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Production of human serum albumin by sugar starvation induced promoter and rice cell culture

Li-Fen Huang[1], Yu-Kuo Liu[2], Chung-An Lu[1,3], Shie-Liang Hsieh[4] & Su-May Yu[1,*]

[1]*Institute of Molecular Biology, Academia Sinica, Nankang, Taipei 115, Taiwan, ROC*
[2]*Department of Chemical and Material Engineering, Chang Gung University, Kwei-Shan, Tao-Yuan, Taiwan, ROC*
[3]*Department of Life Sciences, National Central University, Chung-Li, Tao-Yuan, Taiwan, ROC*
[4]*Department of Microbiology and Immunology, National Yang-Ming University, School of Medicine, Taipei 112, Taiwan, ROC*

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Abstract

Human serum albumin (HSA) is the most widely used clinical serum protein. Currently, commercial HSA can only be obtained from human plasma, due to lack of commercially feasible recombinant protein expression systems. In this study, inducible expression and secretion of HSA by transformed rice suspension cell culture was established. Mature form of HSA was expressed under the control of the sucrose starvation-inducible rice *αAmy3* promoter, and secretion of HSA into the culture medium was achieved by using the *αAmy3* signal sequence. High concentrations of HSA were secreted into culture medium in a short time (2–4 days) by sucrose depletion after cell concentrations had reached a peak density in culture medium containing sucrose. The recombinant HSA had the same electrophoretic mobility as commercial HSA and was stable and free from apparent proteolysis in the culture medium. In a flask scale culture with repeated sucrose provision-depletion cycles, HSA was stably produced with yields up to 11.5% of total medium proteins or 15 mg/L per cycle after each sucrose provision-depletion cycle. A bubble column type bioreactor was designed for production of HSA. In the bioreactor scale culture, HSA was produced with yields up to 76.4 mg/L 4 days after sucrose depletion. HSA was purified from the culture medium to high purity by a simple purification scheme. Enrichment of HSA in culture medium simplifies downstream purification, minimizes protease degradation, and may reduce production cost. The combination of a DNA construct containing the *αAmy3* promoter and signal sequence, and the use of a rice suspension cell culture can provide an effective system for the production of recombinant pharmaceutical proteins.

Introduction

Plants have become attractive expression systems for the production of antibodies, vaccines and therapeutic proteins for use as diagnostic and therapeutic tools in health care (Fischer & Emans, 2000; Giddings et al., 2000; Daniell et al., 2001). Many recombinant human proteins produced in plant cells are correctly folded and functional (Daniell et al., 2001). Among various plant expression systems being explored, genetically

*Author for correspondence
E mail: sumay@imb.sinica.edu.tw

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modified plant cell culture offers a promising system for production of recombinant pharmaceutical proteins (Doran, 2000). This system represents a new form of agricultural production that is totally devoid of problems associated with the vagaries of weather, pest, soil and gene flow in the environment. Due to shorter growth cycles, after transformation the time-scale needed for production of recombinant proteins in cell culture can be counted in days or weeks compared with the months needed for whole plant production. When needed for clinical use, recombinant protein production under certified, controllable and sterile conditions with straightforward purification protocols may offer improved safety (Fischer & Emans, 2000), which provides advantages for GMP and regulatory compliance. Compared to other protein production systems, which use microbial fermentation, animal or insect cell cultures, plant cell cultures have several advantages. Microbial systems have a limited capacity for the accurate post-translational modification of eukaryotic proteins. Bacterial fermentation often results in the production of insoluble protein aggregates, and substantial costs are involved in solubilizing and refolding these aggregates into native proteins. Endotoxins produced during bacterial fermentation also add to the cost of protein production. Animal and insect cells are more expensive and difficult to cultivate due to complex nutrient requirements and susceptibility to mechanical stress in bioreactors. The lower cultivation temperatures and inexpensive defined culture media (relatively simple solutions containing no exogenously supplemented protein or serum) make plant cell culture systems competitive with the microbial and animal culture systems.

Recovery and purification of recombinant proteins from plant biomass is an expensive and technically challenging business that may amount to 80–94% of the final product costs (Kusnadi et al., 1997; Evangelista et al., 1998). One additional advantage of plant cell culture is that the recombinant protein fused with a signal sequence could be expressed and secreted into the culture medium, and then recovered and purified in the absence of large quantities of contaminating proteins. The downstream processing of protein from medium is less expensive, which may balance, in part, the higher costs of fermentation (Doran, 2000). Transformed plant suspension cells have been employed for the production of a number of biologically active mammalian proteins and antibodies (Firek et al., 1993; Magnuson et al., 1998; James et al., 2000; Lee et al., 2002; Kwon et al., 2003; Sunil Kumar et al., 2003). In these applications, the constitutive Cauliflower Mosaic Virus 35S RNA (*CaMV35S*) or ubiquitin promoter and a signal sequence were used for expression and secretion of the recombinant proteins. However, the extracellular concentrations of these proteins were low, ranging from 10 to 600 μg/L. Alternative promoters and plant cell culture systems that could significantly enhance the productivity of recombinant proteins are important for the reduction of costs to offset the substantially higher cost of biomass production through cell culture or fermentation.

α-Amylase is an essential enzyme for hydrolysis of starch during germination of cereal grains. The expression of α-amylase genes in germinating cereal grains is induced by both the phytohormone gibberellin and sugar starvation, and in cultured rice suspension cells is activated by sugar starvation and repressed by sugar provision (Yu et al., 1991, 1992). *αAmy3* and *αAmy8* are the two most highly expressed α-amylase genes in sucrose-starved rice suspension cells, with the transcription rate of *αAmy3* approximately 4-fold that of *αAmy8* (Sheu et al., 1996). The *αAmy8* promoter and signal sequence have been used for expression of recombinant β-glucuronidase (GUS) in transformed rice suspension cells, with yields up to 40% of total proteins present in medium (Chan et al., 1994). The *αAmy3* promoter and signal sequence were also used for production of recombinant human α_1-antitrypsin (rAAT) and human granulocyte–macrophage colony stimulating factor (hGM-CSF) in sucrose-starved rice suspension cells, and functional rAAT and hGM-CSF were secreted into the culture medium, with yields up to 200 and 129 mg/L (Terashima et al., 2001; Shin et al., 2003), respectively. The *αAmy3* promoter and signal sequence used for expression of a human lysozyme led to accumulation of this recombinant protein in the cell wall/apoplastic space of rice suspension cells, with yields up to 4% of total soluble proteins (Huang et al., 2002).

Human serum albumin (HSA) is the major protein component of human plasma and consists of a single nonglycosylated polypeptide chain of 585 amino acids (66.5 kDa). The primary function of HSA lies in the maintenance of colloid osmotic pressure within blood vessels. HSA also acts as a carrier for transporting numerous hydrophobic molecules, e.g., steroid hormones, bilirubin and fatty acids. Due to these properties, HSA is used clinically to treat severe hypoalbuminemia or traumatic shock (Peters, 1995). The annual world demand for HSA exceeds 500 tons, representing a market value of more than $1.5 billion. Commercial HSA is obtained from human plasma, which is limited in supply and may contain viral contaminants. As HSA demand continuously increases world-wide, finding alternative ways to produce HSA is highly attractive. Attempts have been made to produce HSA in various microbial systems (Latta et al., 1987; Saunders et al., 1987; Quirk et al., 1989; Fleer et al., 1991; Ohtani et al., 1998); however, none of these systems is yet commercially feasible. The feasibility of expressing HSA in plant systems has also been explored. HSA was first expressed in both transgenic tobacco and potato leaves using the *CaMV35S* promoter (Sijmons et al., 1990), but only very low expression levels were attained (0.02% of total soluble proteins, TSP). More recently, a 10-fold increase in HSA accumulation was obtained (0.2% TSP) by its expression in the apoplast of potato tubers (Farran et al., 2002). Recently, a 500-fold increase in HSA accumulation (11.1% TSP) was obtained by expression in tobacco leaf chloroplasts using a chloroplast *psbA* promoter (Millan et al., 2003). However, HSA synthesized in chloroplasts aggregates and forms inclusion bodies, and after purification and solublization of inclusion bodies and subsequent refolding, only 20% of the initial quantities of HSA in leaves were recovered. Although a final yield of 0.25 mg HSA/g fresh weight of leaves could be achieved, which exceeds industrial estimates of cost-effective yield of pharmaceutical production (0.1 mg/g fresh weight of plant tissues) (Farran et al., 2002), extensive purification was needed. The *αAmy3* promoter, signal sequence, and rice cell culture represent a powerful system for production of recombinant proteins as human therapeutics. In the present study, this system was used for expression of HSA.

Materials and methods

Plant material

Oryza sativa L. cv Tainung 67 was used in this study. Twenty immature seeds were dehulled, sterilized with 50 mL of diluted bleach (the final concentration was 2.4% NaOCl) for 30 min, washed with sterile water, and placed on N6D agar medium (Toki, 1997) for callus induction. After 3 weeks, calli derived from scutella were subcultured in fresh N6D medium for *Agrobacterium*-mediated transformation. Transformed calli were subcultured in a liquid Murahige and Skoog medium, containing 3% sucrose and 10 μM 2,4-dichlorophenoxyacetic acid, to establish a suspension cell culture as described previously (Yu et al., 1991).

Plasmid constructions

For generation of *αAmy3* promoter-signal peptide sequence-*Hsa* chimeric gene, a 1.2-kb DNA fragment containing the promoter and signal sequence of *αAmy3* was PCR-amplified using p3G-132II (Lu et al., 1998) as the DNA template. The DNA fragment was inserted into the *Sal*I and *Hind*III sites in pBluescript SKII+ (Strategene), generating pBS-Amy3PSP. The 315-bp *αAmy3* 3'UTR (Chan & Yu, 1998) was PCR amplified and inserted into the *Hind*III and *Sac*II sites in pBS-Amy3PSP, generating pBS-Amy3PSP3U. A 1758-bp DNA fragment containing the mature HSA coding sequence (*Hsa*) was RT-PCR-amplified (Sambrook & Russell, 2001) from total RNA derived from human blood cells using 5′-GCCCAAGCTTGATGCACACA AGAGTGAGGTTG-3′ and 5′-GCCCAAGCTTAT-AAGCCTAAGGCAGCTTGAC-3′ (*Hind*III site underlined) as forward and reverse primers, respectively. The *Hsa* cDNA was inserted into the *Hind*III site between the *αAmy3* promoter-signal peptide sequence and the *αAmy3* 3′UTR in pBS-Amy3PSP3U to make a translational fusion, generating p3HSA. The correct orientation of the construct was verified by DNA sequencing

analysis. pSMY1H (Ho et al., 2000) is a plasmid containing the Cauliflower Mosaic Virus 35S RNA gene (*CaMV35S*) promoter-hygromycin phosphotransferase coding region (*Hph*)-nopaline synthease gene (*Nos*) terminator in pPZP200 (Hajdukiewicz et al., 1994). The *CaMV35S* promoter in pSMY1H was replaced with the rice *Act1* promoter from pDM302 (Cao et al., 1992), generating pSMY1AH. p3HSA was then linearized with *Sal*I and inserted into the same site in the binary vector pSMY1AH, generating pA3HSA.

Rice transformation

Plasmid pA3HSA was introduced into *A. tumefaciens* strain EHA105 (Hood et al., 1986) with an electroporator (BTX, San Diego) according to the manufacturer's instructions. Calli induced from immature rice seeds were transformed with *A. tumefaciens* containing pA3HSA as described (Toki, 1997).

DNA gel blot analysis

Genomic DNA was isolated from cultured rice suspension cells and gel blot analysis was performed as described (Ho et al., 2000). The ^{32}P random-primer-labeled *Hsa* cDNA was used as a probe.

RNA gel blot analysis

Total RNA was isolated from cultured rice suspension cells and gel blot analysis was performed as described (Ho et al., 2000). The ^{32}P random-primer-labeled *Hsa* cDNA, *αAmy3* 3′UTR (*αAmy3*-specific DNA) (Sheu et al., 1996) and *Act1* cDNA (McElroy et al., 1990) were used as probes.

Protein gel blot analysis

Total cellular proteins were extracted from cultured rice suspension cells with an extraction buffer (50 mM Tris-HCl, pH 8.8, 1 mM EDTA, 10% [v/v] glycerol, 1% [v/v] Triton X-100, 10 mM β-mercaptoethanol, and 0.1% [w/v] sarkosyl). Total medium proteins were collected by centrifugation at 18,000 × *g* at 4°C for 15 min for removal of cell debris. Protein concentration was determined with the protein assay reagent (Pierce) based on the Bradford method with BSA as a standard. Protein gel blot analysis was performed as described (Yu et al., 1991). The commercial HSA purified from human serum (Sigma) was used as a positive control. The polyclonal goat anti-HSA antibodies (ICN Pharmaceuticals) were diluted 1000-fold prior to use.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Falcon) were coated with 50 μL per well of 10 ng/μL goat anti-HSA polyclonal antibodies in 50 mM Na bicarbonate buffer, pH 9.6, at room temperature for 4 h and then replaced by 200 μL per well of phosphate saline buffer containing 1% skimmed milk at 4°C overnight. The blocking buffer was removed and 50 μL of each sample was added to the well and incubated at 37°C for 1 h. Samples were washed with PBST (PBS buffer, containing 2 mM KH_2PO_4, 18 mM Na_2HPO_4, 150 mM NaCl, pH 7.6, plus 0.05% Tween 20) and 50 μL per well of 10 ng/μL rabbit anti-HSA polyclonal antibodies were added. After incubated at 37°C for 1 h, 50 μL per well of peroxidase-conjugated anti-rabbit IgG antibodies (diluted to 1:2000) were added to the well. Samples were incubated at 37°C for 1 h, washed with PBST, and 50 μL of 2,2′-azinobis-(3 ethyl-benzthiazoline sulfonic acid) (Sigma) substrate solution was added to the well. The absorbance of samples at 405 nm was recorded with a microplate reader.

Tetrazolium reduction

Viability or metabolic activity of cells was determined by reduction of triphenyl tetrazolium chloride (TTC) as described (Chen et al., 1994).

Determination of cell dry weight

Culture medium was discarded and cells were washed three times with distilled water. Cells were transferred to a pre-weighed dish and dried at 80°C until a constant weight was obtained.

Culture of rice suspension cells in a bioreactor

The bioreactor consisted of two coaxial glass cylinders. The diameter of the inner cylinder was

12.3 cm, and its height was 29.5 cm. The space between inner and outer cylinders, which served as a water jacket, was filled with water. Ambient air from an air compressor was bubbled through a humidifier, metered through an air flowmeter, and then sterilized by passing through a 0.2 μm autoclavable filter (Millipore). The sterilized air was supplied from the bottom of the bioreactor through a sintered glass filter (160 μm) and introduced into the culture medium.

The bioreactor with 2.5 L working volume was autoclaved and filled with 2 L sterilized sucrose-containing MS medium. Rice suspension cells were cultured in ten 500-mL Erlenmeyer flasks, each containing 100 mL of sucrose-containing medium, for 5 days. A total of 42 mL cells collected from ten flasks, along with 500 mL of old culture medium, were used as inoculum for culture in the bioreactor. The culture temperature in bioreactor was controlled, by circulating water through the water jacket, with a constant temperature of 27°C ($\pm$1°C). The culture pH was maintained at 7 ($\pm$0.1) with 2 N NaOH. The sparging air volume was adjusted using an air flow meter (RK 1150, Kojima Co, Japan) to give a constant flow rate of 4 L/min. The culture was maintained in the dark. Repeated batch cultures were performed several times to build up high cell densities in the bioreactor. After reaching a stable high cell density (sedimented cell volume was 50% of bioreactor working volume), HSA production was induced by replacing the culture broth with sucrose-free MS medium. The culture medium was sampled daily and the yield of HSA was determined.

Purification of HSA from the culture medium

Two milliliter of rice suspension cells were cultured in 25 mL of sucrose-free MS medium for 2 days. The culture medium was collected and ammonium sulfate was added to 40% saturation. The mixture was stirred at 4°C for 30 min and centrifuged at 10,000 $\times$ *g* for 15 min. The supernatant was collected and dialyzed against three changes of 500 mL PBS buffer overnight. The dialysate was applied to 2 mL DEAE-sepharose (Amersham) ion-exchange column that had been pre-equilibrated with 10 mM phosphate buffer (pH 7.2). The column was washed with 10 mM Na phosphate buffer (pH 6.0), and then albumin was eluted from this column with 25 mM Na acetate buffer, pH 4.7. The eluate was dialyzed against PBS buffer and passed through a Centricon 30 column (Amicon), by centrifugation at 4000 $\times$ *g* at 4°C, to concentrate HSA.

Results

Generation of transformed rice cell lines expressing HSA

The expression vector pA3HSA contains the *αAmy3* promoter-signal sequence-*Hsa* cDNA and *Act1* promoter-*Hph* chimeric genes (Figure 1(a)). pA3HSA was introduced into the rice genome and putative transformed rice calli were selected with hygromycin. Identity of transformants was

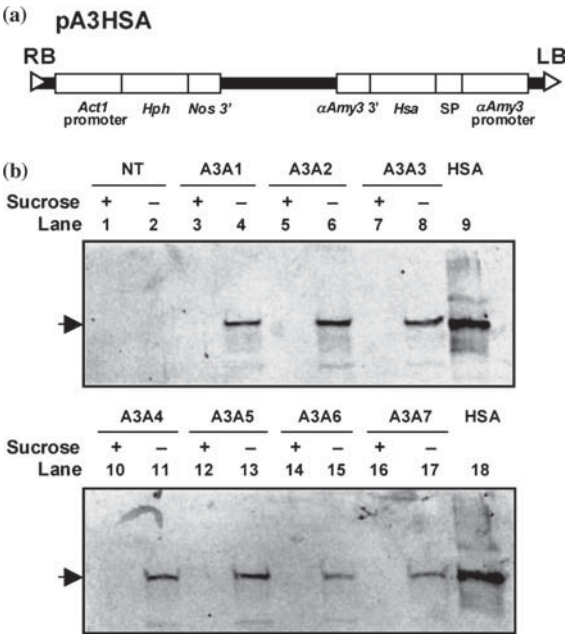

Figure 1. Expression of HSA in transformed rice cell lines. (a) Expression cassette for rice transformation. pA3HSA contains the *Act1* promoter *Hph Nos* 3′ and *αAmy3* promoter signal peptide sequence (SP) *Hsa αAmy3* 3′ chimeric genes. RB and LB, right and left borders of T DNA, respectively. Arrows indicate correct orientations of the border sequences. (b) Protein gel blot analysis of seven independently transformed rice cell lines expressing HSA. T0 transformed calli were cultured in sucrose containing (+) or sucrose free ( ) MS liquid medium overnight. Total proteins were isolated from cells and subjected to gel blot analysis using anti HSA antibodies. Ninety micrograms of total cellular proteins were loaded per lane. Commercial HSA was used as a positive control. NT: non transformed control. Lanes 9 and 18 contain 30 ng of commercial HSA. Arrowheads indicate position of HSA.

confirmed with genomic DNA gel blot analysis (data not shown). Accumulation of HSA in calli of seven T0 transformed cell lines was detected with protein gel blot analysis. HSA was not detected in the non-transformant and transformed cells provided with sucrose, but was detected in transformed cell lines starved of sucrose (Figure 1(b)). The recombinant HSA had the same electrophoretic mobility as commercial HSA. The transformed cell lines were regenerated to T0 plants and T1 seeds were harvested.

Sucrose starvation-induced synthesis of HSA mRNA and protein

Calli were generated from embryos of individual T1 seeds and cultured as suspension cells again. Yield of HSA in culture medium were analyzed and line 3A1 was found to have the highest yield, probably due to homogenization of cells. Therefore, 3A1 was selected for establishment of an inducible suspension cell culture system for production of recombinant HSA. DNA gel blot analysis showed that cell line 3A1 contains 3 copies of *Hsa* (Figure 2(a)). RNA gel blot analysis using the *Hsa* cDNA as a probe, was unable to detect accumulation of *Hsa* mRNA in suspension cells provided with sucrose, but increasing amounts of HSA mRNA were detected within 12 h of sucrose starvation, and remained high until day 4 after the onset of sucrose starvation (Figure 2(b)). By using the *αAmy3* 3'UTR DNA as a probe, accumulation of both endogenous *αAmy3* and *Hsa* mRNAs was detected in sucrose-starved cells. Rice *Act1* mRNA, which served as a control, had an opposite accumulation pattern, with higher mRNA levels in sucrose-provided cells and lower levels in sucrose-starved cells.

To determine whether HSA was synthesized and secreted into the culture medium in a sucrose starvation-dependent manner, total proteins were extracted from rice suspension cells and collected from the culture medium. HSA was accumulated only in cells and culture medium of cell line 3A1 in the absence of sucrose (Figure 2(c), lanes 4 and 8). The amount of HSA synthesized by T1 suspension cells appeared to be higher than that synthesized by T0 suspension cells (compare Figure 1(b) with Figure 2(c)), probably due to T1 suspension cells, which were derived from trans-

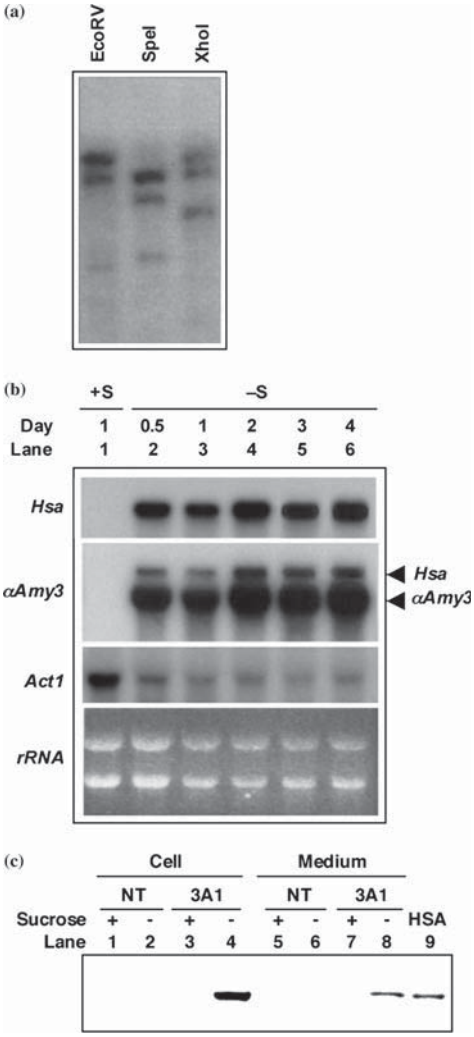

Figure 2. Sucrose starvation induced synthesis of HSA mRNA and protein. (a) DNA gel blot analysis. Genomic DNA of cell line 3A1 was digested with *Eco*RV, *Spe*I or *Xho*I and subjected to gel blot analysis using the ^{32}P labeled *Hsa* coding region as the probe. (b) RNA gel blot analysis. One milliliter of 3A1 suspension cells was cultured in 25 mL sucrose containing medium for 5 days, and then 1 mL of fresh cells were transferred to 25 mL sucrose containing medium (+ S) for 1 day or sucrose free (S) medium for various lengths of time. Total RNA was isolated and subjected to gel blot analysis using *Hsa* cDNA, *Act1* cDNA or *αAmy3* gene specific DNA as a probe. Ten μg of total RNA were loaded per lane. Arrowheads indicate positions of *Hsa* and *αAmy3* mRNAs. (c) Protein gel blot analysis. One milliliter of 3A1 suspension cells was cultured in 25 mL sucrose containing medium for 5 days, and then 1 mL of fresh cells were transferred to 25 mL sucrose containing (+) medium or sucrose free ( ) medium for 2 days. Proteins were extracted from cells or collected from the culture medium. HSA was detected by gel blot analysis using HSA antibodies. Total cellular proteins (20 μg) were loaded in each of lanes 1 4, and total medium proteins (240 ng) were loaded in each of lanes 5 8. NT, non transformed control. Lane 9 contains 30 ng of commercial HSA.

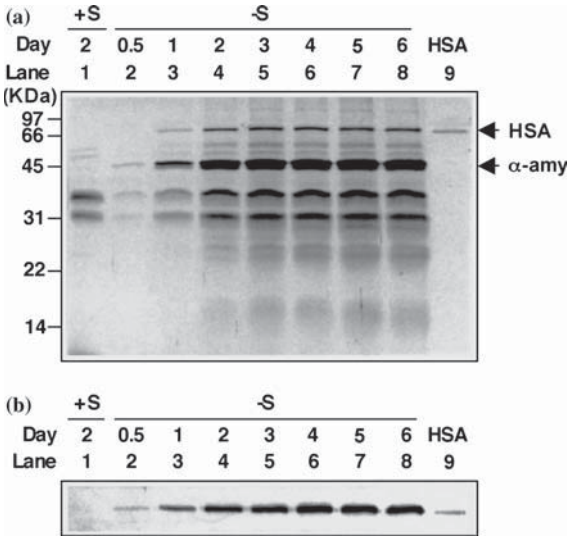

Figure 3. Sucrose starvation induced accumulation of HSA in the culture medium. One milliliter of 3A1 suspension cells was cultured in 25 mL sucrose containing medium for 5 days, and then 1 mL of fresh cells were transferred to 25 mL sucrose containing medium (+S) for 2 days or sucrose free (−S) medium for various lengths of time. Thirty microliters of culture medium was collected daily and loaded in each of lanes 1–8. (a) Protein gel staining analysis. HSA was detected by silver staining after SDS PAGE analysis. Arrowheads indicate positions of HSA and endogenous α amylases (α amy). Thirty ng of commercial HSA was loaded in lane 9. (b) Protein gel blot analysis. HSA was detected by HSA antibodies. Sixty nanograms of commercial HSA was loaded in lane 9.

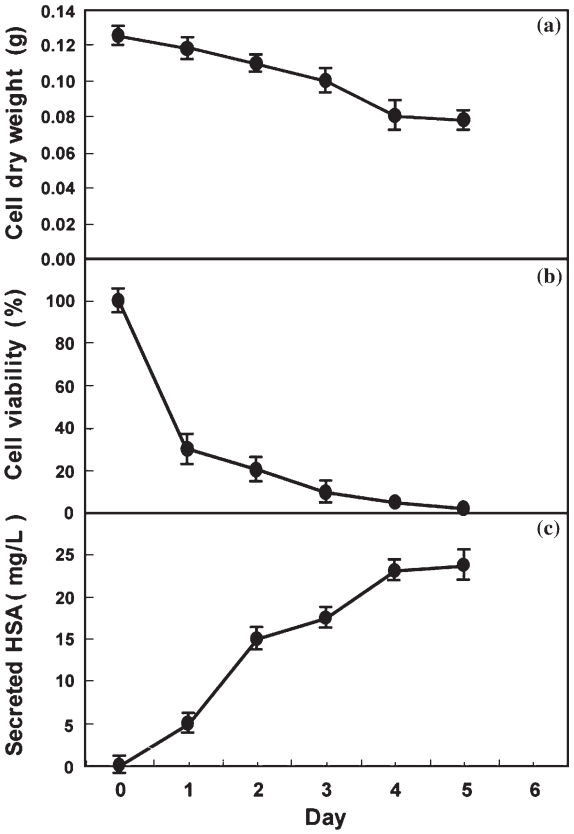

Figure 4. Inverse correlation between HSA production and cell growth and viability. One milliliter of 3A1 suspension cells was cultured in 25 mL sucrose containing medium for 5 days, and then 1 mL of fresh cells were transferred to 25 mL sucrose free medium for various lengths of time. Cells were collected for determination of dry weight and viability. Culture media were collected for determination of HSA concentration. Error bars indicate standard errors from triplicate cultures. (a) Cell dry weight. (b) Cell viability. The capability of TTC reduction in cells cultured for 5 days in sucrose containing medium is expressed as 100%. (c) Concentration of secreted HSA. The concentration of HSA in culture medium was determined by ELISA.

genic seeds, being more homogenous than T0 suspension cells, which contained heterogenous population of transformed cells. The molecular weight of HSA collected from either transformed cells or culture medium was identical to that of the commercial HSA (Figure 2(c), lane 9).

Sucrose starvation-induced accumulation of HSA in the culture medium

Accumulation of HSA in culture media of cell line 3A1, as a function of time under sucrose starvation, was also determined. Analyses of total proteins collected from culture media by SDS-PAGE and silver staining detected accumulation of the 66.5 kDa HSA increasing over the 6-day period of sucrose starvation (Figure 3(a)). The accumulation profile of HSA was similar to that of the 44- and 46-kDa endogenous α-amylases (Chen et al., 1994). The same protein samples were also analyzed with gel blot analysis using HSA antibodies. Accumulation of HSA increased with time, reached a maximal level on day 4, and remained high until day 6 after the onset of sucrose starvation (Figure 3(b)).

Inverse correlation between HSA production and cell growth and viability

To investigate the correlation between cell growth and viability and accumulation of secreted HSA under conditions of sucrose starvation, these parameters were monitored in cell line 3A1. Cell growth, expressed as dry weight,

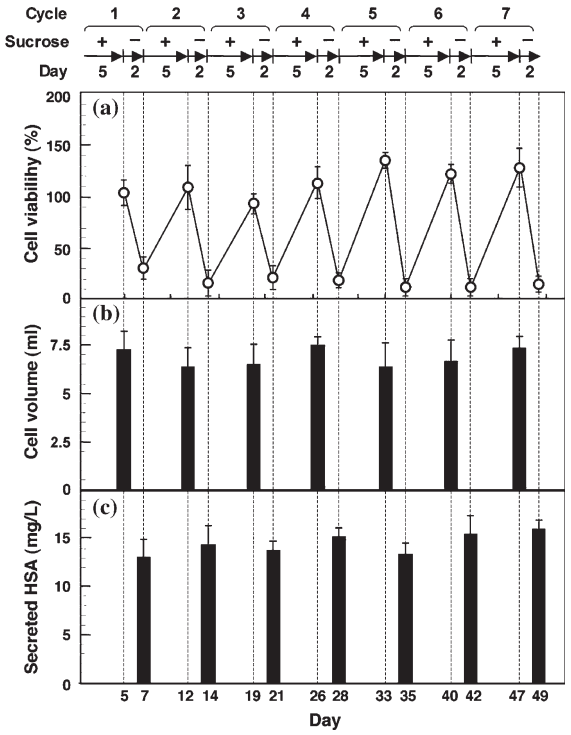

Figure 5. Stable production of HSA under repeated cycles of sucrose provision and depletion. Two milliliter of 3A1 suspen sion cells were cultured in 100 mL sucrose containing (+) medium (growth phase) for 5 days, and then 4 mL of cells were transferred to 100 mL sucrose free ( ) medium (produc tion phase) for 2 days. The 5 day growth phase and 2 day production phase cycles were repeated 7 times (in a period of 49 days). Cells were collected for determination of viability and growth, and media were collected for determination of concentration of secreted HSA. Error bars indicate standard errors from triplicate cultures. (a) Cell viability. The capabil ity of TTC reduction in cells cultured for 5 days in sucrose containing medium in the first cycle is expressed as 100%. (b) Cell volume. Cells collected on each day were placed in a 10 mL graduated tube and fresh MS medium was added to a final volume of 8 mL. The volume of cells was determined by subtracting the volume of medium required to fill up the 8 mL final volume. (c) Concentration of secreted HSA. The concentration of HSA in culture medium was determined by ELISA.

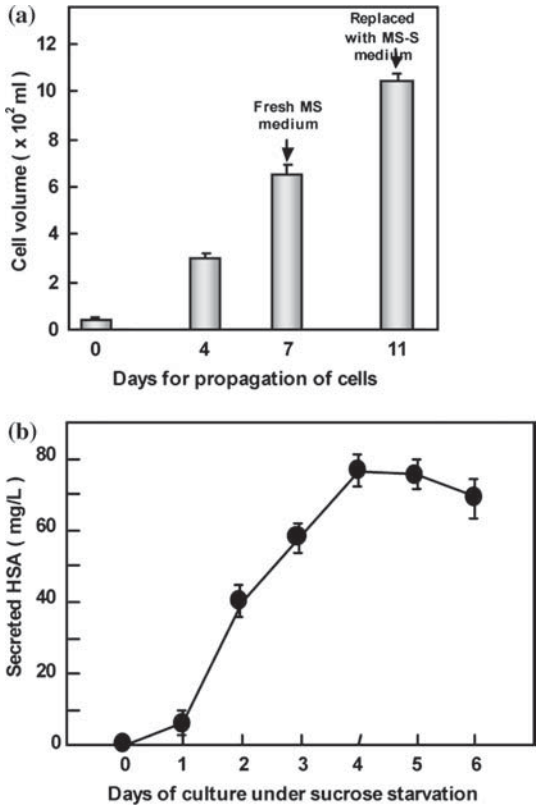

Figure 6. Scale up production of HSA in a bioreactor. (a) Propagation of cells for production of HSA. Forty two millili ter of 3A1 suspension cells were inoculated into the bioreactor and repeatedly batch cultured with 2 L of sucrose containing medium for 11 days. The culture medium was changed once with fresh sucrose containing MS medium on day 7. Sedi mented cell volumes were determined on days 0, 4, 7 and 11. The cell volume (1.25 L) reached 50% of the working volume (2.5 L) of the bioreactor on day 11. The culture medium was then replaced with 2 L of sucrose free MS (MS S) medium and cells were cultured for another 6 days. (b) Concentration of HSA in culture medium. Culture medium was collected daily and the concentration of secreted HSA was determined by ELISA. Error bars indicate standard errors from triplicate cultures.

decreased gradually until only 64% of the original level remained on day 5 after sucrose starvation (Figure 4, panel (a)). Cell viability dropped significantly on day 1 (Figure 4, panel (b)), with almost no living cells left on day 5 after sucrose starvation. By contrast, accumulation of HSA in the culture medium increased steadily and peaked on days 4–5 after sucrose starvation (Figure 4(c)).

Stable production of HSA under repeated cycles of sucrose provision and depletion

Rice suspension cells are normally cultured in sucrose-containing medium (growth phase) and then shifted to sucrose-free medium (production phase) for HSA production. To investigate whether HSA could be produced in cells subjected to repeated cycles of sucrose provision and sucrose depletion, cell line 3A1 were cultured for 5 days in sucrose-containing medium and 2 days in sucrose-free medium for 7 cycles. Cell viability

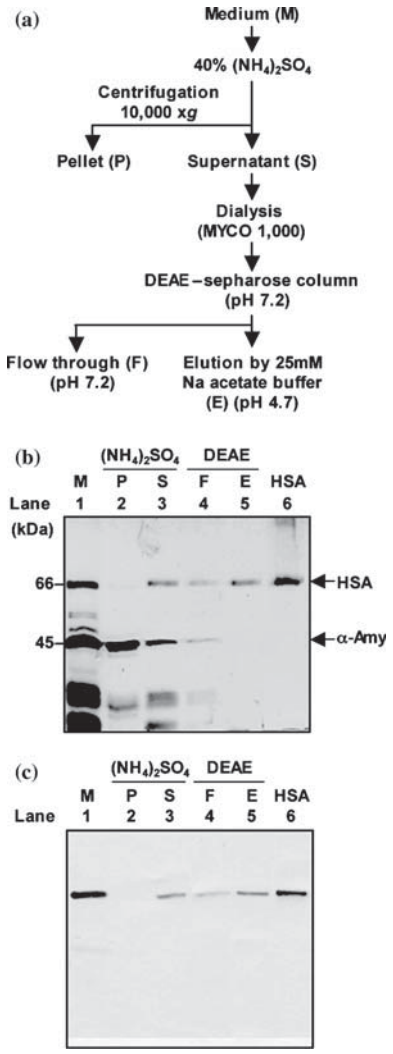

Figure 7. Scheme of purification of HSA from the culture medium. One milliliter of 3A1 suspension cells was cultured in 25 mL sucrose containing medium for 5 days, and then 2 mL of fresh cells were transferred to 25 mL sucrose free medium for 2 days. The culture medium was collected for purification of HSA. (a) Scheme of purification steps. (b) Pro teins from each fraction during the purification process were analyzed by SDS PAGE and silver staining. Lane 1, culture medium (M) prior to purification; lane 2, pellet (P) after ammonium sulfate precipitation; lane 3, supernatant (S) after ammonium sulfate precipitation; lane 4, flow through (F) from DEAE Sepharose column; lane 5, eluted fraction (E) from DEAE Sepharose column; lane 6, 30 ng of HSA stan dard. Arrowheads indicate positions of HSA and α amylases (α amy). (c) Gel blot analysis of proteins in each fraction in (B) using HSA antibodies.

and growth and HSA accumulation in culture media were monitored. In each cycle, the cell viability declined to 10–30% of original level after sucrose depletion but recovered to around 100% or even higher than the original level when sucrose was provided (Figure 5, panel (a)). Cell growth, expressed as cell volume and measured on day 5 after sucrose provision, did not significantly change throughout the 7 cycles (Figure 5, panel (b)). Neither did the concentrations of HSA in the culture medium change when determined on day 2 after sucrose depletion, ranging between 13 and 16 mg/L throughout the 7 cycles (Figure 5, panel (c)).

Scale-up HSA production in a bioreactor

To explore the potential of industrial production of HSA, a modified bubble column type bioreactor with a working volume of 2.5 L was fabricated and tested for HSA production. Repeated batch culture was performed until the sedimentation volume of suspension cells (approximately 1.25 L) reached 50% of working volume (2.5 L) of the bioreactor (Figure 6(a)). The culture broth in the bioreactor was then replaced with the sucrose-free medium, and accumulation of HSA in the culture medium was monitored during the next 6-day production phase. The concentration of HSA in the culture medium reached a maximal level (76.5 mg/L) between days 4 and 5 and declined on day 6 after sucrose starvation (Figure 6(b)).

Simple scheme purification of HSA from the culture medium

HSA was purified from the culture medium by ammonium sulfate precipitation and DEAE chromatography (Figure 7(a)). After 40% ammonium sulfate precipitation of total proteins in culture medium, most α-amylases were removed (Figure 7(b), lane 2) and 70% of HSA was left in the supernatant (Figure 7(b), lane 3). Supernatant containing HSA was then loaded onto a DEAE column and eluted with 25 mM Na acetate buffer, pH 4.7. Approximately 30% of HSA and all non-HSA proteins in the supernatant were removed in the flow through fractions (Figure 7(b), lane 4) and 70% of HSA was eluted (Figure 7(b), lane 5). Protein gel blot analysis using the HSA antibodies confirmed the presence of HSA in each fraction during the purification process (Figure 7(c)). Overall, this scheme achieved a yield of 50% with the final HSA fraction having purity greater than 95%.

Discussion

In this study, using a novel inducible expression system based on a combination of the *αAmy3* promoter and signal peptide sequence and rice suspension cell culture, high-level production of secreted HSA was achieved. Since the *Hsa* gene was linked to a metabolically regulated *αAmy3* promoter and signal sequence, expression and secretion of HSA was controlled by the addition or removal of sucrose from the culture medium.

αAmy3 is the most abundantly expressed α-amylase gene, with its mRNA level constituting 60% of total α-amylase mRNAs, in sucrose-starved rice suspension cells (Sheu et al., 1996). The *αAmy3* promoter activity is also the highest of the 9 known rice α-amylase genes upon sucrose starvation (Sheu et al., 1996). In this study, although the *αAmy3* promoter directed high-level and sucrose starvation-inducible expression of HSA in rice suspension cell culture, accumulation of HSA mRNA was significantly less than that of endogenous *αAmy3* mRNA (Figure 2(b)). The discrepancy in expression levels between foreign and endogenous genes driven by the same promoter could have two explanations. Firstly, the 1.2-kb *αAmy3* promoter used for controlling *Hsa* expression probably does not cover all regulatory sequences necessary for high *αAmy3* promoter activity. Secondly, HSA mRNA could be less stable than *αAmy3* mRNA, even though the *αAmy3* 3'UTR, which is known to stabilize mRNA under sucrose starvation (Chan & Yu, 1998a, b), was fused downstream of the HSA mRNA (Figure 1(a)).

Heterologous proteins appear to be more prone to proteolysis, as they are regarded by cells as foreign (Enfors, 1992). Consequently, proteolytic stability is a significant factor influencing the final yields of recombinant proteins in all expression systems. Significantly lower levels of protease have been detected in the culture medium of rice suspension cells compared to those in the culture medium of tobacco suspension cells (Shin et al., 2003). Additionally, only half the amount of protease is present in the culture medium of sucrose-starved rice suspension cells compared to that in the culture medium of sucrose-provided rice suspension cells (Shin et al., 2003). The relatively low proteolytic activity present in rice cell culture medium is considered an important contributing factor to the 1000-fold increase in yield of secreted recombinant hGM-CSF compared to tobacco cell culture medium (Shin et al., 2003). In this case, yields of secreted recombinant hGM-CSF produced by an *αAmy3* promoter-signal sequence-rice cell culture system (129 mg/L) (Shin et al., 2003) are significantly higher than those by a *CaMV35S* promoter-tomato cell culture system (45 μg/L) (Kwon et al., 2003) and a *CaMV35S* promoter-tobacco cell culture system (783 μg/L) (Lee et al., 2002). HSA is highly susceptible to proteolytic degradation (Sijmons et al., 1990). In this study, the 66.5 kDa HSA accumulated in the culture medium up to the 6th day after sucrose starvation (Figure 3), upon which all cells died (Figure 4(b)), suggesting that HSA is relatively stable in the rice cell culture medium. Our study demonstrates that the rice cell culture system provides a valuable alternative for production of secreted recombinant proteins that are highly susceptible to proteolytic degradation. Use of rice cell culture as a protein expression system offers an additional advantage for their relatively high growth rate, with minimal aggregate clumping, and are easy to cultivate. Significantly, rice is a well characterized food crop that does not produce any toxic secondary metabolites found in some plant cell cultures, such as those derived from tobacco.

Sugar starvation generally leads to a decrease in respiration rate, degradation of reserves, and a decline in the metabolic activities in plant cells (Yu, 1999). In this study, under the control of the *αAmy3* promoter, HSA was synthesized at the expense of cell mass and cell viability under sucrose starvation (Figure 4, panels (a) and (b)). Although accumulation of HSA reached a peak level on the 4th day after sucrose starvation (Figure 3(b) and Figure 4, panel (c)), the cell viability dropped to only 5% of original level (Figure 4, panel B). Viability and growth of cells and production of secreted HSA during a repeated 5-day growth and 2-day production regime were maintained at relatively stable levels (Figure 5), which provides a basis for the large-scale production of HSA using bioreactors. To induce the *αAmy3* promoter, and thus HSA production, would necessitate the use of a bioreactor with medium (sucrose-containing to sucrose-free medium) exchange capabilities. It appears higher yields of secreted recombinant protein could be achieved by increase in cell density in a bioreactor. The highest

yield of HSA in the 25-mL flask scale culture was estimated to be 23 mg/L (11.5% of total secreted proteins) on day 4 after sucrose starvation (Figure 4(c)). Whereas, the highest yield of HSA in the 2-L scale bioreactor was 75 mg/L (0.23 mg/g fresh weight cells) on day 4 after sucrose starvation (Figure 6(b)).

In this study, the N-terminal end of HSA produced in the rice cell culture system has not been determined. However, several studies suggest that HSA and other passenger proteins fused with plant-derived signal peptides are correctly cleaved and secreted extracellularly by transformed plant cells. HSA fused to signal peptides, derived from either tobacco PR-2 protein or potato protease inhibitor II, are properly processed in transgenic potato plants or tubers, giving rise to a mature protein with N-terminal end identical to the native HSA (Sijmons et al., 1990; Farran et al., 2002). rAAT and lysozyme fused with the *αAmy3* signal peptide have also been shown to be properly processed in transformed rice suspension cells, producing mature proteins with N-terminal identical to the native forms of the two proteins (Terashima et al., 1999; Huang et al., 2002). Additionally, HSA expressed and secreted into apoplasts of transgenic tobacco (Sijmons et al., 1990), rAAT and hGM-CSF expressed and secreted into the culture medium of transformed rice suspension cells (Terashima et al., 1999; Shin et al., 2003), and human lysozyme expressed and secreted into apoplasts of transformed rice suspension cells (Huang et al., 2002) all are structurally and/or functionally indistinguishable from their respective native proteins. These studies suggest that human proteins expressed and secreted by plant cells could be correctly folded into biologically active forms.

The availability of a homogenous rice suspension cell line permits HSA production by fermentation. In this study, a simple scheme has been developed to purify HSA from this expression system to high purity. It is anticipated that the production of HSA by rice cell culture could be improved and scaled up with further optimized conditions. Enrichment of HSA in culture medium facilitates detection, simplifies downstream purification, minimizes protease degradation, and would minimize the production cost for this protein. This sugar starvation-inducible expression system could be used for the production of numerous recombinant proteins. Transformed rice suspension cells grow efficiently to a high density in media with a high sucrose concentration. Production of recombinant proteins is then induced by switching transformed rice cells to sucrose-free media. Transgenic rice suspension cell cultures can provide large quantities of recombinant proteins in a matter of weeks or a few months. For recombinant pharmaceutical proteins yielding several hundred mg per L (Terashima et al., 2001; Shin et al., 2003) within 5-day production regime, a 3-L rice suspension cell culture would yield a few grams of these proteins in 3 weeks. This inducible system is particularly advantageous for production of recombinant proteins that could be toxic and affect cell growth. In this case, accumulation of recombinant proteins is not induced when cells are cultured in medium containing sucrose.

In conclusion, a protein expression system based on the α-amylase promoter-signal peptide-rice cell culture provides several advantages: (1) Promoter activity is highly inducible by sucrose starvation, which facilitates production of both non-toxic and toxic recombinant proteins; (2) The rice cell culture secretes relatively lower amount of protease into the culture medium, which facilitates production of unstable recombinant proteins; (3) Recombinant protein is secreted into the culture medium at a high concentration, which facilitates downstream protein purification.

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