2-D Analysis of Leaf Protein Samples Treated with ProteoMiner™ Beads Under Denaturing and Nondenaturing Conditions

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Introduction

One of the fundamental challenges in proteome analysis is the fact that samples are often dominated by a relatively small number of high-abundance proteins whose presence can obscure other less abundant proteins and limit the capacity and resolution of the separation technique(s) employed. This is very apparent in serum and plasma, where some 20 proteins constitute more than 98% of the protein mass. It also applies to the proteome of leaves and other photosynthetic tissues, where over 50% of the protein typically consists of the single enzyme ribulose bisphosphate carboxylase (RuBisCo) (Figure 1).

The removal of highly abundant proteins is addressed by two fundamentally different approaches. First is immunodepletion, which is used to remove species-specific proteins in the case of serum or plasma. Alternatively, one can use a library of combinatorial hexapeptides to bind all possible proteins in a complex mixture. In this approach, which is independent of the sample source, the concentration of high-abundant proteins is reduced and medium- and low-abundance proteins are enriched. ProteoMiner technology is an effective tool for enriching low-abundance proteins realative to high-abundance proteins. It has been developed for biomarker discovery with serum samples, but has the potential to be applicable to a wide range of sample types. ProteoMiner technology is commercially available as ProteoMiner protein enrichment kits from Bio-Rad Laboratories, Inc. (Figure 2).

This study was initiated in order to examine the applicability of ProteoMiner protein enrichment kits for the reduction of RuBisCo and subsequent enrichment of medium- and low-abundance proteins from leaf-derived protein samples. Effective application of ProteoMiner technology requires high concentration of sample proteins and this presents a challenge for plant-derived samples since 1) plant extracts typically have low protein concentration and 2) much of the protein in plants is not soluble in the absence of chaotropes or detergents. We have developed effective methods for enrichment of spinach leaf samples prepared both under native and denaturing conditions that circumvent these constraints, using ProteoMiner beads.

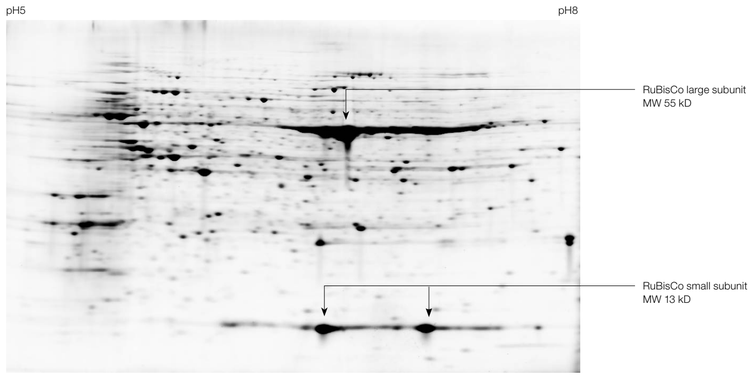

Fig. 1. RuBisCo protein after 2-D separation. The protein has a large subunit (MW 55 kD) and two small subunits (MW 13 kD).

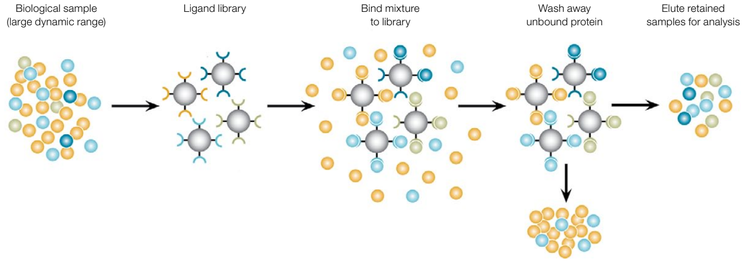

Fig. 2. Illustration of the mechanism of sample binding to ProteoMiner beads.

ProteoMiner technology is based on a combinatorial library of hexapeptides bound to a chromatographic support. Each bead functions as a specific ligand. A complex sample is incubated with the ProteoMiner beads and proteins bind to the ligands to which they have the highest affinity. Unbound proteins consist primarily of high-abundance proteins and are removed during a wash step. Bound proteins are then eluted for analysis.

Nondenatured Protein Sample Workflow

Grind and extract tissue with buffer plus inhibitors

Grind 40 g spinach in blender with 160 ml extraction buffer

Extraction buffer:
- 50 mM Tris-HCl, pH 8.0
- 50 mM NaCl
- 1 mM EDTA (protease inhibitor)
- 1 mM PMSF (protease inhibitor)
- 10 µM leupeptin (protease inhibitor)
- 10 µM E64 (protease inhibitor)
- 10 µM bestatin (protease inhibitor)

Add 140 µl 2-mercaptoethanol and 7.5 g PVPP
Filter through cheesecloth
Centrifuge 10 min 34,000 × g
Reserve supernatant

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Precipitate protein with ammonium sulfate

Add ammonium sulfate to 95% saturation and stir 1 hr at 4°C

Centrifuge 10 min 34,000 × g. Discard supernatant

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Resuspend in small volume

Resuspend protein in 16 ml PBS plus protease inhibitors

Centrifuge 10 min 34,000 × g. Reserve supernatant
Typical sample concentration: 25 mg/ml protein

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Incubate with ProteoMiner beads

Incubate 25 mg spinach leaf sample for 2 hr. Wash with PBS.
Elute 3 x 100 µl acidic urea CHAPS buffer

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Separate using 2-D gel electrophoresis

50 µg sample. First-dimension separation using 11 cm ReadyStrip™ IPG Strips pH 5 – 8, second-dimension separation using Criterion™ Tris-HCl 8 – 16% linear gradient gel

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Staining and imaging analysis

Stain with Flamingo™ fluorescent gel stain, image on a Molecular Imager® PharosFX™ imaging system, image analysis with PDQuest™ software

Fig. 3. Workflow followed for the soluble protein extracts.

Nondenatured Protein Extract

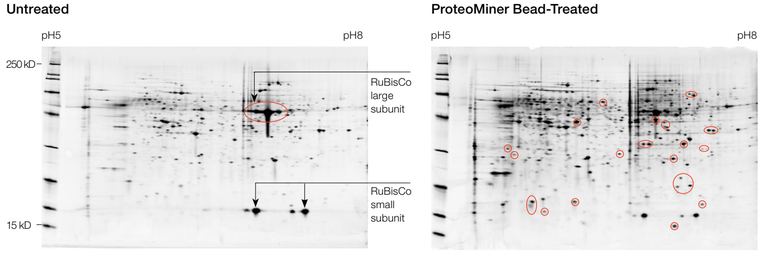

Fig. 4. 2-D separation of soluble protein extract. Samples were loaded on 11 cm pH 5–8 IPG strips for first dimension separation and to a 8–16% Criterion Tris-HCl gel for SDS-PAGE second dimension separation. ○ show proteins that have been enriched in the ProteoMiner bead-treated sample.

Denatured Protein Extract Workflow

Grind and extract tissue with hot 2% SDS

Grind 10 g spinach with 30 ml extraction buffer and heat at 100°C

Extraction buffer: 40% sucrose, 2.7% SDS, 133 mM Tris-HCl, pH 8.0, 6.7% 2-mercaptoethanol

↓

Extract proteins into phenol

Add equal volume of buffer-saturated phenol and mix

Centrifuge 10 min 10,000 × g. Reserve upper (phenol) phase. Repeat extraction and pool phenol phases

↓

Precipitate proteins with salt and methanol

Mix with 6.25 volume of 0.1 M ammonium acetate in methanol

Incubate 1 hr at –20 °C. Centrifuge 10 min 34,000 × g.
Discard supernatant. Typical protein yield: 70 mg protein

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Resuspend denatured protein in HFIP. Dilute into 40% TFE, 150 mM NaCl, 10 mM $NaHCO_3$, pH 9.5.
Remove insoluble protein by centrifugation

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Incubate with ProteoMiner beads

Incubate 50 ml (1.4 mg/ml spinach leaf sample) for 16 hr. Decant liquid, place slurry in a Micro Bio Spin™ column. Wash with PBS. Elute 3 x 100 µl with acidic urea CHAPS buffer

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Separate using 2-D gel electrophoresis

Small format: 50 µg sample load. First-dimesion separation using 11 cm IPG strips pH 5 – 8. Second-dimension separation using Criterion 8 – 16% gels. Large format: 325 µg sample load. First-dimension separation using 24 cm IPG strips pH 5 – 8. Second-dimension separation using hand-cast 12.5% gel

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Staining imaging analysis

Same as nondenaturing workflow

Fig. 5. Workflow followed for denatured proteins.

Denatured Protein Extract

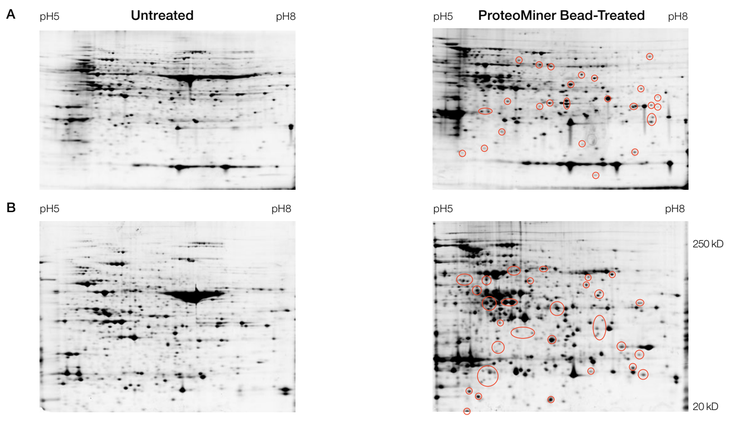

Fig. 6. 2-D separation of denatured protein extract. Panel A shows first-dimension separation using small format, 11 cm pH 5–8 IPG strips, followed by second-dimension SDS-PAGE separation using 8–16% Criterion Tris-HCl linear gradient gel. Panel B shows first-dimension separation using large format, 24 cm pH 5–8 IPG strips, followed by second-dimension SDS-PAGE separation using 12.5% hand-cast gels. Large format gels were used for better resolution, higher sample load and larger spots for protein mass spectroscopy identification. ○ show proteins that have been enriched in the ProteoMiner bead-treated sample.

Conclusions

- Treatment with ProteoMiner beads can be successfully applied to spinach protein that has been extracted under denaturing and nondenaturing conditions. ProteoMiner bead treatment enriches a unique population of proteins not apparent in the untreated sample
- Fluoroalcohols like hexafluoroisopropanol (HFIP) and trifluoroethanol (TFE) can solubilize denatured protein without interfering with the ionic and hydrophobic interaction between ProteoMiner bead ligands and proteins
- RuBisCo large subunit is significantly depleted in both denaturing and nondenaturing conditions

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