A continuum of research projects to improve extraction of oil and proteins in oilseed plants

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Abstract: *A key challenge in the actual context of fossil sources rarefaction, global warming, and of increase of the world global population, is to promote the use of molecules derived from renewable sources such as plants. Among these molecules, lipids and proteins are targets of interest. Plant lipids from oilseeds are attractive substitutes to the use of fossil oil. Till the beginning of the 20th century, numerous products used in the daily life were derived from natural renewable products. For instance, plant oil was commonly used as fuel for vehicles and was entering in the composition of paintings, lubricants etc. Unfortunately, natural oils have been progressively replaced by cheaper fossil oil in the fabrication of these products. Nowadays, fossil oils are becoming increasingly expensive being a finite comodity. It is thus important to reduce our dependence from fossil oil and develop substitution industries. Oilseeds contain important amounts of proteins which are mainly used in feed. As several kilograms of plant protein are needed to obtain one kilogram of animal protein, the interest toward using plant protein in food is reinforced. The developments of the use of plant lipids, as well as proteins are a major stakes for the competitiveness of European agriculture and industry, as well as for sustainable development. Extraction of oil and proteins from rapeseed has a significant cost, in term of energy and solvent uses, and finally affects the ultimate quality of the products (protein digestibility). In order to quantitatively extract seed reserves under mild conditions, it will be necessary to limit the amount of energy needed, and avoid any use of solvents. Ideally, seeds should be processed in a bio refinery. In this paper, we will describe how oilseeds store their reserves, and roadblocks for improving actual oilseed extraction processes. A continuum of research projects aimed at answering targeted questions will be presented, with selected results obtained.*

Key words: *oil, oilseed, rapeseed, oil bodies, protein bodies, oil extraction*

Introduction

Interest for vegetable oil is growing, especially with the increasing demand on fossil oil substitutes. World oil production is close to 135 Mt with palm, soybean and rapeseed oils representing 31%, 24% and 15% respectively. In Europe, rapeseed is the major source of oil (69%). Most of this production is devoted to food uses, followed by emerging uses of oil as bio fuels, and to a lesser extent, as chemicals (250.000 t oil for EU in 2007[1]). Seeds from rape, a major European oil crop (>20,2 Mt in 2007) contain around 45% (w/w) oil and 17 to 25% protein depending on the variety considered. Rapeseed oil has a low saturated fatty acid content and is rich in alpha linolenic acid (ALA, omega-3). It is thus one of the few foods which significantly contribute to the increase of omega-3 fatty acids in diets, by providing ALA[2]. The two main valuable protein fractions in rapeseed meal are seed storage proteins: the 2S albumin-type

[1] http://www.prolea.com/
[2] http://www.anses.fr/

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which is highly basic and rich in sulphur containing amino acids, and the 12S globulin-type which is neutral and of high molecular weight. The 2S albumin fraction contributes largely to reach the recommendations in digestible lysine and methionine for cattle feed which actually represents 70% of the animal feed outlet (Tostain, 2009).

Efficient oil extraction from rapeseed appears rather difficult, by comparison to other seeds currently crushed at the industrial scale (soybean, sunflower). As rapeseed seeds are very rich in oil (45% like sunflower seeds), crushing requires a preparation step consisting in flaking, cooking, prepressing, pelletizing and an extraction step using hexane. The flaking-cooking step allows better efficiency of the prepressing and the solvent treatment lowers the residual oil in the expeller cake from around 15-20% to 2-4%. The deoiled cake is then desolventized by heat and live steam treatment. The meal therefore obtained is a very rich protein source but the various treatments for desolventisation have a negative impact on the protein solubility and digestibility, lowering its use and value, especially for animal feeding. Moreover, these proteins and especially 2S albumins present interesting functional properties (Malabat *et al.*, 2001) such as foaming or emulsifying properties, which are partly or completely lost through the standard oil extraction process. Problems associated with hexane, an inflammable solvent recognized as a volatile organic compound responsible for air pollution, have made extraction plants expensive to build, run and maintain, due to environmental safety issues and regulations (Gros *et al.*, 2003, Campbell *et al.*, 2011). Finally, the emission of hexane and the energy consumption per ton of crushed rape seeds are around 1 liter and 280 kWh, respectively[3]. This energy is mainly thermal (85%), obtained from fossil sources (gas) and a large part (65%) is used to cook and dry the seeds, then to desolventize the meal. An improvement of the ability of rape seeds to be crushed could allow to decrease the necessary energy and consequently, the environmental and economical costs of the crushing operation. The increase of rapeseed economical value therefore relies in our ability to improve oil extraction while preserving the availability and stability of protein by-products, and saving energy during the process.

Organization of oil and protein reserves within seeds

Seed lipids and proteins are stored in specialized sub cellular organelles called oil and protein bodies (OBs, PBs) (Purkrtova *et al.*, 2008a; Herman and Larkins, 1999). OBs are composed of a core of neutral lipids (mainly triacyl glycerols) surrounded by a phospholipid monolayer in which a limited number of proteins is found. The protein complement of seed OBs has been described in various botanical families, among them, Brassicaceae (Jolivet *et al.*, 2004; Jolivet *et al.*, 2006, Katavic *et al.*, 2006; Jolivet *et al.*, 2009) and Euphorbiaceae (Eastmond, 2004; Popluechai *et al.*, 2011). The number of proteins varies from 3 to 33, the reasons for this high variability remaining unknown. Oleosins are the most abundant proteins in seed OBs. These very hydrophobic proteins belong to a multigenic family (Kim *et al.*, 2002). They are involved in seed OBs stability, size and oil yield (Siloto *et al.*, 2006) and freezing tolerance (Shimada *et al.*, 2008). Caleosin, a OB protein capable to bind Ca^{2+} stabilizes OBs *in vivo* and *in vitro* (Froissard *et al.*, 2009; Purkrtova *et al.*, 2008b). Less is known on other minor proteins found at the surface of seed OBs. Biogenesis, senescence, lipid composition, structural organisation, and stabilization of OBs remain largely unknown.

In contrast to their oil counterparts, PBs are almost exclusively composed of proteins that serve as sources of nitrogen, sulfur, and carbon compounds during seed germination (Shotwell and Larkins, 1988). These proteins undergo controlled condensation starting in the endoplasmic reticulum. Storage proteins are found either as proforms in intermediate compartments, called precursor accumulating vesicles and dense vesicles or as maturated proteins in the final protein storage vacuole (Robinson *et al.*, 2005).

Seed reserve extraction

Rapeseed oil extraction using pressing has been studied and optimized by testing various conditions of mechanical and thermal treatments of the seeds, according to

[3] www.creol.fr

an experience gained for a long time (Laisney, 1984). However, it has been observed that whenever a new constraint (quality of the seeds, temperature, etc) emerged, it led to a decrease in performance which was difficult to rally. The crushing of the new double-low rapeseed varieties in the eighties was a well known example. The use of twin-screw extruder for extracting sunflower oil was improved by addition of phosphoric acid and alcohol, which enhanced the lability of the oily spherosomes (Dufaure *et al.*, 1999), thus releasing the oil more easily.

Aqueous Extraction Processing, and Enzyme Assisted Extraction Processing are very attractive. They lead to three distinct fractions. The residual, insoluble material, is rich in cellulose, proteins and entrained soluble materials. The liquid fraction (skim) contains soluble proteins, minerals, carbohydrates and dispersed OBs of small size (Campbell *et al.*, 2011). The oil in water emulsion (cream) is stabilized by proteins and phospholipids. Stabilization by mucilage has also been reported for linseeds (Gros *et al.*, 2003). Recovery of oil from the dispersed OBs and emulsions remains a challenge. High oil extraction yield (up to 99%) from soybean is reported in the literature (see Campbell *et al.*, 2011 for review). However, oil is found either in skim (up to 23%) or in cream (up to 76%), which may need further destabilization for complete oil extraction. Enzyme Assisted Extraction of rapeseed oil and proteins with a set of commercial enzymes improved extraction, but the overall yield remained low (22.2-26% of oil, instead of 16.5% in the absence of enzymes) (Latif *et al.*, 2008), even if the oil quality (in terms of oxidative stability parameters) was better than when solvent extraction was used.

A cognitive approach rather than an empiric one to predict the behaviour of the material during the process would lead to a more efficient fitting of the process to the seed.

According to ''reverse engineering'', a cognitive approach would also allow to suit the composition and the structure of the seed to the need (ability to be destructured, quality of the by-products). It is therefore extremely important to identify the molecular and cellular factors to understand the mechanisms involved in biogenesis of storage oil and protein bodies in seeds, in order to identify key factors for the stability of these storing organelles. This will allow to select rapeseed genotypes with the appropriate traits for easier oil extraction and to develop milder processes which should use as little as possible energy, and ideally no solvent, for safety and toxicity reasons. The ideal products of such extraction should be refined oil and meal devoid of solvent with proteins retaining their initial functional properties.

A continuum of research projects to improve oil and proteins extraction from oilseed plants

Due to the economic and environmental issues associated with seed reserve extraction, it is necessary to have academic laboratories and industries work together. Since 2006, the French National Research Agency[4] has supported different projects aiming to improve extraction of oil and proteins from oilseed plants. Genobodies project (2006-2009) involved five academic laboratories and one industrial partner. It aimed to analyze oil and protein bodies in *Arabidopsis thaliana* and *Brassica napus* seedlings to serve as a basis of knowledge to further improve seed extraction procedure. Genergy project (2008-2012, six academics and two industries) focusses on oil yield increase, nitrogen input reduction and improvement of oil extraction while preserving availability and stability of by-products and saving energy during the extraction process. The genetic variability of a large panel of genotypes, studied in this project, is used to explore several traits (seed yield, oil yield, pressing...). The effect of N supply on the traits is also studied. SOPOL project (2008-2012, four academics) aims at producing generic knowledge, using various biological and physical approaches to solve the three dimensional structure of seed OBs ''structural proteins'', namely oleosins and caleosin, and give a molecular basis to OBs stability.

Selected results from the research projects

Extensive oil and protein extraction will not be achieved without substantially increasing the knowledge on the composition, structure and stability of these complex emulsions (containing lipids, proteins, and polysaccharides). Intraspecies variability, and their associated-biological processes associated (reserve accumulation and mobilization of stored material) will deserve special attention too. Results presented in the upcoming sections have been obtained within the frame of the ANR Genobodies, Genergy and SOPOL programs.

On oil bodies

Description of the protein composition of OBs from double-zero winter-type *B. napus* have been achieved by a combination of proteomic and genomic tools (Jolivet *et al.*, 2006; Jolivet *et al.*, 2009). By comparison with *A. thaliana* OBs, rapeseed OBs contains numerous integral protein isoforms displaying a high level of sequence conservation with their arabidopsis counterparts (Jolivet *et al.*, 2009). This can be explained not only by the polyploidy nature of the *B. napus* genome but also by the presence of numerous duplications of chromosomal portions into the rapeseed genome. Genes coding for some OB proteins of interest are expressed during seed development in a pattern similar to that of oil accumulation, and a sequential deposition of integral OB proteins has been established (Jolivet *et al.*, in press). Mutants for the major oleosins have been constructed in *A. thaliana* and *B. napus* in spite of the fact that the production of null mutants is challenging for polyploidy species such as *B. napus*. Solubilization of oleosins by specific polymers prior to structural determination using powerful

[4] http://www.agence-nationale-recherche.fr/en/project-based-funding-to-advance-french-research/

Synchrotron Light has provided original data on their fold in solution (Gohon *et al.*, 2011). Calcium ions were capable to affect the solubility of caleosin, and to strongly modify the shape and aggregation state of purified OBs (Purkrtova *et al.*, 2008b). The presence of an hydroxysteroid dehydrogenase (HSD1) activity in *A. thaliana* and *B. napus* OBs has been detected but the biological function and the substrates of this enzyme remain unknown (d'Andréa *et al.*, 2007a).

On protein bodies

The mechanisms responsible for the high degree of reserve protein condensation in PBs remain a matter of debate (Herman and Larkins, 1999; Vitale and Denecke, 1999). We aimed to improve condensation of storage proteins by overexpression of candidate genes involved in transport and/or condensation of storage proteins. Overexpression in *A. thaliana* developing seeds of the receptor VSR1;1, a major vacuolar receptor for storage proteins (Shimada *et al.*, 2003) had no massive impact on oil and on protein quantity and composition. Overexpression of the receptor like protein, RMR (Jiang *et al.*, 2000), could not be tested. Moreover, the absence of homologue of RMR in the rapeseed EST library is not encouraging to pursue with RMR genes. In order to try to modulate expression of 12S globulins and 2S albumins, major constituents of PBs in Brassicaceae with contrasted nutritional values, 12S expression has been silenced. Both 12S and 2S protein expressions are impacted, while oil content was not affected.

Biotechnological outputs

Oleosins represent 2-3 % of the seed mass (d'Andréa *et al.*, 2007b). It is possible to selectively extract oleosins from seeds (d'Andréa *et al.*, 2007c) and to produce fractions enriched in oleosins from cakes using a mixture of organic solvents (d'Andréa *et al.*, 2007b). These fractions are better emulsifiers than phospholipids (PLs), as deduced from interfacial studies and reconstituted OBs studies (C. Lebon, unpublished). Induction of OBs coalescence using ions would be of great interest for the oil extraction. (Purkrtova *et al.*, 2008b). The optimization of pressing in terms of industrial production remains difficult because the crushing facilities are equipped with screw presses with continuous flow capacities of several tons per hour. The need for miniature assays is evident to facilitate the screening of seeds and improve crushing capability. This is especially true for the seeds of the miniature model plant *A. thaliana*. Using a micropress, the static pressing of different seeds has been studied: *B. napus*, *Linum usitasissimum* and *A. thaliana*. During pressing, the behaviour of *A. thaliana* seeds differs according to the ecotype considered (Savoire *et al.*, 2010; Savoire, 2008). The behaviour of the seeds during static and continuous pressing is different since in the latter the material must be rigid enough to form a plug which is necessary to increase the pressure in the barrel and the separation of the oil from the cake. Moreover, when applied to linseed, similar evolution of pressing yield according to harvesting date has been highlighted between static and continuous (Komet) presses. Work is in progress to model the continuous pressing with data from static pressing and other rheological characteristics.

Conclusions and perspectives

The issues of better understanding the reserves biogenesis in oleoproteaginous seeds and the molecular basis of their extractability are yet to be answered. Cognitive work, combining knowledge of genes and the structure of the seed at different levels of organization, study of model pressing, and associated mathematical representations, seems unavoidable to propose more gentle conditions of reserves extraction to obtain desired raw materials. A better comprehension of the biogenesis and the reserves accumulation and extractability in model plants as well as the rules to scale up that knowledge for extrapolation to crops are definite topics of research for the future. The recent publication of patents (i.e. DOW WO 2008/024840 A2) on the extraction of protein reserves from rapeseed for food purposes, or the emergence of new oilseeds for the bio fuel market (i.e. Jatropha, Camelina) are perfect illustrations of these new topics.

REFERENCES

Campbell KA, GItaz CE, Johnson LA, *et al.* Advances in aqueous processing of soybean. *J Am Oil Chem Soc* 2011; 88: 449-65.

d'Andréa S, *et al.* At5g50600 encodes a member of the short-chain dehydrogenase reductase superfamily with 11beta- and 17beta-hydroxysteroid dehydrogenase activities associated with Arabidopsis thaliana seed oil bodies. *Biochimie* 2007a; 89: 222-9.

d'Andréa S, *et al.* Identification of rapeseed oleosins, a family of emulsifying proteins, and optimization of their extraction from seeds and defatted meals using organic solvents. In: *12th Intl Rapeseed Congress*. 2007b. Wuhan, China.

d'Andréa S, *et al.* Selective one-step extraction of Arabidopsis thaliana seed oleosins using organic solvents. *J Agric Food Chem* 2007c; 55: 10008-15.

Dufaure C, Mouloungui Z, Rigal L. A twin-screw extruder for oil extraction: II Alcohol extraction of oleic sunflower seeds. *J Am Oil Chem Soc* 1999; 76: 1081-6.

Eastmond PJ. Cloning and characterization of the acid lipase from castor beans. *J Biol Chem* 2004; 279: 45540-5.

Froissard M, *et al.* Heterologous expression of AtClo1, a plant oil body protein, induces lipid accumulation in yeast. *FEMS Yeast Res* 2009; 9: 428-38.

Gohon Y, *et al.* High water solubility and fold in amphipols of proteins with large hydrophobic regions: oleosins and caleosin from seed lipid bodies. *Biochim Biophys Acta* 2011; 1808: 706-16.

Gros C, Lanoiselle JL, Vorobiev E. Towards an alternative extraction process for linseed oil. *Chem Eng Res Des* 2003; 81: 1059-65.

Herman EM, Larkins BA. Protein storage bodies and vacuoles. *Plant Cell* 1999; 11: 601-14.

Jiang L, *et al.* Biogenesis of the protein storage vacuole crystalloid. *J Cell Biol* 2000; 150: 755-70.

Jolivet P, *et al.* Protein composition of oil bodies in Arabidopsis thaliana ecotype WS. *Plant Physiol Biochem* 2004; 42: 501-9.

Jolivet P, *et al.* Purification and characterization of oil bodies from Brassica napus seeds. *OCL* 2006; 13: 426-30.

Jolivet P, *et al.* Protein composition of oil bodies from mature Brassica napus seeds. *Proteomics* 2009; 9: 3268-84.

Katavic V, *et al.* Protein and lipid composition analysis of oil bodies from two Brassica napus cultivars. *Proteomics* 2006; 6: 4586-98.

Kim HU, *et al.*, A novel group of oleosins is present inside the pollen of Arabidopsis. *J Biol Chem* 2002; 277: 22677-84.

Laisney J, ed. L'huilerie moderne, un art une technique. Paris: Ed. CFDT, 1984: 318.

Latif S, Diosady LL, Anwar F. Enzyme-assisted aqueous extraction of oil and protein from canola (Brassica napus L.) seeds. *Eur J Lip Sci Tech* 2008; 110: 887-92.

Malabat C, *et al.* Emulsifying and foaming properties of native and chemically modified peptides from the 2S and 12S proteins of rapeseed (Brassica napus L.). *J Am Oil Chem Soc* 2001; 78: 235-42.

Popluechai S, *et al.* Jatropha curcas oil body proteome and oleosins: L-form JcOle3 as a potential phylogenetic marker. *Plant Physiol Biochem* 2011; 49: 352-6.

Purkrtova Z, *et al*. Structure and function of seed lipid-body-associated proteins. *C R Biol* 2008a; 331: 746-54.

Purkrtova Z, *et al.* Caleosin of Arabidopsis thaliana: Effect of calcium on functional and structural properties. *J Agric Food Chem* 2008b; 56: 11217-24.

Robinson DG, Oliviusson P, Hinz G. Protein sorting to the storage vacuoles of plants: a critical appraisal. *Traffic* 2005; 6: 615-25.

Savoire R. Etude multi-échelles de la séparation solide-liquide dans la trituration du lin oléagineux. 2008, Université de Technologie de Compiègne : 217.

Savoire R, *et al.* Micro-pressing of rapeseed (Brassica napus L.) and Arabidopsis thaliana seeds for evaluation of the oil extractability. *OCL* 2010; 17: 115-9.

Shimada T, *et al.* Vacuolar sorting receptor for seed storage proteins in Arabidopsis thaliana. *Proc Natl Acad Sci U S A* 2003; 100: 16095-100.

Shimada TL, *et al.* A novel role for oleosins in freezing tolerance of oilseeds in Arabidopsis thaliana. *Plant J* 2008; 55: 798-809.

Shotwell M, Larkins B. The biochemistry and molecular biology of seed storage proteins. In: Marcus A, editor. *The Biochemistry of Plants, A Comprehensive Treatise.* New York: Academic Press, 1988.

Siloto RM, *et al.* The accumulation of oleosins determines the size of seed oilbodies in Arabidopsis. *Plant Cell* 2006; 18: 1961-74.

Tostain S. Les nouvelles perspectives du Colza. In: *Journées AFTAA 2009.* Paris.

Vitale A, Denecke J. The endoplasmic reticulum-gateway of the secretory pathway. *Plant Cell* 1999; 11: 615-28.