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Cross species applicability of abundant protein depletion columns for ribulose-1,5-bisphosphate carboxylase/oxygenase

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Abstract

In plants, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) is an important enzyme in the Calvin cycle, catalyzing the first step of carbon fixation. Because of its critical role in photosynthesis, RuBisCO comprises 30–60% of the total protein content in green leaf tissue and represents a major protein which can interfere with determination of lower abundance proteins in plant proteomics. A potential solution to aid in the determination of low level proteins in plant proteomics are RuBisCO immunodepletion columns. Two formats, spin and LC, of Seppro™ IgY RuBisCO depletion columns were evaluated for cross species applicability. The spin and LC columns were found to deplete arabidopsis RuBisCO by greater than 90 and 98%, respectively, and automation could be achieved with the LC format. Canola RuBisCO was depleted to a similar extent, and there was evidence suggesting that corn and tobacco RuBisCO were also highly depleted in flow through fractions. Model proteins were spiked into samples to provide insight into the degree of non-specific binding. Finally, improved detection and identification of lower abundance proteins was demonstrated after depletion.
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Keywords: RuBisCO; Ribulose-1,5-bisphosphate carboxylase/oxygenase; Protein; Immunodepletion; Arabidopsis; Tobacco; Corn; Canola; Proteomics; Peptide mass fingerprint; Abundant protein depletion; Depletion

1. Introduction

In proteomic analysis, the large concentration distribution of proteins poses a significant analytical challenge. Proteins exist in cells at mg/mL concentrations down to pg/mL levels, which exceed the dynamic range of most analytical techniques [1,2]. Further exacerbating the problem, many of the high abundance proteins (HAPs) are ubiquitous across tissues and fluids, span a large range of size and isoelectric point (pI), and represent a majority of the total protein content by mass [1–4]. For example, albumin, IgG, IgM, IgA, transferrin, α1-antitrypsin, α1-acid glycoprotein, fibrinogen, haptoglobin, apolipoprotein A-I, apolipoprotein A-II, and α2-macroglobulin make up more than 96% of total protein by mass in mammalian plasma; but they represent less than 0.1% of the total number of proteins [1,3,5]. Although HAPs can offer diagnostic value, low abundance proteins (LAPs) are often more important markers of disease, so their identification can be critical [6]. Often, information about these LAP targets is lost when the analytical technique is overwhelmed by more abundant species. Various methods have been developed to reduce or eliminate these HAPs to enhance detection of low abundant targets. Methods of fractionation such as precipitation, ultracentrifugation, molecular weight separation, pI separation, and affinity chromatography are heavily utilized today; however, they do not always provide specific depletion of the HAPs resulting in loss of lower abundance species [1–4,7].

For more selective depletion, immunoaffinity techniques were developed to selectively remove HAPs from samples prior to analysis [8]. Initially, IgG antibodies were raised against the one or two most abundant HAPs; but recently, IgY technology has been harnessed to create commercial columns capable

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of simultaneous depletion of 12 or more HAPs from raw sera, and 87 medium abundance proteins (MAPs) in a second step [5,8]. The greater specificity offered by immunoaffinity interactions enables HAP depletion with less non-specific loss of lower abundance species. In addition, species selective immunodepletion columns are available for rat, mouse, dog, and human sera [8,9]. This technology has been applied to improve detection of LAPs for a variety of applications, including drug toxicity determination, cancer screening, and investigation of inflammatory response [6,10,11].

In plant proteomics, ribulose-1,5-bisphosphate carboxylase /oxygenase (RuBisCO) represents a major HAP. It comprises 30–60% of the total protein content in green leaf tissue depending on species, and is an important enzyme in the Calvin cycle catalyzing the first step of carbon fixation [7,12–15]. Its active form is an ∼540 kDa hexadecamer comprised of four heavy chain dimers (∼54 kDa ea.) and eight light chains (∼14 kDa ea.) [13]. RuBisCO is expressed predominantly in chloroplasts of green plants, but forms of RuBisCO exist in non-green algae, cyanobacteria, some dinoflagellates and purple non-sulfur bacteria [16,17]. Because of its necessity to photosynthesis, RuBisCO has been estimated to account for approximately 16% of the total protein on earth [14]. RuBisCO is present at high concentrations in green leaf tissue because of its slow reaction kinetics and represents a major HAP that can interfere with MAP and LAP determination in plant proteomics. For instance, roughly 12.5% of the spots on a two-dimensional SDS-PAGE gel of arabidopsis leaf extract have been identified as RuBisCO or its variants [15]. Various fractionation methods have been developed specifically for RuBisCO depletion; however, the methods all suffer from limited selectivity as described above [7].

Recently, commercial immunodepletion technologies emerged for depletion of RuBisCO. GenWay Biotech, Inc. raised polyclonal IgY antibodies against spinach RuBisCO. The antibodies are bound to copolymeric beads with a covalent linker and sold in spin column or packed liquid chromatography (LC) column formats. The Genway columns are rated to bind up to 0.8 mg RuBisCO/mL of bead slurry with specific depletion of greater than 90% of both the small and large chains of RuBisCO [18]. Because RuBisCO structure is highly conserved across many plant species [17], the antibodies should be capable of binding RuBisCO of other species. Cross species applicability has been shown for polyclonal albumin antibodies, but not for RuBisCO [9].

Cross species applicability was evaluated for both depletion formats in this study. Binding of the small and large chains of RuBisCO was determined for arabidopsis and canola, and evidence was found for depletion of corn and tobacco RuBisCO. Additionally, column capacity was determined on both formats in order to estimate binding efficiency towards other species of RuBisCO. Non-specific binding of three model proteins was also measured to determine the specificity of the columns, and a few lower abundance proteins were identified by peptide mass fingerprint (PMF) after RuBisCO depletion. Finally, advantages and disadvantages of the LC and spin columns are compared to facilitate format selection.

2. Experimental

Purified RuBisCO standards and leaf tissue of arabidopsis, canola, corn, and tobacco were obtained from Dow AgroSciences (Indianapolis, IN). RNase A, myoglobin, and ovalbumin from Sigma–Aldrich (St. Louis, MO) were selected as model proteins. Stock solutions were prepared in nuclease-free water, and all dilutions were prepared in dilution buffer (10 mM Tris, 150 mM NaCl, pH 7.4, supplied in a 10× concentrate in GenWay's spin column kit). Stock solutions were stored in aliquots at −20 °C until use, and fresh dilutions were prepared daily. All chemicals, unless otherwise stated, were purchased from Sigma–Aldrich (St. Louis, MO).

2.1. Preparation of RuBisCO standard

RuBisCO standard was extracted and purified from canola leaf. 10 g of leaf tissue was grounded with liquid nitrogen using mortar and pestle to very fine powder, 50 mL of extraction buffer (100 mM Tris, pH 8.0, 100 mM NaCl, 5 mM DTT, 0.1% triton X-100, 0.5 mL protease inhibitor cocktail (Sigma), and 10 μL of Benzonase (Sigma)) was added and mixed well. The mixture was kept on ice for 60 min and then centrifuged at 18,000 revolutions per minute (rpm) for 30 min, at 4 °C. The supernatant was fractionated by gel filtration, ion exchange, and hydrophobic interaction chromatography (HIC). In the first step, supernatant was loaded onto a Superdex 200 column (Amersham Pharmacia Biotech) at flow rate 1.0 mL/min and eluted with 50 mM Tris, pH 8.0, 50 mL NaCl, 2 mM EDTA, 2 mM $MgCl_2$ buffer. Fractions containing RuBisCO (as confirmed by 1D-PAGE) were combined and then further fractionated by ion exchange chromatography using a Mono Q column (Amersham Pharmacia Biotech) with 50 mM Tris, 50 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$ pH 8.0 buffer. Proteins were eluted with a 0–100% gradient of 50 mM Tris, 900 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$ at pH 8.0. The resulting fractions were analyzed by 1D-PAGE, and those containing RuBisCO were combined and then separated on a HIC 10-10 column (Amersham Pharmacia Biotech). 25 mM Tris, pH 8.0, 50 mM NaCl, 2 mM EDTA, 2 mM $MgCl_2$ buffer was employed for separation with a gradient of 1–0 M ammonium sulfate. The final product was verified by PMF. The method employed is described in greater detail below.

2.2. Tissue homogenization and extraction

Leaf tissue was prepared for analysis by freeze drying in 50 mL centrifuge tubes. After drying, two 5 mm diameter tungsten ball bearings were added to each tube, and the tubes were capped. The leaf tissue was crushed and homogenized by shaking the tube until a fine powder was achieved (∼2 min). Leaf tissue homogenates were stored at −20 °C until use.

For the leaf samples, 20 mg of powdered leaf tissue was combined with 1000 μL of dilution buffer and spiked with 6 μL of plant protease inhibitor cocktail (P9599) from Sigma–Aldrich. For corn, 30 mg of tissue was used because of its lower RuBisCO content [19]. The samples were vortexed for 2 min, and then the

samples were centrifuged at 2000 × *g* for 15 min. The resulting supernatant was filtered through a 0.45 μm spin filter. 100 μL of the supernatant was retained for use as a raw sample, and the rest was used for depletion. A Bradford assay was employed to measure total protein in the raw leaf extracts.

2.3. RuBisCO depletion

2.3.1. Spin format

A Seppro™ RuBisCO IgY Spin Column Kit (cat# 28-288-23153-SC) from GenWay Biotech (San Diego, CA) was evaluated. The instructions supplied with the kit were followed for depletion. Briefly, 800 μL of GenWay bead suspension was transferred to a new spin column. The column was capped at the top and the plastic tip was snapped off of the bottom before centrifugation. The column was centrifuged at 2000 rpm for 30 s to remove suspension buffer, and then a cap for the tip of the column was attached to seal it. The beads were resuspended in 500 μL of sample (leaf extract or protein standard) and incubated at room temperature for 15 min with agitation. After 15 min, the cap on the column's tip was removed, and the unbound fraction was collected by centrifugation at 2000 rpm for 30 s. Four wash steps using 500 μL of dilution buffer each were performed to elute remaining unbound proteins. The column was agitated for 1 min with dilution buffer and then centrifuged for each wash step. The wash and unbound fractions were pooled unless otherwise stated. Bound RuBisCO was then eluted off the column with five, 500 μL volumes of stripping buffer (250 mM glycine, pH 2.5, supplied by GenWay as a 1 M glycine concentrate with the spin column kit). The column was agitated for 3 min and then centrifuged for each strip. The stripped fractions were pooled prior to concentration and neutralized with 500 μL of neutralization buffer (250 mM Tris–HCl, pH 8.0, supplied with the kit as a concentrate (1 M Tris–HCl)). The column was then immediately neutralized with 600 μL neutralization buffer under agitation for 5 min. Neutralization buffer was removed by centrifugation, and the beads were resuspended in dilution buffer.

2.3.2. LC format

A Seppro™ IgY-RuBisCO LC-2 Column (cat# 28-288-23153-LC2) from GenWay Biotech was chosen for evaluation. Instructions supplied with the LC column kit were followed; however, the method is outlined below for completeness. The RuBisCO column was connected to an Agilent 1100 HPLC system (Santa Clara, CA) using two 1/4-20 flangeless to 10–32 adapters from Upchurch Scientific (Oak Harbor, WA). A KrudKatcher™ inline filter from Phenomenex (Torrance, CA) was employed to prevent clogging of the column. The Agilent system was equipped with an autosampler, microdegasser, quaternary pump, thermostatted column compartment, and variable wavelength UV detector (VWD). Column temperature was not controlled, and detection was performed by UV absorbance at 220 nm. Although up to 200 μL of sample can be loaded onto the RuBisCO LC-2 column, 100 μL injections were performed because of instrumental constraints. Three mobile phases were necessary for operation: dilution buffer (10 mM Tris, 150 mM NaCl, pH 7.4), stripping buffer (100 mM glycine, pH 2.5), and neutralization buffer (100 mM Tris–HCl, pH 8.0). The buffers were all prepared from 10× concentrates supplied with the kit. It is important to note that the concentrations of stripping and neutralization buffer are different for the spin and LC column formats. Table 1 summarizes the HPLC pump program for depletion.

Fractions of unbound protein were collected with a Gilson FC-203B (Middleton, WI) fraction collector. Fractions were collected based on an experimentally determined time window of 11.5–18.5 min. Greater than 97% of the flow through peak could be collected in that time while keeping the fraction volume to 2.6 mL. The time delay between the detector and fraction collector was neglected because the dead volume was sufficiently low at the flow rates employed to not be a significant contributor to sample loss.

Depletion chromatograms were also used to determine RuBisCO capacity of the LC column by measuring the stripped peak area as a function of RuBisCO load.

Table 1
The HPLC pump program for RuBisCO depletion

Time (min)	Dilution buffer (%)	Stripping Buffer (%)	Neutralization buffer (%)	Flow rate (mL/min)
0.00	100	0	0	0.1
10.00	100	0	0	0.1
10.01	100	0	0	0.2
17.00	100	0	0	0.2
17.01	100	0	0	1.0
22.00	100	0	0	1.0
22.01	0	100	0	1.0
36.00	0	100	0	1.0
36.01	0	0	100	1.0
42.00	0	0	100	1.0
42.01	100	0	0	1.0
47.99	100	0	0	1.0
48.00	100	0	0	0.1

Dilution buffer is 10 mM Tris and 150 mM NaCl at pH 7.4, stripping buffer is comprised of 100 mM glycine at pH 2.5, and neutralization buffer is 100 mM Tris–HCl at pH 8.0. It is important to note that the flow rates are low initially to provide time for protein binding but are ramped up to improve peak shape of the eluting protein bands.

2.4. Fraction concentration

Fractions were concentrated prior to analysis to improve detection of low abundance species. For the spin and LC formats, fraction volumes were 2.5 and 2.6 mL, respectively. They were reduced to 25–50 μL with a Centricon, YM-3 centrifugal filter device from Millipore (Billerica, MA). The ultrafiltration device had a 3 kDa molecular weight cut-off membrane, and could hold up to 3.0 mL of solution. Fractions were loaded into each device and centrifuged at 4000 × *g* for 200 min at 4 °C. Because the concentrate volume varied from device to device, a 100 μL Rainin Electronic Delivery Pipette (Woburn, MA) was employed to measure the retained volume. Dilution buffer was then added to bring the final volume to 100 μL. Samples not analyzed immediately were frozen at −20 °C.

2.5. Sample analysis

2.5.1. Agilent Bioanalyzer

The Agilent Protein 80 kit (part # 5067-1515) was employed for protein separation, detection, and quantification. Kit instructions were followed for sample and chip preparation. Briefly, 4 μL of sample concentrate was combined with 2 μL of Protein 80 Sample Buffer. Denaturing conditions were employed, so the sample buffer contained 3.5% β-mercaptoethanol. The resulting mixture was spun at 2000 rpm for 45 s and then heated at 90 °C for 5 min. Condensate was then spun down at 10,000 rpm for 10 s. 84 μL of nuclease-free water was then added to the samples, and they were vortexed for 5 s. Protein ladder supplied with the kit was prepared in the same fashion.

A new chip was employed for each analysis. Each chip was primed with Gel-Dye Mix, and the reagent reservoirs were loaded appropriately with either 12 μL of Gel-Dye Mix or Destaining Solution. 6 μL of each sample and the protein ladder were loaded into the sample reservoirs of the chip, and it was placed immediately in the Bioanalyzer. The Sample Buffer, Gel-Dye Mix, and Destaining Solution were all provided with the Protein 80 kit.

The Bioanalyzer software automatically integrates peaks and assigns a protein mass based on the area of the upper marker at 95 kDa. This calculated mass was used for quantitation because it provides automatic normalization to the lower molecular weight marker. Contrast of the gel images was tuned manually to enable better visualization of small features, and raw data were exported to enable plotting of electropherograms in Microsoft Excel. Data from the Bioanalyzer were used to determine binding capacity of the spin columns by comparing breakthrough of RuBisCO into the flow through fraction at different RuBisCO loads. It was also used to determine non-specific binding of the model proteins, carryover of those proteins into subsequent runs, and cross species RuBisCO binding.

2.5.2. HPLC-UV

Raw and depleted samples were also analyzed with an Agilent 1100 HPLC system. 100 μL polypropylene autosampler vial inserts were used to minimize protein absorption. Column temperature was controlled at 50 °C on a Waters (Milford, MA) XBridge™ BEH300, C18, 2.1 mm × 100 mm, 3.5 μm column, and a flow rate of 0.3 mL/min was used for separation. 10 μL injections were used, and absorbance was monitored at 280 and 220 nm. Mobile phase A was comprised of 97/3 H_2O/ACN with 0.06% TFA, and mobile phase B contained 20/80 H_2O/ACN with 0.05%TFA. Mobile phase B was ramped from 10 to 45% over the first 5 min of the separation and then brought to 90% over the next 15 min. To facilitate full sample elution, mobile phase B was ramped to 100% over 2 min and held for another 2 min at 100%. Finally, mobile phase B was returned to 10% over the next minute, and 5 min was provided for re-equilibration. Chromatograms were integrated with Chemstation software (revision A.10.02), and the data were used to determine cross species RuBisCO binding, model protein quantitation, and to confirm results from the Bioanalyzer.

2.5.3. Peptide mass fingerprint

Raw leaf extract and flow through fractions of spinach were separated by 1D-PAGE on a Criterion 10.5–14% Tris–HCl gels from BioRad (Hercules, CA). Large chain RuBisCO from raw spinach extract and three bands from the spinach flow through fraction (at ∼22, 45, and 75 kDa) were excised and incubated with trypsin at 37 °C overnight. A Waters Acquity UPLC system equipped with a TUV detector was interfaced with a Waters QTOFmicro mass spectrometer. A BEH C18 2.1 mm × 100 mm, 1.7 mm column (Waters, Milford, MA) was used. Column temperature was maintained at 50 °C. Auto sampler was set to 7 °C. Mobile phase A (MPA) was 0.1% formic acid in deionized water. Mobile phase B (MPB) was 0.1% formic acid in acetonitrile. The flow rate was 100 μL/min. Injection volume was 10 μL. UV signal was detected at 214 nm (sampling rate 40 points/s). The samples (in-gel tryptic digests) were loaded directly on to the analytical column. The column was held at 5% MPB for 5 min prior to the gradient (5–40% MPB over 30.4 min). The column was then washed at 90% MPB and then re-equilibrated to initial conditions (5% MPB).

The QTOFmicro mass spectrometer was operated in the positive ionization mode. Data acquisition was performed with a cycle time of 1 scan/s (scan acquisition time: 0.88 s; interscan delay: 0.1 s) in the MS mode. The lock mass channel was sampled every 7 s during MS analysis. The reference ion used was the singly charged Leucine-Enkephalin ion at *m/z* 556.2771. The following mass spectrometer settings were used. Capillary: 2800 V, sample cone: 15 V, extraction cone: 0.9 V, desolvation temperature: 300 °C, source temperature: 90 °C, desolvation gas: 700 L/h, cone gas: 10 L/h, MCP: 2350 V.

All LC–MS data were processed manually. The spectrum of each chromatographic peak was summed, smoothed (SG, 2 × 3 channels), centroided (4 channels, top 80%, by area) and *m/z* error corrected (lock mass channel: 10 scans, *m/z* 556.2271 ± 0.5 Da). All multiply charged ions were then reduced to their singly charged state prior to subjecting the data to peptide mass fingerprinting. The peptide mass data were searched using the program MASCOT against a non-redundant database (NCBInr) or Swiss-Prot database located at the Matrix Sciences web site (Internet address:

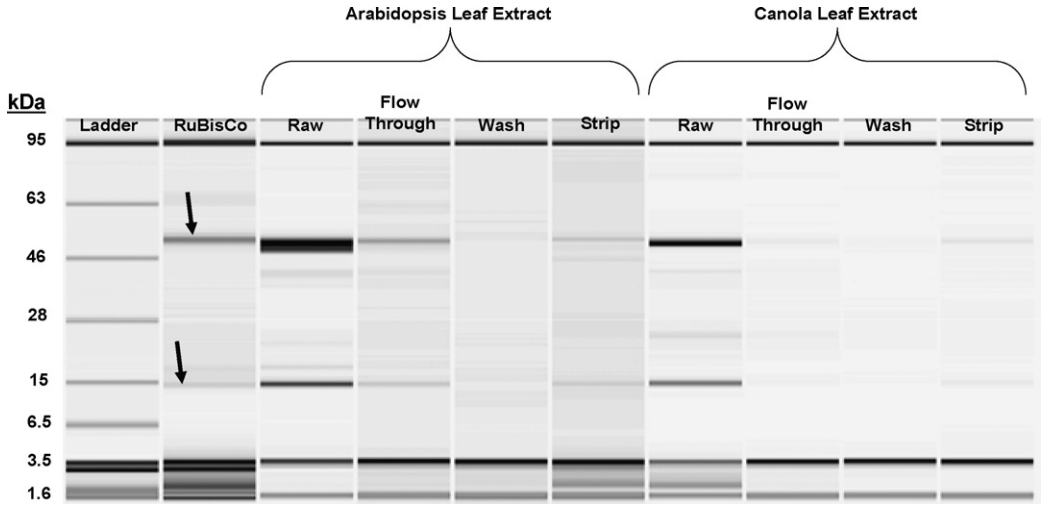

Fig. 1. A gel image showing RuBisCO content of arabidopsis and canola leaf extracts. RuBisCO large and small chain fragments are marked by arrows in the second lane. Greater than 90% of the RuBisCO is removed from the sample in the flow through and wash fractions by the immunoaffinity column. Bound RuBisCO is not fully recovered in the stripped fraction because of sensitivity of the protein to hydrolysis at low pH leads to rapid degradation.

http://www.matrixscience.com). [20] Typical search parameters were as follows: consideration of up to four incomplete cleavage sites per peptide, fixed modification carboxyamidomethylcysteine (CAM), peptide mass tolerance 20 ppm, tryptic peptides and singly charged monoisotopic peaks.

3. Results and discussion

Recently, GenWay Biotech offers a number of Seppro™ RuBisCO depletion products including spin columns, Seppro™ tips, and 2 and 10 mL LC columns. The spin column and LC-2 columns were evaluated in this study because they offer low to mid capacity and throughput, which is more suitable for exploratory research. Although the IgY antibodies were raised against spinach RuBisCO, this HAP depletion technology will be of greater utility if it can be leveraged across many plant species. Because the antibodies are polyclonal and the RuBisCO sequence is highly conserved across species [16,17], binding efficiency of RuBisCO from other species should remain high. In this study, spinach, canola, corn, arabidopsis, and tobacco leaf extracts were depleted and binding capacity was compared using the LC format. In addition, utility of this depletion technology was demonstrated through improved detection of lower abundance species and by enabling identification of proteins eclipsed by RuBisCO.

3.1. Spin column format

For a qualitative evaluation of their utility, spin columns were used to deplete arabidopsis and canola leaf extract with a total protein concentration of 0.6 and 1.0 mg/mL, respectively. With a molecular weight separation on the Bioanalyzer, the RuBisCO small and large chains were readily detected at 14 and 51 kDa in the raw, flow through, and wash fractions (Fig. 1). The flow through and wash fractions were analyzed separately in this case, but they were typically pooled prior to analysis. At this protein load, greater than 90% of the arabidopsis and canola RuBisCO were depleted when comparing the peak area of the raw fraction to the summed peak area of the flow through and wash fractions. Bound RuBisCO should have eluted in the stripped fraction; however, there is little to no RuBisCO detected in the stripped fractions (Fig. 1). Although this could be an indication of incomplete stripping of the bound proteins from the beads, RuBisCO is unstable at low pH, so it likely degraded before analysis [15]. In support of the latter, no loss in binding capacity was observed after more than 30 depletions.

For a more quantitative measurement of cross species binding, the RuBisCO capacity was determined using RuBisCO standards purified from canola. Identity of the protein standard was confirmed by PMF (Table 2). The columns have a capacity rating of 0.8 mg RuBisCO/mL of bead suspension, so a column was challenged with 0.4, 0.6, 0.8, 1.0, and 1.2 mg of RuBisCO standard purified from canola. The columns achieved greater than 90% binding of both small and large chain RuBisCO up

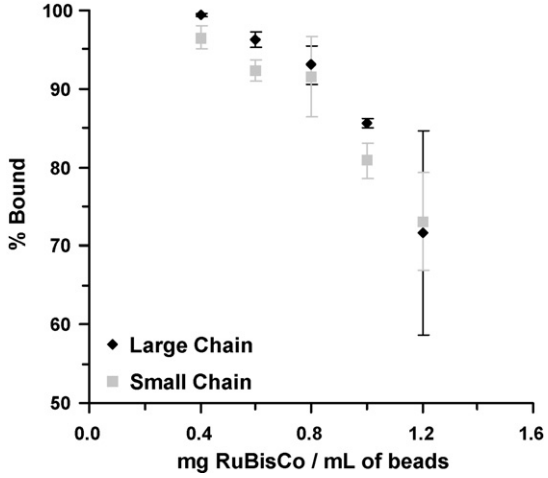

Fig. 2. The spin columns are rated to bind up to 0.8 mg RuBisCO/mL of immunoaffinity beads. At 0.8 mg, RuBisCO binding capacity is greater than 90%, with a loss in capacity seen above 0.8 mg. Error bars represent the standard deviation with $n = 3$.

Table 2
PMF of RuBisCO standard purified from canola

Fragment	Residue #	Sequence	Theoretical *m/z*	Experimental *m/z*
T2	9–14	ASVGFK	608.340	608.339
T3–4	15–21	AGVKEYK	794.441	794.452
T3–6	15–41	AGVKEYKLNYYTPEYETKDTDILAAFR	3198.599	3198.621
T4–6	19–41	EYKLNYYTPEYETKDTDILAAFR	2843.377	2843.421
T5	22–32	LNYYTPEYETK	1420.663	1420.678
T5–6	22–41	LNYYTPEYETKDTDILAAFR	2423.177	2423.202
T6	33–41	DTDILAAFR	1021.531	1021.542
T7	42–79	VTPQPGVPPEEAGAAVAAESSTGTWTTVWTDGLTSLDR	3854.872	3854.946
T7–8	42–81	VTPQPGVPPEEAGAAVAAESSTGTWTTVWTDGLTSLDRYK	4146.030	4146.085
T7–9	42–83	VTPQPGVPPEEAGAAVAAESSTGTWTTVWTDGLTSLDRYKGR	4359.153	4359.189
T11–12	129–139	ALAALRLEDLR	1240.737	1240.748
T11–13	129–146	ALAALRLEDLRIPPAYTK	2011.170	2011.200
T13	140–146	IPPAYTK	789.451	789.462
T14	147–159	TFQGPPHGIQVER	1465.755	1465.776
T14–16	147–164	TFQGPPHGIQVERDKLNK	2064.098	2064.121
T21–22	195–213	GGLDFTKDDENVNSQPFMR	2169.987	2169.978
T26	228–236	SQAETGEIK	962.479	962.492
T37–41	320–358	LSGGDHVHAGTVVGKLEGDRESTLGFVDLLRDDYVEKDR	4255.138	4255.159
T38–41	335–358	LEG DRESTLG FVDLLRDDYVEKDR	2840.406	2840.429
T45–46	432–446	NEGRDLAVEGNEIIR	1684.861	1684.884
T46	436–446	DLAVEGNEIIR	1228.653	1228.672

The protein was identified as ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*Brassica napus*) gi|157326087. 21 peptides were matched for 41% sequence coverage, and the reported mass (51.2 kDa) is consistent with the 1D-PAGE data.

to the capacity rating with increased breakthrough seen above 0.8 mg (Fig. 2).

With immunoaffinity, non-specific protein binding can limit quantitation and recovery of low abundance species. Because the IgY antibodies were raised in chicken, non-specific binding should be less problematic [8,9]; however, protein absorption can still occur on the bead, frit, and column surface. RNase A, myoglobin, and ovalbumin were used as targets to measure non-specific protein loss. RNase A and myoglobin were chosen because they are not of plant origin, and ovalbumin was chosen because the antibodies are avian. In theory, the former should have little non-specific interaction with the depletion resin, while the latter should show some interaction with the avian IgY antibodies. RNase A and myoglobin were recovered in the flow through fraction at 86 ± 10 and $89 \pm 6\%$ ($n = 3$). Neither protein was found in the stripped fractions suggesting that incidental loss was due to a mechanism other than non-specific IgY binding. Ovalbumin recovery was lower at 67 ± 8 and $71 \pm 9\%$ for the two isoforms ($n = 3$). Although the exact mechanism leading to lower ovalbumin recovery was not investigated, some loss is not surprising because ovalbumin is an avian protein and likely interacts with IgY [9].

3.2. LC column format

To enable direct comparison to the spin columns, binding capacity, cross species applicability, and non-specific binding were measured in the LC format. Although the same IgY resin and elution buffers are employed in the LC column, differences in performance were expected because more

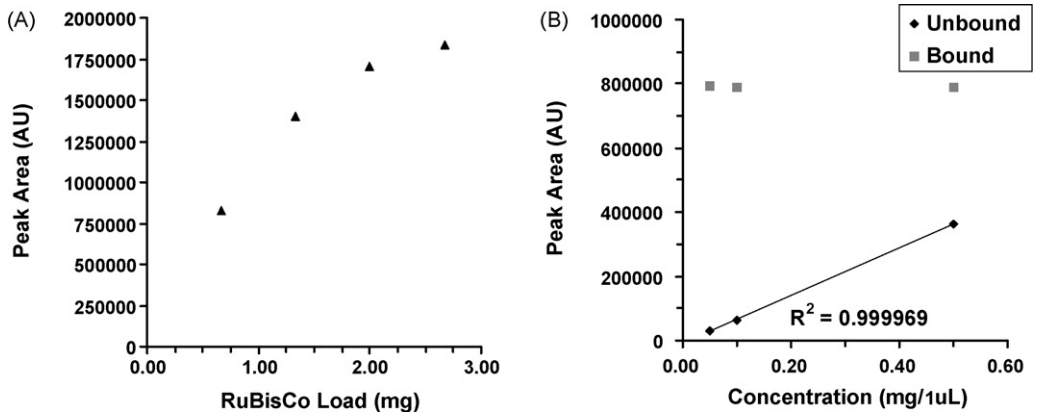

Fig. 3. (A) Area of the stripped peak is plotted as a function of RuBisCO load to demonstrate breakthrough threshold in the LC column. The column binding rating of up to 2.0 mg of RuBisCO is supported by these data because the area does not increase linearly above 2.00 mg/mL. (B) In addition, total protein of the flow through fraction can be quantitated with good linearity.

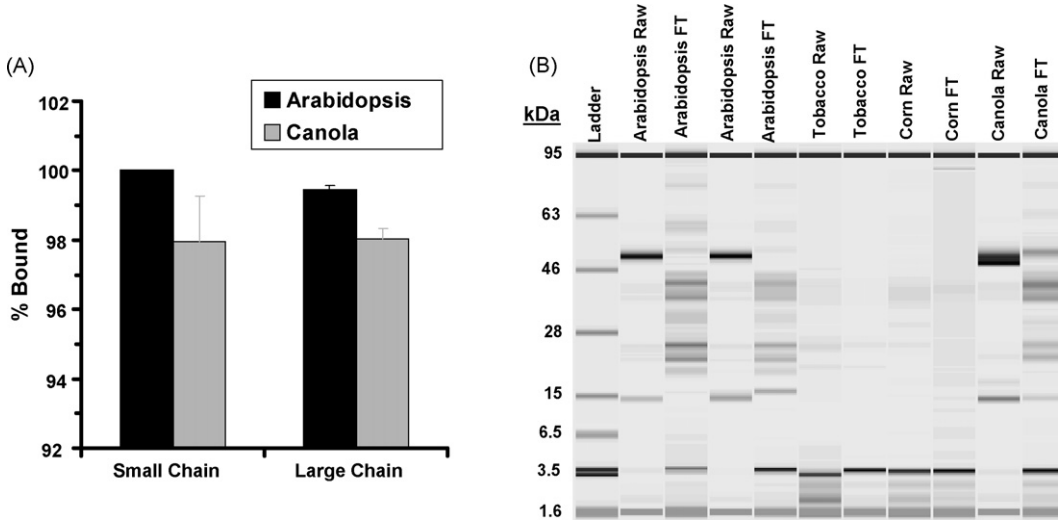

Fig. 4. (A) Arabidopsis and canola RuBisCO bind to the LC column with little breakthrough into the flow through fraction. The arabidopsis small chain fragment appears to be 100% bound because it could not be detected in the flow through fraction. Error bars are plotted as standard deviation with $n = 3$. (B) A gel image showing raw and flow through (FT) fractions of four different plant species is shown. Corn and tobacco RuBisCO was not detected in the raw leaf extracts, so depletion in these species was not measured directly.

beads are present, fractionation is automated, there is little opportunity for bead loss, and sample volume is limited. In addition to the above parameters, target quantitation and run-to-run carryover were also determined in the LC format.

GenWay rates the LC column RuBisCO binding capacity at 2.0 mg. Capacity was measured by monitoring the RuBisCO peak area in the stripped fraction as it eluted from the LC column. Glycine in the stripping buffer leads to a high background in the stripped fraction; however, RuBisCO was quantitated after background subtraction of the signal from a blank run. The area of this peak was plotted as a function of RuBisCO load (Fig. 3A). Above 2 mg, the peak area does not increase linearly with RuBisCO load indicating breakthrough of the HAP. The peak should increase linearly in response to greater protein loads as can be seen with differing concentrations of the three model proteins (Fig. 3B).

Both canola and arabidopsis leaf extracts were tested on the LC format; and in addition, corn and tobacco extracts were depleted as well. Greater than 98% of the large and small chain, canola and arabidopsis RuBisCO was depleted from the leaf extracts with this technology (Fig. 4A). Unfortunately, RuBisCO from corn and tobacco was not detected in the raw leaf extracts, so they cannot be compared directly (Fig. 4B). RuBisCO content is much lower in corn leaf compared to the other three, so the tissue mass used for corn extraction may have been insufficient for adequate detection of RuBisCO [19]. Tobacco should have an abundance of RuBisCO; however, extraction buffer additives were not employed to bind phenolic compounds, which have been shown to cause protein degradation [13]. Nevertheless, HPLC analysis of corn, tobacco, arabidopsis, and canola extracts revealed a peak at 3.5 min in the chromatogram, which was decreased by greater than 97% in all four flow through fractions (Fig. 5A and B). The peak identity was not deter-

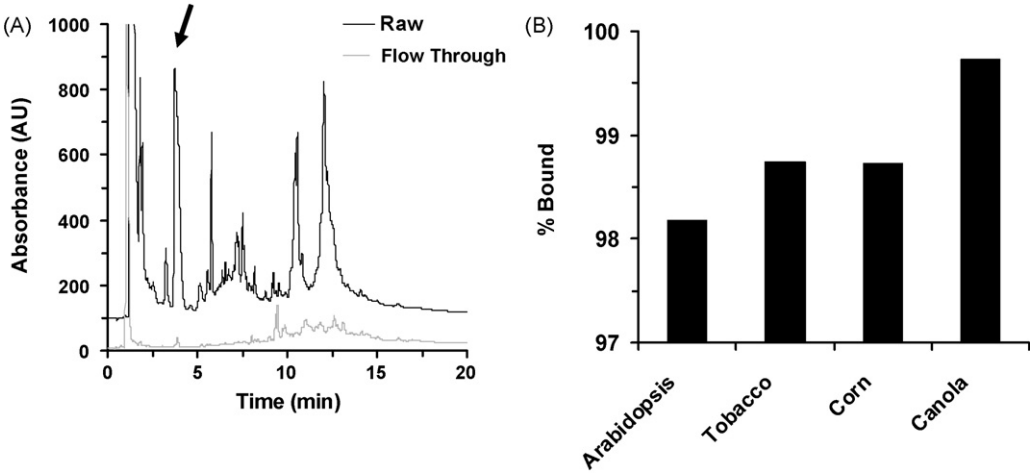

Fig. 5. (A) RuBisCO elutes at 11 and 12.5 min in the HPLC-UV chromatogram above. (B) The peak at 3.5 min is an unknown; however, it is depleted by >98% for all four species tested. This peak may be a RuBisCO degradation product or a member of the RuBisCO interactome.

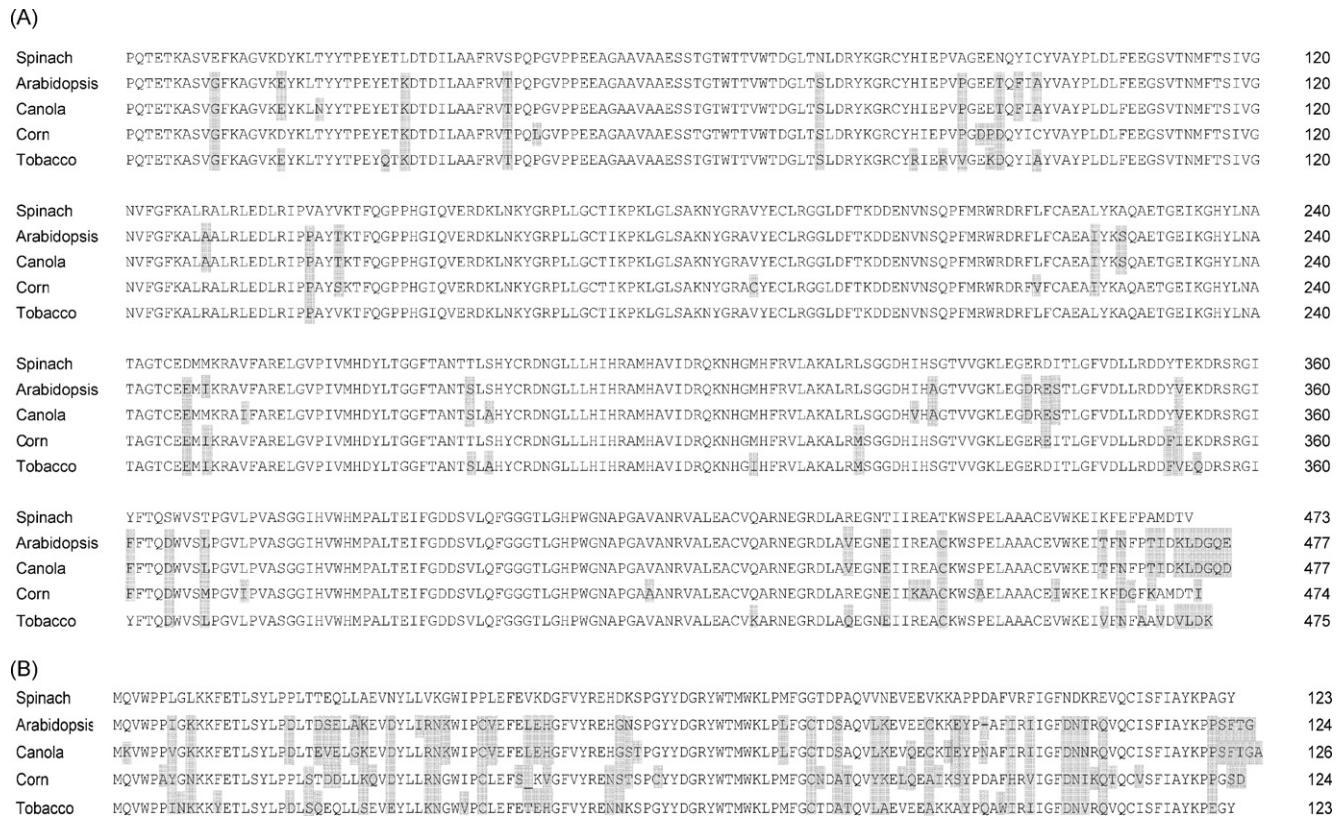

Fig. 6. Sequence alignment for the large and small subunits of RuBisCO is illustrated with differences highlighted with a grey box. Sequence homologies between the large subunit of spinach and those of arabidopsis, canola, corn, and tobacco are 93, 92, 92, and 92%, respectively. Homology between the small subunits is lower at 69, 66, 66, and 72%.

mined, but it is neither small nor large chain RuBisCO based on retention time. Further analysis is required, but the peak could be a RuBisCO degradation product or possibly a member of RuBisCO's interactome. High binding capacity across species is not surprising for the large subunit of RuBisCO as arabidopsis, canola, corn, and tobacco share >90% homology with spinach, and the small subunit shares >65% homology (Fig. 6).

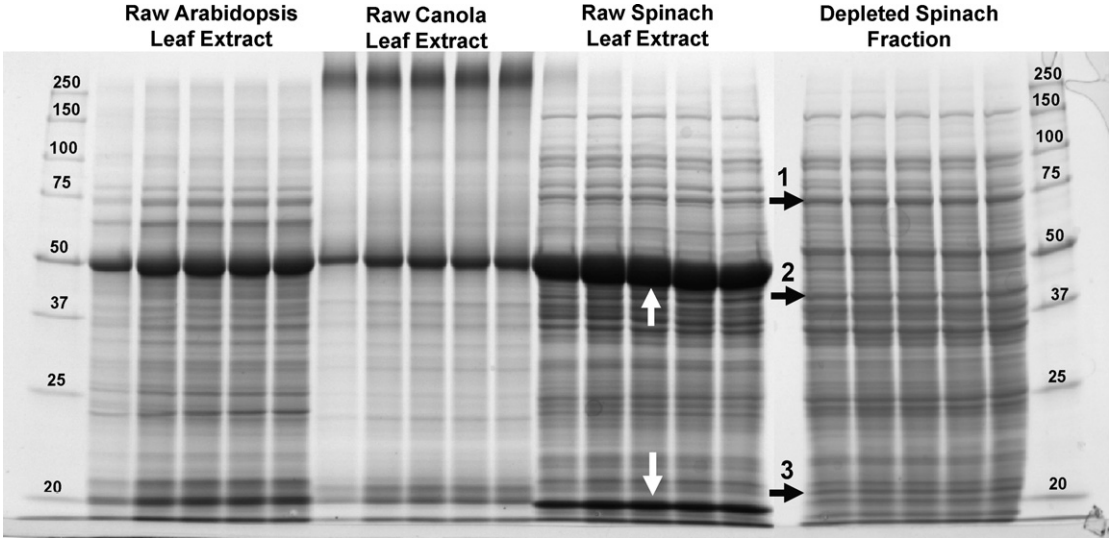

Fig. 7. Images of two, 1D-PAGE separations are overlaid above. The large and small subunits of RuBisCO (white arrows) are the predominant features in the three raw leaf extracts of the first gel. There is little to no RuBisCO in the depleted spinach fractions shown in the partial gel on the right side of the image. The three black arrows were tentatively identified as transketolase (*Spinacia oleracea*) (gi|2529342), phosphoglycerate kinase (*Spinacia oleracea*) (gi|1346698), and oxygen-evolving enhancer protein 3 (*Spinacia oleracea*) (gi|131397) by PMF (arrows 1, 2, and 3, respectively). The reported masses for these proteins (80.7, 45.7, and 24.8 kDa, respectively) are consistent with the MW ladder on the gel.

Table 3
PMF of phosphoglycerate kinase, chloroplast precursor (*Spinacia oleracea*) gi|1346698

Fragment	Residue #	Sequence	Theoretical *m/z*	Experimental *m/z*
T6–7	33–45	SVGDLTSADLKGK	1290.690	1290.712
T6–8	33–46	SVGDLTSADLKGKK	1418.785	1418.802
T8–11	46–70	KVFVRADLNVPLDDSQNITDDTRIR	2900.522	2900.569
T9–10	47–68	VFVRADLNVPLDDSQNITDDTR	2503.242	2503.278
T9–11	47–70	VFVRADLNVPLDDSQNITDDTRIR	2772.427	2772.480
T10	51–68	ADLNVPLDDSQNITDDTR	2001.936	2001.946
T10–11	51–70	ADLNVPLDDSQNITDDTRIR	2271.121	2271.153
T12	71–77	AAIPTIK	713.456	713.453
T12–13	71–85	AAIPTIKHLINNGAK	1560.922	1560.939
T13	78–85	HLINNGAK	866.484	866.478
T14	86–96	VILSSHLGRPK	1206.732	1206.750
T15	97–101	GVTPK	501.303	501.299
T16	102–110	FSLAPLVPR	999.599	999.608
T19	133–149	LVAELPEGGVLLLENVR	1821.048	1821.072
T23–24	164–182	KLASLADLYVNDAFGTAHR	2062.072	2062.104
T24	165–182	LASLADLYVNDAFGTAHR	1933.977	1934.007
T28	219–229	RPFAAIVGGSK	1102.637	1102.656
T34–37	285–311	AKEKGVSLLLPTDVVIADKFAADADSK	2801.529	2801.565
T36	289–303	GVSLLLPTDVVIADK	1539.899	1539.892
T41–43	369–376	KLEEISKK	974.588	974.600
T44	377–393	GATTIIGGGDSVAAVEK	1545.812	1545.828
T46	418–433	QLPGVLALNEADPVPV	1631.900	1631.914

23 peptides were matched for 44% sequence coverage, and the reported mass of this protein (45.7 kDa) is consistent with the 1D-PAGE data.

Non-specific binding of the model proteins was found to be similar for RNase A and myoglobin with ~85% recovery in the flow through fraction. Ovalbumin recovery was not determined because coelution of a system peak in the chromatograms prevented accurate quantitation. In addition to moderate recovery, high linearity ($r^2 > 0.999$) was achieved between 50 and 500 μg/mL for RNase A, ovalbumin, and myoglobin after depletion; and run-to-run carryover was low at ~0.2%.

3.3. Format comparison

The spin and LC columns share identical chemistry for RuBisCO depletion, so there are many similarities between

Table 4
PMF of the large subunit of RuBisCO from raw spinach leaf extract

Fragment	Residue #	Sequence	Theoretical *m/z*	Experimental *m/z*
T2–3	9–18	ASVGFKAGVK	963.562	963.574
T2–5	9–41	ASVG FKAGVKDYKLTYYTPEYETLDTDILAAFR	3745.900	3745.913
T3–5	15–41	AGVKDYKLTYYTPEYETLDTDILAAFR	3156.578	3156.597
T4–5	19–41	DYKLTYYTPEYETLDTDILAAFR	2801.356	2801.388
T5	22–41	LTYYTPEYETLDTDILAAFR	2395.170	2395.180
T6–8	42–83	VSPQPGVPPEEAGAAVAAESSTGTWTTVWTDGLTNLDRYKGR	4372.148	4372.177
T11–12	132–139	ALRLEDLR	985.579	985.582
T11–13	132–146	ALRLEDLRIPVAYVK	1756.048	1756.056
T12–13	135–146	LEDLRIPVAYVK	1415.826	1415.835
T13	140–146	IPVAYVK	789.487	789.484
T14	147–159	TFQGPPHGIQVER	1465.755	1465.764
T14–15	147–161	TFQGPPHGIQVERDK	1708.877	1708.884
T14–16	147–164	TFQGPPHGIQVERDKLNK	2064.098	2064.121
T18–19	178–187	LGLSAKNYGR	1078.600	1078.600
T21–22	195–213	GGLDFTKDDENVNSQPFMR	2169.987	2170.005
T37–38	320–339	LSGGDHIHSGTVVGKLEGER	2048.052	2048.077
T37–41	320–358	LSGGDHIHSGTVVGKLEGERDITLGFVDLLRDDYTEKDR	4313.180	4313.137
T45–46	432–439	NEGRDLAR	930.475	930.482
T45–47	432–446	NEGRDLAREGNTIIR	1713.899	1713.921
T46–47	436–446	DLAREGNTIIR	1257.691	1257.706
T47	440–446	EGNTIIR	802.442	802.446
T47–48	440–450	EGNTIIREATK	1231.664	1231.674

The protein was identified as ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (*Spinacia oleracea*) gi|11497536. 22 peptides were matched for 41% sequence coverage, and the reported mass (53.1 kDa) is consistent with the 1D-PAGE data.

the formats. In both cases, approximately 50 min are required for a single depletion. In addition, fraction volumes are nearly identical in each case. However, differences in bead quantity and sample handling lead to differing performance between the formats. First, the LC column contains greater than 2× more depletion resin, so RuBisCO binding capacity is more than double at 2 mg compared to 0.8 mg for the spin columns. This effect is demonstrated by the higher percent binding of RuBisCO in identical leaf extracts run on the LC (>98%) compared to the spin format (>90%). However, sample volume is limited with the LC column because up to 500 μL (or 2.5×) of sample can be loaded onto the spin column.

Sample handling leads to a number of important differences between the formats. First, the spin columns require repeated capping and uncapping, which leads to bead loss over time and eventually contributes to loss of capacity. This is not a problem in the LC format because frits hold the beads in place. However, the frits make particulates more problematic with the LC format because clogs cannot be reversed. Although it is important to filter the sample prior to depletion with either format, beads in a clogged spin column can be transferred to a new one thus reducing the effect of particulate matter. Third, all depletion steps are performed manually with the spin format; and because of the number of wash and stripping steps, there is little down-time during the depletion process. With the LC column and a fraction collector, depletion can be automated and run overnight. Finally, run-to-run variability is lower with the LC format because automation limits errors associated with manual sample handling.

3.4. Advantages of RuBisCo depletion

One advantage to RuBisCO depletion is illustrated in Fig. 4B. In this gel image, a greater number of protein bands are visible in the depleted (or flow through) fractions. Because a limited amount of labeling reagent is employed with the Agilent Bioanalyzer, the labeling is competitive. By eliminating the high concentration of RuBisCO, detection of less abundant proteins is improved. Although this is not a problem with PAGE separations because of the huge excess of dye, the size of the RuBisCO bands can hinder identification of proteins of similar mass. As a demonstration, three protein bands in a depleted sample of spinach leaf extract (Fig. 6) were identified by PMF. Two of the proteins, transketolase (gi|2529342) and oxygen-evolving enhancer protein 3 (gi|131397), were well resolved from the RuBisCO bands, and can be seen in the raw spinach extract (Fig. 7), phosphoglycerate kinase (gi|1346698), however, is difficult to discern from the raw leaf extract in Fig. 7 because it is virtually eclipsed by the RuBisCO large subunit. However, after depletion there is little to no RuBisCO remaining. The phosphoglycerate kinase band is well resolved and can be more easily excised, digested, and identified. 23 peptides, contributing to over 44% of the total sequence, were identified for this protein after depletion (Table 3), but only the large subunit of RuBisCO was identified from the raw extract. For the RuBisCO, 22 peptides, covering 41% of the total sequence, were identified in the database search (Table 4).

4. Conclusions

Cross species applicability of two formats of Seppro™ IgY RuBisCO depletion columns from GenWay Biotech was evaluated. The spin and LC columns depleted arabidopsis and canola RuBisCO by greater than 90 and 98%, respectively, suggesting that the technology may be applicable across a number of plant species. Sample dilution is the primary drawback of this technology because low level proteins are the principal target; however, sensitive detection or concentration can be employed to help alleviate this problem.

In the future, it may be advantageous to determine applicability to species with less sequence homology. Arabidopsis, corn, tobacco, and canola RuBisCOs all share high sequence homology with spinach, but other species such as red algae do not [16,17]. Applicability of this depletion technology to more distant relatives such as red algae, which has ∼60% sequence homology to spinach, would be interesting [21].

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