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Homogenous Phase Enrichment of Cysteine-Containing Peptides for Improved Proteome Coverage

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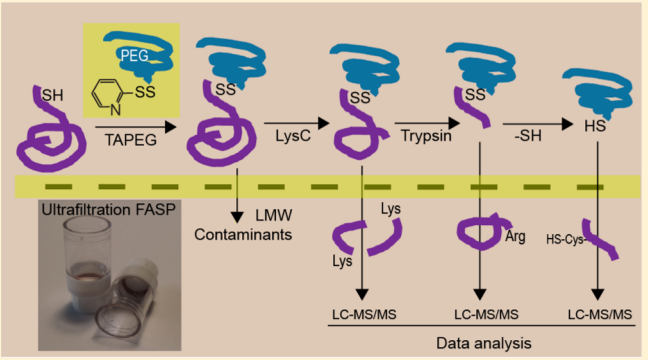
Supporting Information

ABSTRACT: We describe a proteomic reactor-based homogeneous phase enrichment of cysteine-containing peptides in a filter aided sample preparation (FASP) format. In this approach thiol-reduced proteins are derivatized with thiol-activated polyethylene glycol (TAPEG) before protein cleavage. Consecutive digestion with endoproteinase LysC and trypsin allows isolation of two fractions of nonderivatized peptides. After reduction of disulfide bonds between cysteine-containing peptides and the polyethylene glycol moieties, a third fraction of peptides is collected. LC-MS/MS analyses revealed that on average this fraction consists of 95% cysteine-containing peptides. Since 85−93% of all peptides are unique to a single subfraction, the combination of TAPEG and FASP offers an efficient peptide separation strategy. Analysis of whole cell lysates of mouse brain, liver, red muscle fibers, and CaCo-2 cells using the TAPEG FASP approach allowed identification of 6,900, 5,800, 4,200 and 7,900 proteins, 10−30% more than were identified using two-step digestion without isolation of Cys-containing peptides. The fractionation also increased the protein sequence coverage by 10−30%.



Prefractionation of peptides is an essential and often critical step in shot-gun type gel-free proteomics analysis. C_{18}-reverse phase separation prior to mass spectrometry is routinely used. However, additional prefractionation of peptides by different chromatographic methods is usually necessary to achieve the required depth of proteomic analysis. Combination of the reverse phase chromatography with strong cation exchange (SCX) fractionation was introduced by Yates and co-workers.[1] This approach, called the Multidimensional Protein Identification Technology (MudPIT), has been one of the most frequently used. In addition to SCX, alternative chromatographic methods, including strong anion exchange, hydrophilic interaction (HILIC), and basic pH reverse phase chromatography, have been proposed by many authors. For practical reasons the peptide separation preceding the C18 reverse phase chromatography is frequently accomplished using a "cartridge type" fractionation which leads to generation of only a few fractions.[2,3] Recently, we have demonstrated that consecutive digestion of proteins with multiple proteases in the filter aided sample preparation (FASP) format allows generation of two peptide fractions with an overlap of only 2−5%.[4] Compared to traditional digestion with trypsin, the multiple enzyme digestion FASP (MED-FASP) with LysC and trypsin increased the number of identified proteins up to 40%. A similar stepwise protein cleavage fractionation in the filter format can also facilitate identification of low abundant proteins.[5,6]

Reduction of disulfide bridges and consecutive alkylation of the thiols are key steps in proteomic sample preparation protocols. For the alkylation, a number of reagents can be used; however, iodoacetamide and its derivatives are the most frequently used ones. Alternatively, the thiol moieties can be used for covalent chromatography, allowing isolation of cysteine-containing peptides,[7,8] or for covalent attachment of a variety of reagents enabling protein quantitation and/or affinity enrichment.[9−16] In practice, isolation and analysis of the cysteine-containing peptides lead to reduction of sample complexity and facilitate identification of less abundant proteins. In all these methods, enrichment of cysteine-containing peptides is accomplished using affinity capture by suitable resins or magnetic beads.

Covalent attachment of polyethylene glycols (PEGs), known as PEGylation, is a commonly used strategy for enhancing the therapeutic and biotechnological potential of proteins and peptides.[17,18] Most frequently, PEGylation procedures use the epsilon-amino-group of lysine that is usually located on the surface of proteins. In contrast, the cysteine thiols, which are less abundant and preferentially located in the protein interior, are rarely used for PEGylation.[19]

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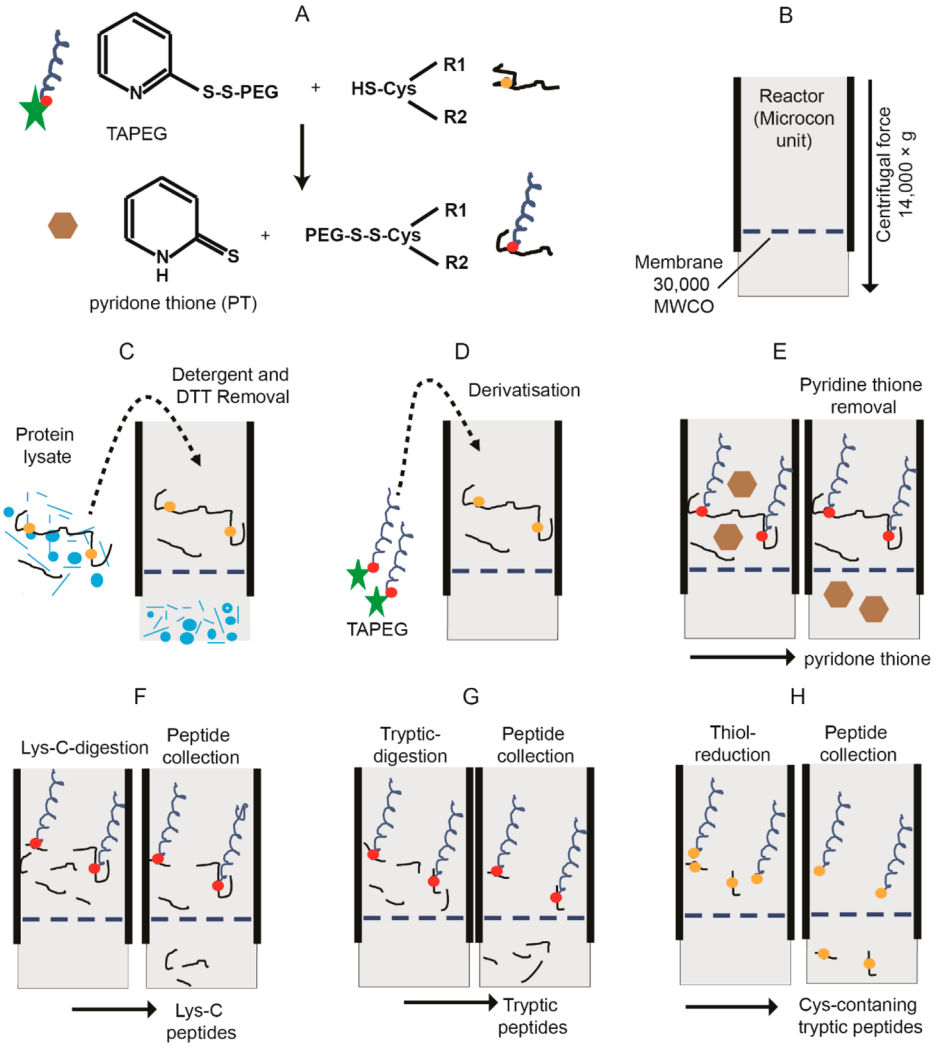

Figure 1. Synthesis of the TAPEG reagent (A) and the protein processing workflow (B–H). (B) The ultrafiltration filter repurposed as a reactor unit. (C) Conditioning of the protein lysate. (D) Derivatization of thiols with TAPEG. (E) Removal of pyridone thione. (F) Digestion with LysC and isolation of peptides. (G) Digestion with trypsin and isolation of tryptic peptides. (H) Reduction of disulfides and isolation of cysteine-containing peptides. Reduced thiols, *orange dots*; disulfide bridges, *red dots*.

Ultrafiltration units repurposed as proteomic reactor devices in the FASP method not only allow complete depletion of reagents used[20] for sample lysis and efficient protein digestion[21] but also allow digestion and release of deoxyribo- and ribonucleotides for nucleic acid quantitation.[21,22] In addition, the device is a useful tool for capturing and selective elution of peptides binding to lectins[23] and antibodies,[24] for analysis of affinity-purified protein complexes,[25] and for isobaric mass tagging.[26] Here, we combine reversible protein PEGylation with MED-FASP. We derivatize proteins at cysteine residues with high molecular weight thiol-activated PEG (TAPEG). We digest proteins consecutively with LysC and trypsin, and we collect separate peptide fractions. Finally, the reduction of disulfide bonds between cysteine-containing peptides and PEG allows collection of a third fraction. Combined analysis of these 3 fractions revealed that on average 90% of the identified peptides were unique to only one of the fractions. The method increases the number of identified unique peptides by 20–30% when compared to the standard MED FASP and offers an alternative peptide fractionation prior to LC-MS/MS.

EXPERIMENTAL SECTION

Synthesis of Thiol-Activated PEG (TAPEG). 200 mg of CH_3O–PEG–SH (RAPP Polymere, Tubingen) was dissolved in 4 mL of Tris-HCl, pH 7.8, and was mixed with 0.25 mL of 50% (v/v) CH_3CN in H_2O containing 10 mg of 2,2′-ditiodipyridine (DTDP; Aldrithiol-2, Sigma-Aldrich). The mixture was dialyzed against several changes of water overnight using Spectra/Por Dialysis Membrane MWCO 10,000 tubing (Spectrum Laboratories). The concentration of the TAPEG reagent was assayed photometrically by treating with a 100-fold molar excess of 2-mecaptoethanol and measuring the absorbance of the reaction product, pyridone thione (PT), at 343 nm. For this purpose 10 μL aliquots of the reagents were mixed with 0.2 mL of 0.1 M Tris-HCl, pH 7.8, and then 1 μL of 2-mercaptoethanol was added. The concentration of PT was calculated using ε_{343} = 8,000. The product yield was >90%. Reagent solutions were stored at 4 °C for several weeks or frozen at −20 °C for more than 1 year without observable deterioration of activity.

Tissue Lysates. Mouse brain, liver and skeletal muscle tissue were homogenized in 0.1 M Tris-HCl, pH 7.8, 0.05 M DTT using a T10 basic Ultraturrax dispenser (IKA). The ratio

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of tissue to the buffer was 1:5−1:10 (g/mL). After addition of SDS to a final concentration of 2% (w/v), the homogenates were sonicated in a Branson type instrument, Heinemann Sonifier 250 (Schwäbisch Gmünd), operating at 20% duty cycle and 3−4 output for 1 min, and were then incubated in a boiling-water-bath for 5 min. After cooling to room temperature, lysates were clarified by centrifugation at 16,000*g* for 10 min. Cultured cells were lysed in 0.1 M Tris-HCl, pH 7.8, 0.05 M DTT, and the lysate was sonicated and incubated at 100 °C for 5 min. The protein concentration in the lysates was determined by the tryptophan fluorescence-based assay.[27]

Protein PEGylation. The whole procedure was carried out at room temperature using an Eppendorf type centrifuge. Aliquots of lysates containing 100 μg of total protein were mixed with 200 μL of UCE buffer containing 8 M urea, 0.1 M Tris-HCl, pH 7.8, and 1 mM EDTA. The mixture was concentrated to less than 10 μL in Microcon-30 centrifugal filters (Merck Millipore) at 14,000*g*. After addition of 100 μL of UCE buffer, the solution was concentrated again. This step was repeated twice. Then the concentrate was mixed with 100 μL of UCE containing 0.05 μmol of the TAPEG reagent. Following 30 min of incubation, the mixture was concentrated and the concentrate washed with an additional 100 μL aliquot of UCE. The concentration of piridone thione in the combined filtrates was measured at 343 nm using ε_{343} = 8,000. Unreacted excess of TAPEG in the retentate was quenched by addition of 0.05 μmol of cysteine in 100 μL of UCE for 30 min. Free cysteine was removed by sample concentration and a wash with 200 μL of digestion buffer (DB) composed of 0.1 M Tris-HCl, pH 8.5.

MED FASP and Release of PEGylated Peptides. The retentate containing PEGylated proteins was incubated with 2 ug of endoproteinase LysC in 40 μL of digestion buffer (DB) at 30 °C overnight. Released peptides were collected by centrifugation.The retentate was washed twice with 80 μL of DB, and then the material remaining on-filter was digested with 1 μg of trypsin in 40 μL of DB at 37 °C for 4 h. Tryptic peptides were collected by centrifugation in the same way as the LysC peptides. Finally, the disulfide bridges between peptides and PEG were reduced by incubation with 50 μL of 10 mM 2-mercaptoethanol in UCE, and the peptides were collected by centrifugation. Peptide in each fraction was quantified using the tryptophan fluorescence-based assay[27] and was desalted on C18 stage tips.[2] The peptide eluates from the reverse phase material were concentrated to a volume of 5 μL and were stored frozen at −20 °C until mass spectrometric analysis.

LC-MS/MS and Data Analysis. Analysis of peptide mixtures was performed as described previously.[4] Briefly, the peptides were separated on a reverse phase column (20 cm × 75 μm inner diameter) packed with 1.8 μm of C18 using a 4 h acetonitrile gradient in 0.1% formic acid at a flow rate of 250 nL/min. The LC was coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific, Germany) via a nanoelectrospray source (Proxeon Biosystems, now Thermo Fisher Scientific). The Q Exactive was operated in data-dependent mode with survey scans acquired at a resolution of 50,000 at m/z 400 (transient time 256 ms). Up to the top 10 most abundant isotope patterns with charge ≥+2 from the survey scan were selected with an isolation window of 1.6 Th, and they were fragmented by HCD with normalized collision energies of 25. The maximum ion injection times for the survey scan and the MS/MS scans were 20 and 60 ms, respectively. The ion target value for both scan modes was set to 10^6. The dynamic exclusion was 25 s and 10 ppm. The spectra were searched using the MaxQuant software version 1.2.6.20.[28]

RESULTS AND DISCUSSION

Reagent Synthesis. Preparation of the TAPEG reagent is easy and does not require specific equipment or skills in compound synthesis. CH_3O−PEG−SH was incubated with a molar excess of DTDP to yield CH_3O−PEG−S-S-2′-pyridine (TAPEG) (Figure 1A).The excess of the DTDP and PT was removed by dialysis. When required, TAPEG solutions were concentrated using ultrafiltration units. The activity of the reagent was assayed by treatment with 2-mercaptoethanol with measurement of PT absorbance at 343 nm.

Analytical Workflow. All steps of the proposed procedure are carried out in the FASP format (Figure 1B). In the first step of the workflow low molecular substances originating from lysed tissue and buffer used for the lysis are removed from thiol-reduced polypeptides (Figure 1C). Removal of substances used for reduction of disulfide bridges, such as DTT and reduced glutathione naturally occurring in tissue, is of particular importance. Following this step, cysteine was derivatized with TAPEG and the reaction yield was analyzed by UV-photometric quantification of PT (Figure 1D and E). Proteins were then consecutively cleaved with endoproteinases LysC and trypsin with collection of the peptides into two separate fractions (Figure 1F and G). Finally, the disulfide bridges were reduced, and released peptides were collected by centrifugation (Figure 1H). Cysteines in the peptides were derivatized with iodoacetamide and desalted on StageTips prior to mass spectrometric analysis.

TAPEP Has High Thiol-Derivatization Efficiency. Efficiency of derivatization of proteins is often limited by accessibility of the reagent to the targeted moieties due to steric hindrance caused by secondary, tertiary, and quaternary structures. We have tested the derivatization efficiency of TAPEG using denatured and thiol-reduced bovine serum albumin (BSA). We compared the reactivity of 3 different types of the reagent with either 10 kDa or 20 kDa PEG moieties (Figure 2A) with the reactivity of DTDP and found no difference between the reagents (Figure 2B). This suggests that the PEG moieties have a negligible effect on thiol-derivatization

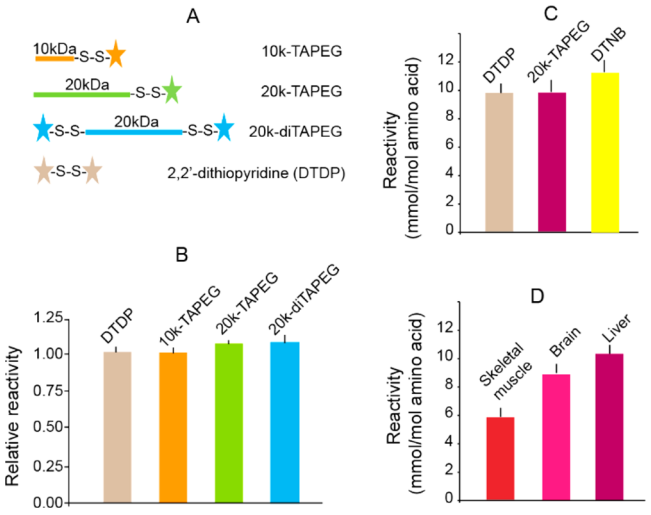

Figure 2. TAPEG reagents (A) and their reactivity with BSA (B), liver (C and D), brain (D), and red muscle fiber lysate (D).

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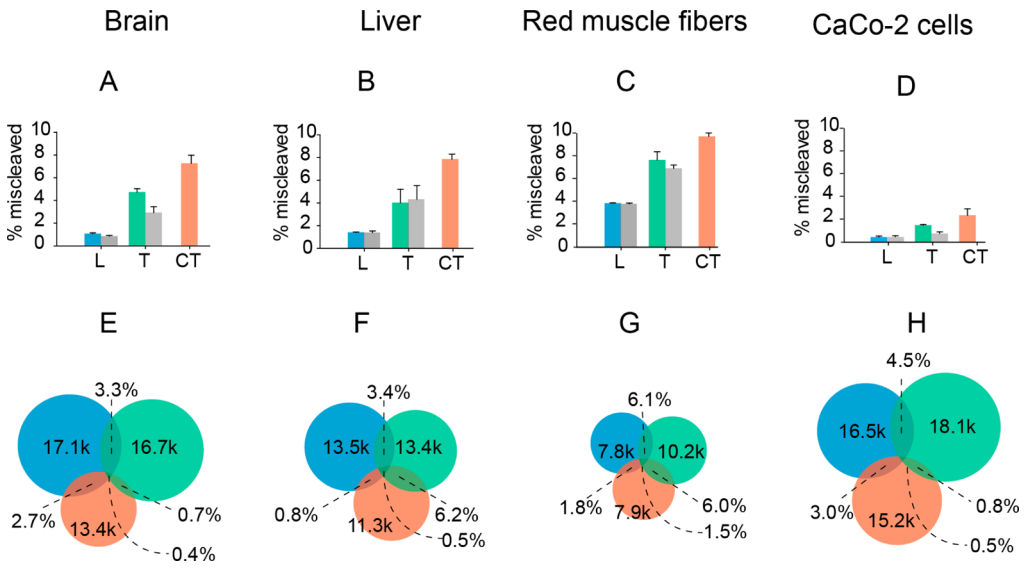

Figure 3. Cleavage efficiencies (A–D) and fractionation effect (E–H). Peptides generated by TAPEG FASP, are in blue, green, and orange for LysC peptides (L), tryptic peptides (T), and cysteine-containing tryptic peptides (CT), respectively. Peptides generated by MED FASP are in gray. (D–F) Values in the circles refer to number of unique peptides identified per fraction in thousands (k) whereas the percent values denote the peptide overlaps between the peptide fractions. Error bars are standard deviation values of 3 and 2 experiments for MED FASP and TAPEG FASP, respectively.

Table 1. Enrichment of Thiol-Containing Peptides

		cysteine-containing peptides sequenced			
sample	% of cysteine-containing peptides	experiment 1	experiment 2	experiment 3	experiment 4
Brain	95.8 ± 1.0	10358	9586		
Liver	95.0 ± 0.3	11090	11615		
RMF	89.7 ± 0.3	5493	5887		
CaCo-2	96.2 ± 0.6	12436	12695	13380	13194
HCT-116	94.6 ± 0.2	13190	13328		
HeLa	95.6 ± 0.6	12220	11742	11186	11306
U87-MG	94.5 ± 0.1	11562	11379		

of the polypeptides chain of BSA. The same was observed when the reactivities of 20k-TAPEG and DTDP were compared using complex protein mixtures originating from mouse liver (Figure 2C). Notably, also using DTNB, another reagent for thiol determination, we found similar reactivity. A comparison of the reactivity of TAPEG across proteins extracted from 3 mouse organs revealed similar contents of reactive thiols in brain and liver proteins, and about 2 times lower reactivity in muscle (Figure 2D).

Efficiency of Protein Cleavage. Efficient protein cleavage with proteases is one of the prerequisites of proteomic analysis. Recently we have reported that MED FASP allows generation of protein lysates with a low number of peptides with miscleaved sites.[21] However, we expected that in the PEG-derivatized proteins a fraction of the protease sites could be inaccessible. To assess this effect, we compared the miscleavage rates in the TAPEG-FASP approach and MED FASP. We found that, in the fractions collected after LysC cleavage and tryptic digestion, the abundances of partially cleaved peptides were similar in both FASP methods (Figure 3A–C) (Supporting Information Table 1). Across the analyzed tissue lysates the lowest miscleavage rate was observed for brain proteins and the highest for muscle proteins. The fractions eluted from the filters after PEG–protein disulfide bond reduction contained between 7 and 9% of partially digested peptides. This higher extent of miscleavages presumably reflects decreased accessibility of trypsin to its cleavage sites located in the vicinity of PEG-derivatized cysteines. Nonetheless, the overall number of miscleaved peptides generated by the procedure can be considered as low when compared to other digestion approaches. Glatter et al.[29] reported up to 25% of miscleaved peptides after a successive in-solution protein digestion with LysC and trypsin whereas Leon et al. found that 10–25% of generated peptides across various trypsin digestion protocols were partially cleaved.[30]

It is well-known that tissue material is more difficult in proteomic analysis than cultured cells. In particular, tissues with high content of fibrillar proteins, such as muscle fibers, are often difficult to analyze. Our data, showing cleavage efficiencies across three analyzed organs and cultured cells, illustrate this well. Whereas peptides generated from cultured cells contained the lowest amount of miscleaved peptides (Figure 3D), the peptides obtained from RMF had the highest miscleavage rate (Figure 3C). We attribute the increasing miscleavage extent to aggregation of proteins during sample processing. Higher miscleavage rates of brain and liver proteins compared to CaCo-2 proteins may reflect occurrence of proteins from stroma in organs.

Protein Fractionation Effect. Previously we have reported that consecutive digestion of proteins in the FASP format increases the number of identified protein peptides and proteins up to 95% and 40%, respectively.[4] This was due to a

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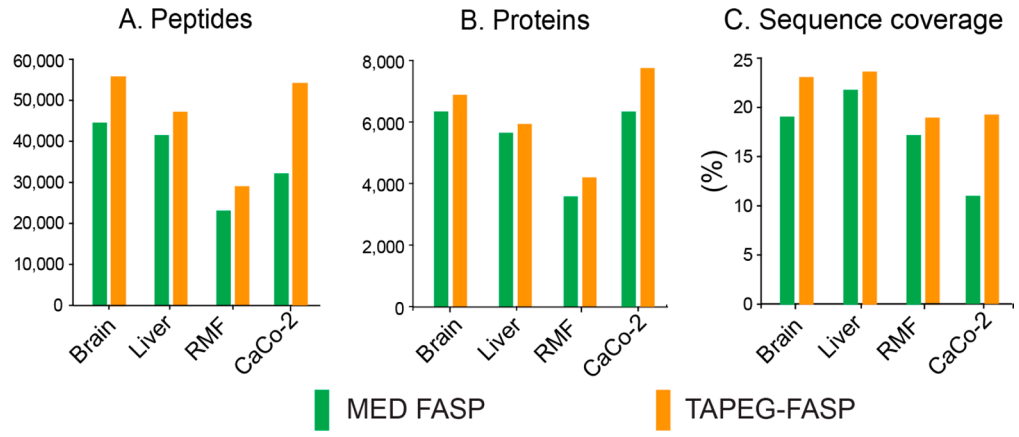

Figure 4. Comparison of the results of proteomic analyses of mouse brain, liver, red muscle fibers (RMF) and CaCo-2 cells attained by MED FASP and PEG-FASP. The bars show values obtained by combined search of data from 3 and 2 experiments for MED FASP and TAPEG FASP, respectively. The total LC-MS/MS data acquisition time used was 24 h per sample. Panel C shows the average sequence coverage of all identified proteins. The sequence coverage values were generated by MaxQuant software and are shown in Supporting Information Table 3.

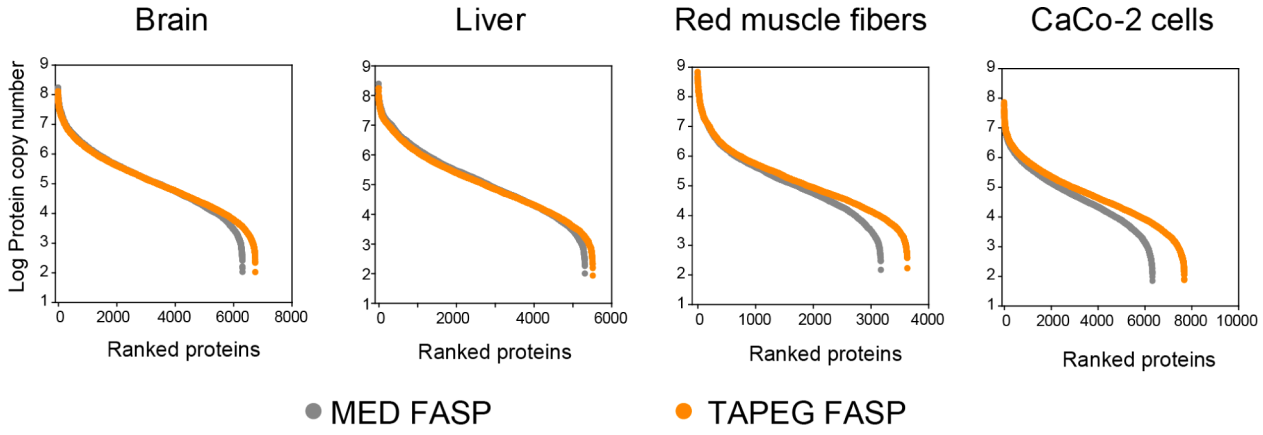

Figure 5. Abundance distribution of proteins identified by MED FASP and TAPEG FASP in whole lysates of mouse tissues and CaCo-2 cells. . The values are from combined searches of data from 3 and 2 experiments for MED FASP and TAPEG FASP, respectively. Protein copy numbers were calculated by the "Proteomic ruler" method.[22]

fractionation effect achieved by collecting two fractions of peptides generated by two or more enzymes. In the TAPEG-FASP approach, we generate 3 fractions. Two are eluted directly after digestions with LysC and trypsin, and the third contains peptides released upon disulfide bond reduction. Analysis of the identified peptides showed a relatively low overlap between pairs of fractions varying from 0.7% to 6.2% of total peptide (Figure 3D–F). The summed amount of peptides that were not identified solely in one fraction was lowest for the brain sample (7.1%) and highest for the red muscle sample (15.4%). The peptide overlaps reflect incomplete proteolytic cleavage and carry-over effects in collection of peptide fractions.

The third fraction obtained by processing liver and brain lysates contained on average 95% of peptides containing cysteine (Table 1). Similarly high enrichments for cysteine-containing peptides were observed for lysates of cultured cells (Supporting Information Table 2) (Table 1).

FASP-TAPEG Increases the Depth of the Proteomic Analysis. Next we compared the number of peptides and proteins that were identified by TAPEG FASP and MED FASP applied to brain, liver, and red muscle fiber samples (Supporting Information Table 3). Since the depth of proteomic analysis often depends on mass spectrometer acquisition time used, we compared two TAPEG FASP preparations, each with 3 fractions, with 3 MED FASP preparations, and with 2 fractions per sample. Therefore, we used the same mass spectrometer acquisition time of 24 h for the analysis of both sample preparation types. We found that across the samples TAPEG FASP outperforms MED FASP. Up to 30% more peptides and up to 17% more proteins were identified using TAPEG FASP compared to MED FASP (Figure 4A and B). This was accompanied by an increase of sequence coverage of 10–20% by TAPEG FASP (Figure 4C). The major reason for the difference between the increase of the number of peptides and the sequence coverage is sequence overlapping between a portion of peptides generated by LysC and trypsin.

Isolation of cysteine-containing peptides for increase of the depth of proteome analysis and identification of lower abundant proteins was already used in the past.[9] Similar effects can be observed comparing MED FASP with TAPEG FASP (Figure 5). The effect of the isolation and analysis of the fractions containing cysteine is higher for muscle and CaCo-2 cells than in brain and liver. The nature of these differences remains unclear.

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CONCLUSIONS

In this paper we describe a procedure for proteomic sample preparation which generates 3 peptide fractions. This approach combines the MED FASP procedure with reversible derivatization of cysteine residues, which allows isolation of a unique fraction of cysteine-containing peptides and two, LysC and tryptic, fractions depleted from cysteine-containing peptides. Due to a low peptide sequence overlap between the fractions, their LC-MS/MS analysis significantly increases the depth of the investigated proteome compared to standard digestion as well as to MED FASP. The method requires the TAPEG reagent, which is easy to synthesize from commercially available and inexpensive chemicals. Alternatively, the reagent can be purchased from "Creative PEGWorks" (Chapel Hill) or "RAPP Polymere" (Tübingen, Germany). We believe that TAPEG FASP will be a useful tool for common proteomic analysis as well as for isolation cysteine-containing peptides. Proteomic analysis using the TAPEG-FASP digestion strategy can be combined with label free or any labeling procedure for protein quantitation.

Classically, isolation of cysteine-containing peptides is achieved by covalent chromatography. In this type of enrichment, peptides are covalently bound to thiol-reactive moieties immobilized on resins. Similarly, for any affinity enrichment of biomolecules, solid supports are used. In contrast, the use of ultrafiltration-based reactor devices for enrichment purposes was only little explored and applied so far. However, the usefulness of the reactor-based approaches was already demonstrated for peptide capturing by antibodies and lectins.[23,24] Here we show that affinity functionalized−high molecular weight PEG can be used for enrichment purposes in the FASP format. Since the high molecular weight PEG can be functionalized in many ways, we believe that various PEG-based reagents can be developed. They will offer a new class of biochemical tools for microrange applications desired in routine as well as cysteine redox proteomics.

ASSOCIATED CONTENT

Supporting Information

Tables of peptides identified by the analysis of mouse tissue lysates and CaCo2-cells, peptides identified by the analysis of human cell lines, and proteins identified by the analysis of mouse tissue lysates and CaCo-2 cells. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b01215.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS

DTDP	2,2′-dithiopyridine
FASP	filter aided sample preparation
PEG	polyethylene glycol
PT	pyridone thione
MED-FASP	multienzyme digestion FASP
TAPEG	thiol-activated PEG (or CH_3O−PEG−S-S-2′-pyridine)

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