Identification of rapeseed oleosins, a family of emulsifying proteins, and optimization of their extraction from seeds and defatted meals using organic solvents

Sabine D'Andréa[1], Pascale Jolivet[1], Alain Quinsac[2], Jacques Evrard[2], Thierry Chardot[1]

[1]*Laboratory of Biochemistry, INRA/AgroParisTech P-G, 78850 Thiverval-Grignon, France*
[2]*Service Transformation et Valorisation des graines oléagineuses, CETIOM, 33600 Pessac, France*
Email: andrea@grignon.inra.fr

Abstract

In oilseeds, lipids are stored in oil bodies, also called oleosomes. They are composed of a core of triacylglycerols surrounded by a monolayer of phospholipids in which different proteins are inserted. Eight proteins were previously identified in *Arabidopsis thaliana* oleosomes, the most abundant ones belonging to the oleosin family. These seed oleosins, named S1, S2, S3, S4 and S5, are amphiphatic proteins containing a long central hydrophobic domain highly conserved and two terminal hydrophilic domains, and therefore display emulsifying properties. In addition, oleosins are expected to be key factors for the stability of oleosomes and their study may contribute, in particular for rapeseed, to improve oil extraction using mild technologies while preserving proteins quality.

Here, we describe the oleosin content of *Brassica napus* oleosomes. These organelles have been purified by 6 flotation centrifugation steps in order to remove proteins non-specifically associated. Protein identification was carried out after SDS-PAGE separation, nano-LC-MS/MS analysis of trypsin peptides and comparison with *A. thaliana* protein database and *B. napus* EST databank. Major proteins were characterized as 15 different oleosins, representing up to 84% of oleosome proteins, and highly homologous to *A. thaliana* oleosins.

Specific antibodies raised against the N-terminal domain of *A. thaliana* oleosins, cross-reacted with rapeseed oleosins. Immunoblot analysis corroborated proteomic results, showing the presence of *B. napus* oleosins orthologous to *A. thaliana* S1, S2, S3, S4 and S5. These antibodies were used to set up a semi-quantitative assay of rapeseed oleosins by dot-blot. This assay allowed the study of oleosin extraction from seeds using organic solvents. One mixture of chloroform/methanol gave optimal extraction of all the oleosins. This protocol was also used on seed defatted meals and allowed the specific extraction of oleosins, identified by immunoblot and proteomic analyses. Surprisingly, oleosins extracted from seed defatted meals displayed the same apparent molecular mass than in seeds, showing that they are not degraded during industrial process. Together with oleosins, napins and cruciferins were also identified in this extract.

This is the first report showing the presence of oleosins in rapeseed defatted meals. A method for their specific extraction is described. The functional properties of this extract need to be further investigated to determine potential biotechnological applications.

Key words: Oleosins, *Brassica napus*, seed, oleosome, protein composition, organic solvent, extraction.

Introduction

Lipids required for energy supply during seedling growth are stored in oleosomes, also referred as oil bodies or lipid droplets. In exalbuminous oilseeds, like *Brassica napus* and *Arabidopsis thaliana*, these organelles are found in cotyledons and the embryonic axis. Oleosomes are often considered just as lipid balls because of their simple structure, but they display a remarkable stability, withstanding dessication, rehydratation and temperature variations. Understanding the molecular composition and organization of this organelle and its biogenesis should lead to identify key factors for its stability. This is a central question to answer in the aim to develop novel and milder technologies of rapeseed oil extraction, with improved efficiency and more preserved quality of protein by-products.

Oleosomes are small spheres with diameter ranging between 0.5 and 2 µm, depending of the specie. This size has been assumed to provide a high surface-to-volume ratio that would facilitate access by lipases during germination (1). They are composed of a core of neutral lipids (triacylglycerols) surrounded by a monolayer of phospholipids in which different proteins are embedded. The major proteins associated with oleosomes are oleosins, which are usually present as two or more isoforms. In *Arabidopsis thaliana*, 4 different isoforms of oleosins, named S1, S2, S3 and S4, are present and accounted for up to 79% of oil body proteins (2). Oleosins form a family with similar structural features that include a highly conserved core of hydrophobic residues flanked by hydrophilic or amphipathic N and C termini of various length. This central hydrophobic domain of ~70 amino acids is the longest hydrophobic fragment known to date and is believed to have an hairpin structure due to a conserved central proline knot (3). Protease protection assays revealed that oleosins are anchored in oil bodies by the hydrophobic domain, exposing the hydrophilic N- and C-terminal ends to the cytoplasm (4). Several indirect observations have suggested that oleosins promote electrical repulsion between oleosomes, preventing them from coalescing. Recently, the relation between oleosin content and oleosome size has been demonstrated using reverse genetic approach (5).

The interfacial properties of oleosins, suspected from their ability to be inserted to lipid particles *in vivo* and *in vitro*, were

analyzed on purified recombinant oleosins of *A. thaliana*. Oleosins decreased the interfacial tension at the oil/water interface more efficiently than β-casein, an emulsifying protein used in food industry (6). As oleosins represent up to 10% of the seed proteins in weight (7,8), they are a potential attractive source of natural emulsifier.

In the present work, we report the exhaustive description of the oleosin content of *Brassica napus* oleosomes. Moreover, we set up a simple protocol for specific extraction of oleosins from *B. napus* seeds and defatted meals. This procedure will be useful to investigate the potential use of oleosins as natural emulsifier.

Materials and methods

Oleosomes purification

Oleosomes were purified from mature seeds of *Brassica napus* hybrid Explus (generous gift of Monsanto, Saint-Louis, Missouri) as previously described (2).

Proteins identification

Proteins were separated by SDS-PAGE and stained with G-250 Coomassie blue. Protein bands were excised from the polyacrylamide gel and digested with trypsin. Trypsin digestion, peptide processing and nano-LC-MS/MS analysis of trypsin peptides were as previously described (2).

Immunoblot and dot-blot

For immunoblot analysis, proteins were resolved by SDS-PAGE before blotting on to PVDF membrane. For dot-blot, protein solubilized in organic solvents were spotted directly on to PVDF membrane. The membrane was probed with rabbit serum at various dilution: 1:5000 dilution for anti-S2 and anti-S3 sera, 1:4000 dilution for anti-S1 serum and 1:2000 for anti-S4 and anti-S5 sera. Peroxidase conjugated goat anti-rabbit IgG (Pierce) was used as secondary antibody. Saturation and incubation with antibodies were carried out for 90 min in TBS (20 mM Tris, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 and 5% skimmed dry milk. After each antibody incubation, the membrane was washed 3 times for 10 min in TBS containing 0.05% Tween-20. Peroxidase activity was revealed using SuperSignal West Dura Extended Duration Substrate (Pierce) according to the manufacturer protocol. Membrane was exposed to Kodak Biomax XAR film. Film was scanned (600 dpi) using an Epson Expression 1680 Pro scanner. The resulting TIFF file of dot-blot was analyzed using the Image Quant software (version 4.2a) from Molecular Dynamics (GE Healthcare).

Extraction of oleosins using chloroform/methanol

Mature seeds of *B. napus* hybrid Explus were grounded at 8 mg/mL using a glass Potter-Elvehjem grinder. Several grinding buffers were assayed: 50 mM Na carbonate pH 11, 10 and 9; 50 mM Tris-HCl pH 9, 8, 7 and 6; 50 mM Na citrate pH 5, 4 and 3. Extraction was performed at room temperature by slowly adding 9 volumes of chloroform/methanol mixture at various ratio: from 0/9 to 9/0. After mixing, the extract was centrifuged and the organic phase collected. For SDS-PAGE analysis, organic solvents were evaporated under a stream of N_2 and proteins were solubilized in non-reducing Laemmli buffer.

Protein measurement

Protein amounts in seeds, defatted meals and organic extracts were determined by amino acid measurement after alkaline hydrolysis, using bovine serum albumin as standard, as previously described (9).

Table I: Identified proteins in purified rape oleosomes.

Band	Relative Intensity (%)	Protein	Apparent Molecular Mass (kDa)	Sequence Molecular Mass (kDa)	Protein coverage (%)	pI	A thaliana orthologues
A	2.8	Contig 1048694	15	15.580	11.5	10.0	At5g51210/S5
B	71.3	Contig 1047406	18	20.645	19.3	8.3	At3g01570/S1
		Contig 1047407		20.576	27.1	8.3	At3g01570/S1
		Contig 1047290		19.871	24.0	9.2	At3g27670/S2
		Contig 1047291		19.748	34.2	7.1	At3g27670/S2
		Contig 1047291		18.913	25.8	9.1	At3g27670/S2
		Contig 1047292		20.117	40.0	7.8	At3g27670/S2
		Contig 1047157 (BnV)		20.434	20.5	9.1	At4g25140/S3
		Contig 1047158		19.870	22.5	9.3	At4g25140/S3
		Contig 1047159 (BnIII)		21.409	12.4	9.3	At4g25140/S3
		Contig 1047156 (NapII)		20.551	18.3	9.4	At4g25140/S3
		Contig 1047155		19.384	20.7	9.2	At4g25140/S3
		Contig 1047162		19.211	14.1	9.2	At4g25140/S3
C	10.1	Contig 1047654	22	22.893	47.5	9.1	At5g40420/S4
		Contig 1047657		22.952	45.2	8.8	At5g40420/S4
D	4.1	Contig 1047992	26	28.133	16.3	6.0	At4g26740/ caleosin
E	6.7	Contig 1048529	42	39.114	52.7	6.6	At5g50600/ steroléosin
F	5.0	β-glucosidase	62	56.283	10.1	6.0	At3g03640

Results and discussion

Oleosin content of rape oleosome

The proteins contained in the oleosomes from the last stage of purification were analysed by SDS-PAGE (figure 1). Upon gel scanning and image analysis, an approximate quantification based on band intensity was obtained (table I). Trypsin peptides obtained from the excised protein bands were analyzed with LC-MS/MS and protein identification was performed using databases not only of *Brassica napus* genome and ESTs but also of *Arabidopsis thaliana* genome. Results summarized in Table I show that oleosins are the major proteins of rape oleosomes, accounting for more than 84% of its protein content. The more intense band (figure 1, band B) contained 12 different oleosins with the same apparent molecular mass of 18 kDa. Comparison with *A. thaliana* oleosins showed that 6 of these rape oleosins were orthologues to S3, 4 to S2 and 2 to S1. Two more oleosins, homologous to *A. thaliana* oleosin S4, were identified in a second band, less intense, at a slightly higher apparent molecular mass (band C at 22 kDa). In addition, one faint band at 15 kDa (band A), accounting for 2.8 % of oleosome proteins, was shown to be the orthologue to *A. thaliana* oleosin S5. This is the first demonstration by proteomic analysis that oleosin S5 is present in oleosomes isolated from mature seeds.

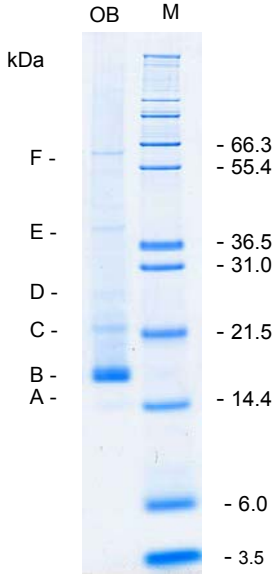

Figure 1: SDS-PAGE of proteins from purified rape oleosomes (OB). Protein bands are lettered as in Table I.

Cross-reactivity of antisera to A. thaliana oleosins with B. napus oleosins

Different sera were obtained by immunizing rabbits with bacterially expressed *A. thaliana* oleosins S1, S2, S3 and S5 (unpublished data). Only the N-terminal domain of each oleosin was used for immunization, as this portion is the less conserved among oleosins. Serum raised against *A. thaliana* oleosin S4 was described previously (6). Specificity of these 5 sera was demonstrated by immunoblot analysis of *A. thaliana* oleosins, either expressed in *E. coli* (unpublished data) or from purified oleosomes (figure 2). As these sera were shown to cross-react with *B. napus* oleosins (figure 2), they can be used to analyse rape oleosin content by immunoblot.

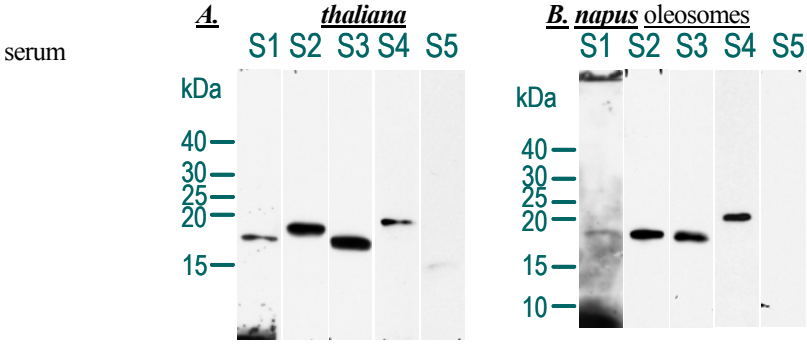

Figure 2: Specific sera to *A. thaliana* oleosins cross-reacted with proteins from purified rape oleosomes.

Optimization of oleosin extraction from seeds using organic solvents

The extraction protocol, adapted from (10), is based on the differential solubilization of hydrophobic proteins in chloroform/methanol mixture. The yield of rape oleosins solubilized in the organic phase was estimated using dot-blot assays with sera raised against oleosins S1, S2, S3 and S4 of *A. thaliana*. The pH effect of the grinding buffer was analyzed. The most

basic buffer gave the best extraction efficiency, whatever the oleosin. In a second step, seeds ground in 50 mM Na_2CO_3 (pH 11) were extracted with mixtures containing various chloroform/methanol ratios (from 0/9 to 9/0) in order to optimize the composition of the organic solvent. In monophasic mixtures (i.e. for chloroform/methanol ratios from 0/9 to 5/4), increasing chloroform content increased extraction efficiency, whatever the oleosin. In biphasic extracts (i.e. for chloroform/methanol ratios from 6/3 to 9/0), the organic phase was free of oleosins. In conclusion, the extraction was optimized as follow: seeds ground in 50 mM Na_2CO_3 and extracted with 9 volumes of chloroform/methanol 5/4. This protocol led to a specific enrichment in oleosins. Preliminary results indicated that oleosins represent around 50% of the proteins extracted from rape seeds.

Protein composition of rape seed and defatted meal extracts

This extraction protocol was applied to industrial rape defatted meals (Saipol, Grand-Couronne, France). The protein content of this extract was analyzed by SDS-PAGE and identified by immunoblot analysis, together with the seed extract one (figure 3). Both extracts contained all the 5 types of oleosin (S1, S2, S3, S4 and S5) in similar proportions to oleosomes, showing that extraction efficiency is within the same range whatever the oleosin. Proteomic analysis confirmed the presence of oleosins and identified contaminating proteins as napins and cruciferins.

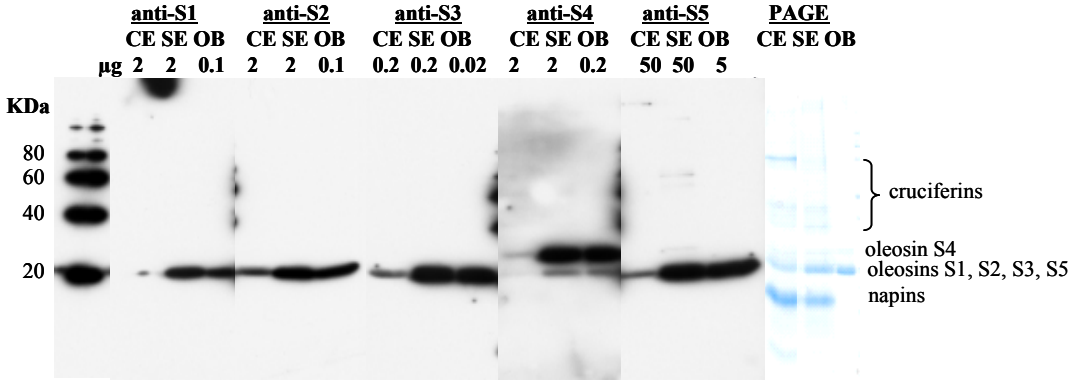

Figure 3: SDS-PAGE and immunoblot analyses of rape oleosomes (OB), seed extract (SE) and defatted meal extract (CE). Different amounts of oleosomes (µg of protein), seed extract (µg of extracted seeds) and defatted meal extract (µg of extracted defatted meals) were probed with serums against *A. thaliana* oleosins.

Conclusion

The protein complement of oleosomes purified from *Brassica napus* mature seeds was exhaustively analyzed, leading to the identification of 15 different oleosins.

As oleosins display original solubility properties, similar to lipid ones, they could be extracted using chloroform/methanol. In the present work, we described the optimization of a novel and rapid procedure for their specific extraction from seeds, without tedious oleosome purification. Moreover, the protocol was successfully applied to rape defatted meals, leading to an extract containing around 10-13% of the total proteins (preliminary results) and enriched in oleosins. In the future, interfacial properties of these seed and defatted meal extracts will have to be characterized.

Acknowledgements

The authors would like to thank Nathalie Nesi (INRA, Le Rheu) for sharing *B. napus* EST database and for helpful discussion. This work was supported by ANR-Génoplante (Genobodies Program GNP05063G to S.A., P.J., A.Q. and T.C.).

References

1. Tzen, J., Cao, Y., Laurent, P., Ratnayake, C., and Huang, A. (1993) *Plant Physiol* **101**, 267-276
2. Jolivet, P., Roux, E., D'Andrea, S., Davanture, M., Negroni, L., Zivy, M., and Chardot, T. (2004) *Plant Physiol. Biochem.* **42**, 501-509
3. Li, M., Murphy, D. J., Lee, K. H., Wilson, R., Smith, L. J., Clark, D. C., and Sung, J. Y. (2002) *J Biol Chem* **277**, 37888-37895
4. Tzen, J. T., Lie, G. C., and Huang, A. H. (1992) *J Biol Chem* **267**, 15626-15634
5. Siloto, R. M., Findlay, K., Lopez-Villalobos, A., Yeung, E. C., Nykiforuk, C. L., and Moloney, M. M. (2006) *Plant Cell* **18**, 1961-1974
6. Roux, E., Baumberger, S., Axelos, M. A., and Chardot, T. (2004) *J Agric Food Chem* **52**, 5245-5249
7. Huang, A. H. (1996) *Plant Physiol* **110**, 1055-1061
8. Murphy, D. J. (2001) *Prog Lipid Res* **40**, 325-438
9. Landry, J., and Delhaye, S. (1996) *Anal Biochem* **243**, 191-194
10. Seigneurin-Berny, D., Rolland, N., Garin, J., and Joyard, J. (1999) *Plant J* **19**, 217-228