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Quantitation of peptides from non-invasive skin tapings using isotope dilution and tandem mass spectrometry

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ABSTRACT

Previous work from our laboratories utilized a novel skin taping method and mass spectrometry-based proteomics to discover clinical biomarkers of skin conditions; these included atopic dermatitis, *Staphylococcus aureus* colonization, and eczema herpeticum. While suitable for discovery purposes, semi-quantitative proteomics is generally time-consuming and expensive. Furthermore, depending on the method used, discovery-based proteomics can result in high variation and inadequate sensitivity to detect low abundant peptides. Therefore, we strove to develop a rapid, sensitive, and reproducible method to quantitate disease-related proteins from skin tapings. We utilized isotopically-labeled peptides and tandem mass spectrometry to obtain absolute quantitation values on 14 peptides from 7 proteins; these proteins had shown previous importance in skin disease. The method demonstrated good reproducibility, dynamic range, and linearity ($R^2 > 0.993$) when n = 3 standards were analyzed across 0.05–2.5 pmol. The method was used to determine if differences exist between skin proteins in a small group of atopic versus non-atopic individuals (n = 12). While only minimal differences were found, peptides were detected in all samples and exhibited good correlation between peptides for 5 of the 7 proteins (R^2 = 0.71–0.98). This method can be applied to larger cohorts to further establish the relationships of these proteins to skin disease.

1. Introduction

Previous work from our laboratory has resulted in the development of a semi-quantitative mass spectrometry-based strategy to study skin diseases such as atopic dermatitis and eczema herpeticum. The strategy uses non-invasively obtained skin taping samples; a collection method that is appropriate for all age groups, including infants and children. In general, a total of 10 tapings from a single sampling results in confident identification of over 100 proteins. These include proteins that have previously been determined to be present in the cornified and granular layers of the skin [1].

When applied to a study on atopic dermatitis (AD), the semi-quantitative strategy successfully revealed novel proteins involved in the pathogenesis of AD [2]. Specifically, it was found that there was a lower expression of skin barrier proteins and enzymes in individuals with AD. These include filaggrin 2, corneodesmosin, and transglutaminase 3. It was further shown that patients colonized with methicillin-resistant *Staphylococcus aureus* (*S. aureus*) had greater amounts of epidermal fatty acid-binding protein (FABP) [3]; it was hypothesized that this might perpetuate the inflammatory response through eicosanoid signaling. While suitable for discovery based experiments, the semi-quantitative approach was deemed inappropriate for larger scale clinical studies. This was attributed to several factors including the time consuming and expensive nature of the approach and the concentration of protein required for technical replicates. Finally, relatively high variance in peptide abundances was observed during discovery experiments; this was considered to be largely due to matrix effects. The quantitative analysis of skin proteins has numerous additional applications, including cancer, psoriasis, and photodamage [4–7]. Therefore, the current study aimed to develop a method that overcame these challenges to produce a rapid, sensitive, and validated method to quantitate skin proteins.

Isotope dilution is a commonly used strategy for obtaining absolute concentrations (e.g. Pg/ml) of molecules in complex matrices. The

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strategy has been applied in multiple examples of peptide quantitation [8–10]. Briefly, a synthetic, isotope-labeled peptide is added during sample preparation and acts as an internal standard; tandem mass spectrometry is used to resolve and quantitate both the endogenous and labeled peptides [11]. The known amount of the labeled peptide is used to determine the concentration of endogenous peptide in the sample. This strategy is sometimes referred to as isotope dilution.

In the current study, we developed an isotope dilution technique to quantitatively measure 14 peptides from 7 proteins; these proteins were previously shown to be related to skin disease [2,3,12]. Overall, the 17 min method exhibited good linearity for all peptides and good reproducibility when standards were spiked into control samples. When applied to a small study evaluating the effect of location on skin proteins, it was determined that only two peptides were consistently different between patients with atopic dermatitis. However, 5 of 7 proteins had good correlation between the two peptides used to quantitate that protein, with R^2 values > 0.71.

2. Experimental

2.1. Sample collection & storage

Six atopic and six non-atopic subjects were consented under National Jewish Health IRB protocol # HS 1962, NJ209. Tapings were obtained for the following locations: bicep, antecubital fossae (anterior elbow), forearm, abdomen, back, thigh, and behind the knee. Sample collection procedures have been described elsewhere in detail [2]. Briefly, 20 standard D-Squame Skin Sampling Discs (CuDerm, Dallas, TX) were applied sequentially to the same location and placed adhesive side up in its own 20 ml borosilicate scintillation vial (Wheaton, Fisher Scientific, Pittsburgh, PA) and frozen in a −80 °C freezer. Participants were advised to not to use soap, lotion, or perfume on the day of collection. In addition, forearm samples were obtained from one non-atopic individual for the purpose of method and assay development; this included the generation of linearity curves for all labeled standards.

2.2. Reagents

Ammonium bicarbonate (ABC), sodium dodecyl sulfate (SDS), LC/MS grade acetonitrile and formic acid were obtained from Fisher Scientific. LC grade water was obtained from Burdick and Jackson. HALT protease inhibitor 100× was obtained from Pierce. Dithiothreitol (DTT) and iodoacetamide (IAA) were obtained from Bio-rad. Trypsin was obtained from Promega. Labeled peptides were custom synthesized from Sigma-Aldrich and New England Peptides. Since the peptides used for quantitation were from a tryptic digest, the C-terminus of the peptide was labeled with U-13C6, U-15N4 arginine (+10 Da shift) or a U-13C6, U-15N4 lysine (+8 Da shift). Individual peptides were reconstituted in either 0.1% formic acid and 30% acetonitrile or 5% ammonium hydroxide based on the solubility of the individual peptide. A mixture of all of the labeled peptides was then prepared at a concentration of 250 pmol/µl in 0.1% formic acid in 30% acetonitrile. Peptide digestion tubes were then prepared by pipetting 20 µl (5 pmol total of each labeled peptide) of the combined mix into a 1.5 ml low retention microfuge tube and frozen at −70 °C until ready for use.

2.3. Protein extraction

Discs (i.e. tape strips) were removed from the freezer and protein was harvested as follows: 500 µl of extraction buffer (1% SDS, 50 mM ABC, 10 mM DTT, 1× HALT) was placed in the well of a 6 well cell culture plate and discs were placed in to the well with the sticky side down using forceps and incubated for 1 min. While holding with forceps, the entire sticky side of the disc was scraped with a methanol cleaned cell scraper; as much sample was recovered as possible. The extracted disc was discarded. This process was repeated for up to 10 discs. The extract from 10 discs was then transferred to a 1.5 ml low retention microfuge tube and stored on ice. The volume was measured and the extraction well was washed with enough extraction buffer to bring the total volume of protein extract to 500 µl. This process was performed separately for each disc obtained at each location.

Following extraction from the disc, the samples were vortexed briefly and centrifuged to collect all liquid. Each sample was sonicated 3 times for 2 s each using a probe sonicator at a frequency of 25%. The samples were stored on ice for ~1 min after each sonication. Between each sonication the probe was washed by sonication in Contrad, followed by rinsing with 70% EtOH, then sonication with Millipore water. The sonicated extracts were then centrifuged at 14,000 RPM for 10 min at 4 °C and the supernatant was placed in a new microfuge tube.

Detergent was removed from the samples prior to protein digestion using a Pierce Detergent Removal Spin Column; the standard Pierce protocol was used with 50 mM ABC as wash buffer. The protein content was then determined using a Bradford protein assay with bovine serum albumin as the standard.

2.4. Protein digestion

Following initial processing, including detergent removal, a volume equivalent to 100 µg of total protein was added to a 1.5 ml centrifuge tube containing 5 pM of each isotope labeled peptide. Samples were dried in a speedvac prior to alkylation and digestion. A volume of 52.5 µl of 50% trifluoroethanol in 50 mM ABC and 0.2 mM DTT was added to the sample; tubes were vortexed for 1 min to denature and reduce the proteins. The sample was then heated for 45 min at 65 °C. 10 µl of 4 mM iodoacetamide was added and the sample was incubated in the dark at room temperature for 1 h to alkylate cysteine residues. 2.5 µl of 4 mM DTT was added and the sample was incubated in the dark at room temperature for 1 h to remove excess iodoacetamide. 3.3 µg of trypsin in 400 µl of 25 mM ABC was added and the sample was digested for 17 h at 37 °C. Following digestion, trypsin was deactivated with 2 µl of neat formic acid; the sample was placed into a speedvac until dry. Digested protein samples were frozen at −80 °C until analysis. On the day of analysis the samples were removed from the freezer and reconstituted in 100 µl of 0.1% ammonium hydroxide in 3% acetonitrile. The samples were analyzed within 24 h of reconstitution.

2.5. Quadrupole time-of-flight mass spectrometry and database searching for method development

For initial experiments designed to select peptides for inclusion in the assay, digested peptides were analyzed using liquid chromatography mass spectrometry (LC/MS) as described previously [13,14]. Briefly, tryptic peptides were analyzed using an Agilent Technologies 1100 series nanoflow HPLC-chip/MS system coupled to an Agilent 6520 Quadrupole Time-of-Flight (QTOF) mass spectrometer. Separation of peptides was accomplished using a Protein ID high capacity chip (0.075 mm × 150 mm 300 Å C18 analytical column with a 160 nl enrichment column). A total of 1 µg total protein was injected onto the column with the following run conditions: nano pump flow rate = 450 nl/min; capillary pump flow rate = 4 µl/min; drying gas temperature = 300 °C; gas flow rate = 4 l/min and; capillary voltage = 1800 V–2100 V. The analytical separation employed a combination of Buffer A (3% acetonitrile, 97% water, 0.1% formic acid) and Buffer B (90% acetonitrile, 10% water, 0.1% formic acid). The linear gradient employed was from 3%–36% Buffer B in 33 min; a linear gradient to 80% Buffer B at 35 min; holding at 80% Buffer B until 40 min and an 8-minute post-run equilibration in 3% Buffer A.

Data analyses were performed using the Spectrum Mill MS Proteomics Workbench software suite (Rev A.03) (Agilent Technologies, Santa Clara, CA). Data were searched against the Swiss-Prot database (UniProt Release 14). Search parameters included setting the species selection to “human”; the missed cleavage allowance to “2”