Dry Eye: A Protein Conformational Disease

Mohammad Azharuddin,[1] Jayesh Khandelwal,[2] Himadri Datta,[2] Anjan Kr. Dasgupta,[1] and Sufi O. Raja[1]

[1]Department of Biochemistry and Centre for Excellence in Biomedical Engineering and Systems Biology, University of Calcutta, Ballygunge Campus, Kolkata, India
[2]Regional Institute of Ophthalmology (RIO), Calcutta Medical College, Kolkata, India

Correspondence: Anjan Kr. Dasgupta, Department of Biochemistry and Centre for Excellence in Biomedical Engineering and Systems Biology, University of Calcutta, 35 Ballygunge Circular Road, Kolkata 700019, India;
adgcal@gmail.com,
adbioc@caluniv.ac.in.

Submitted: November 3, 2014
Accepted: January 21, 2015

Citation: Azharuddin M, Khandelwal J, Datta H, Dasgupta AK, Raja SO. Dry eye: a protein conformational disease. *Invest Ophthalmol Vis Sci*. 2015;56:1423–1429. DOI:10.1167/iovs.14-15992

Purpose. The purpose of this study was to determine whether aqueous-deficient dry eyes (ADDE) is a protein conformational disease. Up to now the therapeutic regimen has been based on empirical results, but these observations may unfold new theranostic approaches for ADDE management.

Methods. Fifty ADDE patients and 46 healthy volunteers were recruited. Schirmer's test, tear breakup time, tear meniscus height, and fluorescein staining tests were conducted on the subjects. Tear protein for ADDE and control patients was collected and extracted using Schirmer's strip. Protein aggregation was studied by appraisal of average protein size, using dynamic light scattering (DLS), fast performance liquid chromatography (FPLC), and synchronous fluorescence spectroscopy (SFS).

Results. Dynamic light scattering data showed a comparatively higher abundance of aggregated proteins in ADDE patients than that in controls. For controls, the size distribution of tear proteins was <50 nm in diameter, whereas the size distribution for ADDE individuals was up to 300 nm in diameter. Fast performance liquid chromatography experiments in native tear proteins exhibited minimal difference in the FPLC profiles for ADDE patients and controls. Denatured tear protein FPLC profiles for patients indicated the presence of protein aggregates which were absent in controls. Our hypothesis was further verified by SFS; lower tryptophan fluorescence in ADDE patients is an indication of oxidative stress, which leads to protein aggregation.

Conclusions. Aqueous-deficient dry eyes is likely to be a protein conformational disease. Unlike other conformational diseases where single proteins are involved, this may be a reflection of structural loss for a significant fraction of the tear proteome.

Keywords: conformational disease, dry eye, dynamic light scattering, protein aggregation

Protein folding has rarely been raised in context with tear proteins. In contrast, emphasis has been on tear proteomics[1] and variations with disease.[2] As we know, most proteins maintain a well-defined three-dimensional structure, whether alone or in a complex mixture, in order to be fully active. Protein misfolding leads to formation of transiently aggregated structure. Such aggregates are seen in diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis, and prion diseases.[3]

Dry eyes seem to be one of the most prevalent diseases for which no well-defined drug is available today,[4] and the mechanism of its emergence is obscure.[5] According to the report of the International Dry Eye Workshop (DEWS) definition and classification,[6] dry eye is a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort,[7–9] visual disturbance,[10–12] and tear film instability,[13–15] with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film[16–19] and inflammation of the ocular surface.[20,21]

Dry eyes are generally subdivided into the categories aqueous-deficient dry eyes (ADDE) and evaporative dry eye (EDE). Aqueous-deficient dry eyes, also known as lacrimal insufficiency, is the condition in which the lacrimal glands do not produce enough of the watery (aqueous) layer of tears. Tears are made up of many substances secreted from various parts of the lacrimal system. The conditions collectively referred to as dry eye can occur for many reasons, the most obvious reason being insufficient quantity of tears. This is in contrast with EDE, where the primary problem is or starts as poor production or secretion of the oil. Biophysical and mass spectrometry techniques have been used previously to analyze or differentiate between dry eyes and control eyes.[22–24] The ocular surface of the human eye consists of a thin layer of tear film which is a three-structured layer composed of lipids, mucins, proteins, and other small organic metabolites. Recent, proteomic studies of tear fluids revealed the presence of 491 proteins including a large number of proteases and protease inhibitors.[25] The major tear proteins include lysozyme, lactoferrin, lipocalin, and lipophilin. Other proteomic studies suggest a spectra of different biomarkers[26–28]; however, there has yet to be a biomarker set that is a clinically reliable diagnostic marker to an ophthalmologist.

There is another perspective (independent of looking for proteins or lipid markers) with which to investigate dry eye disease. The inspiration of this third perspective is already there in published reports. Apart from complex agents like sera, there are reported agents like trehalose that are known to be effective for dry eye disease.[29] Trehalose is also known for its

Table 1. Criteria for Categorizing ADDE and Control Individuals Along With Tear Volume and Total Tear Protein Concentration

Category	Criteria
Schirmer's test	≤5 mm wetting over 5 min (normal > 15 mm wetting)
Tear breakup time	≤10 s
Tear meniscus height	≤0.2 mm
Fluorescein staining	More than 3 of 15[14]
Tear volume	13.78 ± 0.6 μL for control and 2.09 ± 0.09 μL for ADDE patients
Total tear protein concentration	0.69 ± 0.03 mg/mL for control and 0.31 ± 0.01 mg/mL for ADDE patients

chaperone-like activity that disrupts protein aggregates.[30] The question we raised in this study was whether the robust systemic nature of dry eye disease was implied by such observations. Presence of aggregated proteins implies the role of protein misfolding. The obvious question is whether dry eye disease involves aberrations in protein conformations. So far, the approach has been concentrated on finding proteomics or lipid markers rather than the conformational perspective.

Methods

This study was approved by the institutional ethics committee prior to its commencement. The research adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects.

Subjects

Fifty ADDE patients (23 male, 27 female; mean age: 30 ± 1.6 years old) were recruited from the out-patient department. Forty-six (92 eyes) control subjects were recruited from among the hospital staff (21 male, 25 female; mean age: 32 ± 2.5 years old). All other inclusion and exclusion criteria have been described in our earlier work.[31]

Collection and Extraction of Tear Fluid From Schirmer's Strip

Sampling was done at a fixed time for ADDE patients and controls. Total time needed from collection to extraction was 20 minutes. Collection and extraction were performed in adjacent laboratories. The Schirmer's strips were placed in an Eppendorf tube and transferred on ice in order to prevent warming of the samples. Similarly, during extraction, all the steps were carried out on ice and were then centrifuged at 4°C. Following the sampling procedure, the wet portion of the strip was immediately transferred to a 0.5-mL Eppendorf tube punctured at the bottom, with addition of 100 μL extraction buffer (50 mM NH_4HCO_3) on top of the Schirmer's strip. The tube was then placed in a large, 1.5-mL tube and centrifuged at 15,871*g* for 5 minutes. The centrifugal force pulls the tear fluid out of the Schirmer's strip, which was collected in the outer 1.5-mL tube.[32] The tear fluid extract was then stored at –80°C for further analysis.

Standard Curve of Schirmer's Strip Reading to Solution Volume Added

The experiment was carried out to obtain the relationship between the Schirmer's strip score (in millimeters) and the volume of ammonium bicarbonate solution (in microliters) adsorbed onto the strip over a period of 1 minute. The standard curve was further used to calculate the volume of tears adsorbed onto the strip during the collection process.[33]

Total Protein Estimation by Bradford Reagent

Absorption was measured (Evolution 300 UV-VIS; Thermo Fisher Scientific, Waltham, MA, USA) using Bradford reagent (protein kit assay; Bio-Rad Laboratories, Hercules, CA, USA) at 595 nm. Protein concentration was normalized to 0.1 mg/mL for both groups for all assays.

Size Measurement of Tear Fluid by Dynamic Light Scattering (DLS)

A 400-μL sample was used for DLS measurement (Zetasizer Nano Series; Malvern Instruments, Malvern, UK) at 4°C.

Synchronous Fluorescence Spectroscopy of Tear Fluid

Pooled tear proteins from 50 ADDE patients and 46 controls were subjected to polarized synchronous fluorescence spectroscopic measurement. This study was performed using a standard fluorimeter (Quantamaster 40 unit; PTI, Edison, NJ, USA). The offset value ($\Delta\lambda$) was 80 nm. The excitation and emission slit widths were 8 nm, and polarizers (Glan Thompson polarizers; Edmund Optics, Barrington, NJ, USA), both excitation and emission, were fixed at 0°. The excitation wavelength was 280 nm.

ANS Binding Study

An assay using 30-μM 8-anilino naphthalene sulfonic acid (ANS) was used. The proteins were incubated with ANS for 30 minutes at room temperature, and then fluorescence was measured. The excitation wavelength was 420 nm.

Fast Performance Liquid Chromatography Fractionation of tear fluid

Fractionation of pooled tear proteins was done by size exclusion liquid chromatography (Akta purifier fast performance liquid chromatography [FPLC]; GMI, Ramsey, MN, USA) using a Superose 6 column with a bed volume of 24 mL (GE Healthcare, Piscataway, NJ, USA). The column was equilibrated with 0.05 M ammonium bicarbonate, 0.1 M NaCl (Sisco Research Laboratories, Mumbai, India). A 0.5-mL sample of tears with total normalized protein concentration was loaded for each run at a flow rate of 0.5 mL/min. Protein peak fractions were monitored at 280 nm. Tear proteins were denatured with SDS-PAGE sample buffer containing 0.06 M Tris-HCl, pH 6.8, 5% glycerol, 2% SDS, 4% β-mercaptoethanol, without Bromophenol blue. A sample containing tear proteins along with the sample buffer was heated at 95°C for 10 min and then loaded onto an FPLC column.

Results

Table 1 shows the criteria for categorizing ADDE patients and controls and along with them the amount of tear volume and total tear protein concentration present. Figure 1 shows a standard curve between volume added to the Schirmer's strip relative to Schirmer's score; from these data, the volumes of tear fluid present in controls and ADDE patients were calculated as shown in Table 1.

Investigative Ophthalmology & Visual Science

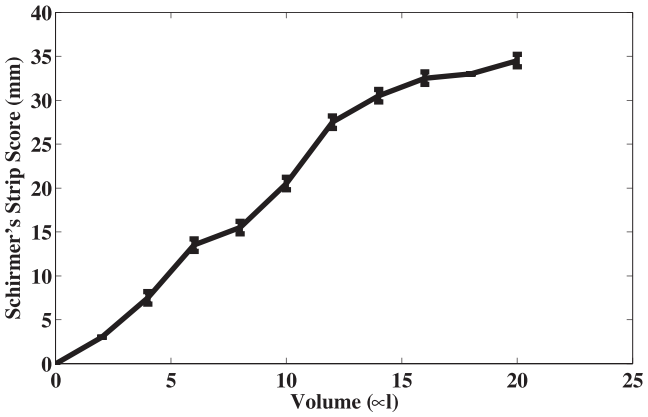

FIGURE 1. Standard curve between volumes added to the strip against Schirmer's score.

Figures 2A and 2B show size distribution of tear proteins in which the ordinate represents frequency (in percentage) at various size limits. The control (Fig. 2A) shows a size range (<70 nm), whereas for ADDE patients (Fig. 2B), the overall size distribution is wide and extends to as high as 300 nm. Dynamic light scattering data suggest the presence of large protein aggregates for ADDE patients in contrast with that for controls. The presence of aggregates suggests interplay of incorrect folding, and such misfolding is known to result in protein aggregation.[34]

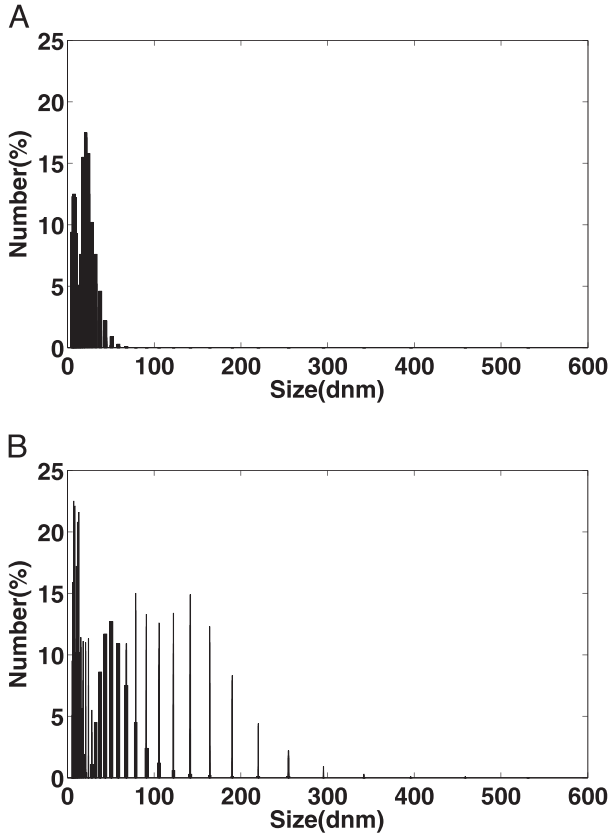

FIGURE 2. (**A**) Dynamic light scattering data representing the size of tear proteins for normal control. (**B**) Dynamic light scattering data representing the size of tear proteins for ADDE patients.

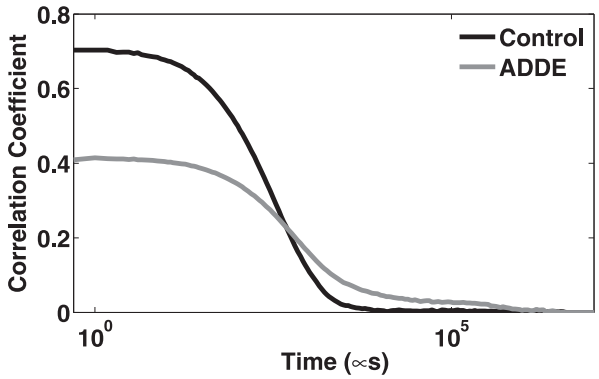

FIGURE 3. Correlation coefficient graph for pooled tear proteins of control (*dark line*) and ADDE (*light line*).

Dynamic light scattering data were analyzed on the basis of autocorrelation function.[35] The slope of the semilog plot of the autocorrelation function plotted against time provides the hydrodynamic diameter; the intercept is a measure of the scatterer density. Figure 3 shows the representative autocorrelation functions of control and ADDE subjects. We found that the ADDE profile (Fig. 3, gray line) has a lower light scatter density than that of controls (Fig. 3, dark line). This is consistent with the presence of aggregation (assuming protein concentration is taken identically in the two cases) because aggregation would lead to a reduction of scatter number. The stiffness of the slope of the autocorrelation is inversely proportional to the diffusion coefficient (Fig. 3, slope of the linear portion of the semilog plot). The lower value of the slope in ADDE implies higher diffusion coefficient of the ADDE proteins, which again, implies the presence of aggregated proteins.

To examine functional difference between control and ADDE, we opted for synchronous fluorescence spectroscopy (SFS), which we have recently shown to be useful[36] for analyzing the oxidative status of a complex protein mixture (e.g., serum). Figure 4A shows that the fluorescence intensity of free tryptophan is lower in ADDE patients (Fig. 4A, light lines) than in controls (Fig. 4A, dark line). A higher rate of tryptophan catabolism is an indicator of oxidative stress.[36] Hence, in ADDE patients, the observed lower value of free tryptophan fluorescence may be due to higher oxidative stress, which results in protein aggregation. We did not obtain significant changes in kynurenine but did find a lowering of tryptophan fluorescence. This essentially ruled out existence of oxidative stress. The lowering of tryptophan fluorescence can be explained in terms of lesser accessibility of tryptophan-containing residues as a result of the presence of aggregated structures. Figure 4B, on the other hand, shows enhancement of ANS fluorescence, which implies higher availability of hydrophobic sites. Such hydrophobicity enhancements may be again a signature of presence of unfolded protein whose hydrophobic collapse (seen for native structure) is absent.

The results discussed earlier point out that tear proteins of ADDE patients exhibit a tendency to form large-molecular-weight aggregates; this hypothesis was further verified by FPLC results.

Figures 5A and 5B show FPLC profiles of tear proteins in control and ADDE patients. The volume of the injected sample as well the concentration was normalized before the run. It can be seen from the figure that there are negligible differences in FPLC profiles for ADDE and controls, except that there is a decrease in the concentration of lower-molecular-weight protein in ADDE patients. Figure 5B shows the results obtained

Investigative Ophthalmology & Visual Science

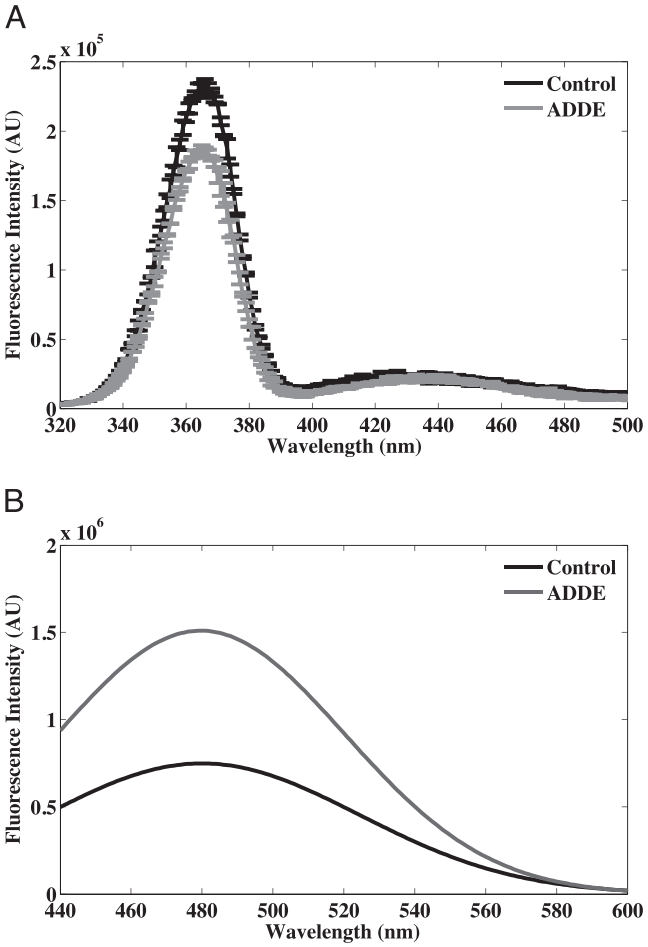

FIGURE 4. (**A**) Synchronous fluorescence scan with an offset value ($\Delta\lambda$ = 80 nm). There is the presence of free tryptophan in the case of control (*dark line*), which is absent in ADDE patients (*light line*), indicative of oxidative damage. The excitation wavelength was 280 nm. (**B**) ANS binding for control and ADDE. Excitation wavelength was 420 nm.

after pooled tear proteins for ADDE patients was injected into FPLC for fractionation before and after denaturation. The figure indicates that in patient samples there was an increase in the concentration of low-molecular-weight protein as monitored at 280 nm. This increase in the concentration could be due to the fact that prior to denaturation of proteins, large-molecular-weight-aggregate proteins, as measured by DLS (Fig. 1B), were not able to enter the FPLC column. When tear proteins were denatured, large protein aggregates are fragmented into smaller molecules and, hence, an increase in their concentration compared to that in controls (range, 70–14 kDa, left to right, which corresponds to the 20- to 25-mL range of the FPLC).

Similarly, Figure 5A show native and denatured tear protein FPLC profiles for controls. The results once again validate our hypothesis that ADDE is a protein conformational disease.

Table 2 shows molecular weight range for different fractions of native tear proteins for controls and ADDE patients (Table 2, entries may represent the proteins lysozyme [16.6 kDa], lactoferrin [78 kDa], lipocalin-1 [19 kDa], and lacritin [23 kDa]; such proteins are abundant in tear fluid). Notably, the last entry, lacritin, is absent in the ADDE samples, and this protein is known to form cross-linked structures in ADDE that would prevent its column entry.[37]

DISCUSSION

Tear proteins provide valuable sources of information regarding not only the general health status of the eye but also many other physiological diseases like diabetes and breast cancer, to name a few.[38–41] Tears were collected with Schirmer's strips as opposed to the capillary tube method. It has been reported that total tear protein concentration obtained by Schirmer's strip is much higher than that with the capillary method.[42] In practical experience, sampling by strip was rapid, reliable, and without any risk to the subjects. On the other hand, the capillary tube method requires a greater precision and skill. Also, the strip can be used for diagnosis of dry eye. The patients also feel relatively comfortable with Schirmer's strip rather than the capillary method. Proteins retained on the strip after the extraction process are in negligible concentrations, or there might be no proteins present. We did a simple test to determine the presence of proteins on the strip after the extraction process. We immersed the strip in protein estimation reagent (Bio-Rad Laboratories) and found that the strip color remained identical for three sets (blank, control, and ADDE), indicating the fact that there were insufficient quantities of proteins on the strip (Supplementary Fig. S1).

The subjects were chosen primarily by excluding the older age group to avoid interference from age-related complications that might be unrelated to the disease.

Analysis of tear proteins using biophysical, mass proteomics approaches have been reported by many researchers in order to differentiate various pathological conditions of the eye.[22,23] Appropriate treatment of the disease is an area which is debatable. Some researchers have reported that iodide iontophoresis is good as a treatment for this disease[43] because of the oxidative damage to the anterior portion of the eye. Recently, with the emergence of mass spectrometry techniques, we are gaining in depth insight into tear fluid.[44] In this study, we applied different biophysical techniques such DLS, SFS, and FPLC to emphasize the fact that ADDE disease falls under protein conformational problem, which has not been reported previously.

The idea that ADDE could be a protein folding disease originates from the fact that in this disease, patients suffer from oxidative stress and inflammation, as established earlier.[45,46] Oxidative stress occurs as a result of an imbalance between the production of harmful reactive oxygen species and the human body's defense mechanisms required to eliminate the stress. Particularly in humans, oxidative damage plays a pivotal role in progression and development of various protein conformational diseases such as Alzheimer's and Parkinson's disease and other neurodegenerative disorders.[47–49] There have been reports of decreased antioxidant enzymes in dry eye disease.[50]

In our study, we focused on protein aggregation (Figs. 2A, 2B), for which we have shown the DLS data from tear proteins for control and ADDE patients. It can be seen that tear proteins of ADDE patients consist of large-molecular-weight proteins or protein aggregates, which are absent in control individuals (Fig. 2). The presence of protein aggregates was confirmed by correlation coefficient results, which further proved our hypothesis. As shown in Figure 3, the correlation function value of control individuals was higher than in ADDE patients. In other ways, it implies that in case of aggregated protein solution, the correlation coefficient value will persist for a longer time indicating aggregation of proteins, on the other hand correlation function value will correlate more rapidly for protein solution without the presence of any aggregated protein structures.

An alternative way of examining protein aggregation is oxidative damage, which plays a significant role in many protein conformational diseases.[51,52] The change in trypto-

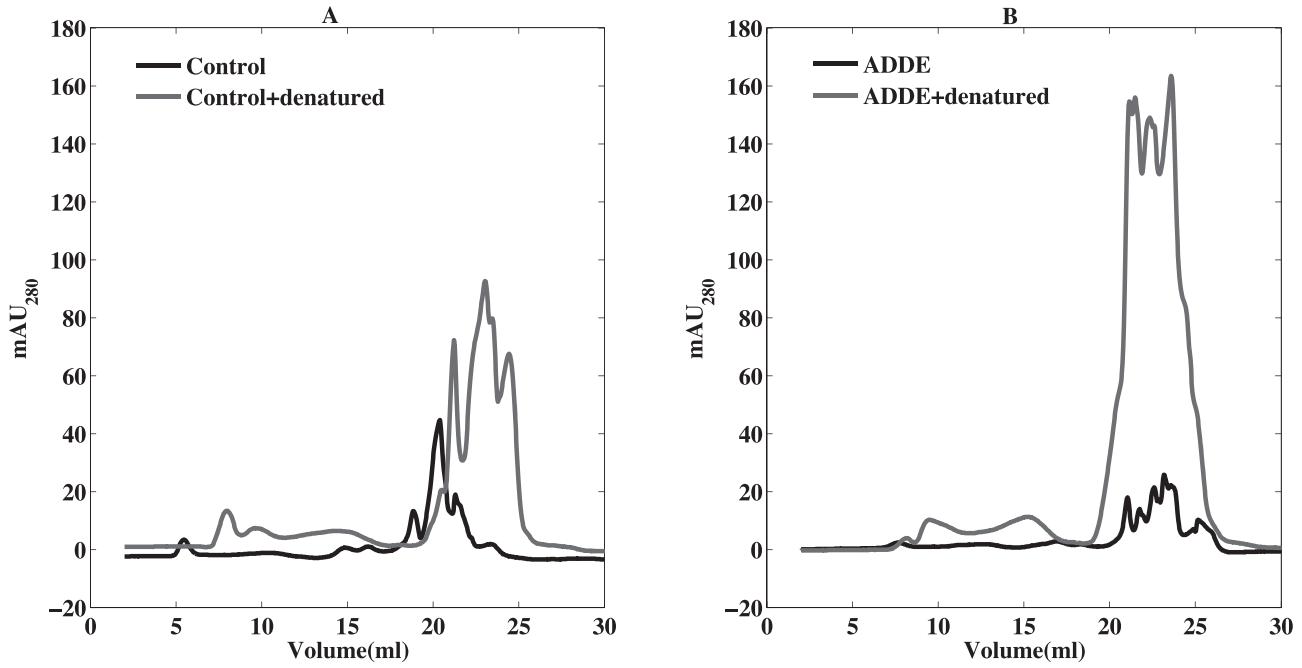

FIGURE 5. (A, B) Fast performance liquid chromatography profile for control and ADDE tear proteins before and after denaturation, respectively.

phan fluorescence intensity (Fig. 4) is indicative of oxidative damage, as reported previously.[53] Synchronous fluorescence spectroscopy has been used previously to differentiate between cataractous and noncataractous lenses.[54] There is evidence that oxidative stress leads to aggregation of proteins,[51,52] which may also be applicable in our case.

We also conducted size exclusion FPLC experiments to validate our results. Data shown in Figure 5 indicate that protein aggregation is one of the factors that determines the disease state. Figures 5A and 5B show fractionation of tear proteins for control and ADDE individuals before and after protein denaturation. It follows that FPLC profiles for both the groups are similar apart from the fact that ADDE subjects exhibit lower concentration of low molecular weight protein compared to control. When we denatured the tear proteins and then injected them, we found completely different results, as shown in Figure 5, that upon denaturation of proteins, there is an increase in the concentration of low-molecular-weight proteins for ADDE individuals in contrast to that for controls, although in control subjects there is also an increment but it is much lower than that in ADDE patients. These results indicate that in its native state, large fractions of ADDE tear proteins were unable to enter the FPLC column because of the formation of protein aggregates, whereas due to protein denaturation aggregated proteins (in case of ADDE) were fragmented into smaller sizes. These fragmented, smaller molecules were then able to enter the size exclusion column, showing an increase in the absorbance value measured at 280 nm.

TABLE 2. Occurrence of Different Proteins in ADDE and Control

Molecular Weight, Mean ± SD, kDa	Control, %	ADDE, %
10000 ± 300	5.65	–
3777 ± 1200	–	4.56
1819 ± 570	10.6	–
589 ± 180	–	9.39
255 ± 78	5.53	–
133 ± 42	4.8	8.08
58.6 ± 18	8.65	3.19
30 ± 9	21.46	–
21 ± 6.3	9.69	9.76
17 ± 5.1	–	6.99
13 ± 3.9	–	10.18
10 ± 3	4.81	15.82
6 ± 1.8	–	11.97

Table 2 shows that certain proteins in the molecular weight range of 12 to 13 kDa may be more abundant in dry eye. As indicated previously,[37] this may imply involvement of lacritin, which may be present in different multimeric states in dry eye patients and may contribute to the overall abundance of extended structure.

The simultaneous lowering of tryptophan fluorescence in SFS studies (Fig. 4A) and enhancement of ANS fluorescence (Fig. 4B) implies that tryptophan-containing residues may be buried, but the hydrophobic sites may be exposed.

To summarize, we may say that the dry eye may involve higher abundance of extended structure. This abundance can be mechanistically linked to the absence of chaperon-like entities that may be either proteins or even lipids binding to proteins, altering multimeric proteins. The objective of proteomics or lipidomics research in dry eye disease, therefore, must focus on the folding aspects rather than on only searching for specific biomarkers. The approach may also be helpful for discovering new therapeutic regimes for the dry eye disease, the preliminary indications being already present in literature (e.g., effectiveness of trehalose-a protein dispersing sugar in treatment of dry eye).

Acknowledgments

Supported by UGC-Research Fellowships in Science for meritorious students under SAP supported departments (no. F.4-1/2006[BSR]/5-19/2007[BSR] May 17, 2013; 2013–2014).

Disclosure: **M. Azharuddin**, None; **J. Khandelwal**, None; **H. Datta**, None; **A.K. Dasgupta**, None; **S.O. Raja**, None

References

1. Pannebaker C, Chandler HL, Nichols JJ. Tear proteomics in keratoconus. *Mol Vis*. 2010;16:1949–1957.
2. Wu K, Zhang Y. Clinical application of tear proteomics: present and future prospects. *Proteomics Clin Appl*. 2007;1: 972–982.
3. Ross CA, Poirier MA. Protein aggregation and neurodegenerative disease. *Nat Med*. 2004;10(suppl):S10–S17.
4. Tsubota K, Goto E, Fujita H, et al. Treatment of dry eye by autologous serum application in Sjögren's syndrome. *Br J Ophthalmol*. 1999;83:390–395.
5. Pflugfelder SC, Solomon A, Stern ME. The diagnosis and management of dry eye: a twenty-five–year review. *Cornea*. 2000;19:644–649.
6. Lemp MA, Foulks GN. The definition and classification of dry eye disease. *Ocul Surf*. 2007;5:75–92.
7. Begley CG, Chalmers RL, Abetz L, et al. The relationship between habitual patient-reported symptoms and clinical signs among patients with dry eye of varying severity. *Invest Ophthalmol Vis Sci*. 2003;44:4753–4761.
8. Adatia FA, Michaeli-Cohen A, Naor J, Caffery B, Bookman A, Slomovic A. Correlation between corneal sensitivity, subjective dry eye symptoms and corneal staining in Sjogren's syndrome. *Can J Ophthalmol*. 2004;39:767–771.
9. Vitale S, Goodman LA, Reed GF, Smith JA. Comparison of the NEI-VFQ and OSDI questionnaires in patients with Sjögren's syndrome-related dry eye. *Health Qual Life Outcomes*. 2004; 2:44.
10. Rieger G. The importance of the precorneal tear film for the quality of optical imaging. *Br J Ophthalmol*. 1992;76:157–158.
11. Liu Z, Pflugfelder SC. Corneal surface regularity and the effect of artificial tears in aqueous tear deficiency. *Ophthalmology*. 1999;106:939–943.
12. Goto E, Yagi Y, Matsumoto Y, Tsubota K. Impaired functional visual acuity of dry eye patients. *Am J Ophthalmol*. 2002;133: 181–186.
13. Holly FJ. Formation and rupture of the tear film. *Exp Eye Res*. 1973;15:515–525.
14. Bron AJ. Diagnosis of dry eye. *Surv Ophthalmol*. 2001;45: S221–S226.
15. Goto T, Zheng X, Klyce SD, et al. A new method for tear film stability analysis using videokeratography. *Am J Ophthalmol*. 2003;135:607–612.
16. Farris RL, Stuchell RN, Mandel I. Tear osmolarity variation in the dry eye. *Trans Am Ophthalmol Soc*. 1986;84:250–268.
17. Gilbard JP. Human tear film electrolyte concentrations in health and dry-eye disease. *Int Ophthalmol Clin*. 1994;34:27–36.
18. Murube J. Tear osmolarity. *Ocul Surf*. 2006;4:62–73.
19. Tomlinson A, Khanal S, Ramaesh K, Diaper C, McFadyen A. Tear film osmolarity: determination of a referent for dry eye diagnosis. *Invest Ophthalmol Vis Sci*. 2006;47:4309–4315.
20. Pflugfelder SC, Jones D, Ji Z, Afonso A, Monroy D. Altered cytokine balance in the tear fluid and conjunctiva of patients with Sjögren's syndrome keratoconjunctivitis sicca. *Curr Eye Res*. 1999;19:201–211.
21. Tsubota K, Fujihara T, Saito K, Takeuchi T. Conjunctival epithelium expression of HLA-DR in dry eye patients. *Ophthalmologica*. 1998;213:16–19.
22. Versura P, Nanni P, Bavelloni A, et al. Tear proteomics in evaporative dry eye disease. *Eye*. 2010;24:1396–1402.
23. Aluru SV, Agarwal S, Srinivasan B, et al. Lacrimal proline rich 4 (LPRR4) protein in the tear fluid is a potential biomarker of dry eye syndrome. *PLoS One*. 2012;7:e51979.
24. Mengher L, Pandher K, Bron A, Davey C. Effect of sodium hyaluronate (0.1%) on break-up time (NIBUT) in patients with dry eyes. *Br J Ophthalmol*. 1986;70:442–447.
25. de Souza GA, de Godoy LM, Mann M. Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. *Genome Biol*. 2006;7: R72.
26. Tomosugi N, Kitagawa K, Takahashi N, Sugai S, Ishikawa I. Diagnostic potential of tear proteomic patterns in Sjögren's syndrome. *J Proteome Res*. 2005;4:820–825.
27. Nichols JJ, Green-Church KB. Mass spectrometry-based proteomic analyses in contact lens-related dry eye. *Cornea*. 2009;28:1109–1117.
28. Dohlman CH, inventor; Massachusetts Eye & Ear Infirmary, assignee. Dry eye treatment. US Patent 7,186,233. March 6, 2007.
29. Matsuo T, Tsuchida Y, Morimoto N. Trehalose eye drops in the treatment of dry eye syndrome. *Ophthalmology*. 2002;109: 2024–2029.
30. Crowe JH. Trehalose as a "chemical chaperone." *Molecular Aspects of the Stress Response: Chaperones, Membranes and Networks*. New York: Springer; 2007; 594:143–158.
31. Azharuddin M, Bera SK, Datta H, Dasgupta AK. Thermal fluctuation based study of aqueous deficient dry eyes by non-invasive thermal imaging. *Exp Eye Res*. 2014;120:97–102.
32. Posa A, Bräuer L, Schicht M, Garreis F, Beileke S, Paulsen F. Schirmer strip vs. capillary tube method: non-invasive methods of obtaining proteins from tear fluid. *Ann Anat*. 2013;195:137–142.
33. VanDerMeid KR, Su SP, Krenzer KL, Ward KW, Zhang JZ. A method to extract cytokines and matrix metalloproteinases from Schirmer strips and analyze using Luminex. *Mol Vis*. 2011;17:1056–1063.
34. Chiti F, Dobson CM. Protein misfolding, functional amyloid, and human disease. *Annu Rev Biochem*. 2006;75:333–366.
35. Berne BJ, Pecora R. *Dynamic Light Scattering: With Applications to Chemistry, Biology, and Physics*. N Chelmsford, MA: Dover Publications, Inc.; 2000.
36. Raja SO, Shaw J, Chattopadhyay A, Chatterjee S, Bhattacharya M, Dasgupta AK. Synchronous fluorescence based one step optical method for assessing oxidative stress and its dependence on serum ferritin. *Anal Methods*. 2014;6:6228–6231.
37. Velez F, Romano JA, McKown RL, et al. Tissue transglutaminase is a negative regulator of monomeric lacritin bioactivity. *Invest Ophthalmol Vis Sci*. 2013;54:2123–2132.
38. Herber S, Grus FH, Sabuncuo P, Augustin AJ. Two-dimensional analysis of tear protein patterns of diabetic patients. *Electrophoresis*. 2001;22:1838–1844.
39. Grus F, Sabuncuo P, Dick H, Augustin A, Pfeiffer N. Changes in the tear proteins of diabetic patients. *BMC Ophthalmol*. 2002; 2:4.
40. Lebrecht A, Boehm D, Schmidt M, Koelbl H, Schwirz RL, Grus FH. Diagnosis of breast cancer by tear proteomic pattern. *Cancer Genomics Proteomics*. 2009;6:177–182.
41. Evans V, Vockler C, Friedlander M, Walsh B, Willcox MD. Lacryglobin in human tears, a potential marker for cancer. *Clin Exp Ophthalmol*. 2001;29:161–163.
42. Farias E, Yasunaga KL, Peixoto RV, Fonseca MP, Fontes W, Galera PD. Comparison of two methods of tear sampling for protein quantification by Bradford method. *Pesqui Vet Bras*. 2013;33:261–264.
43. Horwath-Winter J, Schmut O, Haller-Schober E, Gruber A, Rieger G. Iodide iontophoresis as a treatment for dry eye syndrome. *Br J Ophthalmol*. 2005;89:40–44.
44. Grus FH, Joachim SC, Pfeiffer N. Proteomics in ocular fluids. *Proteomics Clin Appl*. 2007;1:876–888.

Investigative Ophthalmology & Visual Science

45. Augustin AJ, Spitznas M, Kaviani N, et al. Oxidative reactions in the tear fluid of patients suffering from dry eyes. *Graefe's Arch Clin Exp Ophthalmol*. 1995;233:694–698.
46. Wakamatsu TH, Dogru M, Tsubota K. Tearful relations: oxidative stress, inflammation and eye diseases. *Arq Bras Oftalmol*. 2008;71:72–79.
47. Mecocci P, MacGarvey U, Beal MF. Oxidative damage to mitochondrial DNA is increased in Alzheimer's disease. *Ann Neurol*. 1994;36:747–751.
48. Simonian N, Coyle J. Oxidative stress in neurodegenerative diseases. *Annu Rev Pharmacol Toxicol*. 1996;36:83–106.
49. Jenner P, Olanow CW. Oxidative stress and the pathogenesis of Parkinson's disease. *Neurology*. 1996;47:161S–170S.
50. Cejkova J, Ardan T, Simonova Z, et al. Decreased expression of antioxidant enzymes in the conjunctival epithelium of dry eye (Sjogren's syndrome) and its possible contribution to the development of ocular surface oxidative injuries. *Histol Histopathol*. 2008;23:1477–1483.
51. Squier TC. Oxidative stress and protein aggregation during biological aging. *Exp Gerontol*. 2001;36:1539–1550.
52. Tabner BJ, El-Agnaf O, German M, Fullwood NJ, Allsop D. Protein aggregation, metals and oxidative stress in neurodegenerative diseases. *Biochem Soc Trans*. 2005;33:1082–1086.
53. Petrônio MS, Fernandes JR, de Menezes ML, Ximenes VF. Oxidation of bovine albumin by hypochlorous and hypobromous acids: structural and functional alterations. *Br J Pharm Res*. 2013;3:147–160.
54. Rao C. Synchronous scan fluorescence spectroscopy of proteins and human eye lenses. *Biochem Biophys Res Commun*. 1991;176:1351–1357.

Investigative Ophthalmology & Visual Science