

## RESEARCH ARTICLE

# Protein and lipid composition analysis of oil bodies from two *Brassica napus* cultivars

Vesna Katavic<sup>1</sup>, Ganesh Kumar Agrawal<sup>1,2\*</sup>, Martin Hajduch<sup>1\*</sup>, Stefan L. Harris<sup>1,3</sup> and Jay J. Thelen<sup>1</sup>

<sup>1</sup> University of Missouri-Columbia, Department of Biochemistry, Columbia, USA

<sup>2</sup> Research Laboratory for Agricultural Biotechnology and Biochemistry, Kathmandu, Nepal

<sup>3</sup> Prairie View A&M University, Biological Sciences Department, Prairie View, Texas, USA

Oil bodies were purified from mature seed of two *Brassica napus* crop cultivars, Reston and Westar. Purified oil body proteins were subjected to both 2-DE followed by LC-MS/MS and multidimensional protein identification technology. Besides previously known oil body proteins oleosin, putative embryo specific protein ATS1, (similar to caleosin), and 11-beta-hydroxysteroid dehydrogenase-like protein (steroleosin), several new proteins were identified in this study. One of the identified proteins, a short chain dehydrogenase/reductase, is similar to a triacylglycerol-associated factor from narrow-leafed lupin while the other, a protein annotated as a myrosinase associated protein, shows high similarity to the lipase/hydrolase family of enzymes with GDSL-motifs. These similarities suggest these two proteins could be involved in oil body degradation. Detailed analysis of the two other oil body components, polar lipids (lipid monolayer) and neutral lipids (triacylglycerol matrix) was also performed. Major differences were observed in the fatty acid composition of polar lipid fractions between the two *B. napus* cultivars. Neutral lipid composition confirmed erucic acid and oleic acid accumulation in Reston and Westar seed oil, respectively.

Received: January 9, 2006

Revised: April 11, 2006

Accepted: April 28, 2006

**Keywords:**

2-DE / MS / MudPIT / Oil body / Rapeseed

## 1 Introduction

In plants, storage lipids in the form of triacylglycerol (TAG) are deposited in the embryo or endosperm during seed development, and are mobilized upon germination to provide carbon and energy for the developing seedling. At the

cellular level, TAG is synthesized in the endoplasmic reticulum (ER) and stored in small spherical organelles termed oil bodies. *In situ* electron microscopic observations of maturing seed suggest that plant seed oil bodies are formed through the 'budding' of ER. The mechanistic details of ER budding are not entirely understood but it is known that the process involves the accumulation of TAG molecules at the region between the two polar lipid (PL) monolayers of the ER membrane until a nascent oil body, composed of TAG matrix surrounded by a PL monolayer, is produced [1–3].

The major protein component of oil body organelles are oleosins, low molecular weight (15–26 kDa) basic proteins, embedded in the PL monolayer. Oleosins are prominent in plant seed oil bodies [4–6] and together with PL prevent coalescence of oil bodies and attack of unspecific cytosolic lipases and phospholipases by maintaining organelles as small

**Correspondence:** Professor Jay J. Thelen, University of Missouri-Columbia, Department of Biochemistry, 109 Life Sciences Center, Columbia, MO 65211, USA

**E-mail:** thelenj@missouri.edu

**Fax:** +1-573-884-9676

**Abbreviations:** **ATS1**, embryo specific protein; **ER**, endoplasmic reticulum; **FAME**, fatty acid methyl ester; **MudPIT**, multidimensional protein identification technology; **PA**, phosphatidic acid; **PC**, phosphatidylcholine; **PE**, phosphatidylethanolamine; **PL**, polar lipids; **RT**, room temperature; **TAG**, triacylglycerol; **TEM**, transmission electron microscopy

\* These authors contributed equally

particles through surface charge and steric hindrance in desiccating seed [7, 8]. It has also been suggested that oleosins provide a specific binding site for lipases during seed germination [9, 10]. Analysis of maturing seed and microspore embryo cultures showed that in Brassica species TAGs and oleosins accumulate simultaneously during oil body formation [11]. As the fundamental protein found in oil bodies of diverse angiosperms and gymnosperms, oleosins have been studied in numerous plant species including corn, sesame, safflower, sunflower, rapeseed, castor bean, and soybean [12–20].

Oil bodies from sesame and *Arabidopsis thaliana* were reported to contain a small number of proteins other than oleosins [21]. Chen *et al.* [22] identified three minor proteins (Sop1, Sop2, Sop3) in sesame oil bodies. The Sop1 protein was found to be homologous to a rice protein with a calcium binding domain and was named caleosin [23]. Lin *et al.* [24] characterized Sop2 protein from sesame and named it steroleosin for its homology to a sterol binding dehydrogenase/reductase class of proteins involved in signal transduction in diverse organisms. A glycosylphosphatidylinositol-anchored protein of unknown function was recently identified in oil bodies from *A. thaliana* [25]. These discoveries suggested that the composition of oil bodies might be more complex than previously envisioned.

Recent development of high throughput protein identification techniques allows for more thorough systematic proteomic analyses. However, current MS-based approaches have only recently been used to study the protein composition of plant oil bodies. Jolivet *et al.* [25] used SDS-PAGE coupled with MS to resolve and identify proteins from *A. thaliana* oil bodies. In addition to four oleosins, this investigation revealed a 11- $\beta$ -hydroxysteroid dehydrogenase-like protein, an embryo specific protein, (ATS1), a probable aquaporin, and a glycosylphosphatidylinositol-anchored protein of unknown function.

A more thorough analysis of plant oil body proteomes could potentially be obtained by using multiple, complementary proteomic approaches. For this purpose we selected a major oilseed crop, *Brassica napus* (common names: rapeseed; oilseed rape) and adapted previously established protocols to purify and characterize oil bodies from two *B. napus* cultivars: high erucic, low glucosinolate cultivar (cv.) Reston and low erucic, low glucosinolate cv. Westar (canola). *B. napus* oil body proteins were characterized using two independent, complementary approaches: 2-DE followed by in-gel trypsin digestion and LC-MS/MS; and in-solution trypsin digestion of total proteins followed by multidimensional LC-MS/MS, also referred to as Multidimensional Protein Identification Technology (MudPIT) [26]. In addition to major, previously characterized oil body proteins several additional proteins were identified.

The other, non-protein component of oil bodies is lipids. The oil body PL monolayer and TAG matrix were analyzed by TLC and GC-MS. The results support known differences in neutral lipid fatty acid composition between Reston and

Westar cultivars. However, analyses of PLs reveal major, previously uncharacterized differences in PL fatty acid composition between these two cultivars.

## 2 Materials and methods

### 2.1 Plant material

Mature seed from two *Brassica napus* cultivars was used for oil body isolation, high erucic acid, low glucosinolate cv. Reston and spring canola (low erucic acid, low glucosinolate) cv. Westar. Reston seed was kindly provided by Dr. Peter McVetty, University of Manitoba, Winnipeg, Canada. Westar seed was obtained courtesy of Dr. Gerhard Rakow, Agriculture and Agri-Food Canada Research Center, Saskatoon, Saskatchewan.

### 2.2 Isolation of oil bodies from mature seed

Oil bodies were isolated according to the method by Tzen *et al.*, [27] with the following modifications. Approximately 5 g of mature seed was ground in 15 mL cold (4°C) grinding medium one (GMI; 1 mM EDTA, 10 mM KCl, 1 mM MgCl<sub>2</sub>, 2 mM DTT, 0.6 M sucrose, 0.15 M tricine-KOH, pH 7.5) using a mortar and pestle. Crude homogenate was filtered through two layers of Miracloth and added to 15 mL cold (4°C) flotation medium one (FMI; the same composition as GMI with the addition of 0.4 M sucrose). The sample was centrifuged at 10 000  $\times$  g (Sorvall® HB-6 rotor) for 30 min. The top oleaginous layer was carefully removed using a spatula and resuspended in grinding medium two (GMII; the same as GMI plus 2 M NaCl) using a 50 mL glass Dounce homogenizer. The suspension was added to 15 mL of flotation medium two (FMII; the same as FMI plus 2 M NaCl), and centrifuged at 10 000  $\times$  g for 30 min. The top layer was resuspended with a glass homogenizer in 15 mL GMI, added to 15 mL FMI and centrifuged at 10 000  $\times$  g for 30 min. The procedure was repeated and the final oil body layer was either resuspended in 3 mL GMI (purified oil bodies) or subjected to one of the following treatments: oil body layer was resuspended in room temperature (RT, 25°C) medium GMII and shaken at RT for 30 min (salt washed) or the oil body layer was resuspended in RT medium GMI with 8 M urea and shaken at RT for 30 min (urea washed). Suspensions were centrifuged at 10 000  $\times$  g for 30 min. Oil body layers were resuspended with glass homogenizer in 15 mL cold (4°C) GMI, added to 15 mL cold (4°C) FMI and centrifuged as above. The procedure was repeated and final oil body layers were resuspended in 3 mL GMI medium. The final oil body preparations were used for oil body protein isolation.

### 2.3 Protein isolation from oil body preparations

Oil body protein was isolated according to the method by Tzen and Huang [28] with the following modifications. To each sample of 0.5 mL isolated oil bodies, 0.5 mL petroleum

ether was added and sample was vortexed. The sample was centrifuged 5 min at  $13\,000 \times g$ . After centrifugation, the upper petroleum ether layer containing neutral lipids was removed. The procedure was repeated two more times, petroleum ether fractions were pooled and dried under nitrogen gas. The interfacial layer and bottom aqueous phase were sparged with nitrogen gas to remove any remaining petroleum ether. To the interfacial layer and aqueous phase 0.75 mL chloroform/methanol (2:1 v/v) was added and samples were vortexed. The lower chloroform phases containing PLs were washed three times with 1 mL methanol/water (1:1 v/v) pooled, and dried under nitrogen gas. The protein rich interfacial layer was resuspended in 0.25 mL water, 0.75 mL chloroform/methanol (2:1 v/v) was added and samples were vortexed and centrifuged ( $13\,000 \times g$ , 5 min). The procedure was repeated two more times. After washing, oil body protein pellet was resuspended in 0.5 mL water, sonicated 5 min and precipitated in 4 volumes of cold 100% acetone for 16 h at  $-20^\circ\text{C}$ .

#### 2.4 Transmission electron microscopy (TEM)

Samples were fixed in 2% v/v glutaraldehyde, 2% v/v paraformaldehyde in 100 mM sodium cacodylate buffer-NaOH, pH 7.4 for 2.5 h at  $4^\circ\text{C}$  and rinsed three times 20 min with 130 mM sucrose, 10 mM 2-mercaptoethanol in 100 mM sodium cacodylate buffer-NaOH, pH 7.4. Samples were post-fixed with 1% w/v osmium tetroxide in 100 mM sodium cacodylate buffer-NaOH, pH 7.4, rinsed three times 5 min with ultrapure water (Milli-Q), and dehydrated through a graded series of acetone (20%, 50%, 70%, 90%,  $3 \times 100\%$  v/v). After infiltration through a graded acetone/Epon/Spurr's epoxy resin series samples were embedded in 100% w/v Spurr's epoxy resin and polymerized at  $60^\circ\text{C}$  for 24 h. Ultrathin sections were prepared using a Diatome diamond knife on an 8800 Ultratome III (LKB Instruments, Inc., Gaithersburg, MD) and stained with uranyl acetate and lead citrate. The stained sections were examined on a JEM-1200EX transmission electron microscope (JEOL, Ltd., Akishima, Japan). Images were recorded on 4489 film (Eastman-Kodak, Rochester, NY).

#### 2.5 SDS-PAGE and western blot analyses of oil body proteins

After precipitation in acetone, samples were centrifuged 15 min at  $8\,500 \times g$ . Protein pellets were dried and resuspended in  $0.5 \times$  SDS-PAGE sample buffer ( $1 \times$  sample buffer equals: 60 mM Tris-HCl, pH 6.8; 60 mM SDS; 5% glycerol; 100 mM DTT; 30 mM bromophenol blue) by vortexing. Samples were centrifuged 15 min at  $13\,000 \times g$  to precipitate insoluble materials. Protein concentration in the supernatant was determined using protein assay from BioRad (Hercules, CA), based upon modified procedure by Bradford [29]. Protein quantification was performed in triplicate against standard curve of chicken  $\gamma$ -globulin. Ten  $\mu\text{g}$  protein was loaded per lane on 12% SDS-PAGE gel. The

molecular weight marker was Sigma Marker Wide Range (Product no. M4038). After electrophoresis gel was washed three times for 15 min in deionized water and stained 16 h in colloidal Coomassie (20% v/v) ethanol, 1.6% v/v phosphoric acid, 8% w/v ammonium sulfate, 0.08% w/v colloidal CBB G-250). For immunoblot analyses 20  $\mu\text{g}$  of protein was resolved per lane and electroblotted to nitrocellulose membrane. Antibody probing was performed as described previously [30]. Anti-Arabidopsis oleosin D9 mouse monoclonal antibody was raised against bacterial produced 18 kDa Arabidopsis oleosin. Secondary antibody was anti-mouse IgG developed in goat (Sigma).

#### 2.6 Analyses of oil body lipids by TLC and GC

TLC plates (K6 Silica Gel 60 Å from Whatman) were soaked in 0.15 M ammonium sulfate for 30 s, air dried for 3 h and immediately before use were activated by heating at  $120^\circ\text{C}$  for 3 h. PL standards (1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine; 1,2-Dioleoyl-*sn*-Glycero-3-Phosphoethanolamine; 1,2-Dioleoyl-*sn*-Glycero-3-Phosphate; Avanti Polar Lipids, Inc) were diluted in chloroform at 1 mg/mL and 5  $\mu\text{L}$  was spotted on the plate. Oil body PLs were diluted in chloroform and spotted on the plate. Plates were developed in acetone/toluene/water (91:30:8) to allow the separation of PL from the origin. Bands were visualized after development with iodine. For GC analyses, lipid bands were scraped and collected into glass Pasteur pipettes plugged with glass-wool, eluted with 4 mL of acidic chloroform/methanol 1:2 v/v, [31] and washed three times with 2 mL chloroform. Samples were dried under nitrogen gas and transmethylated with 1% v/v sulfuric acid in methanol at  $60^\circ\text{C}$  for 30 min. After transmethylation, fatty acid methyl esters were extracted with hexane (2 mL water and 100  $\mu\text{L}$  hexane) and analyzed by GC on an Agilent Technologies model 689N Network GC System gas chromatograph fitted with a DB-23 column (30 m  $\times$  0.25 mm; film thickness 0.25  $\mu\text{m}$ ; Agilent 122–2332). The GC conditions were: injector temperature and flame ionization detector temperature,  $250^\circ\text{C}$ ; running temperature program,  $150^\circ\text{C}$  for 1 min, then increasing at  $2^\circ\text{C}/\text{min}$  to  $200^\circ\text{C}$  and holding at this temperature for 5 min. Quantification of FAMES was performed using a flame ionization detector and FAME identification was performed using a mass selective detector.

#### 2.7 2-DE of oil body proteins

After acetone precipitation, protein pellets were dried and resuspended in IEF sample extraction medium (8 M urea, 2 M thiourea, 2% w/v CHAPS, 2% v/v Triton X-100, 50 mM DTT) by vortexing at low speed for 1 h. Insoluble matter was removed by centrifugation for 20 min at  $14\,000 \times g$ . The desired amount of protein (0.5 mg) was added to a 1.5 mL tube, and volume was brought up to 450  $\mu\text{L}$  with IEF extraction media. Ampholytes (2.25  $\mu\text{L}$ ) were added, mixed by vortexing, and centrifuged for 5 min at  $14\,000 \times g$  to remove the

remaining insoluble matter. 2-DE of oil body proteins, gel staining, imaging, and analysis was performed as described by Hajduch *et al.* [32].

## 2.8 LC-MS/MS identification of proteins from 2-D gels

Protein spots from 2-D gels were arrayed into 96-well Multi-Screen plate model R5.5  $\mu\text{m}$  hydrophilic PTFE membrane, glass-filled polypropylene plates (Milipore, Bedford, MA) using a 1.5 mm diameter excision pen (Gel Company, San Francisco, CA). In-gel digestion with trypsin and sample preparation for MS was performed according to Hajduch *et al.* [32]. LC-MS/MS analyses of tryptic peptides were performed using an LTQ Proteome X mass spectrometer (Thermo Finnigan, San Jose, CA). The mass spectrometer was operated according to manufacturer's instructions for high throughput protein identification. Briefly, on-line capillary LC included two polymeric sample traps (2  $\mu\text{g}$  capacity each) and a fast-equilibrating C18 capillary column (Micro-Tech Scientific, Cousteau Ct. Vista, CA; 150  $\mu\text{m}$  ID  $\times$  10 cm). The method alternated between loading/equilibration and elution using two peptide traps to reduce time required for each online LC-MS/MS. For analysis, 10  $\mu\text{L}$  of sample in 0.1% v/v formic acid was loaded. For sample elution, a 15 min gradient with 40% of solution A (0.1% formic acid in water) and 60% of solution B (0.1% formic acid in acetonitrile) was followed by a 5 min gradient with 20% solution A and 80% solution B. The column was reset for 2 min and re-equilibrated for 10 min with 100% of solution A before sample previously absorbed onto the second trap was eluted. Eluted tryptic peptides were directly analyzed by LC-MS/MS using 75  $\mu\text{m}$  ID, 360  $\mu\text{m}$  OD, 15  $\mu\text{m}$  tip needles (New Objective, Woburn) with a 1.7 kV nano-spray voltage. Manufacturer's recommended scan method for high-throughput protein identification consisted of double-play analysis mode; a full MS scan (400–1600  $m/z$ ) followed by data dependent triggered MS/MS scan for the most intense ion.

For protein identification, acquired MS/MS spectra were searched against the non-redundant protein National Center for Biotechnology Information (NCBI; ftp://ftp.ncbi.nih.gov/blast/) database (as of June 2005) using the 'SEQUEST Search' algorithm in the BioWorks 3.2 software package (Thermo-Finnigan). Four criteria were applied to SEQUEST searches to obtain high-confidence protein assignments: 1) a minimum of two unique peptides were required for an assignment; 2) cross-correlation number (Xcorr) versus charge state must exceed 1.5, 2.0, and 2.5 for +1, +2, and +3 charged peptides, respectively; 3) a peptide probability of 0.005 was employed; 4) peptide mass search tolerance was 2  $m/z$ .

## 2.9 MudPIT of oil body proteins

After acetone precipitation, dry protein pellet was resuspended in 100 mM Tris-HCl, pH 8.0; 8 M urea, 5 mM DTT, and vortexed for 30 min at RT. The clarified protein super-

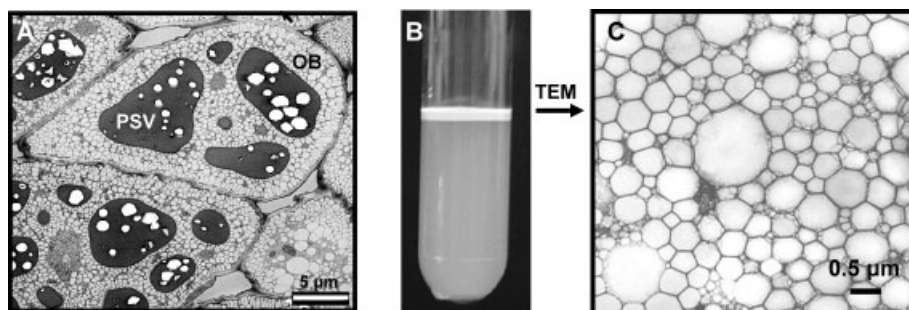
natant following centrifugation at high speed (14 000  $\times$  g, 15 min) was quantified and a total of 0.1 mg protein was reduced with 10 mM DTT at RT for 1 h and alkylated with 40 mM iodoacetamide in the dark for 1 h at RT. For in-solution digestion, the protein solution was diluted to 1 M urea with 100 mM Tris-HCl, pH 8.0, followed by the addition of calcium chloride to a final concentration of 1 mM. Sequencing grade modified trypsin (Promega, Madison, WI, USA) was added to the solution at a 50:1 protein enzyme ratio and incubated at 37°C for 20 h. The reaction was stopped by acidification with formic acid to a final concentration of 5%. Sample was lyophilized and stored at  $-80^{\circ}\text{C}$  until MS analysis.

Before MS analysis, all samples were reconstituted in 60  $\mu\text{L}$  of 0.1% formic acid in water. The MudPIT experiments were performed according to the manufacturer's instructions on a ProteomeX LTQ workstation (Thermo-Finnigan). Peptides (100  $\mu\text{g}$  in a volume of 20  $\mu\text{L}$ ) were loaded onto a strong cation exchange resin (BioBasic SCX, 100  $\times$  0.32 mm, 300  $\text{\AA}$ , 5  $\mu\text{m}$ ; Thermo-Finnigan) and eluted stepwise with six ammonium chloride "bumps" (50, 100, 200, 400, 600, and 800 mM, respectively) onto peptide traps (C18, 5  $\times$  1 mm, Thermo-Finnigan, Bellefonte) for concentrating and desalting prior to final separation by reversed-phase capillary column (BioBasic C18, 100  $\times$  0.18 mm, 300  $\text{\AA}$ , 5  $\mu\text{m}$ ; Thermo-Finnigan) using an ACN gradient (0% to 80% v/v) solvent B in solvent A for a duration of 41 min, Solvent A = 0.1% v/v formic acid in water; Solvent B = 100% ACN containing 0.1% v/v formic acid). Eluted peptides were ionized with a fused-silica PicoTip emitter (12 cm, 360  $\mu\text{m}$  OD, 75  $\mu\text{m}$  ID, 30  $\mu\text{m}$  tip; New Objective, Woburn). The heated PicoTip emitter was held at ion spray 1.7 kV and a flow rate of 250 nL/min. Ions were analyzed in the data-dependent positive acquisition mode. Following each full scan (mass range  $m/z$  400–2000), six data-dependent MS/MS scans, isolation width 2 amu, 35% normalized collision energy, minimum signal threshold 500 counts, dynamic exclusion (repeat count, 1; repeat duration, 30 s; exclusion duration 75 s), of the six most intense parent ions were acquired. For protein identification, acquired MS/MS spectra were searched under the same conditions as described in Section 2.8 for 2-DE analyses.

## 3 Results

### 3.1 *In vitro* isolated oil bodies from *B. napus* seed are homogenous in size, reflecting *in vivo* characteristics

TEM analysis of developing *B. napus* seed revealed a cross-sectional view of a single embryonic cell may contain over 300 distinct oil body organelles which in total comprise greater than 50% of the cell area (Fig. 1A). *B. napus* oil bodies were spherical in shape, ranging in size from 0.2 to 3.0  $\mu\text{m}$ . Homogenization of seed in aqueous media followed by

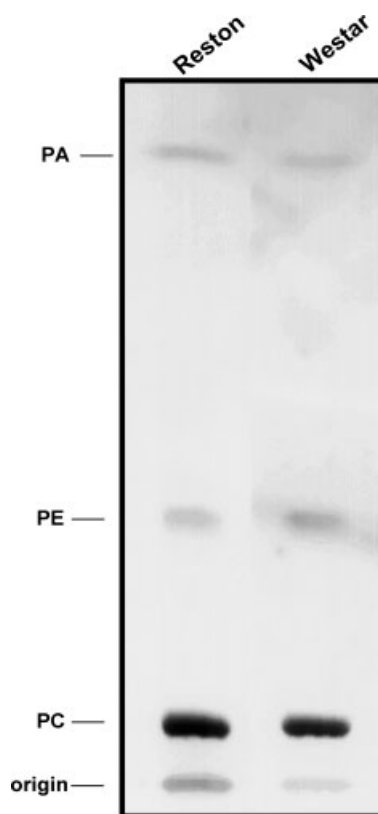


**Figure 1.** Oil body purification and TEM analyses: (A) TEM of embryonic cells from *B. napus* cv. Reston at 6 weeks after flowering. PSV, protein storage vacuole; OB, oil bodies. Bar equals 5.0  $\mu\text{m}$ . (B) Purification of oil bodies from mature *B. napus* cv. Reston seed. In aqueous media oil bodies collect at the surface during centrifugation; after iterative washes, the top layer of oil bodies were collected. (C) TEM of purified rapeseed oil bodies. Bar equals 0.5  $\mu\text{m}$ .

differential centrifugation resulted in the collection of oil bodies at the media surface, appearing as a gelatinous, white layer (Fig. 1B). Analysis of *in vitro* purified oil bodies from mature *B. napus* seed by TEM revealed similar characteristics as TEM analysis of oil bodies from intact seed. The structures were globular and compact in shape, and ranged in size from 0.2 to 2.0  $\mu\text{m}$  with the majority of oil bodies 0.4 to 0.5  $\mu\text{m}$  in diameter (Fig. 1C). The observed size range of *in vitro* oil bodies corresponded to the size of oil bodies in the intact cells of developing embryos at 6 weeks after flowering.

### 3.2 PLs from isolated *B. napus* oil bodies are primarily phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidic acid (PA)

Analysis of PLs from *B. napus* cv. Reston and Westar oil bodies revealed in order of abundance: PC, PE, and PA; based upon co-migration with lipid standards (Fig. 2). Differences in fatty acid composition were detected among different PL components as well as between the same PLs from two different cultivars (Table 1); Reston contained 5% and 11% oleic acid in PC and PA, respectively, while Westar contained 21% and 16%. Reduced 18:1 content in PC and PA from Reston was compensated by increased levels of stearic acid (18:0). No linoleic acid (18:2) was detected in FAMES from PC and PE isolated from Reston oil bodies while both PC and PE from Westar oil bodies had 1–2% linoleic acid. Analysis of neutral lipids confirmed known fatty acid composition of TAGs from these two rapeseed cultivars (Table 1). Reston TAGs contained 26% erucic acid (22:1) and *ca.* 30% 18:1 while Westar TAGs contained 63% 18:1 and no 22:1. Since seed fatty acid composition varies with environmental conditions and also within the Reston cultivar, as a control we analyzed the composition of whole Reston seed used in this study and determined 22:1 to be at 29% and 18:1 at 21% (data not shown).



**Figure 2.** TLC of PL fraction from oil bodies isolated from *B. napus* cv. Reston and Westar. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid.

### 3.3 SDS-PAGE analysis of *B. napus* oil bodies revealed several other protein bands in addition to oleosin

Proteins isolated from crude seed extracts, purified oil body preparations, and purified oil bodies washed with salt or urea were separated by SDS-PAGE and visualized by CBB

**Table 1.** GC analyses of polar and neutral (TAGs) lipids from oil body membranes from *B. napus* Reston and Westar cultivars.

Polar Lipids	Fatty Acid Composition %			
	16:0	18:0	18:1	18:2
PC-R	33.9	57.6	4.9	nd
PC-W	29.4	47.7	21.1	1.8
PE-R	35.3	51.5	13.2	nd
PE-W	26.2	57.6	14.9	1.3
PA-R	24.5	64.7	10.8	nd
PA-W	34.7	49.2	16.1	nd

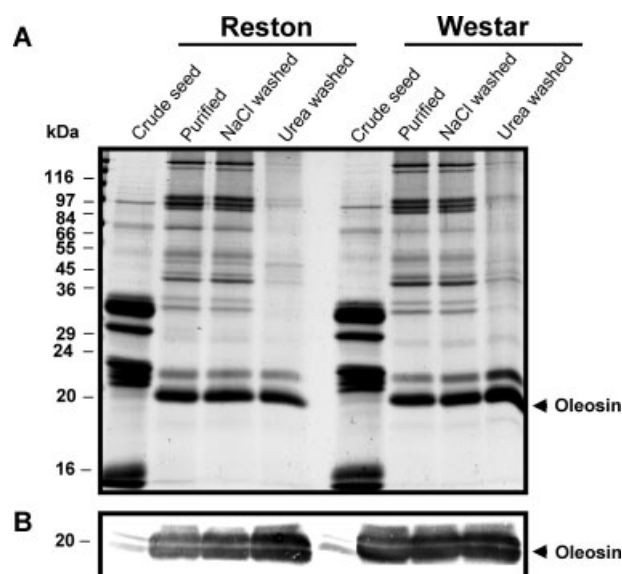
Neutral Lipids	Fatty Acid Composition %										
	16:0	16:1	16:2	18:0	18:1	18:2	18:3	20:0	20:1	20:2	22:1
TAG-R	4.9	nd	nd	1.1	30	15.5	9.6	0.3	12.1	0.2	26.2
TAG-W	4.7	0.2	0.1	1.7	63.4	20	8.3	0.2	0.8	nd	nd

nd, not detected; R, Reston; W, Westar.

staining. Protein samples from crude seed extracts were rich in proteins corresponding to the molecular weights of major storage proteins cruciferin and napin. In contrast, proteins recovered from untreated, salt washed, or urea washed oil bodies were significantly enriched in a protein migrating at 20 kDa, corresponding to oleosin. Although oleosin was the most abundant protein in all three different oil body preparations, at least fifteen other protein bands were also detected. The overall SDS-PAGE profile of purified oil body proteins was almost identical to purified oil bodies washed with 2 M sodium chloride. Purified oil body preparations washed with 8 M urea contained prominent oleosin bands but reduced levels of nearly every other protein (Fig. 3A). Enrichment of oleosin in purified oil body preparations was confirmed by immunoblot analyses with mAbs raised against *Arabidopsis* oleosin (Fig. 3B). Bands corresponding to oleosin were faint in crude seed protein fractions, but very intense in purified oil body preparations indicating enrichment in these fractions. Enrichment of oleosin was highest in urea washed oil body preparations (Fig. 3B). Overall, SDS-PAGE analysis of oil body protein fractions from Reston and Westar yielded nearly identical profiles, suggesting oil body protein composition and association is unaffected by the aforementioned differences in acyl lipid composition between these cultivars.

### 3.4 Experimental design for *B. napus* oil body proteome analyses

To systematically analyze proteins isolated from oil bodies of *B. napus* cvs. Reston and Westar two different proteomic approaches were employed: 1) 2-DE followed by in-gel tryptic digestion and C18 reversed-phase LC-MS/MS; and 2) in-solution tryptic digestion followed by MudPIT analyses (Fig. 4).

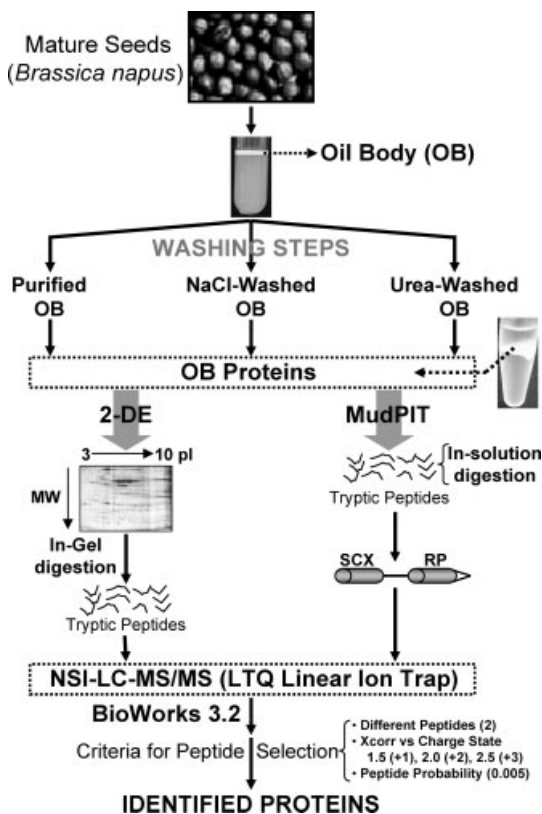


**Figure 3.** (A) CBB stained SDS-PAGE of total protein (10 µg) from crude seed, purified oil body, 2 M NaCl washed oil body, and 8 M urea washed oil body preparations from *B. napus* Reston and Westar cultivars. Molecular weight markers are shown in kDa. (B) Immunoblot analysis of oleosins in oil body fractions isolated from Reston and Westar seed. Twenty micrograms of protein from each fraction described in (A) was resolved in each lane and probed with mouse mAbs raised against recombinant *Arabidopsis* oleosin.

In both cases, tandem MS data were searched against the NCBI non-redundant database using SEQUEST within the BioWorks 3.2 software program using protein assignment criteria listed in Fig. 4. Stringent criteria were used for protein assignments to minimize false-positives, a concern when complete genome data are not available for data mining.

### 3.5 2-DE and LC-MS/MS analyses

High resolution 2-DE of proteins from mature seed oil bodies revealed approximately 100 protein spots for both Reston and Westar cultivars (Fig. 5). The oil body 2-DE maps for the two cultivars were nearly identical, and based upon the similarities by SDS-PAGE, this was predictable (data not shown). The 96 most intense spots were excised from gels of each cultivar (192 total protein spots), digested with trypsin and analyzed by LC-MS/MS. This analysis resulted in a total of 91 identified 2-DE spots from both sets of gels (Table 2). In addition to oleosins, identified proteins could be classified into groups including 11- $\beta$ -hydroxysteroid dehydrogenase-like proteins, ATS1, short chain dehydrogenase/reductase, myrosinases, myrosinase binding proteins, myrosinase-associated proteins,  $\beta$ -glucosidases, storage proteins, and heat shock proteins (Table 2, Fig. 5). Three oleosin proteins (oleosin type 4, 1803528A and oleosin BN-V) with experimental molecular masses of 24, 21 and 19 kDa and pI values



**Figure 4.** Experimental design for oil body purification and washing, protein extraction, and analyses using 2-DE and MudPIT proteomics approaches. After 2-DE, protein-containing gel spots were excised and subjected to in-gel trypsin digestion. For MudPIT analyses, in-solution protein mix was reduced and alkylated before digesting with trypsin. Tryptic peptides from 2-DE spots were resolved and analyzed by online RP-LC, nanospray ionization-MS/MS. Tryptic peptides from in-solution digest were resolved and analyzed by online multidimensional chromatography (strong cation exchange followed by C18 RP) nanospray ionization-MS/MS. Resulting MS/MS spectra were searched against the NCBI database using SEQUEST. Criteria for peptide and protein assignment were designated within the BioWorks™ 3.2 software.

of 9.5, 10, and 9.5 respectively, were identified. Two other proteins showing high homology with seed oil body proteins described in other species were identified including 11- $\beta$ -hydroxysteroid dehydrogenase-like protein similar to steroleosin from sesame oil bodies and putative ATS1 similar to caleosins described in oil bodies from sesame and rice. In addition, a short chain dehydrogenase/reductase was identified from Reston oil bodies. This protein has an experimental molecular mass of 32 kDa and *pI* value of 6.0 which is in good correlation with theoretical values for the same protein from *A. thaliana* ( $M_r$  31.3; *pI* 6.0; Acc. No. 21700875). Surprisingly, MS analyses revealed myrosinase binding proteins, myrosinase associated proteins, several myrosinase enzymes, and  $\beta$ -glucosidases associated with oil body preparations from both *B. napus* cultivars. The remainder of

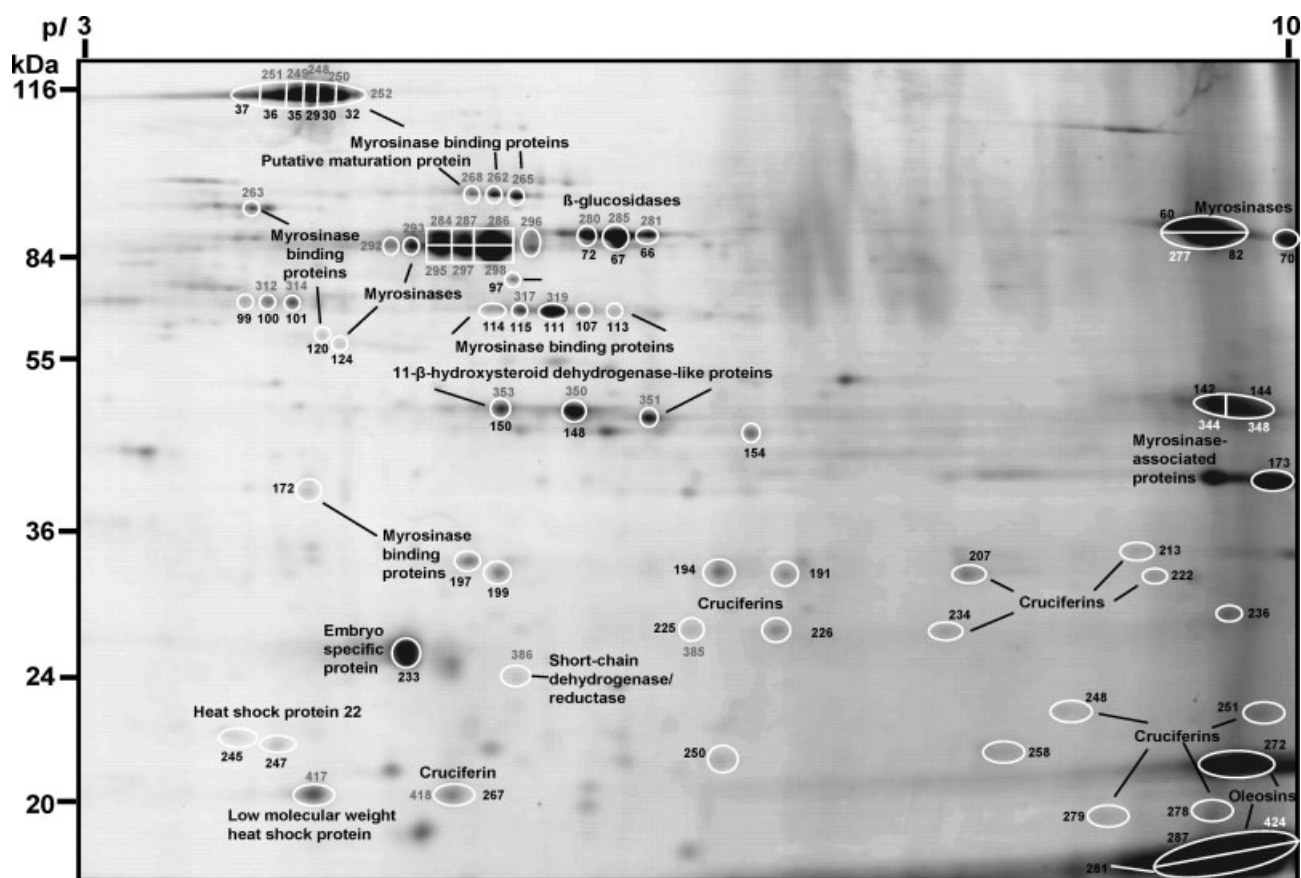
identified proteins are prominently expressed seed proteins, but previously shown to be associated with non-oil body organelles and are therefore possible contaminants. These protein contaminants included storage proteins cruciferin and napin (found in protein storage vesicles) and import inner membrane translocase and ATP synthase  $\alpha$ -chain (mitochondrial proteins).

### 3.6 MudPIT analyses

Since 2-DE is well documented to be biased against high molecular weight and hydrophobic proteins, MudPIT was also applied to oil body protein preparations. Analysis of Reston and Westar oil body protein preparations by MudPIT revealed the same group of proteins identified by 2-DE as well as aspartic protease, protein disulfide isomerase, luminal binding protein, and a LEA domain-containing protein (Supplementary Tables 1 and 2). MudPIT analyses were performed on protein samples isolated from (i) purified oil bodies, (ii) salt-washed oil bodies, and (iii) urea-washed oil bodies to determine the nature of association for each protein. Although MudPIT data are not quantitative, based upon the protein assignments it was apparent that washing purified oil bodies with 2 M sodium chloride had minimal effect on protein association. In contrast, 8 M urea completely abrogated the association of protein disulfide isomerase, LEA proteins, ATPase subunits, and heat shock proteins. A partial disruption of myrosinases, myrosinase binding proteins, beta-glucosidase, aspartate proteases, cruciferins and napin seed storage proteins was also observed. Oleosin, ATS1, beta hydroxysteroid dehydrogenase, and short-chain dehydrogenase/reductase proteins were apparently resistant to urea dissociation. These conclusions based upon qualitative MudPIT analyses are in strong agreement with the quantitative analyses by SDS-PAGE indicating that non-ionic interactions are responsible for the association of most proteins with the PL monolayer of oil bodies (Fig. 3).

## 4 Discussion

The major structural components of plant oil bodies are proteins and lipids, both storage and membrane. Since oil body proteins can either be embedded within the lipid monolayer, associated with lipid head groups, or peripherally associated with either of the aforementioned classes it is possible that membrane lipid composition could influence protein association with oil bodies. Two oilseed rape cultivars, Reston and Westar, are known to differ in the amount of elongated fatty acids within storage lipids. However, it is not known if the polar membrane lipids of oil bodies also differ in composition as a result of their genetic background. Therefore, in addition to comparing protein composition of oil bodies between these two cultivars, PLs were also analyzed and compared.



**Figure 5.** Analysis of proteins isolated from rapeseed oil bodies by 2-DE in combination with CBB staining. Proteins (0.5 mg) were analyzed using linear, wide-range IPG strips with pH range from 3 to 10. pI and molecular mass (in kDa) are noted. Protein spots identified by LC-MS/MS are circled and numbered in accordance with Table 2.

#### 4.1 Lipid analysis of rapeseed oil bodies reveals differences in PL composition

The main pathway for TAG biosynthesis (Kennedy pathway) involves three sequential transfers of acyl groups from acyl-CoA to a glycerol backbone, with the last reaction being the acylation of diacylglycerol catalysed by acyl-CoA:diacylglycerol acyltransferase [33]. In addition to acyl-CoA dependent TAG bioassembly TAG can also be synthesized in the absence of acyl-CoA by phospholipid:diacylglycerol acyltransferase, also referred to as PDAT. The reaction involves the transfer of an acyl group from PLs (*e.g.* PC) to diacylglycerol to form TAG and lyso-PC [34, 35]. During oil body formation, the PC pool in ER could be used for membrane lipid synthesis as well as TAG assembly. In addition to differences in the fatty acid composition of TAGs, differences in the fatty acid composition of PL components (PC in particular) between Reston and Westar could be the result of microsomal elongase enzyme (FAE1 condensing enzyme) activity in *cv.* Reston which catalyzes elongation of 18:1-CoA to 20:1-CoA and 22:1-CoA. Microsomal FAE1 enzyme from Westar is inactive due to a point mutation in the coding region of the

corresponding gene [36–39] which leads to accumulation of 18 carbon fatty acids, with negligible levels of very long chain fatty acids being synthesized. It is possible that because PL pool is shared between two pathways, storage and membrane lipid synthesis, besides affecting fatty acid composition of neutral lipids the activity of FAE1 condensing enzyme in Reston could alter the fatty acid composition of PL membranes of ER and consequently the composition of oil body PL monolayers. Thus, in Reston a portion of available 18:1-CoA pool is further elongated to 20:1-CoA and 22:1-CoA, while in Westar it is channelled to further desaturation and 18:2 formation.

#### 4.2 Proteomic analysis of rapeseed oil body proteome reveals a tightly associated novel short-chain dehydrogenase with similarity to a TAG-associated factor

2-DE in combination with LC-MS/MS analyses resulted in the assignment of 91 proteins spots (Table 2). Oleosin proteins, 11-beta hydroxysteroid dehydrogenase-like protein and ATS1 were previously reported as integral components of oil

**Table 2.** Protein assignments from LC-MS/MS analyses of 2-D gel spots from *B. napus* cv. Reston and cv. Westar purified oil body proteins

Protein name	Spec.	Acc. No	Theor. MW/pi	Reston				Westar			
				Spot No.	P (pro)	Exper. MW/p/	Pept./Cov.	Spot No	P (pro)	Exper. MW/p/	Pept./Cov.
Oleosin type 4	<i>At</i>	1592686	20.3/6.9	424	4.43E-04	20/9.5	2/13.1	287	1.28E-02	19/9.5	2/12
1803528A Oleosin	<i>Bn</i>	228416	20.7/9.5					281	2.90E-04	21/9.5	3/19.8
Oleosin BN-V	<i>Bn</i>	808944	20.3/9.2					272	1.27E-06	21/9.5	2/13.1
11-Beta-hydroxysteroid deh.	<i>At</i>	62320743	39.1/5.9	350	5.99E-05	45/5.5	2/3.1	148	2.33E-05	47/5.5	2/7.4
11-Beta-hydroxysteroid deh.	<i>At</i>	62320743	39.1/5.9	351	4.88E-04	44/6.0	5/15.8				
11-Beta-hydroxysteroid deh.	<i>At</i>	62320743	39.1/5.9	353	7.05E-04	43/5.0	2/6.3	150	8.06E-10	45/5.1	6/19.8
Embryo-specific protein 1 (ATS1)	<i>At</i>	7269526	28.0/5.8					233	6.57E-04	30/4.5	3/15.1
Short-chain dehydrogenase/ reductase	<i>At</i>	21700875	31.3/6.0	386	1.09E-06	27/5.5	2/9				
Myrosinase-assoc. prot.	<i>Bn</i>	1216389	41.8/8.5	344	5.85E-06	45/9.5	5/19.9	142	2.86E-12	47/9.3	14/37.7
Myrosinase-assoc. prot.	<i>Bn</i>	1216389	41.8/8.5	348	6.43E-07	44/9.6	6/21	144	3.55E-12	47/9.6	15/38.3
Myrosinase-assoc. prot.	<i>Bn</i>	1216389	41.8/8.5					173	2.50E-04	43/9.7	2/5.4
Myrosinase	<i>Rs</i>	11034734	62.9/8.3	277	2.72E-06	93/9.4	2/5.5	82	2.85E-11	92/9.3	11/22.3
Myrosinase	<i>Rs</i>	11034734	62.3/8.3	287	2.35E-04	90/5.1	2/4.7				
Myrosinase	<i>Rs</i>	11034734	62.9/8.3	292	5.71E-05	90/4.5	3/6.4				
Myrosinase	<i>Rs</i>	11034734	62.9/8.3	293	6.61E-05	90/4.7	3/7.7				
Myrosinase	<i>Bn s p</i>	56130949	62.96/6.7	284	4.3E-08	89/4.9	3/8				
Myrosinase	<i>Bn s p</i>	56130949	62.96/6.7	286	3.3E-08	90/5.3	4/11.3				
Myrosinase	<i>Bn s p</i>	56130949	62.96/6.7	295	1.1E-09	89/4.9	5/10.9				
Myrosinase	<i>Bn s p</i>	56130949	62.96/6.7	297	1.9E-06	90/5.1	4/10.2				
Myrosinase	<i>Bn s p</i>	56130949	62.96/6.7	298	1.1E-10	90/5.3	7/16				
Myrosinase	<i>Bj</i>	12621052	62.7/7.1	296	1.55E-04	90/5.5	2/5.8				
Myrosinase	<i>Bj</i>	12621052	62.7/7.1					124	6.03E-06	69/4.4	2/5.8
Myrosinase	<i>Bn</i>	840725	62.8/8.7					60	1.57E-09	93/9.4	2/3.8
Myrosinase	<i>Bn</i>	840725	62.8/8.7					70	1.78E-06	93/10	3/6.8
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	248	1.5E-09	115/4.3	19/25.6	29	9.14E-10	116/4.4	16/25.4
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	249	8.3E-11	115/4.2	20/24.6	35	1.40E-10	114/4.3	18/23.3
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	250	4.4E-08	115/4.5	6/9.5	30	5.05E-11	115/4.5	20/31.4
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	251	5.0E-08	115/4.1	5/7.8	36	9.43E-10	113/4.2	14/19.5
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	252	4.8E-09	115/4.5	17/22.2	32	2.74E-07	115/4.5	7/11.3
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	262	2.8E-07	95/5.2	7/13.7				
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	263	6.3E-10	93/4.0	6/11.4				
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	265	8.0E-11	95/5.3	4/8.8				
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	312	1.2E-09	75/4.1	7/9.8	100	2.21E-07	75/4.0	7/7.3
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5	314	1.2E-07	75/4.2	7/8.5	101	7.10E-10	75/4.1	6/9.9
Myrosinase-binding protein	<i>Bn</i>	1655824	99.4/5.5					172	9.14E-06	45/4.0	5/6.5
Myrosinase-binding protein	<i>Bn</i>	1655824						37	2.12E-08	116/3.9	7/9.5
Myrosinase-binding protein	<i>Bn</i>	1655824						99	8.80E-10	75/3.9	8/10
Myrosinase-binding protein	<i>At</i>	62321136	49.7/6.0	317	2.79E-06	72/5.5	2/5.6	115	2.19E-10	73/5.3	4/10.2
Myrosinase-binding protein	<i>At</i>	62321136	49.7/6.0	319	1.45E-06	72/5.8	3/8.5	111	3.71E-10	73/5.3	5/13.9
Myrosinase-binding protein	<i>At</i>	62321136	49.7/6.0					107	1.11E-09	73/5.6	5/12.2
Myrosinase-binding protein	<i>At</i>	62321136	49.7/6.0					114	4.72E-09	73/5.1	2/7.4
Myrosinase-binding protein	<i>At</i>	62321136	49.7/6.0					120	1.71E-09	65/4.2	4/10.2
Myrosinase-binding protein	<i>At</i>	62321136	49.7/6.0					197	3.98E-06	40/5.0	2/8.5
Myrosinase-binding protein	<i>At</i>	62321136	49.7/6.0					199	9.07E-09	38/5.2	3/8.5
Myrosinase-binding protein	<i>At</i>	62321136	49.7/6.0					113	4.10E-07	72/5.8	2/7.6
Beta glucosidase	<i>Bn</i>	757740	58.5/6.2	280	1.62E-06	89/5.8	6/14.5	72	4.13E-09	90/5.8	8/19.5
Beta glucosidase	<i>Bn</i>	757740	58.5/6.2	281	4.57E-06	91/6.1	3/8.2	66	1.65E-13	90/6.0	13/39.7
Beta glucosidase	<i>Bn</i>	757740	58.5/6.2	285	5.49E-11	85/6.0	9/22	67	1.41E-08	90/5.9	9/26.6
Cruciferin	<i>Bn</i>	33284988	51.3/6.6	385	5.31E-05	32/6.1	2/6.2	225	1.27E-09	31/6.3	4/13.3
Cruciferin	<i>Bn</i>	33284988	51.3/6.6	418	7.36E-12	20/5.0	2/9	267	1.29E-07	21/4.9	4/12.9
Cruciferin	<i>Bn</i>	33284988	51.3/6.6					191	2.01E-07	30/6.5	3/9.9
Cruciferin	<i>Bn</i>	33284988	51.3/6.6					194	1.15E-06	30/6.2	4/12.2
Cruciferin	<i>Bn</i>	33284988	51.3/6.6					222	5.62E-04	29/9.0	2/6.2

Table 2. Continued

Protein name	Spec.	Acc. No	Theor. MW/pi	Reston				Westar			
				Spot No.	P (pro)	Exper. MW/pi	Pept./Cov.	Spot No	P (pro)	Exper. MW/pi	Pept./Cov.
CRU4_BRANA Cruciferin	<i>Bn</i>	461841	51.4/7.7					226	2.81E-09	27/6.6	2/7.3
CRU4_BRANA Cruciferin	<i>Bn</i>	461841	51.4/7.7					234	1.19E-09	28/7.9	2/8.4
CRU4_BRANA Cruciferin	<i>Bn</i>	461841	51.4/7.7					278	6.42E-12	22/9.5	4/14.2
CRU4_BRANA Cruciferin	<i>Bn</i>	461841	51.4/7.7					279	2.40E-08	22/8.9	6/14.4
Cruciferin subunit	<i>Bn</i>	12751302	54.4/8.1					207	1.70E-06	31/8.0	2/5.1
Cruciferin subunit	<i>Bn</i>	12751302	54.4/8.1					213	1.77E-06	36/9.0	3/6.1
Cruciferin subunit	<i>Bn</i>	12751302	54.4/8.1					248	3.80E-11	23/8.5	9/25.2
Cruciferin subunit	<i>Bn</i>	12751302	54.4/8.1					251	1.00E-12	23/9.7	12/26
Low molecular weight heat-shock protein	<i>Br</i>	2465461	17.7/5.6	417	2.98E-07	21/4.2	2/12.7				
Heat shock protein 22.0	<i>At</i>	7267721	22/5.9					245	1.51E-06	22/3.8	2/12.8
Heat shock protein 22.0	<i>At</i>	7267721	22/5.9					247	1.40E-05	22/4.0	2/12.8
Putative seed maturation protein	<i>At</i>	4559335	67.2/6.0	268	2.31E-04	95/5.1	2/4.1				
Glyceraldehyde-3-phosp. dehydrog., cyt.	<i>Dc</i>	462137	36.9/6.5					154	3.69E-11	40/6.7	7/27.2
Mitoch. import inner membr. transloc. sbu.	<i>At</i>	30683558	18.7/7.6					250	4.51E-06	21/6.3	2/12.4
Mitoch. import inner membr. transloc. sbu.	<i>At</i>	30683558	18.7/7.6					258	5.12E-07	21/8.0	2/18
ATP synthase alpha chain, mitochondrial	<i>At</i>	14916970	55.0/6.0					97	1.49E-06	80/5.5	2/4.5
Voltage-dep. anion-select. ch. prot.	<i>Br</i>	42601787	29.4/8.7					236	3.50E-08	30/9.5	5/26.4

Acc.No, accession number in database; *At*, *Arabidopsis thaliana*; *Bj*, *Brassica juncea*; *Bn*, *Brassica napus*; *Bn s p*, *Brassica napus subspecies pekinensis*; *Bo*, *Brassica oleracea*; Cov., percentage of coverage; *Dc*, *Dianthus caryophyllus*; Exper. MW/PI, experimental values for molecular weight and isoelectric point; P (pro), peptide probability; Pept., number of unique peptide matched; *Rn*, *Raphanus sativus*; Spec, plant species; Theor. MW/PI, theoretical values for molecular weight and isoelectric point;

body membrane monolayer from rice, soybean, and recently *Arabidopsis* [21, 23, 25, 40, 41]. In addition to these known oil body proteins, this proteomic investigation of oil body proteins from *B. napus* Reston and Westar revealed the presence of a protein annotated as a short-chain dehydrogenase/reductase enzyme from *A. thaliana* (Acc. No. 21700875). This protein was observed in both the 2-DE and MudPIT analyses and was resistant to 8 M urea dissociation. The high homology (77% identity, 88% similarity, 0% gaps) with a putative TAG-associated factor from narrow-leaved lupin, (*Lupinus angustifolius*; Acc. no AY143339.1) suggests this novel oil body protein may have an as yet unknown function in oil body structure, synthesis or degradation.

#### 4.3 Myrosinases, myrosinase binding proteins and myrosinase associated proteins in rapeseed oil body proteome

Proteomic analyses of oil bodies purified from seed of two *B. napus* cultivars revealed a surprising abundance of myrosinases, myrosinase binding proteins, and myrosinase associated proteins. Myrosinases, also referred to as thioglucoside glucosylhydrolases, catalyze the hydrolysis of glucosino-

lates. The study of *A. thaliana* ecotype WS oil body proteins reported recently by Jolivet *et al.*, [25] is the only proteomic analysis of isolated oil bodies. Based on their results no myrosinases were detected in the protein fraction of *Arabidopsis* oil bodies. Although both *A. thaliana* and *B. napus* are cruciferous plants, *A. thaliana* has only two myrosinase genes, and they are expressed exclusively in the phloem parenchyma, whereas the larger *Brassicaceae* members have approximately 20 genes expressed in both the ground tissue and phloem parenchyma [42–44]. In *Arabidopsis* seed, myrosinases are poorly expressed, whereas in *B. napus* seed members of all three myrosinase gene families are transcribed. Although glucosinolates are abundant in *Arabidopsis* seed there is a paucity of myrosinase activity, which is necessary to release the insecticidal thiocyanate compounds. Thus, in *Arabidopsis*, glucosinolates have been regarded as storage compounds used during later stages of germination [44].

It is known that myrosinases form myrosin grains present in idioblasts called myrosin cells [45]. In seeds of oilseed rape the myrosin cells are scattered throughout the tissue and constitute 2% to 5% of the total number of embryonic cells. They contain fewer storage lipids and have a high content of ER with myrosin grains forming continuous reticular

system, a “myrosin body” [46, 47]. Yet, myrosinases could be detected even in protein fractions of very stringently washed oil body preparations (buffer with 8 M urea, 30 min at RT on shaker). This could indicate that oil bodies in myrosin cells could be tightly associated with myrosinases *in vivo*. Alternatively, myrosin grains may adhere to oil bodies after being released from myrosin cells during oil body preparation, although we did not observe these structures in any TEM micrographs of isolated oil bodies.

Using 2-DE for protein separation prior to analysis by MS, multiple isoelectric species of myrosinases, myrosinase binding proteins, and myrosinase associated proteins were detected and characterized in purified oil body protein preparations from both rapeseed cultivars (Table 2). Some of them could be isoforms resulting from polymorphisms in coding regions of corresponding genes. For example, there are at least three different families of myrosinase genes in *B. napus* (MA, MB, and MC, [42]) that could have emerged because of the amphidiploid nature of this species. However, it is more likely that the existence of several myrosinase isoelectric species is due to glycosylation. All known myrosinases are glycosylated and the sites for N-linked glycosylation are not conserved which leads to myrosinase isoforms with different amino acid sequences as well as multiple posttranslational modifications due to varying degrees of glycosylation [48].

A protein annotated as myrosinase associated protein (GI 1216389) was identified by both 2-DE and MudPIT proteomic analyses of oil body proteins from both cultivars (Table 2, 3, 4). This protein shares homology (70% identity, 81% similarity, 5% gaps) to an *A. thaliana* enzyme (GI 4587543) that belongs to the PFI 00657 lipase/acylhydrolase family containing a GDSL-motif. The function of myrosinase associated proteins and myrosinase binding proteins is unknown at present, except that certain myrosinase binding proteins were found to be important for complete formation of myrosinase isoenzymes in *B. napus* [49, 50]. Thus, it is possible that at least some of the proteins classified as myrosinase associated or myrosinase binding proteins could function as lipases (as sequence homology indicates) and as such be associated with oil bodies while others could be associated with other organelles in plant cells. In support of the notion, myrosinase binding protein GI 7488496 was previously identified as an integral part of a “crystalloid” fraction of *B. napus* protein storage vacuoles [51]. Furthermore, a GDSL-acylhydrolase, believed to be involved in oil body break down, was recently characterized to be associated with the surface of oil bodies in Arabidopsis seed [52].

#### 4.4 Seed storage protein contamination of oil bodies

As it is always difficult to purify any organelle to homogeneity it was not unexpected that storage proteins from protein storage vesicles were present even in protein samples from urea washed oil bodies (Table 3 and 4). This

is not surprising, considering that napin and cruciferin constitute approximately 20% and 60% of total protein in mature rapeseed, respectively [53]. Moreover, it was previously reported that storage lipids were detected in significant amounts within *in vitro* preparations of protein bodies from rice and soybean endosperm [54]. Recently, Gillespie *et al.* [55] reported that protein storage vesicles from *Brassicaceae* contain internalized membranes in crystalloid like structure of protein bodies. It is therefore possible that hydrophobic internal protein body membranes adhere to oil bodies, which makes it very difficult or even impossible to isolate oil bodies without contamination with storage proteins even after stringent washing with salt and urea.

#### 4.5 Conclusion

This study investigates the oil body proteomes from two *B. napus* cultivars, Reston and canola cv. Westar, along with systematic analyses of polar and neutral lipid components of this organelle. To analyze oil body protein fractions, two different proteomic approaches, in combination with stringent database search conditions, were applied. While 2-DE allowed for the identification of multiple isoelectric species of numerous oil body proteins in a semi-quantitative manner, MudPIT analyses mostly confirmed the results achieved with 2-DE and also allowed for the identification of some proteins that were not identified using a 2-DE approach. Overall, the proteins identified using the MudPIT approach had higher values for protein coverage and probability than proteins identified from 2-DE gels. However, by most assessments these two protein identification approaches yielded similar results and therefore appear more redundant rather than opposing or complementary, as they are frequently portrayed in the literature.

Proteomic analysis of Reston and Westar cultivars of *B. napus*, both 2-DE and MudPIT, revealed a similar set of proteins. Although a more quantitative protein comparison of oil bodies from these two cultivars may reveal subtle differences, it is apparent that the overall protein composition of oil bodies from these two cultivars is very similar. The high degree of similarity suggests the differences in membrane PLs and matrix TAG lipids between these two cultivars has a minimal effect on the protein structure of oil bodies as a whole. Although no apparent protein compositional differences were detected, systematic analysis of two cultivars served as confirmation for the novel oil body associated proteins reported here.

In conclusion, this study gives insight into possible associations between cell proteins and oil body organelles in rapeseed embryonic cells. The high level of similarity of one of the identified proteins to a lipase and the other one to a putative TAG-associated factor strongly indicates possible association of these novel

proteins to rapeseed oil bodies *in vivo*. Further biochemical analysis of these proteins are underway to verify this association and what role, if any, they have in oil body function.

*Drs. Maurice Moloney and Elizabeth Murray from Sem-BioSys Genetics Inc., Calgary, AB, Canada are gratefully acknowledged for providing mAb aroleom D9 (anti-Arabidopsis oleosin D9). We thank Cheryl Jansen (Electron Microscopy Core, University of Missouri-Columbia) for processing the samples for EM.*

## 5 References

- [1] Cummins, I., Murphy, D. J., in: Quinn, P. J., Harwood, J. L. (Eds.), *Plant Lipid Biochemistry, Structure, and Utilization*, Portland Press, London 1990, pp. 231–233.
- [2] Jacks, T. J., Hensarling, T. P., Neucere, J. N., Yatsu, I. Y., Barker, R. H., *J. Am. Oil Chem. Soc.* 1990, **67**, 353–361.
- [3] Frey-Wyssling, A. E., Grieshaber, E., Muhlthaler, K., *J. Ultrastruct. Res.* 1963, **8**, 506–516.
- [4] Herman, E. M., *Planta* 1987, **172**, 336–345.
- [5] Huang, A. H. C., Qu, R., Lai, Y. K., Ratnayake, C. et al., in: Emes, M. J. (Eds), *Structure, Synthesis and Degradation of Oil Bodies in Maize in Compartmentation of Plant Metabolism*, Cambridge University Press, Cambridge 1990, pp. 43–58.
- [6] Murphy, D. J., Cummins, I., Kang, A. S., *Biochem. J.* 1989, **258**, 285–293.
- [7] Leprince, O., van Aelst, A. C., Pritchard, H. W., Murphy, D. J., *Planta* 1998, **204**, 109–119.
- [8] Vance, V. B., Huang, A. H. C., *J. Biol. Chem.* 1987, **262**, 11275–11279.
- [9] Rosnitschek, I., Theimer, R. R., *Planta*, 1980, **148**, 193–198.
- [10] Huang, A. H. C., *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 1992, **43**, 177–200.
- [11] Holbrook, L. A., Van Rooijen, G. J. H., Wilen, R. W., Moloney, M. M., *Plant Physiol.* 1991, **97**, 894–899.
- [12] Aalen, R. B., *Plant Mol. Biol.* 1995, **28**, 583–588.
- [13] Lee, K., Ratnayake, C., Huang, A. H. C., *Plant J.* 1995, **7**, 603–611.
- [14] Peng, C.-C., Tzen, J. T. C., *Plant Cell Physiol.* 1998, **39**, 35–42.
- [15] Lacey, D. J., Wellner, N., Beaudoin, F., Napier, J. A., Shewry, P. R., *Biochem. J.* 1998, **334**, 469–477.
- [16] Milichip, M., Tatham, A. S., Jackson, F., Griffiths, G. et al., *Biochem. J.* 1996, **314**, 333–337.
- [17] Murphy, D. J., Cummins, I., *Plant Science* 1989, **60**, 47–54.
- [18] Moreau, R. A., Liu, K. D. F., Huang, A. H. C., *Plant Physiol.* 1980, **65**, 1176–1180.
- [19] Kalinski, A., Loer, D. S., Weisemann, J. M., Matthews, B. J., Herman, E. M., *Plant Mol. Biol.* 1991, **17**, 1095–1098.
- [20] Beisson, F., Ferte, N., Vouloury, R., Arondel, V., *Plant Physiol. Biochem.* 2001, **39**, 1–8.
- [21] Frandsen, G. I., Mundy, J., Tzen, J. T. C., *Physiol. Plantarum* 2001, **112**, 301–307.
- [22] Chen, E. C. F., Tai, S. S. K., Peng, C.-C., Tzen, J. T. C., *Plant Cell Physiol.* 1998, **39**, 935–941.
- [23] Chen, E. C. F., Tsai, S. S. K., Tzen, J. T. C., *Plant Cell Physiol.* 1999, **40**, 1079–1086.
- [24] Lin, L.-J., Tai, S. S. K., Peng, C.-C., Tzen, J. T. C., *Plant Physiol.* 2002, **128**, 1200–1211.
- [25] Jolivet, P., Roux, E., d'Andrea, S., Davanture, M. et al., *Plant Physiol. Biochem.* 2004, **42**, 501–509.
- [26] Washburn, M. P., Wolters, D., Yates, J. R. III, *Nat. Biotechnol.* 2001, **19**, 242–247.
- [27] Tzen, J. T. C., Peng, C.-C., Cheng, D.-J., Chen, E. C. F., Chiu, J. M. H., *J. Biochem.* 1997, **121**, 762–768.
- [28] Tzen, J. T. C., Huang, A. H. C., *J. Cell Biol.* 1992, **117**, 327–335.
- [29] Bradford, M. M., *Anal. Biochem.* 1976, **72**, 248–254.
- [30] Thelen, J. J., Mekhedov, S., Ohlrogge, J. B., *Plant Physiol.* 2001, **125**, 2016–2028.
- [31] Bligh, E. G., Dyer, W. J., *Can. J. Biochem. Physiol.* 1959, **37**, 911–917.
- [32] Hajdich, M., Ganapathy, A., Stein, J. W., Thelen, J. J., *Plant Physiol.* 2005, **137**, 1397–1419.
- [33] Stymne, S., Stobart, K., in: Stumpf, P. K. (Eds), *The Biochemistry of Plants: A Comprehensive Treatise*, Academic, New York 1987, pp. 175–214.
- [34] Dahlqvist, A., Ståhl, U., Lenman, M., Banas, A. et al., *Proc. Natl. Acad. USA*, 2000, **6**, 6487–6492.
- [35] Ståhl, U., Carlsson, A. S., Lenman, M., Dahlqvist, A. et al., *Plant Physiol.* 2004, **135**, 1324–1325.
- [36] Han, J., Lühs, W., Sonntag, K., Borchardt, D. S. et al., *Plant Mol. Biol.* 2001, **46**, 229–239.
- [37] Roscoe, T. J., Lessire, R., Puyaubert, J., Renard, M., Delseny, M., *FEBS Lett.* 2001, **492**, 107–111.
- [38] Katavic, V., Mietkiewska, E., Barton, D. L., Giblin, M. E. et al., *Eur. J. Biochem.* 2002, **269**, 5625–5631.
- [39] Katavic, V., Barton, D. L., Giblin, M. E., Reed, D. W. et al., *FEBS Lett.* 2004, **562**, 118–124.
- [40] Nuccio, M. L., Thomas, T. L., *Plant Mol. Biol.* 1999, **39**, 1153–1163.
- [41] Takahashi, S., Katagiri, T., Yamaguchi-Shinozaki, K., Shinozaki, K., *Plant Cell Physiol.* 2000, **41**, 898–903.
- [42] Rask, L., Andréasson, E., Ekblom, B., Eriksson, S. et al., *Plant Mol. Biol.* 2000, **42**, 93–113.
- [43] Eriksson, S., Ek, B., Xue, J., Rask, L., Meijer, J., *Physiol. Plant.* 2001, **111**, 353–364.
- [44] Andréasson, E., Jørgensen, L. B., Höglund, A.-S., Rask, L., Meijer, J., *Plant Physiol.* 2001, **127**, 1750–1763.
- [45] Thangstad, O. P., Evjen, K., Bones, A. M., *Protoplasma*, 1991, **161**, 85–93.
- [46] Höglund, A. S., Lenman, M., Falk, A., Rask, L., *Plant Physiol.* 1991, **95**, 213–221.
- [47] Höglund, A. S., Lenman, M., Rask, L., *Plant Sci.* 1992, **85**, 165–170.

- [48] Peškan-Berghöfer, T., Shahollari, B., Huong-Giong, P., Hehl, S. *et al.*, *Physiol. Plantarum*, 2004, 122, 465–477.
- [49] Eriksson, S., Andréasson, E., Ekbohm, B., Granér, G. *et al.*, *Plant Physiol.* 2002, 129, 1592–1599.
- [50] Lenman, M., Falk, A., Rödin, J., Höglund, A-S. *et al.*, *Plant Physiol.* 1993, 103, 703–711.
- [51] Taipalensuu, J., Falk, A., Ek, B., Rask, L., *Eur. J. Biochem.*, 1997, 243, 605–611.
- [52] Körner, M., Rudolph, M., Wang, X., Athenstaedt, K. *et al.*, *Proceedings of the Second European Symposium on Plant Lipids*. 2006, Copenhagen, Denmark.
- [53] Höglund, A-S., Rödin, J., Larsson, E., Rask, L., *Plant Physiol.* 1992, 98, 509–515.
- [54] Pernollet, J-C., *Phytochemistry* 1978, 17, 1473–1480.
- [55] Gillespie, J., Rogers, S. W., Deery, M., Dupree, P., Rogers, J. C., *Plant J.* 2005, 41, 429–441.