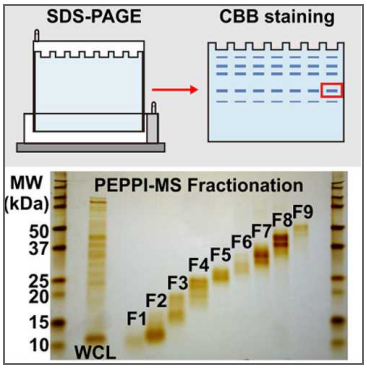

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Protocol status: Working
This protocol has been published in a peer-reviewed journal and used successfully used by other research groups - see citations on journal article.

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PEPPI-MS

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ABSTRACT

This protocol is derived from the version originally published in the Journal of Proteome Research (https://doi.org/10.1021/acs.jproteome.0c00303).

Ayako Takemori was instrumental in developing the original PEPPI-MS protocol, but not involved in the creation of the version hosted on protocols.io.

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PROTOCOL integer ID: 58228

GUIDELINES

Concentrations and volumes of protein solutions have been optimized for bacterial cell lysate in the range of 0.5 - 1.0 mg/mL. Adjust as needed for different sample types.

We recommend against preconcentrating the sample using an Amicon or similar filter unit, as a significant amount of sample can be lost to adsorption to the cellulose membrane. If preconcentration is needed, use of a vacuum concentrator is recommended.

We recommend the use of Eppendorf Protein LoBind microcentrifuge tubes to minimize loss of sample through adsorption.

MATERIALS

Materials from specific manufacturers listed below are recommendations and can be freely replaced. We recommend the use of Bis-Tris or similar gel chemistries for electrophoresis due to the close-to-neutral pH during separation. Ammonium bicarbonate buffer and iodoacetamide solutions should be prepared fresh.

Consumables

- TaKaRa or Kimble-Chase Biomasher II Tissue Homogenizers
- Costar 8163 0.45 µm centrifugal filters
- Amicon Ultra 0.5 mL centrifugal filters, 3 kDa MWCO
- Invitrogen NuPAGE 4-12% Bis-Tris gel, 1 mm, 10 well or 2D well format
- Invitrogen NuPAGE 4X LDS loading buffer
- Invitrogen NuPAGE 20X MES-SDS running buffer
- BioRad Precision Plus Dual Color Standard
- BioRad BioSafe coomassie blue stainn or Atto EZstain Aqua
- 100 mM ammonium bicarbonate, pH ~9 with 0.1% sodium dodecyl sulfate
- 0.10 M dithiothreitol stock solution
- 0.20 M iodoacetamide stock solution (add 3.7 mg of IAA to 100 µL of water)
- Gel fixing solution: 10% acetic acid, 30% ethanol, 60% water (HPLC-grade or better)
- 1.5 mL microcentrifuge tubes

Equipment

- Heating block with 1.5 mL microcentrifuge tube adapter
- Vortexer or tube shaker with 1.5 mL microcentrifuge tube adapter
- Electrophoresis chamber for mini format gels (10 by 10 cm)
- Microcentrifuge capable of 14000G
- Rocking platform

SAFETY WARNINGS

Iodoacetamide poses a significant health risk! Handle it carefully, use appropriate PPE, and dispose of it properly.

BEFORE START INSTRUCTIONS

Set heating block to 50 °C.

1 Prepare a volume of protein solution (*e.g.* whole cell lysate) sufficient to load 10 gel lanes with 11 µg of protein in a volume no greater than 18.75 µL per lane (e.g. 110 µg of protein in ≤187.5 µL) in a 1.5 mL microcentrifuge tube.

2 Add DTT stock solution to protein sample to a final concentration of 1 mM (e.g. 1 µL of 0.1 M DTT per 100 µL of lysate). Incubate sample at 50 °C for one hour then allow to cool to room temperature.

3 During incubation, prepare fresh iodoacetamide (IAA) stock solution. Keep IAA solution in the dark. A 0.2 M stock is recommended.

4 Add IAA stock solution to protein sample to a final concentration of 2 mM (1 µL of 0.2 M stock per 100 µL of sample). Incubate at room temp. in the dark for 45 minutes.

5 Add a 1/3 volume of 4X NuPAGE LDS loading buffer to sample (e.g. add 62.5 µL to 187.5 µL sample).

6 Carry out SDS-PAGE according to manufacturer's protocol using NuPAGE 4-12% Bis-Tris gradient gels and NuPAGE MES SDS running buffer. Recommended loading: One marker lane with 20 µL of 0.5x diluted BioRad Precision Plus Dual Color Standard and nine lanes with 20 - 25 µL of reduced & alkylated protein sample containing 11 µg of protein each.

7 Immediately after run, remove gel from cassette and rinse VIGOROUSLY three times with deionized water for 5, 10, and 15 minutes each to remove SDS. SDS contamination will interfere with protein staining.

8 If staining with BioRad Bio-Safe coomassie blue stain (Bio-Safe CBB), rinse gel with fixing solution for 10 minutes. Replace fixing solution and rinse gel for another 45 minutes. Replace fixing solution with water (HPLC-grade or better) and rinse for two hours, changing water twice. Gels can be left in fixing solution or water overnight at 4 °C if necessary.

9 Stain using Bio-Safe CBB (or Atto EZstain Aqua if available) for at least 60 minutes. Protein bands should be clearly visible. Afterwards do several 5 minute water rinses to reduce background.

10 Take pictures of gel then designate and number sections to be cut for each PEPPI fraction (it will not be possible to tell which bands were cut after the PEPPI process).

11 Using a scalpel, cut lanes into sections and deposit into numbered TaKaRa BioMasher tissue homogenizer tubes.

11.1 Ensure cuts are straight across the gel to avoid cross-contamination of adjacent PEPPI fractions. When cutting the gel, bands from adjacent lanes should be married to increase total protein content. Original Takemori protocol calls for marrying all nine lysate lanes to get enough protein for MCW precipitation. Butcher recommends two 1.5 mL homogenizers for a nine-lane gel section of approximately 5 mm in width.

12 Add 300 µL of freshly-prepared 100 mM ammonium bicarbonate, pH ~ 9 with 0.1% w/v SDS to each tube.

13 Crush gel pieces in each tube extensively using homogenizer until mixture is completely homogenous and no large pieces are left.

14 Shake tubes at 1500 RPM for 10 minutes at room temp. During shaking time, rinse two 0.45 µm pore size centrifugal filter units (Costar 8163) and one 3 kDa MWCO Amicon Ultra centrifugal filter unit per fraction with 100 mM ammonium bicarbonate with 0.1% w/v SDS.

15 Use 1000 µL micropipette to transfer tube contents to pre-rinsed centrifugal filter units with 0.45 µm pore size (Costar 8163). Rinse each BioMasher tube with 100 µL of 100 mM ammonium bicarbonate with 0.1% SDS and add rinsate to centrifugal filter. Centrifuge for 10 - 15 minutes at 14,000G to separate gel pieces from recovered protein solution. - No more than half of a nine-

lane section should be added to each filter or the filter disc will clog and no solution will flow through.

16 Transfer filtrate to pre-rinsed 3 kDa MWCO Amicon Ultra centrifugal filter unit. Centrifuge at 14,000G to reduce total volume per PEPPI fraction to ~150 µL. Two spins of 6 and 4 minutes each is usually sufficient to reduce volume to ~150 µL. Note that protein will be in the retentate (the liquid left behind in the filter unit) and not in the filtrate (the liquid that flows through to the bottom tube). Multiple aliquots of filtrate from the same fraction can be spun down in the same tube to produce more concentrated protein solutions. At this point it may be worthwhile to perform a BCA assay to determine fraction of protein that was recovered. PEPPI fractions can be frozen at -80 °C for later processing.

17 Carry out methanol-chloroform-water precipitation to remove SDS and prepare samples for analysis.