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Human tear viscosity: An interactive role for proteins and lipids

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Abstract

Human tear viscosity is poorly understood. Tears need to remain on the ocular surface for lubrication without causing damage to the surface epithelia due to drag when blinking. Whole tears are shear-thinning (non-Newtonian), which cannot be explained by the amount of mucin present, nor by individual proteins. Whole tears minus lipids become Newtonian. Though no free lipids had previously been found in collected tears, tear lipocalin (TL), a major tear protein, is known to bind lipids. In this study, we aimed to confirm whether there are any free lipids in collected tears, and to clarify the combined contribution of tear proteins to viscosity, including experiments on recombinant TL, both without (apo-TL) and with (holo-TL) bound lipid. We also investigated possible oligomer formation by holo- and apo-TL as a mechanism for viscosity using SDS-PAGE and analytical ultracentrifugation (AU). For comparison, we have included results for β-lactoglobulin, a well-characterised lipocalin protein. No free lipids were detected in whole tears. Rheology showed that any protein combination that included lysozyme or lactoferrin was shear-thinning, as was apo-TL, though holo-TL was Newtonian (linear). Results from SDS-PAGE and AU showed apo-TL to be entirely monomeric, but holo-TL showed some dimerization. Both apo- and holo-β-lactoglobulin exhibited a monomer–dimer equilibrium. We conclude that hetero-protein interactions, possibly electrostatic, involving lipid-binding-induced structural changes to TL, significantly contribute to the viscosity of human tears.

Keywords: Analytical ultracentrifugation; β-lactoglobulin; Lipid binding; Protein structure; Tear lipocalin; Viscosity

1. Introduction

The mechanisms underlying human tear viscosity remain poorly understood. Natural whole tears show non-Newtonian shear-thinning behaviour, with higher viscosities at lower shear rates, as opposed to the linear, shear-independent viscosity of Newtonian liquids [1]. The tear film is suggested to consist of a mucous layer, secreted by the corneal/conjunctival epithelium, an aqueous layer, and a much thinner lipid layer covering the surface at the aqueous–air interface. The aqueous layer contains four major proteins (secretory IgA, lysozyme, lactoferrin, and tear lipocalin—also known as tear-specific prealbumin or TSP), several minor proteins, lipids, many electrolytes, and (though not in all cases [2,3]) a small amount of dissolved mucin.

Mucins are very large, linear molecules, with molecular masses often greater than one million Daltons, which are able to interact and aggregate, and are therefore capable of producing solutions with high viscosity [4]. However, the amount of soluble mucin in collected human tears is insufficient, on its own, to account for their viscosity [3]. Moreover, individual proteins are often globular in structure, and too small to cause high viscosities. There is some evidence though that combinations of tear proteins may have a role in producing high tear viscosity. It has been shown that in vitro mixtures of two model tear proteins give non-Newtonian shear-thinning, but only when lysozyme was present [5].

Added to this is the observation from our preliminary studies that if the lipids in tears are removed, tear viscosity becomes Newtonian (shear-independent). However, when the tear lipids are added back to the original tear sample, non-Newtonian viscosity returns [6], suggesting a definite role for lipids, though no mechanism has yet been identified. From studies of surface tension, Nagyová and Tiffany [7] detected no free lipids in collected aqueous tears when passed through a 5-kDa cut-off cellulose triacetate filter, though the possibility remains that the lipids may have bound to the filter itself. However, one of the major tear proteins, tear lipocalin, is known to bind a broad array of lipids including cholesterol,

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fatty acids, phospholipids and glycolipids [8] and therefore has a potential role in tear viscosity.

Tear lipocalin (TL) is a member of the lipocalin protein family, whose members are characterised by their ability to carry hydrophobic ligands, and include retinol-binding protein, odorant-binding protein, bovine β-lactoglobulin (BLG), mouse major urinary protein, α_{2u}-globulin and insecticyanin [9]. As well as being one of the most abundant human tear proteins [10], TL has also been found in human saliva [11,12], pituitary [13], sweat [12], nasal and respiratory tract mucosae [12,14–16] and prostate [17].

The exact function of human TL is unknown, but several potential roles in the tear film have been identified. It is thought to function as an anti-viral and anti-microbial agent via endonuclease activity [18] and cysteine protease inhibition [19,20], and also to act as a lipid scavenger [8,21,22] and an aid to tear film stability [7,8,23–25]. The lipid-binding properties of TL are implicated in all of these functions.

Lipid binding by TL may therefore have a role in homo- and hetero-protein complex formation within the tear film [26]. Evidence on the oligomeric state of TL is inconclusive [27]. Some reports suggest that it is monomeric [19,22,28–30], others that it is a dimer [12,31], while yet further studies say it may exist in both forms [19,22].

The present work aims to clarify which factors in the tear film are responsible for producing high tear viscosity. Specifically, we aim to: (1) confirm whether there is any free lipid in collected human aqueous tears. (2) Compare the viscosities of whole human tears with tears minus lipids. (3) Assess the contribution of individual model tear proteins and protein mixtures to solution viscosity. (4) Measure the viscosity of recombinant TL, with (holo-TL) and without (apo-TL) bound lipid. (5) Investigate possible oligomer formation of holo- and apo-TL using SDS-PAGE and analytical ultracentrifugation. For the purpose of comparison, we have included results for BLG, a well characterised member of the lipocalin family and the main protein in the whey fraction of cows' milk.

2. Materials and methods

2.1. Materials

Glass capillary tubes (Intramark) were bought from VWR International Ltd., Lutterworth, UK. Millipore 400 μl regenerated cellulose centrifugal filters (5 kDa MW cut-off) were purchased from Sigma-Aldrich Ltd., Poole, UK. Thin layer chromatography (TLC) plates (Whatman, 250 μm silica gel 60 Å) were obtained from The Labsales Company, Cambridge, UK. Recombinant human tear lipocalin was kindly provided by Professor B. Redl, University of Innsbruck, Austria. Briefly, human cDNA was expressed in *E. coli* M15 using the pQE system [17]. The recombinant TL isoform had an M_r of 17,577 kDa (neglecting the His tag), with an amino acid sequence identical to that given by the database Swiss-Prot for TL (accession number: P31025). The protein had identical biochemical properties to the native TL protein [17] and was found to be lipid-free.

All other proteins (lysozyme, lactoferrin, human sIgA and BLG), cholesterol, palmitic acid, stearic acid, sphingomyelin, and iodoacetic acid were obtained from Sigma-Aldrich Ltd., Poole, UK. The BLG sample was tested and found to be lipid-free (i.e. apo-BLG). Bis(sulfosuccinimidyl)suberate (BS^3) was obtained from Pierce, Cheshire, UK. Molecular weight markers were bought from New England Biolabs, Herts, UK. Standard chemicals used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and a silver staining reagent kit were obtained from Sigma-Aldrich, UK. Proteins were used at 1.2 mg/ml (typical TL concentration in human tears collected with some degree of stimulation [10]), and dissolved in PBS (Sigma-Aldrich, UK, 0.01 M phosphate, 0.137 M NaCl), pH 7.6 (typical salt concentration and pH of human tears).

2.2. Tear collection

Tear samples were taken from the lower meniscus of human volunteer subjects, who had given informed consent, using a narrow glass capillary tube, flamed at both ends for sterility and to eliminate sharp edges. Tearing was stimulated by moving nasal hairs with a narrow glass rod. The end of the collection tube was placed within the swollen meniscus, thus contamination from aqueous/air interfacial meibomian oils was negligible. During the taking of the sample, care was taken to not touch the globe with the end of the tube. Samples were pooled and stored in glass vials at −20 °C.

2.3. Extraction of tear lipids

Pooled tears were shaken with an equal volume of chloroform/methanol (2:1 v/v) for 1 min, then centrifuged at 3000 rpm for 10 min to separate the two phases. After recovery of the aqueous phase, nitrogen was bubbled through it to remove any dissolved solvent.

2.4. Investigation of the presence or absence of free lipids in tears

2.4.1. Centrifugal filtration

A pooled sample (200 μl) of human tears was put into a 5-kDa cut-off centrifugal filter, and spun in a bench-top centrifuge at 13000 rpm for 20 min, until all the fluid had passed through the filter. The filtrate was then extracted for lipids with chloroform/methanol 2:1. Prior to use with tears, the centrifugal filters were rinsed with the chloroform/methanol solvent. A mixture of three standard lipids, tristearin, stearic acid and sphingomyelin (1.3 mg/ml total, in relative concentrations of 1.2:1.4:1 respectively) was then prepared in chloroform/methanol (2:1 v/v). A series of 2-fold dilutions were made from this. One 200-μl aliquot (1.32 mg/ml) was put into a 5-kDa cut-off centrifugal filter. A 100-μl aliquot containing 0.132 mg/ml was dried down and 1 ml of normal saline added and the test tube sonicated to disperse the lipid. As with the protein samples, the lipid mixtures were spun at 13000 rpm for 20 min, and the filtrate retained.

2.4.2. Semi-quantitative estimation of tear lipids and standard lipid mixture

The above lipid samples in chloroform/methanol were reduced under nitrogen to 50 μl volumes, and then each sample in turn was spotted, with multiple applications to minimise spreading, onto the same TLC plate. The plate was then immersed in a solution of ammonium sulphate (10% w/v), removed and put in an oven at 150 °C, rising to 200 °C, for approximately 30 min, to char the lipids.

2.5. Rheometry

Variable-shear viscosity measurements were taken using a Contraves Low-Shear 30 rheometer, which is of the concentric-cylinder Couette type. The sample fluid filled the annular space between a stainless-steel bob suspended from a torsion wire, and the inner wall of a stainless-steel sample cup. The cup rotated at pre-determined speeds, inducing torque on the bob. The servo system recorded the counter-torque required to maintain the position of the bob. Measurements were carried out at room temperature (22–24 °C), and at high humidity to prevent evaporation of the sample, which can affect viscosity. The counter-torque readings for each shear rate were converted to viscosity measurements using tables supplied with the rheometer.

2.6. Binding lipids to lipocalins (holo-TL and holo-BLG)

This was achieved using the method previously described by Gasymov et al. [24] with minor modifications. Briefly, cholesterol, palmitic acid, stearic