Applying Magnetic Bead Separation / MALDI-TOF Mass Spectrometry to Human Tear Fluid Proteome Analysis

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Abstract

The proteins and peptides in tears play an important role in preserving the integrity and stability of the ocular surface. Proteomic analysis of tear films will enable us to detect early biological markers of eye diseases, however, it is often hampered by the small amount of tear volume and the low protein concentration. Here we adopted magnetic bead-based purification (ClinProt system) followed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) to profile human tear proteins. Basal and reflex tear fluids were collected from normal healthy volunteers using glass microcapillary tubes. Reversed phase (C8) and weak cation exchange (WCX) magnetic beads were applied to obtain multiple components detected as clear signals. Principal component analysis showed a clear differentiation between basal and reflex tears. Among the key alterations, two markedly increased peaks in the reflex tear fluids at m/z 2422.12 and m/z 2721.29 were subsequently analyzed by tandem MS analysis and their source to be proline-rich protein 4 (PRP4). We conclude that magnetic bead-based separation combined with MALDI-TOF-MS (ClinProt MALDI-TOF) appears to be ideally suited for the first-line screening of peptides and proteins in tears.

The search for biomarkers of human diseases has been increasingly successful because of emerging new techniques in the field of proteomics (Hu et al., 2006; Villanueva et al., 2004; Zhang et al., 2004; Ketterlinus et al., 2005; Koo et al., 2005; Cheng et al., 2005; Mirr et al., 2005). Proteins and peptides in tears are reported to play important roles in preserving the integrity and stability of the ocular surface, and changes in tear proteins eins are associated with various pathological eye conditions (Koo et al., 2005). Among the molecules identified are candidates for biomarkers of dry-eye diseases (Gruns et al., 2005; Tomosugi et al., 2005). Earlier investigations of tear film proteins have included extensive analysis using high-performance liquid chromatography (HPLC) or two-dimensional (2-D) gel electrophoresis, combined with mass spectrometry-based protein identification (Koo et al., 2005; Cheng et al., 2005; Mirr et al., 2005; Gruns et al., 2005; Tomosugi et al., 2005; Kijlstra et al., 1989; Zhou et al., 2006; Li et al., 2005; Fung et al., 2004; de Souza et al., 2006), but these protocols are sometimes hampered by the small amount of tear fluid and its low protein concentration. For high-throughput analysis, surface-enhanced laser desorption / ionization time-of-flight (SELDI-TOF) MS analysis was developed (Gruns et al., 2005; Tomosugi et al., 2005). With this technique, very small sample volumes can be directly applied to chip-based array surfaces; however, its limitations include the identification. Here we show that the combination of difficulty of further protein magnetic bead separation and MALDI-TOF MS spectrometry (ClinProt system) is a reasonably efficacious, simple method for profiling and identifying proteins from eluted tear fluids.

Open-eye basal tear fluids were collected from twenty normal healthy volunteers who did not wear contact lenses and had no evidence of ocular disease. The subjects ranged in age from 20 to 29 years, old enough to collect properly physiological tears as described below. Informed consent was obtained from all volunteers participating in the study, and the protocols were approved by the institutional ethics committee and conformed to the provisions of the declaration of Helsinki. The ophthalmic examination included subjective symptoms, Schirmer's test, biomicroscopy with careful examination of the lid margin and meibomian glands, and tear break-up time. Each volunteer was questioned about subjective symptoms such as burning, itching, foreign body sensation, dryness, and photophobia. Tear fluid was collected in the afternoon using 1-µL glass micro-capillary tubes (Corning, New York, NY, USA) without touching the lid margins or eye-lashes. After basal tear fluids were collected, reflex tear fluids were elicited by nasopharyngeal scrub and collected. The collected samples were stored at -80 °C until analysis.

For analysis, the tear fluid samples were thawed and purified with a reagent set that included two kinds of chemically coated magnetic beads: reversed phase (C8) and weak cation exchange (WCX) (ClinProt™ Bruker Daltonics). We used œ-cyano-4-hydroxycinnamic acid as the matrix solution. All these procedures were performed at room temperature with moderate humidity. The eluted samples were then dropped onto a MALDI sample plate (600 µm Anchorchip™: Bruker Daltonics), and spectra were obtained by an Autoflex II MALDI-TOF mass spectrometer (Bruker Daltonics) operated in positive-ion linear mode. All spectra were obtained randomly over the surface of the matrix spot. The criteria for peak detection were: signal-to-noise ratio >5 and 2-Da peak-width filter. Approximately 10-20 peaks were produced after the treatment with the WCX or C8 beads (Figure 1A). Multiple components were detected as clear signals in the mass range of 0-20 kDa, which includes proteins such as lysozyme and lipocalin. Inducible secreted tear proteins are believed to consist primarily of three entities that account for 85% of the total protein content: lysozyme, lactoferrin, and the tear-specific lipocalins (Kijlstra et al., 1989). Lysozyme and lipocalin were previously identified as protein fragments at m/z 14,687.8 and m/z 17,438, respectively (Fung, 15 2004). In the present study, we detected signals with the same m/z ratios in the basal tear fluid samples (Figure1A). However, we could not detect lactoferrin by MALDI-TOF MS analysis with WCX or C8 beads, or by electrospray ionization (ESI)-MS analysis, for unknown reasons (Kijlstra et al., 1989).
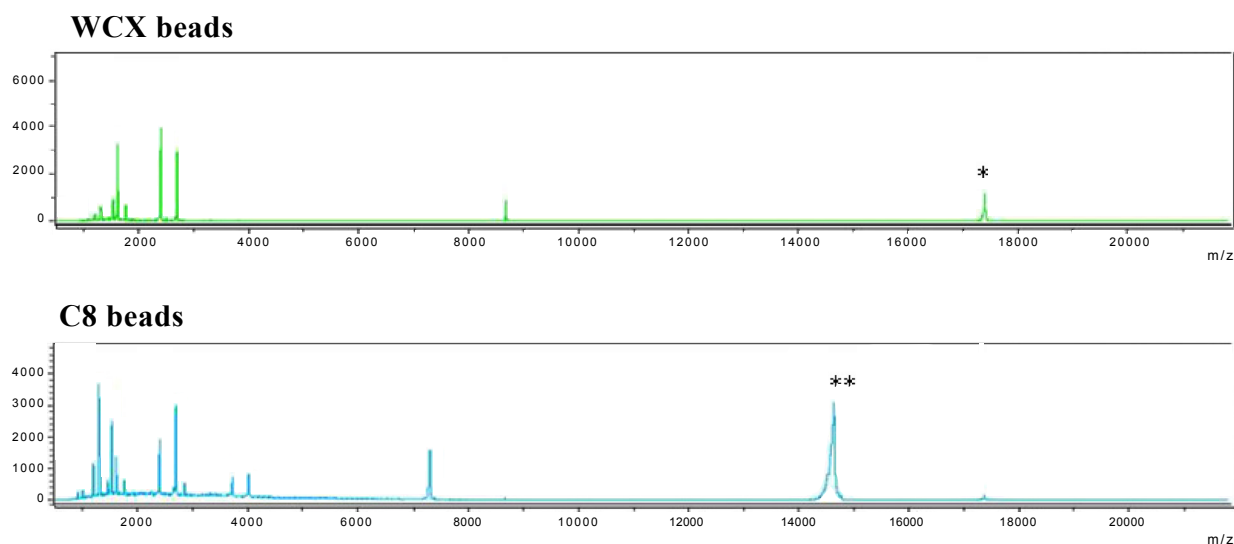

Figure 1: Protein/Peptide profiling of tear fluid samples from twenty healthy volunteers using ClinProt Mass Spectrometry.

(A) Typical ClinProt profiles of basal tear fluids eluted from WCX and C8 beads in the mass range 0-20 kDa m/z and subjected to flexAnalysis™. Multiple components were detected as clear signals, including lipocalin (*: m/z 17438) and lysozyme (**: m/z 14687.8).

The obtained data were graphed as columns representing normalized peak intensities (Figure1B; pseudo-gel view) and further analyzed by a multivariate statistical analysis including principal component analysis (PCA) by the *ClinProTools*TM software (Bruker Daltonik) (Zhang et al., 2004; Ketterlinus et al., 2005). The results showed a differential distribution of samples from basal tears from reflex tears (Figure1C).
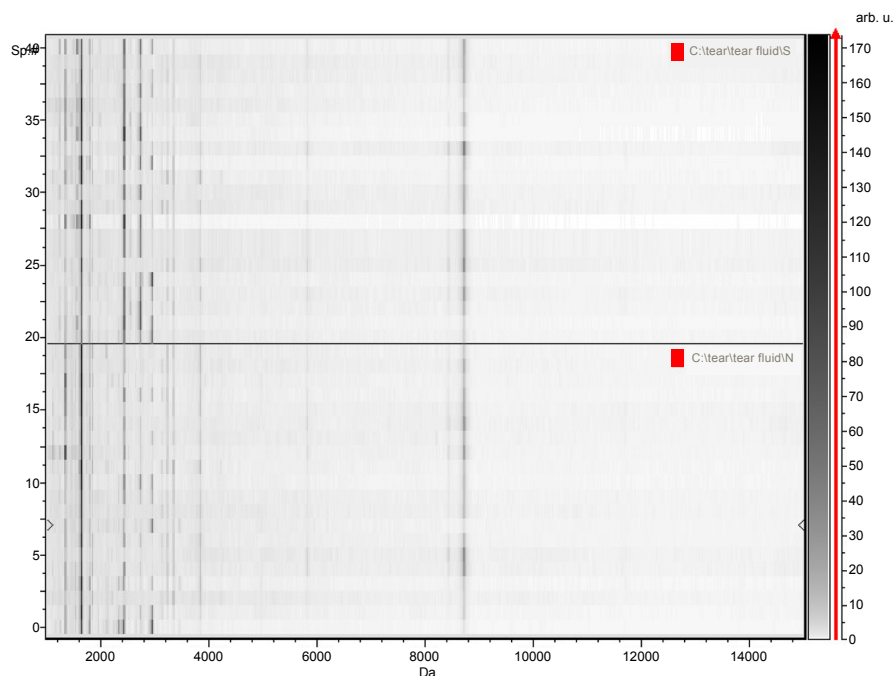

Figure 1: (B) Psuedo-gel views of the mass spectrum of basal tear fluids (lower column) and the reflex tear fluids (upper column) were shown with the calculated molecular weight (m/z values) along the x-axis and relative intensity along the y-axis using ClinProtools™.
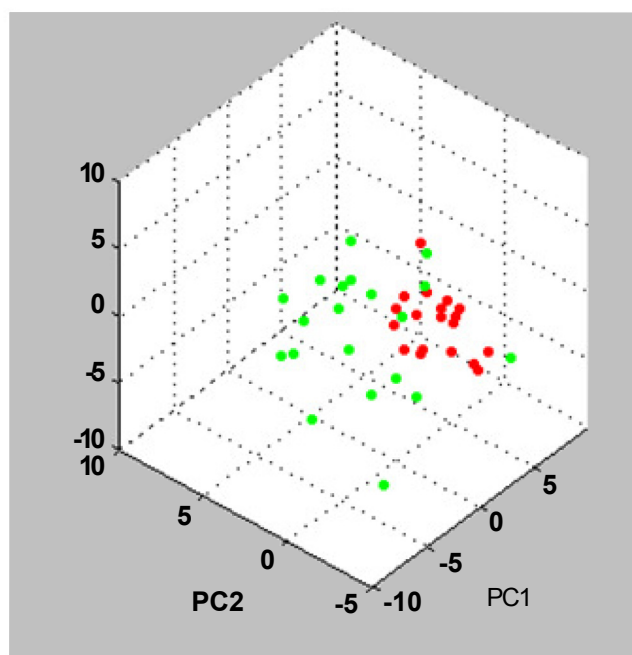

Figure 1: (C) 3-D view of PCA scores plot analyzed by *ClinProtools™*. Green spots represent reflex tears and the red spots represent basal tears.

We next examined the profiles of proteins smaller than 3.5 kDa obtained from seven representative samples each of basal and reflex tear fluid (Figure1D). For this purpose, the selected peak must have sufficient intensity to generate a valuable MS/MS fragment spectrum, and a spectrum is acquired in the high-resolution reflectron mode to determine the exact mass of the molecule of interest. Although it is clear from visual inspection (Figure1D), the two peaks in the spectra obtained from the reflex tears seemed to be the key protein / peptides peaks contributing the most towards the group selection by PCA loading plots as well (data not shown). Subsequently, the TOF/TOF fragment spectrum is acquired from the same sample spot and used for de-novo sequencing or database search. Before the analysis, the tear fluids were concentrated using a ZipTip (Millipore). In the MALDI-TOF/TOF mode, precursor ions were accelerated to 8 kV and selected in a timed ion gate. The fragments were further accelerated by 19 kV in the LIFT cell and their masses were analyzed after the ion reflector passage. S/MS spectra were searched against the human NCBI database using the *MASCOT* search algorithm (http://www.matrixscience.com/home.html), with a mass tolerance of 0.2Da for MS and 0.75 for MS/MS. No enzyme was selected and methionine oxidation and acetylation of the N terminus were used as variable modifications
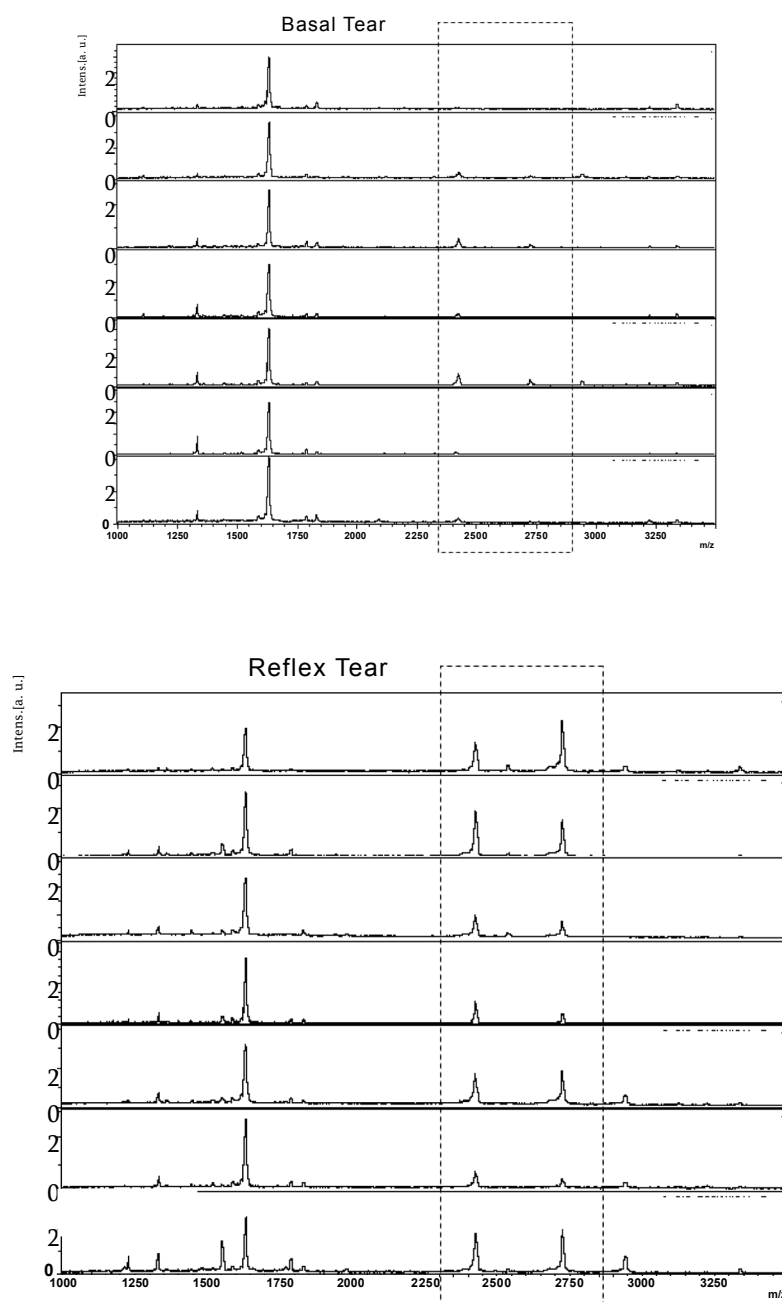

Figure 1: (D) ClinProt profiles of basal and reflex tear fluids eluted from WCX beads in the mass range 1000-3500 Da m/z (n=7). In the reflex tear fluid samples, the height of two peaks increased markedly (m/z 2422 and 2721) (inside the dotted squares).

The Mascot probability score for the peak with m/z =2,422 was 66, indicating a reasonably high confidence in identifying the peptide sequence. The sequence was determined to be QEASSFFRRDRPARHPQEQP, which matched the C-terminal fragment of proline-rich protein 4 (aa. 113-132) (locus number AAB26584) (Supplementary data A) (Fung et al., 2004). A complete MS/MS spectrum of the peak with m/z =2,721 was not obtained; however, the peptide sequence was determined to be RRDRPARH ~W, which partially matched the C-terminal fragment of proline-rich protein 4 (aa.120-134) in the NCBI BLAST protein-protein database (locus number AAB26584) (Supplementary data B).

We also analyzed the protease-digested HPLC fractions of our samples by 15 ESI-LC/MS/MS (esquire HCT; Bruker Daltonics). Not surprisingly, several well-known abundant tear proteins, such as lysozyme, lacritin, lipocalin, and secretoglobin, were detected, and a total of proteins, including PRP4, were identified in the reflex tear fluids. In this study, lactoferrin was not among the abundant proteins detected in tear fluids (Kijlstra et al., 1989).

Proline-rich proteins (PRPs) are believed to play a significant role in the oral mucosal defense system, in which they affect the aggregation of microorganisms, thereby decreasing the organisms' capacity to colonize tissue surfaces (Fung et al., 2004; de Souza et al., 2006). In addition, bacterial proteases are known to clip the N-5 -terminus of PRPs, releasing two peptides that have cytokine-like properties, by which they up-regulate the host defense against potential pathogens. PRP4 is expressed in the lacrimal acinar cells and other anterior exocrine glands (Dickinson and Thiesse, 1995). Since the reflex tear fluids were collected soon after the nasopharyngeal scrub, the PRP4 detected in the reflex tear fluids may have been stored in the acinar cells and released quickly after the stimulation. In addition to PRPs, lysozyme is reported to mediate protective functions in the eye (Kijlstra et al., 1989; Zhou, et al., 2006; Li et al., 2005; Fung et al., 2004; de Souza et al., 2006). Lysozyme serves as a non-specific innate opsonin by binding to the bacterial surface, reducing the negative charge, and facilitating phagocytosis of the bacterium before opsonins from the acquired immune system arrive at the scene. In contrast to PRP4, the peak height of lysozyme showed no remarkable difference between the basal and reflex tear fluids. Thus, it is possible that PRP4 is the first molecule that rapidly confronts foreign antigens at the ocular surface.

In conclusion, the key finding of this study is the up-regulation of a C-terminus of PRP4 in the reflex tear fluids from normal healthy subjects. Accordingly, the magnetic bead separation and MALDI-TOF analysis in combination with bioinformatics software is useful for the high-throughput protein profiling of tear fluids. This is the first study demonstrating the usefulness of the *ClinProt beads system* for this purpose. This simple and easy approach may be applicable to the discovery of biomarkers in ocular diseases as well.

Acknowledgements

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