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# Generation of a transgenic silkworm that secretes recombinant proteins in the sericin layer of cocoon: Production of recombinant human serum albumin

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**Abstract**

In this study we produced germline transgenic silkworms that spin cocoons containing recombinant human serum albumin (rHSA) in the sericin layer. A *piggyBac*-based transformation vector was constructed that carried HSA cDNA driven by sericin-1 gene promoter, viral enhancer *hr*3, and gene encoding viral *trans*-activator IE1. Isolated silk glands were bombarded with the vector and transplanted into host larvae. Three days later, the transplants were immunohistochemically analyzed, which showed that middle silk gland (MSG) cells expressed rHSA and secreted it into the MSG lumen. Then, silkworm eggs were injected with the vector and developed to larvae. The obtained transgenic silkworms spun silk threads whose sericin layers contained rHSA at 3.0 μg/mg of cocoons. Most (83%) of the rHSA in cocoons was extracted with phosphate buffered saline, which was then subjected to ammonium sulfate precipitation and affinity chromatography. Finally, we obtained 2.8 mg of 99%-pure rHSA from 2 g of cocoons. Measurements of circular dichroism spectra of rHSA, and equilibrium dissociation constants of rHSA to warfarin and naproxen indicated that rHSA was conformationally and functionally identical to natural plasma HSA. Germline transgenic silkworms will be useful for producing various recombinant proteins in the sericin layer of cocoons.
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*Keywords:* *piggyBac*-based transformation vector; Viral *trans*-activator IE1; Circular dichroism spectrum; *Bombyx mori* nuclear polyhedrosis virus

*Abbreviations:* HSA, human serum albumin; rHSA, recombinant HSA; PSG, posterior silk gland; MSG, middle silk gland; BmNPV, *Bombyx mori* nuclear polyhedrosis virus; CD, circular dichroism; nHSA, native HSA; AA, amino acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; G1, the first generation; SPR, surface plasmon resonance; RU, resonance unit

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## 1. Introduction

Human serum albumin (HSA) is a non-glycosylated monomeric protein with a molecular mass of 66.5 kDa and the most abundant protein in plasma. This protein plays important homeostatic roles to maintain colloidal osmotic pressure, and also functions as a transporter of numerous metabolites and chemicals. HSA is clinically used for treating patients with severe hypoalbuminaemia, traumatic shocks, and nephrotic syndromes to maintain an appropriate circulating volume and osmotic pressures in blood (reviewed by Peters, 1995). Contrasting with other clinically used proteins such as cytokines, hormones, and growth factors, HSA is usually administrated with large amounts ranging from 5 to 12.5 g per dose. Currently, HSA for clinical uses is mostly prepared from pooled human blood, which has a potential risk of the contamination of blood-derived pathogens. Therefore, alternative source of the pathogen-free bulky HSA has been sought for. Several attempts have been reported to produce HSA with recombinant DNA technologies using *Bacillus subtilis* (Saunders et al., 1987), yeasts *Pichia pastroris* (Kobayashi et al., 1998, 2000; Watanabe et al., 2001), and mice (Barash et al., 1993) as hosts. Recombinant HSA (rHSA) was expressed in *Pichia* yeast under the control of an alcohol oxidase-2 gene promoter. The recombinant protein was secreted in culture at a level of a gram order per litter (Kobayashi et al., 1998). In mice, rHSA was expressed in mammary gland cells under a β-lactoglobulin gene promoter and secreted into milk at 2.5 mg $ml^{-1}$ in the highest yield strain, and this ability was transmitted to its progeny (Shani et al., 1992).

A silkworm, *Bombyx mori*, synthesizes vast amounts of silk proteins in silk gland and spins out them as a cocoon. This prominent capability must be useful for mass production of recombinant proteins. In addition, knowledge on silkworms and know-how for rearing them have been accumulated because silkworms have been traditionally used as a main producer of silk in sericultural industry for a very long period of time. Silk fibers are mainly composed of two types of proteins, fibroin and sericin. The former constitutes the core of silk fibers, makes up 70–80% of total silk proteins, and is synthesized in the posterior silk gland (PSG). Sericin is a group of hydrophilic glue proteins surrounding the fibroin core, making up 20–30% of the silk proteins. All sericin subtypes are synthesized in the middle silk gland (MSG). Two sericin genes, *Ser*1 and *Ser*2, have been known, the former encoding the majority of sericin (Couble et al., 1987; Takasu et al., 2002).

Previously, we generated a transgenic silkworm that spins a cocoon containing a recombinant protein, human type III collagen fragment, as a fusion protein with fibroin (Tomita et al., 2003). The fusion protein was secreted from PSG cells and incorporated into fibroin fibers in the silk gland lumen, resulting in spinning the fusion proteins into cocoons. However, the solubilization of the fusion protein from fibroins required a strong chaotropic reagent that generally denatures proteins, because the recombinant fusion protein was embedded in highly hydrophobic and insoluble fibroin threads. Thus, it is thought that the method to produce recombinant proteins as fusion proteins with fibroins might not have wide applications to other proteins. In this study, we developed a method to produce a recombinant protein in the sericin layer of cocoons instead of the fibroin layer and as an "independent" protein from fibroin not as a fusion protein with it. This study was initiated as a result of the following considerations. First, the recombinant protein was thought to be more easily extractable from the sericin layer than from the fibroin layer due to its superficial location in a cocoon and the hydrophilic nature of sericin. Second, we expected that the recombinant protein can be secreted from MSG cells into the lumen even if the target protein is not fused with sericin. In the case of PSG cells, recombinant proteins were required to be fused with fibroin protein for their secretion into the lumen (Tomita et al., 2003).

We developed a method to generate a transgenic silkworm that secretes rHSA into the sericin layer using the following transgene. A *Ser*1 promoter was inserted into a *piggyBac*-based vector at a upstream region of HSA cDNA and at a downstream region of *B. mori* nucleopolyhedrosis virus (BmNPV) enhancer, *hr*3. *Trans*-activator gene, IE1, was also introduced to enhance the transcriptional activity of the *Ser*1 promoter. The transgene was injected into silkworm eggs to yield the transgene-bearing worms as reported previously (Tomita et al., 2003; Adachi et al., 2006). The obtained silkworms secreted rHSA into the sericin layer of silk fibers as expected. rHSA was extractable from the fibers with saline, and purified

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from the extracts through simple and moderate biochemical procedures. Characterization of rHSA with circular dichroism (CD) spectroscopy and with drug-binding tests of the rHSA showed that the rHSA was conformationally and functionally identical to native HSA (nHSA). This study demonstrated the validity to utilize MSG cells as host cells in transgenic silkworm technology for producing clinically precious recombinant proteins.

## 2. Materials and methods

### 2.1. Experimental animals

A strain of silkworm, pnd-w1, was obtained from National Institute of Agrobiological Science (Tsukuba, Ibaraki, Japan). Larvae were reared at 25 °C on an artificial diet (Mukin Yosan System Lab.).

### 2.2. Cloning of HSA cDNA

A HSA cDNA was amplified by PCR from a human liver cDNA library (Invitrogen) with the primer set nos. 1 and 2 shown in Table 1. PCR was carried out using KOD-plus-DNA polymerase (TOYOBO) in a thermal cycler PTC-200 (MJ Research). The reaction was consecutively performed with one cycle of incubation for 1 min at 95 °C, and 20 cycles of incubation for 30 s at each of 94 °C and 60 °C, and for 2 min at 68 °C. The PCR product was cloned into a pENTR/D-TOPO vector (Invitrogen). Sequencing of the cloned PCR product was performed with a BigDye terminator v3.0 cycle sequencing kit (Applied Biosystems) using ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). The cloned HSA cDNA encoded 18 amino acid (AA)-long secretion signal peptide, 6 AA-long propeptide, and 585 AA-long mature peptide (Fig. 1).

### 2.3. Vector construction

A *piggyBac*-based transformation vector pMSG2 was constructed by modifying pLE (Tomita et al., 2003). pMSG2 contained three expression units between right- and left-arms of *piggyBac*: DsRed cDNA under the control of 3*xP*3 promoter; BmNPV IE1 cDNA under the control of *Ser*1 promoter and BmNPV *hr*3 enhancer; and the sequence of *attR*1-Cm$^R$-*ccdB-attR*2 under the control of *Ser*1 promoter and BmNPV *hr*3 enhancer (Fig. 1). A transformation vector, pALBM2, was made by replacing "*attR*1-Cm$^R$-*ccdB-attR*2" with HSA cDNA from pENTER/D-TOPO *in vitro* by using a Gateway LR Clonase Enzyme Mix (Invitrogen). The vector was amplified in *Escherichia coli* using a Gateway LR Clonase Enzyme Mix. The above sequences were amplified as follows. *Ser*1 promoter was obtained by PCR from silkworm genomic DNA with the primer set nos. 3 and 4 (Table 1). BmNPV IE1 cDNA, BmNPV *hr*3 sequence, and *attR*1-Cm$^R$-*ccdB-attR*2 were obtained from pXINSECT-DEST 38 (Invitrogen). The direction of BmNPV IE1 cDNA was opposite to those of DsRed and HSA cDNA.

Table 1
List of oligonucleotides

| No. | Sequence (5′-3′) |
|---|---|
| 1 | CACCATGAAGTGGGTAACCTTTATTTCC[a] |
| 2 | TTATAAGCCTAAGGCAGCTTGACTTG |
| 3 | AGAAGACTATCTATCCGTGGTCC[b] |
| 4 | CAAGGATTCTGTGCAGCATTTG[b] |
| 5 | FAM-CCAGTTATGTGTGTTGCATGAGAAAACGCC-TAMRA[b] |
| 6 | CGTCATCTGAAGACGGTTTCTG[b] |
| 7 | GGTGCTCGTCTTATCGGTTGT[b] |
| 8 | FAM-TAGAAGGAAATCAGGATCCGGTCACAAAAG-TAMRA[b] |
| 9 | CATGCCACAACTGCTACACAGA[b] |
| 10 | CACCAGTAGAGGCAGGAATGATG[b] |
| 11 | FAM-CTGGAAAATTATGGCGTGATGGCCG-TAMRA[b] |

[a] The underline indicates a sequence for directional cloning.
[b] Primer sets, nos. 3 and 4, and the probe, no. 5, for measuring mRNAs for HSA. Primer sets, nos. 6 and 7, and the probe, no. 8, for measuring mRNAs for sericin-1. Primer sets, nos. 9 and 10, and the probe, no. 11, for measuring mRNAs for G3PDH. The 5′-ends of oligonucleotides, nos. 5, 8, and 11, were conjugated with fluorencin (FAM), and their 3′-ends were conjugated with tetramethylrhodamine (TAMRA).

### 2.4. Transient expression of rHSA in MSGs

pALBM2 was bombarded into MSGs using a particle delivery device, HELIOS Gene Gun (Bio-Rad Laboratories) as described previously (Adachi et al., 2006) with a few modifications. pALBM2 was precipitated onto gold particles at a density of 4 μg of DNA per mg of particles. MSGs were dissected from larvae at

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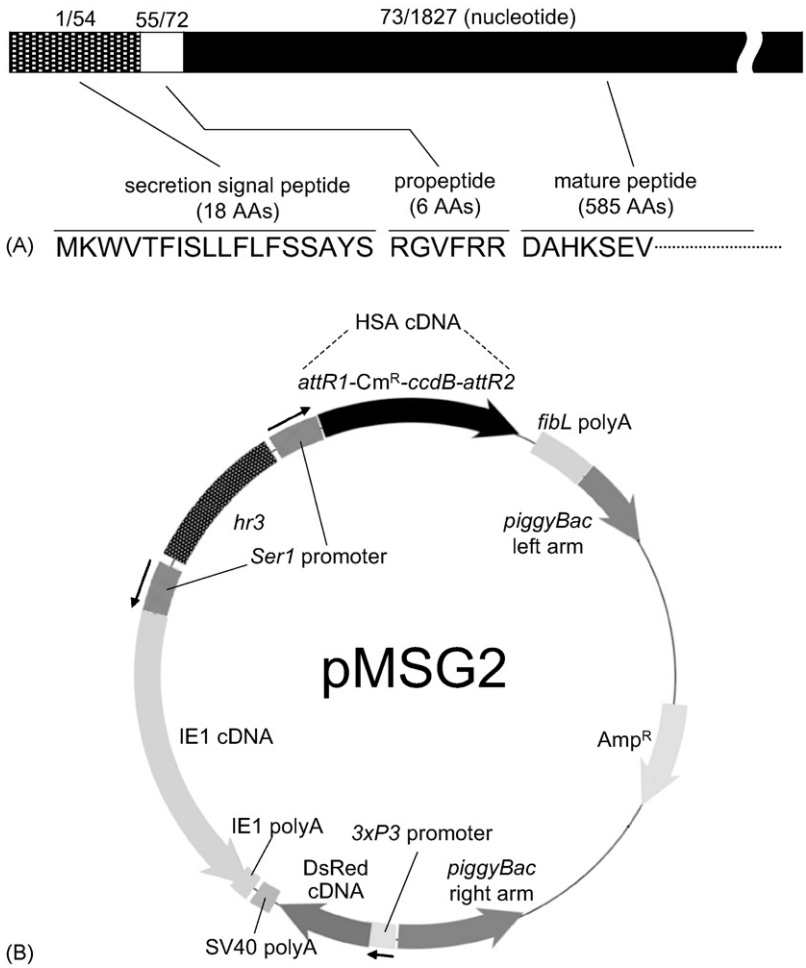


Fig. 1. The structure of HSA cDNA and the transformation vector pMSG2. (A) HSA cDNA. The cloned HSA cDNA encoded 18 amino acid (AA)-long secretion signal peptide (hatched box), 6 AA-long propeptide (open box), and 585 AA-long mature peptide (filled box). (B) Transformation vector pMSG2. pMSG2 contained three expression units between right- and left-arms of *piggyBac* as follows: (1) *3xP3* promoter-driven DsRed cDNA with a SV40 polyA signal sequence (SV40 polyA); (2) *Ser1* promoter-driven BmNPV IE1 cDNA with a IE1 polyA signal sequence (IE1 polyA); (3) BmNPV *hr3* enhancer-*Ser1* promoter upstream of a cDNA entry site, *attR1*-Cm$^R$-*ccdB-attR2*, where HSA cDNA was cloned later. A fibroin light chain gene polyA signal sequence (*fibL* polyA) was downstream of *attR1*-Cm$^R$-*ccdB-attR2*. Arrows indicate the direction of genes. pALBM2 was constructed by transferring the HSA cDNA from pENTER/D-TOPO to a *piggyBac*-based transformation vector pMSG2.

day 2 of the fifth instar and bombarded with the DNA-conjugated gold particles and were transplanted into the haemolymphatic cavities of host larvae at day 2 of fifth instar. Dissected silk glands without DNA-transfection were also transplanted as negative controls. After 3 days, the transplants were removed from host larvae. Transplants for SDS-polyacrylamide gel electrophoresis (PAGE) analysis were homogenized in 200 μl of Passive Lysis Buffer (Promega) and centrifuged at 10,000 × *g* for 15 min to obtain supernatants. Proteins in the supernatants were electrophoresed on 5–20% polacrylamide gradient gels (Atto). The gels were stained with CBB R250 or used for immunoblot analysis, in which proteins on the gels were transferred to nitrocellulose membranes. The membranes were sequentially reacted with mouse anti-HSA monoclonal antibodies (HSA-11, Sigma), horseradish peroxidase-conjugated rabbit anti-mouse IgG antibodies, and chemiluminescent substrates (ECL Western-blotting detection system, Amersham). Chemiluminescent sig-

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nals were recorded with an image analyzer LAS-1000 (Fuji Film). Fatty acid free- and essentially globulin free-HSA was obtained from Sigma and used as a standard. MSGs from some transplants for immunohistochemistrical analysis were fixed with 10% formalin in phosphate buffer overnight, frozen in O.C.T. compound (Ted Pella), and sectioned 10 μm thick. The sections were washed with phosphate buffered saline (PBS) for 15 min, blocked with 0.5% skimmed milk in PBS for 40 min, and incubated with rabbit anti-HSA polyclonal antibody A-0001 (DAKO) in the blocking solution for 2 h. Bound antibodies were detected with Alexa Fluor 488-labeled goat anti-rabbit IgG antibodies (Molecular Probes), and observed under a fluorescence microscope (Nikon).

### 2.5. Generation of germline transgenic silkworms

We generated transgenic silkworms "ALBM2" that synthesized rHSA in MSGs by germline transformation as described previously (Tomita et al., 2003). Over 12,000 eggs were injected with a mixture of pALBM2 and a helper plasmid, pHA3PIG (Tamura et al., 2000), which transiently expressed *piggyBac* transposase. Among them, 2057 eggs (16.5%) were hatched to larvae, 665 larvae of which were metamorphosed to moths. These moths were mated within the same family, which yielded 355 broods. We called a brood as a positive brood when the brood contained at least one DsRed-positive egg. We obtained 28 positive broods in the first generation (G1). Seventeen larvae from five positive G1 broods spun cocoons during the final larval instar and were metamorphosed to moths, which produced eggs of the second generation. Genomic Southern blot analysis indicated that these 17 G1 moths were divided into 12 different strains with respect to chromosomal insertion positions and copy numbers of the transgene, from which we selected strains with single copy transgene. As a result, 10 strains were obtained for further analysis. Proteins were extracted with PBS from small pieces of cocoons (about 5 mg) of the 10 single transgene strains. Their rHSA contents in cocoon extracts were determined by enzyme-linked immunosorbent assay with HSA ELISA kit (Bethyl), which ranged from 0.6 to 2.5 $\mu g\ mg^{-1}$ cocoon. A worm with the highest content was chosen and crossed with a wild-type worm. The offspring called ALBM2-171-6 strain were used in following experiments.

### 2.6. Quantification of HSA mRNAs

Total RNAs were prepared from MSGs and PSGs of larvae at day 3 of the fifth instar using ISOGEN (Nippongene). cDNAs were synthesized from the RNAs using SUPERSCRIPT first-strand synthesis system for RT-PCR (Gibco BRL). Primers and TaqMan probes for measuring mRNAs of *HSA*, *Ser*1, and *G3PDH* were designed by software, Primer Express (Applied Biosystems), and are listed in Table 1. cDNA fragments of *HSA*, *Ser1*, and *G3PDH* were amplified with the above primers and were inserted into a cloning vector pCR4 (Invitrogen). These plasmids were serially diluted and used for making the standard amplification curves. Reaction mixtures for the quantitative PCR were prepared according to the instruction supplied by the manufacturer (Applied Biosystems). PCR amplification and real time detection of amplicons were performed on 50 μl of the mixture using ABI PRISM 7700 Sequence Detector (Applied Biosystems) with the standard program (15 min-denaturation at 95 °C, followed by 50 cycles of incubation of "95 °C, 15 s" and "60 °C, 1 min"). Measurements were performed three times for each sample.

### 2.7. Immunocytochemial and immunoblot analyses of rHSA in cocoons spun by germline transgenic silkworms

Cocoons produced by transgenic animals were sectioned 5 μm thick and was immunohistochemically examined with anti-HSA antibodies as in MSGs of the transient expression experiments described above. After observing through a fluorescence microscope, sections were stained with Azure B, and viewed under a bright field microscope (Nikon).

Proteins were extracted from small pieces of cocoons (about 5 mg) with PBS (100 μl per 1 mg cocoons) overnight at 4 °C. Proteins were also extracted with saturated LiSCN containing 2% 2-meracptoethanol (100 μl per 1 mg cocoons) at room temperature. Most silk proteins remained insoluble in PBS, but dissolved in the LiSCN solution. The extracts were subjected to SDS-PAGE and immunoblotting as described above. nHSA was purified from Human Reference Serum (Bethyl) according to the method as described below, and used as a standard in SDS-PAGE and immunoblot analyses.

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### 2.8. Purification of rHSA from cocoons

All manipulations were performed at 4 °C unless otherwise noted. Fresh cocoons containing rHSA were ground in liquid nitrogen by a homogenizer and its aliquots (2 g) were extracted with 100 ml of PBS overnight. HSA was purified according to a previous report (Watanabe et al., 2001). Proteins in the PBS-extracts were precipitated by 60% saturated ammonium sulfate at pH 4.4, collected by centrifugation at 27,800 × *g* for 20 min, and resuspended in 5 ml of the starting buffer, 200 mM acetate buffer (pH 5.5). The proteins (ammonium sulfate-precipitates) were loaded onto a Blue-Sepharose CL-6B column (Amersham) at room temperature. The column was washed with five bed volumes of the starting buffer and then of the starting buffer containing 0.5 M NaCl. rHSA was eluted with 3 ml of 1.5 M NaCl in the starting buffer. The eluate was dialyzed against deionized water twice for 2 days and treated with charcoal SA-2 (Norit) to remove fatty acids bound to rHSA as described previously (Chen, 1967). Charcoal was removed by centrifugation at 27,800 × *g*, and then the supernatant was dialyzed against deionized water and then 67 mM phosphate buffer at pH 7.4, each twice. The dialysates were used as the purified rHSA. Aliquots (1/500 vol.) of PBS-extracts, ammonium sulfate-precipitates, and purified rHSA were analyzed by SDS-PAGE. Proteins on the gels were stained with CBB R250, and quantified with an image analyzer LAS-1000 (Fuji Film). rHSA in each purification step was quantified by dot-immunoblotting. The test samples and standard nHSA solutions (1 μl each) were spotted onto nitrocellulose membranes and dried up. The membranes were immunoblotted as above. Signals of HSA on the membranes were quantified with the image analyzer. Measurements were performed three times for each sample.

The N-terminal sequences of the purified rHSA were determined with a protein sequencer G1000A (Hewlett Packard) using a standard protocol of Edman degradation. For the degradation reaction, 36 pmol of HSA was loaded onto the sequencer and the reaction was carried out for seven cycles.

### 2.9. CD measurement

CD of HSA was measured using a spectropolarimeter J-820 (Jasco). HSA was dissolved in 50 mM phosphate buffer (pH 7.4) at the concentration of 0.75 μM for measurement in a far ultra-violet (UV) region, 195–250 nm, which was placed in a cell with 2 mm-pass length. For near UV, 250–300 nm, HSA was dissolved in the same buffer at 1.5 μM in a cell with 10 mm pass length. The slit width was set at 1 nm and dynode voltage never exceeded 0.7 kV. The temperature was kept at 25 °C during the procedures. Measurements were performed 10 times and the averaged values were plotted. Similarly, CDs of fatty acid free- and essentially globulin free-nHSA were measured.

### 2.10. Binding characteristics between HSA and ligands

Equilibrium dissociation constants ($K_D$) for binding between HSA and ligands were determined according to Rich et al. (2001) by monitoring the binding with surface plasmon resonance (SPR) which was measured using BIACORE S51 optical biosensors equipped with a research-grade CM5 chip (BIACORE AB), following the BIACORE application note 30 (http://www.biacore.com/lifesciences/service/application_notes). rHSA and nHSA were immobilized to the CM5 chip by amine-coupling in 10 mM citrate buffer (pH 4.7) at densities of 7800 resonance units (RU) and 8400 RU, respectively. Warfarin and naproxen (both Sigma) as analytes were dissolved at 30 mM in running buffer (10 mM phosphate buffer, pH 7.4, containing 3% dimethylsulfoxide) and stocked. Immediately prior to analysis, the two compounds were diluted with running buffer to make a series of concentrates (0, 0.78, 1.5, 3.1, 6.2, 12.5, 25, 50, and 100 μM).

Sample cycles consisted of a 60 s sample injection, a 30 s buffer flow (=dissociation phase), a wash with 50% dimethylsulfoxide, a 30 s regeneration pulse of 10 mM glycine–HCl (pH 1.5), a 30 s stabilization period, and finally, a 60 s buffer injection to check for carry over. All injections within the sample cycles were made at a flow rate of 90 μl $min^{-1}$. All assays were carried out at 25 °C. RU values of the compounds bound at equilibrium at each concentration were measured at three times.

The RU values were processed for reference subtractions and solvent corrections using BIACORE S51 evaluation software ver. 1.2.1 and were plotted at each concentration. Binding models and equilibrium dissociation constant ($K_D$) of warfarin and naproxen were

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obtained by fitting the obtained curves with the theoretical "ligands versus analytes saturation curves", using the above software. In this process RU values were converted to "%-bound figures" and $K_D$ was evaluated using the follow equilibrium equation: $K_D = (y - xp)(x - xp)/xp$, where $x$ is the total concentration of the compound, $y$ the total concentration of HSA, and $p$ is the fraction bound (%-bound/100).

## 3. Results

### 3.1. Synthesis and secretion of rHSA in transient expression system

Isolated MSGs were transfected with pALBM2, transplanted into haemolymphatic cavities of host larvae, harvested 3 days post-transplantation, and subjected to immunoblot analysis and immunohistochemistry for HSA. Immunoblotting on the extracts from the explants detected the major band that corresponded to the predicted molecular weight (66.5 kDa) of HSA (Fig. 2A). Transverse immunohistochemical sections of MSGs showed that rHSA was mostly secreted into sericin layers and slightly present in MSG cells (Fig. 2B). These results indicate that MSG cells are able to express rHSA and secrete it to the lumen of the glands.

### 3.2. Generation of transgenic silkworms

Plasmid mixtures consisting of pALBM2 and pHA3PIG were injected to more than 12,000 eggs. As a result, 10 HSA-expressing strains were obtained. Transgenic worms spun the cocoons during final instar. These cocoons were normal in appearance, size and weight, but they did not repel water, but were wettable differently from wild-type cocoons, which was explainable by the presence of rHSA in the sericin layers as described below.

The expression level of rHSA was determined by RT-PCR on silk glands of 5th instar larvae of transgenic silkworms (Table 2). Transgenic and wild-type worms expressed *G3PDH* mRNAs at similar levels. *HSA* mRNA was mainly expressed in MSGs. Unexpectedly, PSGs also expressed *HSA* mRNA, the level being approximately one third of that in MSGs. It was conceivable that IE1 and *hr3* may disrupt tissue-specific expression of the transgene driven by *Ser1* promoter. It should be noted that the amounts of *Ser1* mRNA in transgenic worms were significantly lower than those in wild-type animals. This might be due to the competition for transcription factors involved in the promoter activity of *Ser1*.

### 3.3. Localization, contents and solubility of rHSA in cocoons

Immunohistochemical examinations with anti-HSA antibodies were done on sections of both transgenic and wild-type cocoons (Fig. 3). rHSA proteins were present in sericin layers of the transgenic, but not wild-type silk threads. Cocoons were extracted with PBS and with saturated LiSCN that completely dissolves cocoon proteins (Takasu et al., 2002). These extracts were separated by SDS-PAGE and stained with CBB (Fig. 4, left panel). The band with the predicted molecular mass of HSA (66.5 kDa) was seen only in transgenic cocoons. Comparison of the staining between extracts of PBS and LiSCN showed that most of rHSA was extractable with PBS as with LiSCN. The quantity of rHSA in extracts was determined by immunoblotting (Fig. 4, right panel). The PBS and LiSCN extracts of 1 mg of cocoon powders contained 2.5 and 3.0 μg, respec-

Table 2
Expression levels of *HSA* mRNAs in MSGs of the transgenic silkworms

| mRNA | Transgenic | | Wild-type | |
|---|---|---|---|---|
| | MSG ($\times 10^3$) | PSG ($\times 10^3$) | MSG ($\times 10^3$) | PSG ($\times 10^3$) |
| *HSA* | 1320.3 ± 258.9[a] | 409.2 ± 92.2 | 0.0 | 0.3 ± 0.4 |
| *Ser1* | 5608.9 ± 1011.0 | 2.5 ± 4.6 | 7793.5 ± 875.6 | 1.2 ± 1.5 |
| *G3PDH* | 2353.3 ± 342.8 | 1972.8 ± 248.7 | 2203.8 ± 295.1 | 2061.1 ± 517.5 |

Copy numbers of mRNAs of *HSA*, *Ser1*, and *G3PDH* were determined in MSGs and PSGs of larvae at day 3 of the fifth instar by RT-PCR and expressed per 10 ng of total RNA.

[a] The mean ± S.D. of three samples.

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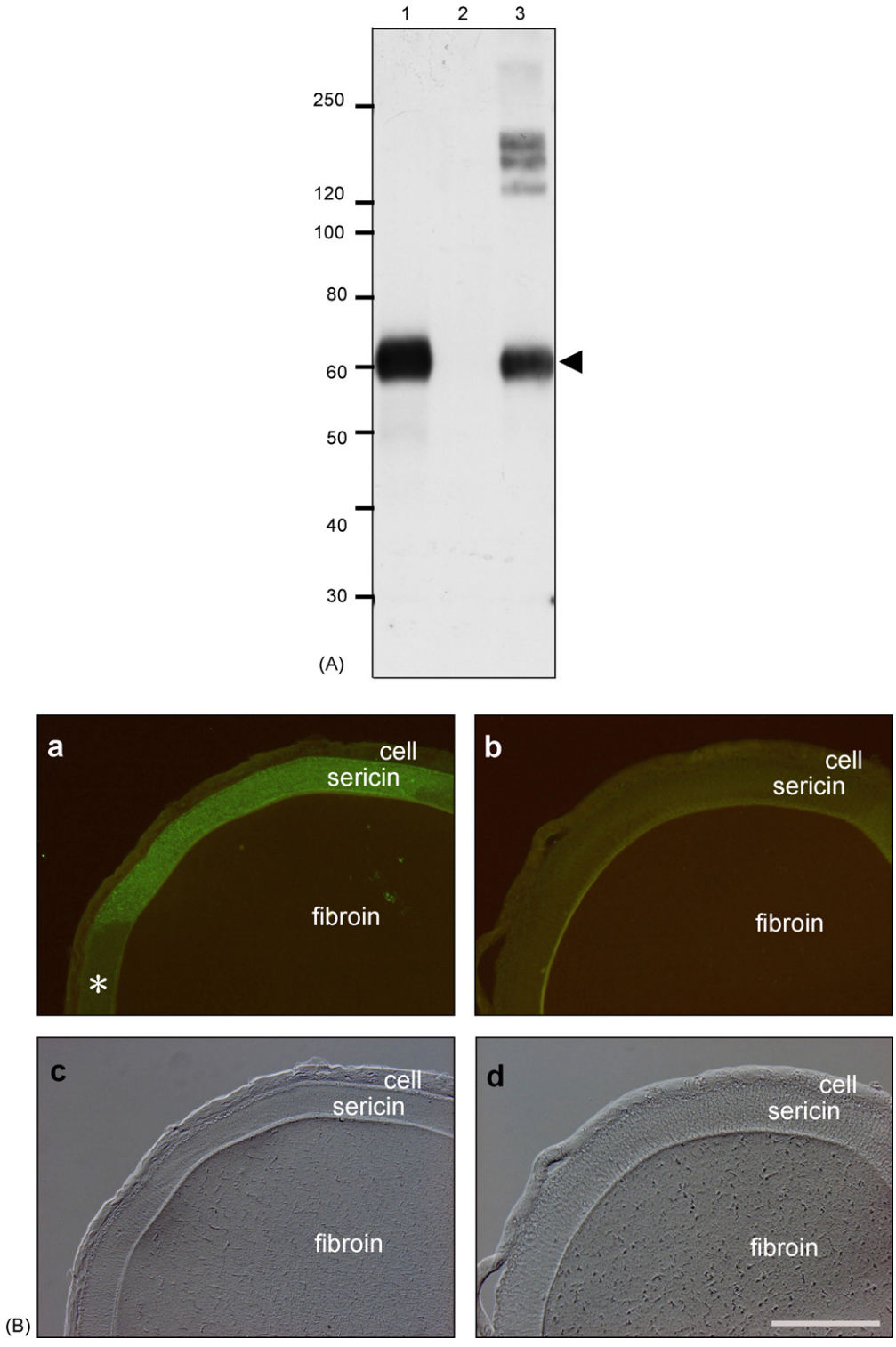


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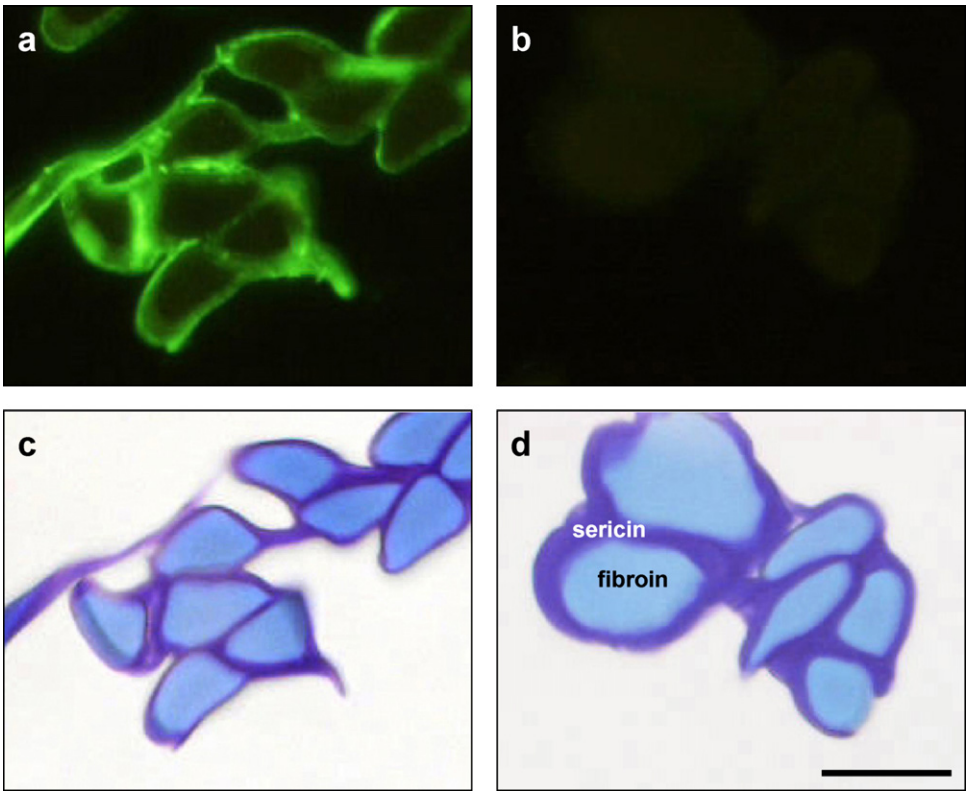


Fig. 3. Distribution of rHSA in cocoons of transgenic silkworms. Cocoons of transgenic (a and c) and wild-type (b and d) silkworms were sectioned, immunostained with anti-HSA antibodies, and viewed through a fluorescence microscope (a and b). Then, the sections were stained with Azure B and viewed through a bright field microscope (c and d). HSA (green in panel a) was located in the sericin layer which stained deep blue in panel c and d, whereas no HSA in the fibroin layer which was stained sky blue in panel c and d. Scale bar, 10 μm.

tively, indicating that 83% of rHSA in a cocoon was extractable with PBS.

### 3.4. Purification of rHSA from cocoons

We attempted to isolate and purify rHSA from 2 g cocoon powders as a starting material that had been estimated to contain 6 mg rHSA from the value of the above rHSA content per unit weight of the cocoon. The isolation procedure included three steps, PBS-extraction, ammonium sulfate-precipitation, and the final preparation of purified rHSA as detailed in Section 2. Aliquots of these three preparations were analyzed with SDS-PAGE for the protein composition. The gels were stained with CBB (Fig. 5) or subjected to dot immunoblot analysis with anti-

Fig. 2. Transient expression of rHSA in MSG. (A) Detection of rHSA in MSGs by immunoblotting. Isolated MSGs were transfected with pALBM2 vectors (lane 1) and without transfection (lane 2), transplanted into haemolymphatic cavities of host larvae, and kept there for 3 days. Extracts prepared from MSGs of the transplants were separated with SDS-PAGE together with a standard HSA (lane 3) and immunoblotted with anti-HSA antibodies. The Arabic numerals at the left side indicates molecular weights in kDa and the arrowhead at the right side points to the immunopositive band with 66.5 kDa. (B) Immunohistochemistry of HSA on MSGs transfected with pALBM2 vector. MSGs transfected with pALBM2 (a and c) or without transfection (b and d) were sectioned, and immunostained with anti-HSA antibodies. Then sections were viewed through a fluorescence (a and b) and a differential interference contrast microscope (c and d). Most of the HSA (green) was located in the sericin layer (sericin) in the lumen and small amounts of HSA were observed in MSG cells (cell), but no signals were detected in the fibroin layer (fibroin). Immuno-staining of the region marked by the asterisk in the sericin layer of panel a was weak, because this region tightly adhered to the glass plate and, thus, was not fully exposed to anti-HSA antibodies. Scale bar, 40 μm.

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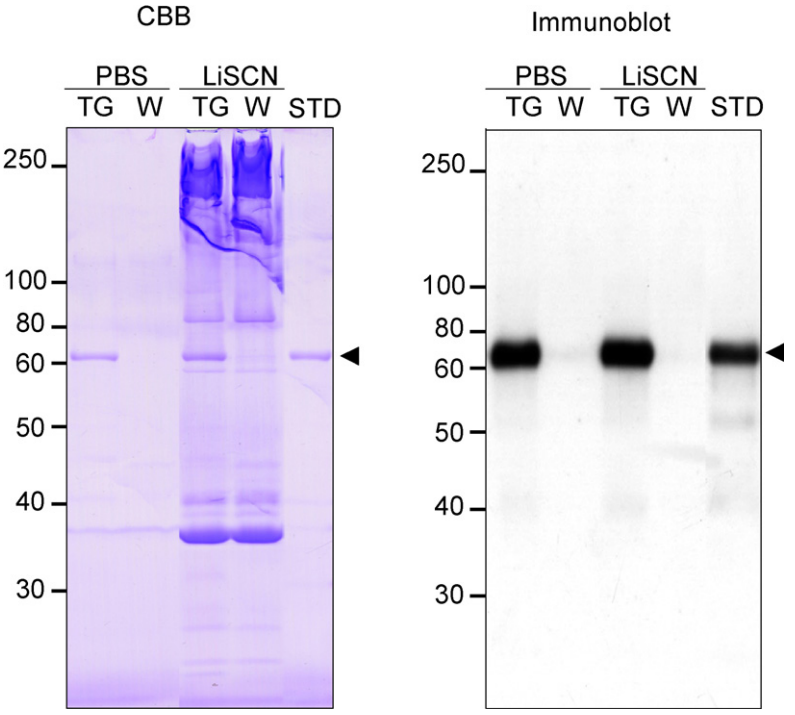


Fig. 4. Extraction of rHSA from cocoons of transgenic silkworms. Proteins were extracted from cocoons of transgenic (TG) and wild-type (W) silkworms with PBS and saturated LiSCN (LiSCN). The extracts and standard HSA (STD) were subjected to SDS-PAGE. Some gels were stained with CBB (left panel). The others were immunoblotted with anti-HSA antibodies (right panel). The arrowheads point to the band of HSA. Arabic numerals are molecular masses in kDa.

HSA antibodies. Purity and contents of rHSA in the three preparations were determined by densitometry on CBB-stained SDS-PAGE gels and dot immunoblots, respectively (Table 3). PBS-extracts contained 5.3 mg rHSA with a purity of 65%. The purity increased to 93 and 99% in the ammonium sulfate-precipitates and the final preparation that contained 3.3 and 2.8 mg rHSA, respectively, indicating that 59% of rHSA in PBS-extract was recovered in the final preparation.

The purified rHSA was subjected to an amino acid sequencer with seven cycles of Edman degradation. N-terminal amino acid sequencing of rHSA detected two amino acid peaks in each cycle as shown in Table 4. Intensities of the two peaks were at a nearly equal level. Considering the sequence of HSA shown in Fig. 1 (Dugaiczyk et al., 1982), we concluded that rHSA in cocoons was a mixture of mature albumin (upper raw in Table 4) and proalbumin (lower raw).

Table 3
Yield and purity of rHSA in the fractions obtained during purification

| Fraction | Yield (mg)[a] | Purity (%)[b] |
|---|---|---|
| PBS-extracts | 5.3 ± 0.4[c] | 65.1 ± 4.7 |
| Ammonium sulfate precipitates | 3.3 ± 0.3 | 93.2 ± 0.8 |
| Blue-Sepharose binding | 2.8 ± 0.2 | 99.0 ± 0.1 |

[a] Yield of rHSA were determined by dot blot analysis. The starting material (2 g of powdered cocoons) was calculated to contain 6 mg of rHSA.

[b] Purity of rHSA was determined by densitometric analysis of the CBB-stained gel shown in Fig. 5. Determinations were repeated three times.

[c] The mean ± S.D. of three samples.

Table 4
N-terminal sequencing of rHSA

| | Cycle | | | | | | |
|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Peak | R | G | V | F | R | R | D |
| | D | A | H | K | S | E | V |

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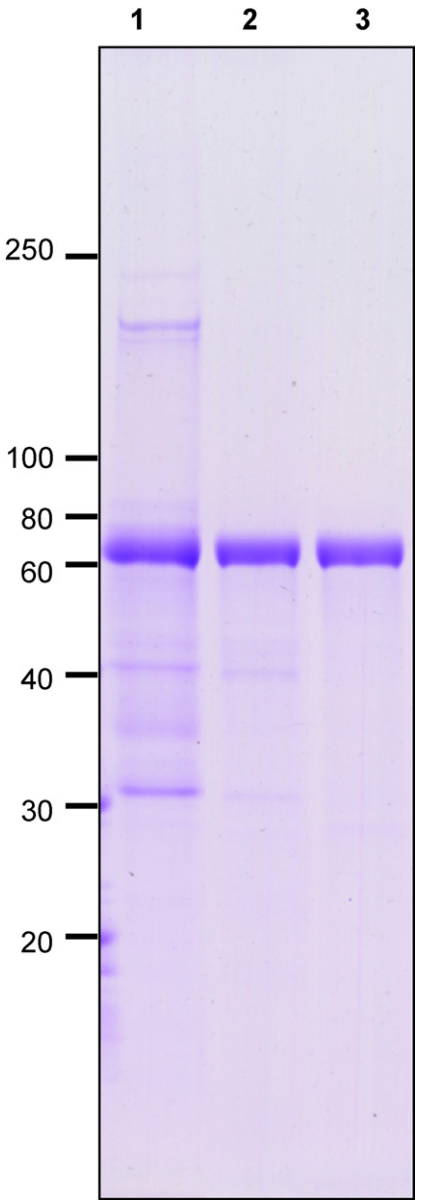


Fig. 5. Purification of rHSA from cocoons of ALBM2 transgenic silkworms. Cocoon proteins were extracted with PBS, precipitated with ammonium sulfate, and subjected to Blue-Sepharose affinity-chromatography. PBS-extracts (lane 1), ammonium sulfate-precipitates (lane 2), and purified rHSA (lane 3) were subjected to SDS-PAGE by loading 1/500 volume aliquots. Arabic numerals at the left side indicate molecular masses in kDa.

### 3.5. Structural characterization

CD spectra were obtained for rHSA and nHSA from 195 to 250 nm (far-UV) and from 250 to 300 nm (near-UV), which represents secondary (Horwitz et al., 1969) and tertiary structures of peptides (Yang et al., 1986). The spectra of rHSA were nearly identical to those of nHSA in both ranges of wave length (Fig. 6), including ellipticity peaks at 222 nm in the shorter range, representing the α-helix structure, and the negative maxima at 262 and 268 nm in the longer range, which are characteristic peaks of HSA (Sjoholm and Ljungstedt, 1973; Dockal et al., 1999). In addition, a CD spectrum was predicted using a calculation program (http://www.embl-heidelberg.de/~andrade/k2d/) by assuming that the contents of α-helix and β-structure were 66 and 11%, respectively (Wetzel et al., 1980). The above spectra for rHSA and nHSA showed a complete match to this predicted one (data not shown). Thus, it was concluded that rHSA was identical to nHSA in the secondary and tertiary structures.

### 3.6. Ligand binding properties

$K_D$s of rHSA for the binding to warfarin and naproxen were determined and compared to those of nHSA. Fitting curve analyses revealed that warfarin bound to rHSA following the multiple-site steady state (one specific site and one low affinity site) model as in the binding to nHSA. The binding of naproxen to rHSA followed the one-site steady state model also as in the binding to nHSA. $K_D$s for the binding of rHSA to warfarin and naproxen were 1.32 and 48.0 μM, respectively, which were close to those for nHSA, 1.12 and 33.8 μM, respectively. These data together indicated that rHSA was very close to nHSA in the ligand binding properties.

## 4. Discussion

In the present study, we developed a method to yield transgenic silkworms secreting rHSA into sericin layers of silk fibers. The weight content of rHSA in a cocoon was variable among the transgenic strains, 0.3% being highest. Most (83%) of rHSA was easily extracted from cocoons by immersing the powdered

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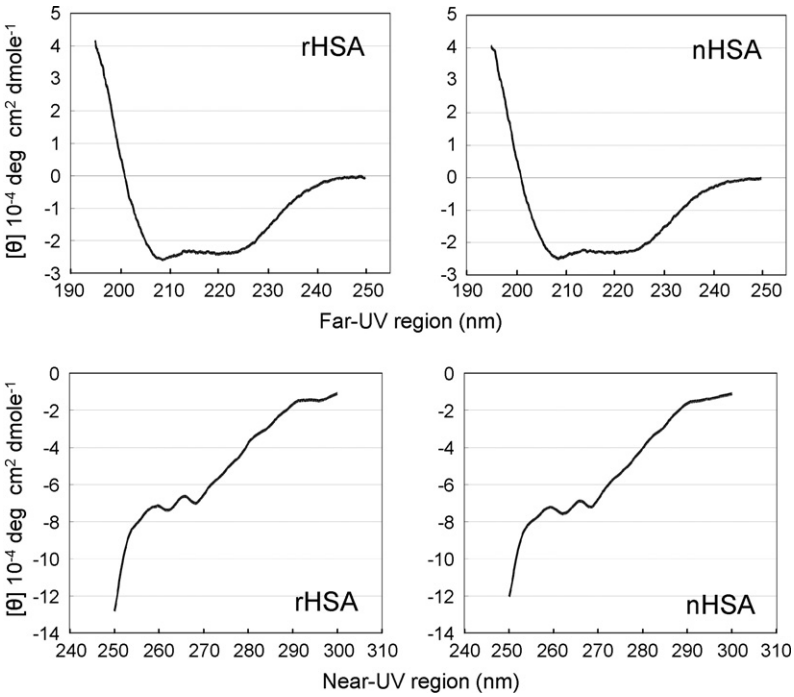


Fig. 6. CD spectra of HSA. Far-UV (195–250 nm) and near-UV (250–300 nm) spectra of rHSA and native HSA (nHSA) are shown. Measurements were performed 10 times and averaged values were plotted.

cocoons in PBS at 4 °C for 24 h, under which endogenous silk proteins were hardly extracted. CBB-stained gels of SDS-PAGE of PBS-extracts showed that the extract did not contain sericin, but contain minor cocoon proteins such as seroins and protease inhibitors (Nirmala et al., 2001). From the extracts, rHSA was purified by ammonium sulfate precipitation and Blue-Sepharose affinity chromatography. This simple method permitted us to obtain rHSA with 99% purity and 53% yield.

Sericin-1 protein has 38 amino acid residue-long repetitive motifs in its sequences. A predicted secondary structure of the protein suggested that these motifs form anti-parallel β-sheets (Garel et al., 1997). Sericin molecules formed insoluble meshwork structure comprised of random coils and locally formed β-sheets in a sericin film prepared from hot water-soluble sericins (Komatsu, 1975). Sericin molecules in the sericin layer of native silk fibers possibly take such structures. This assumption allows us to consider that rHSA proteins in silk fibers produced by transgenic silkworms are tightly trapped in insoluble sericin meshwork, but easily released from it when the fibers are soaked in PBS.

In mammalian hepatocytes, serum albumin was synthesized in endoplasmic reticulum as proalbumin with the six amino acid residue-long propeptide at the N-terminus. After transported to Golgi apparatus, the propeptide is removed by a processing enzyme, furin, and then is secreted from the cells as the mature albumin (Quinn et al., 1975; Ikehara et al., 1976; Peters and Reed, 1980; Peters and Davidson, 1986). N-terminal sequencing showed that the purified rHSA preparation was a mixture of mature HSA and proalbumin, suggesting that the propetides were incompletely removed from proalbumins in Golgi apparatus of MSG cells. The incomplete processing of rHSA could be explained by the assumption that amounts of recombinant proalbumin was relatively excessive compared to the processing enzyme present in Golgi apparatus and/or by differences of the substrate specificity between human furin and its silkworm homolog, the latter having not been identified to date. It was reported that deletion of the propeptide inhibited transport of

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albumin through the secretion pathway (McCracken and Kruse, 1989). However, *Pichia pastoris* infected with mature HSA cDNA that lacked the sequence of the propeptide was able to translate its message into the protein with proper structures and functions, and secrete the protein adequately (Watanabe et al., 2001). Thus, completely processed HSA might be synthesized also in the transgenic silkworms by introducing the cDNA lacking the propeptide-coding sequence.

The conformation of rHSA was studied by CD spectroscopy. We concluded that rHSA synthesized by the transgenic silkworms was identical to nHSA in secondary and tertiary structures, because the spectra of rHSA were identical to those of nHSA. Thus, rHSA is considered to take the native conformation in the sericin layer of the silk fibers, and this conformation was not destroyed during the extraction performed in this study.

One of the important functions of HSA is to transport a variety of small molecules with low affinities. According to "Kragh-Hansen's" model, there are five major binding sites for ligands in HSA. Long-chain fatty acid, diazepam, bilirubin, salicylate, and warfarin each is known to interact with each of five sites (Kragh-Hansen, 1990). We investigated affinities of drugs toward two binding sites of rHSA, the warfarin site and the benzodiazepine site (Sudlow et al., 1975), using warfarin and naproxen as ligands for the warfarin and benzodiazepine sites, respectively. $K_D$ values of rHSA and nHSA against warfarin and naproxen were determined by fitting analysis of SPR sensorgrams as reported previously (Frostell-Karlsson et al., 2000; Rich et al., 2001). From this analysis, it was concluded that ligand binding properties of rHSA were very similar to those of nHSA at both binding sites. Thus, we concluded that rHSA is identical to native HSA in its function on drug binding.

The present study demonstrated that rHSA was produced in the sericin layers of silk fibers spun by germline transgenic silkworms, and that rHSA was selectively extracted with PBS from cocoons with a small amount of contaminated silk proteins. This technology is applicable to production of a wide range of clinically valuable recombinant proteins including blood coagulation proteins, cytokines, growth factors, and peptide hormones. At present, the yield of recombinant proteins in the sericin layer is not high enough for industrial purposes. Development of a technology to increase the yield is a future target of research, which will permit us to produce precious clinically valuable recombinant proteins in bulk.

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