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Extraction and purification methods in downstream processing of plant-based recombinant proteins

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ABSTRACT

During the last two decades, the production of recombinant proteins in plant systems has been receiving increased attention. Currently, proteins are considered as the most important biopharmaceuticals. However, high costs and problems with scaling up the purification and isolation processes make the production of plant-based recombinant proteins a challenging task. This paper presents a summary of the information regarding the downstream processing in plant systems and provides a comprehensible overview of its key steps, such as extraction and purification. To highlight the recent progress, mainly new developments in the downstream technology have been chosen. Furthermore, besides most popular techniques, alternative methods have been described.

1. Introduction

A broad array of plants has been used in medicine for thousands of years. However, thanks to rapid progress in genetic engineering; 25 years ago, it was confirmed that plants are capable of producing recombinant proteins. In 1990, the first recombinant protein with potential therapeutic use—human serum albumin—was expressed in potato and tobacco leaves, as well as in cell suspension cultures [1]. Since then, hundreds of recombinant proteins have been successfully expressed in immensely diverse plants [2,3], but the development of molecular farming, which was supposed to revolutionize the production of biopharmaceuticals, started to slow down. As a result, only few plant-derived recombinant proteins are currently registered as pharmaceuticals [4]. Compared to animal, bacterial, and yeast cells, the commercial use of plant cells for protein production has only started recently. However, thanks to their advantages in the area of protein processing; plant cells are becoming an accepted alternative to mammalian and microbial platforms [5]. The lack of plant pathogens, which are similar to the ones affecting animals and humans, results in higher safety. Compared to other hosts, the recombinant proteins in plants show more stability. Furthermore, their expression systems are highly scalable and rapid [6]. Therefore, plant-based protein production systems may play an important role in the fast production of large amounts of medicine, such as vaccines during influenza epidemic [7]. Unlike microbes, higher plants are capable of producing proteins with desired N-glycosylation (human-like glycomodification) and folding [8,9]. Moreover, plant cells can produce substances that would be toxic for mammalian or bacterial cells [10] In addition, unlike mammalian cells, plants are insensitive to slight changes of conditions, such as pH, temperature, availability of metabolites, and their protein yield is high. Compared to mammalian or animal cells, upstream processing in plants and plant cells offers a wider range of methods and higher diversity of species [11]. The cultivation of plants demands lower infrastructural costs because it can use an existing agricultural base. Moreover, the storage of plants with recombinant proteins (e.g. seeds) is easier and cheaper than the storage of host cells from different organisms. Seeds can be stored at room temperature for long periods of time [12]. Compared to other recombinant protein platforms, such a production can be scaled up to agricultural levels by people with lower qualifications. Using plant platforms is economically justified [13] by their lowest cost of production among the recombinant protein hosts [14]. It is estimated that, compared to other systems, costs of protein production in plants could be 10–50 times lower [15].

In spite of many advantages of the recombinant protein

Abbreviations: DSP, downstream processing; GMP, good manufacturing practice; HCP, host cell proteins; ELPs, elastin-like polypeptides; mAb, monoclonal antibodies; EBA, expanded bed adsorption; POI, protein of interest.

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production in plants, biotech industry still relies on the small number of standardized technologies [16]. Such a situation is caused by few barriers constricting clinical development and commercialization of plant-derived pharmaceutical proteins [17]. However, few pharmaceuticals derived from plants, including the enzyme glucocerebrosidase, interferon alpha 2b, and insulin, are proceeding toward commercialization [18]. The economic efficiency is strictly connected to the type of protein produced in plants [19]. To bypass regulatory difficulties, several companies focused on the production of non-clinical proteins, such as technical reagents, enzymes, and diagnostic proteins, which are commercially successful [20]. Because of the novelty of the process, its efficacy still requires improvements, especially in the area of biotechnological procedures: accelerating and optimizing the process, as well as generating new products [21]. Furthermore, problems with meeting the high, aseptic standards of biopharmaceutical production, tailored to platforms based on animal and microbial cells, still occur [22]. During the production process of proteins in whole plants, maintaining Good Manufacturing Practice (GMP) rules is usually challenging due to the differences in cultivation. There is a big demand for the methods allowing plant cultivation in highly standardized conditions, with the reduction of pollutants and with an improved logistic. Vertical farming and hydroponic medium cultivation could become promising solutions to this dilemma, but further developments are needed [23]. This way of plant cultivation could possibly tackle one of the main issues hindering the development of commercial plant-based protein production—high cost of downstream processing (DSP). Existing techniques of DSP are efficient in small scale but are usually uneconomical on a large scale (e.g. affinity chromatography). Some approaches are designed to offset the expenses of protein purification with profits from the extraction of other plant components (e.g. starch from potatoes producing human serum albumin). While the development of edible pharmaceuticals (mainly vaccines) could make purification redundant, the scientists using this approach currently face many obstacles.

2. Downstream processing strategies

DSP can be described as a group of steps leading to recovery of highly purified product from biological matrix. The need for highly regulated and exceptionally pure biopharmaceuticals makes downstream processing a critical component of the overall process. At the beginning of genetic engineering era, scientists have been focusing mainly on demonstrating that plants can produce the recombinant proteins, thus neglecting the importance of development of extraction and purification methods. Nonetheless, currently, DSP in plants is the most expensive part of the recombinant protein production and is estimated to account for up to 80% of total costs [24]. Its development is also a crucial part in ensuring the GMP compliance. Not only molecular farming, but also conventional systems struggle to overcome DSP bottleneck. To obtain homogeneous and pure product, the presence of contaminants must be removed or lowered to acceptable levels. Characterizing the nature of contaminant and proving the GMP compliance are the main challenges of plant purification. In other protein production platforms, such as mammalian cells or bacterial cells, powerful methods have been developed to remove animal viruses or endotoxins. In case of plant viruses, despite the lack of reports of their harmfulness to humans, unquantified risk is capable of halting the development of GMP processes. Another issue is the difference between plant cells, which must be purified from cellulose, fibers, oils, or metabolites (such as nicotine from tobacco or oxalic acid from alfalfa), and their bacterial and mammalian counterparts [25]. This barrier can be alleviated by using innovative strategies for maximizing yield, improving product recovery, and reducing costs. Many strategies have been proposed to optimize and shorten the downstream processing by utilizing lower-cost materials or reducing the number of processing steps. The main steps of downstream processing usually contain tissue harvesting, protein extraction, purification, and formulation (Fig. 1) [26]. The general rule for designing DSP steps is that first steps of biological product processing must conform to the production platform, while later steps must be adapted to the properties of protein of interest (POI) [27]. The intention is to maximize purity and this goal is achieved by increasing the amount of recovered recombinant protein that reduces the cost and time to complete the process.

2.1. Protein extraction

During this phase, the target protein is released from biological matrix. This step is critical in defining target protein quality, its concentration, and the total volume of extract. Moreover, the extraction stage is largely responsible for the quantity and type of impurities. The extraction method depends primarily on its type of expression in plant and normally includes plant harvesting and tissue maceration. In addition, in seed-based expression systems, first step comprises milling. Milled seed or leafy biomass can be homogenized by press or blade-based homogenizers. To make protein recovery easier, buffer is usually added to received biomass; moreover, it provides sufficient pH and salinity, adds antioxidants, and maintains solubility [28]. Protease inhibitors are usually added in this step due to the threat of proteolysis of target protein. The type of buffer depends on the type of extracted protein. Buffers widely vary between each other, but aqueous buffers are the most common. Sometimes other substances, such as supercritical or ionic fluids, are also used. Organic solvents (hexane or phenol) can be used for non-protein targets, membrane proteins, and fat removal from biomass.

2.2. Alternative extraction methods

Another mechanical method known as vacuum infiltration-centrifugation is used to obtain extracellular-targeted products from apoplast. Such method can potentially lower the costs of downstream processing, as recovery of proteins is achieved without homogenization of whole tissue [29]. The release of proteins and contaminants (e.g. proteolytic enzymes, which can destroy the product) is the main obstacle during tissue maceration [30]. Secretion-based systems are another alternative for traditional, mechanical extraction because the product is secreted into hydroponic medium or cell culture supernatant. To date, many scientists proved the possibility of collecting the recombinant proteins with this method. For example, in the growth medium of genetically modified tobacco cell suspensions [31], heavy chain of monoclonal antibody and single chain Fv have been recovered [32]. IgG1 antibody has been secreted by hairy roots of genetically transformed tobacco [33].

An important advantage of this method is the ability to

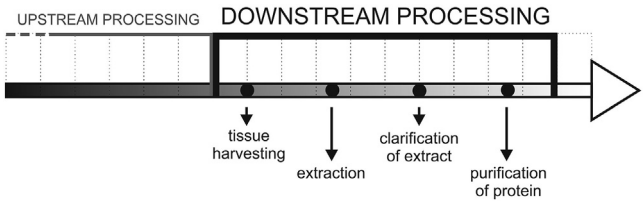

Fig. 1. Timeline of general strategy for downstream processing in main steps.