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MISCELLANEOUS

Quantification of tear proteins by SDS-PAGE with an internal standard protein: A new method with special reference to small volume tears

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Abstract
Background Quantitative determination of tear proteins is critical to our understanding of those ocular diseases with tear protein changes, but remains technically complex due to the small sample volumes available from patients. The aim of this study was to efficiently quantify the tear proteins by SDS-PAGE with an internal standard protein in small tear volumes.
Methods Schirmer test paper and capillary tubes were used to collect tear samples. Soybean trypsin inhibitor (SBTI) was used as an external standard or an internal standard to analyze tear samples in 15% SDS-PAGE gel. The total tear protein and its major components were quantified by band densitometry. Total tear protein concentrations were also measured by Bradford assay. Using this internal standard method, we compared differences between tear samples collected by the Schirmer test paper and capillary tube, and then examined the differences detected between tear samples obtained from young and elderly people.
Results Using SBTI as an internal standard in SDS-PAGE, the total tear protein concentrations were determined to be 12.03±0.45 mg/ml, showing no difference from the result determined by the Bradford assay ($P>0.05$). The quantities of the eight major tear protein bands were 2.26±0.07 (18.8%), 0.10±0.01(0.8%), 0.63±0.13(5.3%), 0.52±0.07 (4.3%), 1.14±0.18(9.5%), 1.33±0.21(11.1%), 2.76±0.16 (23.0%), and 2.95±0.13 mg/ml (24.5%), similar to the result obtained by using SBTI as an external standard ($P>0.05$). Comparing the methods of collection, we identified that the Schirmer test paper sampling induced increased concentrations of band 2 (mainly HSA) in tears, and decreased five other major tear protein bands. Comparing total protein concentrations among different age groups, we noted a higher total protein concentration in young people ($P<0.05$). This high protein level was contributed equally by all major tear protein components.
Conclusion Using SBTI as an internal standard, we can simultaneously quantify total tear protein and the major tear protein components by SDS-PAGE densitometry using small volume tears. This method appears promising for use as a diagnostic tool for identifying the occurrence of ocular diseases with tear protein changes.

Keywords SDS-PAGE · Tear protein · Internal standard · Protein quantification · Densitometry

Introduction

Changes in tear protein concentrations are associated with many systemic and ocular diseases [1–6]. Quantitative determination of tear proteins is of increasing interest in ophthalmology, but remains a technical challenge due to the small sample volumes available and the complexity of its composition. Many methods have been previously described for quantification of individual proteins in tears, including immunological techniques, high-performance liquid chromatography (HPLC), and the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) densitometry method [7–11]. The immunological techniques are generally complicated, expensive and time-

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consuming. Furthermore, each of these techniques usually is restricted to testing a single protein in the tears. These disadvantages make it challenging to effectively translate the approaches to clinical application in ophthalmology.

The most abundant proteins detected in tear samples include lactoferrin (1350–6300 μg/ml), lysozyme (500–4500 μg/ml), sIgA (186–2420 μg/ml), lipocalin (500–1500 μg/ml), CuZn superoxide dismutase (760 μg/ml), IgA (186 μg/ml), cystatins (120.7±19 μg/mg), and α1-protease inhibitor (106.1±41.7 μg/mg), and account for more than 90% of the total tear proteins [12–14]. Using a combined HPLC-ELISA procedure, Fullard et al. measured 13 specific proteins in non-stimulated and stimulated tears produced by normal subjects, and found these proteins represented 77% of the total tear protein that was determined by the Bradford assay with 10 μl tears [15]. However, the procedure was technically complicated, and 10 μl tears were hard to obtain without stimulation. SDS-PAGE has been used for many years to efficiently analyze human tear proteins. The advantages of this technique remain in the fact that it allows visualization of the whole tear protein profile, is capable of analyzing several tear samples in one experiment, and requires a minimal amount of sample volume. According to previous studies, whole tear sample separated by SDS-PAGE gel yielded a defined profile of 7–10 major tear protein bands [16–20]; in addition, as little as 20 ng/band could be visualized by this procedure [21, 22]. Accordingly, proteins above 100 μg/ml in tear samples may be visualized on SDS-PAGE from as little as 0.2 μl tear volume loaded. Some investigators have used SDS-PAGE densitometry method to quantify the protein bands visualized in the gels [7, 23–25]. According to previous reports, the main drawback of this technique is that it's difficult to accurately and reproducibly quantify stained proteins in the gel [11, 26].

The aim of this study was to improve the accuracy and reproducibility of SDS-PAGE densitometry by adding an internal standard in tear protein samples. Using this method, each tear protein band within a gel was normalized to the internal standard. This enabled accurate quantification of tear protein bands and accurate comparison between gels.

Materials and methods

Tear sample collection

Tear samples were collected from young and elderly people by Schirmer test paper and capillary tube. All the subjects were not contact lens wearers, had normal ocular surface, and were generally healthy. Informed consent was obtained from each subject, and ethics approval for this work was obtained from the Zhongshan Ophthalmic Center, Sun Yat-sen University.

Schirmer test paper (Tianjin Jingming New Technological Development Tianjin Co., Ltd, Tianjin, China) tear collection was performed by placing the Schirmer test paper at the junction of the middle and lateral thirds of the lower eyelid. The subjects were instructed to close their eyes for 5 min during which the collection took place. Capillary tube tear collection was carried out by placing a 100 mm length by 0.5 mm inner diameter capillary tube (Huaxi Medical University Instrument Factory, Chengdu, China) on the inferior meniscus under slit-lamp microscopy with soft illumination. Typically, 2–5 μl tears were collected within 2 min. Care was taken to avoid touching either the corneal or conjunctival surfaces.

For comparison of (1) using bovine serum albumin (BSA), soybean trypsin inhibitor (SBTI) and egg-white lysozyme as standards in Bradford assay, and (2) quantification of tear protein bands using SBTI as an external and internal standard in SDS-PAGE gel, tears were repeatedly collected from one subject (25 years old, female) by Schirmer test paper without anesthesia. For the comparison of tear samples collected by Schirmer test paper and capillary tube, tears were collected from ten healthy young people (20.5±0.5 years old, five males and five females), first by capillary tube without anesthesia, and then following 30 minutes of rest, by Schirmer test paper under topical anesthesia. For the comparison of tear protein between the young and the elderly people, tears were collected from 12 elderly individuals (74±10.5 years old, five males and seven females, pre-cataract surgery patients without other ocular diseases) by capillary tube without anesthesia.

For tears collected by Schirmer test paper, volumes of tears were determined by: (1) a standard curve constructed by the amount of 1 mg/ml BSA applied to the Schirmer test paper against the wetted length of the Schirmer test paper, and (2) differences in the weight of the Schirmer filter paper before and after wetting. The average tears in 10-mm wetted Schirmer test paper were 7 μl, which agreed with the previous report [27].

Protein sample preparation

Tears collected by capillary tube were blown into Eppendorf tubes with an ear-washing bulb and centrifuged for 10 min at 14000×g. The supernatants were diluted 10 times by addition of ultra-pure water, stored at −70°C, and used within 2 months.

For the samples collected by Schirmer test paper, the flexed part that contacted the conjunctiva was cut off, using the first 10-mm strip with scale. The 10-mm strip was placed in a 1.5 ml Nanosep MF 0.2 μm centrifugal device (Pall Corporation P/N ODM02C33, Ann Arbor,MI, USA). 100 μl of 100 mM ammonium bicarbonate was added into

the device and incubated at room temperature for 1 hour [20]. Then, the tear protein solution was eluted by centrifugation for 10 min at 14000×g. The eluted tear protein solutions were stored at −70°C and used within 2 months.

One mg/ml of BSA (Shanghai Shengzheng Biotechnology Co., Ltd, Shanghai, China), SBTI (Sigma, St. Louis, MO, USA) and egg-white lysozyme (Chongqing Kerun Biomedical R&D Co., Ltd, Chongqing, China) were prepared and used for Bradford assay and SDS-PAGE.

Establishing the optimum standard

According to image analysis, the blank area between the tear protein bands would be a suitable place to add an internal standard protein. Several commercially available single protein markers were compared with the tear protein pattern in different concentration of SDS-PAGE gels; STBI was chosen as the most suitable internal standard protein candidate for use in 15% SDS-PAGE gel. Then the affinity for Coomassie Brilliant Blue G250 (CBB G250) of SBTI compared to BSA and lysozyme was investigated by the Bradford microassay system [28]. Total tear protein concentrations were determined by Bradford assay, using BSA, SBTI and lysozyme as standards. Next, the responses of SBTI, BSA and lysozyme to CBB R250 in gel were investigated.

Quantification of tear protein bands with SBTI as external and internal standards

SDS-PAGE was performed on a mini-vertical electrophoresis system (Bio-Rad Mini-PROTEAN® 3 Cell, Bio-Rad Laboratories, Hercules, CA, USA), in accordance with the discontinuous buffer system of Laemmli [29]. The gel was 8.3 x 7.3 cm^2 large and 1 mm thick, with 15 wells. Each sample was mixed with 2×SDS-PAGE sample buffer and heated to 100°C for 5 min before loading. Tear proteins were separated on a 15% separating gel (0.1% SDS, 1.5 M Tris-HCl, pH 8.8) with a 5% stacking gel on top (0.1% SDS, 0.5 M Tris-HCl, pH 6.8), under reducing conditions. Typically, 8–10 μl sample solution containing 0.21 μl tears (calculated from 7 μl tears/10 mm wetted Schirmer test paper) was loaded. For constructing a standard curve, increasing amounts of SBTI (20, 50, 100, 200, 300, 400, 500, 600, 700 and 800 ng respectively) with or without 0.21 μl tears were added in the different lanes.

Electrophoresis was performed in electrode buffer (0.1% SDS, 0.25 M glycine, 0.025 M Tris-HCl, pH 8.3) at 60 V for 10 min, and then switched to 120 V for 120 min. Each experiment was repeated in three gels. After SDS-PAGE, the gels were stained and destained according to the rapid CBB R250 staining method following the procedures reported by Wong [21].

Digital images of the SDS-PADE gels were captured with the G:BOX system (Syngene, Cambridge, England) using GeneSnap acquisition software in a transillumination mode. The images were analyzed by Quantity One (Bio-Rad Laboratories, USA). Quantity One calculated the "trace quantity" (densitometric value) of the band according to the Quantity One manual. As described in the manual, each identified band is defined by brackets above and below the band. The width of each set of brackets is determined by the lane sampling width. The height of the brackets is determined automatically, using a band-finding formula together with parameters selected. When a band is quantitated, the average intensity of each horizontal row of pixels within the brackets is calculated. Next, the number of pixel rows between the top and bottom brackets is determined. Taken together, these result in an intensity profile for the band. Finally, the area under the profile curve to the baseline is integrated, resulting in units of intensity x millimeters. This is the "trace quantity" of the band. Standard curves used for quantification of the major tear protein bands (tears repeatedly colleted from one subject) were plotted by the trace quantity of SBTI band against the quantity of SBTI loaded. The total tear protein concentrations were estimated by calculating the sum of the individual tear protein bands.

Comparison of different tear samples

For the comparison of tear sample methods and tear protein concentrations between different age groups, 400 ng SBTI was added to different tear samples as an internal standard. The tear protein band quantities were calculated by:

$$\frac{\text{The trace quantity of the tear protein bands}}{\text{The trace quantity of 400 ng SBTI}} \times 400\text{ng}$$

Statistics

The comparison of BSA, SBTI, and lysozyme as standards in Bradford assay was assessed by one-way ANOVA. The comparison of total tear protein concentrations determined by band densitometry and Bradford assay was assessed by independent sample *t*-test for tear samples repeatedly collected from one subject and paired sample *t*-test for different tear samples. The comparison of using SBTI as an external and internal standard in SDS-PAGE gel was assessed by independent sample *t*-test. The differences between the tear samples collected by Schirmer test paper and capillary tube were assessed by paired sample *t*-test. The differences between the tear proteins of the young and elderly people were assessed by independent sample *t*-test. For all statistical tests, $p<0.05$ was considered significant.

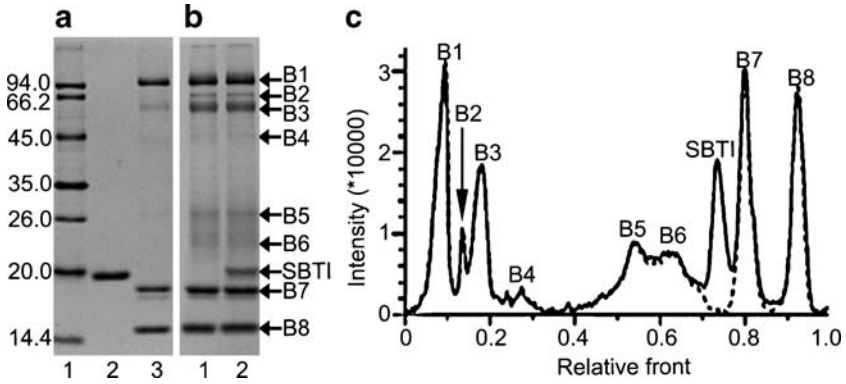

Fig. 1 SBTI did not overlap the major tear protein bands. **a** Lane 1 depicts the protein marker (94.0, 66.2, 45.0, 35.0, 26.0, 20.0 and 14.4 kDa). Lane 2 depicts 400 ng SBTI. Lane 3 was loaded with 8 μl sample solution containing 0.20 μl tears collected by capillary tube. **b** Lane 1 was loaded with 10 μl sample solution containing 0.21 μl tears collected by Schirmer test paper without anesthesia. Lane 2 was loaded with 10 μl sample solution containing 400 ng SBTI and 0.21 μl tears collected by Schirmer test paper without anesthesia. **c** Lane comparison between the tear proteins (dotted line, lane 1 in **b**) and the mixture of tear proteins with SBTI (solid line, lane 2 in **b**). *B1*, Band 1; *B2*, Band 2; *B3*, Band 3; and so forth

Results

SBTI was identified as a suitable internal standard

In 15% SDS-PAGE, 10 μl sample solution containing 0.2–0.3 μl tears was loaded, and eight major bands were visualized (Fig. 1). Bands 1–8 were about 90, 66, 60, 44, 26, 22.2, 18, 14.8 kDa respectively. There was a blank area between Band 6 and Band 7 around 20 kDa. SBTI, a protein marker with molecular weight of 20.1 kDa, didn't overlap the major tear protein bands, and appeared likely to serve as an effective internal standard protein.

In Bradford assay, 1 ml 0.01% CBB G250 reagent solution was added to equal amounts of BSA, SBTI or lysozyme (range from 1–10 μg in 0.1 ml water solution). The absorbances of the three proteins at 595 nm were nearly the same (Fig. 2a). The total tear protein concentrations determined by the Bradford assay using BSA, SBTI and lysozyme as standards were 12.09±1.06 mg/ml, 12.26±1.09 mg/ml, and 11.70±1.08 mg/ml respectively ($P>0.05$, Fig. 2b).

In SDS-PAGE gel, when equal amounts of BSA, SBTI and lysozyme were loaded in the same lane (Fig. 3), the trace quantities (densitometric value) of the three protein bands were nearly equal to each other. Each of them evidenced a good linear relationship (Pearson $r>0.98$, $P<0.01$) between the trace quantity of the protein band and the known amount of protein loaded in the range from 20~1000 ng. These results suggested that SBTI can be used as a standard to quantify protein levels by SDS-PAGE densitometry.

Quantification of tear protein on SDS-PAGE

As mentioned above, total tear protein concentration determined by the Bradford assay using SBTI as a standard was 12.26±1.09 mg/ml. We quantified the same tear

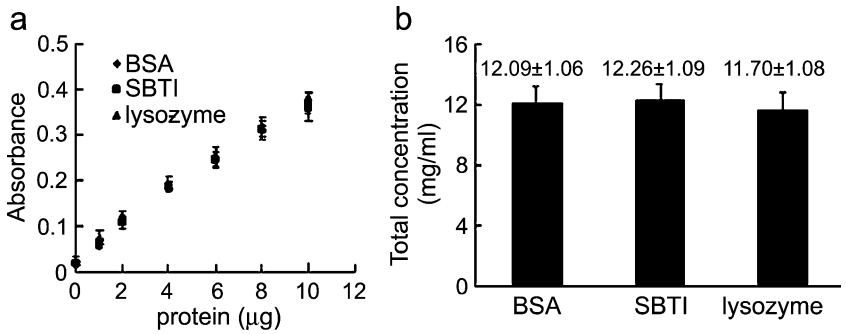

Fig. 2 Total tear protein concentrations as determined by the Bradford assay. **a** Equal amounts of BSA, SBTI and lysozyme (ranging from 1–10 μg in 0.1 ml water solution) were mixed with 1 ml 0.01% CBB G250 reagent solution. Absorbance was recorded on a spectrophotometer at 595 nm. **b** Total tear protein concentrations were determined by the Bradford assay using BSA, SBTI and lysozyme as standards (data represented three individual experiments)

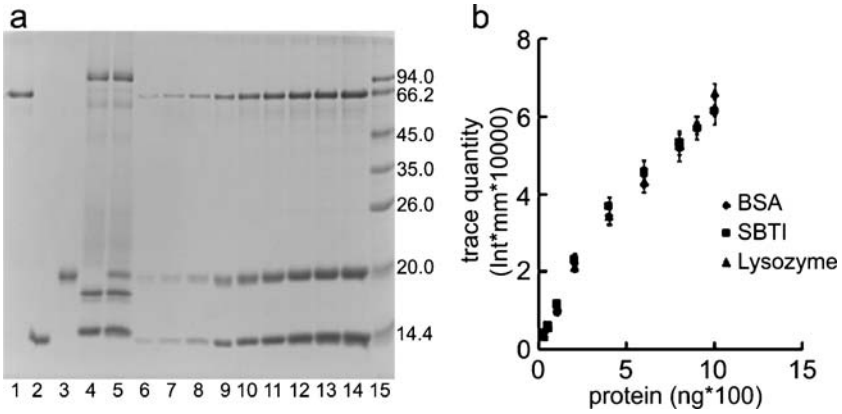

Fig. 3 Response of proteins to CBB R250 in gel. **a** SDS-PAGE of BSA, SBTI, lysozyme and tear proteins. Lanes 1–3 were loaded with BSA, SBTI or lysozyme respectively. Lane 4: tear proteins. Lane 5: the mixture of tear proteins (same as lane 4) and SBTI. Lanes 6–14 were loaded with different amounts of equal mixtures of BSA, SBTI and lysozyme (25, 50, 100, 200, 400, 600, 800, 900 and 1000 ng respectively). Lane 15 was loaded with protein marker (same as Fig. 1). **b** The linear relationship between the trace quantity of the protein bands and the amount of the protein loaded. The trace quantity was calculated from the SDS-PAGE by the Quantity One software package

sample by SDS-PAGE densitometry using SBTI as a standard (Fig. 4, Table 1). With SBTI as an internal standard, the total tear protein concentration as estimated by the sum of the individual tear protein bands was 12.03± 0.45 mg/ml, similar to the value determined by the Bradford assay ($P>0.05$). The quantities of the eight major tear protein bands were 2.26±0.07(18.8%), 0.10±0.01 (0.8%), 0.63±0.13(5.3%), 0.52±0.07(4.3%), 1.14±0.18 (9.5%), 1.33±0.21(11.1%), 2.76±0.16(23.0%), and 2.95± 0.13 mg/ml (24.5%).

With SBTI as an external standard, the eight major tear protein band quantities were approximately the same as the result obtained by using SBTI as an internal standard ($P>0.05$, Table 1). And the total tear protein concentrations were almost equal to the result determined by the Bradford assay ($P>0.05$).

Comparison of tear samples collected by Schirmer test paper and capillary tube

Using SBTI as an internal standard, we analyzed tear samples collected by Schirmer test paper and capillary tubes from ten young people (Fig. 5, Table 2). For samples collected by capillary tube, the total protein levels (12.28± 1.53 mg/ml) were higher than that of the samples collected by Schirmer test paper (9.75±0.90 mg/ml, $P < 0.05$). By

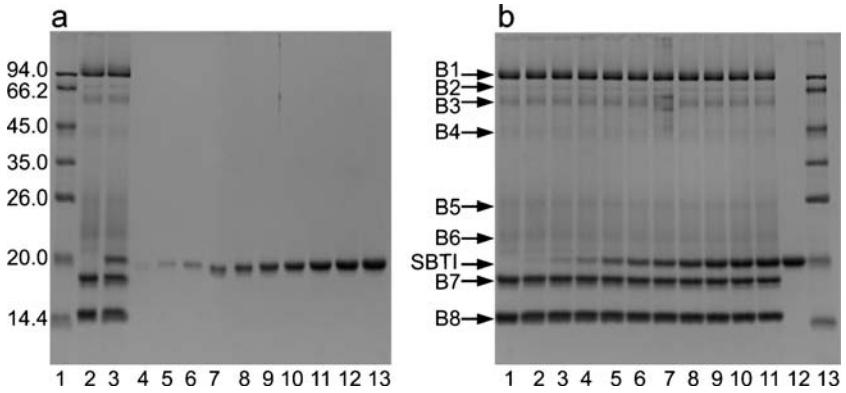

Fig. 4 SDS-PAGE of tear samples with SBTI as external and internal standards. **a** SDS-PAGE of tear samples with SBTI as an external standard. Lane 1 was loaded with protein marker (same as Fig. 1). Lane 2 was loaded with 10 μl sample solution containing 0.21 μl tears. Lane 3 was loaded with 10 μl sample solution containing 0.21 μl tears and 400 ng SBTI. Lanes 4–13 were loaded with increasing amounts of SBTI (20, 50, 100, 200, 300, 400, 500, 600, 700 and 800 ng respectively). **b** SDS-PAGE of tear samples with SBTI as an internal standard. Lane 1 was loaded with 10 μl sample solution containing 0.21 μl tears. Lanes 2–11 were loaded with 10 μl sample solution containing 0.21 μl tears and increasing amount of SBTI (20, 50, 100, 200, 300, 400, 500, 600, 700 and 800 ng respectively). Lane 12 was SBTI only. Lane 13 was protein marker. All the tear proteins used in this experiment were collected from one subject by Schirmer test paper without anesthesia. *B1*, Band1; *B2*, Band2; *B3*, Band3; and so forth

Table 1 The quantities of tear protein bands[a]

Band no.	External standard		Internal standard		*P*-value*
	(*n*=6, in mg/ml)	CV[c]	(*n*=11, in mg/ml)	CV	
1	2.27±0.18 (18.7%)	7.9%	2.26±0.07 (18.8%)	3.1%	0.843
2	0.10±0.01 (0.8%)	10.0%	0.10±0.01 (0.8%)	10.0%	0.615
3	0.69±0.05 (5.7%)	7.2%	0.63±0.13 (5.3%)	20.6%	0.214
4	0.49±0.18 (4.1%)	36.7%	0.52±0.07 (4.4%)	13.5%	0.71
5	1.16±0.17 (9.6%)	14.7%	1.14±0.18 (9.5%)	15.8%	0.811
6	1.39±0.18 (11.4%)	12.9%	1.33±0.21 (11.1%)	15.8%	0.572
7	2.74±0.19 (22.5%)	6.9%	2.76±0.16 (23.0%)	5.8%	0.892
8	2.92±0.12 (24.0%)	4.1%	2.95±0.13 (24.5%)	4.4%	0.687
Other bands	0.39±0.06 (3.2%)	–	0.34±0.05 (2.8%)	–	–
SUM[b]	12.17±0.56	4.6%	12.03±0.45	3.7%	0.597

Values are presented as mean ± standard deviation (%).
[a] Percentages were determined by: tear protein band quantities / SUM*100%. [b] SUM (the total tear protein concentration) was the sum of individual bands.
[c] CV, coefficient of variation= standard deviation/mean*100%.
**P*-values determined by independent sample *t*-test. Tear samples were repeatedly collected from one subject by Schirmer test paper, no anesthesia.

performing band analysis, we found that the overall increases were due to increases in Bands 1, 3, 5, 6, 7 in tear samples collected by capillary tube ($P < 0.05$). On the other hand, Band 2 was much higher in tear samples collected by Schirmer test paper ($P<0.05$). The quantities of Bands 4 and 8 showed no statistically significant differences between two sampling methods ($P>0.05$). For the two sampling methods, the total protein concentrations determined by band densitometry and Bradford assay showed no significant differences ($P>0.05$).

Comparison of tear proteins between young and elderly people

Using SBTI as an internal standard, we analyzed tear proteins from young and elderly people (Fig. 6, Table 3). The total protein concentrations of the young people (12.28± 1.53 mg/ml) were much higher than those of the elderly people (8.12±1.07 mg/ml, $P<0.05$). The quantities of the eight major tear protein bands from the young people's samples were all much higher than those from the elderly people ($P<0.05$). However, when we compared the percentages of each band, the percentage contents of Band 5 and Band 8 were much higher in the elderly people's samples. On the other hand, the percentage content of Band 1 was higher in the young people's samples. The percentage contents of Bands 2, 3, 4, 6 and 7 from the young and the elderly did not show significant differences. Within each group of people, the total tear protein concentrations determined by band densitometry and Bradford assay showed no obvious differences ($P>0.05$).

Discussion

Human tear fluid, characterized by very small volumes and very large orders of magnitude of complex protein constituents, is important for proper physiological function of the ocular surface, and alterations to this fluid are associated with various systemic and ocular diseases.

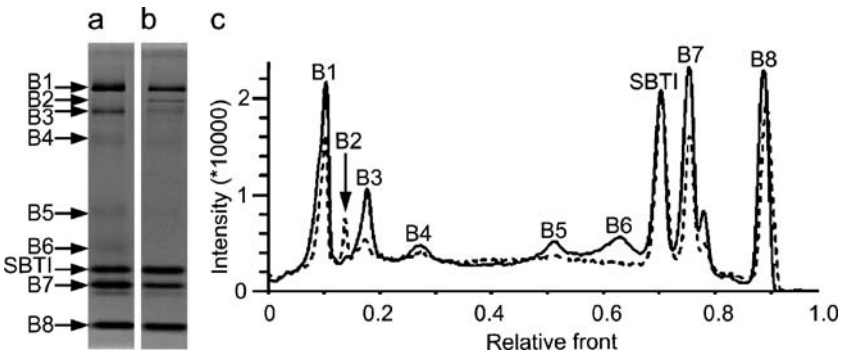

Fig. 5 The comparison of tear collection methods with SBTI as an internal standard. **a** Loaded with 8 μl sample solution containing 400 ng SBTI and 0.20 μl tears collected by capillary tube. **b** Loaded with 10 μl sample solution containing 400 ng SBTI and 0.21 μl tears collected by Schirmer test paper under anesthesia. **c** The comparison of protein profiles between tears collected by capillary tube and Schirmer test paper from the same subject. (solid line, tears collected by capillary tube; dotted line, tears collected by Schirmer test paper). *B1*, Band1; *B2*, Band2; *B3*, Band3; and so forth

Table 2 Comparison of tear proteins between the samples collected by Schirmer test paper and capillary tube[a]

Band no.	Schirmer test paper (n=10, in mg/ml)	Capillary tube (n=10, in mg/ml)	**P**-value
1	1.79±0.35 (18.4%)	2.64±0.60 (20.6%)	<0.001*
2	0.53±0.24 (5.4%)	0.26±0.07 (2.0%)	0.002*
3	0.88±0.16 (9.0%)	1.61±0.47 (12.6%)	<0.001*
4	0.82±0.13 (8.5%)	0.88±0.13 (6.9%)	0.311
5	0.89±0.10 (9.1%)	1.23±0.25 (9.6%)	0.002*
6	0.44±0.10 (4.5%)	0.89±0.19 (7.0%)	<0.001*
7	1.65±0.27 (16.9)	2.41±0.32 (18.8%)	<0.001*
8	1.93±0.30 (19.8)	2.05±0.25 (16.0%)	0.316
Other bands	0.81±0.10 (8.3%)	0.83±0.14 (6.5%)	–
SUM[b]	9.75±0.90	12.28±1.53	<0.001*
Total concentration (Bradford assay)	9.08±1.50	13.47±1.63	0.001*

Values are presented as mean ± standard deviation (%). [a] Percentages were determined by: tear protein band quantities / SUM*100%. [b] SUM (the total tear protein concentration) was the sum of individual bands. *P-values determined by paired sample t-test. P<0.05 was considered significant.

Although tear proteome studies demonstrate there are hundreds of proteins in human tears, only a few are those most highly abundant proteins [12, 19]. To date, very few tear protein tests have been applied to clinical practices [30, 31]. The clinical applications of tear tests are limited by the minimal tear volume and complex tear components. In the present study, we used SBTI as an internal standard to determine both the total tear protein levels and the major tear protein components by SDS-PAGE densitometry. The advantages of our method are the abilities to: (1) reveal the total tear protein content and tear protein profile, (2) quantify the major tear protein components, (3) analyze more than 10 different tear samples in one measurement (SDS-PAGE), and (4) perform efficiently using only a very small tear sample volume (≥0.2 μl). Although one band may represent a complex of different proteins, the major bands on the gel represent the recognized highly abundant tear proteins. Therefore, the present method provides accurate information for the major tear protein constituents and the recognized profile of highly abundant proteins, making this method particularly useful in the clinical setting.

The choice of the internal standard protein

On the SDS-PAGE gel, an internal standard must not overlap with the major tear protein bands. We investigated the tear protein pattern as obtained by varying concentrations of polyacrylamide gels, and compared with the results of other published studies [19, 20, 24, 32, 33].

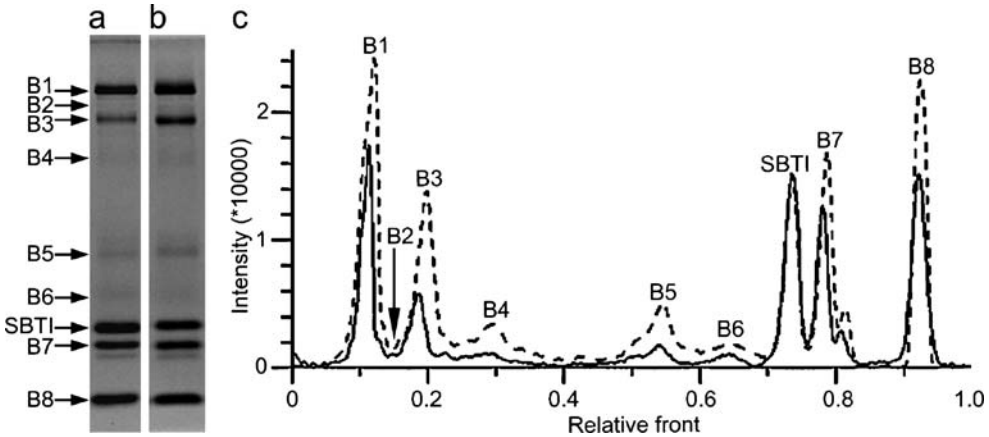

Fig. 6 The comparison of tear proteins between elderly and young individuals using SBTI as an internal standard. In SDS-PAGE gel, 8 μl sample solution containing 400 ng SBTI and 0.20 μl tears from the elderly (**a**) or young people (**b**) was loaded. **c** The comparison of tear protein profiles from the elderly and the young people (solid line, tears collected from elderly individuals; dotted line, tears collected from the young). Tear samples were collected by capillary tube. *B1*, Band1; *B2*, Band2; *B3*, Band3; and so forth

Table 3 Comparison of tear proteins from young and elderly people[a]

Band no.	Young people (n=10, in mg/ml)	Elderly people (n=12, in mg/ml)	P-value
1	2.64±0.60 (20.6%)	1.48±0.26 (18.2%)	<0.001*
2	0.26±0.07 (2.0%)	0.15±0.04 (1.9%)	<0.001*
3	1.61±0.47 (12.6%)	0.91±0.27 (11.2%)	<0.001*
4	0.88±0.13 (6.9%)	0.55±0.07 (6.8%)	<0.001*
5	1.23±0.25 (9.6%)	0.92±0.17 (11.3%)	0.002*
6	0.89±0.19 (7.0%)	0.61±0.10 (7.5%)	0.001*
7	2.41±0.32 (18.8%)	1.46±0.26 (17.9%)	<0.001*
8	2.05±0.25 (16.0%)	1.44±0.30 (17.8%)	<0.001*
Other bands	0.83±0.14 (6.5%)	0.60±0.11 (7.4%)	–
SUM[b]	12.28±1.53	8.12±1.07	<0.001*
Total concentration (Bradford assay)	13.47±1.63	8.16±1.07	<0.001*

Values are presented as mean ± standard deviation (%). [a] Percentages were determined by: tear protein band quantities / SUM*100%. [b] SUM (the total tear protein concentration) was the sum of individual bands. *P-values determined by independent sample t-test. $P<0.05$ was considered significant.

We identified a commercially available protein standard marker, SBTI, with a molecular weight of 20.1 kD, that did not overlap with any of the major tear protein bands in the 15% gel (Fig. 1). The commercial form of SBTI (Sigma, Catalog Number T9767) was pure enough and stable for electrophoresis.

Next, we tested the chemical properties of SBTI to see if it was suitable for use as a standard to quantify tear proteins in solution and in SDS-PAGE gel. Although there are controversies regarding the responses of CBB G250 to different proteins, the Bradford assay is widely used and accepted [28, 34]. In our experiment, the results of the Bradford assay showed that the affinity for CBB G250 of SBTI was very similar in solution to that of BSA and lysozyme, which agreed with the report by Bradford [28]. The total tear protein concentrations determined by the Bradford assay, using BSA, SBTI and lysozyme as standards, were significantly close to each other. Thus, SBTI can be used as an efficient standard to calculate the concentrations of other proteins by the Bradford assay.

In SDS-PAGE gels, Ng et al. had used human serum albumin (HSA) and lysozyme as standard proteins to calculate the concentration of HSA, lipocalin and lysozyme in tears by means of band densitometry [7]. Our experiment showed that the affinities of CBB R250 to BSA, SBTI and lysozyme were sufficiently close to one another. These results suggest that SBTI can serve as a standard to calculate the quantities of tear proteins by SDS-PAGE densitometry.

Quantification of major tear proteins

With SBTI as an internal standard, we were able to simultaneously calculate the quantities of total tear protein and the major tear protein components for more than ten samples in one experiment, requiring as little as 0.2 μl tears from each tear sample. The intra-gel and inter-gel varia-

Table 4 The quantities of major tear proteins in literatures and current study

Studies	Subjects	Tear collection	Standard used	Band 1	Band 7	Band 8	Total concentration
Berta [32] (1986)	60 Caucasians (age unknown)	Capillary tube (nasal ethanol instillation)	Unclear	2.24±0.84	1.84±0.43	2.01±0.68	Unclear
Ng et al. [19] (2000)	30 HK[b]-Chinese (18–34 years)	Capillary tube (yawn reflex)	Lactoferrin Lysozyme	2.73±0.82	2.89±0.88	2.46±0.44	11.48±2.32*
Current study	10 Chinese (19–20 years)	Capillary tube	SBTI	2.64±0.60	2.41±0.32	2.05±0.25	13.47±1.63* (12.28±1.53)[a]
		Schirmer strip	SBTI	1.79±0.35	1.65±0.27	1.93±0.30	9.08±1.50* (9.75±0.90)[a]

Values are presented as mean ± standard deviation in mg/ml. *Total tear protein concentrations were determined by the Bradford assay using IgG as a standard in the study by Ng et al., and SBTI as a standard in current study. [a] Total tear protein concentrations were determined by the sum of individual bands. [b] HK, Hongkong.

bilities for intense bands (Bands 1, 2, 7, 8) and for total tear protein concentration were small (CV≤10%), while variabilities for weak bands (Bands 3, 4, 5, 6) were much larger (Table 1). This may be due to the weak intensity and diffusion of Bands 3, 4, 5, 6, which would produce more variations during gel image analysis.

We summarized the major tear protein contents measured by band densitometry previously [7, 35] and in our current study in Table 4. In the study by Ng et al., lactoferrin was used as an external standard for the quantification of Band 1 (mainly lactoferrin), while lysozyme was used as an external standard for the quantification of Band 7 (mainly lipocalin) and Band 8 (mainly lysozyme). Since each band may represent a mixture of different proteins, using corresponding pure standard protein to calculate every single band is inefficient [19, 20]. Therefore, we used a single standard protein (SBTI) added to tear samples as an internal standard in the present study. Each tear protein band within a gel could be accurately normalized to the internal standard. We were then able to calculate the relative quantities of the major tear protein bands and total tear protein concentration, and compare different samples either in one gel or in different gels. Our approach is much more convenient, as more than ten samples can be simultaneously analyzed in one gel, which may be completed without the need for external standards. Furthermore, the tear sample volume required is as little as 0.2 μl, which is ideal for quantitative analysis of small volume tear samples.

The application of the internal stand method

Stuchell et al. had used an immunochemical method to compare the differences and correlations between the tear samples collected by Schirmer test paper and capillary tubes [36]. They found the concentrations of HSA (mainly in Band 2), IgG and transferrin in the tears collected by Schirmer test paper were significantly higher than those collected by the capillary tube, and that lysozyme (mainly in Band 8) and lactoferrin (mainly in Band 1) were not significantly different among samples collected by either method. In the present study, we compared the differences between the tear samples collected by Schirmer test paper and capillary tubes with SBTI as an internal standard on SDS-PAGE gel. We found similar results, except in the case of Band 1 (mainly lactoferrin), where concentration was higher in tear samples collected by capillary tube. This may be due to the non-specific nature of our method. However, when each individual sample was investigated, the quantity of Band 1 still showed significant correlations between the two tear sample methods (data not shown). In addition, there was a low total protein concentration in samples collected by Schirmer test paper. This observation may be a result of the elution procedure we used, and the fact that we discarded the flexed part of test paper that contacted with the conjunctiva. Compared to the immunochemical method, we overcame their technical shortcomings by using the internal standard method, which was much simpler to perform and required significantly less tear sample volume.

Using SBTI as an internal standard, we found the quantities of the major tear protein bands from young people were much higher than those from the elderly individuals. The results were in agreement with McGill's study [9], which used ELISA to study the normal tear protein profiles and age-related changes. McGill reported finding a linear and corresponding decline of lysozyme (mainly in Band 8) and lactoferrin (mainly in Band 1) with advanced age, while IgA (heavy chain of IgA mainly in Band 3) levels gradually declined with age. We obtained similar results. However, our method was much easier to perform and allowed for the visualization of the entire protein profile in one experiment using a small tear volume. We also found that the percent contents of Band 5 and Band 8 from the elderly were much higher than that from the young people. Our method showed great superiority for comparison of different tear samples.

In conclusion, our experiments showed that, using SBTI as an internal standard, we wer able to simultaneously quantify the total tear protein levels and the major tear protein components by SDS-PAGE densitometry with small volume tears. Our approach may be used as a promising diagnostic tool for diseases characterized by tear protein changes.

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