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Review

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A Unique Strategy for Recovering Recombinant Proteins from Molecular Farming: Affinity Capture on Engineered Oilbodies

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Abstract

Molecular faming has the potential to provide large amounts of recombinant protein for use in diagnostics and as therapeutics. Various strategies have been developed to enhance the expression level, stability, and native folding of recombinant proteins produced in plants. Few investigations into the subcellular distribution of recombinant proteins within plant cells have been published despite the potential to increase the expression level and impact the purification process. This review article discusses the current strategies used for targeting recombinant proteins to various subcellular locations and the advantages of targeting to seed oil bodies for molecular farming applications. Specifically, the affinity capture of antibodies using recombinant oilbodies is discussed.

Recombinant Protein Production in Transgenic Plants

Production of recombinant proteins in transgenic plants is emerging as a competitive and safe alternative to classical mammalian cell culture expression systems. Transgenic plants require minimal investment in their cultivation, have a reduced risk of contamination by human or animal pathogens and are capable of producing large amounts of fully functional mammalian proteins. Bacteria are also often used for the production of recombinant proteins due to reduced production costs, straight forward molecular biology and ease of handling. However, plant systems are more attractive for producing eukaryotic proteins as plant cells carry out many of the post-translational modifications therapeutic proteins require for optimal activity or functionality.

Companies listed in Table 1 represent examples of systems used to produce recombinant proteins, especially in the field of therapeutic products. So far, the majority of therapeutic proteins have been produced in mammalian cell culture systems, such as Chinese hamster ovary (CHO) cells, grown in bioreactors. Although mammalian cell culture has been the standard for producing active therapeutic proteins, this production process is limited in scale.

In part, because nutrients, heat, and gases must diffuse evenly throughout the culture, there is a physical limit on the size of the bioreactor. When large scale production is required the capital cost to build and operate a cell culture facility of sufficient size makes this approach financially challenging for many companies. There is also a growing concern for safety when using animal cell culture or animalderived products, such as serum enriched media, in a production process. Co-purification of viral or prion pathogens is a major safety issue and accounts for a substantial amount of the cost involved in manufacturing, particularly the purification, validation, and quality control systems required to ensure removal or inactivation of such pathogens. Plants do not propagate animal viruses or prions and are expected to have a higher degree of safety.

Molecular farming is the growing and harvesting of crop plants genetically engineered to synthesize 'recombi-

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nant' proteins. The proteins expressed can be therapeutics, vaccines, blood substitutes, enzymes or diagnostic reagents which are extracted and purified prior to use. Transgenic plant systems potentially allow the manufacture of biologic drugs too expensive or required in amounts too great for other systems to produce. Cultivation of plants can be scaled through increased acreage providing manufacturing capacity unmatched by any other system. Alternatively, plant cell suspensions or organ cultures grown in a bioreactor could be used as a production system, although they have certain limitations in production scale, production yield and cost compare to agricultural production. Advances and improvements resulting from experience producing secondary metabolites (e.g. taxol (Son et al., unpublished) or ginseng culture), such as fast production cycles and easy handling of harvest and purification steps could make commercial protein production using these systems more attractive. Recently, expression of a single chain antibody in tobacco suspension cultures (Firek et al., 1993) and a murine immunoglobulin G1 in hairy root cultures (Doran, 2000), leading to secretion of products into the media was reported.

Use of Secretion Pathways for Subcellular Targeting

To understand the factors controlling stability and accumulation of heterologous proteins, it is important to know where the protein of interest is located within specific plant cells or tissues and how this localization changes during development and as a result of environmental conditions. Isolation and purification of the desired protein may be greatly facilitated by sequestering the protein into a particular cellular compartment. However, cell fractionation and purification of proteins from particular tissues or cell types (Table 2) is often technically challenging, and can be time-consuming and laborious. The secretion pathway in plants regulates and determines the passage of polypep-

Table 1. Companies and technologies in bio-manufacturing

Major technology	Mammalian (CHO) cells	Transgenic mammal milk		Molecular farming (transgenic plants)	
Companies	Amgen (www.embrel.com)	GTC Biotherapeutics (www.transgenics.com)	Goat	CropTech (www.croptech.com)	Tobacco
	Genetech (www.gene.com)	PPl Therapeutics (www.PPL-therapeutics.com)	Sheep	Epicyte (www.epicyte.com)	Corn
	Crucell (www.crucell.com)	BioProtein (www.bioprotein.com)	Rabbit	SemBioSys Genetics (www.symbiosys.ca)	Safflower
				Meristem Therapeutics (www.meristem-therapeutics.com)	Tobacco, corn
				Large Scale Biology (www.lsbc.com)	Tobacco
Cost(g raw material)	>$150		$1-$2		$0.05

Table 2. Examples of recombinant proteins targeted to subcellular compartments in transgenic plants.

Proteins	Host plants	Tissue expression	Subcellular targets	References
α-Amylase	Tobacco	Leave	Apoplast	Pen et al. 1993
Avidin	Corn	Seeds	Apoplast	Hood et al. 1997
Bivalent	Tobacco	Leave	Apoplast	Hiatt at al. 1989
β-Glucuronidase	*Brassica*	Seeds	Oil bodies	van Rooijen & Moloney 1995
Anti-oxazolone	Tobacco	Leave	ER	Fiedler et al. 1997
Anti-ABA	Tobacco	Leave	ER	Fiedler et al. 1997
Xylanase	*Brassica*	Seeds	Oil bodies	Liu et al. 1997
Anti-phytochrome	Tobacco	Leave	cytosol	Owen et al. 1992
Anti-β-1.4-endoglucanase	Potato	Roots	cytosol	Schouten et al. 1997
Hirudin	*Brassica*	Seeds	Oil bodies	Parmenter et al. 1995

tides to tonoplast-derived protein bodies, ER derived protein bodies or secretion into the apoplastic space. The advantages of using the secretion pathway are manifold. First, many therapeutic polypeptides are normally secreted in their source organism.

They may undergo specialized folding and post-translational modification that requires components of the ER such as BiP chaperones or glycosylation enzymes. By including the appropriate signal peptide sequence or fusion responsible for directing expression and deposition, it is possible to target recombinant proteins to the lumen of the ER, vacuole or other cellular compartments. Second, secretion into one of the cellular compartments may separate the desired protein from proteases likely to catalyze its breakdown. Secretion has also been found to enhance protein stability by facilitating proper folding. Targeting signals can be used to intentionally retain recombinant proteins within distinct compartments of the cell to protect them from proteolytic degradation, preserve their integrity and to increase accumulation levels (Moloney and Holbrook, 1997). Third, sequestering proteins into certain cellular compartments may facilitate their recovery upon extraction, if the compartment can be preferentially enriched during processing. Secretion into the extracellular media or periplasmic space has proven to be very useful for facilitating purification of foreign proteins from yeast and bacterial systems.

An issue affecting secreted glycoproteins produced in transgenic plant systems is that the glycosylation pattern which occurs in plants is similar, but not indentical, to that found in humans. In plants, the modification to complex glycans includes the addition of xylose and a novel linkage to fucose. The unique plant-specific sugar linkages can be immunogenic in mammals (Faye, 1988). Thus, it is important to confirm for each plant-derived glycoprotein whether or not an undesirable immune response is elicited in the intended host species. It is important to note that Chargelegue et al. (2000) demonstrated that a murine monoclonal antibody produced in transgenic tobacco plants with plant-specific glycans is poorly immunogenic in mice even when administrated parenterally with adjuvant. Strategically, elimination of undesirable steps in the glycosylation pathway within the production species could be made either through the use of a mutant strain or by an alternative form of gene knock-out such as antisense RNA or co-suppression (von Schaewen et al., 1993).

Expression of Recombinant Proteins in Seeds

Seeds are natural storage organs which accumulate high levels of protein during development and following desiccation store this material for long periods without degradation. Strong transcriptional promoters from seed storage protein genes, such as phaseolin, have been used successfully to express recombinant genes at a high level. The β-phaseolin promoter from soybean and the *Arabidopsis* oleosin promoter are some of the strongest promoters available for seed-specific expression. The *Arabidopsis* oleosin sequence has been used in several molecular farming applications including the production of hirudin (Parmenter et al., 1995) and xylanase (Liu et al., 1997). In addition, seeds are relatively free of biotic and abiotic contamination, which provides substantial economical advantage for commercial applications (Moloney, 2000).

Targeting to Oil Bodies

Although many cellular compartments are amendable to cell fractionation (Bejarano and Gonzalez, 1999), few are suitable for large scale production and economical separation. In contrast, seed oilbodies are a unique and promising carrier for protein production and isolation. Seeds which store neutral lipid as an energy source for germination encapsulate the oil into cellular organelles called oleosomes or oilbodies. Oilbodies are comprised of a triacylglyceride core surrounded by a half-unit membrane of phospholipids and embedded with a unique class of protein known as oleosin (Figure 1).

Oleosins are highly expressed seed proteins comprising between 2-10% of total seed protein and are ubiquitous in all common oilseeds such as canola, sunflower, soybean, safflower and peanuts. Oleosins are targeted to oilbodies through their highly hydrophobic central domain and tolerate translational fusions with heterologous protein sequences to either of the hydrophilic N- or C-terminal ends (Figure 2) without apparent loss of oilbody targeting

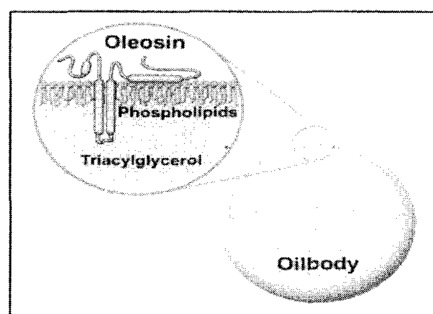

Figure 1. Illustration showing on the three major components of seed oil bodies.

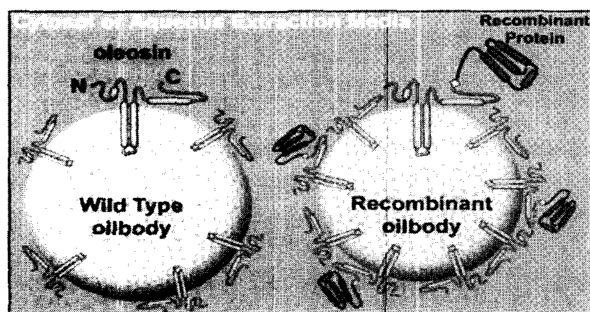

Figure 2. Schematic diagram showing configuration of wild type oleosin and recombinant protein created as a fusion with oleosin on seed oil bodies.

efficiency and structural modification (Moloney and van Rooijen, 1996). Oleosin protein accumulates only on oilbodies and can therefore be easily separated from other cellular contents by floatation centrifugation of aqueous seed extracts (van Rooijen and Moloney, 1995; Moloney, 2000). Therefore, oilbody targeting can be used as an excellent carrier for recombinant proteins produced in oilseeds.

Strategies for Targeting of Recombinant Antibody Production

Antibodies are a large family of glycoprotein that all have a common basic structure consisting of two identical copies of a polypeptide known as the heavy chain (H) and two identical copies of a polypeptide called the light chain (L) held together by disulfide bridges and non-covalent bounds. Antibody molecules have three protein domains: two of the domains form the arm of the Y-shape and contain sites that can bind to antigen, and the third domain associated with immune response forms the base of the Y. Functional expression of recombinant antibodies in transgenic plants was first reported by Hiatt et al., in 1989. Functional full-size antibodies, Fab (fragment antigen binding) and scFv (single-chain fragment variable) fragments have since been shown to express in leaves and seeds without loss of binding specificity or affinity.

Expression of recombinant antibodies in the cytoplasm has only been achieved using single-chain fragments. Single-chain Fv (scFv) antibodies are formed by joining the variable heavy (Vh) and variable light (Vl) chain sequences as a translational fusion. Correct association of the two variable domains can result in the same binding specificity as the original full length immunoglobulin. ScFv fragments do not require post-translational glycosylation associated with full-size antibodies but most scFv sequences benefit from targeting to the ER, where the oxidative-reductive catalysts which facilitate correct disulfide bond formation are present. Further stability and accumulation occurs when an ER retention signal (KDEL) is attached to the scFv to prevent continued secretion into the apoplast. Extended retention of scFv proteins in the ER is thought to promote correct folding leading to higher scFv stability and accumulation. The ER also contains different molecular chaperones to aid in the folding of nascent proteins, preventing aggregation and the formation of incorrect three dimensional structures. ScFvs targeted to compartments other than the ER also appear to have a higher turnover rate or greater susceptibility to proteases localized to the apoplast or present outside the ER (Conrad U and Fiedler, 1998). Selecting for scFv sequences with improved folding kinetics and stability in the reducing environment of the cytosol is important when using scFvs for immunomodulation of metabolic, hormonal, or disease resistance in plants. The accumulation of scFvs in the cytosol is variable and dependent on the particular sequence, particularly important residues within the structural framework of the scFv. Unexpectedly, the attachment of a KDEL motif to the C-terminal end of an anti-cutinase scFv expressed in the cytosol also resulted in a significant improvement in accumulation level despite the understanding that KDEL is an ER lumun-specific sequence (Schouten A et al., 1996; 1997). When targeted to the cytoplasm or apoplast in plants, the maximum scFv yield is 0.01-0.5% with an average of 0.1% of total soluble protein.

In contrast, the yield for scFvs targeted to and retained in the ER is 1.0-6.8% total soluble protein showing 10 to 20 fold or even 100 fold increases over other targeted compartments (Conrad and Fiedler, 1998). When secretion signal peptides are used to direct scFvs into the secretion pathway, the protein has been found localized to the ER, ER vesicles and the nuclear envelope. All forms of the protein appear to be correctly folded and processed as these antibodies have high antigen-binding activity (Fiedler et al., 1997). So far, seed specific accumulation of scFvs in transgenic tobacco plants was achieved by two seed specific promoters, the LeB4 (Baumlein et al., 1991a) and USP promoter (Baumlein et al., 1991b) from *Vicia faba.*

ScFvs could be stored in the seeds for at least 1 year at room temperature without any detectable loss in amount or specific activity of antibodies. The initial strategy used to co-produce and assemble the two full length light and heavy immunoglobulin chains was to express each chain in separate transgenic lines followed by cross-pollination. The resulting progeny were screened to identify lines which had inherited both transgenes and consequently co-express both chains. A critical requirement for the expression of full-size antibodies is the correct folding and assembly of the individual chains. In higher plants this has

been achieved by targeting the individual chains to the ER under direction of proper signal sequences. Without signal peptide sequences, the recombinant antibodies are accumulated in cytosol which is the most difficult compartment to obtain high expression level although several successful examples in the cytosol has been reported (Owen et al., 1992).

It is believed that individual chains of full-size antibody cannot be correctly assembled in the cytosol because of lack of chaperons and reducing environment of the cell compartment, which prevents the formation of disulfide bridges between individual chains (De Jaeger et al., 2000). After assembly and post-translational processing at ER, antibodies are secreted to the intercellular space under the cell wall (apoplast) of specific organs when no retention signals such as KDEL or other intrinsic targeting sequences are present. Secreting full-size antibodies to the apoplast has advantages for the recovery processing using Protein-A matrix which is described in next section.

Recovery Strategies

For essentially all biologic systems used to produce recombinant therapeutic proteins, a substantial portion of the manufacturing cost is due to the expense of purification and downstream processing. The cost advantages of a plant-based production system are only truly realized when the downstream purification process used to recover the recombinant product can also be accomplished economically. The established method for purification of antibodies uses Protein A affinity chromatography as an effective early stage purification step. Fischer et al. (1999) showed that full-size antibodies can be purified from plant cell extracts on Protein-A affinity matrices. Protein A is naturally found on the surface of *Staphylococus aurueus* and shows high affinity and selectivity for IgG immunoglobulins. It has been adapted for use in the purification of IgG, immunoblotting and immunoprecipitation procedures. Using affinity chromatography on a protein-A matrix is very efficient for removing contaminants and can be used to accomplish a 100 fold concentration of the recombinant protein. With this method, they reported that more than 80% of expressed full size IgG can be recovered from suspension cultured tobacco cells.

Transgenic oilbodies have been engineered to display Protein A as an oleosin fusion (Figure 3). Protein A oilbodies have been shown to bind human IgG1 and IgG2, but not IgG3; A pattern analogous to native Protein A. Protein A oilbodies also capture human IgG1 in the pH range of 5-8 and release the antibody below pH 4. Once antibody protein has bound the Protein A oilbodies, unlike solid phase matrices, the complex can be separated from the majority of cellular and tissue components using phase partitioning techniques (Figure 4). This allows the development of continuous flow liquid /liquid handling systems at a reduced capital expense, yet still allows large scale volume and product handling. When used as part of the extraction and isolation procedure for recovering recombinant antibodies from transgenic seeds, the eluted antibody material is of sufficient purity to bypass the first Protein A chromatography column normally employed, and hence eliminate one

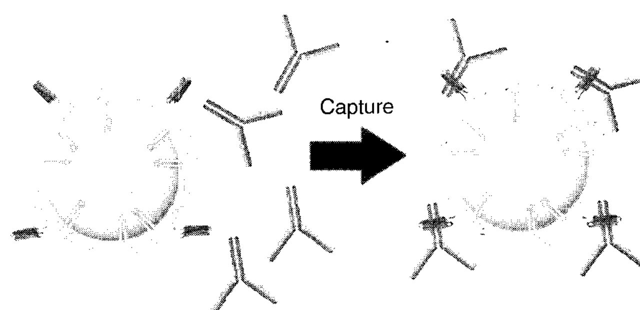

Figure 3. Illustration showing soluble antibody protein binding Protein A displayed on oilbodies. Following washing and elution, antibodies are released in a highly purified form.

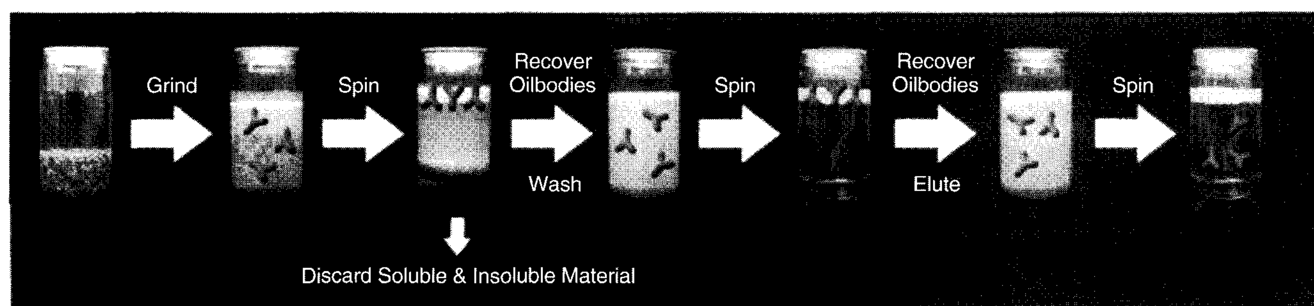

Figure 4. Illustration showing the process of antibody capture on oilbodies, the isolation of oilbodies by floatation centrifugation, and subsequent elution of antibodies into a highly purified soluble fraction.

of the most expensive steps in the purification of antibodies. This material may also potentially be used as the first step in antibody capture and purification from other production systems, such as mammalian cell culture systems.

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