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Analysis of proteins in microsamples of rat airway surface fluid by capillary electrophoresis

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Abstract

A thin layer of airway surface fluid (ASF) lining the pulmonary airways plays an important role in the primary defense mechanisms of the lung against bacterial infection. However, little is known about the composition of ASF due to the thinness (typically 5–30 μm in healthy animals) of the fluid layer and its relative inaccessibility, which causes considerable difficulties in sample collection and subsequent analysis. We have used a novel technique of capillary sampling coupled with capillary electrophoresis (CE) to analyze the protein composition of rat ASF. CE analyses were performed under two different conditions: a borate buffer, pH 9.1, or a phosphate buffer, pH 2.5, with 0.5 m*M* spermine. The different selectivities afforded by the two methods aid in peak identification, and quantitation of most of the major species was possible using both separation conditions. Albumin, transferrin and globulins are observed to be the major protein components in rat ASF, at concentrations of 28 mg ml^{-1}, 4.0 mg ml^{-1} and 34 mg ml^{-1} respectively, in comparison to 31 mg ml^{-1}, 3.1 mg ml^{-1} and 40 mg ml^{-1}, respectively, in rat plasma.

Keywords: Proteins; Albumin; Transferrin; Globulins

1. Introduction

Airway surface fluid (ASF) is a thin (5–30 μm) liquid layer covering the pulmonary airways. So far, little is known quantitatively about the composition of ASF due to the thinness of this fluid layer, and its relatively inaccessible position within the lung airways, which causes considerable difficulties in sampling and subsequent analysis. Certainly various inorganic ions, mucins, glycosaminoglycans and proteins of local or plasma origin are present in ASF [1]. Bronchoalveolar lavage (BAL) [2] has been widely used for the collection of ASF and alveolar fluid. In BAL, a physiological saline solution is forced into all or part of a lung, and then sucked out. By this method, a diluted solution of ASF can be obtained from all or part of the lung. Protein components of the lavage fluid can then be analyzed by conventional methods [3,4]. This procedure is semiquantitative due to large, unknown dilution factors involved in the sampling, and furthermore it is not site specific, providing information on at best a region of the airways.

Development of methods for the quantitative analysis of ASF is important, since it has been

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suggested that ASF inorganic ion composition regulates bactericidal defense mechanisms [5,6], whilst hypersecretion of proteins, glycoproteins and polysaccharides is observed in many obstructive airway diseases [7]. Recently, we have developed a technique involving capillary sampling coupled with analysis by capillary electrophoresis (CE) [8,9] for the determination of inorganic ions in rat ASF. In these methods, a sampling capillary (<300 μm I.D.) is inserted into the trachea, and allowed to rest against the epithelial surface to collect a small volume of ASF. The sampling capillary is then removed, and the separation and sampling capillaries are interfaced to allow injection of 1–2 nl of the ≈100–300 nl ASF sample into the CE system. We now report an adaptation of the same technique for the analysis of proteins in rat ASF. In the present study, two CE methods were developed using either a high ionic strength borate buffer (pH 9.1), or a pH 2.5 phosphate buffer with spermine as an additive to dynamically coat the capillary surface. Both methods proved suitable for quantitation of the major ASF proteins, which in healthy rats were found to be albumin, transferrin and globulins. The different selectivity provided by the low and high-pH separations aided in peak identification.

2. Experimental

2.1. Chemicals

Sodium dihydrogenphosphate was purchased from Fisher Scientific (Montreal, Canada). Boric acid was purchased from Anachemia (Montreal, Canada). Spermine (N,N′-bis[3-aminopropyl]-1,4-butanediamine, 97% pure), sodium dodecyl sulfate (SDS, molecular biology reagent), sodium tetraborate, albumin (rat, human), holo-transferrin (human), α-, β- and γ-globulins (human), lysozyme (chicken egg white), lactoferrin (bovine) and submaxillary mucins (bovine) were purchased from Sigma (St. Louis, MO, USA). Buffer solutions were prepared from distilled and doubly deionised water (Milli-Q50 unit, Millipore, Montreal, Canada). All background electrolyte (BGE) components used were of analytical grade unless otherwise stated.

2.2. Animals

Fisher male rats (6–8 weeks in age, 350–450 g) were purchased from a commercial source (Harlan Sprague Dawley, Indianapolis, IN, USA) and housed in a conventional animal care facility at the Meakins-Christie Laboratories. Protocols were approved by the local animal ethics committee.

2.3. Instrumentation

A CE unit from Applied Biosystems (Foster City, CA, USA), Model 270 A was used. Fused-silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 145 μm O.D., 50 μm I.D. and 72 cm total length were used. The polymer coating was burned off 22 cm from the cathodic end of the capillary to form a detection window. Before use, new capillaries were washed with 0.5 *M* sodium hydroxide for 30 min, deionised water for 10 min, and BGE for 25 min. The oven was thermostatted at 30°C, and UV absorbance detection was carried out at 200 nm. Data were collected with an integrator (Model SP4600, Spectra-Physics, San Jose, CA, USA), and Spectra-Physics Winner software was used for data storage and manipulation.

2.4. Sampling

ASF samples were collected from Fisher male rats by the method described in our previous publications [8,9]. Briefly, the rats were first sedated with xylazine (0.08 ml/100 g body mass, intraperitoneal) and then anaesthetized using pentobarbital (0.053 ml/100 g body mass, intraperitoneal). An intubation tube (6 cm×1.67 mm I.D., 2.42 mm O.D.; Becton-Dickinson, Sparks, MD, USA) was inserted into the trachea and the animal placed supine. The polyethylene sampling capillary (10 cm×280 μm I.D., 610 μm O.D.; Becton-Dickinson) was passed through the intubation tubing, which protects it from picking up liquid in the upper airways, and was then left in the trachea with the end in contact with the epithelium approximately 0.5 cm below the level of the main carina for a period of 3–5 min before being pulled out. ASF samples obtained in this way were typically around 100–300 nl in volume per collection, and were analyzed immediately.

To inject the sample onto the CE, the sampling capillary (280 μm I.D.) was fitted over the end of the separation capillary (145 μm O.D.), until the separation capillary was just touching the ASF sample. The sampling capillary was open at both ends, so the liquid is not forced into the separation capillary when the insertion takes place. After insertion, ≈2 nl of ASF was directly introduced onto the separation capillary by vacuum injection (17 kPa for 1.0 s).

2.5. Protein analysis

Protein analyses were carried out under two different conditions. A pH 2.5 phosphate buffer was prepared by adjusting a 50 m*M* solution of sodium dihydrogenphosphate with concentrated phosphoric acid. Spermine was then added to this solution, to a concentration of 0.5 m*M*. A pH 9.1 buffer was made by titrating a 100 m*M* sodium tetraborate solution with a 100 m*M* boric acid solution. The buffer solutions were degassed by sonication and passed through a 0.45 μm membrane filter (Millipore, Montreal, Canada) before use. Separations using the borate BGE were carried out with an applied electric field of 167 V cm^{-1} (i=40 μA), whilst with the phosphate electrolyte the field strength was 250 V cm^{-1} (i=36 μA). In between runs, the capillaries were washed for 2 min with 100 m*M* SDS in BGE followed by 2 min with 0.5 *M* sodium hydroxide (the preceding washes remove adsorbed protein [10]) and then with BGE (6 min), using a vacuum of 68 kPa to draw solutions through the capillary.

Protein peaks in rat ASF were identified by running standard protein solutions. Peak areas were used for quantitation; these were compared to calibration curves prepared from analyses of aqueous solutions of protein standards. Calibration solutions were introduced into sampling capillaries similar to those used for ASF collection, and introduced onto the separation capillary in the same way as ASF microsamples.

To compare the protein composition of ASF with plasma composition, protein components in rat plasma were also analyzed by using the same protocols. Rat plasma was prepared from the blood of the same rats used for ASF sampling. Blood was obtained via cardiac puncture, and collected into sterile, heparinised containers (Vacutainer, Becton-Dickinson) and was then centrifuged at 1000 *g* for 10 min. Thereafter, plasma was removed and introduced into capillaries similar to those used for ASF collection, and then injected into the separation capillary in the same way as the ASF microsamples.

3. Results and discussion

Since we expected the ASF protein profile to have some similarity to plasma proteins, an approach similar to that used for the CE analysis of plasma proteins seemed appropriate. In general, the CE analysis of proteins requires separation conditions such that binding of the proteins to the capillary surface is reduced or eliminated [11]. Therefore, one BGE used was a borate buffer at moderately high ionic strength and high pH [12,13]. Under these conditions, all except the most basic proteins bear a net negative charge, as do the capillary walls. Binding interactions are further reduced because of screening of the protein's remaining positive charges by the buffer counterions. In the analysis of rat ASF at pH 9.1, the major components observed were a peak corresponding to the transferrin standard which comigrated with the γ-globulin region, followed by β- and α-globulins and albumin (Fig. 1A). This profile is broadly similar to that observed in the analysis of human plasma proteins (e.g., [12,13]), although transferrin is generally found to migrate after the γ-globulins; this inconsistency may reflect slight differences in the separation conditions used. A small peak at the beginning of the separation corresponded to the migration time of lysozyme.

Since we were attempting to determine endogenous compounds in microsamples of a poorly-characterized biofluid, it was not possible to unambiguously identify components based solely on their mobility relative to standards under one set of separation conditions. Therefore, an analysis at pH 2.5 was also performed. Each of the proteins present in the sample would have a very different charge and thus mobility under the acid or alkaline conditions, and thus if a given peak can be shown to coelute with a standard under both sets of conditions, this is a much stronger indication that it is indeed the same component as the standard. The spermine additive used at low pH coats the capillary wall, reducing the

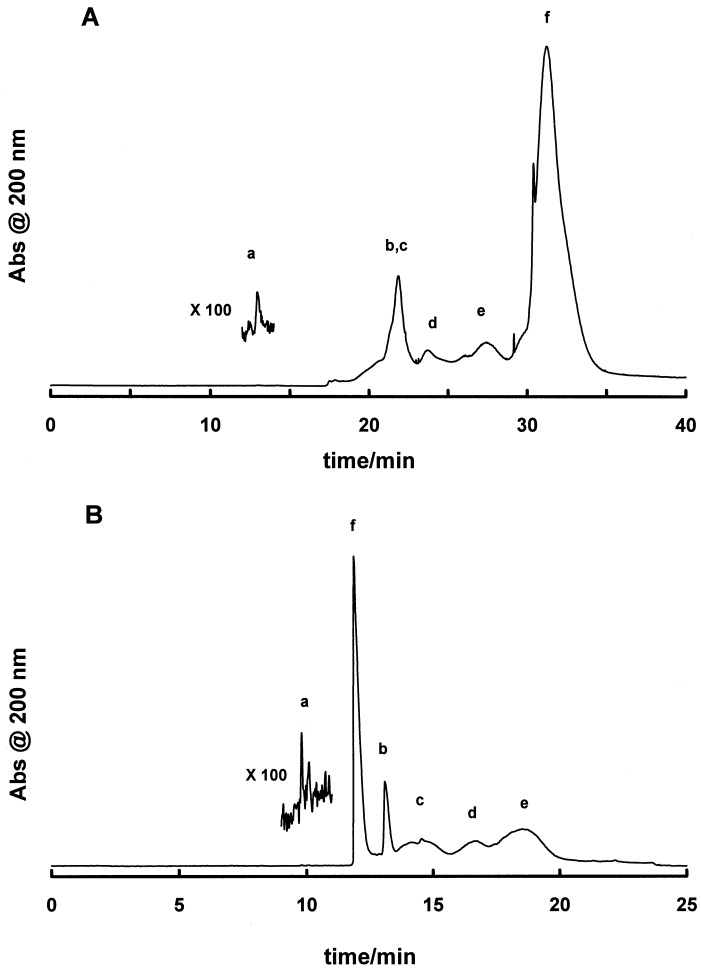

Fig. 1. Separation of major proteins in rat ASF. (A) High-pH separation using uncoated capillaries. Conditions: fused-silica capillary [72 cm (effective length 50 cm)×50 μm I.D., 150 μm O.D.]; field strength, 167 V cm^{-1} (i=40 μA); BGE, borate, pH 9.1; detection, UV at 200 nm. The inset shows the electropherogram from 12 to 14 min magnified 100 times in the vertical axis. Peaks: (a) lysozyme, (b) transferrin, (c) γ-globulin (the sharper transferrin peak is superimposed on the broad γ-globulin peak), (d) β-globulin, (e) α-globulin and (f) albumin. (B) Low-pH separation using capillary dynamically coated with spermine. Conditions: as above except field strength, 250 V cm^{-1} (i=36 μA); BGE, phosphate, pH 2.5 with spermine (0.5 mM) as capillary surface modifier. Peak identification as in (A). The inset shows the electropherogram from 9 to 11 min magnified 100 times in the vertical axis.

electroosmotic flow somewhat [14], and reducing interactions of the proteins with the capillary surface [15]. In the separation at pH 2.5 (Fig. 1B) using phosphate buffer, lysozyme migrates first followed by the major peaks; albumin, followed by transferrin and globulins (γ, β and α). All peaks were identified by comparing their migration times with standards, and the migration order is consistent with that observed in BAL samples analyzed under similar conditions by Gurley et al. [4]. Furthermore, the relative peak areas of each resolved component in both the high- and low-pH separations of ASF were similar.

In comparing the two separations, one should remember that at pH 9.1 all the proteins except lysozyme are above their isoelectric point (pI) values, bear a net negative charge and migrate counter to the electroosmosis. At pH 2.5 they are all below their pI values, bear a net positive charge and thus are migrating in the opposite sense compared to

pH 9.1, moving in the same direction as a weak electroosmotic flow. At both pH values albumin is found to have a high effective mobility relative to the rest of the analytes with the exception of lysozyme at pH 2.5. Transferrin, slowly-migrating along with γ-globulins at pH 9.1, migrates faster than the globulins at pH 2.5. The order of effective mobility of the globulins is reversed between the two separations, although because of the change in electroosmosis between the separations their order of migration remains the same. As well as changes in the ionization of the different functional groups in the proteins, differences in binding of buffer ions and the spermine additive may also modulate the mobilities of the analytes. Clearly the selectivity in these two separations is not similar, and so they possess a reasonable degree of orthogonality by which to make confident peak assignments, based on coelution of ASF peaks with standards under both conditions.

At pH 2.5 with dynamic modification of capillary surface by spermine, the peaks for albumin and to a lesser extent for transferrin are sharper than at pH 9.1. However, the globulins migrate as broad bands under both sets of conditions. Heterogeneity of the proteins is likely to account in large part for the peak shapes observed.

Identification of albumin, transferrin and globulins as major proteins in rat ASF is consistent with similar studies by Gurley et al. [4]. They have used CE and HPLC to characterize proteins in fluid lining of the bronchial tree and alveoli of the lungs of rats using BAL in healthy animals and in those exposed to lung injury from perfluoroisobutylene. They identified albumin, transferrin and globulins (IgG) as major components in the lavage fluid and also found increased levels of albumin and transferrin after inducing lung injury due to leakage of plasma components; absolute quantitation was not possible because of the BAL sampling method. Their CE separation involved the use of a pH 2.5 BGE, with a hydrophilic coated capillary.

We attempted to quantitate the major proteins in rat ASF. Calibration curves were made, typically, over the range of 2–40 mg ml^{-1} for albumin, 1–25 mg ml^{-1} for transferrin and 1–30 mg ml^{-1} for globulins. With the protein concentration expressed in mg ml^{-1}, and the area being the time-normalised peak area, the equations of typical standard curves were as follows. At pH 9.1, area(albumin)=(34 787±1561)[albumin]+(82 236±20 786), r^2=0.996; area(transferrin)=(58 306±1664)[transferrin]+(45 103±13 047), r^2=0.998; area(globulin)=(29 893 ± 1754)[globulin] + (44 501 ± 30 142), r^2=0.993. At pH 2.5, area(albumin)=(31 237±790)[albumin]+(71 658±14 932), r^2=0.998; area(transferrin) = (53 740 ± 1631)[transferrin] + (38 447±5531), r^2=0.998; area(globulin)=(17 945±291)[globulin]−(2074±1766), r^2=1.000. The regression coefficients (r^2) confirm adequate linearity over the range of protein concentrations observed in ASF. Standard curves were generally constructed using only four points, because we wished to make the standard determinations and analyse the ASF samples on the day of collection to limit the possibility of evaporation of stored microsamples. The differences in slope of the lines at pH 2.5 and pH 9.1 reflects a pH-dependent change in extinction coefficient for the proteins.

Triplicate measurements were carried out for standard proteins and for each sample of ASF, to determine the method precision. The relative standard deviations (R.S.D.s) for peak area of triplicate analyses of standard samples at approximately the concentration of interest of each protein were: (1) pH 2.5, 9% for albumin (40 mg ml^{-1}), 12% for transferrin (4.0 mg ml^{-1}) and 14% for γ-globulin (8.0 mg ml^{-1}). (2) pH 9.1, 11% for albumin (25 mg ml^{-1}), 4% for transferrin (2.0 mg ml^{-1}) and 4% for γ-globulin (15 mg ml^{-1}). It seems that imprecision comes about mainly due to errors in injection (since there is apparently little correlation between precision and sample concentration); this is consistent with our previous measurements of inorganic ions [8,9]. We have also previously shown that errors due to viscosity differences between samples or between samples and standards are not significant [9]. Replicate analyses of ASF samples (under a given set of separation conditions) gave R.S.D.s in the range 4–7% for albumin, 11–23% for transferrin and 3–18% for globulins. For migration times, the R.S.D.s were typically <2% for all analytes at both pH values. Because only pure γ-globulin standards were available, the time-normalised peak areas for the α- and β-globulins were compared to the γ-globulin calibration curve for quantitation.

Lysozyme, lactoferrin and mucins are also thought to be present in ASF [1]. In the present studies lysozyme was identified in ASF samples, a small peak being detected at the appropriate migration time (see insets in Fig. 1). This was quantitated to be ≈91 μg ml^{-1}. The value is an approximate one due to the limitations of UV detection. A lactoferrin standard was found to migrate between albumin and transferrin at pH 2.5, but was not observed in rat ASF. Mucins are heterogeneous glycoproteins associated with obstructive airway diseases [1,7]; under the present separation conditions at pH 2.5, bovine submaxillary mucin standards gave broad, poorly-defined peaks which migrated late in the electropherograms. Mucin peaks could not be identified in rat ASF (although this is perhaps not surprising in healthy animals), and alternative separation conditions will probably be necessary for these very large molecules.

Rat plasma was also analyzed (Fig. 2). The elution patterns of rat ASF proteins are very similar to that of plasma proteins under acidic as well as basic

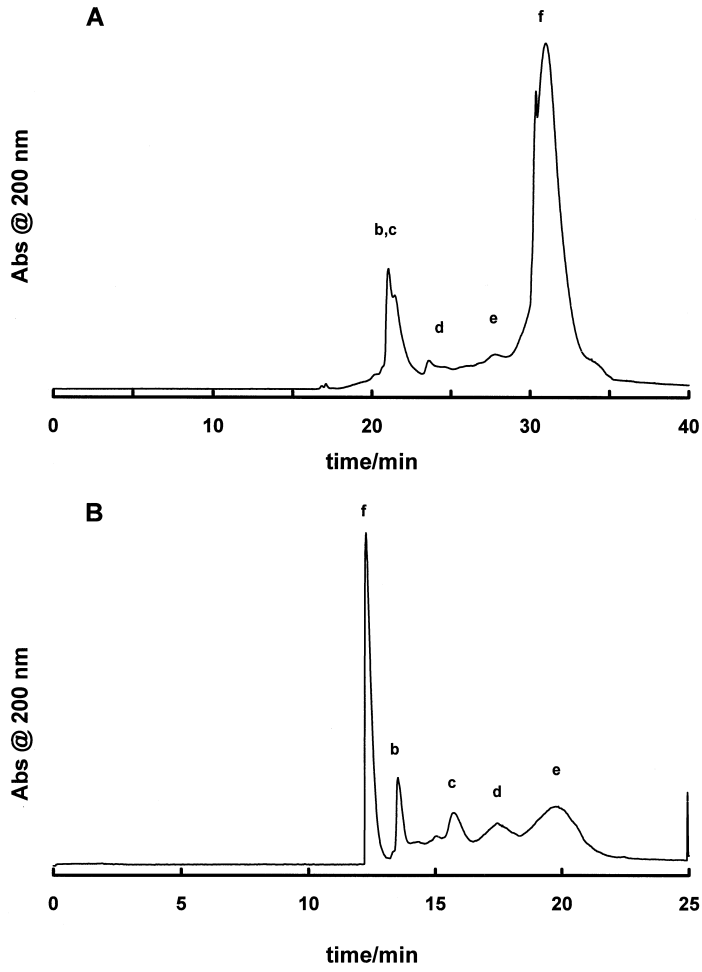

Fig. 2. Separation of major proteins in rat plasma. (A) High-pH separation using uncoated capillary. Conditions: fused-silica capillary [72 cm (effective length 50 cm)×50 μm, 150 μm O.D.]; field strength, 167 V cm^{-1} (i=40 μA); BGE, borate, pH 9.1; detection, UV at 200 nm. (B) Low-pH separation using capillary dynamically coated with spermine. Conditions: as above except field strength, 250 V cm^{-1} (i=36 μA); BGE, phosphate, pH 2.5 with spermine (0.5 mM) as capillary surface modifier. Peak assignments as in Fig. 1.

Table 1
The composition of major proteins determined in ASF and plasma from Fisher rats

Protein	Rat ASF (mg ml^{-1})	Rat plasma (this work) (mg ml^{-1})	Rat plasma (literature values) (mg ml^{-1})
Albumin	27.6±5.3[a]	30.9±4.7[e]	37.3[g]
Transferrin	4.0±0.8[a]	3.1±0.5[e]	3.0[h]
α-Globulin	17.5±3.2[b]	21.8±3.4[f]	17.1[g]
β-Globulin	8.0±2.5[c]	9.5±1.4[f]	10.7[g]
γ-Globulin	8.6±1.2[d]	8.1±2.4[f]	10.5[g]

Error values are the standard deviations on the mean of the measurements.
[a] n=7, 5 at pH 2.5 and 2 at pH 9.1.
[b] n=4, 3 at pH 2.5 and 1 at pH 9.1.
[c] n=3, 2 at pH 2.5 and 1 at pH 9.1.
[d] n=3, all at pH 2.5.
[e] n=5, 4 at pH 2.5 and 1 at pH 9.1.
[f] n=4, all at pH 2.5.
[g] Male albino rat plasma, Ref. [16].
[h] Human plasma value, Ref. [17].

conditions, at least in terms of major protein components. In general, the concentrations of protein in ASF and in plasma were similar (Table 1). Quantitative data for all proteins were obtained at pH 2.5. At pH 9.1, albumin and transferrin could be routinely quantitated (the sharper transferrin peak was integrated by skimming the baseline across the top of the broad γ-globulin peak), and α- and β-globulins also usually gave distinct, quantifiable peaks. Data presented in Table 1 comprises both results obtained at pH 2.5 and pH 9.1, as indicated. Our preference for quantitative measurements is pH 2.5, because of the better resolution achieved.

The similarity between the protein composition of rat ASF and plasma is quite surprising due to the fact that in healthy animals, plasma leak across the airway epithelium is considered to be relatively minor. Furthermore, although the amount of albumin and globulins observed in rat ASF is very similar to the values obtained for plasma, this is not the case with transferrin. In rat ASF, transferrin was found to be at higher concentrations than those observed in plasma. In contrast, inorganic ion concentrations in rat ASF using an identical sampling technique were generally much lower than plasma values [8,9]. This indicates that damage to the airway epithelium due to the sampling technique and subsequent leak of plasma proteins does not account for our findings; since under such conditions, the inorganic ion concentrations would be expected to approach plasma levels. Rather, our results are consistent with the notion that these ASF proteins are produced in the submucosal glands or the surface epithelium. Clearly, further investigation is needed to understand the sources of these proteins and the different regulatory processes which control protein concentrations in ASF.

In summary, the major proteins of rat ASF can be successfully separated and quantitated using our capillary sampling technique coupled with CE analysis. The protein separations were carried out under two different conditions with different selectivities, and both analyses confirm that albumin, transferrin and globulins are the major protein components in rat ASF. The direct sampling technique employed allows quantitative measurements to be made from discrete locations, unlike BAL sampling.

Acknowledgements

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