Original Article

Characterisation of Human Tear Proteins Using High-resolution Mass Spectrometry

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Abstract

Introduction: The proteins found in tears play an important role in maintaining the ocular surface and changes in tear protein components may reflect changes in the health of the ocular surface. Proteomics provides a comprehensive approach for cataloguing all the proteins of the tear proteome, which will help to elucidate disease pathogenesis, make clinical diagnoses and evaluate the influence of medications on the structure, composition and secretion of tear proteins. In this study, an alternative proteomic strategy was investigated to explore the human tear proteome. Materials and Methods: Tear samples were obtained from patients who had pterygium and were collected on the first day and third day after pterygium surgery. Tears pooled from 6 patients were used in the analysis. Reverse-phase high-pressure liquid chromatograph (RP-HPLC) was used as the first step to separate intact proteins into 21 peaks. Each fraction was then tryptic-digested and analysed by nanoLC-nano-ESI-MS/MS to characterise the protein components in each fraction. Results: In total, 60 tear proteins were identified with high confidence, including well-known abundant tear proteins, and tear-specific proteins such as lacritin and proline-rich proteins. Among them, proline-rich protein 5 was found for the first time in tear fluid. A large number of plasma proteins were also observed in tear fluid. Conclusions: The results showed that the proteomic strategy used in this study was successfully applied to analyse tear proteome.

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Key words: Human tear proteins, Human tear proteome, NanoLC-nanoESI-MS/MS, Tear proteomics

Introduction

Over the last few years, with advances in mass spectrometry, there has been increasing interest in exploring the proteome of body fluids such as plasma,[1] urine,[2] cerebrospinal fluid (CSF)[3] and saliva.[4] An important objective is to search for potential biomarkers for the diagnosing and monitoring the progression of disease. A tiny drop of tear fluid may hold a clue to certain eye diseases and even systemic diseases.

Tears are a complex biological mixture, containing electrolytes, proteins, lipids, mucins, some small organic molecules and metabolites.[5] The functions of the tear film include lubrication, protection from disease and nutrition of the cornea. It also plays a critical role in the optical properties of the eye.[6] Normal tear volume is around 6 μL and the secretion rate is about 1.2 μL per minute, with a turnover rate of approximately 16% per minute.[7] The tear film consists of 3 layers: an inner mucin layer; a middle aqueous layer which contains electrolytes, proteins and various metabolites; and an outer lipid layer. Normal tears contain 6 to 10 mg/mL of total proteins.[8] More than 100 different tear proteins have been reported so far and it is believed that there is much more than that.[9] Major tear proteins include lysozyme, lactoferrin, secretory immunoglobulin A (sIgA), serum albumin, lipocalin (previously called tear-specific prealbumin) and lipophilin.

The proteomic analysis of tear fluid is of fundamental interest in eye research. There is no doubt that proteomics will provide a comprehensive approach for cataloguing all tear proteins of the tear proteome, which will help to

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elucidate disease pathogenesis, make clinical diagnoses and evaluate the influence of medications on the structure, composition and secretion of tear proteins. Recently, many efforts have been made to explore tear proteins using proteomic approaches. These include direct analysis with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS),[10,11] 2-dimensional gel electrophoresis followed by MALDI-TOF-MS,[12] "shotgun" [whole tear sample digested by trypsin, followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS)],[9] surface-enhanced laser desorption/ionisation time-of-flight mass spectrometry (SELDI-TOF-MS),[13-15] and intact protein analysis by LC-MS.[16] MS-based methods have yielded both qualitative and quantitative data on tear proteins.

In this preliminary study, a different proteomic strategy was used to analyse tear proteins. Tear samples were firstly fractionated by reverse-phase HPLC (RP-HPLC). Each HPLC peak was then collected and concentrated. Each fraction was digested with trypsin and subjected to nanoLC-nano-ESI-MS/MS analysis.

Materials and Methods

Human Tear Samples and Collection

All human tear samples were obtained with patient consent. The patient consent forms and sample collection procedures were approved by the Ethics Committee of the Singapore Eye Research Institute. Human tear samples were obtained from patients who had pterygium and were collected on the first day and third day after pterygium surgery. Tears were collected from the inferior cul-de-sac using fire-polished 10 μL calibrated glass microcapillary tubes (Drummond Scientific Co, USA), with special care taken not to touch the ocular surface.[13]

Fractionation of Human Tear Proteins by Reverse-phase HPLC

Tears were pooled from 6 patients (post-surgery days 1 and 3). A total of 80 μL of tear fluid was injected into the HPLC system for fractionation. The HPLC system was a Waters 2695 separations module (Waters Associates, Milford, MA, USA) with an auto-sampler and 996 photodiode array detector (PDA). The fractionation was achieved on a Delta PAK C18 (5 μm particle size, 300 Å pore size, 150 x 3.9 mm, Waters Associates) at a flow rate of 0.2 mL/min. Mobile phase A was 0.02% trifluoroacetic acid (TFA) with 0.1% acetic acid in water and mobile phase B was 0.02% TFA with 0.1% acetic acid in acetonitrile. A linear gradient from 20% B to 30% B was used in the first 10 minutes, followed by 30% B to 70% B in the next 60 minutes, and finally from 70% B to 90% B in the last 10 minutes achieving a HPLC run of 80 minutes in total. The eluent was monitored at 210 nm and 21 peaks were collected. These 21 fractions were then lyophilised individually.

Trypsin Digestion of Individual Tear Fractions

Digestion of the proteins was performed using a trypsin digestion kit (ProteoExtract-All-In-One Trypsin Digestion Kit, Calbiochem, Darmstadt, Germany). Extraction buffer 1 (30 μL) from the kit was added to dissolve each of the 21 lyophilised protein samples. The samples were then centrifuged at 10,000 x g for 15 minutes. A 25 μL aliquot of the supernatant was transferred to a new tube and 25 μL of digest buffer and 1 μL of reducing agent was added. This was repeated for each sample. The samples were then incubated at 37°C for 15 minutes. The blocking agent, 1 mL, was then added to each sample and incubated at room temperature for 15 minutes. Trypsin, 1 μL (1 μg/μL) was added to each fraction and incubated at 37°C for 2 hours. The 21 samples were then lyophilised.

Protein Identification by nanoLC-nano-ESI-MS/MS

Each of the digested tear samples was reconstituted using 2% MeOH with 1% formic acid (FA) in water, and injected onto a 300 mm i.d. x 5 mm precolumn (C18 PepMap100, 5 μm, 100 Å, DIONEX, LC Packings, Sunnyvale, CA) using an automated nano-flow liquid chromatography system (UltiMate, DIONEX, LC Packings, Sunnyvale, CA) with a nano-spray source coupled to a quadrupole time-of-flight tandem mass spectrometer (QSTAR XL, Applied Biosystems, Foster City, CA). After washing and desalting for 5 minutes in mobile phase A (0.1% formic acid in water) at a flow rate of 0.03 mL/min, the peptides were eluted using a linear gradient of 5% to 60% B (0.1% formic acid in acetonitrile) over 45 min at a flow rate of 0.3 μL/min on a 75 μm i.d. x 10 cm fused silica column (New Objective, USA) self-packed with C18 materials (Luna 3u C18, 3 μm, 100 Å, Phenomenex, USA). Mass spectra were obtained in the positive ion TOF mode with scan range m/z 300 to 1200 while the MS/MS scan range was m/z 100 to 2500. The nanospray tip potential used for the analyses was 2200 V.

Database Searching

The data output from the MS/MS was processed and searched against the NCBI non-redundant (National Center for Biotechnology Information) protein database, under the human taxonomy, using Mascot software (Matrix Science, UK). The mass tolerances were ± 0.30 Da for both the parent ions (MS) and the fragment ions (MS/MS). Variable modifications of carbamidomethylation of cysteine and oxidation of methionine, together with a maximum of 1 missed tryptic cleavage site, were used. Only peptides with the ranking of 1 from the search result were considered, unless the lower-ranking peptide had an ion score that was

Table 1. Identified Proteins in Tear Fluid Fractionated by RP-HPLC and Followed by nanoLC-nano-ESI-MS/MS

Peak no.	Accession no.	Protein	Molecular function	Biological process	Mascot protein score	No. of peptide(s) found
1	gi|1335210	Lysozyme	Unclassified	Unclassified	469	10
2		Nothing significant/Probably not protein				
3		Nothing significant/Probably not protein				
4	gi|31968	Histone H1	Unclassified	Unclassified	170	3
	gi|3080461	Histone 1, H1b	Unclassified	Unclassified	147	3
	gi|4507065	Secretory leukocyte protease inhibitor	Unclassified	Unclassified	123	3
	gi|182051	Neutrophil elastase	Serine protease	Proteolysis	99	3
	gi|477072	Mucin 7, salivary	Unclassified	Unclassified	85	1
5		Nothing significant/Probably not protein				
6		Nothing significant/Probably not protein				
7	gi|6573592	Phospholipase A2	Phospholipase	Phospholipid Metabolism	378	6
8	gi|1040978	Ribonuclease 4	Endoribonuclease	RNA catabolism	146	3
9		Nothing significant/Probably not protein				
10	gi|1470343 gi|1470346 gi|1470344	HNP-1 HNP-2 HNP-3	Defense and immunity protein	Immunity and defense	Ref. 13	Ref. 13
11	gi|3704683	Proline rich 4 (lacrimal)	Unclassified	Unclassified	213	4
	gi|1854452	PBI (proline-rich protein 5)	Unclassified	Unclassified	138	2
	gi|22208536	Nasopharyngeal carcinoma-associated proline rich 4	Unclassified	Unclassified	78	3
12	gi|18653448	Coagulation factor II (thrombin)	Serine protease	Blood clotting	104	2
13	gi|56203410	Complement factor H	Complement component	Immunity and defense	155	5
	gi|705389	Proline rich peptide (proline-rich protein 3) P-B	Unclassified	Unclassified	83	2
	gi|2392230	Chain A, Human Cathepsin G	Serine protease	Granulocyte-mediated immunity	73	2
14	gi|32165624	Apolipoprotein H (beta-2-glycoprotein I)	Apolipoprotein	Immunity and defense	980	16
	gi|34616	Beta-2 microglobulin	Major histocompatibility complex antigen	MHCI-mediated immunity	318	5
15	gi|6175096	Lactoferrin	Transfer/carrier protein	Cation transport	3572	48
	gi|38026	Zn-alpha2-glycoprotein	Unclassified	Lipid, fatty acid and steroid metabolism	664	10
	gi|55958120	Lipocalin 1 (tear prealbumin)	Transfer/carrier protein	Chemosensory perception – Taste	512	8
	gi|118188	Cystatin SN (salivary cystatin SA-1) (Cystain SA-I)	Cysteine protease inhibitor	Proteolysis	202	3
16	gi|4502027	Albumin	Transfer/carrier protein	Unclassified	3207	48
17	gi|4107233	Lipophilin C	Unclassified	Developmental process	549	7
			2602742065246	7854311		
	gi|225541	Cystic fibrosis antigen	Calmodulin-related protein	Macrophage-mediated immunity	398	8
	gi|4107229	Lipophilin A	Transfer/carrier protein	Unclassified	260	5
	gi|47479525	Lacritin	Unclassified	Unclassified	274	4
	gi|339685	Transthyretin	Transporter	Transport	206	3
	gi|37852	Vimentin	Cytoskeletal protein (Intermediate filament)	Cell structure	52	1
	gi|56205192	S100 calcium-binding protein A12 (calgranulin C)	Calmodulin-related protein	Immunity and defense	46	1

Table 1. contd.

Peak no.	Accession no.	Protein	Molecular function	Biological process	Mascot protein score	No. of peptide(s) found
18	gi|47479525	Lacritin	Unclassified	Unclassified	274	5
	gi|30366	Cystain S (cystatin SA-III)	Cysteine protease inhibitor	Proteolysis	157	3
	gi|339685	Transthyretin	Transporter	Transport	206	3
	gi|32891795	Clusterin (apolipoprotein J)	Unclassified	Apoptosis	80	2
19	gi|178779	Apolipoprotein A-IV	Apolipoprotein	Lipid and fatty acid transport	441	10
20	gi|4557321	Apolipoprotein A-I G1	Apolipoprotein	Lipid and fatty acid transport	1461	23
	gi|1942629	Alpha-1-antitrypsin	Serine protease inhibitor	Proteolysis	1073	19
	gi|4501885	Beta actin	Actin and actin-related protein	Cell structure	295	7
	gi|106642	Ig lambda chain	Immunoglobulin	B-cell and antibody-mediated immunity	264	3
	gi|230581	Chain H, immunoglobulin	Immunoglobulin	B-cell and antibody-mediated immunity	192	4
	gi|56205191	S100 calcium-binding protein A9 (calgranulin B)	Calmodulin-related protein	Immunity and defense	128	3
	gi|1708182	Hemopexin (beta-1B-glycoprotein)	Unclassified	Unclassified	82	2
	gi|442631	Annexin I (annexin A1)	Annexin	Lipid, fatty acid and steroid metabolism; cell structure and motility	61	1
	gi|179161	Antithrombin III	Serine protease inhibitor	Proteolysis; immunity and defense	49	1
21	gi|24977620	Hepatocellular carcinoma-associated protein TB6	Unclassified	Unclassified	825	15
	gi|49257464	IGHA1 protein	Immunoglobulin	B-cell and antibody-mediated immunity	737	12
	gi|546799	IgA2 H chain	Immunoglobulin	B-cell and antibody-mediated immunity	604	10
	gi|21669351	Immunoglobulin kappa light chain VLJ region	Immunoglobulin	B-cell and antibody-mediated immunity	506	10
	gi|116594	Complement C3	Complement component	Immunity and defense	151	3
	gi|116117	Ceruloplasmin (ferroxidase)	Unclassified	Unclassified	114	2
	gi|3337390	Haptoglobin	Serine protease	Proteolysis; blood circulation and gas exchange activity	65	2

close to the first ranking peptide. All of the top-ranking peptides with an ion score much higher than their individual identity score were accepted. Those peptides with an ion score close to their individual identity score and others that were above the homology score were manually inspected to confirm their identity. The peptides that had more than 50% matches to the theoretical spectra in any of the y ions, y +2 ions, b ions or b +2 ions were accepted. If the identification of the protein was only based on 1 peptide, the sequence of the peptide was unique to that protein.

Results

The strategy for this study was to use RP-HPLC to separate intact proteins into a number of peaks, digest each fraction and follow with nanoLC-nano-ESI-MS/MS to characterise the protein components in each fraction. A

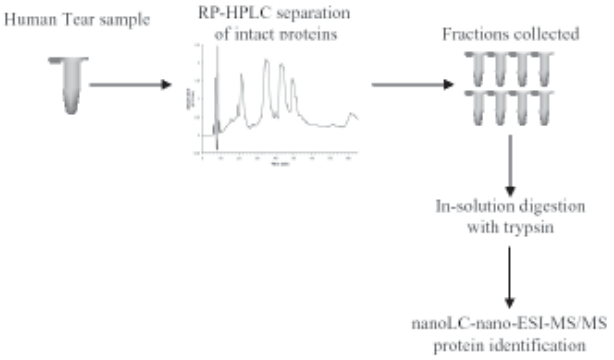

Fig. 1. Schematic representation of the proteomic strategy used in this study. Whole human tear samples were first fractionated with reverse-phase HPLC. The HPLC peaks/fractions were then digested with trypsin and the resulting peptide fragments were analysed using nanoLC-nanoESI-MS/MS.

Table 2. List of Tear Proteins Detected in Many HPLC Peaks

Accession no.	Protein	Molecular function	Biological process	Mascot protein score	No. of peptide(s) found
gi|51094526	Prolactin-induced protein 1	Unclassified	Unclassified	518	8
gi|182424	Alpha-fibrinogen	Extracellular matrix glycoprotein	Blood clotting	207	4
gi|400044	Immunoglobulin J chain	Unclassified	Unclassified	181	3
gi|55962156	Deleted in malignant brain tumours (DMBT1)	Receptor	Ligand-mediated signaling; immunity and defense	179	2
gi|31415705	Transferrin	Transfer/carrier protein	Unclassified	136	3
gi|1836022	Basic proline-rich protein; BPLP (proline-rich protein 1)	Unclassified	B-cell and antibody-mediated immunity	116	2
gi|16041759	IGHM protein	Immunoglobulin	Complement-mediated immunity	102	1
gi|2347133	Complement factor B	Complement component	Unclassified	75	1
gi|1197209	Alpha-1-acid glycoprotein 1 (orosomucoid 1)	Unclassified	Coenzyme and prosthetic group metabolism;	63	1
gi|72105	Vitamin D-binding protein	Non-motor actin-binding protein	Transport	57	1

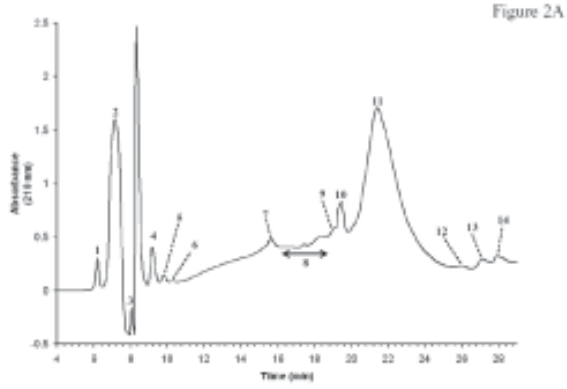

Fig. 2. (A)

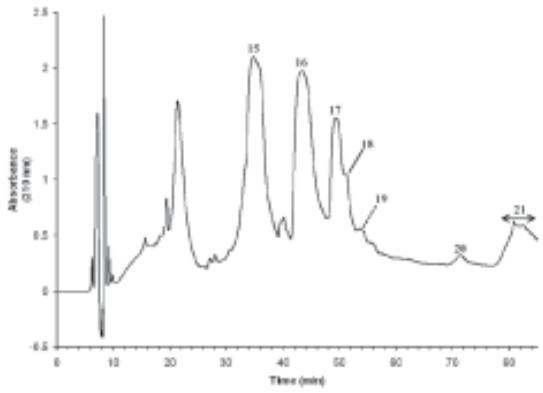

Fig. 2. (B)

Fig. 2. (A) and (B) Chromatograms of intact tear proteins separated by reverse phase HPLC. A total of 21 peaks (fractions) were collected.

schematic workflow of this strategy is given in Figure 1. Figure 2 shows an RP-HPLC separation profile. In total, 21 peaks/fractions were collected. Each fraction was then concentrated and digested by trypsin and subsequently analysed by nanoLC-nano-ESI-MS/MS. Five peaks did not produce significant peptide signals and probably contained no peptides or peptides at an extremely low concentration. The majority of the other 16 fractions contained multiple protein components and are summarised in Table 1. Another 10 proteins listed in Table 2 were detected in many peaks. In total, 60 tear proteins were distinctly identified. All MS/MS spectra were manually validated and the identification of 51 proteins was based on 2 or more distinct peptide matches (9 proteins were identified based on only 1 peptide match, but each had a very high MASCOT score and a peptide sequence unique to that protein).

Not surprisingly, several well-known abundant tear proteins, lysozyme (peak 1), lactoferrin (peak 15), lipocalin – previously called tear-specific prealbumin (peak 15), lipophilin (peak 17), serum albumin (peak 16) and immunoglobulin (fragments appear in several peaks) were all observed. Those were well characterised previously by 1D/2D gel[17-20] and various HPLC techniques (size exclusion,[21-23] reverse phase[16,24] and ion exchange). Several proteins, such as zinc-α-2-glycoprotein, alpha-1-antitrypsin, cystatin SN and cystatin S, which were previously reported by 2D gel[25,26] but not by HPLC, were also detected using the current method.

Proline-rich protein 1 (Basic proline-rich protein; BPLP)
Molecular Weight**: 22871 Da**
Sequence Coverage: **14%**

1 MKLTFFLGLL ALISCFTPSE SQRFSR**RPYL PGQLPPPPLY RPR**WVPPSPP
51 PPYDSR**LNSP LSLPFVPGR**V PPSSFSRFSQ AVILSQLFPL ESIRQPRLFP
101 GYPNLHFPLR PYYVGPIRIL KPPFPPIPFF LAIYLPISNP EPQINITTAD
151 TTITTNPPTT ATATTRHFHK THNDDQLLNS TYLFNTRACH LHISSNPRSI
201 Y

Proline-rich protein 3 (Proline-rich peptide P-B)
Molecular Weight**: 8188 Da**
Sequence Coverage: **65%**

1 MKSLTWILGL WALAACFTPG ESQRGPR**GPY PPGPLAPPQP FGPGFVPPPP**
51 **PPPYGPGRIP PPPPAPYGPG IFPPPPPQP**

Proline-rich protein 5 (Proline-rich protein PBI)
Molecular Weight**: 14117 Da**
Sequence Coverage: **20%**

1 MKSLTWILGL WALAACFTPG ESQRGPR**GPY PPGPLAPPPP PR**FPFGTGFV
51 PPPHPPPYGP GR**FPPPLSPP YGPGR**IPPSP PPPYGPGRIQ SHSLPPPYGP
101 GYPQPPSQPR PYPPGPPFFP VNSPTDPALP TLAP

Lacrimal proline rich 4
Molecular Weight**: 15088 Da**
Sequence Coverage: **37%**

1 MLLVLLSVVL LALSSAQSTD NDVNYEDFTF TIPDVEDSSQ RPDQGPQRPP
51 PEGLLPRPPG DSGNQDDGPQ QRPPKPGGHH R**HPPPPPFQN QQRPPQR**GHR
101 **QLSLPRFPSV SLQEASSFFR RDRPARHPQE QPLW**

Nasopharyngeal carcinoma-associated proline rich 4
Molecular Weight**: 15088 Da**
Sequence Coverage: **26%**

1 MLLVLLSVVL LALSSAQSTD NDVNYEDFTF TIPDVEDSSQ RPDQGPQRPP
51 PEGLLPRPPG DSGNQDDGPQ QRPPKPGGHH R**HPPPPPFQN QQRPPRR**GHR
101 **QLSLPR**FPSV SLQEASSFFQ R**DRPARHPQE QPLW**

Fig. 3. Amino acid sequences, average molecular weights and sequence coverage of 5 proline-rich proteins detected in human tear fluid.

A group of tear-specific proteins were also detected, including lacrimal proline rich 4 (peak 11), proline-rich protein 5 (peak 11), nasopharyngeal carcinoma-associated proline rich 4 (peak 11), proline-rich protein 3 (peak 13), lacritin (peaks 17 and 18), prolactin-induced protein (appeared in several peaks) and basic proline-rich protein (appeared in several peaks). Proline-rich proteins in tears are abundant and diverse. The majority of proline-rich proteins appeared in peak 11. If we use peak area as a rough estimation of the quantity of proteins in an HPLC chromatogram (peak 11), proline-rich proteins in tears should belong to the most abundant tear protein category. In total, 5 proline-rich proteins were found in tear fluid in this study, including proline-rich protein 5, which has not been reported previously. The amino acid sequences, average molecular weights and sequence coverage of these 5 proline-rich proteins are given in Figure 3. Highlighted sections of the peptide fragments were detected by LC-MS/MS. Figure 4 shows the MS/MS spectrum of one of the peptide fragments (GPYPPGPLAPPPPPR) that originated from proline-rich protein 5. The amino acid sequences for lacrimal proline rich 4 and nasopharyngeal carcinoma-

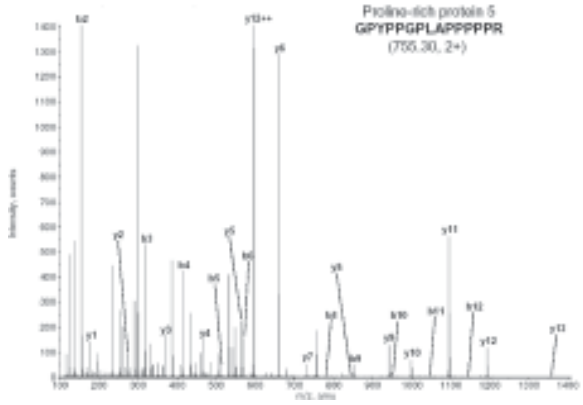

Fig. 4. LC-MS/MS spectrum of a peptide fragment originating from proline-rich protein 5 [GPYPPGPLAPPPPPR, m/z = 755.30, $(M+2H)^{2+}$].

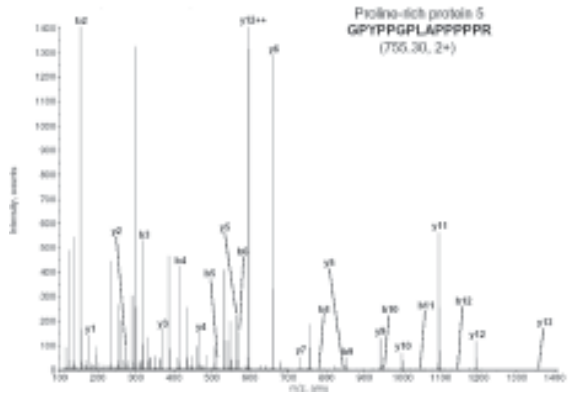

Fig. 5. (A)

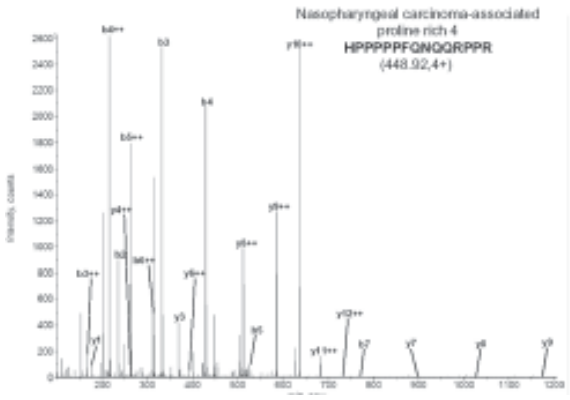

Fig. 5. (B)

Fig. 5. (A) LC-MS/MS spectrum of a peptide fragment originating from lacrimal proline-rich protein 4 [HPPPPPFQNQQRPPQR, m/z = 480.95, $(M+4H)^{4+}$]. (B) LC-MS/MS spectrum of a peptide fragment originating from nasopharyngeal carcinoma-associated proline rich 4 [HPPPPPFQNQ QRPPRR, m/z = 448.92, $(M+4H)^{4+}$].

associated proline rich 4 are very similar (Fig. 3). The differences between them are at 2 sites: Q for lacrimal proline rich 4 whereas R for nasopharyngeal carcinoma-associated proline rich 4 at position 96, and R for lacrimal

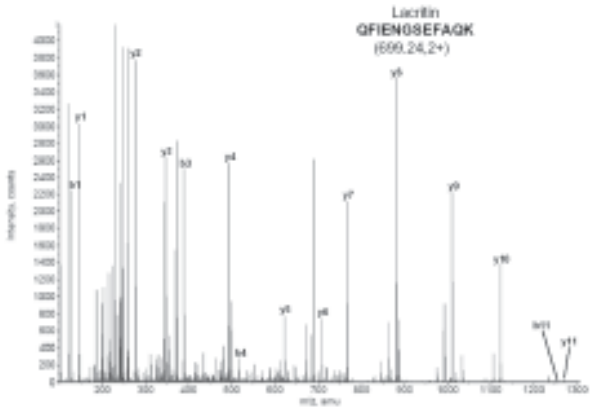

Fig. 6. LC-MS/MS spectrum of a peptide fragment originating from lacritin (QFIENGSEFAQK, m/z = 699.24, $[M+2H]^{2+}$).

proline rich 4 whereas Q for nasopharyngeal carcinoma-associated proline rich 4 at position 120. Figure 5 shows the MS/MS spectra of peptide fragment HPPPPPFQNQQRPPQR and HPPPPPFQNQQRPPRR, which differentiated lacrimal proline rich 4 and nasopharyngeal carcinoma-associated proline rich 4.

MS/MS evidence was uncovered for other representative human tear proteins. Five peptide fragments originating from lacritin were identified. Figure 6 shows an MS/MS spectrum of 1 peptide fragment, QFIENGSEFAQK, which provided confirmatory peptide fragment information for lacritin.

Other important tear proteins which may have antimicrobial or antiviral activity, such as phospholipase A2 (PLA2)[27-29] (peak 7) and secretory leukocyte protease inhibitor (SLPI)[30,31] (peak 7), were also observed in our study.

A group of more hydrophobic proteins eluted late from the column (peaks 20 and 21) included apolipoproteins, actin, annexin, immunoglobulin fragments and haptoglobin.

Discussion

The goal of this study was to explore the tear proteome using an alternative proteomic approach. One popular technique is to digest the whole protein mixture and analyse the digests using 2D-LC coupled with tandem mass spectrometry (often called the "shotgun" approach or MudPIT[32]). Adding a separation step for intact protein before digestion and analysis of the digest by LC-MS/MS may decrease the complexity of analysing whole peptide digests. Another advantage of this approach is that it can tolerate a wide dynamic range of an extremely complex body fluid such as tears [the concentrations of tear proteins can span 9 orders of magnitude, from the most abundant one (mg/mL level) to the low-abundance end (pg/mL level)].

This study revealed diverse forms of proline-rich proteins in tear fluid. A total of 5 proline-rich proteins – lacrimal proline rich 4, proline-rich protein 5, nasopharyngeal carcinoma-associated proline rich 4, proline-rich protein 3 and basic proline-rich protein – were observed. Among them, proline-rich protein 5 was found in tear fluid for the first time and the MS/MS evidence for basic proline-rich protein was also shown in this paper (Fig. 3). Previous work by Grus et al[14] revealed the presence of 3 proline-rich proteins in tears – nasopharyngeal carcinoma-associated proline rich 4, proline-rich protein 3 and proline-rich protein 4. Lacrimal proline-rich protein was well characterised by Fung et al.[33] A truncated form of lacrimal proline-rich protein was also found in their study. Although proline-rich proteins are one of the most abundant proteins in tear fluid, as suggested by the present study and the studies discussed above, this group was not seen using 2D-gels, which may be a technical issue. Proline-rich proteins are not easily stained with standard Coomassie and silver protocols.[34] Proline-rich proteins are also found in saliva.[4] Proline-rich protein 3, basic proline-rich protein and saliva-specific proline-rich proteins (acidic proline-rich phosphoprotein, clones CP3, CP4, CP5 and CP7) have been reported before. The biological functions of proline-rich proteins in tear fluid are still largely unknown. They play an important role in the protection of the ocular surface as antimicrobial proteins and are involved in the pathogenesis of inflammatory and autoimmune diseases.[33]

The tear samples in this study were collected from patients after ocular surface surgery. So proteins identified from these tear samples in this study do not represent the tear proteome under normal conditions. Due to the breakdown of the blood-cornea barrier, more blood leakage proteins (transferrin, complement factors, haptoglobin, etc.) were detected at the high concentration. Inflammatory response factors (for example, S100 calcium-binding proteins A9 and A12)[35,36] were also found in tears.

Low-abundance proteins may be missing in this study because the portions between LC peaks were not collected.

Conclusions

In conclusion, an alternative proteomic strategy – intact protein mixture pre-fractionated with reverse-phase HPLC and fractions subjected to in-solution digestion and analysed using nanoLC-nanoESI-MS/MS – was used to explore the tear proteome. In total, 60 tear proteins were identified with high confidence, including well-known abundant tear proteins and tear-specific proteins such as lacritin and proline-rich proteins. Among them, proline-rich protein 5 was found for the first time in tear fluid. A large number of plasma proteins were also observed in tear fluid. The

advantages of this proteomic approach include the reduction of the complexity of the tear protein mixture and the tolerance of a wide dynamic range of the protein mixture.

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