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Downstream process engineering evaluation of transgenic soybean seeds as host for recombinant protein production

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Abstract

The advantages of using seeds for the production of recombinant proteins with plant-based expression system has been demonstrated by several researchers. The high productivity makes soybean a potential system for large-scale recombinant protein production. However, there is a lack of detailed engineering studies of the downstream process (DSP) of recombinant proteins produced in transgenic soybean. In this work, we evaluated the use of transgenic soybean seeds as hosts for the production of recombinant proteins from a downstream process (DSP) engineering standpoint. Recombinant β-glucuronidase (rGUS), was used as a model for extraction and purification studies. This study showed, that even a protein with acidic p*I* (rGUS) can be successfully separated from native soybean proteins, which also have acidic p*I*. Maximum GUS specific activity (9.5×10^3 U/mg) with high total activity recovery (8.9×10^4 U/mL) was obtained using a simple extraction solution composed of 50 mmol/L citrate buffer at pH 5.25. Purification of rGUS was evaluated by a two-step chromatographic procedure – anion-exchange followed by hydrophobic interaction chromatography – which was compared to the purification of rGUS from transgenic corn and canola. Overall purification factor and activity recovery obtained were 97.3 and 110% (a value higher than 100% probably due to removal of an inhibitor). Comparison of this study with similar ones made with corn and canola seeds indicates that in terms of DSP soybean seeds can be considered a potentially viable plant system for the production of recombinant proteins.

Keywords: Soybean seeds; Extraction; Transgenic plants; Protein purification; Glucuronidase

1. Introduction

A large variety of plant species are being evaluated as host for the production of recombinant proteins with industrial and pharmaceutical applications. The spectrum of plants ranges from cereal crops such as maize, rice, barley, and wheat to legumes like pea and soybean, and also the leafy crops alfalfa and tobacco. According to Stoger et al. [1], seed-based hosts can offer several advantages for the production of recombinant proteins, since the accumulation of proteins takes place in a relatively small volume and stable environment in which they are protected from degradation. Although soybean is considered a potential seed-based bioreactor there are only few reports addressing this issue. In the work of Zeitlin et al. [2], an antibody against herpes simplex virus was expressed constitutively in the plant. The plants were harvested and pressed to extract the recombinant protein that was purified by affinity chromatography. Philip et al. [3] reported the expression of casein in soybean seeds, which was purified also by affinity chromatography. Recently, the expression of the human growth hormone in transgenic soybean was reported by Russel et al. [4]. The reason for relatively few reports on this subject is that a high level expression of a recombinant protein has not been achieved yet in soybean seed. As pointed out by Twyman et al. [5], recombinant protein expression higher than 0.1% of the total soluble protein in seeds is needed for a plant system to be competitive with other expression systems. However, soybean seeds offer the advantages of having a low production cost, and from a regulatory standpoint, a reduced risk of contamination by pollen, since soybean is largely self-pollinating. Therefore, it is a system that needs to be studied in order to have a fully evaluation of its potential.

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Besides the right choice of plant specie, the success of the large-scale use of plants as bioreactors depends also on the downstream processing (DSP) necessary for purification of the recombinant protein. Extraction is a key step in an efficient DSP, since it defines the characteristics of the solution from which the recombinant protein will be purified. Plant species contain different levels of compounds (e.g., native proteins, soluble carbohydrates, phenolic compounds, and lipids) that can be cosolubilized with the recombinant product during the extraction, which are deleterious to the efficiency of the process, separation media, and equipment. Therefore, an efficient extraction condition that minimizes the coextraction of the plant native compounds could simplify DSP operations.

According to Jefferson et al. [6] GUS is a homotetrameric enzyme (monomer molecular mass and p*I* of approximately 68 kDa and 5.5) that cleaves the β-linked terminal glucuronic acids in mono and oligosaccharides and phenols. Although one of the natural sources of GUS is *Escherichia coli*, a recombinant GUS produced in transgenic corn is currently commercialized [7,8]. It is widely used as a visual marker for analysis of gene expression in transgenic plant research. One important application of the expression of GUS is its use as a model protein in studies of process engineering strategies regarding DSP of recombinant proteins produced in transgenic plants [7–11].

Kusnadi et al. [7,8] described the production, purification, and characterization of GUS from transgenic corn seed. An estimated value of 70% of the GUS was extracted with 50 mmol/L sodium phosphate buffer at pH 7.5. The addition of 1% SDS and 2% β-mercaptoethanol resulted in complete extraction of GUS; however, the SDS caused apparent irreversible inactivation of the enzyme. In both reports rGUS was purified with anion-exchange, hydrophobic interaction, and size-exclusion chromatographies. A similar GUS extraction procedure was used by Zhang and Glatz [9] and Zhang et al. [10] with canola as the expression host. Canola extract was obtained by mixing 50 mmol/L sodium phosphate buffer at pH 7.0 with deffated meal at a 1:10 solid-to-liquid ratio for 30 min. Purification of GUS and three other polyaspartate fusions to GUS were studied using anion-exchange chromatography with different elution profiles. Bai and Glatz [11] studied the use of packed and expanded bed ion-exchange adsorption for rGUS purification from transgenic canola. They reported rGUS recovery of 112% and purification factor of 31 using a packed bed of Streamline-DEAE.

In this study we evaluated soybean as a bioreactor by studying the DSP – extraction and purification steps – of a model recombinant protein rGUS produced in the seeds of transgenic plants and comparing the results with reported studies with corn [7,8] and canola seeds [11]. The chromatographic steps (anion-exchange and hydrophobic interaction) were evaluated in terms of purification based on GUS specific activity, as done in the case of corn [7,8] and canola [11]. Aqueous extraction of rGUS, native proteins, carbohydrates, and phenolic compounds was evaluated as a function of pH of extraction solution. The reason to studied extraction of other compounds than rGUS is because native proteins are the major impurities in the extracts, phenolics are known to interfere with DSP since they may cause resin fouling, protein denaturation and degradation, and carbohydrates promote bacterial growth.

2. Materials and methods

2.1. Materials

Transgenic soybean seeds (cultivar BR-16, lines 8–19) expressing the GUS gene were provided by EMBRAPA, Brazil [12]. Genetic transformation was done using the particle bombardment technique, using a constitutive promoter. GUS substrate 4-methylumbelliferyl glucuronide (MUG) and 4-methylumbelliferone (MU) were from Sigma (USA). High-purity water prepared with a Milli-Q System (Millipore, USA) was used in all experiments. All other chemicals used were of at least analytical grade. A DU 650 spectrophotometer (Beckman, USA) and a F-4500 fluorescence spectrophotometer (Hitachi, Japan) were used for the spectrophotometric measurements.

2.2. Methods

2.2.1. Preparation of soybean flour

Soybean seeds were ground in a household coffee grinder by intermittent runs in order to avoid excessive heating (the temperature did not exceed 60 °C). The particles were separated using a set of sieves, resulting in flour with particles smaller than 0.5 mm. This flour was defatted with hexane at 60 °C for 6 h in Soxhlet-type equipment and stored at room temperature until its use in the extraction experiments.

2.2.2. Extraction protocol

In each run, 5 g of soybean flour were mixed with 100 mL of the 50 mmol/L sodium citrate buffer with appropriate pH (1:20 solid-to-liquid ratio) at room temperature for 30 min. Extraction was carried out in a 5.5 cm diameter 250 mL beaker using a mechanical stirrer (Q-251D, IKA Labortechnik, Germany) equipped with an axial-flow impeller (pitched-blade turbine with four blades, 4.0 cm in diameter at 45° angle and positioned 1 cm from the bottom) at a stirring rate of 500 rpm. After 30 min of mixing, each suspension was centrifuged at 15,000 × *g* for 20 min at 5 °C and polished by filtration through a 3 μm filter paper. Extractions for chromatographic experiments were done with 50 mmol/L citrate buffer pH 5.25.

2.2.3. Chromatographic procedures

Chromatographic experiments were carried out with either a Breeze HPLC Waters System (Waters, USA) or a system consisting of a Miniplus 3 peristaltic pump (Gilson, France), an Econo UV Monitor detector, and a fraction collector (both by Bio-Rad, USA) at a constant flow rate of 1.0 mL/min. The purification factor was calculated as the ratio between the specific GUS activity of the pool of eluted fractions and the specific GUS activity of the sample injected.

2.2.3.1. Anion-exchange chromatography. A volume of 5.5 mL of the anion-exchange resin DEAE-Sephadex A25 (Pharmacia, Sweden) was packed into a C10/10 glass column (10.0 cm ×