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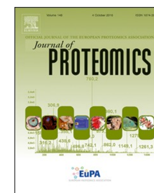
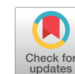


Technical Note

Adding polyvinylpyrrolidone to low level protein samples significantly improves peptide recovery in FASP digests: An inexpensive and simple modification to the FASP protocol



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SUMMARY

Filter-aided sample preparation (FASP) remains a popular choice for proteomic sample preparation, particularly for its ability to produce a 'clean' peptide sample clear of large molecule contaminants. However, sample loss continues to be a problem particularly for sample inputs that contain less than ten micrograms of protein. Here, we describe that the simple addition of a polymer, polyvinylpyrrolidone-40 (PVP-40) to the protein sample prior to FASP digest significantly improves peptide recovery and identifications, especially with lower level sample inputs. PVP-FASP produces clean samples which required no additional sample clean-up prior to nanoLC-MS analysis. In addition, PVP-FASP is compatible with other FASP modifications, including the use of sodium deoxycholate (DOC) to improve trypsin digestion.

Significance: Simple modification to FASP procedure improves sample recovery during proteomic digests in SDS, improving peptide identifications and median peptide intensity.

1. Main text

Many proteomic studies utilize the filter-aided sample preparation (FASP) protocol [1] to prepare samples for mass spectrometry analysis following solubilisation of proteins in SDS. In FASP, smaller contaminants and detergents are washed through an ultrafiltration membrane with a nominal molecular weight cutoff of 10–30 kDa, while proteins and large contaminants are retained at the top of the filter. After trypsin digestion in the filter, the lower molecular weight peptides are spun through the ultrafiltration unit. In this way, the peptide sample is not contaminated by high molecular weight contaminants, such as undigested protein or other polymers that are retained in the top of the filter.

The ability of FASP to process small amounts of protein (< 10 μg) have generally proven less successful than processing larger amounts of protein [2]. Most of this sample loss is likely due to the non-specific binding of proteins to the surfaces of the ultrafiltration unit. Additional sample loss may be caused by irreversible protein aggregation that occurs due to the high protein concentrations that result from the ultrafiltration procedure. Previously, addition of sodium deoxycholate (DOC), deoxycholic acid, polyethylene glycol, dextran, or Tween-20 have been shown to improve FASP yields [3–6]. However, contamination of the final sample has prevented widespread use of these additives. For example, the addition of DOC in FASP protocols generally requires a detergent-removal spin column to maximize recoveries, as acid precipitation fails to remove DOC in its entirety, affecting the ionization of peptide ions and shortening the lifespan of expensive nano-reverse phase columns often used in proteomic analyses [5].

In this work, we explored the use of a stable non-protein polymer, polyvinylpyrrolidone (PVP), to improve recoveries in a FASP protocol. PVP is a linear polymer with a structure somewhat similar to polyethylene glycol (PEG). When compared to PEG, PVP with a molecular weight of approximately 40 kDa is less hydrophobic than PEG, despite having side chains that are more hydrophobic in nature, possibly due to PVP adopting a folded structure in aqueous solutions [7]. PVP is non-toxic and is widely used in pharmaceutical formulations, where it improves solubility and prevents recrystallization in liquid formulations. In molecular biology, PVP has traditionally been used as a blocking agent for Southern blots, and has been occasionally used as a blocking agent for protein Western blots [8]. Since PVP has both favorable biophysical properties and an intrinsic resistance to trypsin, we evaluated whether addition of PVP to protein samples prior to digestion by FASP would improve sample recovery from low level samples, a method we refer to as PVP-FASP.

To evaluate PVP-FASP, we digested both HEK293 cell lysate and rat cerebrospinal fluid (CSF) samples using FASP with or without addition of PVP (Polyvinylpyrrolidone 40 kDa; Sigma). The HEK293 cell lysate

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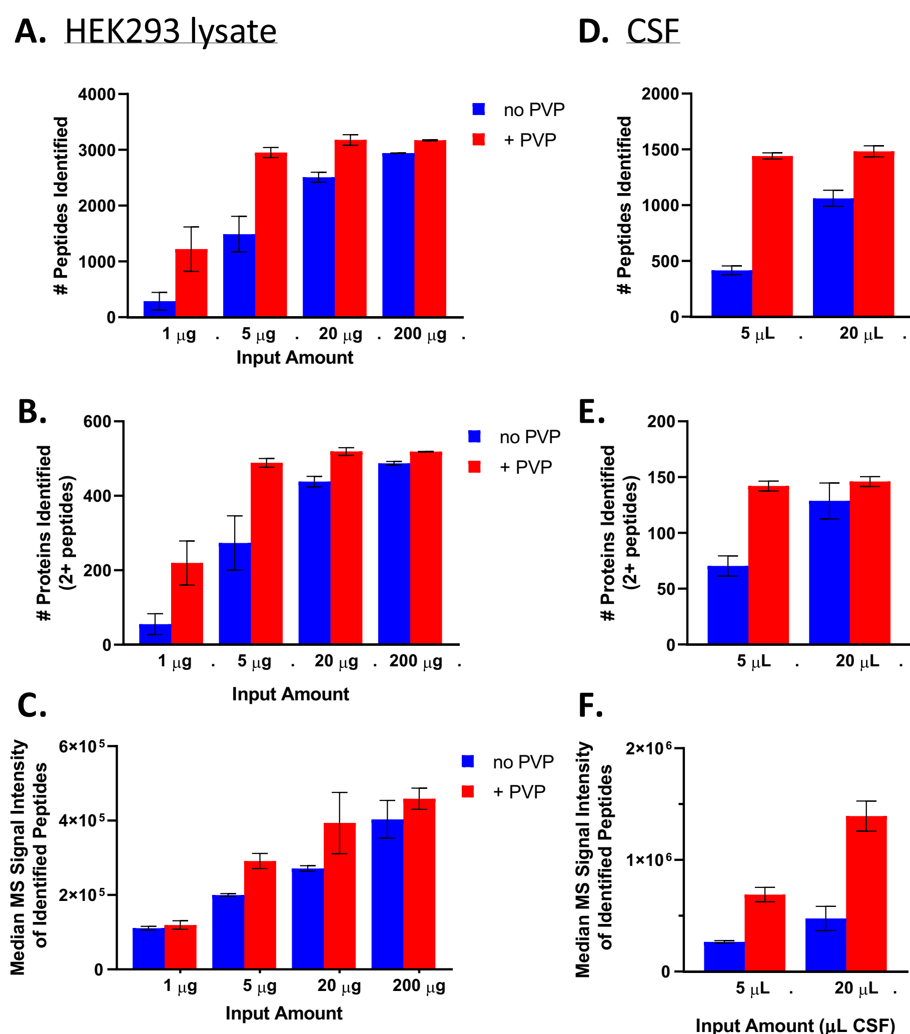
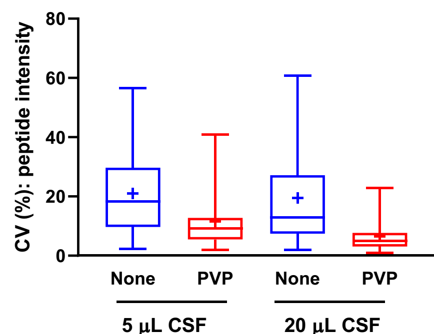


Fig. 1. PVP-FASP significantly improves both the number of peptides and the median peptide signal intensity in low-level protein samples. Different amounts of a (A-C) HEK293 lysate (1, 5, 20 or 200 μg protein) or (D-F) rat CSF (5 μL or 20 μL volume) were digested by FASP without PVP (blue) or with PVP (red) in three separate experiments. The same protein equivalent amount was injected on the LC-MS for all of the samples in each experiment. The number of peptides detected by LC-MS for each sample (A, D), the number of proteins identified by at least 2 peptides (B, E) and the median MS signal intensity of these identified peptides (C, F) are shown. Error bars represent mean ± standard deviation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

was produced by solubilisation in 2% SDS at a concentration of 3 mg/mL protein, as determined by a DC Protein Assay (Bio-Rad). Pooled rat cerebrospinal fluid was purchased from BioIVT. Proteins in both the HEK lysate and CSF were digested by FASP in a 30 kDa MWCO Amicon Ultra-0.5 Centrifugal Filter Unit using published methods [1] with modifications as described in the Supplementary Methods. For PVP-FASP, an excess of PVP-40 that had been pre-cleaned through an Amicon filter (10–30 fold of protein input by weight, minimum = 150 μg PVP, maximum = 2 mg PVP; see Supplementary Methods) was added to the sample just prior to addition to the ultrafiltration unit; all other steps remained unchanged. Samples were analyzed by nano-LC-MS/MS on an LTQ-Orbitrap XL mass spectrometer with a DDA method and MS2 spectra were submitted to Mascot for peptide identification. Analysis of the MS data utilized a label-free approach that produced extracted ion chromatograms (XIC; within a 10 ppm, 40 s retention time window) of all ions that were successfully identified by Mascot in any sample [9,10]. With this approach, all peptide ions were searched for and quantified in all sample runs in that experiment, even if that ion was not selected for MS2. This approach allowed us to correct for the limited MS2 sampling of our mass spectrometer by considering a peptide as 'identified' as long as an XIC peak for the parent ion was detected in the MS1 scans. Each experiment was repeated three times on separate days. Additional methodological details are available in the Supplementary Methods.

We performed FASP and PVP-FASP with 4 different dilutions of the HEK293 cell lysate containing a total of 1 μg, 5 μg, 20 μg, and 200 μg protein. To ensure the general applicability of the improvements seen with PVP-FASP, we also digested cerebrospinal fluid (CSF), starting with either 5 μL or 20 μL of CSF. These volumes of CSF contain a total of approximately 1.5 μg and 6 μg of protein respectively, as determined using a WF assay [11] with a BSA standard curve. In both the HEK lysate and the CSF, we noticed no difference in the sample processing times when PVP was included during the FASP procedure, suggesting that PVP does not clog the filter or significantly increase the sample viscosity at the concentrations used here. Following digestion, peptide samples were diluted such that equivalent protein amounts were analyzed by LC-MS for each sample, assuming 100% recovery. This approach allowed us to compare the relative yield, or recovery, at each input amount. As shown in Fig. 1, sample loss is greater with low-level input samples as evidenced by the lower number of peptide/protein identifications and lower median signal intensity at the smaller protein input amounts for both HEK cell lysate (Fig. 1A-C) and CSF (Fig. 1D-F). PVP-FASP consistently increases the number of unique peptides detected, especially when starting with lower protein amounts, which showed a 2–3 fold increase in detected peptides with PVP. Interestingly, PVP appears to increase the recovery of larger hydrophobic peptides more than smaller hydrophilic peptides, as evidenced by the greater increase in peptide recovery with PVP-FASP in regions that represent a larger *m/z* and later retention time, as demonstrated by visualization of the LC-MS data using MSight [12] (Supplementary Fig. 1). This observation is further supported by a population analysis of the biophysical characteristics of peptides identified in each sample (Supplementary Fig. 2), calculated using the modlamp python module [13]. This analysis shows an average increase in both molecular weight and



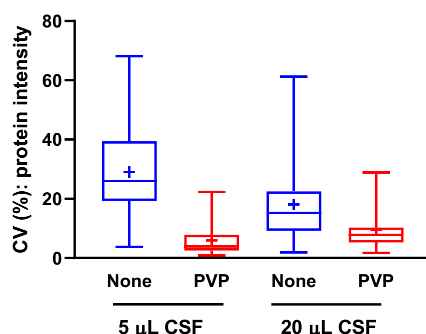


Fig. 2. Quantitative precision of sample preparation technical repeats. Two different amounts of rat CSF (5 or 20 μL) were processed side-by-side in triplicate using standard FASP or FASP-PVP as sample preparation replicates. Box and whiskers plot showing the coefficient of variation (CV) of the XIC intensity across the 3 sample preparation replicates for all (A) peptides or (B) proteins with 2 or more assigned peptides, that were identified and successfully quantified in both the standard and PVP methods in all 3 repetitions. On each plot, the outer box represents the interquartile range, the solid line shows the median CV value, the + represents the mean, and the bars encompass 95% of the data points.

GRAVY index score, a measure of peptide hydrophobicity/hydrophilicity, when PVP is included in the FASP digest, especially in samples with lower protein input amounts. No difference was seen in the isoelectric point (pI) of the identified peptides with PVP. Lastly, the average MS signal intensity of the identified peptide ions was also significantly increased by PVP-FASP (Fig. 1C and F; Supplementary Fig. 1) in all samples except the 1 μg HEK lysate. Based on the MSight visualization data, we suspect the signal intensity is generally higher with PVP-FASP even at the 1 μg level. We speculate that most peptides in this 1 μg sample were at the detection limit for our mass spectrometer, so PVP simply pushed more peptides across this threshold without significantly changing the median intensity of the identified peptides.

Since the majority of FASP digests are used for quantitative comparison between two samples, we repeated the CSF experiment described above using three side-by-side sample preparation replicates with the same input. The identified peptides were quantified using a label-free approach based on the MS signal intensity represented in the XIC for each peptide. The coefficient of variation between the three sample preparation replicates was calculated for all peptides that were successfully quantified in the three replicates using both standard FASP and PVP-FASP, using median scale normalization between samples. As can be seen in Fig. 2, PVP-FASP lowered the variability in peptide quantification significantly (Fig. 2A) and this translated into improved protein level quantification as well (Fig. 2B). Much of this improvement is likely due to the increased peptide intensity values seen with PVP, which make XIC based quantification more accurate by improving signal-to-noise.

We next compared PVP-FASP with another modified FASP procedure (mFASP [5]), referred to here as DOC-FASP since it uses sodium deoxycholate in the digest step. Both PVP-FASP and DOC-FASP were performed in parallel starting with 5 μg of the HEK lysate. We also tested a combination of these two methods by including PVP in the DOC-FASP, referred to here as PVP + DOC-FASP. We found that PVP-FASP and DOC-FASP both showed an increase in the number of peptides and proteins detected and in the median MS signal intensity of those peptides relative to FASP without any additions (Fig. 3A-C). To compare the recovery of specific peptides in each FASP protocol, we

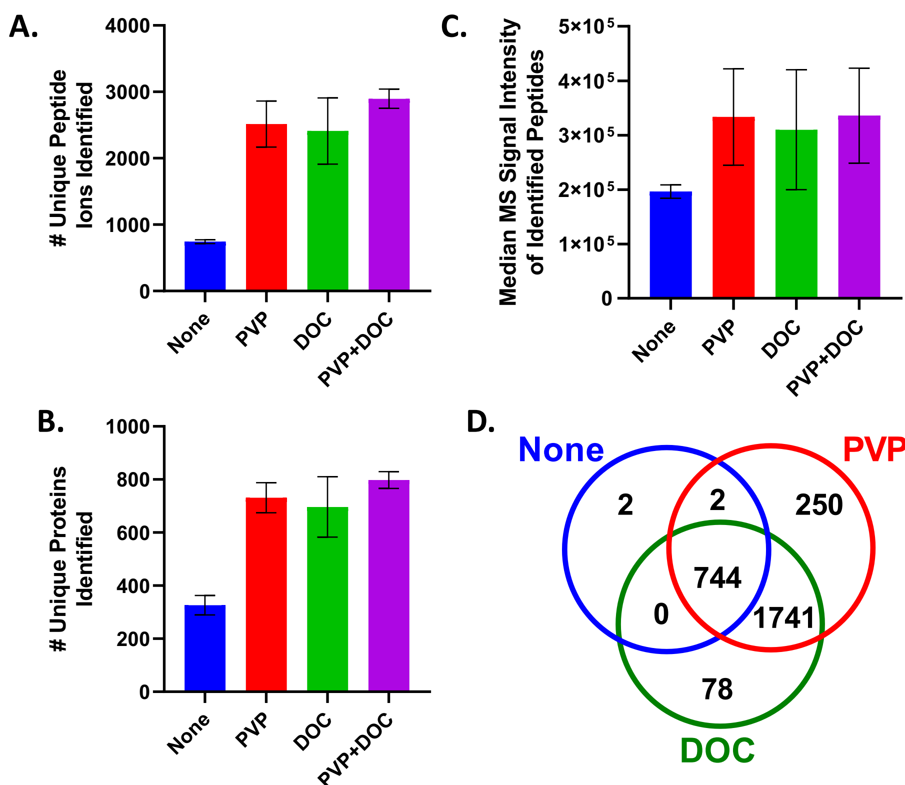


Fig. 3. Comparison of PVP-FASP to DOC-FASP shows similar, but complementary improvements in peptide recovery. HEK cell lysate (5 μg protein) was digested by FASP with no additives (blue), PVP (red), DOC (green), or both PVP and DOC (purple) in three separate experiments. Following LC-MS analysis, the number of peptides (A) and proteins (B) was determined, and the median signal intensity of each peptide (C) was calculated. Error bars represent mean ± standard deviation. (D) Venn diagram of peptides identified in at least 2 of the 3 experimental repeats for each sample. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

focused our analysis on peptide ions that were identified in at least 2 of 3 experimental repeats. A Venn diagram of these peptide identifications with each FASP protocol shows that DOC and PVP both improve detection in the majority of peptides, but that recovery of some peptides is improved more selectively by either PVP or DOC (Fig. 3D). Of these 3053 identified peptides, 748, 2737, and 2563 peptides were detected in the FASP, PVP-FASP, and DOC-FASP samples respectively. Interestingly, 2982 of these 3053 peptides were detected when both PVP and DOC were added to the FASP protocol (PVP + DOC-FASP), suggesting that PVP and DOC additions to FASP are compatible and can be used together to gain the benefits of each modification (Fig. 3, purple bar).

Viewing the peptide LC-MS data by MSight suggests that DOC improves recovery of very large, late-eluting peptides (> 1000 *m/z* with late retention times) relative to PVP, suggesting that addition of DOC may be especially important when large, hydrophobic peptides are of particular interest (Supplementary Fig. 3A). However, several of these larger peptides in the HEK lysate sample may not have been successfully identified by our bioinformatics pipeline, based on analysis of the biophysical properties (pI, molecular weight, and GRAVY index score) for the peptides identified by each sample preparation method (Supplementary Fig. 4A). While DOC seems to work particular well at identifying peptides with a lower pI, both PVP and DOC similarly improve identification of peptides with a larger molecular weight or a higher GRAVY index score, relative to the standard FASP method. Importantly, the rate of missed cleavages was similar for peptides identified in at least 2 of the 3 experimental repeats of each sample preparation method: standard FASP - 13%, PVP-FASP - 15%, DOC-FASP - 15%, PVP + DOC-FASP - 15%, suggesting that trypsin efficiency is similar in all of these FASP methods. The slightly higher missed cleavage rate in the modified FASP protocols likely simply reflects the improved peptide recovery and higher peptide identifications in these samples rather than a true difference in trypsin efficiency; with improved recovery it is more likely that relatively low-level missed cleavage peptides from abundant proteins will be successfully sequenced.

To further support the relative roles that DOC and PVP addition play in FASP digestion, we repeated this experiment using a crude membrane enriched fraction from HEK293 cells that had a higher proportion of hydrophobic proteins. Overall, the results were similar to the results of the total cell HEK lysate, as shown in Supplementary Fig. 5. Of the 3497 unique peptide ions identified in 2 of 3 biological repeats, 1297, 2958, 3311, and 3449 were detected in the FASP, PVP-FASP, DOC-FASP, and PVP + DOC-FASP samples. In this membrane-enriched sample, the population of DOC-FASP peptides had a larger molecular weight and lower pI on average than peptides produced by PVP-FASP (Supplementary Fig. 4B). Further, MSight suggests that DOC appeared to be especially effective at improving the recovery of relatively high molecular weight peptides with late retention times (Supplementary Fig. 3B), although many of these larger peptides were not identified by our workflow. As seen in the HEK lysate results, the percentage of peptides containing missed cleavages in the membrane-enriched fraction was similar for the four FASP methods: standard FASP - 12%, PVP-FASP - 15%, DOC-FASP - 15%, PVP + DOC-FASP - 15%.

The PVP-FASP and DOC-FASP experiments on HEK293 cell lysate and HEK293 membrane-enriched fraction were each repeated three separate times, months apart. Despite the inevitable differences in LC and mass spectrometry performance that occur over such long time periods, the quantitative precision of PVP-FASP, as measured by peptide and protein CV across these three experimental replicates, was improved relative to both standard FASP and DOC-FASP for the HEK293 membrane-enriched samples, as shown in Supplementary Fig. 6B. For the HEK293 cell lysate sample, the median peptide CVs for PVP-FASP was equivalent to the standard FASP results, as shown in Supplementary Fig. 6A; however a subset of peptides showed more variable recovery as evidenced by the higher 75% quartile value. Closer inspection of the individual peptide quantification data, suggests that this increased variability is present in 1 of the 3 experimental repetitions, while the other two replicates are very consistent, and similar to those seen with the CSF and HEK membrane-enriched samples. It is possible that a technical issue during either sample preparation or LC-MS analysis led to this inconsistency in recovery, separation, or ionization of a subset of peptides in one of the PVP-FASP experimental replicates. This hypothesis is further supported by the PVP + DOC-FASP results which show an improvement in CV relative to other FASP methods, as was seen in the HEK membrane-enriched sample. Regardless, even with this issue, the protein level quantification with PVP-FASP shows equivalent CV values to the standard FASP method.

In practical terms, PVP-FASP has several advantages over DOC-FASP, including the fact that sample cleanup to remove lingering detergent is not necessary. Without a cleanup step, PVP-FASP is more easily adaptable to processing large number of samples in a 96-well format, making use of a 96-well ultrafiltration unit with a polyethersulfone membrane [14] where PVP has been shown to similarly improve peptide recovery (data not shown). Unlike DOC-FASP, PVP-FASP is also compatible with FASP-like workflows that utilize the ultrafiltration unit to separate peptides based on binding to a protein, such as glycopeptide enrichment with lectins (N-glyco FASP [15]) or phosphopeptide enrichment by antibodies [16] . It is also expected that PVP-FASP should be compatible with enzymatic digestion using a wide variety of proteases regardless of whether those proteases show activity in the presence of detergents.

Here, we describe how the polymer PVP, a non-protein blocking agent and stabilizing agent, can be added to a protein sample prior to FASP digestion, resulting in significant increases in sample recovery with no contamination. The benefits of PVP addition are especially strong when the total protein being digested is below 20 μg, and no additional sample cleaning steps are required. The clean samples resulting from PVP-FASP might reflect the ability of PVP to fold in aqueous solution [7], which may prevent passage through the ultrafiltration unit pores when compared to a polymer that is more likely to take a linear form. In addition to acting as a carrier to prevent non-specific binding of proteins to surfaces, it is possible that PVP may also stabilize proteins, preventing precipitation at the high protein concentrations that can be reached during ultrafiltration. In conclusion, PVP is an effective, simple, and inexpensive addition to the FASP procedure to improve peptide recovery of lower level samples. PVP can be used alone as a FASP additive, or together with DOC in the digest step to further improve recovery of very large peptides.

CRediT authorship contribution statement

Tammy-Lynn Tremblay: Conceptualization, Data curation, Methodology, Investigation; **Jennifer J. Hill:** Conceptualization, Data curation, Methodology, Investigation, Writing - review & editing, Project administration, Formal analysis, Supervision, Writing - original draft.

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Declaration of Competing Interest

The authors have no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jprot.2020.104000.

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