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Plant Molecular Farming Using Oleosin Partitioning Technology in Oilseeds

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Plant seed oil-bodies or oleosomes ate the repository of the neutral lipid stored in seeds. These organelles in many oilseeds may comprise half of the total cellular volume. Oleosomes are surrounded by a half-unit membrane of phospholipid into which are embedded proteins called oleosins. Oleosins are present at high density on the oil-body surface and after storage proteins comprise the most abundant proteins in oilseeds. Oleosins are specifically targeted and anchored to oil-bodies after co-translation on the ER. It has been shown that the amino-acid sequences responsible for this unique targeting reside primarily in the central hydrophobic core of the oleosin polypeptide. In addition, a signal-like sequence is found near the junction of the hydrophobic domain and ann N-terminal hydrophilic / amphipathic domain. This "signal" which is uncleaved is also essential for correct targeting. Oil-bodies and their associated oleosins may be recovered by floatation centrifugation of aqueous seed extracts. This simple partitioning step results in a dramatic enrichment for oleosins in the oil-body fraction. In the light of these properties, we reasoned that it would be feasible to create fusion proteins on oil-bodies comprising oleosins and an additional valuable protein of pharmaceutical or industrial interest. It was further postulated that if these proteins were displayed on the outer surface of oil-bodies, it would be possible to release them from the purified oil-bodies using chemical or proteolytic cleavage. This could result in a simple means of recovering high-value protein from seeds at a significant (i.e. commercial) scale. This procedure has been successfully reduced to practice for a wide variety of proteins of therapeutic, industrial and food use. The utillity of the method will be discussed using a blood anticoagulant, hirudin, and industrial enzymes as key examples.

Plant seeds typically store part of the energy needed for germination in organelles called oilbodies or oleosomes. Oilbodies are spherical structures, comprising an oil droplet of neutral lipid (most frequently triacylglycerides) surrounded by a half-unit phospholipid membrane. The surface of these oilbodies is surrounded by a unique class of protein called oleosins. These proteins have an extremely hydrophobic core and appear to be lipophilic proteins reported so far in the protein and DNA databases. Their N-and C-termini are more hydrophilic or amphipathic (Huang et al., 1992). Oleosins become associated with nascent oilbodies during oleosome biogenesis on the ER by a co- translation mechanism (Hills et al., 1993, Loer and Herman, 1993). Oleosins in many oilseeds accumulate at relatively high levels. In the *Brassica* species, for example, oleosins may comprise somewhere between 8-20% of total seed protein (Huang, 1996). This level of accumulation reflects the fact that oleosin genes are strongly transcribed during seed development.

When oilseeds containing oleosins are extraxted in aqueous solvents they form a three-phase mixture of insoluble material, aqueous extract and an emulsion of oilbodies which on standing or low-speed centrifugation will result in the flotation of the oilbodies accompanied by their oleosin complement. Successive aqueous washes of this oilbody fraction remove any extraneous proteins bound loosely to oilbodies. The oleosins, conversely, remain tightly associated with the oilbodies due to their highly lipophilic core. Protein analysis of oilbody preparations which have undergone only flotation, centrifugation, and washing reveals that virtually all the other seed proteins are absent, thus the oleosin fraction is highly enriched (Figure 1).

These observations led to the idea that oleosins could serve as vehicles or carriers for heterologous proteins expressed in plant seeds. This would facilitate the expression and simple purification of recombinant proteins in plants. Given that it has been possible to express This establishes that fusions at

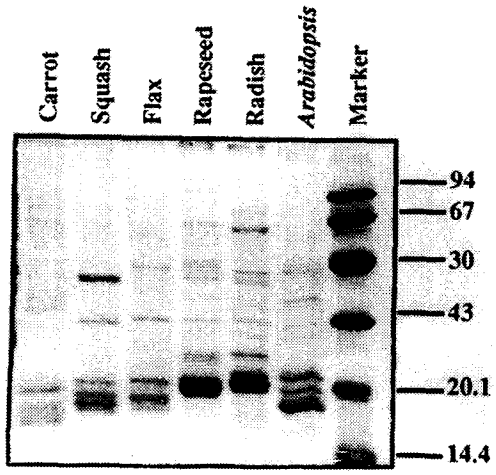

Figure 1. Coomassie blue stained poly acrylamide protein gel showing the oil-body fractions from a variety of common dicotyledonous seeds. The oil-body proteins were enriched only by one round of flotation centrifugation as discussed in the text. This centrifugation separates the rest of the cellular proteins from the oil-body proteins or oleosins. Fusions with these oleosins may be used as vehicle for the subcellular targeting and subsequent purification of desired recombinant proteins in seeds.

the C-terminal of the oleosin are not exposed to the ER lumen and remain on the cytoplasmic side.

This subcellular targeting methodology was further refined by interposing a specific labile cleavage site between the oleosin and recombinant protein domain. In our early experiments, these were 4 amino-acid proteolytic sites hydrolysed by enzymes such as thrombin or factor Xa. Such a configuration should permit the recombinant polypeptide domain to be cleaved from the surface of the oilbodies (See Figure 2). As can be seen from Figure 3 this cleavage can indeed be effected using oilbodies suspended in cleavage buffer and subjected to a specific protease treatment.

Using oleosins for the production of the anti-coagulant, hirudin

While the above experiments demonstrare the basic principles of oilbody-based recombinant protein production, it is of great interest to apply it in cases where the recombinant protein is of high-value. This would test the economics as well as the technical parameters of the system. To this end, we created a translational fusion between an *Arabidopsis* oleosin coding sequence and the coding sequence of the mature form of the blood anti-coagulant, Hirudin. Hirudin is a naturally occurring thrombin inhibitor produced and secreted in the salivary glands of the medicinal leech, *Hirudo*

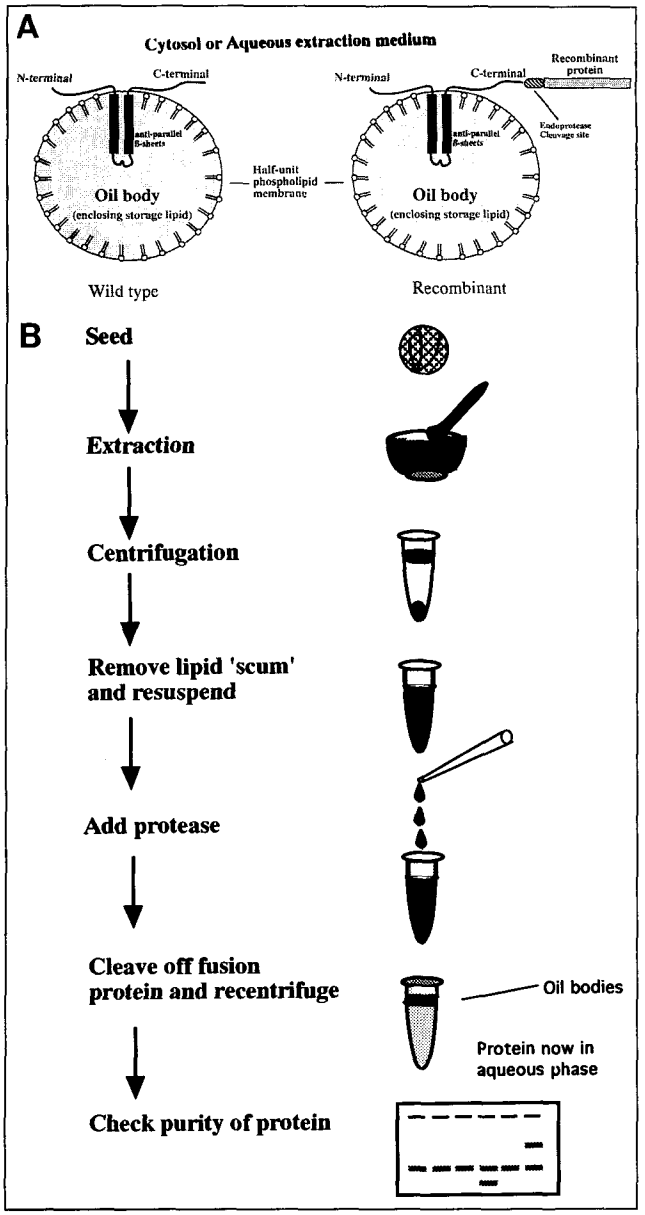

Figure 2. A. Configuration of oleosins on wild-type and recombinant oil based on models from Huang (1992) and van Rooijen and Moloney (1995).
B. Flow diagram indicating extraction and purification steps for recombinant proteins expressed as oleosin fusions.

medicinalis. Hirudin is a powerful anticoagulant with a number of very desirable properties including stoichiometric inhibition of thrombin, short clearing time from the blood and low immunogenicity. The unit cost of production in leeches would be prohibitive for extensive therapeutic applications. Hirudin has been made in a variety of micro organisms including *E. coli* and yeast, but these entail significant fixed costs associated with fermentation. Our objective, therefore, was to test the oleosin expression and purification system as

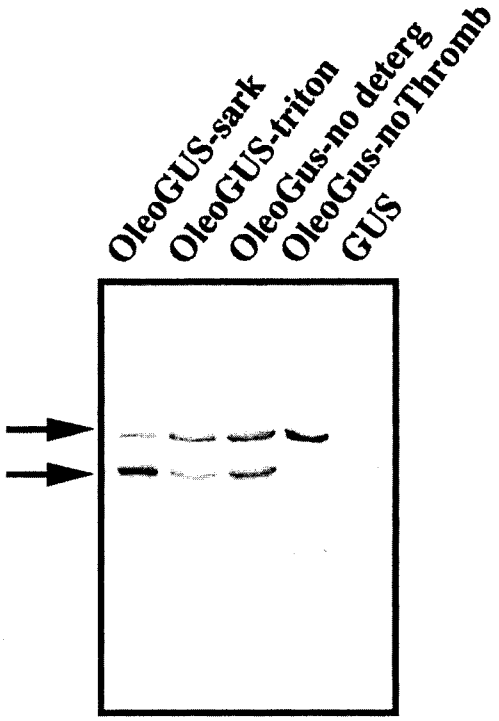

Figure 3. Western blot analysis of cleavage of oleosin-GUS and protein fusions from oil-bodies of transgenic plants.
Oil bodies (5-10 μl) were resuspended in 50 μl thrombin cleavage buffer comprising triethanolamine (TEA) 0.1M (pH 8.4). All oil body samples, except for the controls (lanes 4 and 5: noThromb and GUS) were treated with 0.02 IU thrombin. Oil body samples were supplemented with no detergent (lane 3: no deterg), 2% Triton X-100 (lane 2: triton) or 0.5% sarkosyl (lane 1: sark). Samples were incubated overnight at 37℃ and the reaction completed the next morning by raising the temperature to 55℃ for 30 min. All the protein samples were run on a 7.5% polyacrylamide gel, blotted on PVDF membranes and visualized immunologically using GUS-antiserum (Clontech) as primary antibody with a goat anti-rabbit IgG linked to alkaline phosphatase as secondary. The arrows indicate the molecular weights of full length oleosin-GUS (87,670 Da) and the cleaved/native GUS (68,200 Da). Lane 5 (GUS) comprises a total soluble protein sample (100,000 × g supernatant) from a plant expressing a transcriptional fusion of GUS. This acts as a molecular weight marker and control for plants expressing untargeted GUS.

an alternative and potentially inexpensive source for hirudin.

Constructs containing the translational fusion of oleosin-hirudin under the transcriptional control of an oleosin promoter from *Arabidopsis* were entroduced into *Brassica napus* using *Agrobacterium*-mediated transformation (Moloney et al., 1989). The resulting transgenic plants yielded seed in which a protein of about 25 kDa could be detected. This protein cross-reacted with heterologous proteins in plants for many years, it is interesting to speculate why plants have not been used widely for recombinant protein production. The reasons do not seem to be related to the basic cost of plant production. On a cost-per-kilo basis plant are a very inexpensive source of protein. It appears that the major limitation relates to the cost of extraction and purification. If oilbodies could be used as carriers it would impact favorably upon the economics of production of recombinant proteins in plants.

Subcellular targeting of recombinant oleosins

While native oleosins are targeted with high avidity to oleosomes *in vivo*, it was not clear whether the addition of polypeptide sequences to oleosins would result in aberrant targeting. Such aberrant targeting has been noted previously with modified storage proteins such as recombinant phaseolin (Hoffman et al., 1988) or 2S albumin (Krebbers and Vendekerckhove, 1990). Consequently, experiments were performed to test the idea that modifications of oleosins at either the N-or C-terminal end might affect overall targeing to oilbodies (Van Rooijen and Moloney, 1995a, Van Rooijen and Moloney, 1995b). In that work , the authors showed that both N-and C-terminal translational fusions of oleosin with β-glucuronidase (GUS), did not significantly impair the basic targeting mechanism. Furthermore, the C-terminal oleosin-GUS fusion remained enzymatically active. This activity was followed in oilbody extracts to test whether the oleosin-GUS protein was attached to the oilbody with similar avidity to that of native oleosin. It was found that oleosin fusions targeted and bound to oibodies with similar avidity to native oleosins.

These experiments have exemplified a number of important properties of oleosins. First, they can be extended at either end and will still undergo oilbody targeting. Second, long polypeptide extensions do not seem to pose a problem (GUS has a mw of~67 kDa). Furthermore, these experiments were performed using a β-glucuronidase known to be susceptible to the ER lumen, GUS is inactivated due to glycosylation (Iturriaga et al., 1990). In the case of the oleosin C-terminal extensions, GUS was fully functional indicating that it was not glycosylated. a monoclonal antibody raised against hirudin. This protein proved to be associated tightly with the oilbodies and could not be removed from the oilbodies by successive washings. Attempts to determine whether the oleosin-hirudin fusion protein had antithrombin activity suggested that the fusion protein was completely inactive. As part of the construction of the translational fusion, we had incorporated four additional codons specifying a Factor Xa

cleavage site (I-E-G-R). This configuration was designed to permit release of the hirudin polypeptide sequence off the oilbody by proteolytic cleavage. When the washed oilbodies from these seeds were treated with Factor Xa, hirudin polypeptide was released into the aqueous phase.

Authentic hirudin has three disulfide bridges. These are essential to its activity. Thus, if the plant were to make hirudin polypeptide, but did not allow its correct folding no thrombin inhibition would be detected unless a refolding treatment was applied. In fact, after Factor Xa treatment, the aqueous phase showed strong antithrombin activity. This demonstrates that biologically-active hirudin was released. The specific activity of the inhibitor was similar to that of recombinant hirudin secreted from yeast cells, indicating that the majority of the hirudin released was correctly folded and disulfide bridges were appropriately configured. The released hirudin was then subjected to ion-exchange chromatography (Mono Q). The fractions showing anti-thrombin activity were concentrated and loaded onto a C_{18} reversed phase analytical HPLC column. This showed that the hirudin was substantially pure after the ion-exchange step. The HPLC trace showed two peaks close to the expected retention volume of hirudin. Mass spectrometric analyses of these two peaks using TOF-MALDI revealed that the two peaks were full-length hirudin and a truncated product from which two C-terminal amino-acids were missing. Interestingly, such a truncated from was also found when Hirudin was expressed in yeast (Heim et al., 1994). Both forms of the molecule are potent thrombin inhibitors.

A number of improvements would be required to render this system economical. These are a) expression levels, b) cleavage efficiency and c) cleavage system (use Factor Xa exemplifies the system but would be prohibitively expensive). Expression levels of the recombinant protein have in a few cases been about one-tenth of the native oleosin accumulation. This corresponds to about 1% of total cellular protein. It is, however, expected that using stronger promoters than the native oleosin promoters, higher levels of accumulation will be sustained. Cleavage efficiency varies from one fusion protein to another and may be affected by the conformation of the target protein but also by the spacing between the cleavage site and the oilbody surface. Finally, the use of Factor Xa here was essentially as a convenient experimental system. In practice it would be essential to have an inexpensive cleavage enzyme or chemical to render this process competitive. Recent experiments with inexpensive enzymes such as bacterial clostripain or collagenase suggest that alternative cleavage systems are compatible with this process and would allow the cost of cleavage step to be minimized.

Other proteins or polypeptides expressed as oilbody fusions

The potential for using oleosin fusions as an adaptable means of recombinant protein expression in seeds is under investigation. Table 1 lists a number of examples which illustrate the range of applications that may be amenable to this technology. It is interesting to note that the production of oleosin-polypeptide fusions appears to function for rather short peptides such as IL-1β and Hirudin as well as for much longer polypeptides such as β-glucuronidase. In cases where larger polypeptides are produced there is good evidence that proper foiding occurs. This is evidenced by the fact that in a number of cases including β-glucuronidase and Xylanase, the fusion protein retains its enzymatic activity. This finding has also led to experiments which illustrate the utility of oilbodies as immobilization matrices (Kühnel et al., 1996, Moloney and Van Rooijen, 1996). Using β-glucuronidase as a model it was shown that a dispersion of oilbodies carrying β-glucuronidase on their surface would hydrolyse glucuronide substrates for GUS with catalytic properties indistinguishable from soluble GUS. Furthermore, it was shown that virtually all the enzymatic activity could be recovered and recycled by

Table 1. Proteins and polypeptides which have been expressed in seeds as oleosin fusions.

Fusion Protein (Source)	Size(kDa)	Use
High-methionine Zein fragment (corn)	10 kDa	Alteration of amino-acid content of oilseeds.
Xylanase (Neocallimastix patriciarum, Rumen fungus)	68 kDa	Production of xylanase for aninal feed supplement or in wood pulping.
Single-chain antibodies (synthetic, human/mouse)	30 kDa	Inexpensive production of antigen binding proteins for diagnostic or bioimaging applications.
Proteases (various sources)	30-50 kDa	Cleavage enzymes, meat tenderizers, biological detergents.
Hirudin (Medicinal leech)	6 kDa	Anticoagulant, thrombin inhibitor.
Interleukin 1β (Human)	~1 kDa	Cytokine, antitumour
β-glucuronidase *(E.coli)*	~68 kDa	Marker enzyme. Model for targeting experiments.

the use of flotation centrifugation to obtain the oilbodies. The enzyme in dry seed remains fully active for several years. Once extracted onto oilbo dies the half-life of the enzyme was about 4 weeks (Van Rooijen and Moloney, 1995b).

The expression of the enzyme, xylanase, on oilbodies illustrates several ways in which the basic technology could be used. The enzyme accumulates on seed oilbodies in active form. From here it can be cleaved to release soluble xylanase into solution. Such a preparation could be useful in such processes as de-inking of recycled paper. Alternatively, without significant purification the oilbodies could be mixed with insoluble substrate such as crude wood pulp to help break down the xylan crosslinks. This could be helpful in paper-making. Cost would be minimised as the enzyme itself could be recovered and re-used. On addition to these formulations the enzyme could be delivered in seed meal to animals without purification. In this embodiment, the enzyme would function in the stomach of a monogastric animal to improve digestibility of meal. Clearly, this latter formulation would be created in conjunction with other cellulytic enzymes. The result, however, would be to provide monogastrics with enhanced feed efficiency and reducing biological waste.

Conclusions and future prospects

The use of plant oilbodies and their associated proteins, oleosins as vehicles for recombinant protein product has been illustrated with a number of examples. The major advantage of using oilbodies as carriers in the ease with which proteins can be recovered and purified. Oleosin targeting does not seem to be impaired even by very long polypeptide extensions to the N- or C-termini of the oleosin. This greatly enhances the versatility of this system in contrast to alternative approaches for recombinant protein production in plants. Separation of oilbodies from seed extracts is amenable to scale-up using equipment typical to dairy operations such as cream-separators (Jack et al., 1990). In consequence, it seems likely that this system could be used for the production of a wide range of proteins of therapeutic, industrial and feed or food use. We are investigating production of a wide range of commercially attractive polypeptides in this system and concurrently developing scale-up extraction and purification system which could be applied to different oilseeds engineered for production of such oleosin-polypeptide fusions.

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