Gel-Based Sample Fractionation with SP3-Purification for Top-Down Proteomics

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Abstract

Precise pre-fractionation of proteome samples is a potent method for realizing in-depth analysis in top-down proteomics. PEPPI-MS (Passively Eluting Proteins from Polyacrylamide gels as Intact species for MS), a gel-based sample fractionation method, enables high-resolution proteome fractionation based on molecular weight by highly efficient extraction of proteins from polyacrylamide gels after SDS-PAGE separation. Thereafter it is essential to effectively remove contaminants such as CBB and SDS from the PEPPI fraction prior to mass spectrometry. In this study, we developed a complete, robust, and simple sample preparation workflow named PEPPI-SP3 for top-down proteomics by combining PEPPI-MS with the magnetic bead-based protein purification approach used in SP3 (single-pot, solid-phase-enhanced sample preparation), now one of the standard sample preparation methods in bottom-up proteomics. In PEPPI-SP3, proteins extracted from the gel are collected on the surface of SP3 beads, washed with organic solvents, and recovered intact with 100 mM ammonium bicarbonate containing 0.05% (w/v) SDS. The recovered proteins are subjected to mass spectrometry after additional purification using an anion-exchange StageTip. Performance validation using human cell lysates showed a significant improvement in low-molecular-weight protein recovery with a lower coefficient of variation compared to conventional PEPPI workflows using organic solvent precipitation or ultrafiltration.

Keywords

Polyacrylamide gel electrophoresis; Sample fractionation; Top-down proteomics; PEPPI-MS; SP3

Introduction

A single gene, *in vivo*, produces a variety of translation products with different chemical structures called proteoforms.[1] Proteoforms contribute to the diversity of protein interactions and subcellular localization, thereby expanding the physiological functions of proteins. While the total number of proteoforms is still unknown, it is estimated that the number reaches more than 1 million above that of human genes, which is approximately 22,800.[2] Understanding the entirety of the vast human proteome is one aim of proteomics research today, and the development of comprehensive proteoform analysis technology is urgently needed to realize this goal.

Bottom-up proteomics (BUP), the main analytical approach for large-scale analysis of the human proteome, analyzes peptide fragments obtained by enzymatic digestion and is, in principle, often difficult to apply to highly accurate identification of proteoforms.[3] Top-down proteomics (TDP), which allows direct analysis of intact proteoforms, has therefore been adopted as the main analytical approach for this purpose.[4] In TDP analysis, proteoform identification is generally achieved by separation of intact proteoforms by reversed-phase liquid chromatography (LC) or capillary electrophoresis (CE) followed by fragmentation in an on-line connected mass spectrometer. However, to detect ever more proteoform components from biological samples, sample pre-fractionation prior to LC/CE separation is essential.[5,6]

SDS-polyacrylamide gel electrophoresis (PAGE), a central protein separation method in biochemical experiments, electrophoretically separates linear proteins based on molecular weight (MW) by the addition of the anionic surfactant SDS in a polyacrylamide gel.[7] The properties of SDS-PAGE enable high-resolution separations of complex proteomes in cell lysates, and it is widely used as a powerful sample pre-fractionation method in BUP. In 2020, we developed PEPPI-MS, a highly efficient method for passive extraction of proteins in gels and succeeded in achieving dramatic improvements in the recovery of proteins in gels.[8] Although passive extraction has typically been simple and cost-effective, it has also been associated with low recovery rates, long extraction times, and difficulty in applying to high MW proteins. PEPPI-MS solved these drawbacks by utilizing Coomassie Brilliant Blue (CBB) and SDS as extraction enhancers, achieving high recovery within 10 minutes even for high MW proteins.[8]

In PEPPI-MS, the proteome is fractionated as follows: (1) CBB addition in the gel after separation by SDS-PAGE, (2) excising the sample lane of the MW region of interest, (3) mashing

the gel pieces and passive extraction in 100 mM ammonium bicarbonate solution (pH 8) containing 0.05–0.1 % (w/v) SDS for 10 minutes.[9] Since the fractions obtained contain CBB and SDS, which interfere with MS analysis, protein purification is essential, and either methanol-chloroform-water precipitation (MCW)[8] or anion-exchange disk-assisted sequential sample preparation (AnExSP)[10] is currently used. MCW is suitable for use with small volume solutions of about 300 µL, such as a PEPPI fraction, and is an inexpensive and simple method for protein purification.[11] AnExSP is a method for removing SDS and CBB from samples using an anion-exchange solid-phase extraction (SPE) microspin column called AX-StageTip.[12] AnExSP allows for the purification of PEPPI fractions without the loss of low MW proteins that is often a problem with MCW.[10] For intact proteins, however, the necessary complete removal of SDS from the PEPPI fraction using only the AX-StageTip is difficult and requires a cumbersome urea washing step with a centrifugal ultrafiltration device beforehand.

In this study, we attempted to develop a new purification method for PEPPI fractionation using SP3, which is currently the mainstream sample pretreatment method for BUP.[13,14] In SP3, proteins precipitated by adding organic solvents such as ethanol or acetonitrile are adsorbed onto the surface of carboxylate-modified magnetic beads, and enzymatic digestion of the recovered proteins is performed directly on the beads. Selective recovery of proteins through a reliable and simple operation allows effective removal of SDS and other contaminants and is characterized by high reproducibility in processing trace amounts of protein samples. Combined with commercial automated systems for high-throughput processing of multiple samples that have recently been developed,[15,16] this method can be applied to multi-sample processing.

Despite the excellent sample purification properties of SP3, its application to TDP is currently limited, in part due to the difficulty of recovering intact proteins adsorbed onto the beads. A seminal study by Webb's group reported that incubation with 80% (v/v) formic acid (FA) at –80 °C is effective for recovering intact proteins from the beads, but the conditions are too harsh for high-throughput applications.[17] To adapt SP3 to TDP, we developed new experimental conditions that allow rapid and reproducible protein recovery from the beads at room temperature leading to a simplified yet efficient sample fractionation workflow for TDP that combines PEPPI-based proteome fractionation and magnetic bead-based fraction purification, called PEPPI-SP3. The MW-based high-resolution proteome fractionation achieved by PEPPI-SP3 results in minimal loss of low-MW proteins, allowing high-throughput sample preparation, thorough analysis and eventually multi-sample processing.

Experimental Section

Detailed protocols for previously published procedures such as sample preparation, SDS-PAGE, PEPPI fractionation and protein purification are given in the Supplementary Protocols.

Materials

Unless otherwise indicated, reagents for PEPPI fractionation and protein purification were purchased from FUJIFILM Wako (Osaka, Japan), and reagents for TDP analysis were purchased from Fisher Scientific (Rockford, IL, USA).

Cell Sample

MS-compatible human protein extract (Promega, Madison, WI, USA), a commercially available human cell protein extract (HCPE), was used in this study. Prior to SDS-PAGE, 100 µL of HCPE (1 mg protein) was subjected to reduction treatment by incubation with 1 µL of 500 mM dithiothreitol (DTT) for 90 minutes at 37 °C, followed by alkylation treatment by incubation with 1.5 µL of 1 M iodoacetamide for 30 minutes at 23 °C in the dark. The solvent was replaced with 0.05% (w/v) SDS/100 mM ammonium bicarbonate (ABC) in an Amicon centrifugal 3-kDa ultrafiltration device (Merck Millipore, Darmstadt, Germany) and adjusted to a protein concentration of 2 µg/µL.

SDS-PAGE

SDS-PAGE was performed using precast gel NuPAGE bis-tris 4–12% (1 mm thick, 10 wells) and NuPAGE MES running buffer (Thermo Fisher Scientific, Waltham, MA, USA). Samples were mixed with NuPAGE LDS loading buffer (Thermo) and then applied to the wells of the gel. After electrophoresis was performed at a constant voltage of 180 V, the gels were removed from the cassette and stained with EzStain AQua (ATTO, Tokyo, Japan) for 8 minutes, followed by a 30-minute to 1-hour wash with deionized water. For TDP the following adjustments were made: home-made 10% gel (1 mm thick, 10 wells) was used in place of NuPAGE 4–12% gel (1mm thick, 10 wells), and the staining was performed using Bio-Safe Coomassie Stain (Bio-Rad, Hercules, CA) for 60 minutes instead of EzStain AQua for 8 minutes.

PEPPI fractionation

Sample lanes of stained gel were sliced by craft knife at the MW regions of interest using MW markers as indicators. The excised gel pieces were collected in BioMasher II tubes (Nippi, Tokyo, Japan) and finely ground with a plastic pestle. Gels were further mixed with 250 µL of 0.05% (w/v) SDS/100 mM ABC and shaken in a tube mixer at 23 °C, 1500 rpm for 10 minutes. The gel was removed using a centrifugal filter and the resulting solution (approximately 250 µL), i.e., PEPPI fraction, was used for subsequent protein purification: MCW, FASP, or SP3.

MCW

The PEPPI fraction was transferred to a 1.5 mL microtube, into which 600 µL methanol, 150 µL

chloroform, and 400 µL ultrapure water were added, mixed, and centrifuged at 13500 rpm for 3 minutes at 23 °C. Centrifugation results in the formation of a bilayer solution, of which the upper layer was removed, 400 µL of methanol was added to the remaining bottom layer, and centrifuged at 13500 rpm for 3 minutes at 23 °C. The resulting protein precipitate was washed with 400 µL methanol and air dried for 30 minutes.

FASP

The PEPPI fraction was transferred to an Amicon centrifugal 3-kDa ultrafiltration device, followed by solvent replacement with 8 M urea and further solvent replacement with 100 mM ABC. The solution in the device was purified by AnExSP (self-made AX-StageTip) as previously reported[10]: the AX-StageTip was washed with methanol and equilibrated with 100 mM ABC before use; the sample was applied to the AX-StageTip, washed with 40 µL of 100 mM ABC, eluted with 40 µL of 0.5% (v/v) FA/50% (v/v) ethanol, and additionally eluted with 40 µL of 0.5% (v/v) FA/50% (v/v) acetonitrile, with each of the last three steps performed by centrifugation at 7000 rcf for 3 minutes. The resulting eluate was dried in a centrifugal evaporator before LC-MS analysis.

SP3

50 µL each of Sera-Mag SpeedBead carboxylate-modified E7 magnetic particles (50 mg/ml, cat. no. 45152105050250) and E3 magnetic particles (50 mg/ml, cat. no. 65152105050250) purchased from Cytiva (Marlborough, MA, USA), were added to a 1.5 mL microtube. After washing three times with 800 µL of ultrapure water, the beads were suspended in 250 µL of ultrapure water and stored at 4 °C until use. The previously prepared PEPPI fraction was transferred to a 1.5 mL microtube and mixed with 25 µL of SP3-bead suspension (20 µg/µL) and 1.2 mL of ethanol; after shaking at 1200 rpm for 10 minutes at 23 °C, the microtube was placed on a magnetic stand for 3 minutes and the liquid was removed by aspiration. The beads were washed twice with 800 µL of 80% (v/v) ethanol and once with 800 µL of acetonitrile. To recover intact proteins adsorbed onto the beads, the beads were shaken with 20 µL of 0.05 % (v/v) SDS/100 mM ABC in the microtube at 2000 rpm for 5 minutes at 23 °C. The microtube was placed on a magnetic stand for 3 minutes and the solution containing the recovered protein was subjected to AnExSP purification as described above in the latter half of FASP.

ContamSpot assay

Residual SDS concentration of the PEPPI fraction after purification was determined by ContamSpot assay.[18] The purified PEPPI fraction was dissolved in 10 µL of 0.1% (v/v) FA/2% (v/v) acetonitrile and used for the assay. 2 µL of sample, 2 µL of 0.1% O-toluidine blue and 5 µL of ethyl acetate were mixed in a 0.2 mL PCR tube and centrifuged at 2000 rpm for 15 seconds. From the solution separated in two layers by centrifugation, 1.5 µL of the ethyl acetate layer was spotted onto a thin layer chromatography (TLC) plate.

Trypsin/Lys-C digestion

Prior to LC-MS analysis, PEPPI fractions were solubilized with RapiGest (Waters, Milford, MA, USA) and digested with MS-grade Trypsin/Lys-C mix (Promega). Purified PEPPI fractions from the different four MW regions were dissolved in 10 µL of 0.1% (w/v) RapiGest/100 mM ABC and pooled in a 1.5 mL microtube. Pooled fractions (40 µL total) were mixed with 0.2 µg of Trypsin/Lys-C Mix and digested for 16 hours at 37 °C. To degrade RapiGest, 10 µL of 2.5% (v/v) trifluoroacetic acid (TFA) was added to the digested samples and incubated for 30 minutes at 37 °C. After centrifugation at 13500 rcf for 10 minutes at 23 °C, the supernatant was purified by self-made SDB-StageTip and subjected to LC-MS analysis.

NanoLC/MS/MS of digested peptides

DDA and DIA data were acquired on a timsTOF Pro2 mass spectrometer (Bruker Daltonics, Bremen, Germany) using a CaptiveSpray nano-electrospray ion source (Bruker Daltonics), coupled with nanoElute 2 nanoflow UHPLC system (Bruker Daltonics,). PepSep ULTRA C18 (250 mm × 75 µm, 1.5 µm, Bruker Daltonics) was used as an analytical column at 50 °C. The dried peptide mixtures were dissolved in 30 µL 0.1%(v/v) TFA and 5%(v/v) acetonitrile, and 2 µL of the solution was used for LC/MS/MS per injection. The flow rate was 400 nL/min, and the mobile phases consisted of (A) 0.1%(v/v) FA in water and (B) 0.1%(v/v) FA in acetonitrile. A multi-step linear gradient was employed: 4–20% B in 60 min, 20–28% B in 30 min, 28–40% B in 15 min, 40–100% B in 5 min and held at 100% B for 10 min.

The timsTOF was operated in PASEF (parallel accumulation and serial fragmentation) mode. Capillary voltage of 1,500 V, dry gas of 3.0 L/min and dry Temp of 180 °C were applied. The MS and MS/MS scan range was *m/z* 100–1700, and the $1/K_0$ range 0.6 to 1.6 Vs/cm^2 over a ramp time of 100 ms and accumulation time of 100 ms. The collision energy was linearly ramped according to the ion mobility from 59 eV at $1/K_0$ = 1.60 Vs/cm^2 to 20 eV at $1/K_0$ = 0.60 Vs/cm^2. For DDA analyses, 1 MS scan and 10 PASEF MS/MS scans with scheduled target intensity of 20,000 were performed per cycle. Singly charged and low *m/z* ions were excluded from PASEF precursor selection based on *m/z* and ion mobility using a polygon filter. An active exclusion time was set for 0.4 min. For DIA analyses, the DIA window setting is shown in Table S1. The MS raw data and analysis files have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the jPOST partner repository (https://jpostdb.org) with the data set identifier PXD056413.

DDA and DIA analysis

The DDA data was processed using MSFragger (v. 4.1) FragPipe (v. 22.0)[19–22] to identify peptides and proteins, and generate a spectral library for DIA data analysis. The MS/MS spectra were searched against human SwissProt database (downloaded from UniProtKB on

Apr 16, 2024, canonical only, 40870 entries containing 20435 reverse decoys). Mass accuracy of precursor and product ions was set to 20 ppm, and mass calibration and parameter optimization were enabled. Enzyme specificity was set to trypsin/P allowing for up to one missed cleavage site. Carbamidomethylation of cysteine was set as a fixed modification and oxidation of methionines and acetylation of protein N-terminus were allowed as variable modification. PeptideProphet and ProteinProphet in Philosopher[23] were used to filter the results with FDR < 1%. Spectral library for DIA data analysis was generated based on the result of all DDA data.

For DIA data, proteins were quantified with DIA-NN[24,25] (v. 1.8.2 beta 8) via FragPipe (v. 22.0) with protein-level FDR < 1%. Mass accuracy and scan window were determined separately for different runs. The quantitative results were visualized with MetaboAnalyst (v. 6.0)[26] and PlotsOfData[27].

Top-down sample preparation, PEPPI fractionation, and treatment

Nine HCPE samples of 40 µg were separated on a hand-cast 10% SDS-PAGE and the region corresponding to the 0–30 kDa was excised and subjected to PEPPI as described above. Groups of three were treated by the MCW, FASP, and SP3 methods as described above. Samples were resuspended in 20 µl of 0.1% (v/v) FA, 4.9% (v/v) acetonitirile in LC-MS grade water.

Chromatography and top-down mass spectrometry

Resuspended HCPE fractions (injection volume of 2 µL for FASP and SP3 samples, 3.5 µL injections for MCW) were further separated by nanocapillary high-performance LC online coupled to an EasySpray nanoelectrospray ionization source (Thermo Fisher Scientific, San Jose, CA). Reversed-phase LC was carried out using an Ultimate 3000 chromatographic system (Thermo Fisher Scientific) by applying a gradient of mobile phase B from 5−14% in 2 min, then from 14−42% in 50 min, followed by two consecutive column washes at 85% B for 1 min and a final re-equilibration phase at 5% B for 8.5 min, maintaining a 1.5 µL/min flow rate. Mobile phase A was composed of 4.9% (v/v) acetonitrile in water in the presence of 0.1% (v/v) FA, whereas mobile phase B consisted of 4.9% (v/v) water in acetonitrile and 0.1% (v/v) FA. All mobile phase components were LC−MS purity grade (Fisher Scientific). Samples were directly injected onto a MAbPac EasySpray column with an integrated emitter (Thermo Fisher Scientific; 4 µm particle size, 15 cm length, 150 µm i.d.) heated to 55 °C using the integrated EasySpray column heater. Nanoelectrospray was generated by applying a 2.1−2.2 kV potential. All mass spectrometry measurements were performed on an Orbitrap Eclipse tribrid mass spectrometer (Thermo Fisher Scientific) equipped with a FAIMS Pro unit. Top-down experiments were carried out in “protein mode” with an ion-routing multipole pressure of 3 mTorr. Source region parameters included a temperature of 320 °C for the heated transfer capillary, 30% RF amplitude, and 15 V in-source fragmentation to promote desolvation and the removal of labile adducts. For data-dependent acquisition, precursor ions were identified by survey mass spectra (MS1) collected in the Orbitrap mass analyzer at a resolving power of 120,000 (at 200 *m/z*) within a 400−2000 *m/z* window without spectral averaging (i.e., single microscan), using an

automatic gain control (AGC) target of 5×10^5 charges and a maximum injection time of 50 ms. Precursors were quadrupole selected (3 *m/z* isolation window) for HCD MS^2 fragmentation (35% normalized collision energy) with an intensity threshold of 2.5×10^4 within a charge state range of 5−50+. Fragmentation spectra were collected in the Orbitrap mass analyzer with a resolving power of 60,000 (at 200 *m/z*) within a 400−2000 *m/z* window without spectral averaging, with an AGC target of 2.5×10^5 charges and a maximum injection time of 500 ms. FAIMS compensation voltages (CVs) varied within a single run with 3 separate runs per sample (for a total of 9 distinct CVs per sample), similarly to a previous report[28]; Run 1: −40, −20, and 0 V; Run 2: −60, −50, and −30 V; Run 3: −10, 5, and 15 V; with a 1.2 second cycle time was used for each CV. Dynamic exclusion was applied (30 s duration). All mass spectrometry data files have been uploaded to MassIVE (repository number MSV000095992)

Top-down data analysis

RAW files from all three treatment methods were searched together with ProSight PD v. 4.2 (Proteinaceous, Inc.) run as a node within the Proteome Discoverer 3.0 environment (Thermo Fisher Scientific) against human database. Data analysis was carried out using the provided processing and consensus workflows for High−High. For FAIMS files, the FAIMS CV setting in the Spectrum Selector node was left unspecified to accommodate the use of multiple CVs per file. All spectra were deconvoluted within the High−High cRAWler node: precursor masses were deconvoluted from MS^1 spectra using Xtract utilizing a sliding window algorithm, with a 1 scan offset and a merge tolerance of 30 ppm while requiring the detection of a minimum of 3 charge states in a minimum of 3 sliding window detections. Feature groups required a 2.2 Da tolerance before quantification in the consensus workflow. Two database searches were applied: an Annotated Proteoform search using 2.2 Da precursor mass tolerance, and a Subsequence search based on a 15-ppm precursor tolerance. For both searches, the fragment tolerance was set at 10 ppm. The number of proteoforms arising from a single precursor was limited to 1. Identified proteoforms and UniProt accession numbers from ProSight PD were filtered at 1% FDR (with three separate FDR calculations applied to the proteoform, isoform, and UniProt accession levels). Upon completion of the database searches, the resulting tdReport was filtered for each treatment method and Q-values were recalculated based upon the recommendation of the developer. Plots were generated using GraphPad Prism 10 (GraphPad Software).

Results and Discussion

Purification of PEPPI fraction by SP3

We attempted to establish an SP3 protocol for TDPs, based on previously published protocols for BUP[14], at a sample scale of 250 µL, a typical PEPPI fraction volume. The magnetic beads used were an equal weight mixture of two different carboxylate-modified magnetic beads, i.e. hydrophylic and hydrophobic versions of Cytiva's Sera-Mag™ SpeedBead Carboxylate-Modified

Magnetic Particles. It is generally recommended in SP3 sample preparation for BUP that 10 times the weight of beads be added in proteins.[14] For application to TDP, we generated PEPPI fractions in four different MW ranges from 10 µg of HCPE and examined the amount of protein bound to the beads when different amounts of beads were added (Figure S1). In the MW range from 250 to 20 kDa, the recovery was similar regardless of the amount of beads added, but in the range below 20 kDa, the recovery decreased slightly when the amount of beads added was less than 500 µg. Therefore, we set the amount of beads to 500 µg in this study.

For rapid recovery of tightly adsorbed proteins on beads at room temperature (23–25 °C), we deemed surfactant assistance necessary and chose to use SDS, which can be removed by AnExSP. Specifically, we mixed 20 µL of 0.05% (w/v) SDS/100 mM ABC, which has been previously proven applicable for AX-StageTip purification,[12] with washed beads and shook the mixture at room temperature with a tube shaker (Figure 1A). Analysis of HCPE showed that 0.05% (w/v) SDS/100 mM ABC could recover the protein from the beads within 5 minutes (Figure 1B). As alternatives to SDS, we also examined the use of RapiGest, urea, NDSB-195, and octyl glucoside which can be easily removed before MS analysis. Urea, NDSB-195 and octyl glucoside did not yield any significant protein recovery after 10 minutes of shaking (Figure S2), and the acid degradable surfactant RapiGest yielded the equivalent of SDS, but subsequent acid treatment resulted in degradation of the recovered proteins (Figure S3). We thus decided to continue the use of SDS for protein recovery in this study.

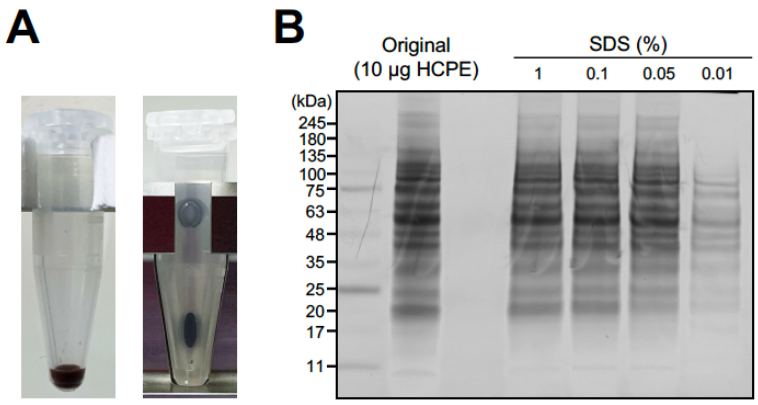

Figure 1. Protein recovery from SP3 beads using SDS solution. (A) Protein recovery from SP3 beads. HCPE (10 µg) adsorbed onto SP3 beads (500 µg) was recovered by shaking the beads in 20 µL of 0.05% (w/v) SDS/100 mM ABC for 5 minutes at 23 °C (left). After protein recovery, the tube was set on a magnetic stand to remove the beads (right). (B) SDS-PAGE image of the proteins recovered from the beads at different SDS concentrations (0.01–1% (w/v)). Bands were stained with CBB.

PEPPI-SP3 workflow

Based on the above results, we established a new experimental workflow, PEPPI-SP3, to purify PEPPI fractions by combining SP3-based protein recovery after AnExSP purification (Supplementary Protocols). The scheme of the PEPPI-SP3 workflow is shown in Figure 2. In the workflow, proteomic samples are separated by SDS-PAGE and then fractionated with PEPPI. Ethanol is added to 500 µg of SeraMag beads and the resulting PEPPI fraction to create an 80% (v/v) ethanol solution and shaken vigorously to adsorb precipitated protein components onto the beads (Figure S4A and S4B). The beads are isolated using a magnetic stand and washed twice with 80% (v/v) ethanol and once with acetonitrile to remove of CBB and SDS (Figure S4C–E). Proteins are recovered from the beads by shaking the beads with 0.05% (w/v) SDS/100 mM ABC for 10 minutes and subjected to LC-MS after AnExSP purification.

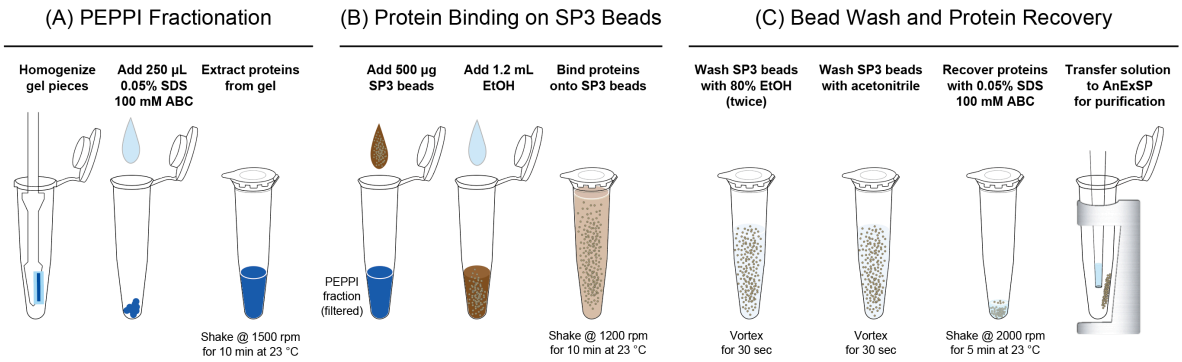

Figure 2. PEPPI-SP3 Workflow for top-down proteomics. (A) PEPPI fractionation. After SDS-PAGE separation of samples, the separated proteins were stained with CBB and the desired molecular weight range in the sample lane was excised. The excised gel pieces were homogenized using disposable plastic masher tubes, and the proteins in the gel were passively extracted with 0.05% SDS/100 mM ABC. (B) Protein binding on SP3 beads. The protein recovery solution (PEPPI fraction) was mixed with SP3 beads and ethanol, stirred, and the precipitated proteins were adsorbed onto the beads. (C) Bead wash and protein recovery. The beads were washed twice with 80% ethanol and once with acetonitrile, then mixed with 20 µL 0.05% SDS/100 mM ABC to recover the protein from the beads; after stirring for 5 minutes, the beads were removed with a magnet and the resulting protein solution was purified with AX-StageTip.

Beads adsorbed on the magnet were air-dried for 10 min.Conventional PEPPI workflows have used (1) PEPPI-MCW with MCW precipitation or (2) PEPPI-FASP combining ultrafiltration filter-assisted urea washing (FASP) and AnExSP. The time from electrophoresis to purification is 2.3 hours for PEPPI-MCW and 5.3 hours for PEPPI-FASP, while PEPPI-SP3 takes 3.6 hours (Figure S5); FASP is a relatively time-consuming process that has been greatly improved by the use of SP3.

We next validated the performance of PEPPI-SP3 through a comparison with PEPPI-MCW and PEPPI-FASP. PEPPI fractions from 10 µg HCPE were processed according to the respective workflows and subjected to SDS-PAGE and quantitative proteomics by data-independent acquisition (DIA) using nano-LC-MS. Figure 3 shows the SDS-PAGE results of the PEPPI fractions obtained from each workflow, and the CBB-stained images show large differences between the workflows in the high MW region above 100 kDa and the low MW region below 20 kDa. Our previous studies have already shown that AnExSP is not suitable for high MW proteins[12]; compared to MCW, workflows using AnExSP for protein purification (FASP and SP3) tend to have lower recovery of proteins above 100 kDa. In the MW region below 20 kDa, MCW processing, which is known to often result in losses in the recovery of low MW proteins, shows an expected decrease in the amount of protein recovered compared to FASP and SP3. Finally, comparing FASP and SP3, it was noted that the fraction treated with SP3 had higher band density than that of FASP, suggesting an absolute advantage of SP3 in the less than 20 kDa region, which is the main target region of current TDP analysis. The level of SDS in the purified fractions was evaluated by the ContamSpot assay,[18] which showed that the SP3/AnExSP-treated fractions had SDS levels below 0.002% (w/v), and the purification results were comparable to MCW (Figure S6). Even when HCPE was increased from 10 µg to 40 µg (Figure S7), the results of purification of the fractions by each workflow were similar to those at 10 µg, with SP3 performing markedly much better below 20 kDa.

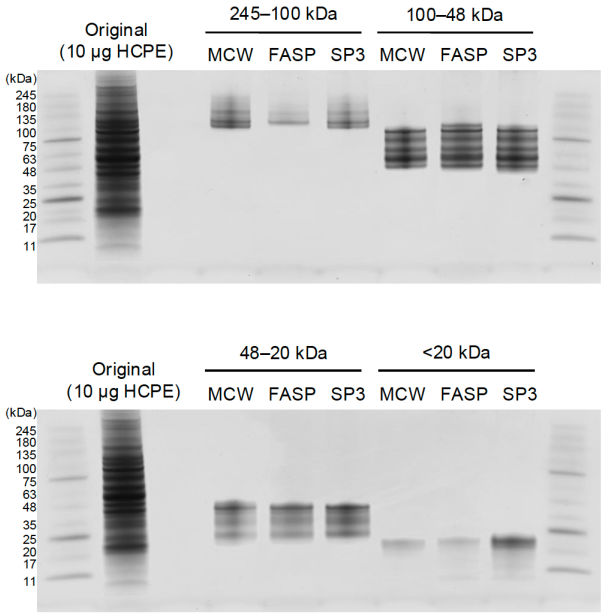

Figure 3. SDS-PAGE images of PEPPI fractions purified by three different methods. Four PEPPI fractions (245–100 kDa; 100–48 kDa; 48–20 kDa; <20 kDa) derived from 10 µg of HCPE were purified by three different methods (MCW, FASP, and SP3) and the fractions were separated again by SDS-PAGE. Gel-separated fractions were stained with CBB.

For DIA analysis, the fractions were pooled, digested with trypsin/Lys-C, and proteome-wide quantification was performed (Figure 4A). Our analyses detected a total of 5379 proteins in all three workflows (Table S2), with little difference in the number detected in each workflow (Figure 4B), and many components (5016) were detected in all three. Figure S8 shows the results of the comparison of DIA quantification between two different workflows (SP3 vs. MCW or SP3 vs. FASP): in the case of SP3 vs. MCW, the number of proteins showing a significant change (>3-fold, $p<0.05$) was similar for each method (MCW: 229 and SP3: 197). Among these proteins, those detected in MCW showed more proteins with longer amino acid lengths while those detected in SP3 showed more proteins with shorter amino acid lengths (<300 amino acids) (Figure 4C), a trend that is consistent with that seen in the SDS-PAGE results. In the case of SP3 vs. FASP (Figure S8), the proteins showing significant differences were three times more abundant in SP3 than in FASP (FASP: 76 and SP3: 220), and most of the proteins abundant in SP3 were proteins with fewer than 300 amino acid residues (Figure 4C). These results indicate that SP3 is the workflow of choice when the protein of interest is a low MW protein. In fact, when considering histones, an important target for proteoform analysis in TDP, SP3 showed better recovery results than the other two for all 15 histone proteins detected in this study (Figure S9). In contrast, for high MW proteins, for which TDP analysis is still underdeveloped, MCW will be the workflow of choice. We also evaluated the quantitative accuracy of each workflow using DIA data. Figure 4D shows the distribution of the coefficient of variation (CV) for each workflow in a violin plot, indicating that SP3 is superior to the other workflows in quantitative accuracy. FASP resulted in less accurate quantitation than the other workflows, which may be due to the loss of protein adsorption on the ultrafiltration filter.

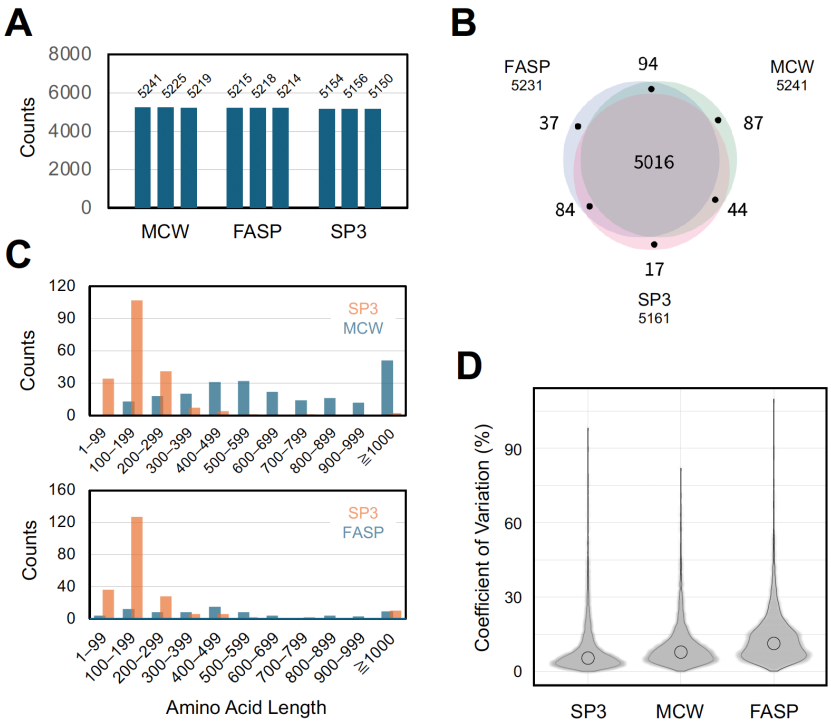

Figure 4. Quantitative evaluation of different PEPPI workflows by DIA. (A) Comparison of proteins identified by DIA. Fractions from 10 µg of HCPE using the three PEPPI workflows, PEPPI-MCW (MCW), PEPPI-FASP (FASP), and PEPPI-SP3 (SP3), were pooled, Lys-C/Trypsin digested, and subjected to DIA analysis by LC-MS. (B) The relationship between the proteins identified in each workflow is shown in the Venn diagram. (C) Comparison of amino acid lengths in proteins showing significant differences in Figure S8. (D) CV distributions of protein groups identified in each workflow. Violin plots were prepared with PlotsOfData[27]. A circle indicates the median.

TDP analysis of PEPPI-SP3 fractions

Next, we validated the performance of the PEPPI-SP3 method for TDP by comparing it to PEPPI-MCW and PEPPI-FASP protocols. For this comparison, three technical replicates of 40 µg HCPE were PEPPI fractionated to generate 0–30 kDa fractions for each treatment method (Figure S10). Following treatment with the respective method (MCW, FASP, and SP3), the samples were analyzed using a FAIMS-HiHi method relying on internal compensation voltage (CV) stepping,[29] with each of the three runs using three different CV values (i.e., each sample was subjected to a total of nine CVs) to increase the depth of the proteome surveyed.

Overall, the PEPPI-SP3 method demonstrated significant superiority over the other two methods across multiple metrics. It notably increased the number of UniProt accessions by 60% and the number of proteoform identifications by 85% relative to the MCW method (Figure 5A). While the FASP method does provide an increase in identifications compared to the MCW method, it falls short of the number of identifications of the SP3 by ~20%. The difference in the number of identifications is unsurprising based on visual inspection of the total ion chromatogram (TIC) of the −40 V compensation voltage, the CV value that led to the identification of most proteoforms for each method (Figure S11). By normalizing the global TIC intensities to the SP3 sample, the FASP sample displays a ~35% reduced signal. However, when a similar comparison is made between SP3 and MCW, the maximum intensities are ~50% lower in MCW despite loading a 75% higher volume of the MCW samples. This indicates that MCW has reduced proteoform recovery compared to both the FASP and SP3 methods. Furthermore, the complexity of the SP3-treated sample, estimated based upon the number of distinct elution peaks present in the chromatogram, is greater than that of either of the two other treatment methods.

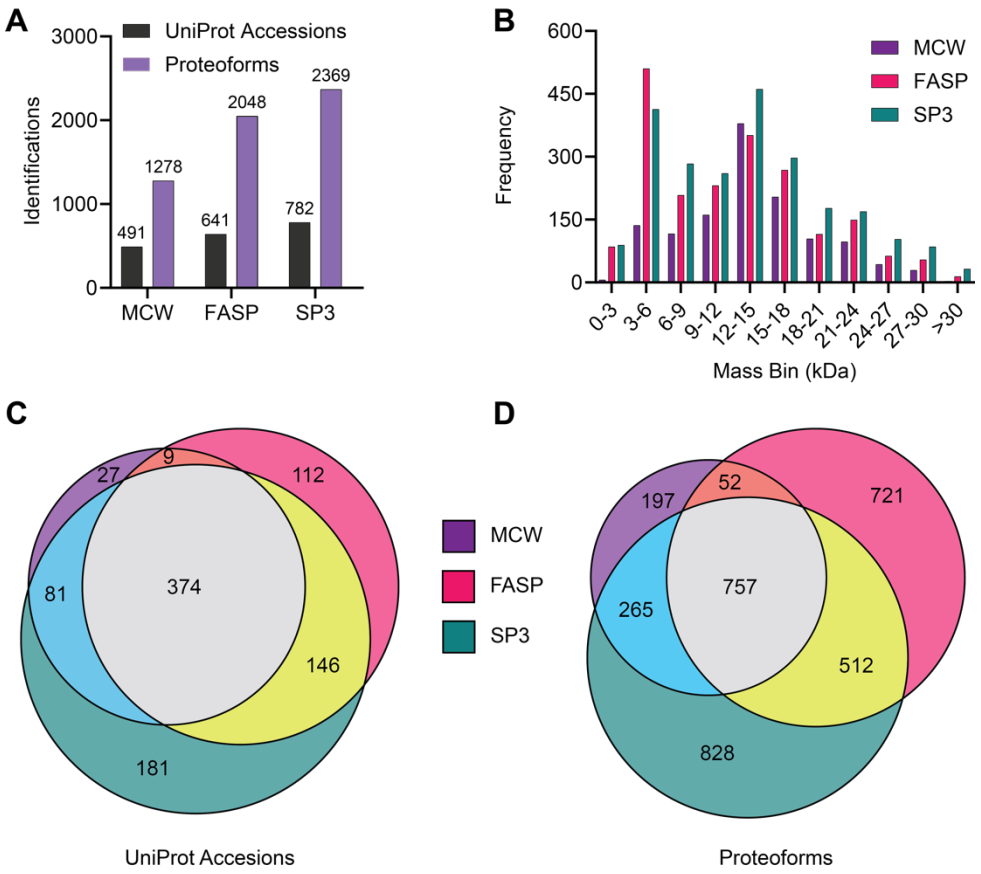

Figure 5. Summary of the comparison of three treatment methods. (A) Global count of identified UniProt accession numbers and unique proteoforms. (B) Mass distribution of identified proteoforms. (C) Venn diagram of identified UniProt accession numbers. (D) Venn diagram of identified proteoforms. Results of the three treatment methods are color-coded based on the legend included in the figure.

Our investigation into the main differences between the three treatment methods revealed unique advantages for the FASP and SP3 methods. In general, the SP3 method outperformed both MCW and FASP methods across the whole 0–30 kDa mass range (Figure 5B). The only notable exception was the 3–6 kDa mass bin, where the FASP protocol identified ~25% more proteoforms. FASP and SP3 methods held substantial advantages for the proteoforms below 9 kDa, leading to the identification of >200% more proteoforms in that range than in MCW-treated samples. In the aggregate 0–9 kDa range, the FASP protocol identified marginally more proteoforms than the SP3 protocol (803 vs 785, respectively). However, the SP3 method maintained a sizeable advantage (~80% increased identifications) over the MCW protocol at the higher molecular weight ranges (>15 kDa)), where previously the MCW method had performed comparably to the FASP protocol when investigating human serum proteoforms.[30] Over the same 15–30 kDa mass range, 30% more proteoforms were identified in the SP3 samples than in the FASP samples.

While the PEPPI-SP3 method achieves more identifications throughout the 0–30 kDa range, we wanted to determine the degree of overlap between the identifications of the three methods. When looking at the number of UniProt accessions identified, the SP3 method identified 93% of those identified by the MCW method and 81% by the FASP method (Figure 5C). However, as we move to the proteoform level, we observe a higher degree of uniqueness between the three methods. The percentage of proteoforms identified in MCW and FASP samples that were in common with SP3 reduced to 80% and 62%, respectively (Figure 5D).

Next, we examined the technical variability of the mass spectrometry analysis. We began by comparing the chromatograms of the technical replicates for a given treatment method. When examining the various CVs, a visual inspection did not display substantial differences between the technical replicates (an example of the CV −40 V for the SP3 technical replicates can be found in Figure S12A). Since the differences in the chromatograms were minor, we investigated the overlap of proteoform identifications between the MS runs for each treatment method (Figure S12B). When comparing the number of proteoforms identified in all three technical replicates per treatment method for each MS run, we observed that the MCW technical replicates have a slightly higher percentage of the identified proteoforms shared across its three technical replicates than either the FASP or SP3 technical replicates. This trend was observed for each MS run. We attribute at least part of the technical variability to the stochastic nature of precursor selection in data-dependent acquisition combined with the increased complexity of samples treated with the FASP and SP3 methods (i.e., larger proteoform heterogeneity, lower identification reproducibility). Multiple actions can be used to mitigate this limitation, among them specifically increasing the number of molecular weight-based PEPPI fractions to analyze and/or using longer chromatographic gradients.

While technical variability at the mass spectrometry level could account for some of the high degree of uniqueness, it is unlikely to be the sole cause. The next line of inquiry was to determine if there was a bias for or against specific post-translational modifications in the various treatment methods. Since the number of proteoforms identified is substantially different between the three methods, we normalized the frequency of a given PTM to the number of proteoforms identified to generate a rate per 100 proteoforms identified. We then performed pair-wise comparisons based on the Log2 transformation of the ratio between two methods for the ten most commonly identified PTMs (Figure S13). In summary, the FASP method could better identify truncation products than the MCW method, but not as well as the SP3 method. At the same time, the FASP method performed unexpectedly poorly at identifying acetylation events at the protein N-terminus and lysines as well as phosphorylation events at serine, threonine, and tyrosine residues compared to both MCW and the SP3 methods. These observations indicate that while FASP excels at identifying small proteoforms (i.e., truncation products), it struggles to hold onto PTMs that are important for signal transduction. Since the difference between the FASP and SP3 methods occurs before the shared use of the AX-StageTip, one can speculate that the membrane of the spin concentrator

used in FASP is absorbing some of these proteoforms. Regarding PTM identification, the SP3 method performs as well as or better than the MCW method for the ten most common modifications, particularly with truncation products.

Conclusions

In the first PEPPI workflows, which are already widely used as sample prefractionation methods for TDP, purification media such as MCW, which often results in the loss of low MW proteins, or FASP, which is time consuming, have been used to purify PEPPI fractions. In this study, we developed PEPPI-SP3, the latest and third PEPPI workflow that incorporates robust and simple protein purification using SP3 beads. The establishment of a process to recover intact proteins adsorbed onto SP3 beads was essential to realize PEPPI-SP3, which was achieved by combining rapid recovery at room temperature with 0.05 % (w/v) SDS and SDS removal with AX-StageTip. The fractions were successfully purified by AnExSP to a level comparable to MCW, and no problems were observed with TDP measurements by LC-MS. PEPPI-SP3, which outperforms conventional methods in recovering proteins in the low MW range below 20 kDa, lends itself well to TDP and, owing to the on-bead processing enabled by SP3, PEPPI-SP3 can also enhance sample pretreatment in BUP and middle-down proteomics.[31] Despite the establishment of SP3 as a versatile sample preparation method in BUP, its application to proteins in gels after SDS-PAGE has remained elusive. The SDS-PAGE to SP3 pathway established in this study finally allows the use of SP3 in gel-based top-down analysis.

With the successful development of PEPPI-SP3 in this study, there are now three potent methods for purifying PEPPI fractions (MCW, FASP, and SP3), but selection of an appropriate workflow for TDP is essential for optimum results. In terms of operating time, purification by SP3 can be completed in less time than FASP, although it is still slower than MCW. In terms of operability, MCW is challenging in its use when the amount of protein is small because it is often difficult to visually confirm the protein pellets formed, and FASP is easy to perform but time-consuming, whereas SP3 has the ideal combination of being robust and simple regardless of the amount of sample. Comparative evaluation by quantitative DIA analysis in this study also supports the superiority of SP3 in quantitative accuracy, making SP3 the workflow of choice for purification of PEPPI fractions in TDP, especially with regard to compatibility with low MW proteins such as histones. For TDP analysis of proteoforms below 30 kDa, the SP3 method provides that greatest number of identifications while also having capability to retain PTMs relevant to signal transduction pathways when compared to both MCW and FASP.

Although high MW proteins above 100 kDa are difficult to analyze with current TDP, PEPPI can still be applied, but for such high MW proteins, MCW is a better choice than SP3. MCW may also have a cost advantage as it does not require SP3 beads or ultrafiltration equipment. The nature of PEPPI allows the use of two different methods when the number of samples is large.

For example, it is possible to use both SP3 and MCW on their respective appropriate MW ranges to reduce cost. There is still room for cost reduction in SP3, however. In recent years, the SP4 protocol,[32] which recovers beads by centrifugation instead of magnetic stands, has been reported as an improved protocol for SP3, allowing the replacement of magnetic beads with low-cost non-magnetic beads. Improvements based on a lower-cost protocol will make a potential PEPPI-SP4 even less expensive.

Clinical application of TDP is likely to accelerate in the near future, and the need for high-throughput processing of large numbers of samples will increase accordingly. The tedious operation of MCW and FASP can be a major obstacle as the number of samples increases. In contrast, commercially available automated equipment is available for SP3 and has demonstrated excellent performance in multi-sample processing in BUP.[15] The elemental technology for automated processing of SP3 in BUP should be transferable to PEPPI fractionation processing for TDP.

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Notes

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