

Expression of human serum albumin in *Pichia pastoris* protease-deficient host and conjugation with gadolinium-diethylenetriamine pentaacetate for application as a contrast agent

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ABSTRACT

Human serum albumin (HSA) is a plasma protein with a molecular weight of 66.5 kDa, which is most widely used in the pharmaceutical and clinical fields. One application of HSA in a drug delivery system is to increase the relaxation time on magnetic resonance imaging. The combination of contrast agent, gadolinium-diethylenetriamine pentaacetate (Gd-DTPA), with HSA can produce better imaging. This study aims to express the recombinant HSA (rHSA) in the *Pichia pastoris* protease-deficient host, SMD1168, purification using chromatographic techniques and conjugation with Gd-DTPA. Optimization of rHSA expression by *P. pastoris* was done by varying the methanol inducer, 0.75%, 1.0%, 1.25%, and 1.5%, as well as characterization by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, the rHSA was purified using gel filtration, characterization by Size Exclusion Chromatography- High Performance Liquid Chromatography (SEC-HPLC), and conjugation with Gd-DTPA. The rHSA (~66.5 kDa) was characterized from broth medium of *P. pastoris* SMD1168. The optimum conditions for rHSA expression were 1.5% methanol at 48 hours incubation, which resulted in 0.44 g/l of rHSA. The rHSA with 81.84% purity was obtained by gel filtration, and conjugation with Gd-DTPA produced high-purity Gd-DTPA-rHSA, demonstrated by a single peak ($t_r = 10.493$ minutes). The protease-deficient *P. pastoris* was successfully used to express intact rHSA and the rHSA was successfully conjugated to Gd-DTPA with high purity.

INTRODUCTION

Human serum albumin (HSA) is a globular protein with a molecular weight of 66.5 kDa, which consists of 585 amino acids and 17 disulfide bonds in a single polypeptide. HSA is a primary protein found in blood plasma. It functions as a carrier for endogenous and exogenous hydrophobic molecules such as fatty acids, bilirubin, and hormones. Furthermore, it also acts as a flexible carrier protein for small lipophilic drug molecules (Charter and Ho, 1994; Raoufinia *et al.*, 2016). HSA can act as a surfactant

and maintain active protein conformation during storage (Mead *et al.*, 2007; Shahin *et al.*, 2020; Taguchi *et al.*, 2012).

The application of albumin in therapy and diagnosis has developed into nanosized drug carriers over the last few years. Albumin has been used as a nanodelivery drug for insulin molecules, tumor drugs, cancer therapy, rheumatoid arthritis, and infectious diseases (Chai *et al.*, 2006; Elsadek and Kratz, 2012; Karimi *et al.*, 2016; Kratz, 2008; Pilati and Howard, 2020). One technique for early detection of cancer is the magnetic resonance imaging (MRI) tool. Detection using MRI usually uses gadolinium-diethylenetriamine pentaacetate (Gd-DTPA) contrast agents to obtain a cross-section of the body using magnetic fields (Meade, 2004; Na *et al.*, 2009; Pierre *et al.*, 2014; Zou and Lu, 2013). HSA reversibly can bind to Gd-DTPA, resulting in significantly higher relaxivity and intravascular retention than the existing contrast agent used in magnetic resonance (Wu *et al.*, 2007; Goyen, 2008; Zhen *et al.*, 2012). The HSA-Gd complex has

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been widely studied. One of the contrast agents containing HSA, gadofosveset, is approved for clinical use (Caravan *et al.*, 2002).

Conventional production of plasma HSA (pHSA) has been done through fractionation of human plasma, which is limited by the availability of blood donations and may carry a risk of viral infections, e.g., hepatitis and human immunodeficiency virus; however, pHSA manufacturing has increased rapidly in recent years (Caravan *et al.*, 2002; Raoufinia *et al.*, 2016; Zhen *et al.*, 2012). The issue of availability of blood donations and the safety of using pHSA underline the importance of producing recombinant HSA (rHSA) to replace pHSA (Erstad, 1996; MacLennan and Barbara, 2006). rHSA is an alternative to obtain authentic HSA in large quantities (Chen *et al.*, 2013; Mead *et al.*, 2007; Nguyen *et al.*, 2020). rHSA has been expressed successfully in various host expression systems and appears to be indistinguishable from pHSA. The physicochemical and immunochemical properties of rHSA demonstrate the same properties as pHSA (Chen *et al.*, 2013; Ohnishi *et al.*, 2008; Ohtani *et al.*, 1998). rHSA has been expressed using the *Escherichia coli* expression system (Latta *et al.*, 1987; Lawn *et al.*, 1981; Nguyen *et al.*, 2020; Sharma *et al.*, 2017), *Saccharomyces cerevisiae* (Kalman *et al.*, 1990; Okabayashi *et al.*, 1991; Sleep *et al.*, 1990), *Pichia pastoris* (Belew *et al.*, 2008; Kobayashi *et al.*, 2000; Prevatt and Sreekrishna, 1994; Zhu *et al.*, 2018), and transgenic plants or animals (He *et al.*, 2011; Moghaddassi *et al.*, 2014; Zhang *et al.*, 2013). Yeast appears to be an efficient host to produce rHSA with the same property as pHSA proven in preclinical and clinical trials (Kobayashi, 2006). Meanwhile, rHSA produced in a rice seed transgenic plant demonstrates great potency for more cost-effective production (Chen *et al.*, 2013).

The expression of rHSA in *E. coli* produced an insoluble fraction, which may be due to the high molecular weight of HSA and the lack of a protein folding factor in *E. coli* to facilitate folding of HSA, which has many disulfide bonds; therefore, it requires a time-consuming and expensive renaturation process (Latta *et al.*, 1987; Lawn *et al.*, 1981). However, recently Nguyen *et al.* (2020) showed that the *E. coli* Origami host significantly improved the expression and solubility of rHSA fused to the maltose-binding protein tag. Around 9.46 mg rHSA with 97% purity was obtained from a 500 ml cell culture. Meanwhile, the expression of rHSA in *S. cerevisiae* hosts results in a low expression level, possibly due to the considerable molecular weight of rHSA. Some of the rHSA is retained in the periplasmic cavity (Okabayashi *et al.*, 1991). The *P. pastoris* expression systems seem to be most suitable for the expression of rHSA that has 17 disulfide bonds, which require posttranslational modification (Zhu *et al.*, 2018).

Pichia pastoris demonstrates a remarkably attractive host for HSA production due to several benefits of this expression system. *Pichia pastoris* is a widely used host for recombinant protein expression due to the fact that it is easy to manipulate, its genetic structure is stable, it has a high cell density when growing in an inexpensive basal salt medium, it has strong inducible alcohol oxidase 1 (*AOX1*) promoter, it has the ability to perform posttranslational modification, and it has a high capacity of secretory expression and low endogenous protein secretion (Ahmad *et al.*, 2014; Baghban *et al.*, 2018; Gaffar *et al.*, 2019). Recently, Zhu *et al.* (2018) have reported the production of rHSA

in *P. pastoris* GS115 hosts in a shake flask and fermenter culture yielded 1.6 and 8.86 g/l, respectively.

Secreted expression of rHSA by either *P. pastoris* or *S. cerevisiae* has always resulted in proteolytic degradation of rHSA, particularly in continuous cultures with periodic feeding (Kang *et al.*, 2000; Kobayashi *et al.*, 2000). Although manipulations of medium were undertaken to control the protease's expression, the protease activity still existed in the culture broth. During fermentation, the leakage of the protease can occur in lysed cells. The protein degradation was reported caused by an endogenous acidic protease that acts at an acidic pH. Kobayashi *et al.* (2000) observed a degradation rate of rHSA equal to 660 mg of rHSA/l/hour at pH 4.3, which caused a significant decrease in rHSA production in yeast. Another strategy to reduce HSA degradation has been achieved by interrupting the gene encoding with an aspartyl protease [Yeast Aspartic Protease3 (YAP3)]. However, the suspicion of proteases that cause such degradation of rHSA in yeast is still unclear (Kerry-Williams *et al.*, 1998).

In this study, we report the production of rHSA in *P. pastoris* protease-deficient SMD1168 extracellularly using the α -factor signal peptide. rHSA production using this host has never been reported previously. SMD1168 is a host that lacks vacuole peptidase A (pep4) which is responsible for the activation of carboxypeptidase Y and protease B1 (Gleeson *et al.*, 1998). Protease-deficient *P. pastoris* is an alternative host to avoid the effects of proteolysis of target proteins by the host system. Protease-deficient strains have been shown to be effective in reducing the degradation of several foreign proteins, particularly in secreted recombinant protein in fermenter cultures, because of the combination of high cell density and lysis of a small number of cells results in relatively high concentrations of vacuolar proteases. However, these protease-deficient cells were not as strong as the wild-type strain with respect to PEP4. These strains have lower viability and slower growth rates, are more difficult to transform, and are more difficult to handle in shake flasks and fermenter cultures (White *et al.*, 1994). The rHSA produced furthermore was conjugated to Gd-DTPA which can later be applied as a contrast agent. This study will provide information on the expression level of HSA in the *P. pastoris* protease-deficient host and its conjugation with Gd-DTPA that has not been previously reported.

MATERIALS AND METHODS

Strains, plasmids, and growth medium

Escherichia coli TOP10F' was used for DNA manipulation, cloning, and sequencing procedures. *Pichia pastoris* SMD1168 [Histidinol dehydrogenase4 (*his4*), *pep4*] was purchased from Invitrogen Co. (Carlsbad, Germany). Plasmid pD912 was obtained from DNA2.0 (Hong Kong). The *E. coli* recombinants were grown at 37°C in low salt Luria-Bertani medium plates (0.5% yeast extract, 1% tryptone, 0.5% NaCl, and 1.5% agar) containing 10 µg/ml tetracycline and 25 µg/ml Zeocin™. *Pichia pastoris* SMD1168 strain was cultured in minimal dextrose (MD) medium plates [20 g/l dextroses, 10 g/l ammonium sulfate, 3.4 g/l yeast nitrogen base (YNB) without amino acids and ammonium sulfate, 400 µg/l biotins, and 15 g/l agar] and yeast extract peptone dextrose (YPD) medium plates (10 g/l yeast extract, 20 g/l tryptone, 20 g/l dextroses, and 15 g/l agar) containing 100–1,000 µg/ml. The yeast recombinants were

screened based on resistance to the Zeocin antibiotic. The medium used for rHSA expression was buffered minimal glycerol medium (BMGY) (10 g/l yeast extract, 20 g/l tryptone, 10 g/l ammonium sulfate, 3.4 g/l YNB without amino acids and ammonium sulfates, 100 mM potassium phosphate buffer pH 6.0, 400 µg/l biotins, and 10 g/l glycerol) and buffered minimal methanol histidine medium (BMMH) (3.4 g/l YNB, 400 µg/l biotins, 40 mg/l histidines, 100 mM potassium phosphate pH 6, and 0.75% methanol).

Reagent

Diethylaminoethyl (DEAE)-cellulose, diethylenetriamine pentaacetate (DTPA), gadolinium trichloride hexahydrate, trifluoroacetic acid (TFA 1%), DTPA anhydride, (PBS) Phosphate Buffer Saline buffer, sodium hydroxide, gadolinium chloride hexahydrate in 2 ml of sodium acetate buffer (pH 5.5), and 1% sodium chloride were purchased from Sigma Aldrich. The grade of all of the reagents was pro analysis.

Apparatus

The equipment used was as follows: High Performance Liquid Chromatography (HPLC) Shimadzu LC 10 ADVP with C-18 column, Sephadex G-25 gel filtration column (1 m × 2 cm), autoclave sterilizer (Hirayama Autoclave HVE-50), SDS-PAGE Mini-Sub Cell (Bio-Rad) and SDS-PAGE (Bio-Rad PowerPac™ Basic), magnetic stirrer (HMS-79 Magnetic Heated), freeze dryer, ice bath, conductivity meter, Millipore, MiniSpin (Eppendorf), Balance (Mettler Toledo AL204), UV-lamp $\lambda_{312\text{ nm}}$ (Viber Lourmat TCP-20M), pH meter (Mettler Toledo InLab pH combination polymer electrodes), automatic micropipette (Thermo Fisher), centrifuge (Tomy MX-305), shaking incubator (DAIHAN Scientific), UV-Vis spectrophotometer (Ultrospec 3000 Pro UV/Vis), vortex (Boeco V-1 Plus), and water bath (Poly Science).

Construction of expression vector containing HSA gene

The sequence of the native HSA gene was obtained from GenBank (ACC: AF190168.1). The sequence was optimized according to the *P. pastoris* preference codon, using the gcua program (www.gcua.org). The HSA gene was then synthesized by DNA2.0 (Hong Kong) and inserted after the α -factor signal sequence in the pD912 expression vector to produce a recombinant plasmid pD912-rHSA. The recombinant plasmid was propagated in the *E. coli* TOP10F' and subsequently isolated by TIANprep Mini Plasmid Kit following the procedure.

Pichia pastoris transformation and screening of transformants with high expression

Briefly, 80 µl of the electrocompetent *P. pastoris* SMD1168 was transformed with 500 ng *SacI*-linearized pD912-rHSA (10 µl) by electroporation (25 µF, 200 Ω, and 1.0 kV), using a MicroPulser Electroporator (Bio-Rad, CA). The cells were mixed with 1 ml prechilled sorbitol (1 M) and incubated at 28°C for 2 hours. The transformants were subsequently selected on the MD plates after growing the cells at 28°C for 48–72 hour and followed by screening on the YPD plates containing various concentrations of Zeocin (100–1,000 µg/ml) at 28°C, up to the point of colony formation (Zhu *et al.*, 2018). The transformants were analyzed by colony Polymerase Chain Reaction (PCR) to confirm the HSA gene's presence in the *P. pastoris* genome,

using universal primers for pD912. The nucleotide sequence was confirmed by the Sanger dideoxy method by using primer pairs: pD912F (5'-ACGTCGCTGTTTGGCC-3') and pD912R (5'-CTGCATCTCTCAG GCAAAT-3').

Expression of rHSA by *P. pastoris*

Pichia pastoris transformants were grown in 20 ml of BMGY at 28°C and 230 rpm, through a period before the culture reached an OD₆₀₀ of 15–20 (approximately 18 hours). The cells were harvested by centrifugation at 3,000 g and 25°C for 20 minutes. These were subsequently resuspended in a 100 ml flask containing 20 ml Buffered Methanol- Complex Medium (BMMY) and allowed to grow under similar conditions as before. Furthermore, methanol p.a. was added to the flasks every 24 hours to achieve a final concentration of 0.75% (v/v), required to induce the AOX1 promoter. The *P. pastoris* transformed with empty vector were exposed to the same treatment and used as a negative control. Also, samples were taken every 24 hours, and then the cultures were centrifuged at 12,000 g for 5 minutes after 144 hours. The presence of rHSA in the supernatant was confirmed using SDS-PAGE carried under moderated conditions using 10% running gel and 4% stacking gel. Then 25 µl of supernatant was analyzed. The protein bands present on the gel were stained with Coomassie Brilliant Blue R250.

Optimization of rHSA expression with various inducer methanol concentrations

Pichia pastoris transformants were grown in four flasks containing 20 ml BMGY and subsequently transferred to BMMH, following the procedure stated above. The rHSA expression was optimized using variations of methanol concentrations (0.75%, 1.0%, 1.25%, and 1.5%), and samples were collected every 24 hours up to the 144th hour. These cultures were then centrifuged at 12,000 g for 5 minutes, followed by detection of rHSA in the supernatant using SDS-PAGE.

Purification of rHSA with Sephadex G-25 gel filtration chromatography

The supernatants obtained were respectively placed into the Sephadex G-25 gel filtration column (1 m × 2 cm), previously equilibrated with a 1% sodium chloride solution. Furthermore, 5 ml of each eluate was taken for up to 57 fractions. The absorptions were measured using a UV-Vis spectrophotometer at 280 nm. The conductivity was also measured. A curve of absorption and conductivity value of the fractions was prepared. The fraction with a maximum peak was characterized by SDS-PAGE.

Characterization of rHSA by RP-HPLC

About 20 µl of HSA standard was injected into the C-18 column. The column was eluted using water-acetonitrile containing 0.1% TFA as a mobile phase. The flow rate was set to 1 ml/minutes for 20 minutes, and the peak was detected using a UV detector at 215 nm. The chromatogram showed the retention time of the HSA standard. The column was washed with the mobile phase to flush the remaining impurities. After cleaning the column, the rHSA sample was injected using the same protocol as a standard injection. This was used to determine sample purity based on the chromatogram generated. The protein concentration

was determined using the Lowry method (Lowry *et al.*, 1951) by using bovine serum albumin as a standard.

Synthesis and characterization of Gd-DTPA-rHSA

Gd-DTPA-rHSA was synthesized using the protocol described by Kundu *et al.* (2011). About 10 mg of rHSA was dissolved in 1 ml of 0.01 M PBS (pH 7.4), followed by the direct incorporation of 10 mg DTPA anhydride powder (mol ratio of rHSA:DTPA = 1:200). The pH was maintained at 7.0 using 0.1 M NaOH, and the mixture was then stirred at 4°C for 2 hours. Subsequently, 10 mg of GdCl₃·6H₂O in 2 ml Acetic acid/Sodium acetic (HAc/NaAc) (pH. 5.5) was added to DTPA-rHSA and stirred slowly for 4 hours at room temperature. The purity of the Gd-DTPA-rHSA generated was determined using Reverse Phase- High Performance Liquid Chromatography (RP-HPLC). The HPLC was run using a C-18 column, and water-acetonitrile containing 0.1% TFA served as the mobile phase. The flow rate was set to 0.5 ml/minute, and the peaks were monitored using a UV detector at 215 nm.

RESULTS

Generation of recombinant *P. pastoris* harboring rHSA

The sequence of the native HSA (GenBank Acc: AF190168.1) was optimized according to the *P. pastoris* preference codon using the Gene-GPS technology from DNA2.0 (Supplement material 1). The amino acid sequence of HSA was confirmed using the database on Protein Data Bank (Acc 1AO6 or 6JE7). The HSA gene was constructed in the pD912 plasmid (DNA2.0) to form recombinant plasmid pD912-rHSA under AOX1 promoter control with an α -factor signal sequence and subsequently cloned in *E. coli* TOP10F⁺. The clone was characterized by electrophoresis agarose as shown in Figure 1.

Plasmid pD912-rHSA was used to transform *P. pastoris* SMD1168 by electroporation. Colony transformants were

analyzed by colony PCR to confirm the HSA gene's presence in the *P. pastoris* genome, using universal primers for pD912 (data not shown). Confirmation of rHSA sequence by using the dideoxy Sanger method using universal primers for pD912 shows 100% homology to HSA sequence (Supplement material 2). The expression of HSA by *P. pastoris* was carried out extracellularly by using methanol as an inducer (Gaffar *et al.*, 2019).

Optimization and production of rHSA in *P. pastoris* SMD1168

The expression of rHSA was induced by variation of methanol concentration (0.75%, 1.0%, 1.25%, and 1.5%). The result showed that 1.5% of methanol provided the best result (Fig. 2). Furthermore, rHSA was characterized on SDS-PAGE with a consistent band (66.5 kDa) starting 24 hours until 144 hours after induction. The expression level of rHSA in *P. pastoris* SMD1168 seems to be lower compared to a previous study. However, no proteolysis products were detected.

Figure 3 shows that the increase in methanol concentration does not correlate directly with protein levels. The highest protein concentration was obtained in a 1.5% methanol inducer at 96 hours after induction. However, the level is not very high compared to 0.75% methanol. Determination of protein concentration using the Lowry method calculates all the total protein in the sample. Indeed, only an rHSA band appears on the gel, and there is no other protein on the SDS-PAGE in Figure 2. The production of rHSA in 100 ml expression media induced by methanol every 24 hours until the 96th hour shows that the level of rHSA expression in *P. pastoris* SMD1168 was 0.44 g/l.

Purification of rHSA by Sephadex G-25 gel filtration chromatography

Figure 4 shows the result of purification of rHSA using gel chromatography filtration, with a Sephadex G-25 column (1 m × 2 cm), with sodium chloride 0.1% as the eluent. There are two

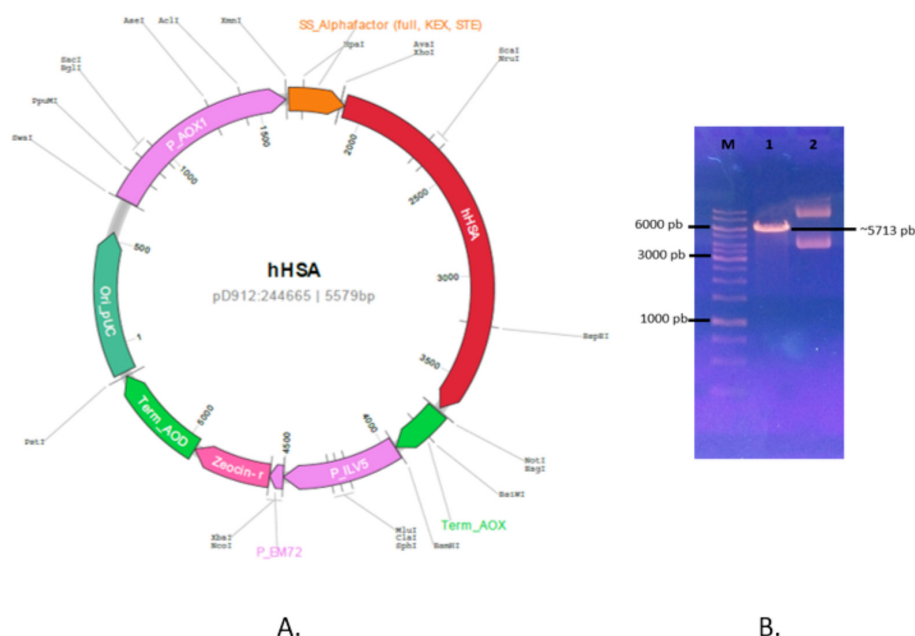


Figure 1. Map of recombinant plasmid and characterization. (A) Map of pD912-HSA. (B) Characterization of pD912-HSA. Line (1) DNA marker, line (2) linear pD912-HSA/*Sac*I, and line (3) pD912-HSA circular.

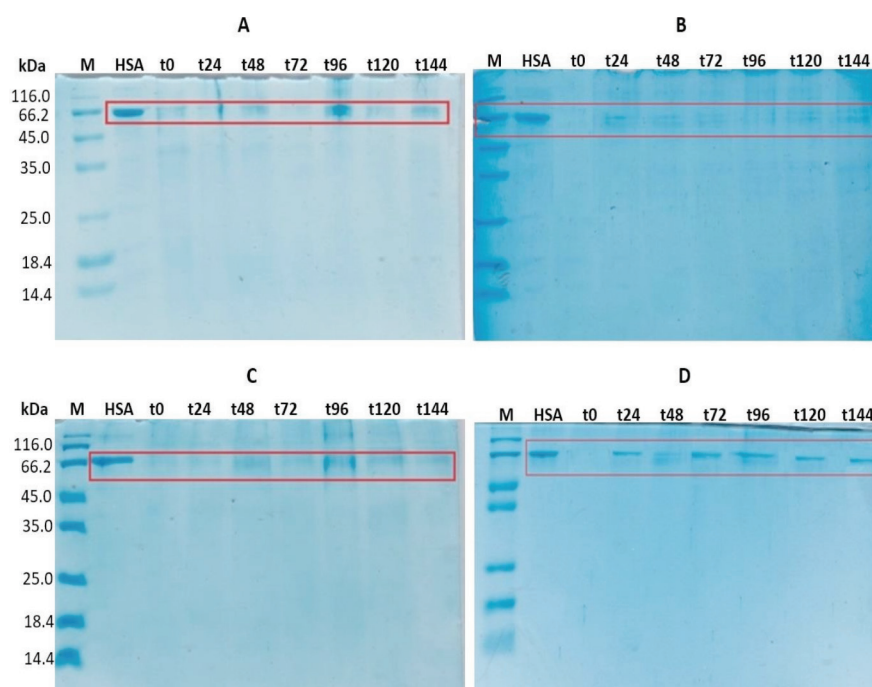


Figure 2. SDS-PAGE of *P. pastoris* SMD1168 transformant culture broth supernatant, with methanol inducer concentration: (A) 0.75%, (B) 1.0%, (C) 1.25%, and (D) 1.5%. M: marker; HSA: HSA standard. The sample was taken every 24 hours, from *t*-0 (0 hour) to *t*-144 (144 hours). 20 μ l of supernatant was loaded to each well.

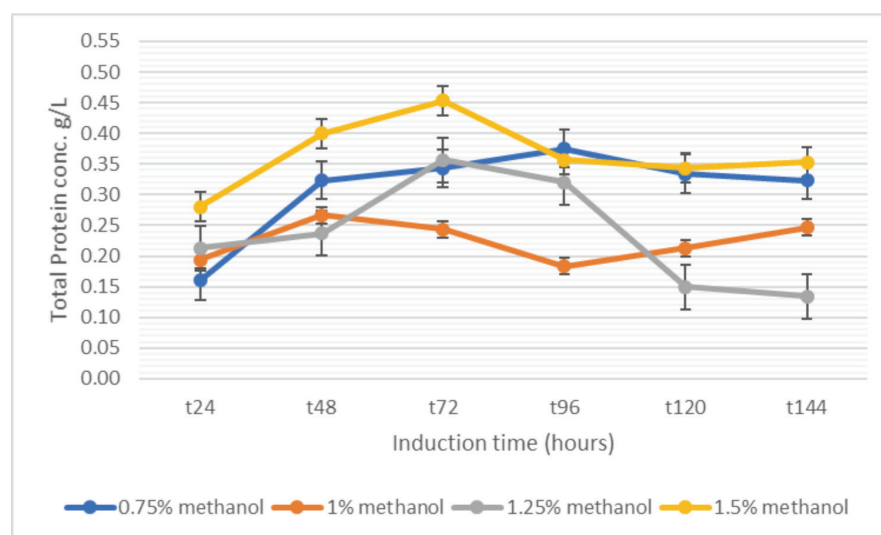


Figure 3. Total protein concentration in culture supernatant *P. pastoris* SMD1168 transformant after induction with 0.75%, 1%, 1.25%, and 1.5% methanol.

major peaks. The first peak (fractions 14–19) was combined into fraction A, while the second peak (fractions 21–25) was combined into B. Figure 3 indicates a relatively higher absorbance of the last peak. At the same time, conductivity was increased after the protein was eluted, as shown in Figure 4. Both samples (fractions A and B) were concentrated by freeze-drying, removing water, and redissolution in 10 ml double-distilled water. SDS-PAGE analysis shows that the A fraction contains the HSA, whereas the B fraction does not show the protein band. The concentration of

rHSA purified by gel filtration chromatography was 0.249 g/l, significantly dropping compared to the amount of HSA present in the culture supernatant (0.44 g/l). This was probably due to the presence of fraction B, which did not contain rHSA.

Characterization of the purity of rHSA by RP-HPLC

Characterization of rHSA using RP-HPLC (with a C-18 column) showed the peak of standard HSA appearing at 1.984 minutes, while the sample displayed a high peak at 2.122

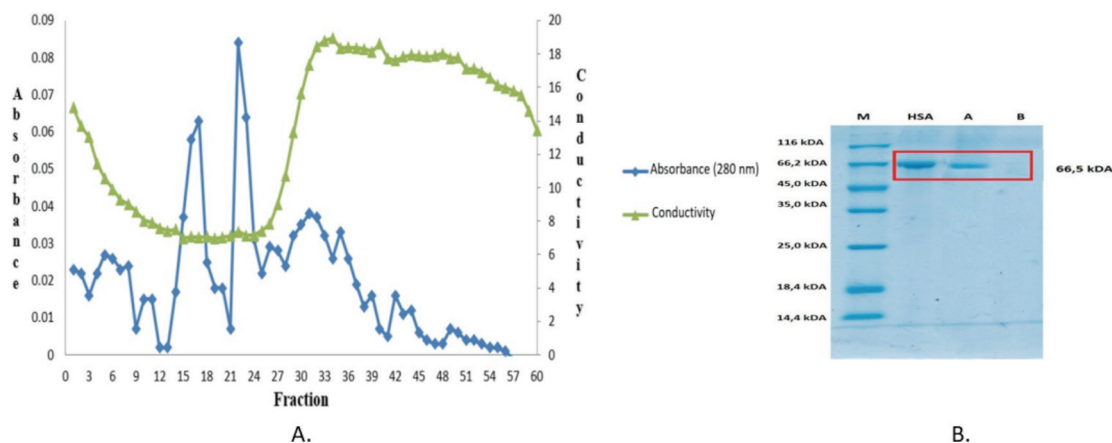


Figure 4. (A) Chromatogram of gel filtration. The metrics used were Sephadex G-25 (1 m × 2 cm), using 1% sodium chloride as an elution buffer, at 5 ml/5 minute flow rate. (B). SDS-PAGE analysis gel filtration chromatography fractions results. Lane (a) fractions of 14–19 and (b) fractions of 21–25. 20 µl of the fraction was loaded, with pure HSA as the standard.

minutes, with an area of 81.84% that indicates the purity of rHSA (Supplement material 3). The 0.138 minute difference in retention time was likely due to the effect of the solvent/medium used in this experiment, which might affect the solution's polarity during separation. SDS-PAGE characterization confirms that the peak contains a 66.5 kDa band.

Synthesis of Gd-DTPA-rHSA

The Gd-DTPA was covalently linked to rHSA molecules via amide formation. The formation of DTPA-rHSA requires a reaction between the carboxyl groups in DTPA-dihydride and lysine residue of rHSA molecules to form an amide. Subsequently, the gadolinium (III) complexation proceeded by reaction Gd^{3+} ion of gadolinium trichloride hexahydrate with DTPA-rHSA (Kundu *et al.*, 2011). Characterization using RP-HPLC (in a C-18 column; water-acetonitrile containing 0.1% TFA as the mobile phase) shows three single peaks: an unconjugated rHSA, rHSA-DTPA, and rHSA-DTPA-Gd with retention times of 7.155, 9.951, and 10.493 minutes, respectively (Fig. 5).

DISCUSSION

The *P. pastoris* SMD1168 (his4, pep4) is a protease-deficient strain that can grow in methanol at a similar rate to the wild type (Mut⁺). The Mut⁺ strain requires a higher concentration of methanol inducer than the Mut^s and Mut⁺ strain. In general, methanol with a concentration of 0.5%–1.5% is used for recombinant protein expression. SMD1168 (his4, pep4) is widely used on fermenter cultures expression of heterologous proteins because the high cell density fermentation causes lysis of a small percentage of cells resulting in the secretion of a relatively high concentration of vacuolar proteases such as proteinase A (pep4) and proteinase B (prb1) (Ahmad *et al.*, 2014; Baghban *et al.*, 2018; Gleeson *et al.*, 1998). However, the protease-deficient strains commonly exhibit slower growth rates subsequently producing low yields. The accumulation of methanol can negatively affect cell growth and later reduce expression levels. Hence, the optimum methanol inducer concentration needs to be evaluated for each expressed protein (White *et al.*, 1994).

The results obtained in this study are one-third lower compared with the results reported by Zhu *et al.* (2018), which expressed rHSA in *P. pastoris* GS115. This was probably because

the strain used in this study was a protease-deficient strain. The SMD1168 strain does not have vacuole peptidase A (pep4). This enzyme is responsible for the activation of carboxypeptidase Y and protease B1 (White *et al.*, 1994). The shortcoming of the protease deficiency strain is that it has lower viability, possesses a slower growth rate, and is more difficult to handle in a shake flask and fermenter cultures. This is likely the cause of the low level of rHSA production in the *P. pastoris* SMD1168 strain. The protease-deficient *P. pastoris* strain produces intact HSA but a low expression level.

DTPA molecule binds covalently to the amine group present in the albumin and Gd (III) molecules through chelate ligands forming Gd-DTPA-HSA complexes (Liu and Chen, 2016). It is most likely that only one DTPA molecule can link to HSA through Lys199. According to Wang *et al.* (2020), this Lys residue can serve as a drug linking site. The partial conjugation possibly happens because there are multiple lysine residues that are available for linkage formation between the HSA and DTPA. The HPLC method that we used should be able to separate different degrees of conjugation since a different degree of conjugation will possess a different degree of polarity and the column used to separate samples based on the polarity of the sample. The albumin-(Gd-DTPA) conjugate was also synthesized by the reaction of the cyclic anhydride of DTPA with albumin and subsequent addition of an excess of Gd^{3+} ions before purification by extensive dialysis (Schmiedl *et al.*, 1987). The DTPA was covalently bound to the amine group of the albumin, followed by chelating the Gd^{3+} to the DTPA ligand (Rinck *et al.*, 2010; Wallnöfer *et al.*, 2020).

We succeeded in conjugating the rHSA from *P. pastoris* SMD1168 with Gd-DTPA to form Gd-DTPA-rHSA with high purity. This serves as a contrast agent and is required in high purity since free DTPA molecules and Gd^{3+} ions are dangerous and toxic (Fauzia *et al.*, 2015; Gallo *et al.*, 2020; Rinck, 2018; Schmiedl *et al.*, 1987; Wallnöfer *et al.*, 2020). The results of this study indicate that the deficient protease host *P. pastoris* SMD1168 can be used to express rHSA, and conjugation with Gd-DTPA produced a complex with high purity. However, the low expression level of rHSA in the host *P. pastoris* SMD1168 needs to be increased. All HPLC chromatograms showed a single peak indicating single species in the sample analyzed (Fig. 5 A–C). Only a small amount of impurities was detected before the main peak of rHSA (Fig. 5A), which most

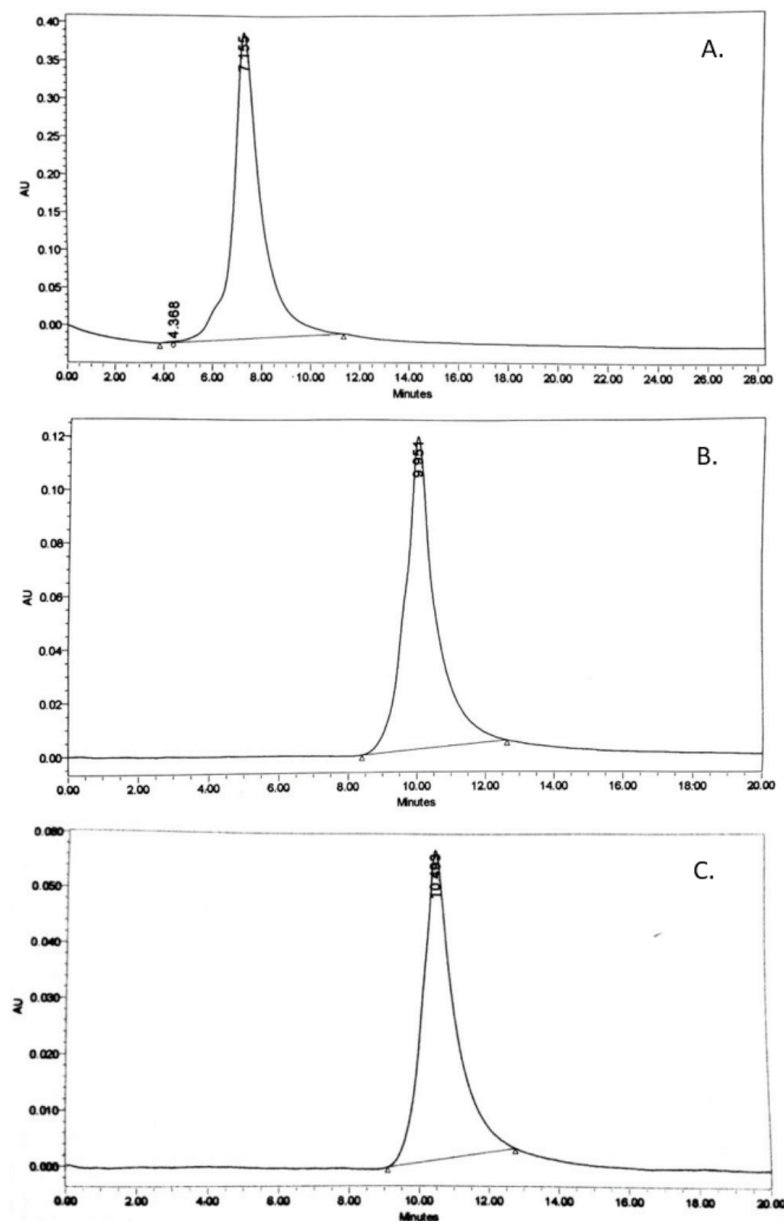


Figure 5. Result of RP-HPLC analysis. (A) rHSA. (B). DTPA-rHSA. (C). Gd-DTPA-rHSA. RP-HPLC has performed at λ_{215} nm. Flow rate of 1 ml/minute. Column C-18, a mobile phase of water-acetonitrile containing 0.1% TFA. Flow rate 0.5 ml/minute. Peak was monitored using a UV detector at 215 nm. <AQ>

likely is a minor protein still present in the sample. However, the impurities did not interfere with the formation of the DTPA-HSA conjugate since only one peak appeared in the chromatogram of the DTPA-HSA sample. No other peak was observed in the chromatogram (Fig. 5B). Finally, the final product Gd-DTPA-HSA (Fig. 5C) was detected as a single peak indicating a product with high purity (~99%). Since a high-purity product was obtained in our study, the product from our current study can be used as a contrast agent in clinical research to determine the influence of rHSA on Gd-DTPA's performance as a contrast agent.

The application of Gd-DTPA-rHSA is to enhance imaging diagnosis with an MRI apparatus. The advantage of using serum protein is based on its biocompatibility, stability, and extended

intravascular retention. The application of contrast agents, including the complex compound Gd-DTPA, has been adopted to improve image quality (Goyen, 2008). The contrast agents enhance internal body structures' visibility in MRI. The most commonly used contrast agent enhancement is a gadolinium-based compound. Such contrast agents shorten the relaxation times of nuclei within body tissues following oral or intravenous administration in MRI (Bumb *et al.*, 2010; Ouyang *et al.*, 2020). After the intravenous administration, Gd-DTPA is reportedly distributed from the intravascular (inside a blood vessel) to the extracellular (outside a blood vessel) areas and is quickly removed from the body through renal excretion. This phenomenon reduces user preference, especially during blood analysis (Gallo *et al.*, 2020). However, the conjugation with a

control compound, e.g., serum protein, HSA, as seen with the Gd-DTPA-HSA complex, provides better imaging results (Bumb *et al.*, 2010; Rinck *et al.*, 2010; Wallnöfer *et al.*, 2020). This complex compound can be synthesized through a reaction between DTPA and albumin molecules, followed by a reaction with excess Gd (III) ions. However, MR imaging contrast applications may extend beyond these typical radiographic applications (Ouyang *et al.*, 2020; Rinck *et al.*, 2010). Combining monoclonal antibodies and MR imaging contrast media technologies may improve the detection and characterization of malignant lesions and other antigenically distinct pathologic lesions, which is an exciting possibility for MR imaging contrast investigation (Ouyang *et al.*, 2020).

CONCLUSION

rHSA was successfully expressed in protease-deficient *P. pastoris* SMD1168. The optimum yield of 0.44 g/l was obtained using a 1.5% methanol inducer at 96 hours after induction. Purification by gel filtration chromatography resulted in 81.84% purity. RP-HPLC with a C-18 column showed that the conjugation of rHSA with Gd-DTPA was successfully performed, evidenced by the presence of a single peak on the chromatogram.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Shabarni Gaffar conceived, designed, and performed the experiments, analyzed and interpreted the data, and wrote and revised the manuscript. Rina Anggraeni conceived and designed the experiments and analyzed, interpreted, and conducted analysis of the data. Mia Tria Novianti analyzed and interpreted the data. Safri Ishmayana analyzed and interpreted the data and wrote and carried out a review of the manuscript. Ukun MS Soedjanaatmadja conceived and designed the experiments, analyzed and interpreted the data, and wrote, compiled, and revised the manuscript.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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SUPPLEMENT MATERIALS

10	20	30	40	50	60	70	80	90	100	110
GTA	ACTGG	CTTCAG	CAGAGCG	CAGATAC	CAAAAT	ACTGTT	CTTCTAG	TGTAG	CCCTAG	TAGT
120	130	140	150	160	170	180	190	200	210	220
TGT	TACCA	GTGGCT	GCTGCC	AGTGG	CGAT	AAGT	CGTGT	CTAC	CGGGT	TGGACT
240	250	260	270	280	290	300	310	320	330	340
GCCC	AGCTT	GGAGCG	AAAGCT	ACAC	CGA	CTG	AGAT	ACCT	ACG	CGT
360	370	380	390	400	410	420	430	440	450	460
GTC	CGA	ACAG	GAGAG	CGCG	CACG	AGGG	AGCTT	CCAG	GGG	AAAC
480	490	500	510	520	530	540	550	560	570	580
GGG	CGCG	AGC	CTT	GGAAA	AAAC	CGCAG	CAAC	CGCG	CGCTT	TTT
600	610	620	630	640	650	660	670	680	690	700
TG	ACTG	CGCT	GAAAT	CTCC	ATCG	CTAC	AGAT	GATG	ACAT	T
720	730	740	750	760	770	780	790	800	810	820
ATT	TAA	TAAT	CAAT	CGAA	AGCA	AAAG	GTGA	ATGA	AACT	TTTT
840	850	860	870	880	890	900	910	920	930	940
AG	ACG	GTG	CAAC	CGAG	CGCT	CTCT	CTCT	CAAC	CGCC	ATTT
960	970	980	990	1000	1010	1020	1030	1040	1050	1060
ATT	AGG	CTA	CTAAC	CACT	AGCTT	TAT	TAG	CCCT	GTCT	AT
1080	1090	1100	1110	1120	1130	1140	1150	1160	1170	1180
CAG	ATG	AGG	CGCTT	GA	GTG	GGG	GTCA	ATAG	T	TT
1200	1210	1220	1230	1240	1250	1260	1270	1280	1290	1300
AAG	ATG	AACT	TAAG	TTGG	TCG	TGAA	ATGCT	AA	CGCG	CAGT
1320	1330	1340	1350	1360	1370	1380	1390	1400	1410	1420
TA	ATCT	CAAT	TAAT	GGCT	TAC	GCAG	CTCT	CT	CAAT	CGCG
1440	1450	1460	1470	1480	1490	1500	1510	1520	1530	1540
TT	GTAT	GCT	CCCA	AGAT	CTG	GTGG	GAAT	ACTG	CT	CAAT
1560	1570	1580	1590	1600	1610	1620	1630	1640	1650	1660
TG	CTT	AAAC	CGCTT	TTTT	TAT	CACT	ATT	AGCT	T	TT
1680	1690	1700	1710	1720	1730	1740	1750	1760	1770	1780
AA	CAAT	TAAT	T	GAAG	AAAT	CGA	AGAT	AGAT	AGAT	AGAT
1800	1810	1820	1830	1840	1850	1860	1870	1880	1890	1900
GA	AGAG	AACT	CGA	AGAT	AGAT	AGAT	AGAT	AGAT	AGAT	AGAT
1920	1930	1940	1950	1960	1970	1980	1990	2000	2010	2020
CG	TAT	CGCT	GA	AGAT	AGAT	AGAT	AGAT	AGAT	AGAT	AGAT
2040	2050	2060	2070	2080	2090	2100	2110	2120	2130	2140
CG	TAT	CGCT	GA	AGAT	AGAT	AGAT	AGAT	AGAT	AGAT	AGAT
2160	2170	2180	2190	2200	2210	2220	2230	2240	2250	2260
GCT	AAAC	AAAG	CGAG	AGAA	TAAT	GAAT	GCTT	TT	GCA	ACAT
2280	2290	2300	2310	2320	2330	2340	2350	2360	2370	2380
AT	GAG	AGAG	CTT	CT	GAAA	AAAT	ACIT	TT	GAC	GAAT
2400	2410	2420	2430	2440	2450	2460	2470	2480	2490	2500
TT	CG	AGG	CAG	CAG	ATA	AGCT	GCT	AT	TT	GCT
2520	2530	2540	2550	2560	2570	2580	2590	2600	2610	2620
GG	CGA	AGCT	GTG	CTT	CAAG	CGCT	TGAG	CGCG	TTTT	GAG
2640	2650	2660	2670	2680	2690	2700	2710	2720	2730	2740
GT	GT	CAG	GT	AT	CT	GT	CT	GT	CT	GT
2760	2770	2780	2790	2800	2810	2820	2830	2840	2850	2860
GCT	A	AAAA	AAAT	CCCA	CTGC	ATCG	AGAG	GTG	AAAA	AT
2880	2890	2900	2910	2920	2930	2940	2950	2960	2970	2980
GC	AAA	AGAT	GT	CTT	CT	GT	GT	AT	TT	CT
3000	3010	3020	3030	3040	3050	3060	3070	3080	3090	3100
GT	TG	CG	CGCG	CTG	ACCT	CT	GAAT	TT	TAAC	CACT
3120	3130	3140	3150	3160	3170	3180	3190	3200	3210	3220
GG	GT	GAG	TATA	AGTT	TCAGA	ATG	CTT	GGT	GAG	AT
3240	3250	3260	3270	3280	3290	3300	3310	3320	3330	3340
TT	GT	CAAG	AGCT	CCG	AGG	CGT	AGG	AGT	AGT	AGT
3360	3370	3380	3390	3400	3410	3420	3430	3440	3450	3460
AG	T	GT	GT	AT	CAAG	AT	CACT	GT	TT	CT
3480	3490	3500	3510	3520	3530	3540	3550	3560	3570	3580
TT	T	CACT	AT	CAG	AG	AG	AG	AG	AG	AG
3600	3610	3620	3630	3640	3650	3660	3670	3680	3690	3700
CG	CG	CTT	TT	GT	GAAG	GT	TT	GAAG	AG	AG
3720	3730	3740	3750	3760	3770	3780	3790	3800	3810	3820
GC	GG	CGCT	CAAG	AGG	ATG	TCAGA	ATG	CCAT	TT	GC
3840	3850	3860	3870	3880	3890	3900	3910	3920	3930	3940
TT	CT	CT	AG	AG	CT	AT	CT	CT	AG	AG
3960	3970	3980	3990	4000	4010	4020	4030	4040	4050	4060
TC	AG	AG	TCAGA	AGAT	TAA	GT	GAAG	CTT	CGT	TT
4080	4090	4100	4110	4120	4130	4140	4150	4160	4170	4180
TT	GT	TT	CCAG	AGG	CCAA	CTT	CCAC	CGT	AGT	AGT
4200	4210	4220	4230	4240	4250	4260	4270	4280	4290	4300
TACT	AG	CAG	ATA	AGAT	GT	CAAC	AAAT	GT	ACCG	GT
4320	4330	4340	4350	4360	4370	4380	4390	4400	4410	4420
GT	G	AC	AGG	TT	GT	CGAT	T	CGCG	TA	AG
4440	4450	4460	4470	4480	4490	4500	4510	4520	4530	4540
TG	AA	AGT	AAAA	T	CGA	AC	CGCG	ACT	CAAC	CG

Supplement material 1. The nucleotide sequence of HSA optimized by gene GPS (DNA 2.0) using *P. pastoris* codon table.

MSA

The multiple sequence alignment result as produced by T-coffee.

T-COFFEE, Version_11.00.d625267 (2016-01-11 15:25:41 - Revision d625267 - Build 507)

Cedric Notredame
SCORE=1000*
* BAD AVG GOOD*
HSA : 100
HSA_1 : 100
cons : 100HSA ATGGACGCCCACAAATCAGAAGTCGCACATCGTTTTAAGGATTTAGGTGAAGAGAACTTTAAGGCTCTTGACTG
HSA_1 ATGGACGCCCACAAATCAGAAGTCGCACATCGTTTTAAGGATTTAGGTGAAGAGAACTTTAAGGCTCTTGACTG

cons *****

HSA ATCGCTTTTCGCACAATATCTACAACAGTGTCCGTTTCGAGGATCATGTTAAGCTTGTTAATGAGGTTACGGAATTT
HSA_1 ATCGCTTTTCGCACAATATCTACAACAGTGTCCGTTTCGAGGATCATGTTAAGCTTGTTAATGAGGTTACGGAATTT

cons *****

HSA GCTAAAACATGCGTTGCAGACGAATCGGCTGAGAATTGTGATAAAAGCTTGCATACTTTGTTCCGAGATAAGTTA
HSA_1 GCTAAAACATGCGTTGCAGACGAATCGGCTGAGAATTGTGATAAAAGCTTGCATACTTTGTTCCGAGATAAGTTA

cons *****

HSA TGTACGGTGGCAACCTTGAGAGAAACATATGGAGAGATGGCAGACTGCTGTGCTAAACAAGAGCCAGAGAGAAAT
HSA_1 TGTACGGTGGCAACCTTGAGAGAAACATATGGAGAGATGGCAGACTGCTGTGCTAAACAAGAGCCAGAGAGAAAT

cons *****

HSA GAATGCTTTTTGCAACATAAAGACGACAATCCTAACTTACCAAGACTGGTTAGGCCAGAGGTGGACGTCATGTGT
HSA_1 GAATGCTTTTTGCAACATAAAGACGACAATCCTAACTTACCAAGACTGGTTAGGCCAGAGGTGGACGTCATGTGT

cons *****

HSA ACAGCTTTTCACGATAATGAAGAGACTTTCTGAAAAAGTACTTGTACGAAATCGCCCGTAGACATCCATACTTT
HSA_1 ACAGCTTTTCACGATAATGAAGAGACTTTCTGAAAAAGTACTTGTACGAAATCGCCCGTAGACATCCATACTTT

cons *****

HSA TATGCACCTGAGTTACTGTTCTTCGCGAAAAGATATAAGGCTGCATTTACTGAATGTTGCCAGGCAGCAGATAAA
HSA_1 TATGCACCTGAGTTACTGTTCTTCGCGAAAAGATATAAGGCTGCATTTACTGAATGTTGCCAGGCAGCAGATAAA

cons *****

HSA GCTGCATGTTTGCTGCCTAAGTTGGATGAGTTAAGAGATGAAGGTAAGGCCAGCTCAGCCAAACAGCGATTAAAG
HSA_1 GCTGCATGTTTGCTGCCTAAGTTGGATGAGTTAAGAGATGAAGGTAAGGCCAGCTCAGCCAAACAGCGATTAAAG

cons *****

HSA TGTGCCAGCTTACAGAAATTCGGCGAACGTGCATTCAAAGCCTGGGCTGTAGCCCGTTTGAGTCAGCGTTTTCT
HSA_1 TGTGCCAGCTTACAGAAATTCGGCGAACGTGCATTCAAAGCCTGGGCTGTAGCCCGTTTGAGTCAGCGTTTTCT

cons *****

HSA AAGGCTGAATTCGCCGAAGTCTCAAATTGGTAACGGACCTTACGAAAGTTCATACTGAGTGTGTCACGGTGAT
HSA_1 AAGGCTGAATTCGCCGAAGTCTCAAATTGGTAACGGACCTTACGAAAGTTCATACTGAGTGTGTCACGGTGAT

cons *****

HSA CTGTTAGAATGTGCTGATGACAGAGCTGATCTTGCTAAATACATATGCGAGAATCAAGACTCCATATCGAGTAA
HSA_1 CTGTTAGAATGTGCTGATGACAGAGCTGATCTTGCTAAATACATATGCGAGAATCAAGACTCCATATCGAGTAA

cons *****

HSA TTGAAAGAGTGTGTGAAAAACCATTTGCTAGAAAAATCCCACTGCATCGCAGAGGTGGAAAAATGACGAAATGCC
HSA_1 TTGAAAGAGTGTGTGAAAAACCATTTGCTAGAAAAATCCCACTGCATCGCAGAGGTGGAAAAATGACGAAATGCC

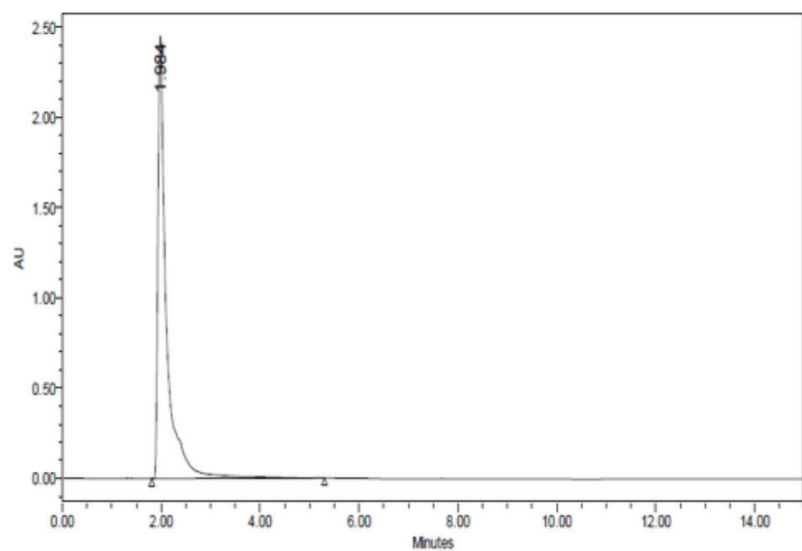
cons *****

HSA GCTGATTTGCCTAGTCTAGCTGCTGATTTGTTGAATCGAAGGATGTGTGTAATACTACGCAGAAGCAAAAGAT
HSA_1 GCTGATTTGCCTAGTCTAGCTGCTGATTTGTTGAATCGAAGGATGTGTGTAATACTACGCAGAAGCAAAAGAT

Continued

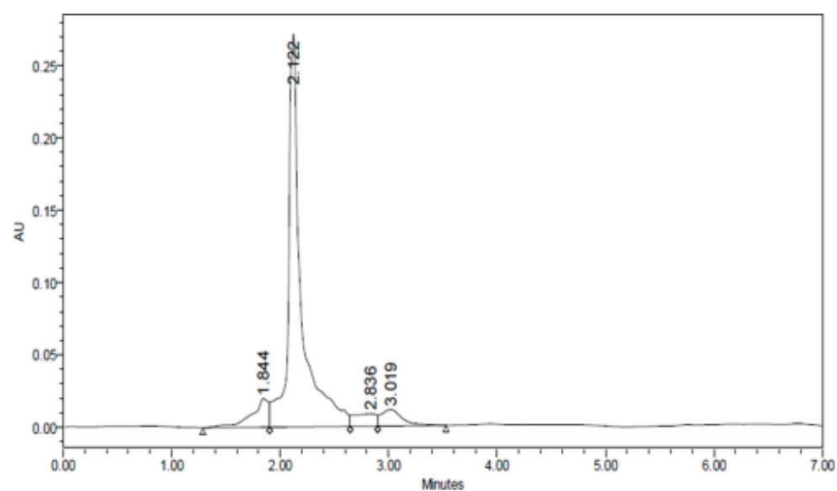
HSA	GTCTTTCTTGGTATGTTTCTGTACGAATATGCTAGAAGGCACCTGACTATTTCAGTGGTTTTGTTACTTAGACTA
HSA_1	GTCTTTCTTGGTATGTTTCTGTACGAATATGCTAGAAGGCACCTGACTATTTCAGTGGTTTTGTTACTTAGACTA
cons	*****
HSA	GCTAAGACATACGAGACAACCTTTGGAAAAATGTTGCGCAGCGGCTGACCCTCATGAATGTTATGCAAAAGTGTTG
HSA_1	GCTAAGACATACGAGACAACCTTTGGAAAAATGTTGCGCAGCGGCTGACCCTCATGAATGTTATGCAAAAGTGTTG
cons	*****
HSA	GACGAGTTTAAACCACTTGTAGAGGAACCCGAGAATCTGATTAAGCAAACTGTGAATTGTTTGAACAATTGGGT
HSA_1	GACGAGTTTAAACCACTTGTAGAGGAACCCGAGAATCTGATTAAGCAAACTGTGAATTGTTTGAACAATTGGGT
cons	*****
HSA	GAGTATAAGTTTCAGAATGCATTGCTGGTGAGATACACTAAAAAGGTGCCACAAGTTAGCACACCAACCTGGTT
HSA_1	GAGTATAAGTTTCAGAATGCATTGCTGGTGAGATACACTAAAAAGGTGCCACAAGTTAGCACACCAACCTGGTT
cons	*****
HSA	GAGGTGTCCAGAAATCTGGGAAAGGTTGGATCTAAGTGTGCAAGCATCCGGAGGCTAAGAGGATGCCCTGTGCA
HSA_1	GAGGTGTCCAGAAATCTGGGAAAGGTTGGATCTAAGTGTGCAAGCATCCGGAGGCTAAGAGGATGCCCTGTGCA
cons	*****
HSA	GAGGACTACCTTTCCGTAGTCCTCAATCAATTGTGTGTACTGCACGAGAAAACCTCCAGTGTCCGATAGAGTCACT
HSA_1	GAGGACTACCTTTCCGTAGTCCTCAATCAATTGTGTGTACTGCACGAGAAAACCTCCAGTGTCCGATAGAGTCACT
cons	*****
HSA	AAGTGTGTACAGAATCACTGGTTAATCGTAGGCCATGTTTTCTGCATTGGAAGTGGATGAGACTTACGTTCCC
HSA_1	AAGTGTGTACAGAATCACTGGTTAATCGTAGGCCATGTTTTCTGCATTGGAAGTGGATGAGACTTACGTTCCC
cons	*****
HSA	AAGGAATTCAATGCTGAGACATTTACTTTTACGCTGATATTTGTACACTATCAGAGAAGGAACGTCAAATCAAA
HSA_1	AAGGAATTCAATGCTGAGACATTTACTTTTACGCTGATATTTGTACACTATCAGAGAAGGAACGTCAAATCAAA
cons	*****
HSA	AAGCAAACCGCTCTGGTCGAATTGGTAAAACATAAGCCTAAGGCTACAAAAGAGCAACTCAAAGCTGTTATGGAT
HSA_1	AAGCAAACCGCTCTGGTCGAATTGGTAAAACATAAGCCTAAGGCTACAAAAGAGCAACTCAAAGCTGTTATGGAT
cons	*****
HSA	GACTTCGCCGCATTTGTTGAGAAGTGCTGCAAAGCTGATGACAAGGAAACATGTTTTGCAGAAGAGGGTAAAAAG
HSA_1	GACTTCGCCGCATTTGTTGAGAAGTGCTGCAAAGCTGATGACAAGGAAACATGTTTTGCAGAAGAGGGTAAAAAG
cons	*****
HSA	TTGGTTGCCGCCTCACAAGCTGCTCTAGGATTGTAA
HSA_1	TTGGTTGCCGCCTCACAAGCTGCTCTAGGATTGTAA
cons	*****

Supplement material 2. Alignment of rHSA (HSA_1) with *HSA* sequence.



A.

	RT	Area	% Area	Height
1	1.984	29561395	100.00	2450784



B.

	RT	Area	% Area	Height
1	1.844	222064	8.05	19891
2	2.122	2256411	81.84	271479
3	2.836	123583	4.48	8644
4	3.019	155005	5.62	11390

Supplement material 3. Result of RP-HPLC. (A) HSA standard; (B) rHSA. Column; C-18, mobile phase; water-acetonitrile containing 0.1% TFA. Flow rate 0.5 ml/minute. Absorption is measured by a UV detector at 215 nm.