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Research review paper

## Recovery and purification of plant-made recombinant proteins

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## ABSTRACT

Plants are becoming commercially acceptable for recombinant protein production for human therapeutics, vaccine antigens, industrial enzymes, and nutraceuticals. Recently, significant advances in expression, protein glycosylation, and gene-to-product development time have been achieved. Safety and regulatory concerns for open-field production systems have also been addressed by using contained systems to grow transgenic plants. However, using contained systems eliminates several advantages of open-field production, such as inexpensive upstream production and scale-up costs. Upstream technological achievements have not been matched by downstream processing advancements. In the past 10 years, the most research progress was achieved in the areas of extraction and pretreatment. Extraction conditions have been optimized for numerous proteins on a case-by-case basis leading to the development of platform-dependent approaches. Pretreatment advances were made after realizing that plant extracts and homogenates have unique compositions that require distinct conditioning prior to purification. However, scientists have relied on purification methods developed for other protein production hosts with modest investments in developing novel plant purification tools. Recently, non-chromatographic purification methods, such as aqueous two-phase partitioning and membrane filtration, have been evaluated as low-cost purification alternatives to packed-bed adsorption.

This paper reviews seed, leafy, and bioreactor-based platforms, highlights strategies for the primary recovery and purification of recombinant proteins, and compares process economics between systems. Lastly, the future direction and research needs for developing economically competitive recombinant proteins with commercial potential are discussed.

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## 1. Introduction

Plants have been used as a source of natural products throughout history, dating back thousands of years. Plant-based products have numerous, diverse industrial applications that continue to expand with the development of new bioprocess and plant molecular biology technologies. One such technology was the development of plant transformation which led to the use of a variety of plant hosts for the expression of human therapeutics (Giddings et al., 2000; Stöger et al., 2005), nutraceuticals (Adkins and Lönnnerdal, 2004), antibodies (Arntzen et al., 2005; De Muijnck et al., 2010; Fischer et al., 2009; Nikolov et al., 2009; Stöger et al., 2002a, 2002b), industrial enzymes (Hood et al., 2007; Howard et al., 2011), vaccine antigens (Ling et al., 2010; Sala et al., 2003), and biopolymers (Mooney, 2009; Sharma and Sharma, 2009). Since the first successful transformation of a higher plant in 1983 (Fraley et al., 1983) and after many years of intense research activity in academia and industry, plants have been recognized as a viable alternative for recombinant protein production.

Plant production platforms were initially dominated by agricultural crops with established production infrastructure such as corn, rice, and tobacco (Fox, 2006; Spök and Karner, 2008). Corn and tobacco are typical representatives of seed- and leaf-based protein expression systems, respectively. Fittingly, the first commercial products were corn-derived avidin and trypsin (Hood et al., 1997; Woodard et al., 2003) and tobacco-derived secretory IgA (Ma et al., 1998). By 2000, there were more than 180 organizations working in the area of molecular farming including 60 to 100 plant biotechnology companies (Fox, 2006; Kaiser, 2008; Twyman et al., 2003). However, the number of plant biotechnology companies has declined since 2005 along with a shift in both preferred protein products and selected plant host systems.

Although transgenic plants are efficient protein producers, free of human pathogens, and capable of post-translational modifications, the transgenic plant production time (Twyman, 2004) along with regulatory uncertainty (Doran, 2000) and purification challenges were initially cited as disadvantages. Some challenges have been addressed in the last 5 to 10 years. For example, the use of transient expression drastically reduced the development time needed to produce gram-level quantities (Hiatt and Pauly, 2006) from >24 months to 14 to 20 days. Regulatory issues have been addressed in different ways; some companies adapted safer product development strategies by focusing on animal-free protein alternatives for less regulated markets. Others embraced standard protein production technologies (bioreactors and other contained systems) that are similar to existing mammalian or microbial cell culture platforms with in-place regulatory processes (Paul and Ma, 2010). Although upstream processing for these platforms is similar to traditional cell cultures, substantially more effort is needed to develop viable downstream process methods and tools that are suitable for plant-derived proteins.

Past and current achievements in expression, targeting, and glycosylation have been recently reviewed by Karg and Kallio (2009). The purpose of this review is to highlight recent advances in downstream processing of transgenic plants and to identify future research needs with a focus on promising products and platforms.

## 2. Review of production platforms

Protein expression platforms developed and evaluated in the past 20 years include a variety of plant host systems including seed crops (corn, canola, soybeans, rice), leafy crops (tobacco, alfalfa, lettuce), aquatic plants (*Lemna minor*), plant cell cultures (tobacco, carrot, rice), and hairy root cultures (Daniell et al., 2009b; Franconi et al., 2010; Karg and Kallio, 2009; Sharma and Sharma, 2009). The last three systems, in addition to moss and green algae, have recently been

categorized as *in vitro* contained production systems (Franconi et al., 2010). We prefer using the term bioreactor-based systems instead of contained systems for this review because seed and leafy plant systems could also be grown in containment (greenhouse). In this review, bioreactor-based systems terminology will be used to describe all liquid-medium supported systems such as hairy root culture, aquatic plants, plant cell culture, moss, and algae. In the next several sections, we describe general advantages and disadvantages of each system (seed-, leafy-, and bioreactor-based systems) and strategies for the recovery and purification of recombinant proteins.

### 2.1. Seed-based systems

The seed-based platform for recombinant protein production evolved naturally from expression and transformation technologies first developed for crop input traits (Davies, 2005). Recombinant proteins successfully expressed in plant seeds include cytokines (Zhu et al., 1994), cell culture proteins (Huang et al., 2002, 2005; Nandi et al., 2002), therapeutic antibodies (Stöger et al., 2000), vaccine antigens (Nochi et al., 2007; Streatfield and Howard, 2003; Wu et al., 2007), and industrial enzymes (Hood et al., 2007). Since 2008, the United States Department of Agriculture (USDA) has approved field release of transgenic seeds expressing human lysozyme, lactoferrin, and serum albumin in rice, apolipoprotein in safflower, and hepatitis B surface antigen and brazzein in corn (<http://www.isb.vt.edu/search-release-data.aspx>).

Seed crops offer many advantages over microbial fermentations, mammalian cell cultures, and transgenic animals as host systems including an extensive knowledge base regarding its cultivation, harvesting, storage, and processing (Kusnadi et al., 1997; Nikolov and Hammes, 2002). Initially, seeds were the preferred host choice for recombinant proteins because expressed proteins accumulate within naturally desiccant storage organelles (Nikolov and Hammes, 2002) that are devoid of protease and other enzymatic activities (Fischer et al., 2009). Endogenous protease inhibitors ensure the stability of the recombinant protein throughout extraction and purification (Menkhaus et al., 2004a). The stability of seed-expressed proteins allows decoupling of upstream and downstream processing (Boothe et al., 2010), so the location of the downstream processing facility can be apart from the growing fields and seed storage facility. In specific cases, such as oral vaccines, nutraceuticals, and industrial enzymes, using generally regarded as safe (GRAS) seed crops (e.g. rice and corn) minimizes processing and purification requirements (Hood and Howard, 2009; Nandi et al., 2002; Yang et al., 2008).

Compared to leafy crops, seed crops have lower biomass yields per unit surface area but if crops are grown in open-fields, the economy of scale, established agriculture practices, and protein stability outweighs the biomass yield disadvantage (Nikolov and Hammes, 2002; Schillberg et al., 2005). Aqueous seed extracts inherently lack alkaloids and chlorophyll pigments but do contain small amounts of phenolics (primarily ferulic and coumaric acids), phytic acid, lipids, and lectins that may interfere with protein purification (Farinas et al., 2005a; Stöger et al., 2002a, 2002b; Wilken and Nikolov, 2006). Perceived disadvantages for seed systems include fear of potential cross contamination, inadvertent mixing of transgenic and non-transgenic grain, and public and food industry aversion to producing biopharmaceuticals in feed or food crops.

### 2.2. Leaf-based systems

Advantages of leafy crops, such as alfalfa and tobacco, include high biomass yield, possibility for multiple growth cycles per year, and established agricultural infrastructure like seed systems but with less risk of pollen spreading (flowering can be prevented). Tobacco has

been the leading leaf-based system for commercial production of recombinant proteins (Twyman et al., 2003) since the first report of monoclonal antibody-expressing tobacco (Hiatt et al., 1989). The advantages and disadvantages of using tobacco and alfalfa systems for producing therapeutic and diagnostic antibodies have been recently reviewed by Fischer et al. (2009). Significant advancements have been achieved using transient expression in leafy crops and applied specifically to expression of monoclonal antibodies (mAbs), vaccines, and other therapeutics products in tobacco (*Nicotiana benthamiana*) (Conley et al., 2011; D'Aoust et al., 2010; Joensuu et al., 2010). Transient expression alleviates transgenic regulatory concerns but is currently best suited for low-volume protein production such as personalized therapeutics, seasonal vaccines, and other specialized markets (Fischer et al., 2009; Pogue et al., 2010). Transient expression of leafy tissues provides a distinct advantage for greenhouse-produced tobacco, compared to other plant-based systems, when rapid production of pandemic vaccines is required (D'Aoust et al., 2010). Possible limitations of transient expression technologies that are not yet well documented include the ease of scaling up, cost at scale, and batch-to-batch expression reproducibility, aspects critical to process development and manufacturing. The main disadvantages of leafy tissues are high water content, storage stability of harvested biomass, and related to these, recombinant protein stability which does not allow decoupling of upstream and downstream processing.

The ease of protein extraction from leafy plants is sometimes seen as a benefit over extraction from seed material, which may require additional unit operations such as seed grinding, soaking, or deoiling (Schillberg et al., 2005). This perceived advantage of leafy systems is offset by potential instability of expressed proteins in metabolically active tissues and their extracts (De Muynck et al., 2010; Doran, 2006). Leaf extracts contain proteolytic activities, phenolic compounds, and chlorophyll-derived pigments that could pose difficulties during downstream processing of leafy-based systems (Barros et al., 2011; Woodard et al., 2009; Yu et al., 2008). The level of phenolic compounds released during extraction varies with tissue type (leaves, roots, seeds) and plant species. Cell membrane and chloroplast debris dispersed in leaf homogenate could be a nuisance during extract clarification. All of these extract characteristics could result in difficult, multi-step purification processes, affecting both quality and quantity (yield) of purified proteins.

In spite of cost advantages of open-field production of seed and leafy crops, expression of biopharmaceuticals has shifted toward production in confined environments such as greenhouses and bioreactor-based systems (Spök and Karner, 2008).

### 2.3. Bioreactor-based systems

From a regulatory and safety perspective, bioreactor-based systems resemble established microbial and mammalian systems and are more likely to gain public acceptance before other growth methods. Tobacco, mainly *N. benthamiana*, carrot, and rice cell cultures and the aquatic plant, *L. minor*, have been evaluated primarily for the production of injectable biopharmaceuticals and other products that necessitate controlled growth conditions for highly regulated recombinant proteins. Notable accomplishments for bioreactor-based systems include the use of tobacco suspension cell culture to produce a USDA-approved Newcastle virus vaccine (Miller et al., 2009) and the production of a high-value, therapeutic protein (glucocerebrosidase) using carrot cell culture (Aviezer et al., 2009a, 2009b); the latter has already been approved in Europe and is expected to obtain U.S. FDA approval this year (Xu et al., 2011). The main advantages of bioreactors are controlled growth conditions with complete containment, consistency in product yield, quality and homogeneity, and speed of production from gene to protein in a matter of weeks (Fischer et al., 2009; Huang et al., 2009). Disadvantages include bioreactor scale-up

limitations similar to those using mammalian cell cultures, relatively low concentrations (10–250 mg/L) of secreted products (Weathers et al., 2010; Xu et al., 2011), instability of secreted proteins (Hellwig et al., 2004), and higher capital costs compared to open-field and greenhouse production. From a downstream processing perspective, the potential advantage of a secreted product is a simpler and less costly purification (Doran, 2006; Hellwig et al., 2004) with the caveat that secretion efficiency may be limited by protein size, hydrophobicity, and/or charge (Fischer et al., 2009). For proteins that are not secreted, plant cell homogenates have similar complexities to that of leafy plants and bioreactor-based systems. Thus, they are less commercially attractive compared to other heterologous protein expression systems since potential processing savings (compared to secreted products) are lost (Doran, 2006).

### 3. Downstream processing: current practices and future opportunities

As the transgenic plant platform continues to mature, research and development interests will likely shift from upstream to downstream processing to improve overall productivity, which has been the case for more established biotechnology (Gottschalk, 2008). Depending on product type and application, downstream processing can account for a significant portion of the total operating costs for product manufacturing. Thus, the development of efficient and selective extraction and purification processes as early as possible is essential for favorable economics (Basaran and Rodriguez-Cerezo, 2008).

Downstream processing efficiency is influenced by the recombinant protein concentration, the complexity of the plant extracts or cell-free culture media, and required final product purity. Downstream processing can be divided into two phases: primary recovery and purification (Harrison et al., 2003). An overview of downstream processing steps for bioreactor-, leafy-, and seed-based systems is given in Fig. 1. Representative extraction and purification processes published since previous reviews in 2004 (Menkhaus et al., 2004a; Nikolov and Woodard, 2004) have been summarized in Tables 1 and 2. Any novel products and/or platforms that did not report individual recovery and purification steps, extraction conditions, product yields, and purities were not included in the tables.

#### 3.1. Primary recovery

The objective of primary recovery is to maximize product titer and yield in extract or cell homogenate, reduce extract or media volume, and to prepare a clarified feed stream for purification. Primary recovery steps are to a greater degree platform dependent (Fig. 1). The primary recovery steps for leafy- and seed-based production systems consist of: 1) product release from the biomass by homogenization or aqueous extraction; 2) solid–liquid separation; and 3) conditioning, pretreatment, and clarification. Downstream processing for bioreactor-based systems starts with biomass harvesting by centrifugation, membrane filtration, or by pumping the liquid media out of the bioreactor vessel. Because intracellular proteins require disintegration of harvested biomass for product release, the primary recovery steps after biomass harvesting are the same as those for leafy- and seed-based systems. For media-secreted proteins, the culture media is typically concentrated, clarified, and conditioned prior to the first chromatography (capture) step.

Some primary recovery unit operations, such as centrifugation, cross-flow filtration, and dead-end filtration, are multi-functional, and their strategic applications are defined by the extract composition, particle size, extract properties, and product stability. Optimization of primary recovery conditions is typically done on a case-by-case basis but only few studies reported the effect of extraction methods and

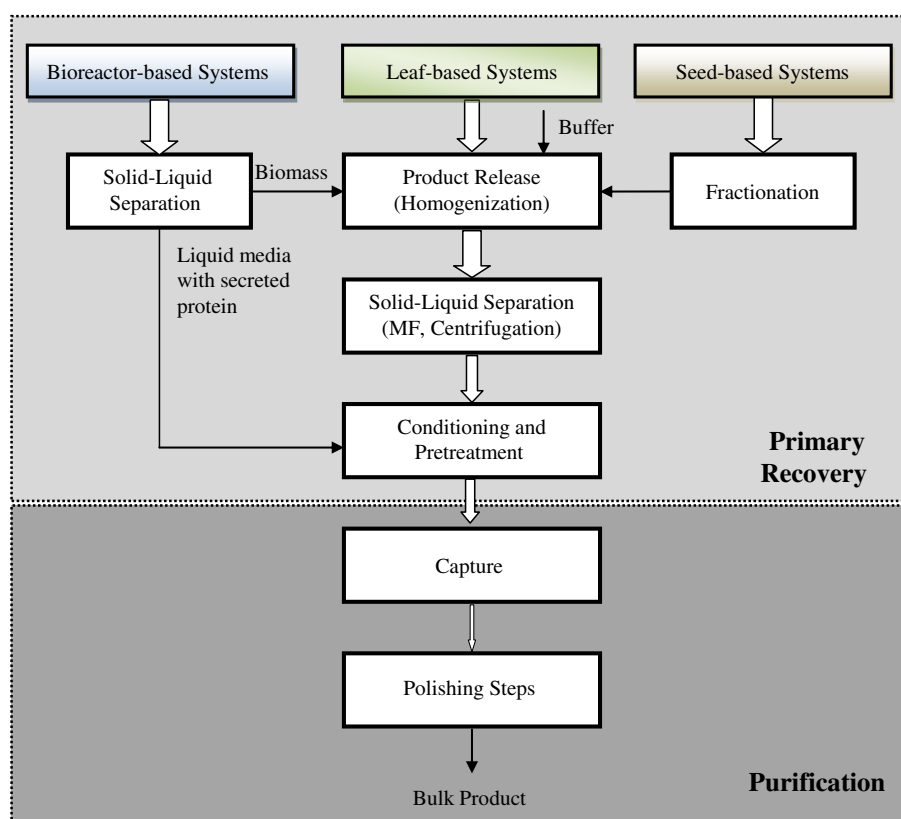


Fig. 1. Downstream processing of recombinant proteins from bioreactor-based, leaf-based, and seed-based systems.

extract composition on protein separation (Gu and Glatz, 2007; Holler and Zhang, 2008).

### 3.1.1. Fractionation

Seed fractionation uses established processing methods, such as dry milling, dry fractionation, and wet milling, to reduce total processing volume and solids content, to enrich the recombinant protein, and to generate co-product revenues for seed-based systems (Moeller et al., 2010; Paraman et al., 2010a, 2010b; Zhang et al., 2009b). For the case of edible vaccines where high purity is not required (for edible plant tissues), fractionation can significantly reduce dosage requirements (Lamphear et al., 2002; Moeller et al., 2010). Oil removal after degermination for a germ-expressed product further enhances recombinant protein concentration and improves purification efficiency (Zhang et al., 2009a). Oil extraction from oilseeds is also very desirable before protein extraction to prevent oil emulsification and reduce recombinant protein losses.

Course fractionation using traditional dry milling provides separate germ-, endosperm-, and bran-rich fractions for further purification or for use in a partially purified form (Moeller et al., 2010; Shepherd et al., 2008a, 2008b; Zhang et al., 2009b). Wet milling provides better separation and thus, higher purity fractions for co-product use (starch) and higher enrichment of recombinant protein compared to dry milling. Recent studies indicate that traditional wet-milling conditions would likely need to be modified to retain recombinant product quality during fractionation (Zhang et al., 2009b).

### 3.1.2. Product release (tissue disruption and product extraction)

The protein release/extraction process is a critical recovery step because it dictates the total extract volume, recombinant protein concentration and purity, and the type and quantity of impurities that have to be removed during purification (Hassan et al., 2008; Nikolov

et al., 2009). Efficient homogenization of plant tissue and disruption of plant cell walls are prerequisites for maximum release of the recombinant protein into the extraction buffer. Physical disruption of high-water content biomass, such as *Lemma*, plant cell culture, and algae, can be accomplished by using high-shear mixers and/or high-pressure homogenizers in the presence of buffer at a tissue-to-buffer ratios ranging from 1:1.5 to 1:8 (Table 1). Ground seed is extracted with low-shear mixers using 4 to 5 L of buffer per kg dry seed. Extraction of oil bodies for oleosin-fusion technology requires much greater water-to-tissue ratios (Boothe et al., 2010; Nykiforuk et al., 2011). Using lower ratios reduces process volume but may also decrease the percentage of recombinant protein extracted. Leafy whole plants (tobacco, alfalfa) contain significant amounts of fiber from stems and stalks that can be screw pressed with or without prior hammer milling (Bratcher et al., 2005; D'Aoust et al., 2004) to produce an extract that is frequently referred to as "green juice" due to the presence of chlorophyll or other pigments. Screw presses are used on pilot-plant and manufacturing scales whereas fruit juice processors are adequate options for bench-scale pressing. Screw pressing minimizes buffer usage and, thus, minimizes the overall extract volume that must be processed in the subsequent steps. Wet grinding (homogenization) can also be used with seed-based systems with concomitant protein extraction, although the preferred method for low-oil-containing seeds is dry grinding/pulverization followed by low-shear mixing with an aqueous buffer. Grinding frozen tissue with mortar and pestle and hand held mixers (polytron) are convenient for initial screening of extraction conditions and evaluating recombinant protein stability on lab-scale, but do not reflect conditions on pilot-plant scale. Ideally, homogenization and extraction should be evaluated using kilogram quantities (not several grams) of transgenic tissue because mixing pattern, shear rate, removal of generated heat, and time required for maximal product release are volume (vessel size) dependent. Milder procedures for product release include

vacuum infiltration (Turpen et al., 2001) and enzymatic digestion of plant cells (Fischer et al., 1999). These methods are attractive but their efficiencies and economic viabilities for manufacturing of biotherapeutics have not yet been demonstrated.

Maximizing recombinant protein release is a critical objective that can have unintended consequences. Recombinant protein extraction is often accompanied by the release of a variety of indigenous proteins and other water-soluble plant cell components such as DNA, chlorophyll and other pigments, alkaloids, phenolics, polysaccharides, and proteases. These components can bind or degrade the recombinant protein affecting final product quality and yield or reduce purification efficiency due to resin or membrane fouling. Extraction optimization may require screening and evaluation of the tissue disruption technique, particle size distribution, buffer composition, plant tissue-to-buffer ratio, and subcellular compartment expression (Azzoni et al., 2005a; Farinas et al., 2005b; Hassan et al., 2008; Menkhaus et al., 2004b; Wilken and Nikolov, 2006; Woodard et al., 2009; Zhang et al., 2005, 2010). Modifying these conditions can enhance extraction by reducing molecular interactions between the recombinant protein and plant debris, organelles, and variety of insoluble plant compounds.

Protecting a recombinant protein from degradation during extraction is important to reduce purification costs since removal of degradation products with similar properties is difficult. To minimize proteolysis and phenol oxidation of the product during primary recovery, extraction buffers often contain a mixture of protein stabilizing agents such as protease inhibitors, metal chelators, and antioxidants (Table 1). Buffer additives such as  $\beta$ -2-mercaptoethanol (B-ME), dithiothreitol (DTT), polyvinyl polypyrrolidone (PVPP), ascorbic acid, and sodium metabisulphite can be used to reduce phenolics interference on adsorption processes, phenolics–protein interactions, or phenolics oxidation, (Holler et al., 2007; Holler and Zhang, 2008; Peckham et al., 2006). However, the use of complex buffer formulations is undesirable at manufacturing scale due to reagent cost and the necessity to remove these components during the product capture step. Consequently, the benefits of using complex buffers should be carefully evaluated since buffer disposal costs could become a significant operating expense (Fischer et al., 2007).

Plant protease activities in homogenates and clarified extracts can be minimized by controlling extraction temperature, pH, and time or by adding protease inhibitors. Identifying the class of proteases responsible for recombinant protein degradation is essential for choosing a protease inhibitor strategy. Many extraction buffers are formulated with protease inhibitors such as EDTA and PMSF (Table 1). An alternative strategy for protecting expressed proteins both *in planta* and in the crude and clarified homogenates is the co-expression of protease inhibitors which can reduce *in planta* degradation, increase overall yield, and decrease proteolytic activity in the crude extracts (Komarnytsky et al., 2006; Rivard et al., 2006). For plant cell culture, modification of media components reduced the presence of proteases two-fold (Huang et al., 2005).

Extraction of membrane-associated proteins could also benefit from addition of detergents. The concentration of membrane-bound IgG increased three-fold from addition of a detergent (Triton X-100) whereas the same antibody targeted to the apoplast and endoplasmic reticulum did not benefit from detergent addition (Hassan et al., 2008). Chloroplast-expressed proteins apparently require detergents to reduce hydrophobic interaction with thylakoid membranes (Boyhan and Daniell, 2011; Daniell et al., 2009a; Tran et al., 2009).

The ratio of recombinant protein to native protein is another critical factor for reducing the purification burden that can be easily manipulated by adjusting the extraction buffer pH and ionic strength and plant-tissue-to-buffer ratio (Azzoni et al., 2005a; Bai et al., 2002; Bai and Nikolov, 2001; Farinas et al., 2005b, 2007; Menkhaus et al., 2004b; Woodard et al., 2009; Zhang et al., 2005). For example, extraction of corn seed with pH 3 buffer instead of pH 9 buffer

resulted in an eight-fold decrease in native protein concentration (Azzoni et al., 2002; Zhong et al., 2007) and a two-fold decrease for the same pH range for tobacco leaves (Balasubramaniam et al., 2003). Low pH extraction (pH 3) allowed easier purification of collagen and aprotinin from transgenic corn extracts (Azzoni et al., 2002; Zhang et al., 2009a; Zhong et al., 2007).

### 3.1.3. Solid–liquid separation

Centrifugation is a common method for solids removal and/or clarification of plant extracts and homogenates because continuous or semi-continuous processing is feasible, the process is easy to scale up, and can handle a variety of solid–liquid suspensions. Seed extracts have large differential solid–liquid densities and are therefore easily clarified by using a low-G-force decanter centrifuge. The presence of insoluble starch in the seed extracts of rice and corn permits using pressure filters or basket centrifuges. The green juice (extract) from screw-pressed leafy plants (tobacco and alfalfa) can be clarified by using a disk-stack centrifuge (Bratcher et al., 2005; D'Aoust et al., 2004) or vacuum drum filter. Algae, *L. minor*, and plant cell cultures are too small for screw pressing and their homogenates can be clarified directly by centrifugation followed by depth filtration. For plant cell culture products, dead-end and cross-flow filtration are common methods employed for clarification. Using cross-flow filtration gives the best clarified stream for subsequent packed-bed chromatography (Hellwig et al., 2004).

### 3.1.4. Conditioning and pretreatment of extracts

To remove impurities after extraction, a variety of clarification and pretreatment steps can be added prior to purification. Plant extracts and plant cell homogenates have a unique composition of native proteins, cell metabolites, and process-derived impurities, and the presence of these potentially harmful impurities may require distinct handling or conditioning of the extract. The purpose of conditioning is to maximize product binding by capture chromatography, reduce binding of plant components to the recombinant protein, and stabilize extracts for purification. Traditional conditioning methods, also referred to as pretreatment methods, for any protein production platforms include adjustment of extract or media pH, ionic strength, buffer composition, and volume reduction by cross-flow filtration. However, process development scientists and engineers quickly realized that conditioning of plant extracts could not always rely on traditional methods and that soluble plant components could severely affect the performance and lifetime of the capture chromatography resins, especially expensive affinity resins (Jervis and Pierpoint, 1989; Woodard et al., 2009). As a result, additional platform-tailored conditioning and pretreatment methods were developed for one or more of the following reasons: 1) to remove plant impurities that interfere with the capture purification step; 2) to reduce the overall plant protein burden; and 3) to reduce recombinant protein exposure to phenolics and proteases.

As indicated in Table 2, several approaches have been implemented specifically for pretreatment of plant extracts including aqueous two-phase partitioning, adsorption, precipitation, and membrane filtration. Aqueous two-phase partitioning has been an effective way of removing plant phenolics and alkaloids from plant extracts prior to capture chromatography (Platis et al., 2008; Platis and Labrou, 2006, 2009). This method reduced the concentration of native tobacco proteins and resulted in a two-fold purity increase of two monoclonal antibodies (Platis et al., 2008). Similarly, Ross and Zhang (2010) reported 94% removal of tobacco protein from an acidic recombinant protein,  $\beta$ -glucuronidase. The successful removal of plant impurities permitted the development of less expensive, non-protein A purification protocols for a monoclonal antibody from transgenic tobacco (Platis et al., 2008) and one from corn extract (Ramessar et al., 2008).

Low percentage ammonium sulfate (25–30%) precipitation is another common method (Table 1) for pretreatment of cell culture

**Table 1**

Selected examples of recombinant protein extraction methods and results.

| Platform                      | Recombinant protein                     | Application                                                    | Amount      | Method         | Tissue to buffer ratio | Time, temp    | Selected buffer                            | Buffer additive(s)   | Product concentration                                  | Reference                  |
|-------------------------------|-----------------------------------------|----------------------------------------------------------------|-------------|----------------|------------------------|---------------|--------------------------------------------|----------------------|--------------------------------------------------------|----------------------------|
| <i>Seed-based systems</i>     |                                         |                                                                |             |                |                        |               |                                            |                      |                                                        |                            |
| Corn seed                     | Aprotinin                               | Protease inhibitor                                             | 20 g        | Mixing         | 1 to 5                 | 1 h at RT     | 200 mM NaCl, pH 3.0                        | None                 | 0.7 µg/mL                                              | (Azzoni et al., 2002)      |
| Corn germ                     | Aprotinin                               | Protease inhibitor                                             | 30 g        | Mixing         | 1 to 5                 | 60 min at RT  | Deionized water, pH 3.0                    | None                 | NR                                                     | (Zhong et al., 2007)       |
| Corn seed (defatted germ)     | Collagen                                | NR                                                             | 5 g         | Mixing         | 1 to 5 (2 times)       | 2 x 3 h at RT | 0.1 M phosphoric acid, 0.15 M NaCl, pH 1.8 | None                 | 120 mg/kg germ fraction                                | (Zhang et al., 2009b)      |
| Corn meal                     | IgG                                     | NR                                                             | 50 g        | Mixing         | 1 to 10                | 8 h at 4 °C   | 150 mM NaCl                                | None                 | 0.52 mg/g (0.052 mg/mL)                                | (Lee and Forciniti, 2010)  |
| Corn seed                     | IgG (2 G12)                             | Anti-HIV                                                       | 5 g         | Mixing         | 1 to 4                 | 60 min at RT  | 20 mM NaPhosphate, pH 6.0                  | None                 | >75 mg/kg DW                                           | (Ramessar et al., 2008)    |
| Rapeseed                      | Chymosin                                | Industrial / food enzyme                                       | 40          | Homogenization | 1 to 10                | NR            | Water with 250 mM NaCl                     | None                 | 4.2% TSP (47 mg/kg)                                    | (Van Rooijen et al., 2008) |
| Rice seed                     | Human lysozyme                          | Gastrointestinal infections, formula and cell culture additive | 20 g        | Mixing         | 1 to 5                 | 60 min at RT  | 50 mM NaAcetate, 50 mM NaCl, pH 4.5        | None                 | 4.4 mg/g (3.3 mg/g for selected extraction conditions) | (Wilken and Nikolov, 2006) |
| Rice seed                     | Human lactoferrin                       | Gastrointestinal infections, formula and cell culture additive | 1 g to 2 kg | Mixing         | 1 to 10                | 1 h at RT     | 0.02 M NaPhosphate, 0.3 M NaCl, pH 7.0     | None                 | 6 mg/g (4.2 mg/g for selected extraction conditions)   | (Nandi et al., 2005)       |
| Rice seed                     | Transferrin                             | Cell culture media additive for regulation; drug carrier       | NR          | Mixing         | 1 to 10                | 30 min at RT  | 25 mM Tris-HCl, pH 7.5                     | None                 | NR, 10 g/kg expression level                           | (Zhang et al., 2010)       |
| Soybean seed (defatted flour) | β-Glucuronidase (GUS)                   | Model protein                                                  | 5 g         | Mixing         | 1 to 20                | 0.5 h at RT   | 50 mM citrate buffer, pH 5.3               | None                 | NR, 3 mg/mL TSP                                        | (Robic et al., 2006)       |
| <i>Leaf-based systems</i>     |                                         |                                                                |             |                |                        |               |                                            |                      |                                                        |                            |
| Tobacco leaves                | Aprotinin                               | Protease inhibitor                                             | NR          | Homogenization | NR                     | NR            | NR                                         | NR                   | ~750 mg/kg (greenhouse), ~300 mg/kg (open field)       | (Pogue et al., 2010)       |
| Tobacco leaves                | β-Glucuronidase                         | Model protein                                                  | NR          | Homogenization | 1 to 5                 | 1 min at 4 °C | 50 mM NaPhosphate, pH 7.0                  | 10 mM BME, 1 mM EDTA | NR, 1.4 mg/mL TSP                                      | (Holler and Zhang, 2008)   |
| Tobacco leaves                | Influenza virus like particles (H5-VLP) | Avian influenza vaccine (A/H5N1)                               | 25 kg       | Homogenization | NR                     | RT            | NR                                         | NR                   | NR                                                     | (D'Aoust et al., 2010)     |
| Tobacco leaves                | IgG (patient 69)                        | Idiotypic vaccines for non-Hodgkin's lymphoma                  | 5 kg        | Homogenization | NR                     | NR            | NR                                         | NR                   | 1.5 g/kg FW                                            | (Bendandi et al., 2010)    |

|                                 |                                    |                                                           |              |                                    |                      |                 |                                                                         |                                                                                             |                                                     |                                                |
|---------------------------------|------------------------------------|-----------------------------------------------------------|--------------|------------------------------------|----------------------|-----------------|-------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|-----------------------------------------------------|------------------------------------------------|
| Tobacco leaves                  | IgG                                | Anti-WNV                                                  | 10 g to 5 kg | Homogenization                     | NR                   | NR              | PBS                                                                     | 1 mM EDTA, 0.3 mg/mL PMSF, 10 mg/mL NaAscorbate, 10 µg/mL leupeptin                         | 0.8 g/kg FW                                         | (Lai et al., 2010)                             |
| Tobacco leaves                  | IgG (H10)                          | anti-tenascin-C                                           | 250 g        | Homogenization                     | 1 to 3               | NR              | PBS, pH 7.4                                                             | 5 mM EDTA, protease inhibitor cocktail (1%v/v)                                              | 600 mg/kg                                           | (Lombardi et al., 2010)                        |
| Tobacco leaves                  | IgG                                | Antibody to hepatitis B surface antigen                   | 10–600 kg    | Hammer milling                     | NR (wetted leaves)   | NR              | 150 mM PBS                                                              | 0.56 mM ascorbic acid                                                                       | 22–46 mg IgG/kg biomass (lowest for 600 kg process) | (Padilla et al., 2009)                         |
| Tobacco leaves                  | IgG (4E10) and IgG (2G12)          | Anti-HIV                                                  | 5 g          | Homogenization and mixing          | 1 to 3               | 60 min at 4 °C  | 50 mM NaPO <sub>4</sub> , pH 5.0                                        | None                                                                                        | 0.5–1.0% of TSP                                     | (Platis et al., 2008, Platis and Labrou, 2009) |
| Tobacco leaves                  | IgG (αCCR5)                        | Anti-HIV activity                                         | 40–60 kg     | Homogenization and screw pressing  | NR (probably 1 to 2) | NR              | NR                                                                      | NR                                                                                          | 250 mg/kg after purification                        | (Pogue et al., 2010)                           |
| Tobacco leaves                  | IgG (C5-1)                         | Diagnostic                                                | 100–150 g    | Homogenization                     | 1 to 3               | 2–3 min at RT   | 20 mM NaPhosphate, 150 mM NaCl, pH 5.8                                  | 2 mM sodium metabisulphite                                                                  | 570 mg/kg FW                                        | (Vézina et al., 2009)                          |
| <i>Bioreactor-based systems</i> |                                    |                                                           |              |                                    |                      |                 |                                                                         |                                                                                             |                                                     |                                                |
| Carrot suspension cell culture  | Glucocerebrosidase (intracellular) | Gaucher's disease                                         | NR           | Homogenization                     | NR                   | 4 °C            | 20 mM NaPO <sub>4</sub> , pH 7.2                                        | 20 mM EDTA, 0.1 mM PMSF; 1% Triton X-100; 20 mM Ascorbic acid; 0.1 mM DL-dithiothreitol     | NR                                                  | (Shaaltiel et al., 2007)                       |
| Lemna fronds                    | IgG1 (anti-CD 30)                  | Non-Hodgkin's lymphoma and anaplastic large-cell lymphoma | NR           | High-shear homogenization          | 1 to 5               | 30 min at 10 °C | 50 mM NaPhosphate, 300 mM NaCl, pH 7.2                                  | 10 mM EDTA                                                                                  | 2% of TSP                                           | (Cox et al., 2006, Nikolov et al., 2009)       |
| Lemna fronds                    | IgG1 (CNTD)                        | NA                                                        | 50 g         | High-shear homogenization          | 1 to 8               | 30 min at 10 °C | 100 mM NaAcetate, 300 mM NaCl                                           | 10 mM EDTA                                                                                  | 50% of TSP at pH 4.5; 10% TSP at pH 7.5             | (Woodard et al., 2009)                         |
| Tobacco suspension cell culture | Anti-rabies Mab (intracellular)    | Human anti-rabies virus Mab for treatment                 | 1 g          | Mixer mill then homogenization     | NR                   | 2 min           | 40 mM Tris–HCl, pH 8.0, 20% glycerol, 2% SDS, and 4% β-mercaptoethanol) | Protease Inhibitor Mixture, 0.1% Triton-X, 2% SDS, 4% B-Me                                  | 3–10 µg/g leaf FW                                   | (Girard et al., 2006)                          |
| Tobacco suspension cell culture | Anti-rabies Mab (intracellular)    | Human anti-rabies virus Mab for treatment                 | 1.45 kg      | French press                       | 1 to 10              | NR              | 20 mM NaPhosphate, pH 7.2                                               | None                                                                                        | 4-fold less than that with 1 g extraction           | (Girard et al., 2006)                          |
| Tobacco suspension cell culture | GFP-fusion protein (intracellular) | Reporter tag                                              | 20 g         | Microson ultrasonic cell disruptor | 1 to 2               | 5 min           | 0.25 M Boric acid, pH 8, 0.25 M NaCl                                    | 1% caffeine, 1% ascorbic acid, 0.1% Triton X-100, 1 mM DTT, 1 mM PMSF, 1% protease cocktail | 10.7 mg TSP                                         | (Peckham et al., 2006)                         |

NR, not reported; RT, room temperature; TSP, total soluble protein.

**Table 2**  
Conditioning, pretreatment, and recombinant protein purification methods.

| Plant system              | Recombinant protein                     | Solid–liquid separation, conditioning and pretreatment                                                                                               | Capture                                              | Intermediate purification               | Polishing                                                    | Purity      | Yield  | Reference                  |
|---------------------------|-----------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------|-----------------------------------------|--------------------------------------------------------------|-------------|--------|----------------------------|
| <i>Seed-based systems</i> |                                         |                                                                                                                                                      |                                                      |                                         |                                                              |             |        |                            |
| Corn seed                 | Aprotinin                               | – Dead-end filtration<br>– Adjust to pH 7.8<br>– Dead-end filtration                                                                                 | Trypsin affinity                                     | IMAC-Cu                                 | None                                                         | 79%         | 49%    | (Azzoni et al., 2002)      |
| Corn germ                 | Aprotinin                               | – Centrifugation<br>– Dead-end filtration                                                                                                            | Weak cation exchange (CM-Sepharose)                  | HIC (Phenyl-Sepharose)                  | None                                                         | 75%         | 34%    | (Zhong et al., 2007)       |
| Corn seed                 | Collagen                                | – Centrifugation<br>– Dialysis<br>– Dead-end filtration                                                                                              | Strong cation exchange (SP-Sepharose FF)             | Gel filtration (Sephacryl 200HR)        | None                                                         | >70%        | 60%    | (Zhang et al., 2009a)      |
| Corn seed                 | IgG                                     | – Centrifugation<br>– Dead-end filtration (2-step)                                                                                                   | PEG/PO <sub>4</sub> separation (10%/15%), 11% NaCl   | PEG/PO <sub>4</sub> separation (9%/11%) | PEG/PO <sub>4</sub> (8.2%/14.8%), Precipitation at interface | 72%         | 49%    | (Lee and Forciniti, 2010)  |
| Corn seed                 | IgG (2G12)                              | – Centrifugation<br>– Dead-end filtration                                                                                                            | Strong cation exchange (SP-Sepharose FF)             | IMAC-Zn                                 | None                                                         | 90%         | 50–60% | (Ramessar et al., 2008)    |
| Rapeseed                  | Chymosin                                | – Centrifugation<br>– pH 2 acidification<br>– Adjust to pH 5.6<br>– Centrifugation<br>– Dilution with water to reduce conductivity                   | Weak anion exchange (DEAE)                           | HIC (Butyl Sepharose)                   | None                                                         | NR          | NR     | (Van Rooijen et al., 2008) |
| Rice seed                 | Human lactoferrin                       | – Centrifugation or sedimentation<br>– Dead-end filtration                                                                                           | Strong cation exchange (SP-Sepharose FF)             | None                                    | UF/DF                                                        | 93%         | 68%    | (Nandi et al., 2005)       |
| Rice seed                 | Transferrin                             | – Centrifugation                                                                                                                                     | Weak anion exchange (DEAE)                           | None                                    | None                                                         | >95%        | 60%    | (Zhang et al., 2010)       |
| Rice seed                 | Human lysozyme                          | – Centrifugation and dead-end filtration<br>– pH adjustment from pH 4.5 to 6 with 1 N NaOH<br>– Dead-end filtration                                  | Strong cation exchange (SP-Sepharose FF)             | None                                    | None                                                         | 95%         | 90%    | (Wilken and Nikolov, 2006) |
| Rice seed                 | Human Lysozyme                          | – Centrifugation and dead-end filtration<br>– pH adjustment from pH 4.5 to 6 with 1 M TRIS and 1 M NaOH or pH 4.5 precipitation (for pH 10 extracts) | Strong cation exchange (SP-Sepharose FF)             | None                                    | None                                                         | 98%         | 97%    | (Wilken and Nikolov, 2010) |
| Soybean seed              | β-Glucuronidase                         | – Centrifugation<br>– Dead-end filtration                                                                                                            | Weak anion exchange (DEAE)                           | HIC (Phenyl Sepharose)                  | None                                                         | NR          | 110%   | (Robic et al., 2006)       |
| <i>Leaf-based systems</i> |                                         |                                                                                                                                                      |                                                      |                                         |                                                              |             |        |                            |
| Tobacco leaves            | Aprotinin                               | – Acidification<br>– Centrifugation<br>– Dead-end filtration<br>– Ultrafiltration                                                                    | Cation exchange                                      | Reverse phase chromatography            | UF/DF                                                        | >99%        | ~50%   | (Pogue et al., 2010)       |
| Tobacco leaves            | β-Glucuronidase                         | – Addition of 2% (w/v) pre-hydrated PVPP<br>– Centrifugation<br>– Dead-end filtration                                                                | Polyelectrolyte precipitation (800 mg PEI/g protein) | HIC (Phenyl Sepharose)                  | Ceramic hydroxyapatite, concentration                        | High purity | 40%    | (Holler and Zhang, 2008)   |
| Tobacco leaves            | Influenza virus like particles (H5-VLP) | – Centrifugation<br>– Clarification (chemical and physical treatments)                                                                               | Cation exchange                                      | Affinity                                | Cross-flow filtration, formulation                           | High purity | NR     | (D'Aoust et al., 2010)     |
| Tobacco leaves            | IgG (patient 69)                        | – Precipitation to below pH 5.1<br>– Filtration                                                                                                      | Protein A                                            | Anion exchange (membrane adsorption)    | NR                                                           | >90%        | >50%   | (Bendandi et al., 2010)    |

|                                 |                                            |                                                                                                                                                                                                                                                            |                                            |                                     |                                              |             |            |                          |
|---------------------------------|--------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------|-------------------------------------|----------------------------------------------|-------------|------------|--------------------------|
| Tobacco leaves                  | IgG                                        | <ul style="list-style-type: none"> <li>– Dead-end filtration</li> <li>– Centrifugation</li> <li>– 25% ammonium sulfate precipitation</li> <li>– Centrifugation</li> <li>– 50% ammonium sulfate precipitation</li> <li>– Resuspend pellet in PBS</li> </ul> | 50% ammonium sulfate precipitation         | Protein A (MabSelect)               | Weak anion exchange, flowthrough mode (DEAE) | >95%        | 50%        | (Lai et al., 2010)       |
| Tobacco leaves                  | IgG (H10)                                  | <ul style="list-style-type: none"> <li>– Centrifugation</li> <li>– Adjustment to pH 7.5</li> <li>– Centrifugation</li> <li>– Dead-end filtration</li> </ul>                                                                                                | Protein A                                  | Strong cation exchange (Source 30S) | Buffer exchange                              | 99%         | 7%         | (Lombardi et al., 2010)  |
| Tobacco leaves                  | IgG (4E10)<br>IgG (2G12)                   | <ul style="list-style-type: none"> <li>– Centrifugation</li> <li>– Dead-end filtration</li> <li>– PEG/Phosphate two-phase partitioning</li> <li>– Dialysis</li> </ul>                                                                                      | Strong cation exchange (SP Sepharose FF)   | IMAC-Zn                             | None                                         | 96%<br>97%  | 36%<br>63% | (Platis et al., 2008)    |
| Tobacco leaves                  | IgG                                        | <ul style="list-style-type: none"> <li>– Centrifugation or dead-end filtration</li> <li>– High-speed centrifugation</li> </ul>                                                                                                                             | Protein A (Streamline) EBA                 | Size exclusion (Sephadex G-25)      | Sterile filtration                           | 95%         | 44%        | (Padilla et al., 2009)   |
| Tobacco leaves                  | IgG ( $\alpha$ CCR5)                       | <ul style="list-style-type: none"> <li>– Horizontal screw press</li> <li>– Acidification</li> <li>– Dead-end filtration</li> </ul>                                                                                                                         | Protein A                                  | Multi-ion exchange                  | UF/DF                                        | 97%         | NR         | (Pogue et al., 2010)     |
| Tobacco leaves                  | IgG (C5-1)                                 | <ul style="list-style-type: none"> <li>– Precipitation at pH 4.8</li> <li>– Centrifugation</li> <li>– Adjustment to pH 8</li> <li>– Centrifugation</li> <li>– Dead-end filtration</li> <li>– Concentration (100 kDa cross-flow filtration)</li> </ul>      | Protein G                                  | None                                | None                                         | >90%        | NR         | (Vézina et al., 2009)    |
| <i>Bioreactor-based systems</i> |                                            |                                                                                                                                                                                                                                                            |                                            |                                     |                                              |             |            |                          |
| Carrot cell suspension          | Glucocerebrosidase (intracellular)         | <ul style="list-style-type: none"> <li>– High-speed centrifugation</li> </ul>                                                                                                                                                                              | Strong cation exchange (Macro-prep high S) | HIC (Phenyl)                        | Strong cation exchange                       | High purity | NR         | (Shaaltiel et al., 2007) |
| Lemna                           | IgG1 (anti-CD 30)                          | <ul style="list-style-type: none"> <li>– Acidification</li> <li>– Centrifugation</li> <li>– Precipitation at pH 4.5</li> <li>– Adjustment to pH 7.2</li> <li>– Dead-end filtration</li> </ul>                                                              | Protein A (Mab Select SuRe)                | Ceramic Hydroxyapatite              | None                                         | >90%        | NR         | (Cox et al., 2006)       |
| Rice cell suspension            | HSA (extracellular)                        | <ul style="list-style-type: none"> <li>– 40% ammonium sulfate precipitation</li> <li>– Centrifugation</li> <li>– Dialysis</li> </ul>                                                                                                                       | Weak anion exchange (DEAE-Sepharose)       | Dialysis and concentration          | None                                         | >95%        | 50%        | (Huang et al., 2005)     |
| Tobacco cell suspension         | GFP-fusion protein (intracellular)         | <ul style="list-style-type: none"> <li>– Centrifugation</li> <li>– Dead-end filtration</li> <li>– 30% ammonium sulfate precipitation</li> <li>– Centrifugation</li> <li>– Dead-end filtration</li> </ul>                                                   | HIC (Phenyl Sepharose)                     | Strong anion exchange (Q-Sepharose) | None                                         | >80%        | >70%       | (Peckham et al., 2006)   |
| Tobacco cell suspension         | Human anti-rabies antibody (intracellular) | <ul style="list-style-type: none"> <li>– Fiberglass filtration</li> <li>– 30 kDa membrane concentration</li> </ul>                                                                                                                                         | Hi Trap Protein G                          | None                                | None                                         | >95%        | 25%        | (Girard et al., 2006)    |
| Tobacco cell suspension         | Human growth hormone intracellular)        | <ul style="list-style-type: none"> <li>– Filtration</li> <li>– 2 M NaCl precipitation</li> <li>– Centrifugation</li> </ul>                                                                                                                                 | HIC (Phenyl Sepharose)                     | None                                | UF concentration                             | >85%        | NR         | (Xu et al., 2010)        |
| Tobacco cell suspension         | Interferon $\alpha$ 2b (extracellular)     | <ul style="list-style-type: none"> <li>– Filtration</li> <li>– 2 M NaCl precipitation</li> <li>– Centrifugation</li> </ul>                                                                                                                                 | HIC (Phenyl Sepharose)                     | None                                | UF concentration                             | >85%        | >60%       | (Xu et al., 2007)        |

NR, not reported; UF/DF, ultrafiltration/diafiltration; HIC, hydrophobic interaction chromatography.

and green tissue homogenates (Lai et al., 2010; Peckham et al., 2006). Ammonium sulfate precipitation removes native plant proteins including the photosynthetic protein, ribulose biphosphate carboxylase/oxygenase (rubisco), aggregates, and cell debris (Garger et al., 2000). In some cases, the ammonium sulfate precipitation step can be bypassed by careful selection of a capture chromatography resin (Zhang et al., 2010).

Although there are few reported examples, recombinant protein secreted into culture media would also need conditioning by cross-flow filtration, pH adjustment, and dead-end filtration (depth filtration), but not necessarily in that order (Echelund et al., 2007; Hellwig et al., 2004). In general, seed extracts require pH adjustment followed by clarification by using centrifugation or depth filtration to remove protein and phytic acid precipitates from the extract (Wilken and Nikolov, 2010). A unique case that called for a product-host tailored conditioning of rice seed extracts involved positively charged human lysozyme and negatively charged phytic acid (Wilken and Nikolov, 2010). The authors developed several approaches for reducing the phytic acid concentration in the extract in order to increase the adsorption capacity of human lysozyme to a cation-exchange resin. Phytate hydrolysis, seed extraction at high pH followed by acidic precipitation to pH 4.5, or using TRIS buffer for extract pH adjustment and chromatography were all effective means of improving adsorption capacity while achieving >95% purity.

Adjusting the pHs of leafy extracts and cell homogenates to <5.0 precipitates the most abundant plant protein (rubisco), cell debris, as well as chlorophyll pigments attached to the protein and debris (Bendandi et al., 2010; Cox et al., 2006; Garger et al., 2000; Pogue et al., 2010; Woodard et al., 2009). Similar to ammonium sulfate precipitation, acid precipitation resulted in at least two-fold purification of a recombinant protein prior to the first chromatography column (Woodard et al., 2009). Acidic (pH 4.5) precipitation of *Lemna* extracts containing IgG also removed phenolics and green pigments. However, phenolics were more effectively removed by adsorption to hydrophobic uncharged or anionic resins (Barros et al., 2011; Woodard et al., 2009).

To remove various organic and inorganic precipitates formed during extract conditioning, a second clarification step consisting of centrifugation and/or dead-end filtration is applied. All purification processes highlighted in Table 2 utilize at least one step for conditioning, pretreatment, and/or clarification and in some cases, a series of steps were used.

### 3.2. Purification

The purification phase usually starts with a capture step to concentrate the recombinant protein and most importantly, remove critical plant impurities that would be detrimental to protein yield, quality, and/or purification efficiency (Holler and Zhang, 2008; Menkhaus and Glatz, 2005; Platis et al., 2008; Wilken and Nikolov, 2010; Woodard et al., 2009). For high purity applications (therapeutic, diagnostic), additional purification steps (polishing) are typically needed to remove residual host impurities and to ensure high product quality. Purification of plant-derived proteins, similarly to other protein production systems, is dependent on protein properties and host cell impurities, and utilizes similar strategies for purification development as other heterologous expression systems. After conditioning and pretreatment, purification procedures employ similar techniques already developed for biopharmaceutical products such as monoclonal antibodies (mAb), mAb fragments, vaccines, cytokines, and hormones (Chen, 2008; Menkhaus et al., 2004a; Nikolov and Woodard, 2004). As indicated in Table 2, purification of recombinant proteins relied primarily on adsorption chromatography because of superior resolution power and the variety of resins available.

There are very few studies reporting purification processes for plant-derived recombinant proteins that have reached clinical trials or

been scaled-up to commercial production. The reason for this is twofold. First, relatively few companies have reached later stages of clinical trials (Phases II and III) to have established robust, at-scale downstream processes and, second, newly developed processes are often deemed proprietary (Boothe et al., 2010). Nevertheless, a significant number of scientific publications since 2004 have addressed specific challenges in downstream process development and scale-up, such as low expression level, plant extract complexity and its effect on purification efficiency, product stability during processing, and removal methods for critical impurities. The number of published methods for protein purification from leafy tissue and cell culture seems to outnumber those from seeds, and in terms of products, therapeutic antibodies and vaccine purification have been more extensively studied. The number of reported analytical purifications is even greater than that included in this review, but we elected to focus on potentially scalable processes or those that address downstream processing bottlenecks, process yields, and protein purity. For recent reviews of protein purification and plant hosts, one could also refer to Boothe et al. (2010), Chen (2008), and Xu et al. (2011).

#### 3.2.1. Capture and polishing

Capture chromatography resins have two primary functions, concentration and partial purification, and should be inexpensive, resistant to chemicals needed for resin regeneration, and able to retain capacity and selectivity over multiple cycles. Resin selection is determined by recombinant protein properties such as charge, hydrophobicity, and biospecificity. Selecting a resin based on the property most unique to the recombinant protein compared to the host system can improve purification efficiency by increasing binding capacity and/or product purity. Once suitable chromatography resin functionality is determined (cation/anion-exchange, affinity, hydrophobic), various resins with different particle sizes, surface areas, and resin backbones need to be screened along with binding conditions, such as pH and ionic strength. As would be expected, acidic proteins like  $\beta$ -glucuronidase (Holler and Zhang, 2008; Robic et al., 2006), chymosin (Van Rooijen et al., 2008), and human serum albumin (Huang et al., 2005) were captured by anion-exchange adsorption, and basic proteins aprotinin, human lysozyme, human lactoferrin, and transferrin as well as several IgGs by cation-exchange (Table 2). In several instances product concentration and partial purification have been achieved by salt (Lai et al., 2010) or polymeric precipitation (Holler et al., 2007; Holler and Zhang, 2008) before the next purification step. Because of relatively low recovery yields compared to affinity and ion-exchange resins, hydrophobic interaction chromatography is not usually considered for product capture unless extract pretreatment requires the use of high salt concentrations (Peckham et al., 2006; Xu et al., 2007, 2011).

IgG and IgG-based products expressed in plant systems have been almost exclusively captured on protein A or G columns and further purified with at least one additional chromatography step using resins and process sequences developed for cell culture-derived antibodies (Chen, 2008; Nikolov et al., 2009). Several examples in Table 2 indicate a change in this trend once it was determined that clarified plant extracts could not be directly loaded on protein A columns without affecting resin efficiency and lifetime (Woodard et al., 2009). Platis and Labrou (2009); Platis et al. (2008), and Ramessar et al. (2008) implemented two-phase partitioning processes for tobacco and corn extracts as a pretreatment step that allowed them to efficiently capture recombinant monoclonal antibodies (anti-HIV IgGs) on a strong cation-exchange resin (Table 1). The subsequent purification step was the same for both host extracts. Higher product (2G12) purity was achieved from tobacco than corn extract probably because of degradation and aggregation of corn-derived 2G12 (Ramessar et al., 2008). Purity and yield of the other IgG protein (4E10) was 96% and 36%, respectively. Tobacco expressed 2G12

and 4E10 differed only in the variable regions but 4E10 apparently interacted differently with the immobilized metal affinity resin, which resulted in a lower yield. Another example is the purification of anti-WNV monoclonal antibody (Hu-E16) from tobacco extract (Lai et al., 2010). Clarified tobacco extract was first treated with 25% ammonium sulfate to remove interfering extract impurities and then Hu-E16 was concentrated by precipitation with 50% ammonium sulfate. Solubilized pellet containing Hu-E16 IgG was purified by using protein A (MabSelect) followed by a weak anion-exchange step. At 5 kg scale, IgG purity was >95% with 49% yield, which was very good for the three-step purification process. In the other examples where IgG extracts were captured on Protein A or G columns, acidic precipitation of host protein at pH<5.1 was apparently helpful in protecting the affinity resin (Bendandi et al., 2010; Cox et al., 2006; Pogue et al., 2010; Vézina et al., 2009). Vézina et al. (2009) reported no significant change of purification performance by protein G resin used for at least 10 cycles with tobacco extract clarified by acidic precipitation.

Intermediate purification and polishing (further purification of eluted proteins from the capture step) can include a variety of orthogonal steps such as HIC, IMAC, ion-exchange, and ceramic hydroxyapatite. The selected method is dictated primarily by target protein properties, as a majority of native impurities should have been removed during the capture step. In some instances when capture chromatography did not result in satisfactory removal of impurities (residual DNA, endotoxin, or leached affinity ligand), the subsequent purification step is often operated in flow-through mode to bind major contaminants and collect the target protein in the flow through (Bendandi et al., 2010; Lai et al., 2010).

**3.2.1.1. Use of affinity tags for purification.** The use of various biospecific (affinity) tags for purification of proteins from plant extracts were recently reviewed by Chen (2008). Affinity tags are convenient for bench-top purification and subsequent characterization of a recombinant protein but may not be economical at production scale because tag removal (required for biopharmaceuticals) by enzymatic or chemical cleavage is not efficient and reduces target protein yield. One application where protein fusions seem beneficial is plastid expression. Fusion partners stabilize and protect chloroplast-expressed target proteins from excessive proteolytic degradation and facilitate fusion protein purification. However, fusions that accumulate at levels greater than 10% of TSP form inclusion bodies which require solubilization and refolding prior to purification, similar to *Escherichia coli* inclusion bodies. In spite of significant advancements in plastid transformation and protein expression, few studies report purification development.

The following examples demonstrate the dual role of the fusion partner: protein accumulation and simplified purification. Leelavathi and Reddy (2003) purified GUS-interferon (INF) fusion protein (6% of TSP) by weak anion-exchange chromatography followed by IMAC-Ni chromatography. The presence of His-tagged GUS in the fusion increased expression 60-fold and simplified the purification. INF was cleaved with factor Xa from the fusion protein with 58% efficiency and was subsequently purified by cation-exchange chromatography. Daniell et al. (2009a) used two ammonium sulfate precipitation steps and an IgG-affinity column to purify insulin-like growth factor (IGF) fused to the Z-domain of *Staphylococcus aureus*. The fusion protein (~10% TSP) was chemically cleaved with hydroxylamine to release the Z-domain. The cleavage efficiency was only 40% but was significantly more efficient and less expensive than the proteolytic one (TEV protease). Cholera toxin B-proinsulin fusion, which accumulated in tobacco leaves as inclusion bodies at 47% of TSP, was solubilized and refolded before purifying the fusion protein by an IMAC-Ni packed-bed column. Metal affinity chromatography was chosen because the cholera toxin B domain contains three adjacent His residues, allowing strong interactions between the fusion and Ni-agarose resin (Boyhan and Daniell, 2011).

**3.2.1.2. Overcoming interfering impurities (case studies).** Aprotinin purification is an interesting case that demonstrates how the effect of host impurity interference could be resolved by changing host system. Azzoni et al. (2002), Azzoni et al. (2005b), and Zhong et al. (2007) explored different process schemes for purifying aprotinin from corn seed extracts and achieved >75% protein purity with similar process yields of <40% (Table 2). Azzoni's processes were hampered by co-purification of native corn trypsin inhibitor which also bound to trypsin affinity column and could not be completely removed by subsequent IMAC or cation-exchange steps. Zhong et al. (2007) solved this interference by using acidic extraction of corn germ, instead of whole seed flour, and heat precipitation of host proteins at 80 °C. Weak cation-exchange chromatography removed corn trypsin but subsequent purification with hydrophobic interaction chromatography (HIC) still resulted in an aprotinin purity of only 75%. Pogue et al. (2010) expressed aprotinin in tobacco leaves, which do not naturally produce any trypsin inhibitors, and purified the aprotinin using the same two chromatography steps, cation-exchange and HIC, as did Zhong et al. (2007). The purity of the final product after diafiltration and concentration step was 99% and overall process yield was 50% – a substantial improvement compared to corn host.

A somewhat unique case that called for a product-tailored solution of rice seed extracts involved positively charged human lysozyme and negatively charged phytic acid (Wilken and Nikolov, 2010). The authors developed several approaches for reducing phytic acid concentration in the extract in order to increase adsorption capacity of human lysozyme to a cation-exchange resin. Phytate hydrolysis, seed extraction at high pH followed by acidic precipitation to pH 4.5, or using TRIS buffer for extract pH adjustment and chromatography were all effective means of improving adsorption capacity while achieving >95% purity.

### 3.2.2. Use of non-chromatographic methods

One of the first non-chromatographic methods that appeared in the literature was capturing and partial purifying of oleosin-fusion proteins by centrifugation (Van Rooijen and Moloney, 1995). These fusions have oleosin proteins as N-terminal fusion partners that allow *in vivo* targeting and/or post-extraction capture of the target protein on the surface of oil bodies (Boothe et al., 2010). Purification of oil bodies with attached fusions is done by several washing steps in aqueous solutions using centrifugation. Attached recombinant protein can be released from oil bodies by proteolytic cleavage of the oleosin-target protein linkage or by elution in the case of affinity bound proteins. The affinity binding approach is exemplified by constructs developed recently that contain an N-terminal anti-oleosin single chain antibody (scFv) as the fusion partner. Following their elution from oil bodies these fusions can be cleaved either chemically (e.g. acidic cleavage) or enzymatically to release the recombinant protein (Boothe et al., 2010; Nykiforuk et al., 2011). The potential downside of these two strategies is the need to cleave the recombinant protein from the oleosin or scFv fusion partner. The cleavage precision and efficiency, whether chemical or enzymatic, is typically less than 70% and results in reduced product yield. Subsequent purification of released recombinant proteins from respective fusions is done by standard adsorption chromatography.

An interesting idea that sought to further exploit this platform was the development of inexpensive media for IgG or thrombin purification using oil bodies with covalently attached affinity ligands such as protein A and hirudin (Moloney et al., 2008). Similarly, Werner et al. (2006) attached protein A to the C-terminus of the core protein of turnip vein clearing virus. Expressing this fusion in tobacco leaves produced rod-shaped, nanometer size viral particles that displayed protein A on the surface. The high-surface density of Protein A allowed binding of 2 g IgG per g viral particle.

Other equally good examples of non-chromatographic purification methods utilized unique protein properties (size, hydrophobicity or

stability) and host system properties to accomplish enhanced separation efficiency. Lee and Forciniti (2010) explored the use of aqueous two-phase (PEG/salt) partitioning as a sole recovery and purification method of non-glycosylated mAb expressed in corn seed. By manipulating the system composition, pH, and ionic strength they managed to partially purify the antibody in a three-stage process. The first two stages were typical two-phase partitioning with the target protein concentration enriched in the bottom (aqueous salt phase) and host impurities in the top (PEG) phase. These two-extraction protocols resulted in a rather modest mAb purification of 1.3- and 1.4-fold, respectively. The third stage consisted of mAb precipitation at the two-phase interface and resulted in almost 10-fold purification. Overall, the three-stage processes delivered 72% pure mAb with 49% yield. Clearly, an additional adsorption step, most likely affinity chromatography, would be needed to purify the antibody for biopharmaceutical applications.

Aspelund and Glatz (2010) demonstrated purification of recombinant collagen from low pH corn extracts by cross-flow filtration. Diafiltration of corn endosperm extracts at pH 3.1 by using 100-kDa MWCO membranes removed 96% of host protein and resulted in 89% pure collagen. Improved purification of collagen was achieved by protein precipitation of endosperm extracts with sodium chloride at pH 2.1. Membrane diafiltration of the resuspended precipitate resulted in 99% pure collagen and 87% yield. Diafiltration of corn germ extracts using the same conditions resulted in a collagen purity of only 50% due to inferior protein sieving. Thus, the unique composition of endosperm extract and molecular properties of collagen (high molecular weight and stability at low pH) allowed the development of this extremely attractive and inexpensive purification scheme.

Although much progress has been made over the past 7 years, downstream processing still requires further attention and technological breakthroughs to fulfill the long anticipated goal of low manufacturing cost for plant-derived recombinant proteins. However, improvements in downstream processing alone will not suffice; high protein expression levels and assured product fidelity are also necessary.

#### 4. Process economics

The manufacturing cost for plant-produced proteins consists of upstream (biomass production) and downstream purifications costs. The cost of manufacturing in most cases is a strong function of protein expression, overall process yield, and production scale. As discussed in the previous section, the downstream processing costs are also affected by the ease of product recovery, the complexity of clarified plant or cell culture extracts, protein stability, and required purity (Nikolov and Hammes, 2002). For example, biopharmaceuticals and processing enzymes for cGMP manufacturing may require protein purities as high as 99% and those for diagnostics, 90%. Although in all three cases the downstream manufacturing processes have to be robust (batch-to-batch repeatability), the main difference would be required documentation and regulatory-related activities. These are often “hidden costs” in biopharmaceutical manufacturing that are not readily available in the published literature and often either unaccounted for or underestimated (Farid, 2007). The support activities, such as process and cleaning validation, buffer preparation, equipment cleaning, and quality control and quality assurance (QC/QA), could be a substantial fraction of operating costs. For example, the labor cost for validation and QC/QA activities can easily account for more than 50% of the direct labor cost (Nikolov, unpublished data).

The breakdown of upstream production and downstream purification cost depend primarily on end application with biopharmaceutical and industrial proteins being at the opposite end of the cost and purity spectrum (high to low). In general, the upstream cost for highly-purified proteins (>90%) from seed crops ranges from 5 to 10%

of the total manufacturing cost depending on expression level, purification yield, and annual product output (Evangelista et al., 1998; Mison and Curling, 2000; Nandi et al., 2005; Nikolov and Hammes, 2002). For proteins of similar purity and annual throughput, greenhouse and bioreactor production costs could be up to 20–25% and 35%, respectively, of the total manufacturing cost (unpublished estimates). Therefore, downstream processing contribution to total manufacturing cost of goods could range from 65 to 95%. The production cost (\$/g product) of the same recombinant protein produced in a contained system (greenhouse) would be 3–5 times greater than that produced in an open-field system (Pogue et al., 2010). There is less information and certainty about the cost of bioreactor-based systems, but one should anticipate 30–80 times greater production cost compared to open-field. Upstream production cost for low-purity protein products, such as industrial enzymes and oral vaccines, would be around 50% of the total manufacturing cost since minimal downstream processing would be required as less costly recovery methods such as membrane filtration and protein precipitation are typically used (Arntzen et al., 2006; Nikolov and Hammes, 2002). With regard to overall cost savings, the manufacturing cost for an open-field produced recombinant protein would be 30–50% lower than the cost of a bioreactor-produced protein, if downstream processing costs were similar.

The cost savings would be significantly higher for a non-pharmaceutical product, which indicates that bioreactors are only commercially viable production systems for pharmaceutical products. It is likely that the best way to achieve cost savings for a bioreactor-based system would be to maximize biomass productivity, product concentration, and downstream processing efficiency.

Another important consideration for the three production systems would be the capital investment, which is rarely addressed in published literature. By some estimates, capital investment increases multi-fold and proportionally to the level of containment and sophistication of plant growth controls with bioreactor-based systems approaching that of mammalian culture bioreactors (Spök and Karner, 2008).

The decision-making process for choosing a plant production system is complex and requires case-by-case cost analyses. From a downstream cost perspective, several evaluation criteria could be applied for selecting the best system that matches product characteristics and allows overall manufacturing cost reduction (Nikolov and Hammes, 2002). In addition to protein expression level and stability, the following criteria should be considered: biomass yield and storage stability (allowing processing flexibility and decoupling), selection of off-the-shelf purification tools, biomass disposal cost, and byproduct revenues (e.g. biomass and starch conversion to energy). Other business drivers for consideration include capital investment, production scale and cost, product application and specifications (therapeutics vs. industrial proteins), speed to market, and regulatory requirements (Nikolov and Hammes, 2002; Spök and Karner, 2008).

#### 5. Conclusions and future directions

Several perceived disadvantages of using plant systems for recombinant protein production have been addressed quite well over the past 5 to 10 years, including concerns about recombinant protein glycosylation, development time for biopharmaceuticals, open-field production issues, and expression levels. For example, open-field production safety and regulatory issues have been resolved by using contained systems for plant production and product development time has drastically been reduced by using transient expression in leafy systems. Transient expression and contained systems have benefits but also pose certain limitations on protein production. Batch-to-batch variability of a transiently expressed protein could be a costly nuisance to downstream processing and QA/QC, certainly more than for a stably expressed protein. The use of

contained systems addresses some public and regulatory concerns but eliminates a significant advantage of open-field production, which is production cost savings with easy, inexpensive scale-up. Another development that accelerated the shift to contained systems was the realization that plants cannot compete with mammalian cell culture and microbial systems for “blockbuster” products such as monoclonal antibodies and insulin. Thus, many plant biotechnology companies are now considering difficult to express proteins, personalized therapeutics, products with orphan drug status, and small volume products like interferon and influenza vaccines.

In spite of upstream technological achievements, there are still significant breakthroughs yet to be made in downstream processing since only a few novel and cost-effective downstream processing approaches have been introduced over the past 7 years. There are two main reasons for the lack of processing breakthroughs: 1) the lack of funding for academia and non-profit organizations to target bio-separations development for transgenic systems; and 2) the perception that previously developed methods for other protein expression hosts are also suitable for plant systems. Thus, the plant biotechnology community is limited to adapting technologies developed for microbial and cell-culture separations. Although technology translation may have made sense initially, significantly greater investment in developing novel bioprocessing and purification tools is necessary to exploit the full potential of plant platforms for recombinant protein production.

From a downstream processing perspective, the following considerations would be beneficial to accelerate process development, to reduce the overall manufacturing cost, and to advance plant systems for recombinant protein production.

1. Select the most suitable subcellular compartment or organelle for easy recombinant protein extraction.
2. Whenever possible, judiciously match protein product and plant extract properties for optimal purification. For example, expression of recombinant proteins in oilseeds or full-fat corn germ would be advantageous if using oleosin fusion technology for purification.
3. For oral vaccines, select plants with GRAS status and for industrial enzymes, platforms that provide stability to the product in clarified or partially purified extracts (i.e. absence of proteolytic and phenol oxidase activities).
4. Invest in the development of secreted product platforms, because secreted products are more advantageous from recovery and purification perspectives. For commercial feasibility, a ten-fold improvement in product titers is needed.
5. Take advantage of high expression levels achieved by chloroplast expression by focusing on proteins produced in a soluble form rather than as inclusion bodies. Production of proteins as inclusion bodies eliminates the competitive advantage of plants since non-glycosylated proteins can be efficiently and inexpensively produced by *E. coli*.
6. For intracellular products, develop novel capturing methods that will be less sensitive to fouling by phenolics and chlorophyll-derived pigments. Develop high-throughput strategies to understand the full complexities of plant extracts and the effects of plant components on purification methods.
7. Avoid using affinity tags for biopharmaceutical process development, even for bench-scale development. It is particularly detrimental for start-up companies to wait for clinical trial results to develop a non-tagged protein purification process.
8. Utilize tools, such as experimental design, process simulation, and high-throughput screening methods to reduce experimental design space, to identify process bottlenecks, and to rapidly screen extraction and purification protocols.
9. Evaluate the compatibility of current resin and membrane regeneration methods with plant extracts and tailor these to improve efficiency.
10. Continue to develop methods to simplify purification of recombinant proteins, such as those proposed by Joensuu et al. (2010) and Mee et al. (2008), and to reduce reliance on high-cost chromatography steps.

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