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# Proteomic Analysis of Human Tears: Defensin Expression after Ocular Surface Surgery

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Human tear protein profiles were monitored by surface enhanced laser desorption/ionization-time-of-flight mass spectrometry ProteinChip technology (SELDI-TOF ProteinChip) and liquid chromatography−mass spectrometry (LC−MS). Tears were collected from 21 patients scheduled for surgery to remove an ocular surface neoplasm prior to surgery (day 0) and on days 1, 3, and 30 postoperatively. Using this proteomic approach, we verified that three human α-defensins (HNP-1, HNP-2, and HNP-3) were significantly up-regulated in their expression after surgery and that their levels decreased to approximately normal by day 30 by which time healing was complete. Further confirmation of the identity of the α-defensins in human tears was made by LC purification, trypsin digestion, and ESI−MS/MS analysis of their tryptic digests. The concentrations of HNP-1 and HNP-2 were determined and shown to be markedly increased after ocular surface surgery. The results of the study suggest that human α-defensins HNP-1, HNP-2, and HNP-3 are up-regulated after surgery, and may in addition to their antimicrobial properties have an important role in wound healing.

**Keywords:** human tear proteins • eye • defensins • SELDI-TOF • ProteinChip • proteomics

## Introduction

The ocular surface is covered by a thin tear film that has multiple functions including protection, lubrication, and nutrition and in providing the mirrorlike surface for image formation on the retina.[1] Tears contain mucins, proteins, lipids, and electrolytes in an aqueous environment.[2] Major tear proteins include lysozyme, secretory immunoglobulin A (sIgA), lactoferrin, albumin, lipocalin, and lipophilin.[2] Some of these tear proteins have an important role in protecting the ocular surface from microbial attack since the ocular surface is constantly exposed to environmental pathogens, which is of more concern after injury or surgery. As recent studies have suggested, tear proteins can have a role in protecting the ocular surface and in modulating the wound healing process.[3−6] This has prompted an increased interest in determining how expression levels of functional tear proteins may be controlled after events that compromise the tissue barrier. The determination of a more comprehensive tear protein profile from a single tear sample has been a challenging analytical problem, as the sample volume is usually limited to a few microliters. The conventional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in combination with mass spectrometry (MS) often requires 10 μL or more of tears for one analysis.[7,8] Although 2D-PAGE is a very powerful approach to protein separation, it has limitations when dealing with very large or small proteins, extremely acidic or basic proteins, very hydrophobic proteins, or proteins in low-abundance.

Recently the surface enhanced laser desorption/ionization-time-of-flight (SELDI-TOF) ProteinChip has been introduced[9] as an alternative to 2D-PAGE. This technology utilizes affinity surfaces to retain adherent proteins based on their physical or chemical characteristics, which is then followed by direct analysis using TOF-MS. It is a rapid and reproducible technique to generate protein expression profiles known as "phenomic fingerprints". Furthermore, SELDI-TOF is more sensitive and requires only small amounts of sample (2−3 μL) compared to 2D-PAGE. This system has enabled detection of critical proteins directly from crude mixtures without labor intensive preprocessing and has been proven to be a very useful tool to identify biomarkers in various cancers [10−14] as well as other diseases.[15,16]

The objective of this study was to use a proteomic approach to compare tear protein profiles prior to and following sched-

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uled surgery of the surface of the eye to remove a benign growth. Understanding the role of specific tear proteins on the ocular surface requires the clear association of the expression of the proteins with defined clinical situations, as in the case of the patients selected for this study. The SELDI-TOF ProteinChip technology was used as a rapid profiling method to monitor tear proteins. Candidate peptides/proteins were purified by a reversed-phase HPLC and identified by tandem mass spectrometry.

## Materials and Methods

**Patients.** Tear samples were obtained from both eyes of patients who had an ocular surface disease called pterygium (a vision-obscuring overgrowth onto the clear cornea), which had been determined by the ophthalmologist (DT) to require surgery for removal.[17] All patients received counseling and the tear collection procedure was explained in the patient consent form. The procedure was approved by the Ethics Committee of the Singapore Eye Research Institute. Other than the need for pterygium surgery in one eye only, there were no additional requirements for participation in the study. A total of 21 patients (18 male and 3 female with an average age of 54) were recruited in no special order over an 8-month period. All patients had only unilateral pterygium; the contralateral, control eye had no sign of the disease.

Briefly, tear collection was performed using 10-$\mu$L pipets with fire-polished tips. The tip of the capillary tube was laid into the space between the globe and the lid and tears flowed by capillary action into the pipet.[18] Tears were spun at 8000 rpm to remove cells and frozen at −80 °C. Tears were collected 1−3 days prior to surgery, referred to as "0" time, and on postoperative days 1, 3, and 30. All of the patients healed uneventfully without surgical complications or infections.

**Chemicals and Peptide Standards.** HPLC-grade acetonitrile (ACN) was purchased from Fisher Scientific (USA). Trifluoroacetic acid (TFA), acetic acid, ammonium bicarbonate, α-cyano-hydroxy cinnamic acid (CHCA), DTT and iodoacetamide were purchased from Sigma (St. Louis, MO). Human defensins HNP-1 and HNP-2 standards were also from Sigma (St. Louis, MO). Trypsin was from Roche Molecular Biochemicals (Indianapolis, IN) and RapiGestSF reagent was from Waters Associates, USA. The water used in the mobile phase was Milli-Q grade (Millipore, MA).

**Analysis with ProteinChip SELDI-TOF.** NP20 (normal phase, hydrophilic surface: $SiO_2$) ProteinChip arrays (Ciphergen Biosystems, Fremont, CA) were used in this study. Two microliters of human tears were applied onto each array spot and allowed to air-dry. After that, each target was washed 3× with 5 $\mu$L of deionized water and finally allowed to air-dry. A saturated solution, 0.8 $\mu$L, of the energy absorbing molecule (EAM)-CHCA dissolved in 50% ACN containing 0.5% TFA was added (2×) to the spot and allowed to dry. All ProteinChip arrays were analyzed in a ProteinChip Reader (PBS−II, Ciphergen Biosystems, Fremont, CA) according to an automatic data collection protocol with the following acquisition settings: high mass 20 kDa; digitizer rate 250 MHz; laser intensity 155; sensitivity 10. The instrument was operated in positive ion mode with a source and detector voltage of 1.8 kv. Each spectrum was an average of at least 65 transients and externally calibrated with a mixture of seven known proteins ([Arg-8]-Vasopressin, 1084.247 Da; Somatostatin, 1637.903 Da; DynorphinA [209−225], 2147.500 Da; ACTH [1−24], human, 2933.500 Da; Insulin B-chain, bovine, 3495.941 Da; Insulin, human recombinant, 5807.653 Da; Hirudin BKHV, 7033.614 Da). Data interpretation was analyzed by the use of the ProteinChip Software (version 3.0).

**Analysis with High-Pressure Liquid Chromatography−Mass Spectrometry (LC−MS).** The LC−MS system consisted of a Waters 2690 (Waters Associates, Milford, MA), including an auto-sampler and photodiode array, coupled to a Micromass Platform LCZ (Micromass, UK). The Delta PAK $C_{18}$ analytical column (5-$\mu$m particle size, 300 Å pore, 150 × 3.9 mm) was from Waters Associates. Gradient elution was started at 80% A and 20% B (solvent A: 0.02%TFA-0.1% acetic acid in water, solvent B: 0.02%TFA-0.1% acetic acid in acetonitrile). The proportion of solvent B was increased linearly from 20% to 30% in 10 min, and from 30% to 40% in 30 min. The flow rate was 0.2 mL/min. All mass spectra were recorded under a full scan for positive ions in a continuous mode or in a centroid mode. Optimized mass spectrometer parameters were as follows: capillary voltage at 3.5 kV, cone voltage at 30 V, source block temperature at 140 °C, desolvation temperature at 250 °C, and nitrogen flow rate at 350 l/h. The scan range was from $m/z$ 600 to 3000.

**Protein Purification and Identification.** A total of 90 $\mu$L of human tear samples (pooled from 6 eyes, post-operative day 1 and 3) were used for α-defensin (HNP-1, HNP-2, and HNP-3) purification and identification. All tear samples were centrifuged to remove particles and cells. Similar analytical HPLC conditions were used for purification and 30 $\mu$L of tears was injected at each time × 3 and the peak collected and combined for future protein identification.

The collected fraction was concentrated to about 10 $\mu$L by freeze-drying. A reducing reagent (5 $\mu$L of a mixture of 200 mM DTT in 25 mM ammonium bicarbonate) was added and the sample vortexed. The mixture was incubated for 1.5 h at 56 °C. The sample was alkylated in the dark for 1 h by adding 30 $\mu$L 200 mM iodoacetamide in 25 mM ammonium bicarbonate. The mixture was subjected to HPLC purification again to remove the remaining DTT and iodoacetamide, and concentrated to about 10 $\mu$L by freeze-drying. Trypsin, 5 $\mu$L, (prepared to 0.1% using 25 mM ammonium bicarbonate buffer) and 15 $\mu$L RapiGestSF reagent (0.05%) were added into the sample and incubated for 1 h at 37 °C. Peptide identification was performed on a QSTAR Pulsar quadrupole time-of-flight tandem mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a nano-spray source (Protana/MDS Sciex, Toronto, Canada). All spectra were obtained in the positive-ion TOF mode with the scanned mass ranging from $m/z$ 400 to 1500. All MS/MS spectra were acquired in MCA scan mode at CE values varying between 20 and 75. The nanospray tip potential was maintained at 1000−1200 V throughout the analyses.

**Protein and Peptide Database Search.** The initial identification of tear proteins was based on their measured average molecular weights after searching the SWISS-PROT protein database using TagIdent on the ExPaSy website. The parameters were set to a mass range of less than 0.1% and the p$I$ of the protein to 7 ± 10, since no p$I$ values are available from SELDI or LC−MS. The search was limited to human proteins by specifying the "Organism Species" or "Organism Classification" (OS or OC) as human.

The peptide fragments after trypsin digestion were analyzed by MS/MS and the sequences were determined using Mascot software (Matrix Science, London, UK). The database used for searching was obtained from the National Center for Biotechnology Information (NCBI). In this case, *Homo sapiens* was chosen as the species database.

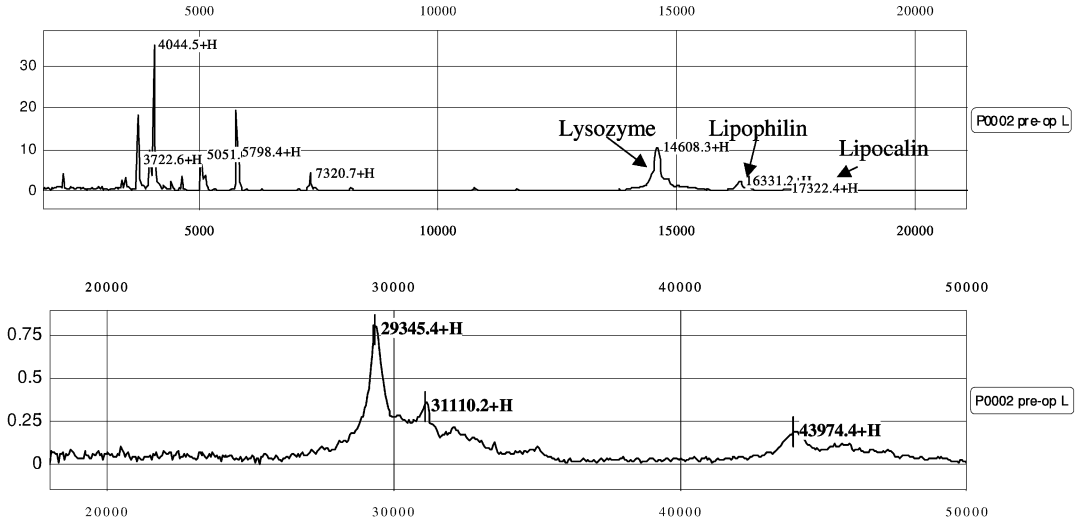


**Figure 1.** Rapid tear protein profiling by SELDI ProteinChip. Samples of human tears were analyzed using the NP20 hydrophilic ProteinChip array. The mass range shown here is from 1500 to 50 000 Da.

**Statistical Analysis.** Data were analyzed using commercial software (STATISTICA ver. 6.0; StatSoft Inc., Tulsa, OK). Data from day 0 (prior to surgery), post-operative day 1, day 3, and 1 month were compared respectively with the control eye group with an unpaired *t*-test. $P < 0.05$ was considered statistically significant, and all values are reported as means ± SD.

## Results and Discussion

**Rapid Monitoring of Protein Profiles of Human Tears by SELDI-TOF ProteinChip Prior to and After Ocular Surface Surgery.** Initially, the SELDI-TOF ProteinChip was used as a high throughput screening technique to capture any change in the protein profiles of human tears prior to and after ocular surface surgery. Each ProteinChip Array has 8 to 24 spots that contain chemical (hydrophilic, hydrophobic, ionic, etc.) or biological (antibody, receptor, DNA, etc.) surfaces designed for the selective capture of proteins/peptides from complex biological mixtures. After the sample was applied to the surface, unbound proteins and other interfering substances (i.e., salts, lipids, detergents, etc.) were washed off, and subsequent steps were similar to sample preparation steps for MALDI-TOF mass spectrometry. Usually 1 to 3 $\mu$L of tear fluid was spotted on the ProteinChip. A typical human tear protein profile (in a mass range of 1500 to 50 000 Da) on a NP20 chip (hydrophilic) is shown in Figure 1. Within this mass range, major tear proteins, such as $\beta$2-microglobulin (11 677.0 Da), lysozyme (14 690.7 Da), lipophilin (16 428.4 Da), and lipocalin (17 428.3 Da) were observed. Unidentified protein peaks were observed at 2129.3, 3371.0, 3440.7, 3486.0, 3700.1, 3722.5, 3955.1, 4044.5, 4408.8, 4615.3, 5051.3, 5778.1, 7322.0, 8184.2, 10 783.5, 29 345.4, 31 110.2, and 43 974.4 Da.

Figure 2 shows a series of mass spectra (in the range of 3000 to 4000 Da) representing tear-protein profiles from the normal, uninvolved control eye as well as the contralateral operated eye. The remarkable changes in the amplitude of peaks $M_1$, $M_2$, and $M_3$ were immediately noticed. Peaks $M_1$, $M_2$, and $M_3$ were barely detectable in either the control eye or operated eye prior to surgery (Lanes 1 and 5). The levels of $M_1$, $M_2$, and $M_3$ were significantly elevated 1 and 3 days after surgery. However, at the end of the 30-day observation period their levels were found to return to approximately normal (Lane 4).

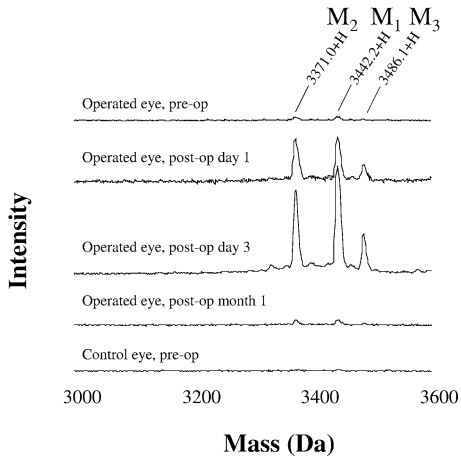


**Figure 2.** Time series from one patient shows the changes in expression levels of tear protein mass spectra in the region of 3000 to 4000 Da, Lane 1: operated eye, prior to surgery; Lane 2: operated eye, 1 day after surgery; Lane 3: operated eye, 3 days after surgery; Lane 4: operated eye, 30 days after surgery; Lane 5, fellow control eye, tears collected 3 days after the other eye's surgery. Signal intensities were normalized so that they directly correlate to the amount of the peptide concentration in the tear fluid.

There were no measurable changes in control eyes at any of the post-surgical times.

The measured molecular weights of peaks $M_1$, $M_2$, and $M_3$ were 3442.2, 3371.0, and 3486.2 Da, respectively. Database searching based on mass weight showed that the mass weights were very close to several of the human α-defensins (HNP-1, HNP-2, and HNP-3). Pure standards for HNP-1 and HNP-2 were analyzed using SELDI-TOF ProteinChip under the same conditions as used for tears and the results were compared with the human tear samples (Figure 3). Measured molecular weights of the standards for HNP-1 (3442.20 Da) and HNP-2 (3371.00 Da) were in good agreement with the theoretical masses (HNP-1: 3442.09 Da, HNP-2: 3371.01 Da). Figure 3 shows representative spectra demonstrating the occurrence of HNP-1 (peak $M_1$, measured mass: 3442.20 Da), HNP-2 (peak $M_2$, measured mass: 3371.00 Da) in a human tear sample. In addition, a peptide with a predicted mass similar to that of

**Table 1.** Amino Acid Sequences of α-Defensins (HNP-1, HNP-2, and HNP-3)

| name | amino acid sequence | $M_w$ (Da) | disulfide bridges positions |
|---|---|---|---|
| human neutrophil defensin-1 | *ACYCR*IPACIAGERRYGTCIYQGRLWAFCC | 3442.09 | 2−30; 4−19; 9−29 |
| human neutrophil defensin-2 | *CYCR*IPACIAGERRYGTCIYQGRLWAFCC | 3371.01 | 1−29; 3−18; 8−28 |
| human neutrophil defensin-3 | *DCYCR*IPACIAGERRYGTCIYQGRLWAFCC | 3486.10 | 2−30; 4−19; 9−29 |

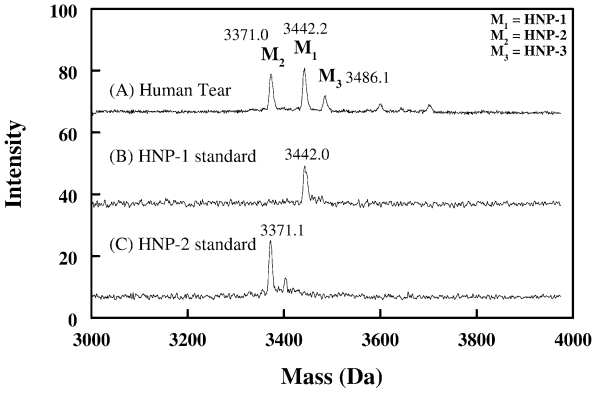


**Figure 3.** Analysis of tear proteins using ProteinChip array SELDI-TOF mass spectrometry. NP20 protein chips were used throughout the study. (A) A representative human tear profile 1 day after pterygium surgery (mass range shown here is from 3000 to 4000 Da). Peaks $M_1$ and $M_2$ are identical to standard HNP-1 and HNP-2, whereas, peak $M_3$ is tentatively identified as HNP-3 with a measured mass of 3486.1 Da. (B) Standard HNP-1 with a measured mass of 3442.0 Da. (C) Standard HNP-2 with a measured mass of 3371.1 Da.

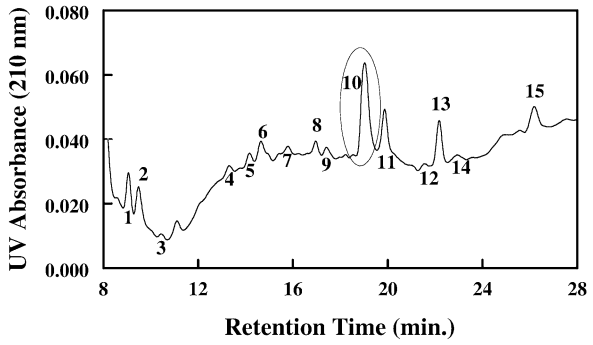


**Figure 4.** HPLC−UV chromatogram of a typical human tear sample. Magnified chromatogram in the region of 8 to 28 min revealed a complex elution pattern. As detailed in the results, peak 10 was shown to contain human α-defensins, HNP-1, HNP-2 and HNP-3.

HNP-3 (peak $M_3$, measured mass: 3486.1 Da, theoretical mass: 3486.10 Da) was also observed in human tears. An HNP-3 standard is not commercially available so comparison of peak $M_3$ to a pure standard was not possible.

We compared the ability of four different chips to bind HNP-1 and HNP-2 standards, i.e., hydrophilic (NP20), hydrophobic (H4), strong anionic (SAX2), and the weak cationic (WCX2). The results showed that NP20 has the highest affinity for α-defensins, which was also similar to the binding behavior of *β*-defensins.[19]

**Purification and Identification of α-Defensins (HNP-1, HNP-2, and HNP-3) in Human Tears.** An LC−MS method was developed to separate and visualize peptides in tear fluids. A typical magnified HPLC chromatogram is shown in Figure 4. The chromatogram from 8 to 28 min revealed a complex elution pattern with over 15 peaks in this region. The corresponding

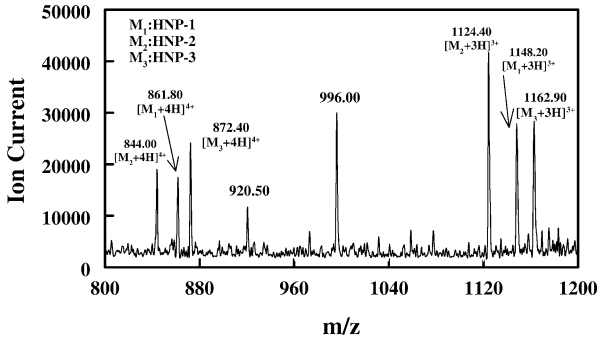


**Figure 5.** Mass spectrum of peak 10 from Figure 4 obtained with the use of online electrospray ionization mass spectrometry.

mass spectrum revealed that peak 10 contained 3 major peptide components ($M_1$, $M_2$, and $M_3$, Figure 5). The retention times ($R_{t\ (M1)}$ = 18.9 min and $R_{t\ (M2)}$ = 19.1 min) of $M_1$ and $M_2$ are identical to those of standards for HNP-1 and HNP-2, respectively. The identification of $M_1$ and $M_2$ in peak 10 were further confirmed to contain HNP-1 and HNP-2 from their mass spectra by comparing them with those of HNP-1 and HNP-2 standards. Triple-charged ($[M+3H]^{3+}$) and quadruple-charged ($[M+4H]^{4+}$) ions dominated the mass spectra and there were no molecular ions ($[M+H]^+$) observed. M3 was tentatively assigned as HNP-3 based on the expected triple-charged and quadruple-charged ion in the mass spectrum.

Peak 10 in Figure 4 (also see Figure 5) was purified by HPLC procedures and analyzed by ProteinChip SELDI-TOF. The results showed the same peptide patterns as Figure 3, which suggested that the peptides monitored by LC−MS (mainly triple-charged and quadruple-charged ions) are identical to those observed using ProteinChip SELDI-TOF (in the form of parent molecular ions).

To verify the identity of HNP-1, HNP-2, and HNP-3, the fraction of peak 10 underwent reduction, alkylation, and trypsin digestion prior to analysis by quadrupole time-of-flight MS/MS. Theoretically, after trypsin digestion, HNP-1, HNP-2, and HNP-3 will each produce four peptide fragments (IPAC*IAGER, YGTC*IYAGR, LAWFC*C*, and AC*YC*R for HNP-1 or C*YC*R for HNP-2 or DC*YC*R for HNP-3, C* represents cysteine treated with iodoacetamide to form carbamidomethyl-cysteine). The only difference in amino acid sequence of HNP-1, HNP-2, and HNP-3 is at the N-terminal position (see Table 1). The expected peptide fragments, i.e., 493.7402 ($[M+2H]^{2+}$ IPAC*IAGER), 559.2401 ($[M+2H]^{2+}$, YGTC*IYAGR), 856.3512 ($[M+H]^+$, LAWFC*C*), 729.2481 ($[M+H]^+$, AC*YC*R), 658.2207 ($[M+H]^+$, C*YC*R) and 773.2492 ($[M+H]^+$, DC*YC*R) were all observed by TOF−MS scan. Ions at $m/z$ of 493.7402, 559.2401, 729.2481, 658.2207, and 773.2492 were also selected and fragmented into smaller ions by MS/MS to generate sequence information (Figure 6A-E). The y-ions observed matched exactly with the expected peptide fragment sequences. Figure 6C−E illustrates the product ion mass spectra at $m/z$ 729.2481, 658.2207, and 773.2492. The $y_4$ ion (at $m/z$ 658.0552) in Figure 6C confirmed that the N-terminal residue of HNP-1 is Ala

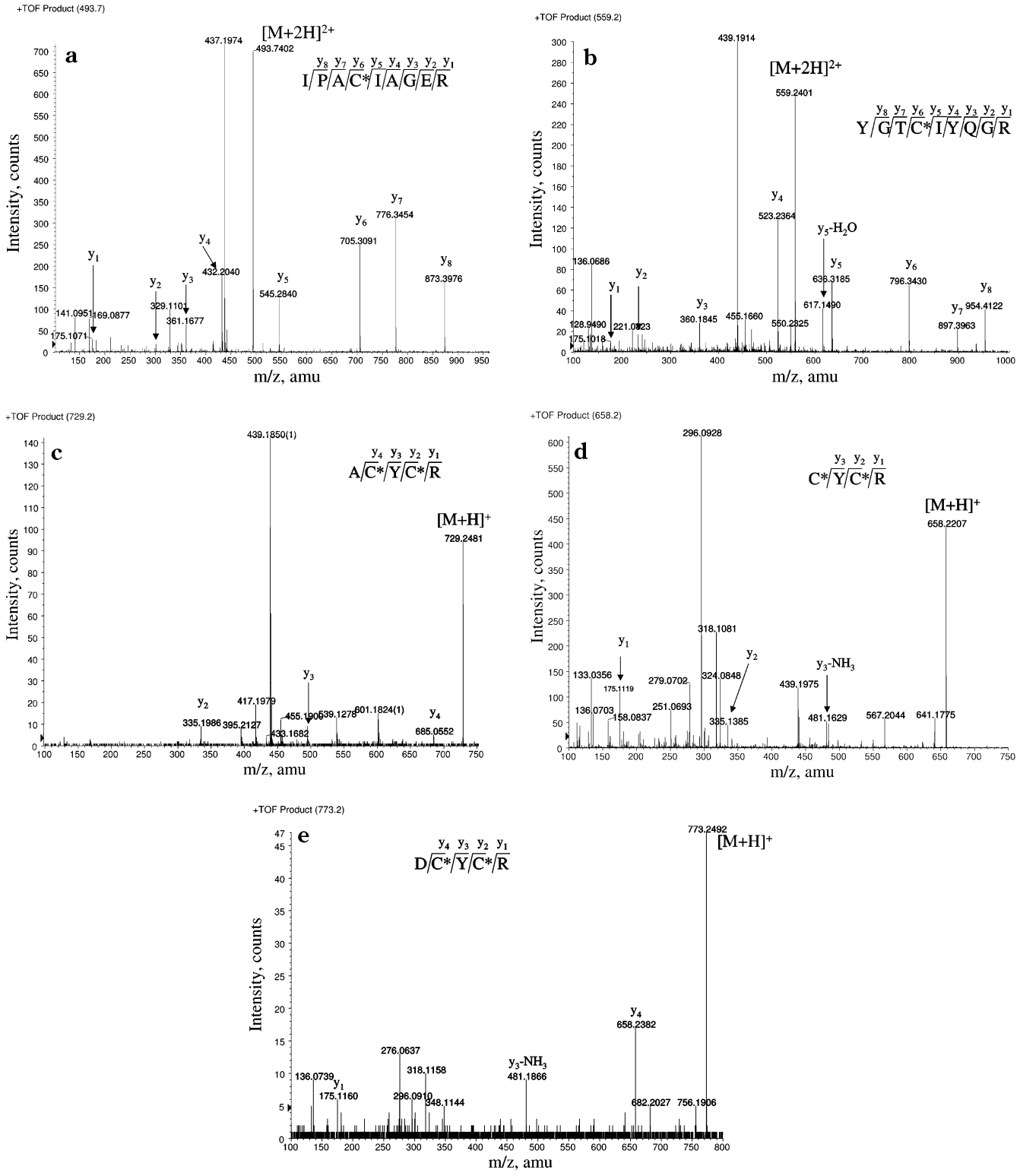


**Figure 6.** Product ion MS/MS spectra of the precursor ions, *m*/*z*, 493.7402, 559.2401, 729.2481, 658.2207, and 773.2492 (A−E) from the tryptic digests of an aliquot of human tear fluids purified by HPLC (peak 10). The MS/MS spectra revealed the peptide sequence information and confirmed the identity of HNP-1, HNP-2, and HNP-3.

(729.2481−658.0552 = 71.1929, the mass of an Ala). Similarly, the $y_4$ ion (at $m/z$ 773.2492) in Figure 6E confirmed that the N-terminal residue HNP-3 is Asp (773.2492−658.0552 = 115.1940, the mass of Asp). The above MS/MS results and Mascot search of the MS/MS data confirmed the identity of HNP-1, HNP-2, and HNP-3.

**Quantitative analysis by LC−MS.** To quantify HNP-1 and HNP-2 concentrations in tears, standards for HNP-1 and HNP-2 were analyzed in the same manner as used for analyzing human tears. The most abundant multiple charged ions, $m/z = 861.5$ ($[M+4H]^{4+}$) for HNP-1 and $m/z = 844.0$ ($[M+4H]^{4+}$) for HNP-2, were monitored simultaneously in selected-ion

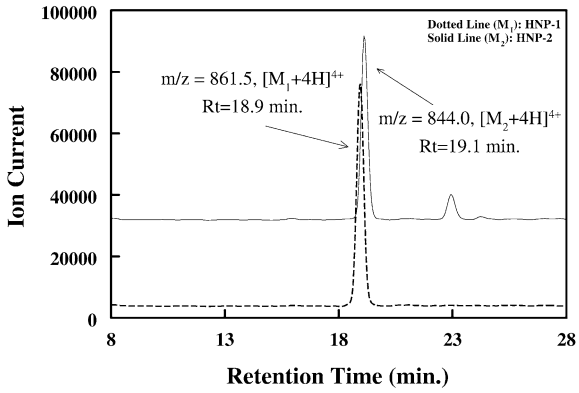


**Figure 7.** Selected-ion recording (SIR) chromatograms of HNP-1 (dotted line) and HNP-2 (solid line) from a human tear sample. The quantification of HNP-1 (*m/z* = 861.5) and HNP-2 (*m/z* = 844.0) was based on peak areas from the SIR chromatograms.

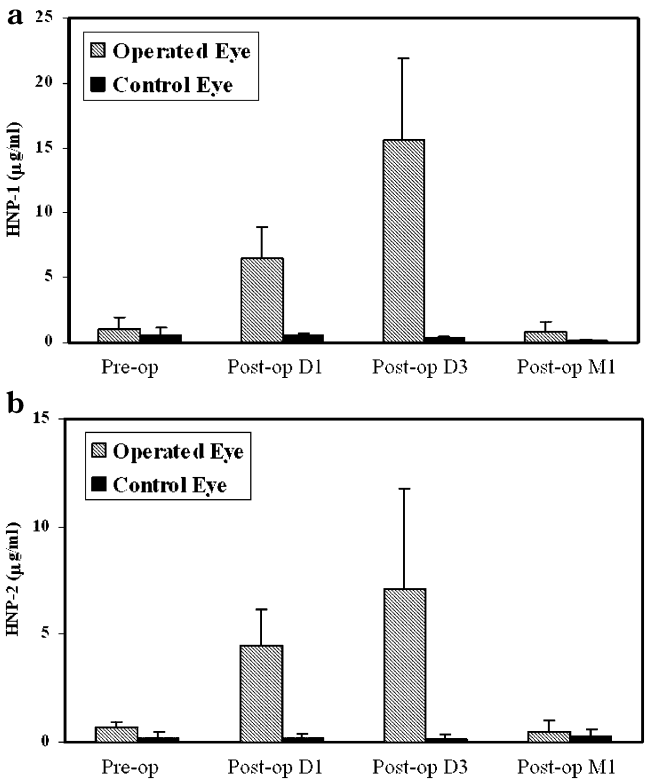


**Figure 8.** Comparison of (A) HNP-1 and (B) HNP-2 concentrations ($\mu$g/mL) in human tear fluid prior to and after pytergyium surgery of the ocular surface.

recording (SIR) for the quantification[20] (Figure 7). Five-point calibration curves (triplicate injections) were developed for the concentration range from 10 to 200 ng on-column for both HNP-1 and HNP-2. The limit of quantification for both HNP-1 and HNP-2 was estimated to be 0.2 $\mu$g/mL at the signal-to-noise ratio of 10.

With the developed method, HNP-1 and HNP-2 tear concentrations from six additional pterygium patients who had undergone surgery were measured. The results of pre-surgical, post-surgical day1, post-surgical day 3, and post-surgical day 30 for both operated and contra-lateral normal eyes are shown in Figure 8. The changes in expression levels of HNP-1 ($P$ = 0.0000) and HNP-2 ($P$ = 0.0000) in tears from operated eyes compared to the control eyes were highly significant at day 3. The tear levels of HNP-1 ($P$ = 0.1535) and HNP-2 ($P$ = 0.3227) returned to approximately normal levels one month after surgery. Since the control samples were from the nondiseased, contralateral eyes of the same patient, the environments for both eyes are considered to be very similar.

Defensins are a family of small, cationic antimicrobial peptides containing an average of 35 amino acids with molecular weights around 3∼4 kDa.[21,22] They possess six cysteine residues that form three intramolecular disulfide bonds. They have broad-spectrum antimicrobial action against gram-positive and gram-negative bacteria, fungi, and some viruses.[23−26] In humans, six α-defensins (HNP-1∼4, HD-5 and HD-6) and three $\beta$-defensins (hBD-1∼3) have been identified.[27] Recent studies have shown that defensins contribute to the host defense mechanisms as part of innate immunity.[28,29] In humans, defensins are not only abundant components of cytoplasmic granules in phagocytes [30] but are also secreted by epithelial cells[22] and present in body fluids including urine, saliva, nasal secretions, and bronchial fluids.[20,31−33] Detection of certain defensins in the human ocular surface, including cornea, conjunctiva, lacrimal gland and tears, has been reported.[34−42]

In a recent report by Zhang et al.,[43] a cluster of peptides isolated from $CD8^+$ T cells were identified as HNP-1, HNP-2, and HNP-3, which accounted for the anti-HIV-1 activity. HNP-1, HNP-2, and HNP-3 belong to one of the sub-families of antimicrobial peptides. The basic biological function of such antimicrobial peptides has been considered to be their activity against bacteria, fungi, and certain viruses. In the paper by Zhang, identification of the α-defensins was made using specific antibodies and SELDI interfaced with a MS/MS spectrometer. In our study, tear proteins were profiled with SELDI. Three human α-defensins (HNP-1, HNP-2, and HNP-3) were found to be significantly up-regulated in their expression in patients' tears after surgery without other complications. The desired peak was purified and concentrated by HPLC and further identified as HNP-1, HNP-2, and HNP-3 by MS/MS. Moreover, mass data differentiated HNP-1, HNP-2, and HNP-3, which only differ at the N-terminal position. However, in the long run, direct mass determination with sequencing is more accurate than relying on antibodies. Our study also supports recent suggestions that human α-defensins (HNP-1, HNP-2, and HNP-3) may have a role in modulating wound healing.

The α-defensins are most probably released into the tear fluids by resident or passing neutrophils or from lacrimal gland secretions. The elevated levels of HNP-1, HNP-2, and HNP-3 after ocular surface surgery may be responses from the mucosal immune system of the eye, which is comprised of the conjunctiva and the corneal epithelium. However, other biological functions of defensins have been reported or suggested such as accelerating epithelial wound healing[44,45] and mediating inflammation processes.[46] In this group of patients, there were no clinical signs of infection; however, healing processes after surgery are accompanied by a certain amount of inflammation and additional studies will be necessary to determine what role and source the α-defensins have in post-surgical healing. Basically, major human tear proteins, lysozyme, lactoferrin, tear-specific lipocalin, and secretory IgA (sIgA) form the peptide defense system of the ocular surface, which suggests that the role of defensins are multiple. It is useful to compare tear protein profiles prior to and after ocular surface surgery to understand the role of tear proteins, and the defensins in

particular in ocular surface wound healing. There are advantages to this determination. If the defensins have both wound healing and natural antimicrobial activity, then it may be useful to study functional components of the defensin molecule to optimize these characteristics for therapeutic purposes. A first step for this kind of study would be to know more about changes in tear protein patterns following an ocular surface surgery or injury.

The absence of the $\beta$-defensins suggests, however, that there is a certain partitioning based on the nature of the ocular surface disturbance. Recent studies in our laboratory have demonstrated that an experimental wound to the rabbit cornea resulted in the up regulation of molecules with defensin-like properties.[47]

## Conclusions

In conclusion, the SELDI-TOF ProteinChip platform provides a rapid and simple method for analyzing limited amount of samples, such as tear fluids, at the proteomic level. Our data showed the presence of three α-defensins HNP-1, HNP-2 and HNP-3 in human tears and revealed that the expression of these defensins significantly increased after ocular surface surgery.

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