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Comparative evaluation of peptide desalting methods for salivary proteome analysis

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ABSTRACT

Background: Reliability and reproducibility are common requirements for high-quality generation of proteome data using mass spectrometry. The aim of this study was to compare four single-step desalting devices to provide a reproducible, high-recovery method for concentrating and purifying tryptic peptides before LC–MS/MS measurements.
Material and methods: Four different methods for peptide purification prior LC–MS/MS analyses (μC18 ZipTip® pipette tips, C18 ZipTip® pipette tips, TopTip C-18 and OASIS® HLB μElution Plate) were tested using whole saliva from healthy volunteers. A number of protein identifications and salivary protein patterns were analyzed comparatively.
Results: Each desalting device facilitated the identification of about 340 proteins. Purification-method dependent variations in protein composition were observed. Nevertheless, the overall inter-approach Pearson correlation coefficients of >0.95 indicate high reproducibility, reliability and recovery of proteins.
Conclusion: The applied devices performed equally well in the removal of low molecular weight contaminants and provide high-quality data for quantitative proteomic analysis. Thus, selection should be primarily based on the amount of peptide extract available and the number of samples to be processed.

1. Introduction

It is well known that sensitivity and accuracy of protein identification and quantification are affected by many factors including sample purity, liquid chromatography coupled mass spectrometry (LC–MS) platform and data analysis system. Substances (e.g. salts, detergents and buffers) that significantly contribute to the content of a biological sample influence the ionization efficiency and the quality of MS data in terms of sensitivity and dynamic range [1,2]. It is necessary to remove undesired contaminants, interfering substances and compounds. Thus, a salt- and detergent-free biological sample preparation still remains a crucial requirement in mass spectrometry based analytics. Numerous techniques and commercial devices are available of which the most commonly used techniques are protein precipitations, liquid–liquid extraction (LLE) or solid-phase extraction (SPE) [3,4]. Protein precipitations (methanol or trichloroacetic acid) are widely used in preprocessing of biological samples in order to concentrate proteins and purify them from various interfering substances [5,6]. In SPE molecules of interest are bound to a surface of a reversed-phase resin through hydrophobic affinity while the unbound fraction will be separated by the washing procedure and the bound clean peptide mixture will subsequently be released using organic solvents. Unwanted chemicals or salts will be removed from samples while analytes will be simultaneously enriched and cleaned. Such chromatography-based methods allow automation and multidimensional separation of complex peptide mixtures. Reversed phase microcolumns have been widely used for peptide treatment to remove interfering substances before LC–MS analysis in proteomics studies [7]. However, few studies have comparatively characterized the effects of different devices on the composition of identified peptides/proteins. The determination of variability in the whole saliva proteome is a pre-requisite for the development of whole saliva as a diagnostic and/or prognostic human biomarker fluid. In this context, it is important that technical variability introduced by sample preprocessing is kept at a minimum to be able to reproducibly assess inter-subject variability.

In this study, we used shotgun LC–MS/MS proteome analyses of a pool of whole saliva of healthy volunteers to assess the impact of four

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different methods for peptide purification: μC18 ZipTip® tips, C18 ZipTip® tips, TopTip C-18 tips and OASIS® HLB μElution microplate. Results were compared with respect to the number of identified proteins, range of dynamic binding capacity, Pearson correlation coefficients and gene ontology protein classification. The aim of this study was to identify a purification procedure suitable for population-based clinical proteome analyses.

2. Material and methods

2.1. Saliva collection and sample preparation

The study was performed with 12 healthy volunteers (mean age 28 years). Subjects had to be free of fever and/or cold and had maintained good oral hygiene. Volunteers were asked to refrain from eating 2 h and drinking 30 min prior to saliva collection. Before saliva collection started, the volunteers were asked to rinse the mouth with clean water. Saliva was collected by Salivette with cotton swabs (Sarstedt, Nümbrecht, Germany) as described [8]. Clear fluid of all volunteer's saliva was pooled and afterwards aliquoted in 1.5 mL tubes. Proteins were precipitated using trichloroacetic acid (TCA) at a final concentration of 10% (v/v) and DTT (0.12% w/v). After vortexing and incubation on-ice for 15 min, precipitated protein was concentrated by centrifugation (16,200 *g*, 15 min, 4 °C). Protein pellets were washed twice with ice-cold 100% acetone and dried with a SpeedVac for 10 min. The protein pellet was solubilized in 8 M urea and 2 M thiourea. Protein pellets were pooled again and the concentration of solubilized protein was determined using a Bradford assay with bovine serum albumin as standard (Bio-Rad, Hercules, CA, USA).

2.2. Description of purification devices

Four different reverse-phase desalting devices were tested: i) μC18 ZipTip® pipette tips (microbed format, recommended loading capacity: 2 μg, spherical silica, 15 μm, 200 Å pore size Millipore, Billerica, MA) have a 0.2 μL bed of chromatography media fixed at the end of the tip, ii) C18 ZipTip® pipette tips (standard bed format, recommended loading capacity: 5 μg with 0.6 μL bed of chromatography media), iii) TopTip C-18 tips (Glygen Corporation Columbia, MD, recommended loading capacity: 75 μg) and iv) OASIS® HLB μElution 96er well plate (Waters, Milford, MA, recommended loading capacity: 30–50 μg). Starting from the recommended protein loading capacity, we prepared for each desalting device five peptide dilutions (one targeted to the recommended loading capacity and two each below and above the recommended protein capacity, respectively) in triplicates (total n = 60). All four devices are advertised for concentrating and purifying samples for downstream LC–MS analyses.

2.3. Sample preparation prior to LC–MS/MS

Protein lysates were reduced (2.5 mM dithiothreitol for 1 h at 60 °C) and alkylated (10 mM iodacetamide for 30 min at 37 °C). Proteolysis was performed using trypsin (Promega, Madison, WI) with an enzyme to substrate ratio of 1:20 at 37 °C overnight. The proteolytic digestion was stopped by adding acetic acid to a final concentration of 1%. After proteolytic digestion, the peptide solutions were desalted using the four different devices. For all four devices the same solutions were used for equilibration, washing and elution except for volume and handling.

μZipTip and ZipTip — solutions were manually pipetted through the tips

(i) microcolumns were conditioned using 3× 10 μL 100% acetonitrile (ACN)
(ii) washing with 3× 10 μL 1% acetic acid
(iii) loading of sample (10 μL) by 15× slowly up and down pipetting
(iv) washing with 3× 10 μL 1% acetic acid
(v) peptide elution with 10 μL 50% ACN followed by 10 μL 80% ACN
(vi) organic solvent was removed by vacuum concentration in a SpeedVac
(vii) resuspension of sample in LC–MS running buffer (2% ACN, 0.1% acetic acid).

TopTip — solutions were pressed through the microcolumn by centrifugation at 10,000 g for 1 min

(i) microcolumns were conditioned using 3× 50 μL 100% ACN
(ii) washing with 3× 50 μL 1% acetic acid
(iii) sample (10–60 μL) was loaded on top of the columns
(iv) washing with 3× 50 μL 1% acetic acid
(v) peptide elution with 50 μL 50% ACN followed by 50 μL 80% ACN
(vi) organic solvent was removed by vacuum concentration in a SpeedVac
(vii) resuspension of sample in LC–MS running buffer (2% ACN, 0.1% acetic acid).

OASIS microplate — solutions were drawn through the microcolumn by a vacuum pump

(i) microcolumns were conditioned using 750 μL 100% ACN
(ii) washing with 500 μL 1% acetic acid
(iii) sample (10–30 μL) was diluted and loaded on top of the columns
(iv) washing with 500 μL 1% acetic acid
(v) peptide elution by 500 μL 50% ACN followed by 500 μL 80% ACN
(vi) organic solvent was removed by lyophilization
(vii) resuspension of sample in LC–MS running buffer (2% ACN, 0.1% acetic acid).

2.4. Analysis by mass spectrometry (nano-LC–MS/MS)

Proteolytically cleaved peptides were separated prior to mass spectrometric analyses by reverse phase nano-HPLC on a 15 cm Acclaim PepMap100-column (C18, 3 μm, 100 Å) using an EASY-nLC Proxeon system (Thermo Scientific, Waltham, MA) at a constant flow rate of 300 nL/min. Separation was achieved using a linear gradient of buffer B from 5% up to 25% within 63 min with 0.1% acetic acid, 2% ACN in water (solvent A) and 0.1% acetic acid in 100% ACN (solvent B). Separated peptides were monitored using an LTQ Orbitrap Velos MS (Thermo Scientific) equipped with a nano-electrospray ion source operated with PicoTip Emitters (New Objective, Woburn, MA, USA). After a first survey scan (r = 30,000) MS/MS data were recorded for the 20 highest mass peaks in the linear ion trap at a collision induced energy of 35%. The exclusion time to reject masses from repetitive MS/MS fragmentation was set to 60 s and the minimal ion signal for MS/MS was 2000. Raw data from the MS instrument were processed using Proteome Discoverer (Thermo Scientific, v1.3.0.339). MS-spectral data were searched against a human FASTA-formatted database (UniProt/SwissProt 12/08 containing 20,022 unique entries) using the SEQUEST algorithm. Database searches were performed with carbamidomethylation on cysteine as fixed modification and oxidation on methionine as variable modification. Enzyme specificity was selected to trypsin with up to two missed cleavages allowed using 10 ppm peptide ion and 0.8 Da MS/MS tolerances. Peptides with a false discovery rate (FDR) of less than 1% were accepted and estimated by Percolator.

Gene ontology (GO) classification of location, biological process, and molecular function was performed using the ProteinCenter software (v3.9.10025, Thermo Scientific).

3. Results and discussion

A pool of whole saliva from healthy volunteers was proteolytically digested and aliquoted for testing four different desalting devices for purification and enrichment of peptide mixtures. The number of identified peptides obtained from analysis of LC–MS/MS spectra varied from 2134 (minimum) to 2395 (maximum) with an average of 2262