

<i>OREGIN Standard Operating Procedure</i>	
Purpose	Extract and separate seed proteins into constituent fragments of different size
Developed by	Clare Hopkins, Jackie Barker
Organisation	Rothamsted Research
Date agreed	August 2008

Procedure for the analysis of *Brassica* seed proteins by SDS-PAGE

Extraction (pages 2 & 3 of 7)

Total soluble proteins are extracted from 5 *Brassica spp.* seed (~20 mg) as described by Shewry et al. (1995), with the exception that bromophenol blue was not added to the extraction buffer. The use of a Hamilton syringe to remove the supernatant prevents oil contamination.

Quantification of protein using the Bradford assay (page 4 of 7)

Protein extracts are quantified using the Biorad Protein microassay (cat# 500-0006), according to the manufacturer's instructions. Bovine serum albumin (BSA) (Sigma A7030) is used for the standard curve.

Protein Separation – using SDS-PAGE (pages 5 & 6 of 7)

Protein extracts (3 µg total protein per sample) are separated by SDS-PAGE according to the method of Laemmli (1970) on a Biorad Protean II system, with 15% resolving and 5% stacking gels, respectively, run at 200 V for 55 min. Benchmark Prestained Ladder (Invitrogen 10748-010) is used as the MW standard.

Staining Gel (page 6 of 7)

Gel is fixed, stained and destained according to the manufacturer's instructions for Brilliant Blue G-colloidal concentrate (Sigma B2025).

Analysing Gel (page 7 of 7)

Gel and Kodak Control Scale T-14 strip are scanned using the HP Scanjet 3970 and saved as 256 greyscale .tif files. Proteins are quantified using Phoretix™ 1D software (Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK).

References

Shewry, P.R., Tatham, A.S., Fido, R.J. (1995). Separation of plant proteins by electrophoresis. In: *Methods in Molecular Biology—Plant Gene Transfer and Expression Protocols*, **49**: 423–437. Jones, H. (ed.). Humana Press, Totowa, NJ.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680–685.

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Brassica seed protein Extraction method

This method is adapted from Shewry et al. (1995).

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Protein Extraction Buffer (+DTT/-Bromophenol Blue; BB)

For 10 mL [final concentration]:

1 M Tris-HCl, pH 6.8	0.625 mL	[62.5 mM]
10% SDS (w/v)	2.000 mL	[2%]
1 M DTT	1.000 mL	[10 mM]
100% Glycerol	**1.000 mL	[10%]
MilliQ water	5.375 mL	-

**suggest weighing 1 g glycerol - easier than pipetting

Protein Extraction Buffer (+DTT/+BB)

Place 1 mL Protein Extraction Buffer (+DTT/-BB; above) into a microfuge tube and add/mix a small quantity of Bromophenol Blue (BB) powder on the tip of a wetted pipette tip. This Buffer is used as a sample buffer for running gels.

Procedure

1. Add 5 seed per sample into a 2 mL safe lock microfuge tube.
2. Weigh and record the weight of each sample.
3. Add one 5 mm stainless steel bead to each tube.
4. Add 20 μ L Protein Extraction Buffer (+DTT/-BB) per mg sample weight to each tube. NOTE: the amount of buffer is dependent on the weight of the sample.
5. Ensure the tubes are closed properly.
6. To grind seed, place tubes in TissueLyser (Qiagen) and operate for 1 min at 30 Hz on either side (2 min total).
7. Centrifuge for 1 min at 13 000 rpm to collect cell debris.
8. Protein can now be extracted.
9. Briefly vortex tubes to loosen the bead.
10. Using a large magnetic flea, remove the bead from the tube by sliding the flea along the outside of the tube. Ensure that no cell debris remains on the bead before removing from tube. If cell debris remains, vortex briefly and pulse spin.
11. Pulse spin sample to re-collect cell debris in tube.

12. Incubate for 1 h at room temperature.
13. After 30 min of incubation, turn on heat block ready for step 15.
14. Pierce lid of tube.
15. Incubate at for 2 min at 100°C for 2 min.
16. Centrifuge sample for 30 min at 13 000 rpm.
17. Using a Hamilton syringe carefully remove supernatant, avoiding the oil layer.
18. Rinse Hamilton syringe 5x in clean MilliQ water between samples.
19. Spin supernatant for 15 min at 13 000 rpm.
20. Using a Hamilton syringe, carefully remove supernatant, avoiding the oil layer.
21. Aliquot 4x 10 µL supernatant into PCR tubes and freeze immediately at -80°C.
22. Proceed to Bradford Assay for protein quantification.

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<i>OREGIN Standard Operating Procedure</i>	
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Quantification of protein using the Bradford assay

This method is according to the manufacturer's instructions for the Bio-Rad Protein Microassay.

Reagent	Company	Catalogue number
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	500-0006
BSA (stock concentration = 1 µg/µL)	Sigma	A7030

Procedure

1. Samples and standards are assayed in duplicate.
2. Add 800 µL MilliQ water to microfuge tube.
3. Remove 1 µL of water using a P2 Gilson pipette. NOTE: this is done as you can't pipette 799 µL using a P1000 Gilson pipette.
4. Remove x µL of water for the other BSA standards. The amount is dependent on the amount of standard that will be added at a ratio of 1:1, e.g. If you require a 7 µg standard, remove 7 µL water and then add 7 µL 1 µg/µL BSA standard.
5. Add 1 µL of sample to a microfuge tube.
6. Vortex and pulse spin.
7. Add 200 µL Bio-Rad Protein Assay Dye Reagent Concentrate.
8. Vortex briefly.
9. Incubate at room temperature for at least 5 min. During this time, turn on the spectrophotometer and set wavelength to 595 nm.
10. Place sample into disposable cuvette.
11. Measure and record the absorbance at 595 nm.
12. Enter values from standard curve and samples into Excel.
13. Generate standard curve from standard values, using an XY scatter plot with linear trendline.
14. Use this to calculate concentration of samples.

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Separation of seed proteins from *Brassica* by SDS-PAGE

Protein extracts (3 µg total protein per sample) are separated by SDS-PAGE according to the method of Laemmli (1970) on a Biorad Protean II system, with 15% resolving and 5% stacking gels, respectively, run at 200 V for 55 min.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, **227**: 680–685.

5x Tris-glycine gel buffer (taken from Sambrook et al., 1989)

For 100 mL [final stock concentration]:

Trizma	1.51 g	[0.125 M]
Glycine	9.40 g	[0.125 M]
10% SDS	5.00 mL	[0.5%]

Add MilliQ water to 100 mL.

Gel preparation

1. Make the following gels in glass vials, without adding the 10% ammonium persulfate and TEMED.

15 % Resolving Gel			5 % Stacking Gel	
Total volume = 10 mL	mL		Total volume = 2 mL	mL
H ₂ O	2.300		H ₂ O	1.400
30% Acrylamide mix	5.000		30% Acrylamide mix	0.330
1.5 M Tris, pH 8.8	2.500		1.0 M Tris, pH 6.8	0.250
10% SDS	0.100		10% SDS	0.020
			10% ammonium	
10% ammonium persulfate	0.100		persulfate	0.020
TEMED	0.004		TEMED	0.002

2. Assemble the Biorad Protean II system using 0.7mm spacers.
3. Add 10% ammonium persulfate and TEMED to the resolving gel solution and quickly pipette into the assembled gel.
4. Pour butan-1-ol across the top of the resolving gel to produce a straight edge.
5. Allow approximately 45 min for gel to polymerise.
6. Rinse the butan-1-ol thoroughly with MilliQ water from the gel edge and dry prior to pouring the stacking gel.
7. Add 10% ammonium persulfate and TEMED to the stacking gel solution and quickly pipette into the assembled gel.
8. Quickly place the comb into the stacking gel.
9. Allow 45 min for gel to polymerise.
10. Remove comb.

11. Rinse wells with 1x Tris-glycine gel buffer to remove any un-polymerised gel.

Preparation of samples for SDS-PAGE

1. Adjust protein samples to a concentration of 3 µg and a volume of 10 µL using Protein Extraction Buffer (+DTT/+BB).
2. Vortex briefly and pulse spin.
3. Heat at 95°C for 2 min.
4. Allow to cool immediately on ice.

Gel loading and running

1. Fill the upper chamber with 1x Tris-glycine gel buffer.
2. Using a 10 µL pipette, load all sample into well carefully.
3. Load 10 µL Benchmark Prestained Ladder (Invitrogen 10748-010) in one lane.
4. Fill the lower chamber with 1x Tris-glycine gel buffer.
5. Run at 200 V for 55 min.

Staining the Gel

The gel is fixed, stained and destained according to the manufacturer's instructions for Brilliant Blue G-colloidal concentrate (Sigma, B2025).

NOTE: Minigels require about 50 mL solution to cover a square Petri dish (100mm x 100mm).

1. Fix the proteins for 30 min in fixing solution (7% glacial acetic acid in 40% (v/v) methanol), rocking gently.
2. Decant the fixing solution into methanol waste.
3. Add staining suspension (40 mL Brilliant Blue G-colloidal concentrate, 10 mL methanol) to gel, rock for 1 – 2 h or overnight if desired.
4. Decant the stain solution into methanol waste.
5. Destain with Destain solution I (10% acetic acid in 25% (v/v) methanol) with shaking for 10-30 sec.
6. Decant the destain solution into methanol waste.
7. Rinse the gel with 25% methanol (Destain solution II).
8. Decant the 25% methanol into methanol waste.
9. Destain the gel with 25% methanol (Destain solution II) for up to 24 h.
10. Decant the 25% methanol into methanol waste.
11. If any precipitated dye remains on the surface of the gel, gently wipe with a clean lab wipe (or cotton wool if you must) soaked in 25% methanol.
12. Add MilliQ water to the gel.
13. Proceed to Analysing Gel.

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<i>OREGIN Standard Operating Procedure</i>	
Purpose	Record SDS-PAGE gel images
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Scanning SDS-PAGE gels with HP Scanjet 3970

1. Open HP Photo & Imaging Gallery.
2. Navigate to the folder you wish to save your gel scans in.
3. Select HP Scanjet 3970 from the list.
4. Place the Kodak Control Scale T-14 strip in the middle of the scanning bed.
5. Place the white card over the top of the strip.
6. Close the scanner lid and press 'NEW SCAN'.
7. The scanner will now do a preliminary scan.
8. Select the area you want (the file size will be too large if you don't narrow down the area to scan). Do this by dragging the square with the dotted lines to select your area of interest (gel or control strip) only.
9. Go to the top menu bar and click on 'OUTPUT TYPE'.
 - Select 256 greyscale (Phoretix will only accept black and white pictures). If you want colour scans, you can leave it as colour, but you will have to also re-scan as 256 greyscale.
 - Select 200 dpi resolution.
10. Press scan.
11. Rename the control with cal_date.tif (.tif is the default file type).
12. Roll the gel carefully onto the scanning bed. If the gel is slightly wet, the liquid will help form a seal and keep out airbubbles.
13. Place the white card on the gel, starting with the card at the end of the scanner nearest you. Bend the card, and roll it down, over the gel.
14. Perform steps 6-12 as above.
15. Use Phoretix™ 1D software (Nonlinear Dynamics Ltd, Newcastle-upon-Tyne, UK) to perform analysis as per manual.
