ELSEVIER

Journal of Chromatography B, 705 (1998) 203–211

JOURNAL OF CHROMATOGRAPHY B

Simplified method for purification of colostrum to obtain secretory component of immunoglobulin A, using secretory component as a reference protein in tracheal aspirate fluid

Sunita Goil, Charles Barnes, Donald Thibeault, William E. Truog*

Sections of Neonatology/Perinatology and of Allergy/Immunology, Department of Pediatrics, The Children's Mercy Hospital, The University of Missouri–Kansas City School of Medicine, 2401 Gillham Road, Kansas City, MO 64108, USA

Received 26 May 1997; received in revised form 8 October 1997; accepted 16 October 1997

Abstract

Many studies employ bronchoalveolar lavage fluid for assessment of biologically active substances secreted from the lung. However, investigators continue to search for a useful reference standard to correct for the inevitable but variable degree of dilution of this fluid. The glycoprotein, soluble secretory component of IgA, may serve as a valid reference protein. We report a simplified method for the purification of secretory component from colostrum. Soluble secretory component was isolated from human colostrum using serial centrifugation, size-exclusion fractionation and ion-exchange chromatography. Secretory component rich fractions were assayed by enzyme immunoassay. They were also evaluated for total amino acid content and distribution and sequence determination with satisfactory agreement with published results. We then demonstrated that soluble secretory component concentration in tracheal aspirate fluid did not correlate with either albumin or with total protein measured in the same samples. Therefore, we conclude that the secretory component of IgA serves as a useful reference marker because its use may avoid errors resulting from leakage of plasma proteins into epithelial lining fluid. Advantages of this method for establishing a standard for secretory component include ready availability of soluble secretory component, simplicity of the method and relative rapidity of the techniques. © 1998 Elsevier Science B.V.

Keywords: Immunoglobulin A; Soluble secretory component

1. Introduction

Airway fluid analysis remains an important tool for assessment of many aspects of pulmonary function. Interpretation of bioactive substances measured in alveolar lavage fluid is limited because of the absence of a reliable reference marker with which to standardize the concentrations of relevant substances in the retrieved epithelial lining fluid (ELF) [1]. This limitation has prompted attempts to identify reliable and reproducible reference substances. Candidate substances have included methylene blue [2], inulin [3], urea [4], total protein [5] and albumin [4,6]. Each of these reference standards can introduce error when values are calculated from epithelial lung fluid [1]. Difficulties encountered with putative reference standards include: diffusion of substances into or from instilled lavage fluid during the process of collection of ELF, uptake of the reference material by the cells lining the airways of the lungs, release of

*Corresponding author.

0378-4347/98/$19.00
PII S0378-4347(97)00523-9

the reference material into lavage fluid because of cell death or because of interruption of the integrity of the alveolar capillary barrier. The latter is a particular problem in diffuse pulmonary diseases in which alterations in permeability are likely to play an important role. This problem is encountered in virtually all of the important neonatal pulmonary disorders. An additional limitation for studies in neonates is the inability selectively to obtain distal airway or alveolar space ELF without variable dilution. Thus, an ideal reference standard would be a substance not found in plasma, but present in or secreted into ELF in a constant concentration relatively unaffected by disease states.

A recent study in preterm neonates [7] supports the use of the glycosolated polypeptide, soluble secretory component (SSC) of immunoglobulin A (IgA), as a useful reference substance. This substance is secreted by the bronchial and bronchiolar epithelium of the lung as early as after 16 weeks of gestation [8]. It is also secreted in the lymphoid tissues of mammary glands and is found in human colostrum. SC of IgA is a single polypeptide chain of approximately 500 amino acid residues with a large glycosolated region. It is found on the basolateral surfaces of secretory epithelial cells where it mediates the transport of polymeric immunoglobulin (pIg) into external secretions. SC is released into secretions either in the free form or bound to a pIg. Secretion of the free peptide appears to occur at a constant rate. In addition, its concentration in serum of newborns is very low and unchanging over the first weeks of life [7]. Thus, SC of IgA may be a useful reference protein for standardizing airway fluid measurements.

The first purpose of this report is to develop a simplified method for the purification of SSC as reference standard. We used commercially available polyclonal rabbit antisera in an enzyme immunoassay (EIA) for the detection of SSC, after SC was purified from a readily available source, human colostrum. The second purpose of the report is to compare values of SSC measured in samples of tracheal aspirate fluids obtained from term infants with respiratory failure with total protein and albumin measurements made in the same ELF layer samples. We thereby provide a simple means of establishing a reference standard for SSC of IgA, which proved useful for its application to the measurement in full term infants.

2. Experimental

2.1. Human volunteers

Clinical studies were performed after obtaining approval from the combined pediatrics institutional review board of the University of Missouri–Kansas City and The Children's Mercy Hospital. Specific written consent was not obtained from the parent(s) of each patient because no experimental manipulation was performed. Secretions obtained in the course of routine respiratory toilet were analyzed.

2.2. Reagents

The following chemicals were obtained from the sources indicated:

Polyclonal rabbit anti SC antibody and Horseradish peroxidase conjugate anti-SC antibody were obtained from Dako (Carpentaria, CA, USA). Sephracryl-S-300 was obtained from Pharmacia (Piscataway, NJ, USA). Bioscale diethylaminoethyl (DEAE) anionic exchange column and Bioscale S2 cation-exchange columns were obtained from BioRad (Hercules, CA, USA). Centricon specimen concentration devices were obtained from Amicon (Beverley, MA, USA). All other chemicals were obtained from Sigma (St Louis, MO, USA).

2.3. Collection of the tracheal aspirate samples

Tracheal aspirate samples were obtained from 20 full term infants with different pulmonary conditions treated with assisted ventilation. Fifteen were treated with a fraction of inspired oxygen (F_IO_2) of 1.0 and five with an F_IO_2 of 0.21. The latter group was intubated for nonpulmonary disorders. Aspirate samples were collected twice from each patient in Leukens trap devices during routine tracheal suctioning, once at 24–48 h of life and once at 48–72 h of life. The trachea was lavaged twice with 0.5 ml 0.9% saline. These samples were immediately frozen at −70°C after collection. The samples were thawed before EIA and an additional 0.5 ml of 0.9% saline

was added to each sample. The samples were emptied into polypropylene tubes and centrifuged at 1500 *g* for 15 min. After centrifugation the supernatant was assayed for SSC, total protein and albumin as described in Sections 2.5 and 2.6.

2.4. Purification of colostrum to obtain SSC component of IgA

To provide a standard for the assay, SSC was purified from 180 ml of colostrum obtained from healthy donors who had delivered their healthy infants at term. SSC was isolated from human colostrum by gel filtration and ion-exchange chromatography following a modified method previously published by Klingmuller and Hilschman [9].

Colostrum was first ultracentrifuged at 55 000 *g* for 30 min and the lipid rich upper layer was removed. Milk proteins were then separated from the fat-poor fluid by size on a Sephacryl-S-300 exclusion column (250×15 mm I.D.) in 0.1 *M* Tris buffer containing 1.0 *M* NaCl, pH 7.5 at a flow-rate of 1.0 ml/min. Eluted proteins were monitored by measurement of the UV responses at 280 nm and collected in 1-ml fractions. The fractions containing the highest concentration of free SC were collected for further purification.

The size-exclusion fractions of the colostrum protein containing high amounts of SC as determined by EIA were exchanged into 0.01 *M* sodium acetate, pH 5.1 using desalting chromatography columns and methods from BioRad and separated on a Bioscale S2 cationic exchange column (80×10 mm I.D.) employing a Model 2350 HPLC system with a Model 2360 gradient maker and a V4 detector all obtained from ISCO, Lincoln, NE, USA. The column was operated at a flow-rate of 1 ml/min.

The sample was injected dissolved in 0.01 *M* sodium acetate, pH 5.1, and eluted with 0.01 *M* sodium acetate, pH 5.1, containing 0.5 *M* NaCl. The gradient program was 5 min of buffer A with a linear gradient up to 100% buffer B in 5 min and an additional 15 min of 100% buffer B before a 5 min wash with buffer A to reequilibrate. Fractions were collected on an ISCO Foxy fraction collector and concentrated by centrifugation using a Centricon spin concentrator (Amicon). Fractions were assayed for SSC by EIA and the fractions with the highest levels were selected for further purification.

Fractions purified by cation-exchange chromatography were exchanged into 0.01 *M* sodium phosphate pH 7.6 using desalting chromatography columns and methods from BioRad and separated on a Bioscale DEAE anion-exchange column (80×10 mm I.D.) employing the same HPLC system as described before. The sample was injected dissolved in 0.01 *M* sodium phosphate pH 7.6 (buffer A) and eluted with 0.3 *M* sodium phosphate pH 4.8 (buffer B). The fractions were collected and concentrated as before. These fractions were assayed for SSC by EIA and the fractions with the highest levels (Fig. 3, fractions 24, 25 and 26) were selected for the use as SSC standard.

A portion of this SSC standard was subjected to total amino acid analysis and amino acid sequence determination at the Core Laboratory Facility of the University of Missouri at Kansas City, MO, USA.

2.5. EIA for determination of the SC of IgA in the colostrum and in the tracheal aspirates

For analytical determination of SSC we employed a standard sandwich EIA technique on 96 well Immulon 2 microtiter plates (Dynatech, Chantilly, VA, USA). Wash buffer, phosphate buffered saline–Tween-20–azide (PTA), contained 0.1 *M* sodium phosphate, 1.3 *M* sodium chloride, 0.2% sodium azide and 1% Tween-20 at pH 7.5. Phosphate–Tween (PT) buffer was also produced without azide for applications involving horse radish peroxidase enzyme. Coating buffer contained 0.05 *M* sodium carbonate and 0.2% sodium azide at pH 9.6. Plates were coated with polyclonal rabbit anti SSC antibody (100 μl per well of the commercial antibody at a dilution of 1:1000 in coating buffer), and kept overnight at 4°C. Next day the plate was blocked for 1 h at 37°C with 0.1% gelatin dissolved in the coating buffer. Blocked plates were washed with PTA buffer. After the blocking step, all use of sodium azide containing buffers was avoided.

A 1:100 dilution of SSC or a 1:10 dilution of defatted colostrum was applied to the top of the standard series column to obtain a calibration curve. The lavage samples containing an unknown amount of SSC were diluted 1:10 in PT buffer and applied to

the top well of each column containing an unknown series. These were then diluted in a half log scale down the plate.

The plate was again incubated for 1 h at 37°C and subsequently washed three times with the PT buffer. One hundred-μl aliquots of the horseradish–peroxidase conjugated antibody solution (diluted 1:1000 in PT buffer) were pipetted into each well. The plate was again incubated for 1 h at 37°C and washed three times with PT buffer. Next, 100 μl of horseradish peroxidase substrate was added (substrate prepared from 0.1% 3,3′-5,5′-tetramethylbenzidine and 0.03% hydrogen peroxide dissolved in 0.1% sodium acetate) into the wells and the plate was allowed to stand for 15 min. The absorbance at 450 nm in each well was measured with a MicroEIA reader (Dynatech MR7000, Dynatech, Chantilly, VA, USA). An 8-point standard curve was plotted in a log/linear scale and unknown values were calculated by the DIAS software (Dynatech). The average least squares correlation coefficient for four assays was 0.98 with a standard deviation of 0.02. Assays were performed in duplicate and all values determined to be in the linear range of the standard were averaged to give a final numerical result. Individual tracheal aspirate samples were assayed for SC of IgA after centrifuging at 2000 *g* for 15 min.

2.6. Measurements of total protein and of albumin in tracheal aspirate samples

Total protein was measured by the dye binding method of Bradford [10] utilizing a reagent kit purchased from BioRad. Specific human albumin was determined using a nephelometric method supplied by Beckman on a Beckman 360 Array nephelometer (Beckman Instruments, Fullerton, CA, USA). These assays were performed by the clinical laboratory of Children's Mercy Hospital.

2.7. Statistical methods

Statistical analysis included a determination of coefficient of variance (C.V.) which is the standard deviation of a set of numbers divided by the mean of the set multiplied by 100. Also, slopes, intercepts and correlation coefficients were calculated for lines constructed graphically from two sets of numbers by the algebraic method of least squares and calculated using the computer program EXCEL (Microsoft Corporation, Seattle, WA, USA).

3. Results

The purification of SSC from human colostrum according to the basic methods of Klingmuller and Hilschman [9] was modified to take advantage of high-pressure liquid chromatography techniques. The initial size-exclusion separation on Sephacryl S-300 (Fig. 1) indicates that the majority of the SSC is in the intermediate molecular mass range of the column. There is a minor amount of SSC in the initial volume indicating a slight propensity of the SSC either to polymerize or to aggregate with other high molecular mass proteins in the colostrum. After cation-exchange HPLC of fractions 13–15 of the size-exclusion chromatography (Fig. 2) two main fractions attributable to SSC proteins were obtained. When fraction 12 from the cation-exchange HPLC was chromatographed on a HPLC DEAE column (Fig. 3), the elution profile of the SSC coincided very nearly with the protein elution profile of the fractions.

A portion of fraction 26 was submitted for amino acid analysis and sequence determination. The results of the amino acid analysis indicated good agreement with previously published values (Table 1). The only unexpected observation is an increase in the Leu content and a concomitant decrease of Asp and Glu. The amino acid sequence determination for the first eight amino acids at the N-terminal end agreed exactly with previously published values [10–13] (Table 2). Quantitative determinations based on amino acid analysis indicated that the purified SSC fraction contained 62.4 μg protein per ml. Using this number it was determined through EIA using purified SSC as a standard that a sample of whole colostrum contained 24 μg SSC per ml. This sample of colostrum was diluted to contain 50% glycerol (as a preservative), aliquoted in 100 μl portions and stored at −20°C until it was used as standard preparation for routine assay. The initially purified SSC was brought to 50% glycerol content and stored at −20°C. The purified SSC standard is used as a primary standard for the production of secondary

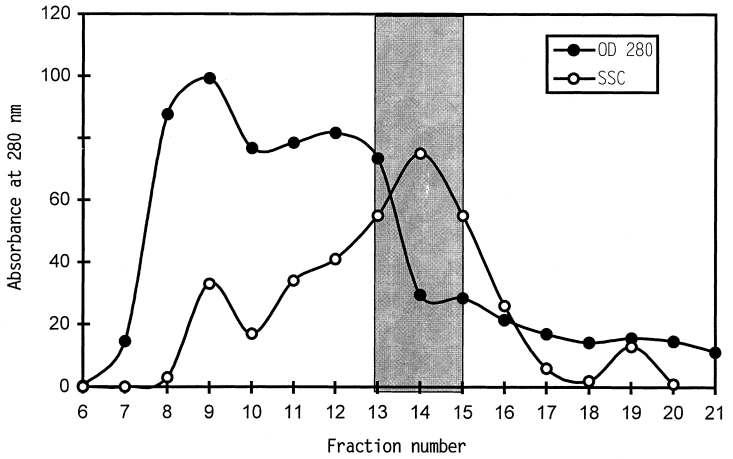

Fig. 1. Size-exclusion chromatography of human colostrum. The trace of optical density at 280 nm is represented by the closed circles and relative SSC concentration as measured by EIA is displayed by the open circles.

standard colostrum preparations which are used to produce standard curves for the individual assays.

The performance of the EIA is essentially the same for ELF samples and colostrum. For three comparisons of purified SSC with defatted colostrum the C.V. of the slopes was 1.7. As indicated in Fig. 4, the line produced by plotting the optical density of the individual wells against the log of the dilution for the standard and the samples is essentially parallel. The C.V. of the slopes of the standard curves for four individual assays was 10%. The typical assay employs eight individual determinations for the standard

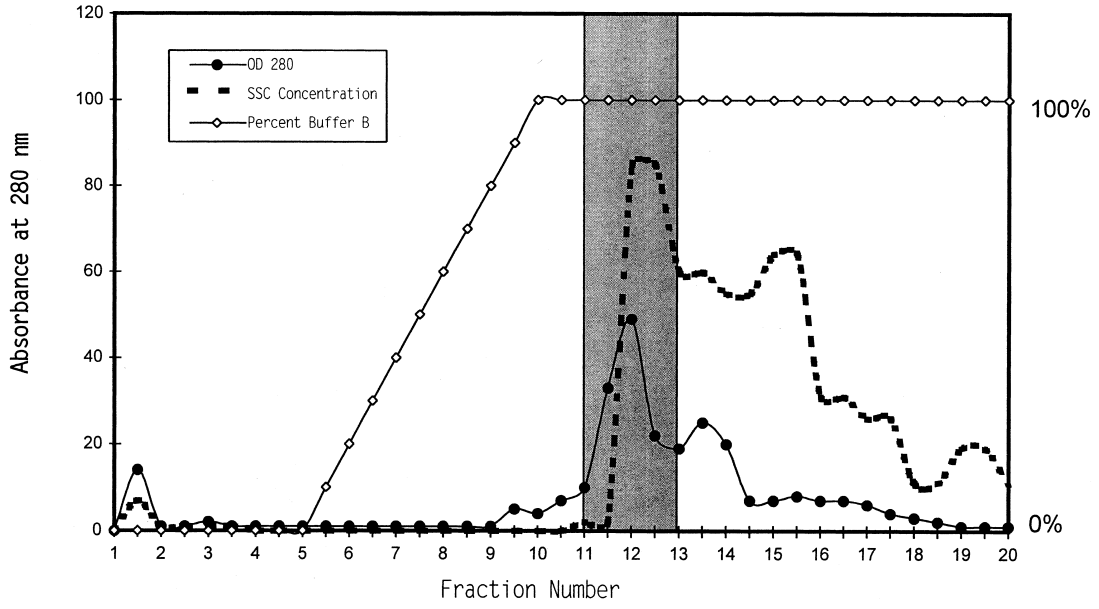

Fig. 2. Cation-exchange chromatography of SSC containing colostrum samples derived from size-exclusion chromatography. The trace of optical density at 280 nm is represented by the closed circles and the relative SSC concentration as measured by EIA is displayed by the dashed line. The percentage of buffer B is represented by the open diamonds.

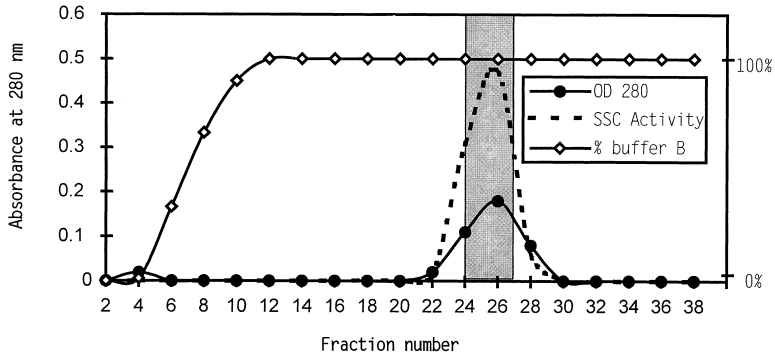

Fig. 3. Anion-exchange chromatography of SSC containing colostrum samples derived from cation-exchange chromatography. The trace of optical density at 280 nm is represented by the closed circles and the relative SSC concentration as measured by EIA is displayed by the dashed line. The percentage of buffer B is represented by the open diamonds.

curve and four individual determinations for each of the unknown samples. The linear range for this method was 5 ng/ml to 624 ng/ml. When the slopes of 10 unknown samples were compared the C.V. was increased to 13 which is acceptable for this type of immunoassay. The inter-assay C.V. was 6.7% ($n=8$ separate assays) and the intra-assay C.V. was 2.5% ($n=4$). The average SSC in 40 samples of ELF was 9.76 μg/ml with a standard deviation of 8.1 μg/ml and a range of 49.78 to 1.2 μg/ml. There was no difference in mean values for the 15 sick infants and for the five infants without pulmonary disorders. The range and mean value for SSC in the first collection was 10.6±9.4 μg/ml. For the second collection the mean±S.D. was 8.9+11.5 μg/ml.

When individual values for SSC were compared with individual values from the same sample of either albumin (Fig. 5) or total protein (Fig. 6), there was no detectable statistical correlation with either of these measurements (correlation coefficients of −0.09 and 0.03, respectively). Analysis of albumin and total protein was carried out in parallel to the measurement of SSC in 30 samples. There was a wide range of values for all three measurements, indicating the effects of dilution and at least in the case of protein and albumin, variable degrees of leakage from vascular to extravascular sites of these

Table 1
Amino acid composition comparison [10–13]

Amino acid	Calculated mole %	Published range
Asx	8.52	9.4–11.0
Glx	9.59	10.3–11.3
Ser	10.71	7.2–12.0
Gly	9.15	9.3–11.0
His	0.89	0.9–1.0
Arg	3.76	3.8–5.0
Thr	5.81	5.3–5.9
Ala	5.60	5.6–6.1
Pro	5.58	4.2–5.0
Tyr	3.03	3.6–4.2
Val	7.56	8.1–8.3
Met	0.00	0.0–0.5
Cys[a]	2.87	2.4–3.5
Ile	4.87	2.9–3.8
Leu	12.46	8.0–9.7
Phe	3.44	2.9–3.3
Lys	3.79	5.0–6.2
Trp[a]	2.36	2.0–3.2

[a] Estimated.

Table 2
Amino acid sequence comparison

Accession number	Sequence
Purified SSC	KSPIFGPE–
130 199	KSPIFGPE
224 300	KSPIFGPE
238 236	KSPIFGPE
255 098	KSPIFGPE
298 788	KSPIFGPE
388 280	KSPIFGPE
56 465	KSPIFGPE

Lys–Ser–Pro–Ile–Phe–Gly–Pro–Glu: Sequence of purified SSC material.
Lys–Ser: Two N-terminal amino acids determined by Klingmuller and Hilschman [9].
Sequence determination of purified SSC and six Gene Bank entries.

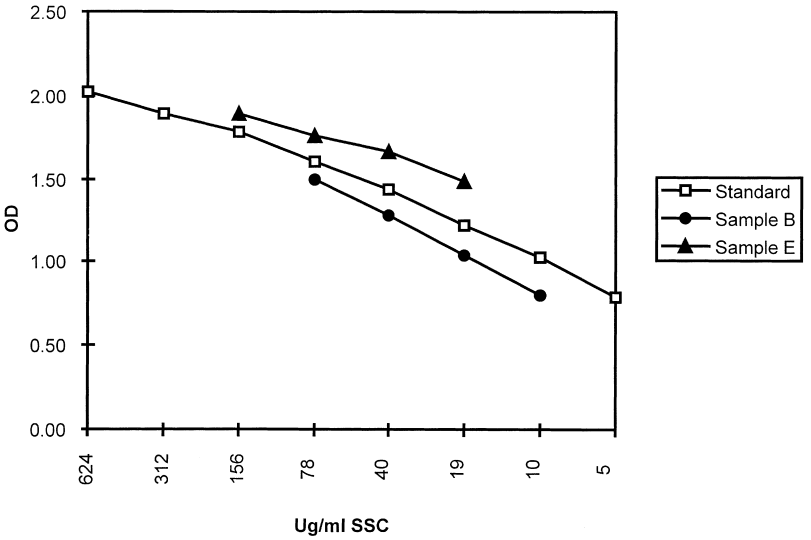

Fig. 4. Graph of optical density at 450 nm versus the log of the dilution of the SSC standard starting concentration of 24 μg/ml and two typical unknown samples. The curves are nearly parallel indicating that the measured component in either reference standard material or samples is identical.

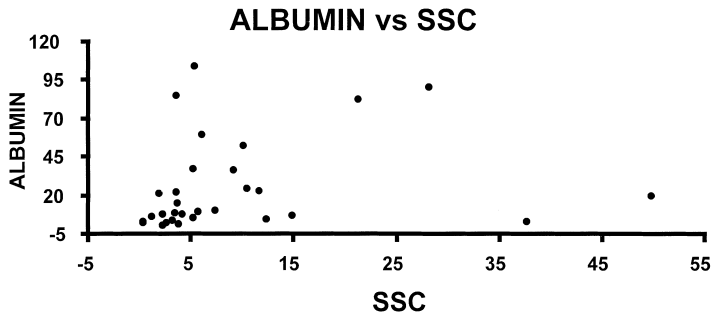

Fig. 5. Graph of SSC and albumin plotted from the same samples: there was no correlation.

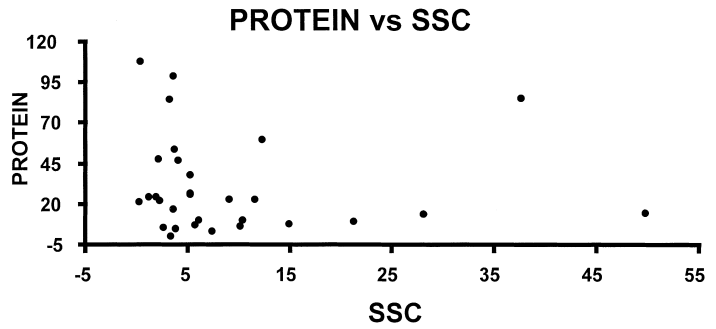

Fig. 6. Graph of SSC plotted against total protein measured in the same samples: there was no correlation.

proteins. Plasma levels of SSCs of IgA were not measured in this study.

4. Discussion

A simplified method to provide a reliable preparation of SSC of IgA, which can serve as a reference protein for measurements of a variety of substances of biological interest in tracheal and pulmonary epithelial secretions is described. The clinical relevance of this substance has recently been set forth by Watts and Bruce [7] and by Goil et al. [14]. In these studies, [7,14] conducted in both preterm and full term infants, both shortly after birth and over a variety of postnatal ages, it was demonstrated that SSC may be a more reliable reference protein than albumin or total protein or other substances which are present in the blood and may leak to a variable degree into pulmonary epithelial secretions. The current results, showing a lack of correlation between tracheal aspirate albumin, and SSC as well as total tracheal aspirate protein and SSC, are compatible with the results of Watts and Bruce [7].

A major focus of the present communication is to demonstrate that using a commercially available antibody and colostrum, a readily available fluid which is rich in SC, it is possible to allow the development of an analytical method to determine SSC for its application to the measurement of biological samples. Using both size-exclusion chromatography and cation-exchange HPLC for protein purification, we achieved a high degree of sample purity. The present method requires an antibody against SC and appropriate chromatographic equipment for purification, but no additional expensive laboratory equipment was necessary. Furthermore, the antibody is commercially available and no radioactive isotopes were required for the final immunoassay step. This method does have many of the inherent problems associated with EIA such as antibody depletion during dilution processes and competition of antibody for available epitopes; however, it performs well in laboratory conditions and has the potential to produce clinically useful results.

To provide further proof that the isolated component represented purified SC, amino acid analysis and amino acid sequence determination (Tables 1 and 2) were performed. The amino acid analysis indicated good agreement with the exception of the increase in Leu content and corresponding decrease in Asp and Glu. An additional proof that the isolated component we are measuring is in fact SSC of IgA, is given by the amino acid sequence determination of the amino acids at the N-terminus. These data agreed exactly with previously published values. This additional confirmatory step provides a high degree of reassurance that the substance measured is in fact SC.

We found that about 10 days elapsed from the start of sample acquisition through protein purification. Therefore, it proved to be reasonable to isolate sufficient amounts of SSC for its subsequent use as an internal reference standard. When prorated over many analyses which would directly or indirectly employ this internal reference standard, the relative initial start up time for making this assay available is modest. This is particularly important because of the current lack of acceptable standards for normalizing these highly variable fluids – especially in newborns in which deep bronchoalveolar lavage is contraindicated.

There exist several advantages of SSC as a reference protein. They include the fact that SSC is secreted into the pulmonary airspace from very early in fetal life, making it useful in even the most premature infants. Because SSC is a relatively large protein, and because it is normally not found in plasma, there is little opportunity for it to leak from airspaces back to the vascular compartment or vice versa. Its rate of secretion appears to be unaffected by disease states, especially those that primarily affect lung acinar areas, such as occur to preterm and term newborns. Its secretion appears to be independent of local IgA production and secretion.

One limitation to the use of a large protein such as SSC is the possible proteolytic loss of the antigenic site of the protein. This would cause falsely low SSC concentrations in ELF and hence boost the apparent concentrations of bioactive substances being measured. However, proteolysis is a risk associated with all protein substances used as references in ELF. The risk of this occurrence cannot be directly ascertained from our data. However, SSC was detected in variable amounts in all the samples assayed in our population, without regard to the degree of underly-

ing pulmonary illness. Thus, we infer the proteolytic loss of SSC was not a major problem in our population.

In summary, using readily available techniques and a readily available source of SC, purification of this material was obtained providing evidence that it should be superior to a use of total protein or of albumin as a reference standard in tracheal aspirate fluids.

Acknowledgements

Supported in part by grants from The Katherine B. Richardson Fund of Children's Mercy Hospital (C.B. and S.G.), The Children's Mercy Hospital Physician Scientist Award (W.E.T.) and The Wyeth Pediatrics Neonatology Fellows Research Award (S.G.).

References

[1] M.A. Berberich, Am. Rev. Respir. Dis. 141 (1990) S168–S202.
[2] R.P. Baughman, C.H. Basken, R.G. Loudon, P. Hurtubise, T. Wessler, Am. Rev. Respir. Dis. 128 (1983) 266–270.
[3] P. Von Wichert, K. Joseph, B. Muller, W.M. Franck, Am. Rev. Respir. Dis. 147 (1993) 148–152.
[4] S.I. Rennard, G. Basset, D. Lecossier et al., J. Appl. Physiol. 60 (1986) 532–538.
[5] T.W. Marcy, W.W. Merrill, J.A. Rankin, H.Y. Reynolds, Am. Rev. Respir. Dis. 135 (1987) 1276–1280.
[6] K.P. Jones, J.H. Edwards, S.P. Reynolds, T.J. Peters, B.H. Davies, Eur. Respir. J. 3 (1990) 152–156.
[7] C.L. Watts, M.C. Bruce, J. Pediatr. 127 (1995) 113–122.
[8] T. Takemura, E. Yoshinobu, Am. Rev. Respir. Dis. 131 (1985) 125–130.
[9] D. Klingmuller, N. Hilschman, Hope-Seyler's Z. Physiol. Chem. Bd. 360 (1979) 1895–1902.
[10] M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[11] P. Krajci, K.H. Grzeschik, A.H. Geurts van Kessel, B. Olaisen, P. Brandtzaeg, Hum. Genet. 87 (1991) 642–648.
[12] P. Krajci, D. Kvale, K. Tasken, P. Brandtzaeg, Eur. J. Immunol. 22 (1992) 2309–2315.
[13] M.A. Kulseth, P. Krajci, O. Myklebost, S. Rogne, DNA Cell Biol. 14 (1995) 251–256.
[14] S. Goil, W.E. Truog, C. Barnes, M. Norberg, M. Rezaiekhaligh, D.W. Thibeault, J. Pediatr., (1997) in press.