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Improved expression and purification of recombinant human serum albumin from transgenic tobacco suspension culture

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ABSTRACT

Most human serum albumin (HSA) for medical applications is derived from human plasma due to the lack of suitable heterologous expression systems for recombinant HSA (rHSA). To determine whether plant cell cultures could provide an alternative source, we employed the hyper-translatable cowpea mosaic virus protein expression system (CPMV-*HT*) to stably express rHSA in tobacco Bright Yellow-2 (BY-2) cells. rHSA was stably produced with yield up to 11.88 μg/ml in the culture medium, accounting for 0.7% of total soluble protein, in a 25-ml flask. Cultivation of transgenic cells in modified Murashige and Skoog medium with a pH of 8.0 improved the yield of rHSA two-fold, which may be the result of reduced proteolytic activity in the modified medium. A simple purification scheme was developed to purify the rHSA from culture medium, resulting in a recovery of 48.41% of the secreted rHSA. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and N-terminal sequence analysis of the purified rHSA revealed that plant cell-derived rHSA is identical to that of the plasma-derived HSA. Our results show that the CPMV-*HT* system, which was originally developed as a transient expression system for use in whole plants, can also be used for high-level expression of rHSA, a protein highly susceptible to proteolysis, in transgenic tobacco cells.

1. Introduction

The use of plants and plant cell suspension cultures is a relatively new strategy for the production of recombinant proteins and has been adopted to express many important pharmaceutical proteins (Brandsma et al., 2010; Shih and Doran, 2009). Compared to whole-plant systems, plant cell suspension cultures have several advantages including the capacity for shorter life cycles, independence from environmental effects such as climate, soil quality, season, day length and weather and the lack of biosafety issues such as gene flow via pollen and the possibility of bacterial contamination from the plant growth environment (Xu et al., 2011). The controlled and sterile conditions of cultivation make plant cell suspension systems amenable to good manufacturing practice (GMP) (Huang and McDonald, 2009). In addition, if the recombinant proteins are secreted into the culture medium, their purification is greatly facilitated by the presence of fewer contaminating proteins and the elimination of the need to collect, disrupt and homogenize the cells (Shih and Doran, 2009). The characteristics of fast growth rate and high biomass yield (Fig. S1) make tobacco Bright Yellow-2 (BY-2) cells (Geelen and Inze, 2001), one of the best characterized and popular plant cell lines, an attractive expression system for heterologous protein expression (Huang et al., 2009; Suen et al., 2010). However, the expression of foreign proteins in BY-2 cells is currently hampered by the low yield (0.5 μg/l–1.0 mg/l in culture medium or 0.01–0.1% of total soluble protein) (Weathers et al., 2010), which is partially the result of proteolytic degradation (Benchabane et al., 2008).

Human serum albumin (HSA) is an important human blood protein (Arroyo, 2009). Aside from its major use as a blood volume expander, HSA is also used as a clinical tool for the delivery of many biological products (Varshney et al., 2010). Since the purified natural product may contain blood-borne pathogens, such as human immunodeficiency virus (HIV) and hepatitis virus, recombinant expression of HSA is a highly attractive way to meet the

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increasing demand of HSA worldwide (Ko et al., 2006). Although several systems for the expression of HSA have been tested, none is yet commercially viable (Desai et al., 2010).

Plants offer a potentially attractive method of producing recombinant HSA (rHSA) in the quantities required to meet current and future demand. rHSA was first expressed in transgenic potato and tobacco; however, this approach resulted in very low yields, accounting 0.02% of total soluble protein (TSP) in buds and young leaves of tobacco, and only 0.004% of TSP in roots (Sijmons et al., 1990). Further experiments showed that when directed to the extracellular compartment of potato roots, the yield of rHSA was 10 times higher, reaching 0.2% of TSP (Farran et al., 2002). The yield of rHSA was increased 500-fold when the protein was expressed in tobacco chloroplasts, reaching 11.1% of TSP. However, this resulted in the formation of inclusion bodies, which needed a further renaturation or refolding process resulting in low recovery of rHSA after purification (Fernandez-San Millan et al., 2003). Furthermore, as plant chloroplasts share a similar mechanism of translation and post-translational modification to bacteria, using plant chloroplasts to express foreign proteins presents similar problems to those encountered with the use of bacterial expression systems. A rice suspension cell-sucrose starvation system, which requires the replacement of the culture medium with one lacking sucrose to induce rHSA production, gave a high yield of rHSA, reaching 15 μg/ml culture medium (Huang et al., 2005). However, the replacement of the medium is labor-intensive and sucrose-deficiency has negative effects on the growth of rice cells, whose growth rate is, in any case, inferior to that of tobacco cells (Hellwig et al., 2004).

The hyper-translatable cowpea mosaic virus protein expression system (CPMV-*HT*), based on a non-replicating deleted version of cowpea mosaic virus (CPMV) RNA-2 which is hyper translatable relative to the wild type RNA-2, has been proven to give high level expression of foreign genes (Sainsbury and Lomonossoff, 2008). The pEAQ-*HT* vector (Sainsbury et al., 2009), based on this system, includes a gene encoding the tomato bushy stunt virus (TBSV) protein P19 (Scholthof, 2007) on the same T-DNA as the target gene. P19 can efficiently suppress posttranscriptional gene silencing (PTGS) that commonly limits the productivity of plant expression systems (Boivin et al., 2010). The pEAQ-*HT* vectors have been extremely useful for transient expression in *Nicotiana benthamiana* leaves (Sainsbury et al., 2010), but there is no report of their use to enhance expression in plant suspension cell cultures.

In this study we demonstrate that rHSA can be stably expressed at high level in cultures of BY-2 cells transformed with a pEAQ-*HT* construct expressing rHSA. Secretion of rHSA into the culture medium was directed by a signal peptide from barley proaleurain, which has already been shown to be correctly processed in transgenic BY-2 cells (Fu et al., 2009; Suen et al., 2010). Purified rHSA was obtained from the culture medium of the transformed BY-2 cells by a simple purification scheme developed during this study. Detailed characterization showed that the plant-expressed protein was indistinguishable from natural HSA.

2. Materials and methods

2.1. Construction of plant expression vectors

For the generation of pART27-*HSA*, *HSA* cDNA (GenBank accession no. BC036003) amplified using primers ZF458 and ZF459 (The PCR primers used in this study are listed in Supplementary Table 1.) from the vector pDNR-LIB was ligated into the pMD18 T-Simple vector (Takara) for sequencing. Subsequently, the *HSA* cDNA was released from the vector by digestion with *Bam* H I and *Kpn* I, and inserted into the pHANNIBAL plasmid. The resulting construct was digested by *Spe* I and *Sac* I and the expression cassette containing the 35S CaMV promoter, the sequence of *HSA* and the 35S CaMV terminator was inserted into the binary vector pART27 (Gleave, 1992). For the generation of the pART27-SP-*HSA* vector, an *HSA* fragment fused to the SP of barley proaleurain (MAHARVLLLALAVLATAAVAVA) was amplified from pDNR-LIB using primers ZF520 and ZF705 and ligated into pMD18 T-Simple vector for sequencing. The SP-*HSA* sequence was released from the vector by digestion with *Bam* H I and *Kpn* I, and then inserted into the pHANNIBAL plasmid. The resulting construct was digested by *Not* I and the expression cassette containing the 35S CaMV promoter, the sequence of SP-*HSA* and the 35S CaMV terminator was inserted into pART27.

For generation of the pCAMBIA-*SaPIN2a* vector, total RNA was isolated from the flowers of American black nightshade (*Solanum americanum* Mill.) plants using the RNeasy Plant Mini Kit (QIAGEN) and total cDNA was synthesized by priming with oligo-dT_{18} and using MMLV Reverse Transcriptase (Promega). The PCR product amplified using primers 2a-full-1 and 2a-full-2 was ligated into the pMD18-T simple vector for sequencing. The resulting vector was digested by *Bam* HI and *Sac* I and the fragment containing *SaPIN2a* cDNA was ligated into pCAMBIA1300. For generation of pEAQ-SP-*HSA*, the SP-*HSA* expression cassette was amplified from pART27-SP-*HSA* using primers ZF721 and ZF722 and the product ligated into pMD18-T simple vector for sequencing. The vector was digested with *Nru* I and *Xho* I and the SP-*HSA* sequence was ligated into pEAQ-*HT*. The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain EHA105 using the freeze–thaw method (Holsters et al., 1978).

2.2. Generation of stable transgenic BY-2 cell lines

Transgenic tobacco BY-2 cells were generated via *Agrobacterium*-mediated transformation as previously described (Tse et al., 2004). Transformed BY-2 cells were selected on MS media containing 250 mg/l carbenicillin and 50 mg/l kanamycin or 50 mg/l hygromycin for 3–4 weeks until resistant cell colonies were formed (Supplementary Fig. S1A and B). Individual transgenic cell lines were identified from resistant cell colonies by PCR. The *HSA*-specific primers ZF713 and ZF718 were used to screen cell lines transgenic for pART27-*HSA*, pART27-SP-*HSA* and pEAQ-SP-*HSA* vectors, while the *SaPIN2a*-specific primers ZF56 and ZF103 were used to screen cell lines transgenic for pART27-SP-*HSA* and pCAMBIA-*SaPIN2a*. The PCR primers used in this study are listed in Supplementary Table 1. The cryopreservation of transgenic BY-2 cell lines was performed according to the published method (Schmale et al., 2006).

2.3. SDS-PAGE and western blot analysis

SDS-PAGE and western blot analysis were carried out as described previously (Wang et al., 2007). The blot was probed with polyclonal antibodies against HSA (Sigma, A4327) and proteins detected using a secondary antibody conjugated to either alkaline phosphatase (AP) or horseradish peroxidase (HRP). The reaction for AP was performed using the colorimetric substrates NBT and BCIP (Roche). The reaction of HRP was performed with chemiluminescent substrates (Pierce).

2.4. rHSA stability in culture medium of transgenic BY-2 cells

To determine the stability of HSA in the culture medium of transgenic BY-2 cells, 5 μg of commercial HSA was incubated for 24 h at 37 °C with 40 μl of BY-2 culture medium taken after a variety of days of growth of the cells. To test the protective effects of different protease inhibitors on HSA stability, 5 mM PMSF (serine protease

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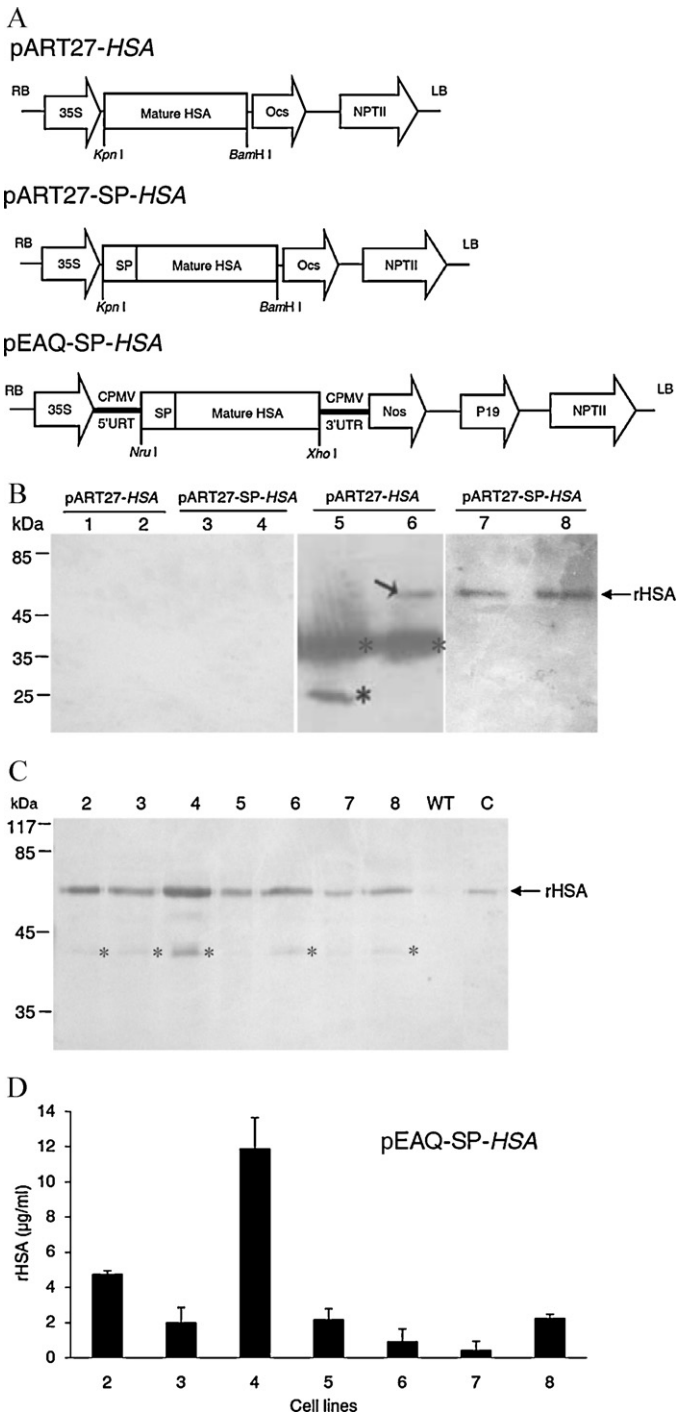

Fig. 1. Accumulation of rHSA in suspensions of transgenic BY-2 cells. (A) Schematic representation of plasmids pART27-*HSA*, pART27-SP-*HSA* and pEAQ-SP-*HSA*. *Abbreviations*: LB, left border of T-DNA; 35S, CaMV 35S promoter; SP, barley proaleurain signal peptide; Nos/Ocs, nopaline/octopine terminator; RB, right border of T-DNA; and P19, a suppressor of gene silencing encoded by tomato bushy stunt virus. (B) Western blot analysis of proteins from BY-2 cells transformed with pART27-*HSA* or pART27-SP-*HSA* with rabbit anti-HSA antibody. Lanes 1–4, 50 μl of the culture medium from suspensions of different transgenic cell lines; lanes 5–8, 15 μg intracellular total proteins of different transgenic cells. The proteins in the culture medium were visualized using an AP-conjugated goat anti-rabbit secondary antibody with a colorimetric method and the intracellular proteins were detected using HRP-conjugated goat anti-rabbit secondary antibody with a chemiluminescent method. (C) Western blot analysis of proteins from BY-2 cells transformed with pEAQ-SP-*HSA* with rabbit anti-HSA antibody using an AP-conjugated goat anti-rabbit secondary antibody with the colorimetric method to detect rHSA. WT, 50 μl of the culture medium from a suspension of wild-type (non-transformed) BY-2 cells; lanes 2–8, 50 μl of the culture medium of different transgenic cell lines; (C) 15 μg of intracellular total protein of the transgenic cell line No. 4. The arrows indicate full-length rHSA and asterisks represent the degradation fragments of rHSA. (D) ELISA result of rHSA from the culture medium of transgenic BY-2 cells overexpressing pEAQ-SP-*HSA*. Three replicates for each sample examined. Each value is the mean ± SD.

inhibitor), 40 μM E-64 (cysteine protease inhibitor), 20 mM EDTA (metalloprotease inhibitor), 16 μM pepstatin (aspartic protease inhibitor) or a protease inhibitor cocktail (Sigma, P9599) were added, respectively, to the HSA-containing culture medium and incubated at 37 °C for 24 h. To determine the influence of pH on HSA stability in the culture medium, 5 μg of commercial HSA were incubated at 37 °C for 24 h with 40 μl of the day 6 BY-2 culture medium adjusted to various pHs between 3.0 and 8.0. The degradation of HSA was examined by SDS-PAGE.

2.5. Quantification of rHSA by enzyme-linked immunosorbent assay (ELISA)

The expression level of rHSA in transgenic BY-2 cell suspension cultures was measured using the ELISA Human Albumin Quantification Kit (Bethyl Laboratories).

2.6. Effects of pH and EDTA on rHSA production

Cultured BY-2 cells were transferred to the different MS media (pH 5.8, pH 3.0 or pH 8.0, supplemented with either 5 mM, 10 mM or 20 mM EDTA) by adding 1 ml cells into 25 ml of the appropriate medium. After 8-day cultivation, the amount of rHSA in the culture medium was analyzed by ELISA and the wet weights (free of medium) of the transgenic and wild type BY-2 cells determined.

2.7. Purification of the rHSA

After the addition of 10 mM N-acetyl-DL-tryptophan and 10 mM sodium octanoate, 15 ml of culture medium was heated at 70 °C for 1 h. After centrifugation at 12,000 rpm for 30 min at 4 °C, the supernatant was loaded onto a Capto blue gel column (GE Healthcare) at a flow rate of 1 ml/min. The flow-through was subsequently loaded on to a DEAE blue gel column (Bio-Rad). Both columns were pre-equilibrated with equilibration buffer (50 mM Tris, 20 mM NaSCN, pH 8.0). rHSA was eluted from the DEAE blue gel column with elution buffer (50 mM Tris, 200 mM NaSCN, pH 8.0). The eluted rHSA was desalted and concentrated by a 30-kDa ultrafiltration tube (Millipore). The recovered rHSA was examined by SDS-PAGE and western blot analysis, and quantified using an image analyzer (Bio-Rad, ChemiDox XRS imager). Protein concentrations were determined using the Bradford method (Bradford, 1976) with commercial HSA as a standard.

2.8. Detection of rHSA glycosylation

Glycoprotein detection was carried out with the GelCode glycoprotein staining kit (Pierce) according to the manufacturer's procedure.

2.9. MALDI-TOF/TOF and N-terminal amino acid sequencing

Purified rHSA excised from the SDS-PAGE gel was digested with trypsin and subjected to MALDI-TOF/TOF analysis using an ABI 4800 mass spectrometer. The N-terminal amino acid sequencing of the purified rHSA was carried out as described previously (Wang et al., 2007).

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3. Results

3.1. Generation of transgenic tobacco BY-2 cells stably expressing rHSA

To investigate the suitability of tobacco BY-2 suspension cells as a platform for HSA production, we initially used the conventional CaMV 35S promoter-based binary vector, pART27 (Gleave, 1992), to express two versions of HSA with or without the signal peptide (SP) of barley proaleurain (Fu et al., 2009), which is known to direct the secretion of recombinant pharmaceutical proteins in tobacco BY-2 cells (Fig. 1A). The accumulation of recombinant HSA (rHSA) in the culture medium of cells transformed with either pART27-*HSA* or pART27-SP-*HSA* was too low to be detected by western blot analysis (Fig. 1B). Consistent with previous observations (Farran et al., 2002; Sijmons et al., 1990), most of the rHSA expressed from pART27-*HSA* (without the SP) which accumulated within the transgenic BY-2 cells was severely degraded to smaller proteins. This degradation occurred to lesser extent in cells expressing rHSA with the SP though the level of accumulation within the cells was much lower (Fig. 1B).

In an attempt to increase the expression levels of rHSA, we inserted the sequence of SP-HSA into plasmid pEAQ-*HT* (Sainsbury et al., 2009) to create a new HSA expression vector, pEAQ-SP-*HSA* (Fig. 1A) which was used to transform BY-2 cells. Western blot analysis revealed significant accumulation of rHSA in culture medium of the resultant cell lines (Fig. 1C), in contrast to the results obtained with pART27-SP-*HSA*. However, some degradation of the secreted rHSA was also noted (Fig. 1C). Enzyme-linked immunosorbent assay (ELISA) was used to assess the levels of rHSA in the culture medium of various transgenic lines and showed that the yield of rHSA varied, with a highest yield (11.88 μg/ml) being found with cell line No. 4 (Fig. 1D).

3.2. Improvement of rHSA yield by reducing proteolytic degradation

Since rHSA is susceptible to proteolysis in BY-2 cell culture (Fig. 1B and C), we hypothesized that the yield of rHSA could be improved by reducing the protease activity of BY-2 cells. To test this hypothesis, we incubated commercial HSA with various culture media from BY-2 cells and analyzed the degradation of HSA by SDS-PAGE. After 24 h incubation, significant degradation of HSA was observed when the protein was incubated with culture medium prepared after 3 days growth of BY-2 cells, and all the HSA had been degraded when incubated with culture medium isolated after 7 days growth (Fig. 2A). The effects of pH and protease inhibitors on the HSA stability in the culture medium of BY-2 cells were evaluated separately. The results indicated that adjusting the pH of the culture medium to either 3 or 8, or adding 20 mM EDTA (a metal chelator) significantly prevented the degradation of HSA in culture medium of BY-2 cells grown for 7 days (Fig. 2B and C). Consistent with this observation, cultivation of the transgenic cells in MS medium adjusted to pH 8 improved the yield of rHSA in the culture medium 2.09-fold (Fig. 2D and E). Attempts to culture BY-2 cells in the MS media with different concentrations of EDTA were unsuccessful as the cells did not grow well (data not shown).

An alternative approach to reduce the proteolytic degradation of rHSA in BY-2 cell culture by the co-expression of a protease inhibitor (Goulet et al., 2010; Miao et al., 2008) was investigated. To this end pCAMBIA-*SaPIN2a* (Fig. 3A), containing the potent serine protease inhibitor SaPIN2a (Xu et al., 2004), was co-expressed in BY-2 cells transformed with pART27-SP-*HSA*. Although the protease activity in the culture medium of BY-2 cells overexpressing *SaPIN2a* was reduced (Fig. 3B), the yields of rHSA in transgenic cells co-expressing *SaPIN2a* and *HSA* did not increase (Fig. 3C).

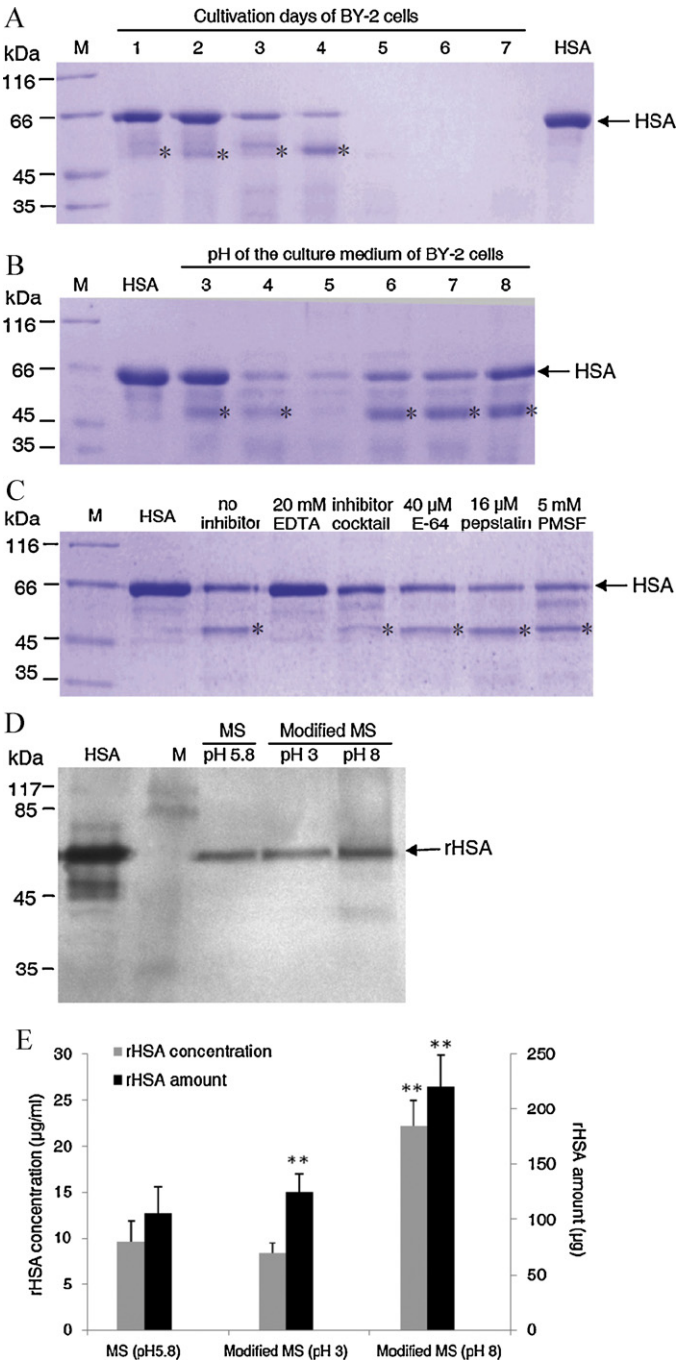

Fig. 2. Analysis of HSA stability. Five micrograms of commercial HSA was incubated in culture medium of BY-2 cells (A) or in the modified culture medium at different pHs (B) or in culture medium with different protease inhibitors (C) at 22 °C for 1 h. Western analysis (D) and the yield (E) of rHSA in the culture medium of transgenic cells transformed with pEAQ-SP-*HSA* during cultivation in the 25-ml modified MS media. Each value is the mean ± SD. Cell line No. 4 was used for the analysis. **Indicates statistically significant differences between the modified MS media and MS medium ($p < 0.01$). The cells used in (B–E) were cultured for 8 days. Asterisks represent the degradation fragments of rHSA.

3.3. Purification of the rHSA from the culture medium of transgenic BY-2 cells

Since heat treatment of protein solutions for 10 h at 60 °C is used to denature viral proteins and inhibit virus replication in plasma-derived HSA products (Burnouf, 2007), we speculated that HSA has a high thermal tolerance. Accordingly, we investigated the use of heat treatment to remove host proteins from the culture medium

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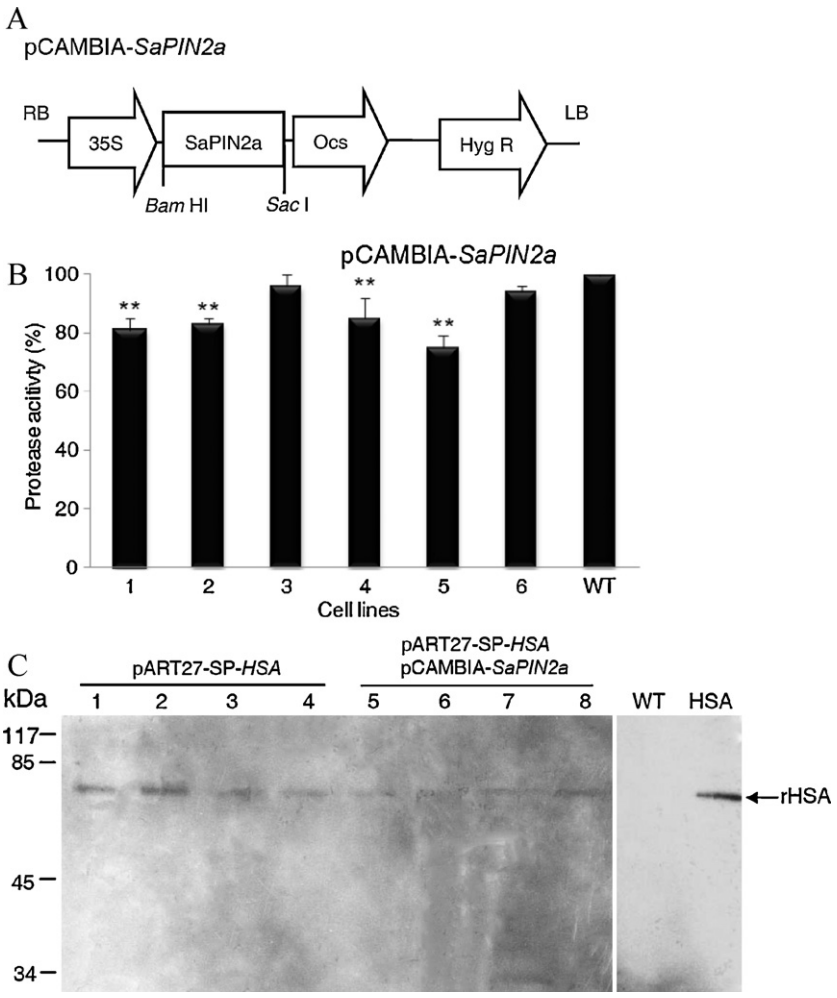

Fig. 3. Co-expression of protease inhibitor SaPIN2a and HSA in BY-2 cells. (A) Schematic representation of the construct pCAMBIA-*SaPIN2a*. *Abbreviations*: LB, left border of the T-DNA; 35S, CaMV 35S promoter; Ocs, octopine synthase terminator; Hyg, hygromycin resistance cassette; and RB, right border of the T-DNA. (B) Protease activity in suspension of BY-2 cells transformed with pCAMBIA-*SaPIN2a*. **Indicates statistically significant differences between various transgenic cell lines and WT ($p < 0.01$). (C) Western blot analysis of rHSA accumulation within BY-2 cells transformed with pART27-SP-*HSA* and pCAMBIA-*SaPIN2a*. The total secreted proteins in the culture medium were precipitated with methanol–chloroform as described previously (Fu et al., 2009). The primary antibody was rabbit anti-human serum albumin and the secondary antibody was HRP-linked with goat anti-rabbit IgG.

of BY-2 cells expressing rHSA. To determine the optimal temperature for removing host proteins, the culture medium was treated at various temperatures and the proteins analyzed by SDS-PAGE and western blot analysis using the anti-HSA antibody. The results indicated that treatment at 70 °C removed most of the host proteins (Fig. 4A). Although treatment at 60 °C resulted in a higher recovery rate of rHSA than that at 70 °C, more host proteins remained associated with the rHSA (Fig. S2). It is also worth noting that treatment at 50 °C resulted in the appearance of a new degradation product (~58 kDa) (Fig. 4A, low panel).

To improve the recovery rate of rHSA, we tested the effects of the protective agents N-acetyl-DL-tryptophan and sodium octanoate (Bartlett et al., 2008) and a buffer (100 mM Tris, pH 8.0) on rHSA stability when incubated at 70 °C for 1 h. The results showed that the addition of 10 mM N-acetyl-DL-tryptophan and 10 mM sodium octanoate to the culture medium was the best way to protect rHSA during the heat treatment (Fig. 4B and C), with a recovery of 81.65% (Table 1). Thus these components were routinely added to the culture medium prior to the heat treatment.

After centrifugation of the heat-treated culture medium, the clarified supernatant was loaded onto a Capto blue gel column (GE Healthcare). Initially we attempted to use this dye-affinity column to capture rHSA as recommended by manufacturer. However, unexpectedly this dye-affinity column captured many other proteins that were not denatured by heat treatment, whereas only a small amount of rHSA was retained by the column (Fig. 5A and B). This observation was confirmed using commercial HSA in water, 0.9% NaCl or MS medium (Fig. S3). Therefore, we used the Capto blue gel as the second step of the purification procedure to remove residual host proteins. As a third step, a DEAE blue column, which proved to be effective for absorbing HSA (Fig. S3) was used to further purify and enrich the rHSA present in the flow-through of the Capto blue gel column. After elution from the DEAE blue col-

Table 1
Purification summary for rHSA from culture medium of transgenic BY-2 cells.

Fractions[a]	Volume (ml)	Total rHSA (μg)	Recovery rate[b] (%)
CM	15	51.20 ± 1.75	100
S(H)	15	40.63 ± 0.7	81.65 ± 3.78
FT	15	25.80 ± 1.21	50.39 ± 2.55
E	5	24.78 ± 0.83	48.41 ± 4.72
H	0.4	22.62 ± 1.28	43.90 ± 2.47

[a] *Fractions*: CM, culture medium; S(H), supernatant after heating at 70 °C; FT, flow through from Capto blue column; E, elution from DEAE Blue column; and H, purified rHSA.
[b] Recovery rate (%) = (total rHSA in each step/total rHSA in CM) × 100.

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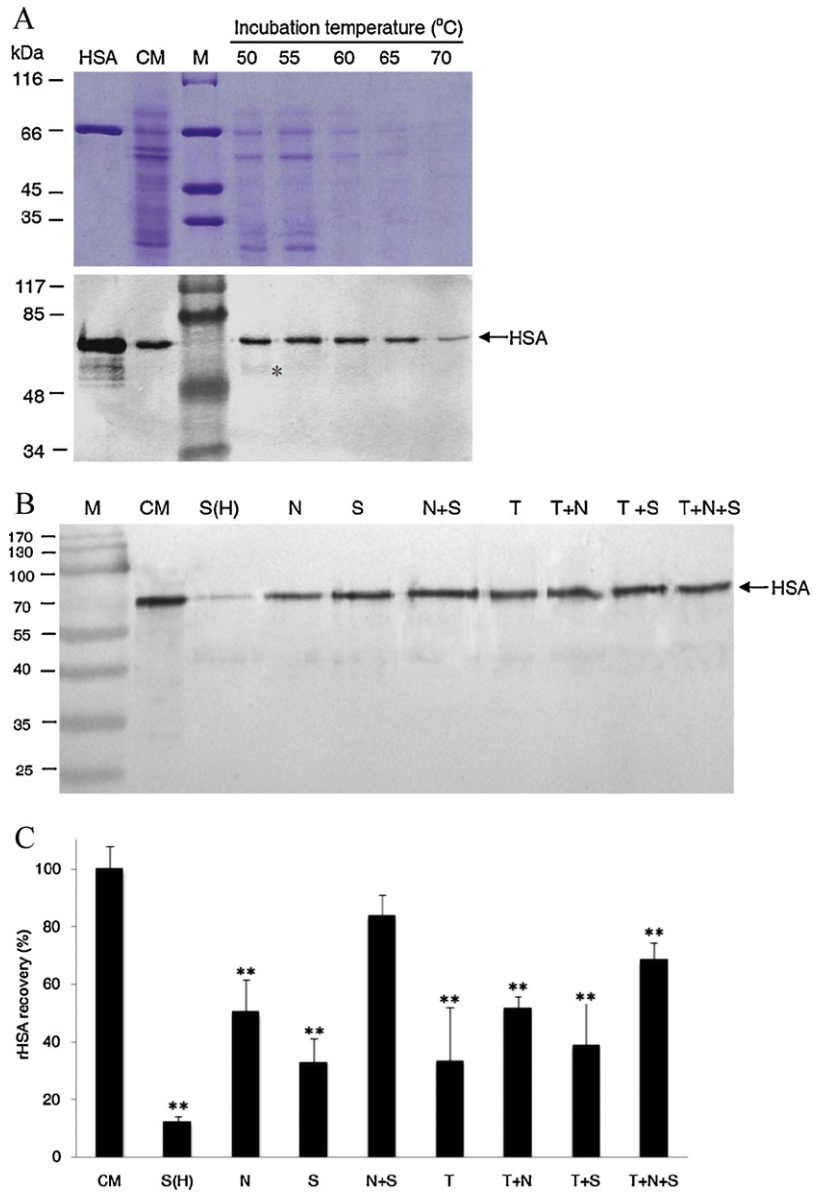

Fig. 4. Thermal stability of rHSA in culture medium of BY-2 cells transformed with pEAQ-SP-HSA. (A) SDS-PAGE and western blot analysis of the proteins that remained in the supernatant after the incubation at the indicated temperatures for 1 h. Asterisks represent the degradation fragments of rHSA. (B) Western blot analysis of the effect of adding protectants on rHSA stability during incubation at 70 °C. CM, culture medium of transgenic BY-2 cells; S(H), supernatant after heat treatment at 70 °C; N, S and T, S(H) with the addition of 10 mM N-acetyl-DL-tryptophan, 10 mM sodium octanoate and 100 mM Tris (pH 8.0) during incubation at 70 °C, respectively. Fifty microliters of each sample were analyzed. The primary antibody was rabbit anti-HSA and the secondary antibody was AP-linked goat anti-rabbit IgG. The position of rHSA is indicated with an arrow. (C) The rHSA recovery determined by ELISA. Three replicates for each sample were examined. Each value is the mean ± SD. **Indicates statistically significant differences between various treatment and control groups ($p < 0.01$). Cell line No. 4 was used for the analysis.

umn using 200 mM NaSCN (Fig. 5C), the purified rHSA sample was desalted by ultrafiltration. The purified rHSA appeared as a single band in SDS-PAGE (Fig. 5C, upper panel), whose purity was greater than 95% as determined by densitometric analysis of the Coomassie brilliant blue R-250-stained gel. Western blot analysis of the purified rHSA confirmed the specificity of the band (Fig. 5C, low panel). The purification scheme of rHSA developed in this study is summarized in Fig. 6.

3.4. Characterization of the purified rHSA from the culture medium of BY-2 cells

To further characterize the purified rHSA, we performed matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis. The results indicated that the tryptic peptide mass fingerprint of rHSA corresponds well to the calculated mass of peptides expected from HSA (Table 2, Fig. S4). The N-terminal amino acid sequence of rHSA was determined and yielded 15 amino acid residues as follows: DAHKSEVA(H)(R)FKDLG (letters in parenthesis indicate uncertain amino acids), which was identical to that expected from native mature HSA. This result showed that the junction between the SP sequence and the mature HSA was correctly recognized and cleaved, exposing the N-terminal amino acid residue of mature rHSA.

Although HSA is believed to be unglycosylated (Lawn et al., 1981), there are some reports indicating that the different post-translational modifications of exogenous proteins can occur in transgenic plants under various conditions (Faye et al., 2005; Yin et al., 2009). Therefore, the glycosylation status of the plant-derived rHSA produced in this study was examined by staining for carbohy-

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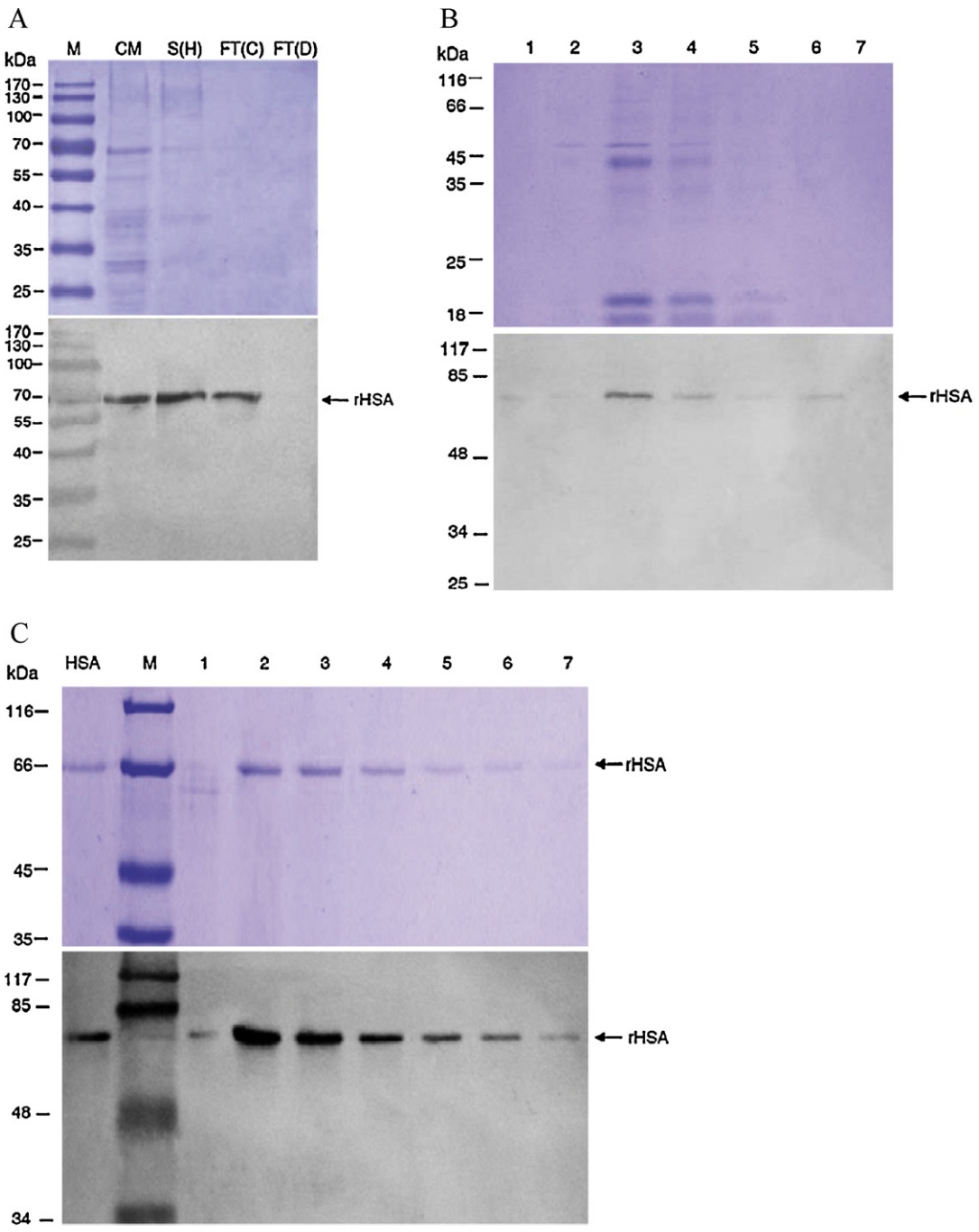

Fig. 5. Analysis of the purification steps of BY-2 expressed rHSA. (A) SDS-PAGE and western blot analysis of the proteins at different stages of the purification process. *Abbreviations*: CM, 50 μl of culture medium of transgenic BY-2 cells; S(H), 50 μl of the supernatant after the heating process; FT(C) or FT(D), 50 μl of the flow though of Capto blue gel or DEAE blue gel. (B) and (C) SDS-PAGE and western blot of the elution fractions of Capto blue gel and DEAE blue gel, respectively. Lanes 1–7 represents the number of elution fraction; lane HSA, 0.5 μg of the commercial HSA. The primary antibody was rabbit anti-HSA and the secondary antibody was AP-linked with goat anti-rabbit IgG. Cell line No. 2 was used for the analysis.

drate with periodic acid-Schiff's reagent. The staining showed that neither the HSA purified from human plasma nor the tobacco BY-2 cell-derived rHSA was glycosylated (Fig. 7).

4. Discussion

The pEAQ vector series was developed as a transient high-level expression system for use in plants (Sainsbury et al., 2009, 2010). In this study, we show that the pEAQ system can also be used for stable high-level expression of rHSA, a protein highly susceptible to proteolysis, in tobacco suspension culture cells. This contrasts with attempts to produce transgenic plants (as opposed to cells in culture) where expression of wild type P19 from the pEAQ vector was found to prevent plant regeneration, a situation which could be relieved only by the deployment of mutant form of P19 (Saxena et al., 2011). Thus the small pEAQ expression vectors (~10 kb) could serve as a viable method for the production of recombinant proteins in transgenic plant cells.

Considering the fast growth and easy transformation characteristics of the tobacco BY-2 cells, the pEAQ vector system in combination with BY-2 cells could be used for the production of numerous recombinant proteins. However, degradation of recom-

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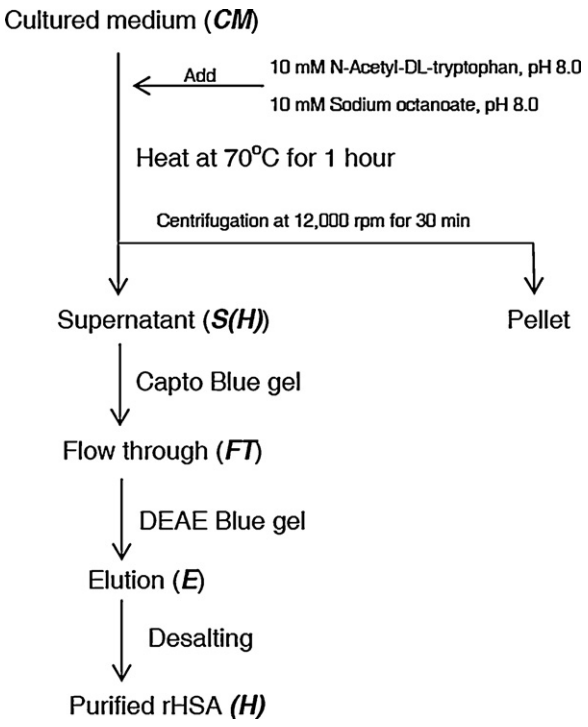

Fig. 6. Scheme of the purification steps of rHSA from the culture medium of transgenic BY-2 cells.

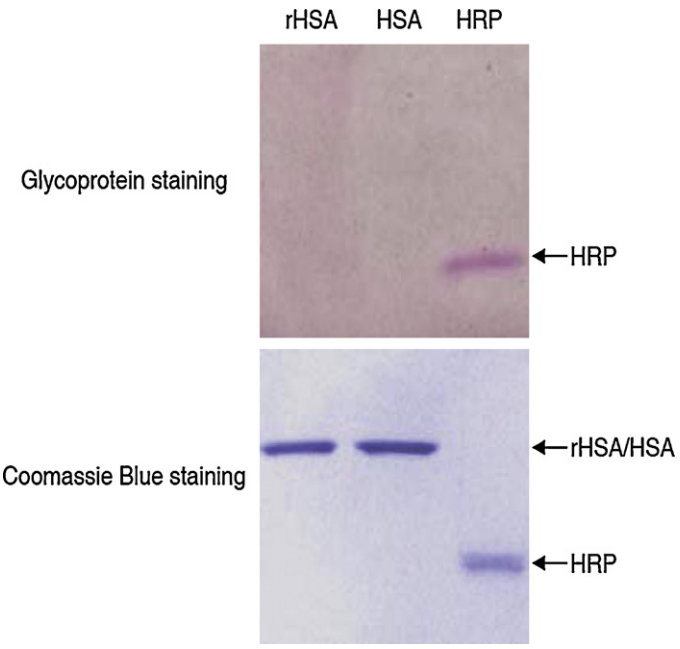

Fig. 7. Glycoprotein staining of rHSA. rHSA, 1 μg of rHSA; HSA, 1 μg of commercial HSA; and HRP, 1 μg of horseradish peroxidase as a positive control.

binant proteins occurs to a greater extent in tobacco than in other species (De Wilde et al., 2000). In addition, since HSA is a highly protease-susceptible protein (Fernandez-San Millan et al., 2003), we suspected that proteolytic stability might be a significant factor influencing its final yields. In this study, we show that rHSA is, indeed, degraded in the culture medium of BY-2 cells. Thus we tested the effects of different protease inhibitors and different pH conditions on rHSA stability in the culture medium of BY-2 cells. Adjusting the pH to either 3 or 8 and adding EDTA to 20 mM in the culture medium of BY-2 cells could significantly prevent rHSA degradation. Furthermore, cultivation of the transgenic cells in modified medium with a pH of 8.0 could improve the yield of rHSA approximately two-fold, reaching 22.1 μg/ml in the culture medium of BY-2 cells in a 25-ml flask scale.

The cost of recombinant protein production using plant bioreactor systems is mostly due to the purification process (Farran et al., 2002). In this study we fused a plant signal peptide to the mature HSA sequence to direct the secretion of rHSA to the culture medium. Directing rHSA to the culture medium greatly facilitates its detection, simplifies downstream purification, minimizes protease degradation and therefore also reduces production costs for this protein. Despite the presence of the signal peptide, measurement by ELISA revealed that 69% of the total rHSA synthesized was still located inside the cells while 31% was found in the culture medium. This result suggested that the signal peptide of barley proaleurain was not very efficient at directing secretion in this system. Identification of a better signal peptide for rHSA secretion in BY-2 cells is in progress.

In this study, a simple scheme was developed for purification of rHSA from the culture medium of BY-2 cells. We used heat treatment at 70 °C as the first step to denature most of the host proteins followed by chromatography on a Capto blue gel to remove the remaining undenatured host proteins. Subsequently, DEAE blue gel chromatography was used to capture and enrich rHSA. The final recovery was 48.41% of the secreted rHSA. The reported purification protocol can be further optimized for industrial production.

In conclusion, we have shown that rHSA, which is highly susceptible to protease degradation, can be successfully expressed and purified from the culture medium of tobacco BY-2 cells transformed with a pEAQ-based construct. Considering the fast growth rate of BY-2 cells and the ease of protein purification due to the secretion of recombinant proteins into the culture medium, the combination of the BY-2 cells and pEAQ vector system can be used as an excellent expression system for production of pharmaceutical proteins.

Table 2
Peptide mass fingerprinting analysis of rHSA by MALDI-TOF MS/MS. The MS/MS spectra of these peptides are shown in Supplementary Fig. S4.

Calculated (*m*/*z*)	Observed (*m*/*z*)	Position of amino acids	Peptide sequence
927.4934	927.5041	162–168	YLYEIAR
960.5624	960.5773	427–434	FQNALLVR
1055.5884	1055.6124	161–168	KYLYEIAR
1074.5426	1074.573	206–214	LDELRDEGK
1083.5946	1083.623	162–169	YLYEIARR
1149.615	1149.6271	66–75	LVNEVTEFAK
1311.7419	1311.7725	362–372	HPDYSVVLLLR
1358.6296	1358.663	570–581	AVMDDFAAFVEK
1467.843	1467.8804	361–372	RHPDYSVVLLLR
1511.8428	1511.8799	439–452	VPQVSTPTLVEVSR
1639.9377	1639.9767	438–452	KVPQVSTPTLVEVSR
1898.9951	1899.032	83–97	RHPYFYAPELLFFAK
1910.9316	1910.976	423–438	RPCFSALEVDETYVPK
2045.0953	2045.1343	311–327	VFDEFKPLVEEPQNLIK
2633.2883	2633.2693	383–404	RMPCAEDYLSVVLNQLCVLHEK

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2011.06.033.

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