% lower than that of SP-B(chl) (data not shown). These results suggested differences in the microenvironments for the tryptophan in SP-B(but) and that in SP-B(chl) which might arise from both chemical and structural modifications of SP-Bs isolated by the two methods.

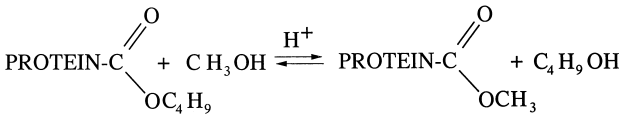
Potential chemical modifications may involve esterification of the carboxyl groups of the proteins. The carboxyl group of C-terminal residues and those of the side chains (aspartic and glutamic acids) of the proteins can undergo organic reactions leading to the formation of esters [31]. A carboxyl group can be converted directly into an ester when heated with alcohol (Fisher esterification); the reaction being reversible with equilibrium constants of about unity [32]:



(1)

A large excess of alcohol can be used to shift the equilibrium towards the products of the reaction and thus to increase the yield of the ester.

During isolation of SP-B(but) and SP-C(but), the proteins dissolved in large excess of butanol were subjected to rotary evaporation at 50°C and this step could have produced butylated proteins (Eq. (1)). These proteins were next redissolved in an excess of acidified chloroform : methanol and subjected to LH-60 chromatography. Under these conditions, it might have been possible for methanol to displace butanol from the protein butyl esters, i.e. a potential transesterification could have led to at least partial exchange of butyl esters with methyl esters (Eq. (2)). This process is catalyzed by acids [33] and the 2 vol% 0.1 M HCl present in the column solvent might have provided the conditions required for this process:



(2)

Therefore, it is likely that at the end of the isolation procedure of the proteins from butanol lipid extract of surfactant the proteins, designated as SP-B(but) and SP-C(but), comprised mixtures of butyl and methyl esters.

During isolation of SP-B(chl) and SP-C(chl), chloroform lipid extracts of surfactant were reduced to dryness by rotary evaporation and the material was redissolved in acidified chloroform : methanol (1 : 1 v/v) and run through LH-60 column. In the presence of acidified methanol methylation of the C-terminal and side-chain carboxyl groups of the proteins might have occurred.

Carboxyl groups of water soluble proteins have been converted to methyl esters in anhydrous methanol containing small amounts of hydrochloric acid [33]. Under such conditions, most proteins experience change in conformation but rather few chemical changes, other than esterification. Only two minor side reactions, methanolysis of amide groups and N → O acyl shift at serine and threonine residues can be detected under typical conditions [34,35].

SP-B (dimer) has ten side amide groups (eight glutamine and two asparagine) as well as four serine and four threonine residues [1]. SP-C (monomer) has one asparagine and no serine or threonine residues [2]. A comparison of the primary structures of the two proteins suggests that the native SP-B would be more prone to undergo the above-described chemical modifications during LH-60 chromatography than would be native SP-C. Since the two sets of SP-Bs and SP-Cs prepared from either butanol or chloroform lipid extracts had been subjected to LH-60 chromatography in acidified chloroform : methanol, one may speculate that this step of the isolation protocol possibly induced similar, if any, chemical changes in the proteins isolated by the two protocols. Therefore, it is reasonable to assume that the differences observed in the properties of the proteins isolated from butanol or chloroform extracts were probably due to conversion of their carboxyl groups to butyl or methyl esters, respectively.

Data from ATR infrared spectroscopy on films of SP-B(but) and SP-B(chl) deposited on zinc selenide crystal revealed the presence of a carbonyl band at $\sim 1735\,cm^{-1}$, consistent with an ester carbonyl (data not shown). Similar bands were detected for the films of SP-C(but) and SP-C(chl) (data not shown). The relative intensity of carbonyl to amide I band was greater for SP-B(but) and SP-B(chl) compared to SP-C(but) and SP-C(chl). These structural data were consistent with a hypothesis that esterification of the proteins occurred during their preparation by the methods utilized in this study.

A variety of chemical analyses have been used for identification and estimation of the products of esterification of water-soluble proteins [34–36]. Since these chemical procedures are usually performed in aqueous media and require large amounts of protein, their utilization for identification of chemical modifications of the hydrophobic pulmonary surfactant proteins was not practical. Both SP-B and SP-C are practically insoluble in water and they are present at very low concentrations in the natural lung surfactant.

The results from MALDI mass spectrometry of SP-Bs (Fig. 5) supported the speculations of potentially variable chemical modifications of the amino acid residues of the proteins during their isolation by the two methods. Comparison of the molecular masses of SP-B(but) and SP-B(chl) revealed a difference of 134 Da, a value consistent with the presence of three butyl groups in SP-B(but) compared to SP-B(chl) which, presumably, was methylated. A simple calculation shows that replacement of a methyl ester with butyl ester would give a rise to an additional 42 Da per carboxyl group. If we assume that SP-B(chl) was not methylated, than the difference of 134 Da between the molecular masses of SP-B(but) and SP-B(chl) could be accounted for by the presence of two butyl and two methyl esters in SP-B(but) compared to non-esterified SP-B(chl) ($2 \times 56 + 2 \times 14 = 140$ Da). The difference in the average masses of SP-B(but) and SP-B(chl), deduced from the centroids of the peaks in the spectra in Fig. 5(A and B), gave a measure of the degree of potential butylation vs. methylation of the proteins. The resolution of the mass measurements, however, was insufficient to permit a resolution of individual peaks in the spectra which would correspond to molecules of different extents of esterification.

A significant difference in the molecular masses was seen for SP-Bs (Fig. 5) but not for SP-Cs (Fig. 6) isolated by the two methods. This is not surprising, given the fact that porcine SP-B (dimeric form) has two glutamic and two aspartic acids [1] which, along with the two C-terminal carboxyl groups, represent a total of six potential sites for esterification per dimer of SP-B. SP-C lacks acidic amino acid residues [2] and, therefore, only the C-terminal carboxyl group could be expected to be chemically modified by alcohols. The molecular masses determined for SP-C(but) and SP-C(chl) were very similar (Fig. 6(A and B)) and, apparently, independent of the method of extraction. However, a potential difference in esterification, butylation vs. methylation, of the two SP-Cs would result in only 42 Da, commensurate with the variability of the measurements for SP-C (± 22 Da).

It is worth noting that the carbonyl bands in the ATR-IR spectra of films of SP-C(but) and SP-C(chl) were very broad and low in intensity. Since the carbonyl stretch from the terminal carboxyl group is usually observed at 1710–1740 cm^{-1} and overlaps the ester carbonyl stretch, it was difficult to determine whether the carbonyl band observed for the SP-C films was due to the presence of ester or carboxyl groups, or both. The differences observed in the properties of the two SP-Cs, however, suggested variances in their structures or chemical compositions, or both.

The results from the surface pressure–area measurements on the two sets of SP-Bs (Fig. 1) and SP-Cs (Fig. 2) revealed that, at any given area per amino acid residue, SP-B(but) and SP-C(but) exerted a higher surface pressure than did SP-B(chl) and SP-C(chl), respectively. The differences in the monolayer properties of the two sets of proteins may reflect differences in the hydrophobicities and charge states of the butylated SP-B(but) and SP-C(but) vs. the non-esterified (or methylated) SP-B(chl) and SP-C(chl). Mita [37] showed that acylation (propionyl and acetyl) of N-terminal and lysine amino groups of bovine serum albumin (BSA) was accompanied by changes in the area occupied by the protein derivatives in the spread monolayers. The observations have been discussed in terms of modifications in the degrees of unfolding of the proteins at the air–water interface induced by changes in their hydrophobicities [37].

The assessment of biophysical activities of the proteins, as measured by the interfacial adsorption, revealed that SP-B(but) and SP-C(but), when present at 5 wt% in DPPC, induced faster adsorption of the reconstituted mixtures than did equal concentrations of SP-B(chl) and SP-C(chl), respectively (curves e and c in Figs. 3 and 4). This observation correlated with the higher intrinsic surface activities of SP-B(but) and SP-C(but) compared to SP-B(chl) and SP-C(chl) (Figs. 1 and 2). Though different mechanisms of interactions and orientations in lipid bilayers have been suggested for SP-B [10,38–40] and SP-C [41–

43], potential esterification of SP-B(but) and SP-C(but) appeared to modify the protein–lipid interactions in a similar way that led to enhancement of the adsorption facility of the lipid–protein mixtures. It is worth noting that Tanaka et al. [9] included a step of heating at 45° for 30 min in 10% ethanol in formulation of their artificial lung surfactants. Yu and Possmayer [44] also found that thermal treatment of phospholipid mixtures with SP-C in chloroform : methanol improved their adsorption abilities. What happens to the lipids or proteins during these preparative steps is unknown, however, the results in the present study suggest that a potential esterification of the proteins during heating in the presence of alcohol may account for the improved performance of the lipid–protein mixtures observed by the authors.

Contrary to the observations of different adsorption rates, surface pressure–area characteristics of the spread monolayers of DPPC plus SP-B or SP-C appeared to be independent of the method of isolation of the proteins. As far as the $A_{mean}(X_r)$ plots are indicators for interactions in the binary monolayers, the similarity between the plots for SP-B(but) and SP-C(but) and those found for SP-B(chl) [23] and SP-C(chl) [27], respectively, suggested that potential changes in the hydrophobicities and charges of the proteins did not substantially affect lipid–protein interactions in the DPPC/protein spread monolayers. The molecular basis of the apparent difference in the effect of the extraction procedure on the $\pi(A_{mean})$ measurements on spread monolayers of DPPC plus SP-B or SP-C from the $\pi(t)$ measurements on similar protein-lipid mixtures is not clear. It may reflect differences in lipid–protein interactions caused by various orientations and structures of the proteins in the lipid monolayer and bilayer environments.

In summary, the method of extraction of surfactant pellet affected the intrinsic surface activities of SP-B and SP-C as well as their biophysical activities in promoting interfacial adsorption of DPPC dispersions. The proteins isolated from butanol lipid extracts showed more pronounced effects than their counterparts isolated from chloroform–methanol extracts. The differences observed in the properties of the proteins were related to chemical modifications of the amino acid residues. ATR-IR and MALDI spectrometry data provided some evidence for esterification of the C-terminal and side-chain carboxyl groups of the proteins by alcohols present in the extraction and purification systems. Since various procedures are being used for purification of SP-B and SP-C [1,5,6,11,45–47] these results suggest that the isolated proteins may be modifications of the ones in vivo, and that differences of effects seen with different preparations might be ascribable at least in part to those alterations.

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