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Original article

Expression of human serum albumin in *Nicotiana benthamiana*

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Abstract

***Backgrounds**:* Human serum albumin (HSA), which is mostly produced from human plasma, is limited by supply limits and safety issues related to potential contamination with blood-borne infections. Thus, choosing a suitable expression system for recombinant HSA synthesis becomes essential for addressing clinical and cell culture application requirements.

***Objective**:* This study explored the use of *Nicotiana benthamiana* as an alternative platform for producing recombinant HSA.

***Methods**:* The *N. benthamiana* plants as an expression platform were used for transient expression of HSA. The agrobacterium solution containing HSA expression vector was infiltrated into the leaves. A single step Ni-NTA affinity chromatography method was used to purify the plant-produced HSA. The liquid chromatography-mass spectrometry (LC/MS) was used to verify the correctness of plant-produced HSA.

***Results**:* We successfully expressed and purified HSA from the leaf of infiltrated *N. benthamiana*. The final HSA yield was estimated to be around 30 mg HSA/kg of *N. benthamiana* lea fresh weight. We identified the intact mass and peptide mapping of plant-produced HSA, the results suggested to be around 69.07 kDa and a total of 93.52% of the HSA sequence was found

***Conclusion**:* These results suggest a promising beginning for the advancement of plant-based HSA in the area of plant molecular farming. Further application needs to be investigated.

Keywords: Human serum albumin, *Nicotiana benthamiana*, plant molecular farming, Solanaceae.

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Human serum albumin (HSA) is a 66.4-kDa multi-domain protein comprising 585 amino acids.[1] The liver produces this plasma protein, which is present in massive quantities in human blood plasma. Up to 50 g/L of HSA can be found in the blood.[2] It is utilized in a diverse array of applications, including as a versatile carrier of ligands such as fusion peptides, drugs, fatty acids, metals, and several bioactive molecules.[3] Additionally, HSA is often used to extend the half-life of therapeutic proteins and drugs, improving their stability and efficacy in the bloodstream.[4, 5] The HSA production is critical for maintaining its supply, which is traditionally derived from blood serum. Recombinant HSA can now produced commercially in a range of protein expression platforms. Thanks to the help of biotechnology advancements. The prokaryotic system provides simple and cost-effective production while lacking proper folding and post-translational modifications (PTMs). The expression platform like plants can perform PTMs, which are essential to their biological function. Although the glycosylation patterns were different among the eukaryotes, these problems can be resolved through glycoengineering techniques.[6] Aside from that, plants reduce the possibility of cross-contamination, which can occur in animal or bacterial cell cultures.[7] Comparing animal and microorganism expression systems, plants offer several distinct advantages. The biosynthetic pathways in plants are largely similar to those in animal cells, although differences exist in the glycosylation process. Additionally, plants can be cultivated on a large scale and are easy to manage. They require minimal input primarily water, soil, and sunlight which makes them a cost-effective platform. Due to these factors, molecular farming in plants is considered an economically viable alternative for the production of recombinant proteins.[8] In 1990s. For the first time, therapeutic proteins have been successfully produced from tobacco and potatoes as the expression model. Several years later, in the mid-1990s, the transgenic plants were introduced.[9] Gastric lipase is one of the first proteins made from transgenic plants that is used to treat cystic fibrosis.[10] The *Nicotiana* species's ability quick growth rate and ease of genetic engineering make them popular in the molecular farming sector. The majority of plant-produced biopharmaceutical products on the market, including growth regulators, hormones, cytokines, vaccines, and medications, are made from tobacco. For the transient and stable expression of recombinant proteins, *N. benthamiana* and *N. tabacum* are frequently used. The plant model including fruits and vegetables like rice seeds, maize, tomato leaves, lettuce, potatoes, etc. was used to express several biologics and recombinant proteins.[6] It has been shown that tobacco plants, especially *N. benthamiana*, can produce pharmaceutically significant proteins in both stable and transient environments. Notably, *N. benthamiana* can generate a range of therapeutic proteins and exhibit high gene expression within the T-DNA plasmid borders.

This system is ideal for rapid prototyping, emergency response in case of biopharmaceutical produce during global pandemics, it enables high protein yields within 5–7 days post-infiltration and does not require stable transformation. Moreover, the availability of deconstructed viral vectors and glycoengineered *N. benthamiana* lines further enhances its capacity for producing complex therapeutic proteins with human-compatible glycosylation.[11] Although it has been shown that rice and stable tobacco systems can produce the recombinant protein at decent yields, their scalability demands extended development times, strict regulatory oversight, and substantial infrastructure.[12] Likewise, it takes months for stable transgenic tobacco lines to develop and validate before they can be produced on a large scale.[13] In this research, we aim to produce the HSA in wild-type *N. benthamiana* plants through agroinfiltration and characterize this plant-derived protein.

Materials and methods

HSA expression vector construction

The nucleotide sequence of HSA (GenBank accession number: AAA98797.1) was *in silico* optimized for suitable expression in *N. benthamiana*. An IEGRMD polypeptide linker was used to attach a Polyhistidine tag that had been fused at the C-terminus end. The commercially synthesized HSA gene (Genewiz, China) was digested with *Xba*I and *Sac*I before ligating with pBYR2e geminiviral vector. The heat shock method was used to transform pBYR2e-HSA-His into *Escherichia coli* DH10B competent cells. The transformants were screened by colony PCR. Positive clones were inoculated with supplemented 50 mg/L of kanamycin (Thermo Fischer Scientific, USA) into Luria-Bertani (LB) broth and shaken at 250 rpm at 37°C overnight. The DNA-spin Plasmid DNA Purification Kit (iNtRON Biotechnology, Korea) was used to extract the plasmids.

Agroinfiltration of pBYR2e-HSA-His

Agrobacterium tumefaciens GV3101 were transformed with pBYR2e-HSA-His via electroporation (MicroPulser, Bio-Rad, USA). Colonies were selected and verified by colony PCR. Positive transformants were cultured in LB broth supplemented with 50 mg/L of kanamycin, gentamicin, and rifampicin overnight at 28°C while constantly shaking (250 rpm). The *Agrobacterium* harboring pBYR2e-HSA-His vector was pelleted (14,000 rpm, 5 min, 4°C) and resuspended in an infiltration buffer (10 mM MES and 10 mM MgSO4, pH 5.5) at a final OD_{600} of 0.2.

Approximately 4 weeks old *N. benthamiana* were grown in a plant room at 28°C with long-day conditions (16 h light/8 h dark cycle). *Agrobacterium*-mediated transformation was performed via syringe without needle in the small-scale expression and vacuum infiltration in the large-scale expression.

The infiltrated leaves were blended in extraction buffer (10 mM Imidazole, 20 mM Tris-HCl buffer pH 7.4, 50 mM NaCl) at 1:2 (w/v). The crude extract was clarified by centrifugation (14,000 rpm, 50 min, 4°C) and 0.45 µm paper filter (Merck, Massachusetts, USA) to eliminate cell debris. The clarified crude was purified by affinity chromatography using Ni-NTA resin (Cytiva, USA). The column was washed with a buffer containing various concentrations of imidazole from 10 mM to 30 mM. The trapped HSA-His was eluted using an elution buffer with 250 mM Imidazole, 20 mM Tris-HCl pH 7.4, and 50 mM NaCl. The purified HSA was desalted and dialyzed with 1X PBS before proceeding with further experiments.

Intact mass analysis by LC/MS

The plant-produced HSA was characterized using liquid chromatography-mass spectrometry (LC-MS) with an Agilent 1290 Infinity II LC system and an Agilent 6545XT Q-TOF mass spectrometer. The plant-produced HSA was desalted and buffer exchanged using a Bio-Gel P6 Micro Bio-Spin column (Bio-Rad, USA). The column was centrifuged for ten minutes at 14,000 rpm. The supernatant was transferred to the vial. 3 uL of desalted pure HSA was added to the Agilent PLRP-S separation column (1000 Å, 2.1 × 50 mm, 5 µm). Mobile phase A included 0.1% formic acid (v/v) in water, while mobile phase B contained 0.1% formic acid (v/v) in acetonitrile.

Results

HSA-His expression and purification

The 21-day-old *N. benthamiana* plants were used for transient expression. The leaves were collected 4 days post-infiltration. The Ni-NTA column chromatography was used for HSA purification from infiltrated leaves. The highest yield of 30 mg of HSA per kilogram of *N. benthamiana* fresh weight was achieved.

After Agroinfiltration, tobacco leaves exhibited necrosis at 4 dpi (**Figure 1A**). This indicates that the HSA was expressed and accumulated in the plant leaves. According to Figure 1A, the HSA protein band in SDS-PAGE (**Figure 1B**) and western blot (**Figure 1C**) appeared in the elution lane. These findings show that HSA was eluted from the column after being trapped in the Ni-NTA resin. Western blot and SDS-PAGE show the HSA estimated size of about 70 kDa.

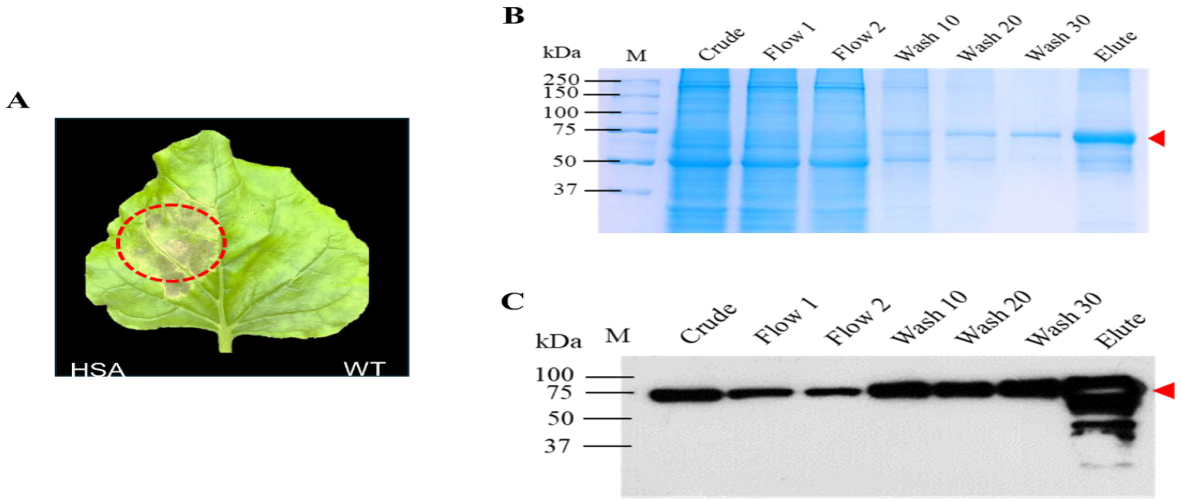

Figure 1. The phenotype of *N. benthamiana* leaf after infiltration (**A**) SDS-PAGE (**B**) and Western blot (**C**) of plant-produced HSA through purification steps. Anti-His-HRP was used to detect the immunoblots of HSA-His protein.

The results indicated an increasing purity after washing the column. SDS-PAGE shows that higher protein purity results from washing the column with a higher imidazole concentration. We can observe that the impurity band reduced after every wash. However, according to the western blot result, throughout the process, HSA is also eliminated along with the protein impurities, which reduces the production yield even though the proteins can be purified by the column.

LC/MS analysis

The plant-produced HSA was further analyzed by LC-MS for intact mass and peptide mapping. The theoretical molecular size was determined using the amino acid sequence compared with BioConfirm Software. Based on the results, the exact mass of the plant-purified HSA was found to be 69 kDa, consistent with the calculated mass (**Figure 2**). Furthermore, peptide mapping confirmed 93.25% amino acid sequence coverage, suggesting a high degree of similarity between the recombinant HSA and native HSA. This extensive sequence coverage confirms that the plant-produced HSA closely matches plasma-derived HSA in the primary structure.

Discussion

HSA is the most abundant protein in human blood plasma, playing a critical role in maintaining blood volume and transporting various substances throughout the body. Given the wide range of applications for HSA, plasma-derived HSA remains insufficient to satisfy demands to treat a variety of illnesses, including liver cirrhosis[14], hypoalbuminemia[15], etc. Using plants as green bioreactors provides cost-effective options by lowering process complexity, and contamination hazards, and allowing large-scale production, particularly through transient expression.[16] Plant expression platform is proven to be effective for the variety production of biopharmaceutical products including subunit vaccine[17] or monoclonal antibodies such as pembrolizumab[18-20] and trastuzumab.[21]

A final yield of 30 mg of HSA per kilogram of *N. benthamiana* fresh weight was obtained. In comparison with other studies on HSA expression, the use of *N. benthamiana* may not achieve the bacterial expression systems (18.92 mg/L of bacterial culture).[22] To compare the expression of HSA in another platform, using the yeast *P. pastoris* showed a high level of protein expression under induced

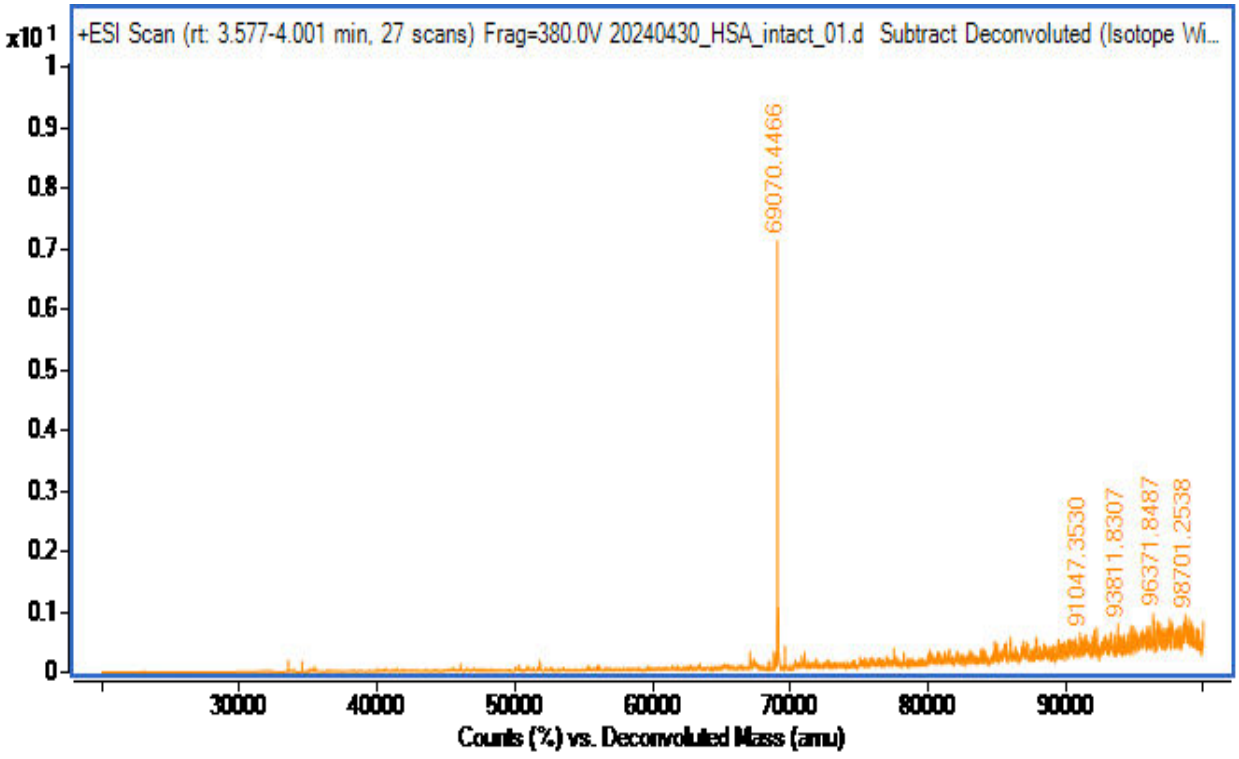

Figure 2. The Characterization of plant-produced HSA by LC-MS: Intact mass analysis of plant-produced HSA.

methanol promoter and the fermentation conditions.[23] Investigating of *Pichia pastoris* to the express rHSA, the final yield can reach up to 1.4 g/L in bioreactor.[24, 25] In contrast, using the stable expression system in rice seeds, the previous study reported that the yield of rHSA can reach 2.75 g/kg in rice seeds. A plant like a potato was also investigated for its ability to express rHSA.[26] However, the final yield is lower than the stable expression in rice seed. Furthermore, the variable depends on the expression cassette, subcellular targeting, and transformation efficiency.[27]

However, the plant transient expression system offers significant advantages in terms of speed and scalability, which could be beneficial for rapid production needs in plants. Previous studies have suggested other methods to enhance protein yield including genetic engineering, improved expression vectors, and co-infiltration of inhibitors of protease.[28, 29] In the previous study, Phelipe and team reported the increased yield of Monoclonal antibody (mAb) expressed in *N. benthamiana* by Co-express cysteine protease inhibitor.[30] There is a study on screening on several protease inhibitors which can be expressed along with the target protein which was reported by Grosse-Holz and colleagues.[31] To address the limitations of protein yield, this method could be promising for the near future works.

The plant-produced HSA was detected as a single band around 70 kDa, aligning with an earlier study that produced recombinant HSA in *P. pastoris* expression system.[32] Nevertheless, size exclusion chromatography should be performed to further compare the purity of the plant-produced HSA. The exact mass of purified HSA protein was determined to be 69.07 kDa, slightly larger than the theoretical molecular weight of 68.77 kDa based on its amino acid sequence. This mass shift from the predicted size may result from post-translational modifications in plants, such as phosphorylation, acetylation, sumoylation, etc.[33] which we have not confirmed. Zhang and colleagues reported the molecular weight of HSA produced from transgenic rice to be around 66.4 kDa.[34] In this case, variations are potentially affected by different post-translational modifications among plant species. Even so, peptide mapping analysis of the plant-purified HSA demonstrated a high amino acid sequence coverage of 93.25%, confirming the protein's primary structure and its similarity to native HSA. Even though, the SDS-PAGE analysis and LC/MS could confirm the success of the expression of HSA in *N. benthamiana*. There are some limitations to be addressed. The size exclusion chromatography could be additionally conducted to precisely confirm the purity of the single-step purification of HSA.[35] Also, with the dynamic light scattering (DLS), DLS could help to confirme the molecular weight of plant-produced HSA, this will be stronger evidence on the integrity of produced protein as reported by Lorber.[36] When combined, these techniques will improve our capability to properly confirm the monomeric purity of HSA and identify any aggregation that might interfere with experimental results.

Conclusion

The successful production and characterization of plant-derived HSA demonstrate the potential of plant expression platforms as cost-effective and efficient protein manufacturers. This proof of concept study confirmed the capability of *N. benthamiana* to produce HSA. Further investigation is needed to exploit its large-scale production and potential applications in medical and pharmaceutical industries.

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Conflict of interest statement

Waranyoo Phoolcharoen is a co-founder and shareholder of Baiya Phytopharm Co., Ltd. Utapin Ngaokrajang, Pipob Suwanchaikasem, Kaewta Rattanapisit, and Christine Joy I. Bulaon are employees of Baiya Phytopharm Co., Ltd. The remaining authors declare no conflicts of interest to report.

Data sharing statement

All data presented in the study are included in the article. Other inquiries can be made available on reasonable requests to the corresponding author/s.

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