Peptidomic analysis of human reflex tear fluid

Eisuke Hayakawa [a,*], Bart Landuyt [a], Geert Baggerman [b,c], Ruud Cuyvers [a], Rob Lavigne [d], Walter Luyten [e], Liliane Schoofs [a]

[a] Research Group of Functional Genomics and Proteomics, KU Leuven, B-3000 Leuven, Belgium
[b] VITO, Boeretang 200, 2400 MOL, Belgium
[c] CFP/Ceproma, University Antwerpen, Groenenborgerlaan 171, 2020 Antwerpen, Belgium
[d] Laboratory of Gene Technology, KU Leuven, B-3001 Leuven, Belgium
[e] Biomedical Sciences Group, Department of Pharmaceutical and Pharmacological Sciences, KU Leuven, B-3000 Leuven, Belgium

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ABSTRACT

Tear fluid is a complex mixture of biological compounds, including carbohydrates, lipids, electrolytes, proteins, and peptides. Despite the physiological importance of tear fluid, little is known about the identity of its endogenous peptides. In this study, we analyzed and identified naturally occurring peptide molecules in human reflex tear fluid by means of LC-MALDI-TOF–TOF. Tandem MS analyses revealed 30 peptides, most of which have not been identified before. Twenty-six peptides are derived from the proline-rich protein 4 and 4 peptides are derived from the polymeric immunoglobulin receptor. Based on their structural characteristics, we suggest that the identified tear fluid peptides contribute to the protective environment of the ocular surface.

1. Introduction

Human tear fluid is a complex mixture of biological compounds. Tear fluid is primarily secreted from the lacrimal gland, and plays important physiological roles such as: keeping the eyes moist, preventing infections, providing nutrients, and serving as a barrier to the outside environment. Moreover, recent studies prove that tear fluid contains chemosignal compounds [18,28,29].

Tear fluid is a rich source and a carrier of biological compounds that are involved in various biological phenomena. It is known that tear fluid contains various molecules, such as electrolytes, lipids, sugars and proteins. Protein concentration in tear fluid is high and prominent tear fluid proteins, such as lysozyme, lactoferrin, secretory immunoglobin A, lipocalin, and lipophilin, are thought to play important roles in various physiological functions [27,40]. Lysozyme, lactoferrin, and secretory immunoglobulin A for instance have shown to act as defense factors against infections from the outside environment. Lipocalin, also known as tear-specific prealbumin, binds and transports lipid molecules. Hence these proteins contribute to maintain the physiological environment of the eye surface. Recently, a more exhaustive study of the entire proteome of human tear fluid has been performed. De Souza et al. detected 491 tear proteins using both a hybrid linear trap, Fourier transform (LTQ-FT), and a linear ion trap, orbitrap (LTQ-Orbitrap) instrument [48]. Based on the annotated biological processes of identified proteins, they revealed that at least 7 proteins responsible for defense against pathogens, 50 proteins involved in immune response, 15 proteins involved in inflammatory response, 31 proteins associated with wounding healing, 12 proteins involved in blood coagulation, and 35 proteins responsible for proteolysis. Green-Church et al. employed a combination of one- and two-dimensional gel electrophoresis and mass spectrometry to analyze the tear film proteome. In total, 97 proteins and a significant number of posttranslational modifications were identified [20].

In contrast to the knowledge available on the tear fluid proteins, information on the content of endogenous peptides present in tear fluid is scarce. Bioactive peptides are often messenger molecules that are derived from larger protein precursor molecules by the controlled action of specific processing enzymes, called

convertases. After cleavage of their precursor, peptides can be further processed and posttranslationally modified before they exert their function(s) [13]. By definition, biologically active peptides are secreted into the extracellular environment, and perform their signaling action on neighboring cells or distant targets after transportation via the bloodstream. Bioactive peptides are found in all metazoan species where they orchestrate a variety of physiological processes, including hormonal signaling, neurotransmission, growth factor signaling, and host defense [7,50].

Recently, several articles have reported on the presence of endogenous peptides in human tear fluid and related tissue. The presence of α-defensins 1–3 was detected by MALDI-TOF analysis in the tear fluids collected from dry eye patients [35]. Based on mRNA expression and Western blot analysis, the tear duct was found to express trefoil factor family peptides, which are involved in mucosal maintenance [41]. Nguyen-Khuong et al. used an LC-MALDI-TOF approach to show the presence of small molecular weight compound in human tear fluid, although no structural identification was carried out [38]. These reports suggest that human tear fluid is a rich source of endogenous peptides. However, a comprehensive analysis to unveil the identity of endogenous peptides in tear fluid has not been conducted so far.

Peptidomics approach allows to simultaneously visualize and identify the repertoire of naturally occurring peptides in a biological sample [5,6,47]. The peptide fraction is extracted using a dedicated protocol, and the entire set of peptides is analyzed by a combination of liquid chromatography (LC) and MS. Recently, peptidomics analyses have been applied to a wide range of animal and plant tissues, and have revealed a great variety of naturally occurring peptides, including several novel forms [11,14,26,42]. In addition to the discovery of novel peptides, the peptidomic approach is currently increasingly applied for diagnostic purposes as well [8].

In this study, we report the identification of peptides from human tear fluid by means of peptidomics. The discovery of endogenous peptides in tear fluid may provide new insights into the physiological function of human tears.

2. Materials and methods

2.1. Sample collection

Human reflex tear samples were collected from four healthy male donors (30–35 years old), not wearing contact lenses. The donors were briefly exposed to onion vapor, and their tears were collected immediately with disposable micro-pipettes (50 μL, Blaubrand). Collected tears (100 μL from each donor) were subsequently centrifuged for 5 min at 4000 × g, to remove cellular components. The supernatants were pooled to increase the sensitivity of the experiment, and transferred to a new tube.

2.2. Sample preparation

Samples were mixed with 3 volumes of 90% methanol containing 1% formic acid, and centrifuged (10,000 × g for 20 min at 4 °C) to remove the protein fraction The supernatants were dried in a vacuum centrifuge (Speedvac concentrator SVC200H, Savant, Holbrook, NY, USA) and stored at −80 °C until analysis.

2.3. Off-line MALDI-TOF–TOF analysis

Just prior to HPLC analysis, samples were dissolved in 300 μL milli-Q water containing 5% acetonitrile and 0.5% trifluoroacetic acid (TFA), and filtered through a 22 μm spin-down filter (Ultrafree-MC, Millipore, Bedford, MA, USA). The sample was fractionated on a Symmetry C18 column (2.1 mm × 150 mm, 3.5 μm, Waters, Milford, MA, USA) using an LC system (Beckmann, Fullerton, USA)

equipped with the solvent module 126 and a Diode Array Detector Module 168 (Gold System). After injection of the peptide extract, a wash step was initiated for 10 min using 2% acetonitrile in 0.1% aqueous TFA. The elution gradient was programmed from 2 to 90% acetonitrile in 0.1% aqueous TFA over 80 min.

Sixty fractions of 1 mL each were collected by an auto-sample collector, and immediately dried. All HPLC fractions were reconstituted in 1.5 μL of water containing 50% acetonitrile and 0.5% TFA, and spotted on a MALDI target plate (ground steel, Bruker Daltonics, Bremen, Germany), then mixed with 1.5 μL of saturated solution of α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile and 0.5% TFA.

MALDI-TOF and MALDI-TOF–TOF analyses were carried out with an Ultraflex II instrument (Bruker Daltonics, Bremen, Germany) in positive ion, reflectron mode. Mass spectra were recorded with the Flex Control software in a mass range from m/z 500 to m/z 4500 in MS mode. Subsequently, peptides were fragmented using LIFT mode.

All fragmentation spectra were loaded and processed using Flex Analysis software (Bruker Daltonics, Bremen, Germany). After smoothing and baseline correction, lists of significant peaks in fragmentation spectra were generated using BioTool (Bruker Daltonics, Bremen, Germany) and used for Mascot MS/MS analysis (Matrix Science, London, UK).

2.4. Peptide characterization

All acquired MS/MS fragmentation spectra were subjected to a Mascot database search against the SwissProt database (version 51.6), where taxonomy was set to *Homo sapiens* (holding 15,720 protein sequences). Mass tolerance was set to 0.4 Da for MS and 0.8 for MS/MS, and no cleavage enzyme for protein digestion was chosen. Search parameters allowed for C-terminal amidation by loss of glycine, oxidation of methionine, as well as formation of pyro-glutamine and pyro-glutamate at the N-terminus. Peptide identifications were considered as positive if (1) the precursor protein was identified with a higher score than identity threshold and (2) the ion score of the peptide was above the homology threshold.

3. Results

In this study, high molecular weight proteins were removed and endogenous (naturally occurring) peptides were extracted from human reflex tear fluid. Subsequently, extracted endogenous peptides were processed by LC–MS/MS (Fig. 1), without any enzymatic treatment in order to identify the native forms of endogenous peptides. In MALDI-TOF–TOF experiments, 136 ions were observed (within the mass range of 500–4500 Da) and selected for fragmentation. From these ions, 30 spectra were successfully characterized with the Mascot search engine against a human protein database (SwissProt 51.6). Table 1 lists the peptide sequences identified in this study. In the present study, peptide identification was performed using large amount of pooled tear sample in order to gain signal intensity and improve fragmentation spectra for reliable peptide characterization. We also conducted LC-MALDI measurement for four individual tear samples to observe the distribution of tear peptides. The molecular masses corresponding to the majority of identified peptides were observed over four individual tear samples (Supplemental Data 1).

3.1. Peptides derived from the PRP4 precursor

Twenty-six peptides were identified from the proline-rich protein 4 (PRP4; UniProt accession number: Q16378). Three of these (DRPARHPQEQPL, DRPARHPQEQP, DRPARHPQE) were reported previously [16]. This protein is 134 amino acid residues in length