ELSEVIER

Research Paper

Enhancement of recombinant human serum albumin in transgenic rice cell culture system by cultivation strategy

Yu-Kuo Liu[1], Yu-Teng Li[2], Ching-Fan Lu[1] and Li-Fen Huang[2]

[1] Graduate Institute of Biochemical and Biomedical Engineering, Chang Gung University, Kwei-Shan, Taoyuan County 320, Taiwan, ROC
[2] Graduate School of Biotechnology and Bioengineering, Yuan Ze University, 135 Yuan-Tung Road, Zhongli, Taoyuan County 320, Taiwan, ROC

Fusion of the sugar-starvation-induced α*Amy3* promoter with its signal peptide has enabled secretion of recombinant human serum albumin (rHSA) into the culture medium. To simplify the production process and increase the rHSA yield in rice suspension cells, a one-step strategem without medium change was adopted. The yield of rHSA was increased sixfold by this one-step approach compared with the two-step recombinant protein process, in which a change of the culture medium to sugar-free medium is required. The one-step strategem was applied to check repeated cycle of rHSA production, and the production of rHSA was also higher in each cycle in the one-step, as opposed to the two-step, production process. The use of the one-step process resulted in fewer damaged cells during the cell sugar starvation phase for recombinant protein production. Furthermore, we scaled up the rHSA production in a 2-L airlift and a 2-L stirred tank bioreactor by the one-step approach, and concluded that rHSA can be enriched to 45 mg L^{-1} in plant culture commonly used MS medium by the airlift-type bioreactor. Our results suggest that rHSA production can be enriched by this optimized cultivation strategem.

Introduction

Pharmaceutical recombinant proteins in the market are expanding rapidly in recent decades, and they have been produced in various host cell types, which differ in terms of their biosafety, biological activity and protein stability. Like mammalian cells, plant cells are capable of protein posttranslational modification, such as glycosylation, but without the risks associated with human/animal pathogens in mammalian cell cultures. Taking advantage of this absence of risk, plant cells have been used to produce complex therapeutic proteins that are required for biosafety, bioactivity and stability.

Rice (*Oryza sativa*), a crop with the third highest level of cultivation globally, is considered a model plant whose genome has been sequenced, and the technologies for gene transformation and tissue culture have been well developed [1,2]. Its hypoallergenic property also makes rice become an excellent host cell for recombinant protein production [3,4]. However, the presence of genetically modified plants in the field has raised public concerns and is regulated strictly by governments. A rice suspension cell system is an alternative option to limit public concern about genetically modified organisms, and it has been developed for the efficient scaling up of recombinant protein production, such as for pharmaceutical proteins [5–12], vaccines [13,14], or antibodies [3,15].

A platform for recombinant protein production in rice suspension cells has been established using a rice α-amylase gene promoter, *RAmy3D*p/*αAmy3*p, the activity of which is induced by sugar starvation [16]; its signal peptide allows the secretion of recombinant proteins outside the cells. These properties motivated the development of a two-step process for producing recombinant protein by using *RAmy3D*p/*αAmy3*p. In the first step, the transgenic rice cells are cultured in sucrose containing medium to increase the cell number and maintain cell viability and cell activity. In the second step, the sucrose-rich medium is removed from the amplified cells, and the sugar-free medium is added to

Corresponding authors: Liu, Y.-K. (ykliu@mail.cgu.edu.tw), Huang, L.-F. (hlf326@saturn.yzu.edu.tw)

http://dx.doi.org/10.1016/j.nbt.2015.03.001

produce recombinant protein. Several recombinant proteins have been produced successfully and secreted into the culture medium from these sugar-starved transgenic rice cells by using this two-step process [7,9,10,14,15,17–20]. However, as described above, exposure to two types of culture medium is required in the two-step production procedure, which increases both the cost of the process and the risk of contamination when changing the medium.

Previously, we reported that the production of a recombinant protein, mouse granulocyte-macrophage colony-stimulating factor (rmGM-CSF), could be achieved in one step by using a cultivation strategy that does not require a change of medium [11]. Transgenic rice suspension cells were cultured in N6D medium with 3% sucrose and rmGM-CSF was produced upon sucrose depletion in the medium. The level of rmGM-CSF production was doubled compared with that in the two-step production process [11]. Furthermore, rmGM-CSF production was scaled up to bioreactor levels by using this cultivation strategy [11]. However, more successful examples of such production are required to determine whether other recombinant proteins or other common plant growth media can also benefit from this cultivation strategy.

Human serum albumin (HSA) is a single unglycosylated protein that contains 585 amino acid residues and forms 17 pairs of disulfide bonds. HSA is the most abundant blood plasma protein and plays important roles in the regulation of osmotic pressure and pH, as well as acting as a versatile carrier for hormones and numerous drugs [21]. HSA is frequently applied in medical treatments, such as surgery and hepatocirrhosis, and also widely used in biochemical applications, such as vaccine formulation and cell culture medium. The current major HSA source is human plasma, but its availability is insufficient to meet the increasing medical demand. Moreover, the use of human plasma is associated with a risk of disease transmission, such as hepatitis B, syphilis, and HIV [22]. In order to meet the increasing demand and assuage biosafety concerns, several platforms for producing recombinant protein, including rice suspension cells, have been developed for producing recombinant HSA (rHSA) [7,23–28]. In our previous work, we generated a transgenic rice cell line by *Agrobacterium*-mediated transformation, and produced rHSA in the culture medium by a two-step process [7].

In the study reported herein, we investigated the effects of various strategies for producing rHSA by culturing transgenic rice suspension cells in flasks. We also demonstrated that the yield of rHSA was increased sixfold when the culture medium was not changed after sucrose was exhausted naturally, compared with that upon changing to sucrose-free medium. Furthermore, we scaled up the production of rHSA successfully in a modified bioreactor by this optimized cultivation strategy.

Materials and methods

Plant material

The genetic background of rice (*O. sativa* L) cell line used in this study is Tainung No. 67. Rice grains were dehulled, sterilized with 2.4% sodium hypochlorite for 1 hour, washed extensively with sterile water, and placed on N6 agar medium [29] containing 3% sucrose and 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) (N6D) at 28°C in the dark to induce calli. Calli were subcultured in liquid Murashige and Skoog (MS) medium [30] containing 3% sucrose and 10 μM 2,4-D at 28°C in the dark to establish suspension-cultured cells. The resultant suspension-cultured cells were agitated on a reciprocal shaker at 120 rpm and incubated at 28°C in the dark.

Rice transformation

The plasmid pA3HSA, which contains an *αAmy3* promoter – signal peptide – HSA fusion construct, was generated in our previously reported work [7]. *Agrobacterium tumefaciens* strain EHA105 [31] was used as a host stain for pA3HSA by electroporation, and rice calli were transformed as described previously [32]. Selection of transformed calli was performed on N6 agar medium with hygromycin (50 mg L^{-1}).

Characterization of the transgenic cell lines

The presence of the transgene insertion in rice calli was analyzed by genomic DNA PCR. Genomic DNA was isolated from putative transgenic calli and then subjected to PCR by using primers HSA-F (5′-GGGCATGTTTTTGTATGAAT-3′) and HSA-R (5′-TTATAAGCCTAAGGCAGCTT-3′).

RT-PCR

Total RNA was isolated from rice transgenic cell lines using Trizol (Invitrogen, Carlsbad, CA, USA) and treated with RNase-free DNase. First-strand cDNA was synthesized by SuperScript III Reverse Transcriptase (Invitrogen) with an oligo-dT primer. A 20-fold dilution of the first-strand cDNA was subjected to PCR with gene-specific primers; the HSA-F and HSA-R were used for *HSA*; the Amy3F (5′-TACAGCGTCTGGGAGAAGGGGTC-3′) and Amy3R (5′-TGCCCCGCAATTAACCTAGAGGC-3′) were used for *αAmy3*; the Actin-F (5′-CTGATGGACAGGTTATCACC-3′) and Actin-R (5′-CAGGTAGCAATAGGTATTACAG-3′) were used for *Act1*.

Protein gel blot analysis

To obtain total soluble secretory proteins from rice suspension cells, the rice cultured medium was filtered through Whatman No. 1 filter paper to remove cells, and then centrifuged at 18,000 × *g* at 4°C for 15 min to obtain the supernatant. The concentration of protein in the supernatant was measured using the Bio-Rad Protein Assay reagent (Bio-Rad, Hercules, CA). Western blotting was performed as described previously [7]. The rabbit antiserum to HSA (ICN Pharmaceuticals, Costa Mesa, CA), applied as the primary antibody, was diluted 1000-fold before use. Then, a 2000-fold dilution of horseradish peroxidase conjugated goat secondary antibody against rabbit IgG (Pierce, Rockford, IL) was diluted before use. Chemiluminescent signals emitted from the complex of primary and secondary antibodies were detected by using the Lumi-light Western blotting substrate (Roche, Basel, Switzerland).

Enzyme-linked immunosorbent assay (ELISA)

The yields of rHSA secreted from transgenic rice cells into cultured medium were measured by a sandwich ELISA method as described previously [7]. Each well in the microtiter plates was coated with 10 μg/mL goat antiserum to HSA at 25°C for 4 hours. Then, 50 μL of each cell-cultured medium sample was added to individual wells and incubated at 37°C for 1 hour. Subsequently, 10 μg/mL rabbit anti-HSA polyclonal antibodies (ICN Pharmaceuticals) was added to the wells and incubated at 37°C for 1 hour. Peroxidase-conjugated anti-rabbit IgG antibodies were diluted 2000-fold and ap-