Selective One-Step Extraction of *Arabidopsis thaliana* Seed Oleosins Using Organic Solvents

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Oleosins are hydrophobic proteins from oleaginous seeds, surrounding and stabilizing oil bodies. They are known to display interesting interfacial properties. Specific sera were raised against four different *A. thaliana* oleosins and used in dot-blot assays for oleosin quantification. These assays were used to set up extraction of oleosins from *A. thaliana* seeds. One mixture of chloroform/methanol gave optimal oleosin extraction. Extracted proteins represented 9% of seed proteins and were identified by immunoblot and proteomic analyses. Oleosins accounted for 79% of the extracted proteins. This simple one-step procedure allows selective extraction and concentration of oleosins from seeds without tedious oil body purification. Oleosin extract was indeed used to demonstrate the presence of the rare oleosin S5 in mature seeds. Moreover, this method will be useful to investigate the potential use of oleosins as emulsifier and to question their possible allergenicity.

KEYWORDS: Oleosin; hydrophobic protein; organic solvent; extraction; seed; *Arabidopsis thaliana*

INTRODUCTION

In cells, lipids are stored in oil bodies, also called lipid droplets or lipid bodies. They are composed of a core of triacylglycerols surrounded by a monolayer of phospholipids in which different proteins are inserted (*1, 2*). In oilseeds, the major proteins present in oil bodies are belonging to the oleosin family. Oleosins are low-molecular-mass amphiphatic proteins (15–26 kDa) containing a long central hydrophobic domain flanked by two terminal hydrophilic domains (*3*). Although the central hydrophobic sequence is well-conserved among oleosin isoforms, the N- and C-terminal domains are more divergent in sequence and length. This hydrophobic domain is the longest known to date, with ∼70 hydrophobic or neutral residues. Even if there is still uncertainty about the structure of the hydrophobic domain, it is generally accepted that it resides within the lipid matrix, forming an hairpin structure consisting of two segments connected by a proline-rich sequence called proline-knot (*4, 5*). Indirect observations have suggested that oleosins maintain the small size (diameters of 0.5–2 μM) and the stability of seed oil bodies and prevent their coalescence during seed dessication and/or imbibition. Using *Arabidopsis thaliana* lines deficient in a major oleosin, Siloto et al. have recently demonstrated that oleosins are effectively controlling oil body size and lipid accumulation (*6*).

As expected from their biological role, oleosins were able to stabilize artificial emulsions (*5, 7, 8*). Moreover, *A. thaliana* oleosins were shown to decrease the interfacial tension at the oil/water interface more efficiently than β-casein (*9*). These results strengthen the interest in exploring potential applications of oleosins as natural emulsifiers in products such as food and cosmetics.

In 2002, Kim et al. published an extensive analysis of oleosin gene expression in *A. thaliana* (*7*). They identified 16 oleosin genes and classified them into three groups according to their tissue-specific expression. The first group includes five genes specifically expressed in maturing seeds, which they named oleosins S1, S2, S3, S4, and S5. The second group consists of three genes expressed in both maturing seeds and florets (microspores) that were named SM1, SM2, and SM3. The eight remaining genes fall into the third group of genes specifically expressed in the florets (tapetum). In 2004, we described the protein composition of purified oil bodies from *Arabidopsis thaliana*, after SDS-PAGE separation and nano-LC-MS/MS analysis of trypsin peptides (*10*). From the eight proteins identified, the most abundant ones were belonging to the oleosin family, but only 4 oleosins (S1, S2, S3, and S4) were detected, whereas 8 were expected in seeds from the mRNA expression data published by Kim et al. (*7*). The 4 missing isoforms S5, SM1, SM2, and SM3 may be either nontranslated or degraded at the mature stage or most probably rare. A method to enrich oleosins would help to test the last hypothesis.

The aim of the present work was to set up a simple and rapid protocol for specific extraction of oleosins from *A. thaliana* seeds. This oleosin extract would be useful in investigating the presence of rare oleosins in seeds. Moreover, such an extract would be expected to display emulsifying properties that could be investigated and compared to data obtained on recombinant oleosins (*9*). As a first step, this study focused on the plant model *A. thaliana*. The final purpose would be to obtain extracts

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Table 1. Plasmids for Expression of Tagged *A. thaliana* Oleosins (complete or truncated) in *E. coli*

		plasmid		recombinant protein	
oleosin	gene (GenBank accession no.)	name	PCR primers (5'—3')[a]	name[b]	fusion[c]
S1	At3g01570 (AAF01542)	pET20/S1	GGAATTCCATATGGCCGACGTTCGCACACACT	rS1	S1–6H
			CCGCTCGAGTGTAGTCTTGGTGTCTCGGACATTATG		
		pGEX4T1/S1N	GTACAAGGATCCATGGCCGACGTTCGCACACAC	rS1N	GST–S1 (M^1–P^{32})
			GGAATTGTCGACTCAAAGGGCCACTCTGAGGATAAAGG		
S2	At3g27660 (BAB02690)	pET20/S2	GGAATTCCATATGGCGAATGTGGATCGTGATCGG	rS2	S2–6H
			CAATCGCTCGAGTGAGCCTCTCCTGGCCTTACC		
		pGEX4T1/S2N	GTACAAGGATCCATGGCGAATGTGGATCGTGATCGG	rS2N	GST–S2 (M^1–Q^{51})
			GGAATTGTCGACTCATTGGTTAGTGGAGGGGCCGC		
S3	At4g25140 (CAA44225)	pET20/S3	see ref *9*	rS3	S3–6H
		pGEX4T1/S3N	GTACAAGGATCCATGGCGGATACAGCTAGAGGAAC	rS3N	GST–S3 (M^1–Q^{42})
			GGAATTGTCGACTCACTGCCTAGACTTGGAGTAGTCAGA		
S4	At5g40420 (AB006702)	pET20/S4	see ref *9*	rS4	S4–6H
S5	At5g51210 (BAA97384)	pET20/S5	GGAATTCCATATGGCGGACCATACAAGAACC	rS5	S5–6H
			CCGCTCGAGAGAAACTTGTTGGTGTTGGACTCC		
		pGEX4T1/S5N	GTACAAGGATCCATGGCGGACCAAACAAGAACCCAT	rS5N	GST–S5 (M^1–Q^{26})
			GGAATTGTCGACTCACTGCCTGGCCCTTCGGATGG		
SM2	At3g18570 (AP001303)	pET20/SM2	GGAATTCCATATGGCGGAAAGGTTCTCTAGCGG	rSM2	SM2–6H
			GGAATTGTCGACAGCACCCAGGGGCCGCATCTTTA		
SM3	At2g25890 (AC005395)	pET20/SM3	GGAATTCCATATGGTAAGCTTATTAAAGCTACAAAAAC	rSM3	SM3–6H
			CCGCTCGAGATGAGTAGTTGTTGTGGTTTGGTGTG		

[a] Restriction sites are underlined, stop codons are in italics, and gene sequences are shown in bold. [b] Underlined text indicates protein used as an immunogen for protein serum production. [c] The fusion indicates the expressed oleosin sequence and the tag position. The tag is glutathione-S-transferase (GST) or poly histidine (6H).

enriched in oleosins from various oilseeds like rape seeds and to evaluate their potential use as a plant emulsifier.

To specifically solubilize oleosins from seeds, we adapted the method originally developed by Seigneurin-Berny (*11*) to extract the most hydrophobic proteins present in chloroplast envelope membranes. This method was based on the differential solubilization of membrane proteins in chloroform/methanol mixtures, which allows extracting and concentrating the most hydrophobic proteins. It was successfully used to decipher plant membrane proteomes, as reviewed by Rolland (*12*). This chloroform/methanol extraction was preferred to methods based on differential solubilization in detergents, as we were interested in studying interfacial properties of the oleosin extract. In addition, even if oleosin solubilization was described in 1.5% SDS (*13*), detergents are not suitable for systems like seeds or oil bodies that contain a high lipid to protein ratio. Furthermore, when methods for differential solubilization of membrane proteins were compared, chloroform/methanol extractions seemed to be the best possible compromise to combine enrichment of highly hydrophobic proteins and extensive elimination of the hydrophilic ones (*14*).

So far, only one report has described the purification of oleosins using organic solvent (*15*). This extraction protocol was set up on almond seeds. In a previous study, our attempts to extrapolate this procedure to *A. thaliana* seeds failed: oleosins were not solubilized in the organic phase but present at the interface (*10*). This observation is consistent with almond oleosin having shorter hydrophilic domains (and consequently being more hydrophobic) than *A. thaliana* oleosins. In the present work, we optimized the selective extraction of *A. thaliana* oleosins using a chloroform/methanol mixture. The optimized one-step protocol allows their specific enrichment directly from seeds, without tedious oil body purification. The amount of extracted oleosins was estimated by dot-blot using specific antibodies developed in our laboratory against recombinant S1, S2, S3, S4, or S5.

MATERIALS AND METHODS

Biological Materials and Reagents. Genomic DNA and developing seed cDNA of *A. thaliana* ecotype Wassilewslija (WS) and mature seeds of *A. thaliana* ecotype Columbia (Col-0) were a generous gift from M. Miquel (Versailles, France). Plasmid PAP003 encoding oleosin S5 isolated from dry seeds of *A. thaliana* ecotype Col-0 was obtained from the *Arabidopsis* Biological Resource Center (OH). Plasmids pET20/S3 and pET20/S4, encoding *A. thaliana* oleosins S3 and S4, respectively, and rabbit serum raised against purified recombinant oleosin S4 were obtained previously, as described by Roux et al. (*9*).

Chemicals were from Sigma-Aldrich (Saint Quentin Fallavier, France), unless stated otherwise. Enzymes and kits for molecular biology and competent *Escherichia coli* cells were from Promega (Charbonnières-les-Bains, France).

Oil Body Purification. The oil body fraction CF4 was prepared from mature WS *A. thaliana* seeds according to ref *10*. Protein content of the fraction was quantified according to Lowry (*16*) using bovine serum albumin as standard, after protein precipitation with 2 vol of cold acetone and resuspension in 0.1 M NaOH containing 0.1 % (w/v) SDS.

Plasmid Constructions for Bacterial Expression of *A. thaliana* Oleosins. DNA fragment encoding oleosins S1 (Genbank accession no. AY059936) and S2 (Genbank accession no. NM_113682) were obtained by PCR amplification using cDNA isolated from *A. thaliana* WS ecotype developing seeds as template. Template for PCR amplification of oleosin S5 (Genbank accession no. X91913) was plasmid PAP003. The intronless SM2 (Genbank accession no. AP001303) and SM3 (Genbank accession no. AC005395) genes were amplified using *A. thaliana* genomic DNA (WS ecotype) as template. Amplification was performed in the Mastercycler Gradient thermocycler from Eppendorf (Le Pecq, France) using the high-fidelity *Pfu* polymerase and specific primers described in **Table 1**. PCR fragments were cloned into *Nde*I and *Xho*I sites of pET-20b(+) vector (Novagen, Fontenay, France), so as to add a histidine tag at the C-terminal end of the recombinant protein. The sequence of the resulting plasmids, herein referred to as pET20/S1, pET20/S2, pET20/S5, pET20/SM2, and pET20/SM3, were assessed by DNA sequencing. When compared to the expected sequence from the Genbank database, plasmids pET20/S5, pET20/SM2, and pET20/SM3 showed no difference, whereas pET20/S2 displayed one silent mutation and one substitution (G152S) and pET20/S1 displayed one silent mutation. The substitution on oleosin S2 may result from polymorphism, as it was present on another independent clone and found in two GenBank sequences.

PCR amplification of DNA fragments encoding the N-terminal hydrophilic domain of oleosins S1, S2, S3, and S5 were obtained using respective pET20 plasmids as template and specific primers (**Table 1**). PCR fragments were cloned into *Bam*H I and *Sal*I sites of the

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pGEX4T1 vector (GE Healthcare, Orsay, France), so as to add a glutathione-S-transferase (GST) tag at the N-terminal end of the recombinant protein. The sequence of the resulting plasmids, herein referred as to pGEX4T1/S1N, pGEX4T1/S2N, pGEX4T1/S3N and pGEX4T1/S5N, were assessed by DNA sequencing and shown to be identical to the expected sequence from database.

Bacterial Expression of *A. thaliana* Oleosins. Recombinant protein expression was induced in BL21 *E. coli* strain with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Bacteria were quantified by measuring absorbance at 600 nm (A_{600}). After centrifugation at 5000 g for 10 min, bacterial pellets were used for SDS-PAGE analysis or for protein purification (see following sections).

Production of Sera. Sera anti-rS1N, anti-rS2N, anti-rS3N, and anti-rS5N were produced by immunizing rabbits with bacterially expressed proteins encoded in pGEX4T1/S1N, pGEX4T1/S2N, pGEX4T1/S3N, and pGEX4T1/S5N, respectively. These antigens were GST fusion proteins containing the N-terminal hydrophilic domain of each oleosin (see **Table 1** for fusion description). They were affinity-purified on glutathione-Sepharose (GE Healthcare, Orsay, France) as previously described (*17*). A second serum against oleosin S3, named anti-rS3 serum, was raised using inclusion bodies of rS3 as immunogen. These inclusion bodies were prepared as follow: IPTG-induced bacteria were centrifuged, resuspended at 20 A_{600}/mL in resuspension buffer (100 mM NaH$_2$PO$_4$, 10 mM Tris, 140 mM NaCl, pH 8) containing 1 mg/mL lysozyme, lysed by addition of 1 volume of lysis buffer (1 % Triton X-100, 1 % Tween-20 in resuspension buffer), sonicated, and centrifuged (15 000 g, 15 min, 4 °C). Inclusion bodies containing rS3 were prepared by washing the pellet with 1% *n*-dodecyl α-D-maltoside in resuspension buffer and with 1.5 M urea in resuspension buffer without NaCl, successively. Rabbits were immunized by three successive subcutaneous injections at 2 week intervals. At monthly intervals, three boosters were administered subcutaneously and blood was collected 2 weeks after the last injection. Antigen (150–300 μg) was emulsified by sonication in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for the following ones.

Rabbit polyclonal serum against 2S napin was obtained by subcutaneous immunization with the purified protein obtained according to Bérot et al. (*18*). One mg of 2S in PBS (10 mM Na$_2$HPO$_4$, 150 mM NaCl, pH 7.2), emulsified with complete (first injection), or incomplete (boost injections) Freund's adjuvant was injected every 15 days. The serum was collected after the 4th injection.

SDS-PAGE. Protein electrophoresis was carried out in Tris-glycine gels containing 16% acrylamide, according to Laemmli (*19*). Alternatively, ready-to-use NuPAGE Novex 12% Bis-Tris gels and NuPAGE MES SDS running buffer from Invitrogen (Cergy, France) were used. Samples were prepared in Laemmli buffer (65.2 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 0.005% bromophenol blue) including 5% β-mercaptoethanol when reduced. Gels were stained with G-250 Coomassie blue according to Neuhoff et al. (*20*). Molecular mass markers were Mark12 (for Coomassie staining) and MagicMark XP (for immunoblot), both from Invitrogen. Gels were scanned (600 dpi) using an Epson Expression 1680 Pro scanner, and the TIFF resulting gels were analyzed using the Image Quant software (version 4.2a) from Molecular Dynamics (GE Healthcare, Orsay, France).

Immunoblot and Dot-Blot. For immunoblot analysis, proteins resolved by SDS-PAGE were blotted on to Immobilon-P PVDF membrane (Millipore, Molsheim, France). For dot-blot, proteins solubilized in organic solvents were spotted directly on to PVDF membrane. Usually, 2.5–10 μL of organic extract was spotted drop by drop using a transferpettor (Brand, Wertheim, Germany) and letting the membrane dry after each drop. Proteins solubilized in aqueous buffer were spotted after wetting the membrane in ethanol and in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) successively. The membrane was probed with rabbit serum at various dilutions: 1:5000 dilution for anti-rS2N, anti-rS3N and anti-rS3 sera, 1:4000 dilution for anti-rS1N serum, 1:2000 for anti-rS4 and anti-rS5N sera, and 1:1000 for antinapin serum. Rabbit antibodies were revealed with peroxydase-conjugated goat antirabbit IgG from Pierce (Perbio Science, Brebières, France). Saturation and incubation with antibodies were carried out for 90 min in TBS containing 0.05% Tween-20 and 5 % skimmed dry milk. After incubation with antibodies, the membrane was washed three times in

TBS containing 0.05% Tween-20. Peroxidase activity was revealed using SuperSignal West Dura Extended Duration Substrate from Pierce according to the manufacturer protocol, and the membrane was exposed for a short period (from 10 s to 5 min) to Kodak Biomax XAR film. Film was scanned (600 dpi) using an Epson Expression 1680 Pro scanner. The TIFF resulting files of dot-blot were analyzed using the Image Quant software (version 4.2a) from Molecular Dynamics (GE Healthcare). Alternatively, luminescence from peroxidase activity was recorded using the LAS-3000 imaging system and MultiGauge software from Fujifilm (St Quentin en Yvelines, France).

Extraction of Oleosins from Seeds. Mature seeds of *A. thaliana* ecotype Col-0 were ground with a glass Potter-Elvehjem grinder at 8 mg of seeds/mL. Several grinding buffers were assayed: 50 mM Na carbonate at pH 11, 10, and 9; 50 mM Tris-HCl at pH 9, 8, 7, and 6; 50 mM Na citrate at pH 5, 4, and 3. Extraction was performed at room temperature by slowly adding 9 volumes of chloroform/methanol mixture at various ratios, from 0/9 to 9/0 (v/v). After being mixed and sonicated for 30 s using a bath sonicator, the extract was centrifuged (20 000 g, 15 min, 20 °C) and the organic phase was collected. For dot-blot analysis, the organic phase was directly spotted on to PVDF membrane, as described above. For SDS-PAGE analysis, organic solvents were evaporated under a stream of N$_2$ and proteins were solubilized in Laemmli buffer.

Seeds were also ground at 8 mg/mL in the following media: nonreducing Laemmli buffer, reducing IEF buffer (7 M urea, 2 M thiourea, 2% Chaps, 25 mM dithiothreitol, 1% pharmalytes,) and TFE/TFA 50/0.01 (50% trifluoroethanol and 0.01% trifluoroacetate in water). Supernatants obtained after centrifugation were analyzed by SDS-PAGE after appropriate dilution in Laemmli buffer.

Protein Measurement. Protein amounts in ground seeds and seed extracts were determined by amino acid measurement after alkaline hydrolysis, using bovine serum albumin as standard. The protocol was adapted from Landry & Delhaye (*21*) as follows. Briefly, proteins were hydrolyzed in 3 M NaOH at 130 °C for 45 min. After centrifugation, 100 μL of supernatant was incubated for 15 min at 100 °C with 100 μL of 35% (v/v) acetic acid, 400 μL of 60% (v/v) 2-methoxyethanol, 150 μL of 0.2 mM KCN in 2.6 M Na acetate, 150 μL of 3% nihydrin in 2-methoxyethanol. After cooling, absorbance was measured at 570 nm.

Protein Identification by LC-MS/MS. Protein bands stained with Coomassie blue were excised and digested as described in ref *10*.

HPLC was carried out with a Spectra System equipment (Thermo Separation Products, Riviera Beach, USA) using a reversed-phase BioBasic-18 column (1 × 150 mm, 300 Å pore size, 5 μm film thickness, Thermo Electron Corporation). The elution was proceed at a flow-rate of 0.1 mL min^{-1} at 20 °C with 5% of solvent B (acetonitrile + 0.1% formic acid) in A (water + 0.1% formic acid) for 2 min and then with a linear gradient of B in A from 5 to 45% over 40 min and then 45 to 95% over 5 min before re-equilibration. Eluant from the column was introduced in the electrospray ionisation source of a Thermo Electron LCQ Deca ion-trap mass spectrometer operating in the positive ion mode. Instrumental parameters were capillary temperature 280 °C, capillary voltage 30 V, spray voltage 4.5 kV, sheath gas flow 80 a.u., auxiliary gas flow 5 a.u. Mass spectra were acquired scanning from m/z 200 to 2000. MS/MS experiments were carried out using a normalized collision energy of 35 a.u. Peptide ions were analyzed using the data-dependent "triple-play" method as follows: (i) full MS scan, (ii) ZoomScan (scan of the major ions with higher resolution to determine their charge), (iii) MS/MS of these ions.

Protein identification was performed with Bioworks 3.1 software using *Arabidopsis* protein sequences downloaded from the National Center for Biotechnology Information FTP site. No enzyme specificity was set for the query. The output data were evaluated in term of (i) trypsin nature of peptides, (ii) Xcorr magnitude up to 1.5, 2.2, and 3.3 for mono-, di-, and tricharged peptides, respectively, to minimize false positives, (iii) dCN higher than 0.1.

RESULTS AND DISCUSSION

Cloning and Expression of *A. thaliana* Seed Oleosins. The two seed-specific oleosins S3 and S4 were cloned for bacterial

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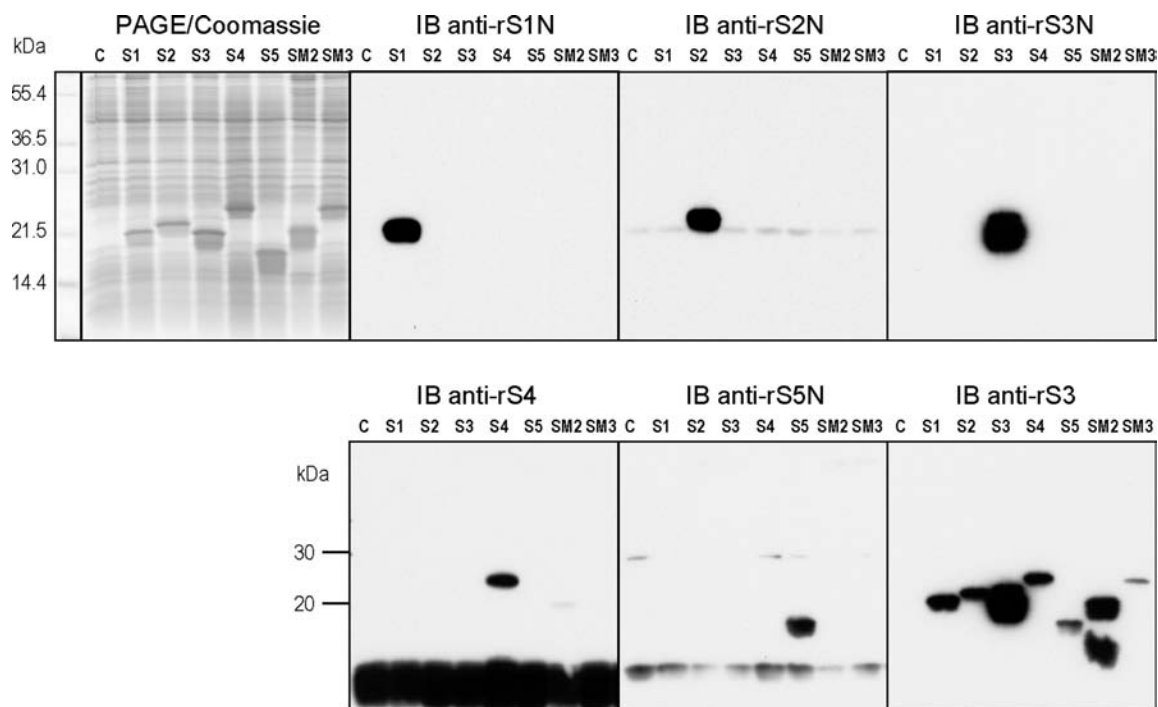*J. Agric. Food Chem.,* Vol. 55, No. 24, 2007 **10011**

Figure 1. Specificity of antioleosin sera towards recombinant oleosins. Recombinant *A. thaliana* oleosins S1, S2, S3, S4, S5, SM2, and SM3 were expressed in *E. coli*, after IPTG induction. Bacterial lysates in Laemmli buffer were resolved by SDS-PAGE before Coomassie blue staining (PAGE/Coomassie; 0.1 A_{600}/lane) or blotting (IB; 0.001 A_{600}/lane). The blots were probed with anti-rS1N, -rS2N, -rS3N, -rS4, -rS5N, or -rS3 sera. Detection was performed using chemiluminescence. Molecular masses are indicated in kDa. This experiment is representative of two.

expression in our laboratory, as reported in a previous work (*9*). In the current study, we describe the cloning and expression of the three seed-specific oleosins S1, S2, and S5, obtained after PCR amplification on developing seed cDNA. In addition, two oleosins out of the three expressed in both maturing seeds and floral microspores (SM oleosins) were selected for cloning and bacterial expression, based on their gene expression during seed development. The selected oleosins SM2 and SM3 were shown to display maximal mRNA expression in mature seeds, contrary to SM1 (*7*). Therefore, from the eight *A. thaliana* oleosins expected to be present in seeds according to their gene expression analysis (*7*), seven were produced as His-tagged protein in transformed BL21 *E. coli* after IPTG induction and referred as to rS1, rS2, rS3, rS4, rS5, rSM2, and rSM3. On SDS-PAGE (**Figure 1**, PAGE/Coomassie), rS1, rS2, rS3, rS4, rS5, and rSM2 displayed an apparent molecular mass in close agreement with the predicted mass taking the poly His tag into account (21.0, 21.4, 19.8, 22.5, 16.1, and 19.4 kDa, respectively), whereas the rSM3 apparent molecular mass was slightly higher than the calculated one (23.7 instead of 20.4 kDa).

Antibodies Allowing Specific Detection of *A. thaliana* Seed Oleosins. Different sera were obtained by immunizing rabbits against bacterially expressed *A. thaliana* oleosins (**Table 1**). Some sera were raised against the most divergent fragments of S1, S2, S3, and S5 oleosins, i.e., their N-terminal domain. They are referred as to anti-rS1N, anti-rS2N, anti-rS3N and anti-rS5N sera. Specificity of these sera was assessed by immunoblot analysis against nonpurified rS1, rS2, rS3, rS4, rS5, rSM2, and rSM3 produced as described above. Anti-rS1N serum recognized one single band at the apparent molecular mass of rS1 (20.8 kDa) only in bacteria producing rS1 (**Figure 1**, IB anti-rS1N), showing its high specificity towards S1 oleosin. Similarly, anti-rS2N, anti-rS3N, and anti-rS5N sera were shown to be strictly specific, as they recognized immunizing oleosin without any

cross-reactivity towards others oleosins (**Figure 1**, IB anti-rS2N, -rS3N, and -rS5N).

Another serum was developed against S3 oleosin, using the full protein rS3 as immunogen. Recombinant S3 expressed in *E. coli* was produced in inclusion bodies (*9*) and contaminant bacterial proteins were removed from these inclusion bodies using detergents and urea, leading to an immunogen containing more than 80% of rS3. The resulting serum, herein referred as to anti-rS3 serum, not only recognized rS3 but also rS1, rS2, rS4, rS5, rSM2, and rSM3 (**Figure 1**, IB anti-rS3). This cross-reactivity is most probably due to antibodies raised against the central hydrophobic core, which is highly conserved among the oleosin family (25–85% identity between hydrophobic domains of S and SM oleosins). Recombinant SM2 is the unique oleosin showing two bands reactive in immunoblot, one at the predicted molecular weight of rSM2 (20 kDa) and another at a lower mass (15 kDa). This additional band could be due to some degraded or truncated rSM2 but not to some bacterial protein, as the band was not present in control bacteria. It is noteworthy that this serum, although raised against a nonpurified bacterial antigen, showed no cross-reactivity against bacterial proteins under our experimental conditions.

The anti-rS4 serum was obtained previously in our laboratory by immunizing a rabbit with purified rS4 (*9*). As the full protein was used as immunogen, we addressed the question of its specificity towards the six other recombinant oleosins. Contrary to anti-rS3 serum, the anti-rS4 serum was specific, showing no cross-reactivity towards other oleosins (**Figure 1**, IB anti-rS4).

Specificity of anti-rS1N, anti-rS2N, anti-rS3N, and anti-rS4 sera was confirmed by immunoblot analysis of proteins from purified *A. thaliana* oil bodies (**Figure 2**). For each serum, one single band was detected at the predicted molecular weight, i.e., 19.8, 20.3, 18.6, and 21.3 kDa for S1, S2, S3, and S4, respectively. Therefore, these sera could be used in dot-blot for
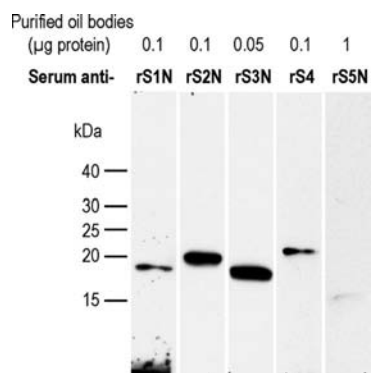
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Purified oil bodies
(µg protein) 0.1 0.1 0.05 0.1 1
Serum anti- rS1N rS2N rS3N rS4 rS5N

kDa

40 —
30 —
25 —

20 —

15 —

Figure 2. Specificity of antioleosin sera towards oleosins from seed oil bodies. *A. thaliana* proteins from purified oil bodies were solubilized in Laemmli buffer and resolved by SDS-PAGE before immunoblot analysis. The blots were probed with anti-rS1N, -rS2N, -rS3N, -rS4, or -rS5N sera. Detection was performed using chemiluminescence. Molecular masses are indicated in kDa.

semiquantitative detection of each four seed oleosins. Anti-rS5N serum revealed a faint band in immunoblot (**Figure 2**) at a molecular weight close to the expected one (14.9 kDa). This weak signal was obtained with 1 µg of oil body proteins, whereas the four other oleosins were detected in 0.1 µg of proteins, indicating that S5 protein is probably present in mature seeds at a much lower level than S1–S4 oleosins.

This observation agrees with Jolivet et al. (*10*), who could detect S1-S4 proteins but not S5 protein in oil bodies from *A. thaliana* mature seeds by proteomic analysis. It is also consistent with the finding that S5 mRNA was present in developing seeds but was rather undetectable in mature seeds (*7*).

Optimization of Oleosin Extraction from *A. thaliana* Seeds Using Chloroform/Methanol. The extraction protocol was adapted from the original work performed by Seigneurin-Berny et al. (*11*). This procedure is based on the differential solubilization of hydrophobic proteins in chloroform/methanol mixtures. *A. thaliana* seeds ground in aqueous buffer were extracted by addition of 9 volumes of chloroform/methanol mixture. The yield of oleosins S1, S2, S3, and S4 solubilized in the organic phase was estimated using dot-blot assays, in order to set up the best extraction conditions.

First of all, the effect of the grinding buffer pH on extraction efficiency was analyzed. As shown in **Figure 3A**, the most basic buffer, i.e., sodium carbonate at pH 11, gave the best extraction efficiency, whatever the oleosin considered. Therefore, pH effect on oleosin extraction is not dependent on oleosin pIs as these proteins differ in their pI (S3 and S4 display basic pI at 9.4, whereas S1 and S2 have more neutral pI, around 7.0). Sodium carbonate is well known to disrupt membranes and to remove peripheral membrane proteins (*22*). In the present experiment, sodium carbonate treatment is likely to act by removing peripheral proteins from oil bodies and unmasking proteins embedded in the phospholipid monolayer, like oleosins, for organic solvent extraction. It is worth noting that, at pH 9, carbonate buffer was more efficient than Tris-HCl buffer, showing that not only pH but also ionic composition of the buffer are important for optimal extraction.

In a second step, composition of the organic solvent was optimized. Different mixtures containing various chloroform/methanol ratios (from 0/9 to 9/0, v/v) were added to ground seeds in 50 mM Na_2CO_3 at pH 11. The ternary mixtures containing 50 mM Na_2CO_3/$CHCl_3$/CH_3OH from 1/0/9 to 1/5/4 (v/v/v) were monophasic but displayed 2 phases of unequal volumes when chloroform was increased (proportions 1/6/3 to
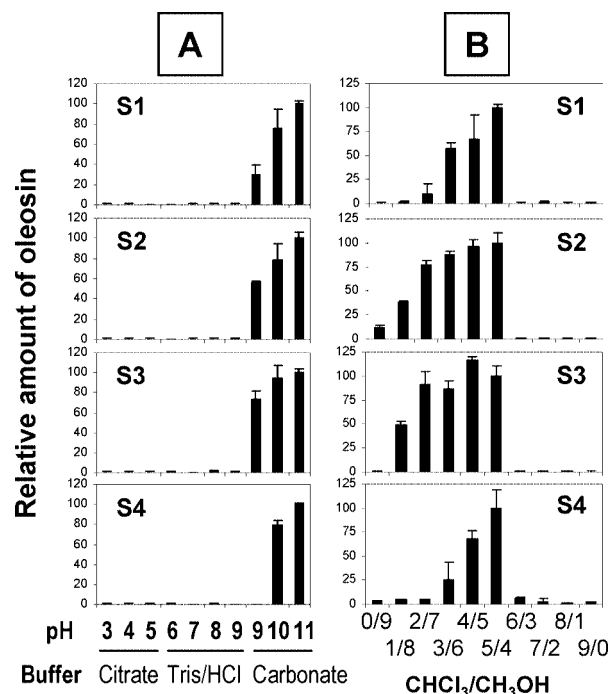

Figure 3. Optimization of oleosin extraction from *A. thaliana* seeds using chloroform/methanol. Seeds were ground in aqueous buffer before addition of chloroform/methanol. The relative amounts of oleosins S1, S2, S3, and S4 present in each extract were measured using semiquantitative dot-blot assays as described in Materials and Methods. Results (mean ± s.d. of duplicate or triplicate) are expressed as relative percentage of the highest value (corresponding to the extract obtained on seeds ground in 50 mM Na_2CO_3 pH 11 and extracted with 9 volumes of chloroform/methanol 5/4 (v/v)). (**A**) Effect of pH. Seeds were ground in buffers at different pH: citrate buffer (pH 3, 4, and 5), Tris/HCl buffer (pH 6, 7, 8, and 9) and carbonate buffer (pH 9, 10, and 11). Ground seeds were extracted with chloroform/methanol 5/4. Experiment representative of three. (**B**) Effect of chloroform/methanol ratio. Seeds ground in 50 mM Na_2CO_3 pH 11 were extracted with 9 volumes of chloroform/methanol mixtures at various ratios, from 0/9 (v/v) to 9/0. Experiment representative of three.

1/9/0). With biphasic mixtures, the main phase was the lower organic phase that was collected carefully without carrying away the interface and the pellet. Oleosins solubilized in the organic phase were detected using dot-blot assays. **Figure 3B** shows that, among monophasic mixtures, the efficiency of oleosin extraction increased with chloroform content, whatever the oleosins. For $CHCl_3$/CH_3OH ratio higher than 5/4, the extract was biphasic and oleosins were absent from the organic phase. In this situation, oleosins were most probably present at the interface, as was demonstrated when *A. thaliana* oil bodies were extracted with $CHCl_3$/CH_3OH 6/3 (*10*).

In summary, the highest rate of oleosin extraction was obtained when seeds were ground in 50 mM Na_2CO_3 at pH 11 and extracted using 9 volumes of chloroform/methanol 5/4 (v/v).

Attempts to solubilize oleosins by grinding seeds directly in the ternary mixture 50 mM Na_2CO_3/chloroform/methanol 1/5/4 (v/v/v) were unsuccessful (data not shown). Most probably, organic solvents in the grinding buffer were leading to denaturation of peripheral oil body proteins and these protein aggregates were masking oleosins, preventing their extraction. Therefore, seeds have to be ground in sodium carbonate prior to chloroform/methanol addition.

Dot-blot analysis was also used to evaluate the efficiency of oleosin extraction by $CHCl_3$/CH_3OH 5/4 compared to TFE/TFA

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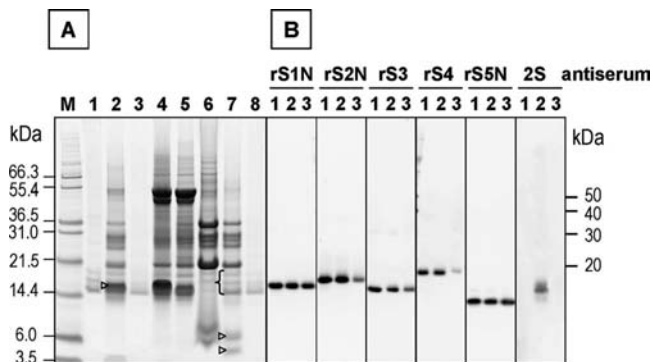*J. Agric. Food Chem.,* Vol. 55, No. 24, 2007 **10013**

Figure 4. Protein analyses of chloroform/methanol extract from *A. thaliana* seeds. (**A**) SDS-PAGE profiles of seed extracts obtained using various solvents: chloroform/methanol 5/4 (lanes 2 and 7), chloroform/methanol 11/7 (lanes 3 and 8), nonreducing Laemmli buffer (lane 4), TFE/TFA 50/0.01 (lane 5), and reducing IEF buffer (lane 6). Extracts obtained from 80 μg of seeds were solubilized in nonreducing Laemmli buffer (lanes 1 to 5) or reducing Laemmli buffer (lanes 6–8) before gel loading. Sixteen micrograms of proteins from purified oil bodies were resolved in lane 1. Protein separation was performed on NuPAGE 12% Bis-Tris gel. Bracket indicates the position of S1, S2, S3, and S4 oleosins; triangle shows 2S albumin. (B) Immunoblot analysis of proteins extracted from seeds using chloroform/methanol 5/4 (lane 2), chloroform/methanol 11/7 (lane 3) and proteins from purified oil bodies (lane 1). Proteins were separated on NuPAGE 12% Bis-Tris gel without reducing agent. The amount of chloroform/methanol extracts loaded on the gel was obtained from 1.5, 0.75, 0.125, 1.5, 15, and 0.75 micrograms of seeds for S1, S2, S3, S4, S5, or 2S immunoblots, respectively. The mass of oil body proteins loaded was 300, 150, 25, 300, 3000, and 150 ng for S1, S2, S3, S4, S5, or 2S immunoblots, respectively. Blots were probed with rabbit sera raised against oleosins rS1N, rS2N, rS3N, rS4, or rS5N, or against napin (2S albumin). Detection was performed using chemiluminescence. Experiment representative of two.

50/0.01, a solvent frequently used to solubilize hydrophobic proteins. While this semiquantitative method gave reproducible data when used to compare different chloroform/methanol extracts, it gave inconsistent data when used to compare aqueous and organic extracts, probably because of the difference in PVDF spotting for aqueous samples compared to organic samples. This semiquantitative method was not able to discriminate between the different solvent systems as organic solvent extraction displayed efficiency ranging from 60 to 120% of the aqueous one.

Protein Characterization of Seed Extract in Chloroform/Methanol 5/4. The protein content of seed extract in chloroform/methanol 5/4 was analyzed by SDS-PAGE (**Figure 4A**, lane 2), showing the presence of several protein bands. The major band at around 16 kDa (indicated with a triangle) was identified as 2S albumin (also called napin) using antibodies against rapeseed napin (**Figure 4B**). In mature seed, this storage protein is composed of two subunits linked by a disulfide bridge (*23*). As expected for 2S albumin, this 16 kDa protein disappeared when reduced (**Figure 4A**, lane 7), whereas 2 bands at 7 and 5 kDa were visible (shown with triangles). This reducing electrophoresis unmasked three bands between 15 and 19 kDa (indicated with a bracket). They were identified as S1–S4 oleosins by comparison with oil body protein profile (**Figure 4A**, lane 1) and by immunoblot analyses using anti-rS1N, -rS2N, -rS3N, and -rS4 antibodies (**Figure 4B**). In NuPAGE gels, the apparent molecular masses of oleosins were smaller than expected from their sequence, as previously shown (*10*). Besides oleosins and albumin, some unidentified bands ranging from

20 to 54 kDa were also present in this chloroform/methanol extract. These proteins were more efficiently extracted by Laemmli buffer, reducing IEF buffer and TFE/TFA 50/0.01 (**Figure 4 A**, lanes 4–6). Consequently, chloroform/methanol 5/4 extracted more selectively oleosins than buffers classically employed for solubilization of hydrophobic proteins.

Chloroform/methanol extract was used to question the presence of oleosin S5 in seeds. This oleosin, the most hydrophobic one, was detected using immunoblot in chloroform/methanol extract (**Figure 4B**). We also succeeded in detecting this oleosin in purified oil bodies as far as 3 μg of proteins were analyzed instead of 1 μg (**Figures 4B** and **2**). This is the first demonstration of the presence of oleosin S5 protein in *A. thaliana* seeds.

Higher Selectivity of Chloroform/Methanol 11/7 for Oleosin Extraction. Extraction with chloroform/methanol 5/4 was not selective enough, as oleosins accounted for only 16% of extracted proteins (**Table 2**). To improve extraction selectivity, we slightly increased the chloroform/methanol ratio. When chloroform/methanol ratio 11/7 was used instead of 5/4, a tiny upper phase appeared and the protein profile of the organic lower phase was dramatically modified (**Figure 4A**, lanes 3 and 2). Proteins soluble in chloroform/methanol 11/7 were not only analyzed by SDS-PAGE (**Figure 5**) but also identified by micro-LC-MS/MS (**Table 3**). Nonoleosin proteins, mainly storage proteins, were almost completely removed in chloroform/methanol 11/7 extract, increasing consequently the purification factor of oleosins from 2.5 to 12.3 (**Table 2**). Oleosin content of chloroform/methanol 11/7 extract was estimated on Coomassie blue stained gel using Image Quant software and averaged 78.7 ± 6.0%, very close to the oleosin content of purified oil bodies (**Table 2**). Besides oleosins, few proteins were solubilized in chloroform/methanol 11/7: several cruciferins (proglobulin; α and β subunits) and one aquaporin. From this study, we estimated that oleosins represent about 6–7% of *A. thaliana* seed proteins and about 2–3% of seed mass (**Table 2**), in agreement with previous reports (*3, 24*). The oleosin content in seeds was calculated not only from data obtained on seeds but also from data obtained on extracts in chloroform/methanol 5/4 and 11/7. The three calculated values, presented in the last column of **Table 2**, are very close (from 2.2 to 2.8% of seed mass). Even if these values suffer from a poor accuracy, this result indicates that oleosin extractions in chloroform/methanol 5/4 and 11/7 are efficient.

Although chloroform/methanol 11/7 is more selective than chloroform/methanol 5/4 for oleosin extraction, it was not as efficient for all five oleosins. The efficiency of chloroform/methanol 11/7 extraction compared to chloroform/methanol 5/4 extraction was determined from the immunoblot shown in **Figure 4B**, after analysis using MultiGauge software of the LAS-3000 image record. It ranges from 1.0 for oleosin S5 to 0.2 for oleosin S4. From these data, we propose the following classification of oleosin hydrophoby: S4 < S2 < S3 < S1 < S5. This experimental classification slightly differs from the classification predicted using hydrophobic indexes. As an example, the Grand Average of Hydropathy (GRAVY) value, calculated using ProtParam on ExPASy server (http://www.expasy.ch/tools/protparam.html), gave the following classification: S3 < S4 < S2 < S1 < S5. One possible explanation of this discrepancy is that the calculated hydrophoby is an average value that does not take into account the blocky structure of oleosins. Partitioning of hydrophobic proteins between the organic phase and the interface when dissolved in chloroform/methanol mixtures at various ratios could be an experimental approach for classification of protein hydrophoby.

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Table 2. Protein and Oleosin Contents in *A. thaliana* Seed Extracts

	protein content (% of seed mass)	protein recovery (%)	oleosin content (% of total proteins)	oleosin purification factor	oleosin content (% of seed mass)
seed	33.6 ± 3.4 (n = 9)	100	6.4 ± 0.6 (n = 3)	1	2.2
extract in CHCl₃/CH₃OH 5/4	17.9 ± 2.5 (n = 13)	53	15.7 ± 1.1 (n = 4)	2.5	2.8
extract in CHCl₃/CH₃OH 11/7	2.9 ± 0.5 (n = 7)	9	78.7 ± 6.0 (n = 14)	12.3	2.3
purified oil body	not determined		91.3 ± 3.9 (n = 3)	14.3	
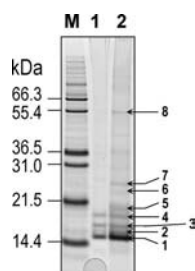
Figure 5. Protein profiles of chloroform/methanol 11/7 seed extract (lane 2) and purified oil bodies (lane 1). Proteins (20 μg) from purified oil bodies and proteins extracted from 250 μg of seeds using chloroform/methanol 11/7 were solubilized in Laemmli buffer before electrophoresis on NuPAGE 12% Bis-Tris gel. Gels were stained with G250 Coomassie blue. M, molecular mass marker in kDa.

Table 3. Proteins Identified by Micro-LC-MS/MS in Chloroform/Methanol Extract from *A. thaliana* Seeds

band	name	MM (kDa)	coverage (%)	accession number	gene
1	S3 oleosin	18.6	15.0	CAA44225	At4g25140
2	S1 oleosin	19.7	29.0	AAF01542	At3g01570
3	S2 oleosin	20.3	30.9	CAA63011	At3g27660
4	S4 oleosin	21.1	32.2	BAB11599	At5g40420
5	12S cruciferin CRU3 subunit β	21.2	67.0	AAB17379	At4g28520
6	probable aquaporin TIP 3.2	28.2	10.5	NP_173223	At1g17810
7	12S cruciferin CRB subunit α	29.8	37.9	CAA32494	At1g03880
8	12S cruciferin CRU3	58.2	11.4	AAB17379	At4g28520

Conclusions. Oleosins display the longest hydrophobic stretch known to date. Their original solubility properties, close to lipid ones, probably originate from this distinctive feature. These properties explain the selectivity of this extraction method that excludes most of the seed proteins (91%). The specific enrichment in seed oleosins obtained by chloroform/methanol extraction allowed the first demonstration of the presence of oleosin S5 protein in mature *A. thaliana* seeds. As oleosin S5 was previously shown to be transiently transcribed in developing seeds (7), our result indicates that S5 mRNA is translated into protein and that S5 protein is still present in mature seeds, whereas mRNA is absent.

Although the selectivity of this method towards oleosins was demonstrated in the present study, we did not succeed in accurately measuring the extraction efficiency. However, the two methods used to evaluate this efficiency (i.e., quantification of extracted oleosin using dot-blot and determination of oleosin content in extracted proteins after SDS-PAGE and Coomassie staining) were both consistent with a value higher than 60%.

The method optimized in the present work is not only selective but also very fast, as this one-step protocol gave a 12-fold enrichment of oleosins. Moreover, it is simple and cheap, as no expensive chromatographic step is required. When compared to the purification method of almond oleosin previ-

ously published, this optimized protocol is more rapid and efficient but gives less pure oleosin extract.

Proteins extracted in chloroform/methanol are easy to handle for interfacial studies (Langmuir balance) and for detection/quantification (dot-blot, immunoblot). If this extraction method can be adapted to *Brassica napus* seeds and to defatted rapeseed meals using nontoxic solvents, the emulsifying properties of these rapeseed extracts will be characterized to question their potential use as surfactant. This characterization could be performed not only by reconstituting stable emulsions, as did Beisson et al. with purified almond oleosin (*15*), but also by measuring the surface tension of the extracted oleosins at different interfaces (pendant drop method). Beside this application, oleosin extraction using organic solvents could be implemented to other seeds, like peanuts and tree nuts, as a better alternative to oil body purification for studying the involvement of oleosins in seed allergies. Few recent studies have indeed shown IgE-binding with oleosins from purified oil bodies in sera of patients with allergic reaction to peanuts, sesame seeds or hazelnuts (*25–27*). As chloroform/methanol extracts are much easier to obtain than purified oil bodies, it should be possible to question the allergenicity of oleosins from different species. Because oleosins from various seeds differ in their hydrophoby, the extraction procedure will probably have to be adapted for each species by optimizing the chloroform/methanol ratio within the 5/4 to 6/3 range.

ABBREVIATIONS USED

IPTG, isopropyl-β-D-thiogalactopyranoside; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulphate; TFA, trifluoroacetic acid; TFE, 2,2,2-trifluoroethanol.

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