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High-level production of recombinant trypsin in transgenic rice cell culture through utilization of an alternative carbon source and recycling system

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ABSTRACT

Productivity of recombinant bovine trypsin using a rice amylase 3D promoter has been studied in transgenic rice suspension culture. Alternative carbon sources were added to rice cell suspension cultures in order to improve the production of recombinant bovine trypsin. It was demonstrated that addition of alternative carbon sources such as succinic acid, fumaric acid and malic acid in the culture medium could increase the productivity of recombinant bovine trypsin 3.8–4.3-fold compared to those in the control medium without carbon sources. The highest accumulated trypsin reached 68.2 mg/L on day 5 in the culture medium with 40 mM fumaric acid.

The feasibility of repeated use of the cells for recombinant trypsin production was tested in transgenic rice cell suspension culture with the culture medium containing the combination of variable sucrose concentration and 40 mM fumaric acid. Among the used combinations, the combination of 1% sucrose and 40 mM fumaric acid resulted in a yield of up to 53 mg/L five days after incubation. It also increased 31% (W/W) of dry cell weight and improved 43% of cell viability compared to that in control medium without sucrose. Based on these data, recycling of the trypsin production process with repeated 1% sucrose and 40 mM fumaric acid supplying-harvesting cycles was developed in flask scale culture. Recombinant bovine trypsin could be stably produced with a yield of up to 53–39 mg/L per cycle during five recycling cycles.

1. Introduction

Commercial production of recombinant therapeutic proteins has primarily relied on bacterial fermentation or mammalian cell culture systems. To date, over 100 proteins in the form of human pharmaceuticals have entered the market [1], and over 370 recombinant proteins are under development [2]. The use of transgenic plants or plant cell cultures for the production of pharmaceuticals, functional proteins, industrial enzymes, and functional secondary metabolites has recently been named molecular farming. These processes are inexpensive, safe, and scalable, and have been studied as an alternative system to animal and microbial production systems [3–6].

Plant molecular farming is expected by some to challenge already established production technologies for pharmaceuticals that currently use bacteria, yeast, and cultured mammalian cells because plants lack human pathogens, oncogenic DNA sequences, prions, and endotoxins [7]. Although still in its initial stages, the plant molecular farming industry has recently demonstrated considerable growth in research and development activity around the world [8]. More than 120 small companies, universities and research institutes that are active in plant molecular farming have been identified [8]. Advanced plant systems have evolved from the concept stage to commercial production of industrial enzymes such as avidin, aprotinin, and trypsin. At least six therapeutic proteins are currently in preclinical testing or clinical trials [9].

The advantages of plant cell cultures in human-like protein production have long been recognized. Plant cell culture systems have advantages and disadvantages compared to whole transgenic plant systems. Even though both systems use the plant cell as a bioreactor, the plant cell culture system has advantages in terms

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of production time, contamination risk, and purification cost. The whole transgenic plant system has advantages in terms of overall cost and scalable capacity [2]. Plant cells can perform nearly all post-translational modifications because they are eukaryotic cells. Consequently, the plant cell system has been recognized as an alternative even though there are some disadvantages, including low yield and a slightly different glycan modification process from humans [2]. The most significant advantage the plant cell system has its mammalian counterpart with the lower contamination risk because plant cells do not harbor human pathogens. The infection of Chinese hamster ovary (CHO) cells with calicivirus, which produces CerezymeR used in the treatment of Gaucher disease, enables an alternative and safe platform for the production of therapeutic recombinant proteins [10]. Pfizer, the world's largest drug company, recently bought licensing rights from Protalix Biotherapeutics (Israel), paying $60 million upfront as well as a milestone payment of up to $55 million for a production system using a carrot cell suspension culture system of glucocerebrosidase, which is an orphan drug for Gaucher disease [11,12]. This event highlights the interest of large international pharmaceutical companies in the commercial viability of plant molecular farming for the production of human pharmaceutical proteins [2]. This plant-made glucocerebrosidase was approved for the treatment of Gaucher disease in France by the French regulatory authority in July 2010 and in USA by the FDA in 2011 and was the first plant-made pharmaceutical treatment for human disease to enter the commercial market [2].

Trypsin is a serine protease that plays a key role in the activation of pancreatic enzymes involved in digestion. It is released from zymogen granules stored in pancreatic acinar cells and is activated by enterokinase following secretion. Enterokinase recognizes the $(Asp)_4$-Lys sequences in the propeptide and cleaves after the lysine residue to release active trypsin [13]. Trypsin is one of several multi-purpose enzymes and is used as a detergent for animal cell cultures and the processing of leather, as a dietary supplement in the food industry, and as a substitutive therapy for patients with pancreatic disease. Currently, bovine trypsin is produced by extraction from cow pancreas. However, contamination of the enzymes with human pathogens, including bovine spongiform encephalopathies, has received significant attention, highlighting the urgency of continued efforts to improve plant cell systems as an alternative expression system for therapeutic recombinant proteins. As a result, there is demand for the production of recombinant trypsin using a non-animal expression system. Recombinant bovine trypsin was produced and used as commercial protease from transgenic maize (*Zea mays*) seed [14]. However, the recovery and purification of recombinant proteins from plants are expensive and technically challenging business that may account for 80% of the final product cost [4,15–17]. One additional advantage of plant cell culture is that the recombinant protein fused with a signal peptide can be expressed and secreted into the culture medium and then recovered and purified without large quantities of contaminating proteins. As a result, the downstream processing of recombinant protein from medium is less expensive. We have already explored the use of a transgenic rice cell suspension culture as an alternative to transgenic whole plants to produce recombinant bovine trypsin and reported production of functional recombinant bovine trypsin in transgenic rice cell suspension cultures [18]. The recombinant bovine trypsin using rice amylase 3D (Ramy3D) promoter, a strong promoter that was induced by sugar starvation was produced and accumulated of 15 mg/L on the fifth day in the culture medium, with yields up to 7.5% of the total secreted proteins [18].

Rice amylase3D promoter systems have been studied as a potential alternative for the high production of some therapeutically interesting cytokines, antibodies and enzymes, though many biological and technological limitations remain before they can be produced commercially. The medium exchange manner that depletes sugar in culture medium may induce cell death because of the loss of carbon sources for the energy metabolism. It may also cause cell rupture as a result of changes in the osmotic pressure and various factors may stimulate apoptosis, and proteases may accumulate in cells and be secreted into the culture medium, thereby reducing the target recombinant protein production during the production phases [19]. Therefore addition of some alternative carbon sources have been tried to improve recombinant protein production. In the production of recombinant α-antitrypsin (AAT), glucose and pyruvate were effected to improve rAAT production in transgenic rice cell suspension culture [20,21]. To improve the synthesis of alkaloid in the *Catharanthus roseus* suspension cell culture, succinic acid, malic acid and citric acid all participate in the citric acid cycle was added in the medium but they showed very different effects on alkaloid production. Succinic acid and malic acid greatly improved alkaloid production but citric acid was not effects [22]. So we checked intermediates in citric acid cycle such as citric acid, succinic acid, fumaric acid and malic acid in addition to fructose and pyruvic acid that had some positive effect in our previous experiment (unpublished results). Among them, fumaric acid was selected for further experiment because it had the most positive effect on production yield of recombinant trypsin. If we can bypass the reaction step that generates those intermediates by adding suitable carbon sources, an energy source for the recombinant protein synthesis can be supplied in high concentration.

In this study, we explored a method to improve the production yield of recombinant trypsin using some alternative carbon source and recycling batch culture system in flask scale and proposed the possibility of using the combination of sucrose and fumaric acid in controlling cost-effective recombinant trypsin production in large-scale culture.

2. Materials and methods

2.1. Expression system and plant cell line

Recombinant bovine trypsin was expressed under the control of the sucrose starvation-inducible rice α-amylase 3D (RAmy3D) promoter, a strong promoter was developed to yield high expression of recombinant protein [23]. A transformed rice cell line, pMYT111-04, was established and maintained in our laboratory as described previously [17].

2.2. Plant cell cultures

The transformed rice cell cultures were propagated and cultured at 28 °C in darkness, using a rotary shaker with a rotation speed of 110 rpm. To maintain the cell line, the cell suspension was cultured in 300-ml flasks using N6 medium containing 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 0.02 mg/l kinetin, and 3% sucrose. A 10-ml inoculum was transferred every seven days for sub-culturing. In order to induce trypsin gene expression under the control of the Ramy3D promoter, the N6 medium was removed from the cell suspension via aspiration, and the cells were transferred to fresh N6 (S-) medium (without sucrose) in 10% amounts (weight of wet cells/volume of medium). The culture supernatant from the culture medium of induced rice cells was collected by pouring the induced cell suspension through 2–3 layers of myracloth (Calbiochem, La Jolla, CA, USA). Proteins in the medium were collected by centrifugation at 18,000 × *g* at a temperature of 4 °C for 10 min in order to remove the debris.

2.3. Screening for alternative carbon sources and measurement of fresh and dry cell weights

To select the best alternative carbon source and incubation time in flask culture, culture medium was removed from the cell suspension via aspiration after seven days of culture (growth stage), and 5 g of fresh cell weight was inoculated in 50 ml of fresh N6 medium without sucrose and containing a different concentration of a different carbon source and incubated for five days (production stage). Fructose, pyruvic acid, citric acid, succinic acid, fumaric acid and malic acid were used to examine the effects of alternative carbon sources. Each alternative carbon source was completely dissolved in N6 medium without sucrose, and each medium was adjusted with 1 N NaOH to the desired concentration at pH 5.8. Recycling of culture with fresh N6 medium containing 1% sucrose with 40 mM fumaric acid was performed. To measure the change of cell weight after adding the alternative carbon