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Expression and purification of recombinant human serum albumin from selectively terminable transgenic rice*

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Abstract: Human serum albumin (HSA) is widely utilized for medical purposes and biochemical research. Transgenic rice has proved to be an attractive bioreactor for mass production of recombinant HSA (rHSA). However, transgene spread is a major environmental and food safety concern for transgenic rice expressing proteins of medical value. This study aimed to develop a selectively terminable transgenic rice line expressing HSA in rice seeds, and a simple process for recovery and purification of rHSA for economical manufacture. An HSA expression cassette was inserted into a T-DNA vector encoding an RNA interference (RNAi) cassette suppressing the *CYP81A6* gene. This gene detoxifies the herbicide bentazon and is linked to the 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) cassette which confers glyphosate tolerance. ANX Sepharose Fast Flow (ANX FF) anion exchange chromatography coupled with Butyl Sepharose High Performance (Butyl HP) hydrophobic interaction chromatography was used to purify rHSA. A transgenic rice line, HSA-84, was obtained with stable expression of rHSA of up to 0.72% of the total dry weight of the dehusked rice seeds. This line also demonstrated high sensitivity to bentazon, and thus could be killed selectively by a spray of bentazon. A two-step chromatography purification scheme was established to purify the rHSA from rice seeds to a purity of 99% with a recovery of 62.4%. Results from mass spectrometry and N-terminus sequencing suggested that the purified rHSA was identical to natural plasma-derived HSA. This study provides an alternative strategy for large-scale production of HSA with a built-in transgene safety control mechanism.

Key words: Recombinant human serum albumin (rHSA), Selectively terminable transgenic rice, Purification
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1 Introduction

Human serum albumin (HSA) is the most widely used human plasma protein. It contains a single unglycosylated polypeptide chain of 585 amino acids (66.5 kDa) in its globular structure (Huang *et al.*, 2005; Belew *et al.*, 2008). HSA is widely used to treat severe hypoproteinemia and hyperbilirubinemia, post-surgery and post-traumatic shock, and hepatocirrhosis (Hastings and Wolf, 1992; Mendez *et al.*, 2005). Aside from its major use as a blood volume expander, HSA is also frequently used in biochemical applications, such as the formulation of protein therapeutics, cell culture media, drug delivery, cryopreservation, in vivo diagnostics, vaccine formulation and manufacturing, and infertility treatments (Hammitt *et al.*, 1991; Marth and Kleinhappl, 2001; Langer *et al.*, 2003; Cai *et al.*, 2006; Subramanian *et al.*, 2007; Kratz, 2008; Tsuchida *et al.*, 2009). Traditionally, the plasma HSA (pHSA) has been obtained from human blood, which is limited in supply and may carry a risk of viral infections, e.g., human immunodeficiency virus (HIV) and hepatitis (Erstad, 1996). As the commercial HSA market increases continuously worldwide, recombinant HSA (rHSA) offers a highly attractive way to meet current and future demand. The physicochemical and immunochemical properties of rHSA have been analyzed previously (Ohtani *et al.*,

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1998; Bosse *et al.*, 2005; He *et al.*, 2011), and found to be comparable in safety and tolerability to those of its natural counterpart, pHSA.

Aside from the traditional yeast expression system (Chuang and Otagiri, 2007), various genetically modified plants have been explored to express heterogeneous HSA, including potato (Farran *et al.*, 2002), tobacco (Dong *et al.*, 2012), and rice (Huang *et al.*, 2005). Among the different plant platforms, transgenic rice seed shows great potential for HSA expression because of its low-cost production feasibility (Huang *et al.*, 2007; He *et al.*, 2011). However, the major concern about using rice to produce rHSA is unintentional spreading of the transgene into the food chain. Although physical containment measures (isolation zones, buffer zones, fencing, etc.) have been taken to contain transgenes, incidents of transgene escape have still occurred (Fox, 2003; Vermij, 2006; Ledford, 2007). No method has yet been reported that involves using chemical traits to prevent transgenes from spreading into regular rice, and which may be applicable to an rHSA bioreactor system. Therefore, a simple and reliable containment method is highly desirable from a biosafety point of view. Since purifying recombinant proteins from plant biomass may represent up to more than 80% of the final product costs (Kusnadi *et al.*, 1997), a simplified downstream scheme is required urgently to refine rHSA for commercially acceptable production.

Based on our previous work (Lin *et al.*, 2008), we have developed a transgenic rice expression system for rHSA with a novel built-in transgene containment technology. An rHSA expression cassette is coupled with a glyphosate resistance gene and an RNA interference (RNAi) cassette rendering bentazon susceptivity. In this study, we report the creation of a transgenic line, which stably expresses high levels of rHSA in seeds and can be selectively terminated by bentazon to contain its spread. We also describe a simple and optimized scheme for rHSA purification.

2 Materials and methods

2.1 Vector construction

The HSA sequence from *Homo sapiens* (gb:CAA01491) was optimized with a rice codon bias and synthesized by Shanghai Sangon Co., Ltd., China. A corn phosphoenolpyruvate carboxylase (PEPC) terminator was added after the stop codon. An *Xba*I site was introduced at the 5′-end and a *Kpn*I site was added to the 3′-end of the synthetic gene. This synthetic gene was linked to a DNA fragment consisting of the rice glutelin Gt1 promoter and its signal peptide to target the rHSA into the rice seeds. The Gt1 promoter and the synthetic HSA gene including the terminator were digested with *Hin*dIII/*Xba*I and *Xba*I/*Kpn*I, respectively, and ligated in a three-way ligation into the pCAMBIA1300 vector backbone (CAMBIA, Australia) pre-digested with *Hin*dIII and *Kpn*I. The resulting vector was named p1300-HSA. The fragment G6-P450-RNAi, consisting of glyphosate tolerance 5-enolpyruvylshikimate-3-phosphate synthase (*EPSPS*) gene G6 (gb:EU169459) directed by the *Zea mays* polyubiquitin-1 promoter (ZmUbi) and the reverse repeat sequence for RNAi against *CYP81A6* (gb:DQ341412) (Pan *et al.*, 2006) directed by the cauliflower mosaic virus 35S promoter (CaMV35S), was released from the plasmid pG6-450i by *Kpn*I and *Xho*I digestion as described previously (Lin *et al.*, 2008). This G6-P450-RNAi cassette was linked to the plasmid p1300-HSA pre-digested with *Kpn*I and *Xho*I. The resulting binary vector for rice transformation, named pCAMBIA1300-HSA-G6-P450-RNAi, includes the HSA expression, glyphosate resistance, and RNAi cassettes (Fig. 1).

2.2 Rice transformation

The T-DNA transformation construct pCAMBIA1300-HSA-G6-P450-RNAi was introduced into *Agrobacterium tumefaciens* (LBA4404) using an electroporator (Eppendorf, Germany) according to the manufacturer's instructions. A local rice cultivar "Xiushui-110" (*Oryza sativa* L. ssp. *japonica*) was transformed using the method reported previously with minor modifications (Hiei *et al.*, 1994). Glyphosate (Sigma, USA) with a final concentration of 2 mmol/L was used as the selection agent.

2.3 Spraying of herbicides

Plants of transgenic rice line HSA-84 and untransformed rice were grown in solution in the greenhouse. Herbicide spray tests were carried out when the height of the rice seedlings reached about 20 cm. The plants were all sprayed with either bentazon or glyphosate using a handheld sprayer.

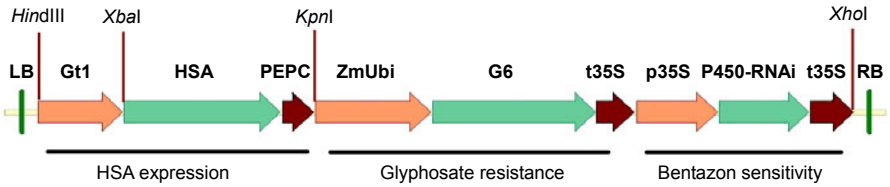

Fig. 1 Diagram of the T-DNA containing the rHSA expression cassette for rice transformation

LB and RB: left and right borders of the T-DNA, respectively; Gt1: rice glutelin Gt1 promoter; HSA: human serum albumin; PEPC: corn phosphoenolpyruvate carboxylase terminator; ZmUbi: *Zea mays* polyubiquitin-1 promoter; G6: 5-enolpyruvylshikimate-3-phosphate synthase isolated from *Pseudomonas putida* fused with chloroplast transit peptide at the N-terminus; t35S: cauliflower mosaic virus 35S terminator; p35S: cauliflower mosaic virus 35S promoter; P450-RNAi: reverse repeat sequence for RNA interference against *CYP81A6*

For the glyphosate tolerance test, 41% propylamine amine salt of glyphosate (Roundup®, Monsanto, USA) was diluted to a final concentration of 0.2% for spraying. For the bentazon susceptibility assay, a 48% bentazon solution (Basagran®, BASF, Germany) was sprayed at a concentration of 2000 mg/L.

2.4 Protein analysis and quantification

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analyses were performed using a Mini-Protean® Tetra Cell Electrophoresis System (Bio-Rad, USA) with 12% gels. The protein concentrations were measured using the Bradford (1976)'s method with 99% purity commercial pHSA (ProSpec, USA) as the standard. The absorbance of the samples at 595 nm was recorded with a VersaMax ELISA Microplate Reader (Molecular Devices, USA). The quantification of rHSA was determined using the ELISA Human Albumin Quantification Kit (Bethyl Laboratories, USA) based on the manufacturer's instructions.

2.5 Isolation and purification of rHSA from transgenic rice seeds

The powder from transgenic rice seeds was milled in 25 mmol/L phosphate buffer (PBS; pH 8.0) at a ratio of 1:5 (w/v) for 2 h to obtain a crude extract. After the addition of 10 mmol/L sodium caprylate, the crude extract was immersed in a thermostatic water bath for 30 min at 68 °C. The mixture was precipitated at pH 4.5 for 4 h at room temperature. The supernatant was adjusted to pH 8.0 before being loaded onto an ANX Sepharose Fast Flow (ANX FF; GE Healthcare, USA) column that had been equilibrated with 25 mmol/L PBS (pH 8.0) prior to sample application. The adsorbed rHSA was eluted with 25 mmol/L PBS containing 150 mmol/L NaCl (pH 8.0). The eluted peak containing the target protein was further applied to a Butyl Sepharose High Performance (Butyl HP; GE Healthcare) column equilibrated with buffer containing 25 mmol/L PBS and 1.2 mol/L ammonium sulfate, at pH 7.0. The binding targeted protein was finally eluted with buffer containing 25 mmol/L PBS and 0.5 mol/L ammonium sulfate, at pH 7.0. The rHSA fraction was desalted and concentrated by ultrafiltration using an Amicon Ultra-15 tube (Millipore, Germany). All chromatographic experiments were performed at room temperature using an AKTA explorer 100 (GE Healthcare) fast protein liquid chromatography (FPLC) system. A linear flow rate of 150 cm/h was used throughout. The final purity of rHSA was determined using a Fast Silver Stain Kit (Beyotime, China).

2.6 Molecular mass determination, N-terminus sequencing, and circular dichroism (CD) spectrum determination

To further characterize rHSA extracted from rice grain, the purified protein was subjected to matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) analysis using a Bruker AutoflexII mass spectrometer (Bruker Daltonic, USA) from Shanghai Applied Protein Technology Co., Ltd., China. The mass spectrometry data of samples were calculated using Mascot software (Matrix Science, UK) to identify proteins in the Swiss-Prot database.

For N-terminal amino acid residue determination, total soluble protein extracted from rice seeds was separated by 12% SDS-PAGE followed by electroblotting onto a polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA). The membrane was stained with coomassie brilliant blue R-250, and the band

corresponding to rHSA was excised for Edman degradation. N-terminal sequencing was carried out using an ABI491A (Applied Biosystems, USA) sequencer from Shanghai Applied Protein Technology Co., Ltd., China.

The CD spectra for rHSA and pHSA were recorded on a JASCO J-815 automatic spectropolarimeter by the Analysis and Measurement Center of Zhejiang University of Technology, China. The concentration of protein was 1 mg/ml in 50 mmol/L PBS (pH 7.4). Data were measured in the range of 190–390 nm at a scanning speed of 50 nm/min.

3 Results

3.1 Creation of transgenic rice stably expressing HSA

A total of 209 independent transformed T0 events were generated and grown in the field to harvest seeds. The total soluble protein extracted from T1 rice seeds was analyzed by SDS-PAGE for selecting transgenic lines with high rHSA expression level. A line named HSA-84 was found to be one of the highest expressing lines (Fig. 2). The transgenic rice seeds of HSA-84 displayed an opaque phenotype compared with non-transgenic control seeds. Due to the obvious visual difference, it was easy to distinguish the transgenic seeds from the regular seeds. We found that the transgenic rice seeds weighed an average of (20.4±1.2) g/1000 grains compared to (23.1±1.6) g/1000 grains for the conventional rice of the same cultivar, suggesting nearly a 10% yield penalty in the HSA-84 line. The germination rate of the transgenic rice was 92%, which was very close to the 94% of the conventional rice. The terminable transgenic rice showed no visible difference in growth and development compared to the non-transgenic control. rHSA was estimated to account for 0.72% of the total dry weight of the dehusked rice seeds. No significant difference was found among the T0–T3 seeds in rHSA expression level, indicating that the rHSA was stably expressed in different generations. Since the genetic cassettes for HSA expression, RNAi of the bentazon detoxifying enzyme, and glyphosate tolerance were all in a single T-DNA fragment, the chance of separation among these three cassettes was extremely low.

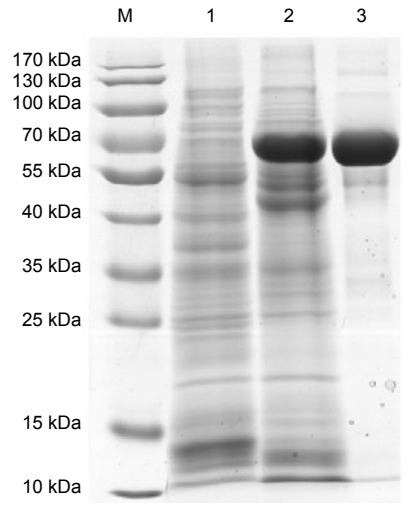

Fig. 2 SDS-PAGE analysis of rHSA in T3 seeds of transgenic rice HSA-84

M: prestained protein ladder; Lane 1: non-transgenic rice seed extract (negative control); Lane 2: seed extract of the T3 transgenic rice HSA-84; Lane 3: pHSA (positive control)

3.2 Selective termination of HSA-84 plants by herbicides

The T3 plants of HSA-84 were tested to determine their susceptibility to bentazon and glyphosate in the greenhouse. We found that one spray of bentazon at 2000 mg/L killed 100% of plants of HSA-84, but none of the regular non-transgenic rice, within 10 d (Fig. 3a). Thus, the regular dose of 2000 mg/L used for normal rice weed control is enough to kill the transgenic rice HSA-84. In contrast, the spraying of 20 mmol/L glyphosate killed all of the conventional rice plants in 10 d but did not affect any transgenic plants, as expected (Fig. 3b). These tests clearly demonstrated that the transgenic event HSA-84 was highly sensitive to bentazon but tolerant to glyphosate, while the conventional rice plants showed the opposite responses. Thus, the termination of the transgenic rice plants could be highly feasible. Moreover, similar results were observed after further tests on T4 and T5 plants, suggesting that the sensitivity to the two herbicides was stably inherited in transgenic line HSA-84. However, more generations of transgenic plants still need to be monitored as the long-term heritability of the RNAi has not yet been well studied.

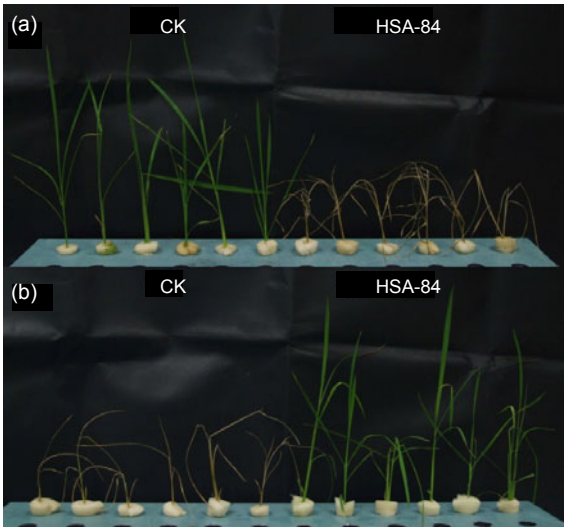

Fig. 3 Susceptibility to bentazon and glyphosate of the transgenic rice HSA-84

The T3 transgenic rice line HSA-84 along with untransformed rice (CK) was cultured in a greenhouse and sprayed with 2000 mg/L bentazon (a) or 20 mmol/L glyphosate (b). The pictures were taken 10 d after spraying

3.3 rHSA purification scheme

A purification scheme comprising crude extract pretreatment and two chromatography steps was developed in this study (Fig. 4). Since HSA has a high thermal tolerance (Sumi *et al.*, 1999; Belew *et al.*, 2008) but high protease-sensitivity (Fernández-San Millán *et al.*, 2003), thermal treatment of crude protein extracts was performed to inactivate proteases and denature some rice seed storage proteins. Initially, we attempted to use Capto MMC (Belew *et al.*, 2008) to capture rHSA. Unexpectedly, we found precipitation at pH 4.5 could significantly remove a large quantity of other proteins (Table 1). Based on this finding, a precipitation procedure, rather than a chromatography step, was used for the initial step in the purification scheme. In the presence of 10 mmol/L sodium caprylate, rHSA was protected from degradation during the heating and precipitation processes with a recovery rate of 90.5% (Table 1). Accordingly, this stabilizer should be dissolved into the crude extract prior to heat treatment. Note that crude extracts with high turbidity could be clarified after heating and precipitating, and thus, centrifugation is not necessary before the chromatography step.

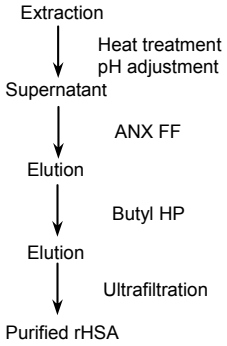

Fig. 4 Scheme showing the steps involved in the purification of rHSA from the transgenic rice seeds

Table 1 Purification summary of rHSA produced by transgenic rice

Purification step	rHSA weight (mg)	Purity (%)	Recovery (%)
Initial extraction	288±19	29.3±3.2	100
Heat treatment & pH adjustment	259±35	59.6±1.9	90.5±3.2
ANX FF	224±24	87.7±2.3	85.8±2.6
Butyl HP	180±36	>99*	80.4±3.9
Final preparation	179±6	>99*	62.4±3.7

Recovery=(rHSA weight in each step/rHSA weight in initial extraction)×100%; Purity=(rHSA weight/TSP weight in the same step)×100%, where TSP is total soluble protein. * Purity of rHSA was determined by SDS-PAGE (silver staining). Values are expressed as mean±standard deviation (SD), n=3

A capture purification step was started with ANX FF, which proved to be effective for rHSA concentration and partial purification. This step enriched rHSA to 85.8% (Table 1), whereas the major low molecular weight proteins in the supernatant were removed in the flow through fraction (Fig. 5). As a polishing step, the Butyl HP column was used to facilitate the removal of the residual non-HSA proteins. The rHSA eluted from Butyl HP column appeared as a single band of pure protein in SDS-PAGE (Fig. 5).

The total recovery of rHSA from triple replications was (62.4±3.7)% (Table 1), indicating that the complete purification process is reproducible. SDS-PAGE silver-staining analysis demonstrated that the final purity of rHSA was comparable to that of a pHSA (>99%) control. Together, these results suggest that the rHSA was successfully purified by the two steps of chromatographic purification.

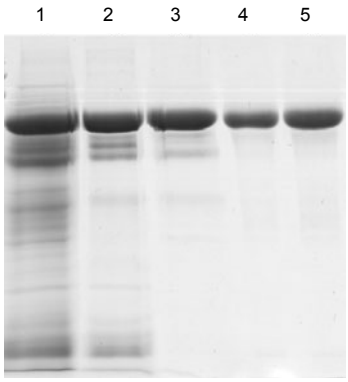

Fig. 5 SDS-PAGE analysis of the main fractions after different stages of the rHSA purification process
Lane 1: crude extract; Lane 2: supernatant after heating and pH adjustment; Lane 3: eluted from ANX FF column; Lane 4: eluted from the Butyl HP column; Lane 5: pHSA

3.4 Characterization of the purified rHSA

Mass spectrometry analysis, N-terminus sequencing, and determination of the CD spectrum were performed to confirm the similarity of rHSA to the natural protein. The *m*/*z* (mass/charge) value showed that the rHSA had an equivalent molecular weight of 66441 Da (Fig. 6), which is very close to the 66531 Da of its natural counterpart from the UniProtKB database. Ten N-terminal amino acid residues were determined as follows: DAHKSEVAHR. This result revealed that the cleavage of the signal peptide sequence was correct and that the mature rHSA was identical to the pHSA in the primary structure. The correct secondary structure is necessary for the functional activity of rHSA. The CD spectrum analysis further confirmed that the recombinant protein had the same conformation as the natural analogue (Fig. 7).

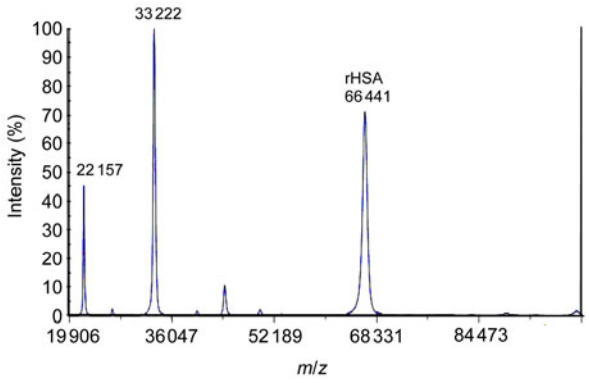

Fig. 6 Molecular weight determination of rHSA by MALDI-TOF

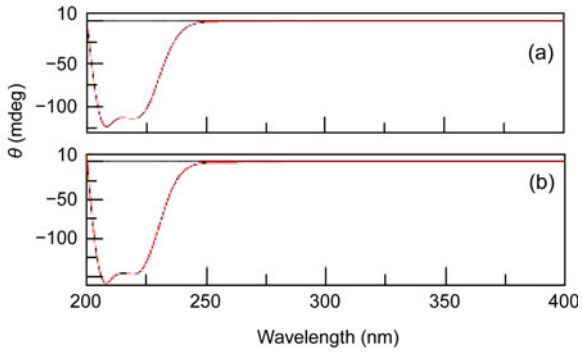

Fig. 7 CD spectra of pHSA (a) and rHSA (b)

4 Discussion

While the technology for producing rHSA using transgenic rice has been well developed, the issue of a transgene containment strategy has not been addressed for transgenic rice for molecular farming. This is a major concern for large scale planting of such transgenic rice in open fields. We have developed a transgenic line for high expression of rHSA with a built-in spread control technology. Such transgenic rice plants could be killed by bentazon during regular weed control if they spread accidently into regular rice. The technology could be used as a preventive scheme to exclude any possibly contaminated transgenic rice expression of rHSA. When a certain area of rice is suspected of being contaminated, we can ensure removal of any rHSA contaminant simply by using bentazon as the herbicide for weed control. The built-in containment strategy illustrated in this study makes the detection and selective termination of the transgenic plants inexpensive and convenient.

Rice-derived rHSA does not require sophisticated equipment, unlike traditional yeast fermentation, and could be scaled up simply by planting a larger acreage of transgenic rice. Rice seeds also offer a low hydrolytic condition for storage of rHSA, which can overcome the limitation of protein stability within the microbial expression system. The concentration of rHSA in T3 transgenic rice seeds was shown to account for 0.72% of dry weight of the dehusked rice seeds in our assays, which is much higher than the estimated cost-effective threshold (0.1 g/kg) for commercial production of rHSA in plants (Farran *et al.*, 2002). Another object of this study was to develop

a good manufacturing practice (GMP)-compliant scheme for isolating rHSA of high purity. The total recovery of our purification process was 62.4% and the final product reached 99% purity, which is likely to be pure enough for use as a cell culture component. The chromatography combination (ANX FF and Butyl HP) designed in this study significantly shortened the purification period and reduced the operational cost. We believe that the simple purification protocol could be further optimized for pilot- or production-scale operations.

We have proved that the rHSA produced by the transgenic rice reported here is identical to native protein from blood in terms of its N-terminal amino acid sequence, molecular mass as measured by MALDI-TOF, and secondary structure as shown by its CD spectrum. Previous research has shown that rHSA is identical to native protein in biological activity (Kobayashi, 2006). Therefore, we expect that the rHSA protein produced by the transgenic rice here is also biologically identical to its native protein.

In conclusion, we have developed a commercially feasible transgenic rice expression system for rHSA with a built-in containment technology. Unlike non-transgenic rice, such transgenic rice with high glyphosate resistance is extremely sensitive to bentazon, and thus can be selectively killed by bentazon. We also designed a simple purification scheme with high efficiency and high yield, which can purify the rHSA to 99% purity.

Compliance with ethics guidelines

Qing ZHANG, Hui YU, Feng-zhen ZHANG, and Zhi-cheng SHEN declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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