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Authors
Erde, Jonathan
Loo, Rachel R Ogorzalek
Loo, Joseph A

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Improving Proteome Coverage and Sample Recovery with Enhanced FASP (eFASP) for Quantitative Proteomic Experiments

Jonathan Erde[†], **Rachel R. Ogorzalek Loo**[‡], and **Joseph A. Loo**[†,‡]

[†] Department of Chemistry and Biochemistry, University of California-Los Angeles, Los Angeles, CA

[‡] Department of Biological Chemistry University of California-Los Angeles, Los Angeles, CA

Abstract

Enhanced Filter Aided Sample Preparation (eFASP) incorporates plastics passivation and digestion-enhancing surfactants into the traditional FASP workflow to reduce sample loss and increase hydrophobic protein representation in qualitative and quantitative proteomics experiments. Resulting protein digests are free of contaminants and can be analyzed directly by LC-MS.

Keywords

Enhanced Filter Aided Sample Preparation; quantitative proteomics; detergent; ammonium deoxycholate

1. Introduction

The integrity of proteomic experiments hinges on consistent and robust protein extraction, solubilization, and digestion. Protocols using anionic detergents and/or chaotropes to extract and solubilize cellular and matrix proteins efficiently, provide samples that must be purified prior to digestion and analysis. Methods such as organic precipitation which remove contaminants, denaturants, and other undesired species (*e.g.*, salts, nucleic acids, lipids, and alkylating reagents), are subject to poor recoveries, re-solubilization problems, and protein-to-protein variation. Enhanced Filter Aided Sample Preparation (eFASP) provides efficient protein extraction, purification and digestion for a variety of samples (1,2).

Traditional Filter Aided Sample Preparation (FASP) circumvents many protein purification challenges by exchanging buffers in spin filter units (ultrafiltration assemblies) that can remove sodium dodecyl sulfate (SDS) and sample contaminants completely (3,4). Proteins are reduced, alkylated, washed, and digested in the filter unit, releasing product free of detergent, reductant, and alkylating agent. Nevertheless, when applied to very small sample sizes, this method can suffer sample losses near 50% (5). Enhanced FASP (eFASP) addresses this challenge by incorporating deoxycholic acid and, optionally, TWEEN®–20 into the FASP workflow.

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Deoxycholic acid (DCA) is a secondary bile acid that, amongst many other uses, is employed as a mild detergent for membrane proteins. It increases the efficiency with which trypsin digests cytosolic and membrane proteins and is easily removed by acidification and phase transfer (PT) to peptide-immiscible ethyl acetate (EA) in liquid-liquid extraction (1,6–11). PT decreases EA-soluble contaminants, including SDS, *n*-octylglucoside, NP-40, and Triton X-100 (12).

The eFASP protocol optionally uses the surfactant TWEEN®–20 to passivate surfaces of Microcon® filter units and collection tubes. TWEEN®–20, as well as SDS, are recognized choices for minimizing protein binding to surfaces and have been recommended by Amicon Centricon for use in filter units (13). Passivation of the filter units and collection tubes used in eFASP can reduce peptide and protein loss due to non-specific surface binding, but care is needed to prevent TWEEN®-related ions from contaminating mass spectra.

Presented here is the eFASP approach, which utilizes 0.2% DCA and (optionally) TWEEN® –20 to quantitatively increase recovery and proteomic coverage of hydrophilic and hydrophobic proteins. An express eFASP method variant is also included, which uses a one-step reduction/alkylation employing tris(2-carboxyethyl)phosphine (TCEP) and 4-vinylpyridine (4-VP) prior to deposition on the Microcon® filter, increasing alkylation specificity and speeding processing (1,14–17).

2. Materials

Prepare all solutions fresh using ultrapure water and MS-grade reagents. Follow all waste disposal regulations and chemical safety guidelines.

2.1 Solutions and Reagents for eFASP (*see* Subheading 3.1)

1. Passivation Solution: 5% (v/v) TWEEN®–20
2. Lysis Buffer: 4% SDS, 0.2% DCA (Sigma, D2510), 50 mM TCEP, 100 mM ammonium bicarbonate (ABC), pH 8 (*see* Notes 1, 2)
3. Exchange Buffer: 8 M urea, 0.2% DCA, 100 mM ABC, pH 8 (*see* Note 1)
4. Alkylation Buffer: 50 mM iodoacetamide, 8 M urea, 0.2% DCA , 100 mM ABC, pH 8 (*see* Notes 1, 2)
5. Digestion Buffer: 0.2% DCA, 50 mM ABC, pH 8 (*see* Note 1)
6. Trypsin Buffer: 0.5 μg / μl trypsin, 50 mM ABC, pH 8

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1. This protocol employs deoxycholic acid rather than sodium deoxycholate, in order to minimize analyte exposure to sodium, which can degrade mass spectrometry analyses. Even with online liquid chromatography to remove much of the Na^+, acidic peptides may appear sodium-adducted. A trade-off in substituting deoxycholic acid for sodium deoxycholate is the former's low solubility; 0.2% DCA is about the maximum solubility achievable in ABC buffer. Dissolving deoxycholic acid in a small volume of ethanol prior to mixing with buffer can facilitate dissolution. Alternatively, ABC buffer may be added to the appropriate quantity of solid DCA immediately before use and vortexed extensively; even if slightly cloudy, the freshly prepared DCA solution can be employed in eFASP. DCA solutions should not be refrigerated; irreversible precipitation may occur. We have found that 0.1% DCA performs almost as well as 0.2% in eFASP; thus, solubility problems may be eased by using the lower concentration. Finally, sodium deoxycholate could substitute for deoxycholic acid, with STAGE tip cleanup prior to injection onto the LC column.
2. Alternatively, Lysis Buffer can employ 5 mM TCEP and/or Alkylation Buffer can be formulated with 5 mM iodoacetamide.

7. Peptide Recovery Buffer: 50 mM ABC, pH 8
8. Ethyl acetate
9. 50% methanol
10. Trifluoroacetic acid
11. MS-grade H_2O

2.2 Solutions and Reagents for Express eFASP (*see* Subheading 3.2)

1. Passivation Solution: 5% (v/v) TWEEN®–20
2. Lysis Buffer: 4% SDS, 0.2% DCA (Sigma, D2510), 50 mM TCEP, 100 mM ammonium bicarbonate (ABC), pH 8 (*see* Notes 1, 2)
3. Exchange Buffer: 8 M urea, 0.2% DCA, 100 mM ABC, pH 8 (*see* Note 1)
4. Alkylation Stock: 500 mM 4-VP in ethanol (*see* Note 3)
5. Quench Buffer: 1 M dithiothreitol (DTT), 100 mM ABC, pH 8
6. Digestion Buffer: 0.2% DCA, 50 mM ABC, pH 8 (*see* Note 1)
7. Trypsin Buffer: 0.5 μg / μl trypsin, 50 mM ABC, pH 8
8. Peptide Recovery Buffer: 50 mM ABC, pH 8
9. Ethyl acetate
10. 50% methanol
11. Trifluoroacetic acid
12. MS-grade H_2O

2.3 Equipment

1. Microcon® UF units (YM-30 30 kDa cutoff limit; Millipore, Billerica, MA).
2. Bench-top centrifuge
3. hermo-mixer (initially set to 90°C)
4. SpeedVac®
5. Squeeze bottle containing MS-grade H_2O
6. Sonicator/homogenizer for disrupting cells
7. Eppendorf LoBind® tube, 2 ml
8. Ultrasonic bath

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[3]. We assume that neat 4-vinylpyridine is 8.8 M in concentration.

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3. Methods

Two eFASP protocols are described. The first is the standard procedure that utilizes in-filter alkylation with iodoacetamide (*see* Subheading 3.1). The second is an express procedure that utilizes in-solution alkylation with 4-vinylpyridine, eliminating some buffer exchange steps to increase speed (*see* Subheading 3.2).

Carry out all procedures at room temperature or as specified and follow instrument and chemical safety guidelines. The passivation steps may be omitted to save time or if TWEEN®-related background ions cannot be minimized in mass spectra.

3.1 eFASP: Standard

3.1.1 Surface Passivation (Optional)

1. On a shaker, incubate filter units and collection tubes overnight in Passivation Solution. Small batches of items may be incubated in 50 mL Falcon centrifuge tubes.
2. With clean tweezers, remove each item and rinse its outer and inner surfaces with MS-grade H_2O dispensed from a squeeze bottle.
3. Transfer items to a clean beaker containing a large volume of MS-grade H_2O; *e.g.*, 250 ml or more. Incubate items for 30 minutes at room temperature, shaking at low speed.
4. epeat step 3 two additional times with fresh MS-grade H_2O.
5. Reserve the passivated collection tubes for peptide recovery from ultrafiltration devices.

3.1.2 Sample Lysis

1. Wash and pellet cells according to established guidelines for cell type.
2. Add sufficient Lysis Buffer to the pelleted cells such that a 25 μl aliquot of lysate will provide the quantity desired for processing by eFASP (or a maximum protein concentration of 10 μg / μl). Ensure that the selected volume and tube size can accommodate the sonicator probe.
3. Place the lysate into a 90°C thermo-mixer and incubate for 10 min, shaking at 600 rpm. Remove lysate and decrease thermo-mixer temperature to 37°C for later use.
4. Sonicate lysate (employing sonicator/homogenization probe) three times for 10-sec each.
5. Centrifuge the lysate at 14,000 × g for 10 min.
6. Repeat sonication and centrifugation once (steps 4 and 5).
7. Sonicate the lysate (including any pelleted material), for 10 sec. Cool to 37°C.

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3.1.3 Sample Processing

1. Transfer 25 μl of lysate to a sample tube containing 200 μl of Exchange Buffer. Vortex briefly to mix.
2. Place a (passivated) filter unit atop a non-passivated collection tube.
3. Dispense the 225 μl lysate/Exchange Buffer sample to the filter unit and centrifuge at 14,000 × g for 10 min. Discard filtrate.
4. Add 200 μl Exchange Buffer to the filter unit and centrifuge at 14,000 × g for 10 min. Discard filtrate.
5. Repeat step 4 two more times.
6. Dispense 100 μl Alkylation Buffer to the filter unit and transfer it to a 37°C thermo-mixer for 1 h, shaking at 300 rpm.
7. Centrifuge the filter unit at 14,000 × g for 10 minutes. Discard filtrate.
8. Add 200 μl Exchange Buffer to the filter unit and centrifuge at 14,000 × g for 10 min. Discard filtrate.
9. Add 200 μl eFASP Digestion Buffer to the filter unit and centrifuge at 14,000 × g for 10 min. Discard filtrate.
10. Repeat step 9 two more times.
11. Transfer the filter unit to a passivated collection tube.
12. Add 100 μl eFASP Digestion Buffer to the filter unit.
13. Calculate the volume of Trypsin Buffer to dispense in order to achieve the desired enzyme-to-substrate ratio; *e.g.*, 1:50 w:w.
14. Deposit the calculated volume of Trypsin Buffer to the filter unit, and place in a 37°C thermo-mixer for 12 h, shaking at low speed. Secure the filter unit cap to minimize evaporation.
15. Remove the filter unit/collection tube assembly from the thermo-mixer and centrifuge at 14,000 × g for 10 min. *Retain peptide-containing filtrate*.
16. Deposit 50 μl of Peptide Recovery Buffer onto the filter unit and centrifuge at 14,000 × g for 10 min.
17. Repeat step 16 step once. *Retain peptide-containing filtrate.*

3.1.4 Phase Transfer

1. To the collection tube with peptide-containing filtrate, add 200 μl of ethyl acetate and transfer to a 2 ml Eppendorf LoBind® tube.
2. Add 2.5 μl TFA and quickly vortex. A white, thread-like precipitate may be visible if a large quantity of peptide is present.
3. Add ethyl acetate to nearly fill the tube, leaving only enough space to agitate without losing liquid.

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4. Agitate the mixture for 10 sec in an ultrasonic bath and centrifuge at 16,000 × g for 10 min.
5. Carefully pipet most of the upper (organic) layer into a tube for discard. Do not disturb the organic/aqueous boundary layer.
6. Repeat steps 3 through 5 two times.
7. Place the uncapped sample tube in a 60°C thermo-mixer, in a fume hood, for 5 min to remove residual ethyl acetate.
8. Remove residual organic solvent and volatile salts by vacuum drying in a SpeedVac®.
9. Resuspend the dried sample in 50% methanol and vacuum-dry.
10. Repeat step 9 two times.

3.2 Express eFASP

3.2.1 Surface Passivation (*Optional*)

3.2.2 Sample Lysis

1. Wash and pellet cells according to established guidelines for cell type.
2. Add sufficient Lysis Buffer to the pelleted cells such that a 25 µl aliquot of lysate will provide the quantity desired for processing by eFASP (or a maximum protein concentration of 10 µg / µl). Ensure that the selected volume and tube size can accommodate the sonicator probe.
3. Place the lysate into a 90°C thermo-mixer and incubate for 10 min, shaking at 600 rpm. Remove lysate and decrease thermo-mixer temperature to 37°C for later use.
4. Sonicate lysate (employing sonicator/homogenization probe) three times for 10-sec each.
5. Centrifuge the lysate at 14,000 × g for 10 min.
6. Repeat sonication and centrifugation once (steps 4 and 5).
7. Sonicate the lysate (including any pelleted material), for 10 sec. Cool to 37°C.
8. Add Alkylation Stock to the lysate to a final concentration of 25 mM 4-VP, and place the sample tube into a 37°C thermo-mixer for 1 h at 300 rpm.
9. Add Quench Buffer to the lysate to a final concentration of 40 mM DTT.

3.2.3 Sample processing

1. Transfer 25 µl of lysate to a sample tube containing 200 µl of Exchange Buffer. Vortex briefly to mix.
2. Place a (passivated) filter unit atop a non-passivated collection tube.

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3. Dispense the 225 μl lysate/Exchange Buffer sample to the filter unit and centrifuge at 14,000 × g for 10 min. Discard filtrate.

4. Add 200 μl eFASP Digestion Buffer to the filter unit and centrifuge at 14,000 × g for 10 min. Discard filtrate.

5. Repeat step 4 two more times.

6. Detach the filter unit from the non-passivated collection tube, and place it on top of a passivated tube.

7. Add 100 μl eFASP Digestion Buffer to the filter unit.

8. Calculate the volume of Trypsin Buffer to dispense in order to achieve the desired enzyme-to-substrate ratio; *e.g.*, 1:50 w:w.

9. Deposit the calculated volume of Trypsin Buffer to the filter unit, and move the filter/collection tube assembly to a 37°C thermo-mixer for 12 h of shaking at low speed. Cap the filter unit to reduce evaporation.

10. Remove the filter/collection tube assembly from the thermo-mixer and centrifuge at 14,000 × g for 10 min. *Retain the peptide-containing filtrate.*

11. Dispense 50 μl of Peptide Recovery Buffer to the filter unit and centrifuge at 14,000 × g for 10 min.

12. Repeat step 11 step once.

3.2.4 Phase Transfer (*see* Subheading 3.1.4)

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