In Vitro and *in Vivo* Properties of Recombinant Human Serum Albumin from *Pichia pastoris* Purified by a Method of Short Processing Time

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Purpose. Recombinant human serum albumin (rHSA), secreted by a *Pichia pastoris* expression system, was purified by a fast and efficient method, the essential feature of which is strong but reversible binding of the protein to Blue Sepharose. The structural characteristics, stability, and ligand-binding properties of the resulting protein were examined, and pre-clinical studies were performed.

Methods. Protein structure was investigated by amino acid sequencing, sodium polyacrylamide gel electrophoresis, CD spectroscopy and chromatography. Stability was examined by denaturation by guanidine hydrochloride and by calorimetry, and ligand binding was studied by ultrafiltration. Rat experiments were performed with ^{125}I-labeled albumin.

Results. Far-ultraviolet and near-ultraviolet CD spectra of rHSA were identical to those of human serum albumin isolated from serum (HSA). Mercaptalbumin and non-mercaptalbumin were separated by high-performance liquid chromatography using an *N*-methylpyridinium polymer-based column. 60% of rHSA existed as mercaptalbumin, a content that is higher than that of a commercial preparation of HSA. Fatty acids, *N*-acetyl-L-tryptophan and pasteurization had similar effects on the conformational stability of rHSA and HSA. Stereoselective ligand-binding properties (warfarin, phenprocoumon, pranoprofen and ibuprofen) of rHSA were the same as those of HSA. The effect of the neutral to base transition on warfarin (site I-ligand) and dansylsarcosine (site II-ligand) binding to rHSA was also similar to HSA. *In vivo* studies showed comparable half-lives, excretion and tissue distributions of the two albumin preparations.

Conclusion. The present yeast expression system and purification procedure result in rHSA with structural and functional properties very similar to those of HSA.

KEY WORDS: recombinant human serum albumin; purification procedure; structural properties; molecular stability; ligand binding; *in vivo* half lives.

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INTRODUCTION

Human serum albumin (HSA), which is the most abundant protein in plasma, is a major antioxidant, transport, and depot protein and is important in maintaining normal osmolarity in plasma and in interstitial fluid (1,2). It is used clinically to treat severe hypoalbuminemia or traumatic shock. Currently, the only source of HSA for clinical application is donated human plasma. However, the source is limited in supply and has the risk of transmission of pathogenic vira such as hepatitis, HIV, and others not yet identified. The vira usually are destroyed by heat treatment of HSA at 60°C for several hours. During this pasteurization process, sodium caprylate and *N*-acetyl-L-tryptophan are widely used as stabilizers. Several groups have attempted to produce recombinant HSA (rHSA) by recombinant DNA technology and by using different host organisms (e.g., Refernces 3,4). rHSA has a number of advantages compared to plasma-derived HSA, because it is free of human-derived infectious agents.

Albumin is a 66.5-kDa single-chain, non-glycosylated polypeptide that organizes to form a heart-shaped protein having approximately 67% α-helix but no β-sheet (5–7). The protein is composed of three homologous domains (I–III), and each domain has two subdomains (A and B) that possess common structural elements. It contains 35 cysteinyl residues, of which 34 form 17 stabilizing disulfide bridges. However, HSA is a heterogeneous mixture of mercaptalbumin (HMA) and nonmercaptalbumin (HNA) (8,9). In HMA the last cysteine residue, at position 34, has a free SH group (10). In HNA, the residue is not free but forms a mixed disulfide with cysteine or glutathione (10) or undergoes oxidation (11) during circulation in body.

HSA is able to bind reversibly a large number of endogenous and exogenous compounds, an ability that implies that the protein can serve as an almost universal transport and depot protein in the circulation (1,2). The capability to bind aromatic and heterocyclic compounds resides to a great extent on the existence of two major binding regions, namely Sudlow's site I and site II (12), which are located within specialized cavities in subdomain IIA and IIIA, respectively (5–7). It is well known that a pH-dependent conformational change occurs in HSA around physiologic pH (2). This conformational change is called neutral to base or N-B transition. In phosphate buffer at about pH 6, almost all the protein is in the N conformation, whereas at pH 9 the B form is predominant. Binding of some ligands, especially site I-ligands, are effected by the N-B transition (13,14).

In the present work, rHSA was overexpressed and secreted by using the yeast species *Pichia pastoris*. The protein can be purified from the culture medium by a combination of ion exchange, hydrophobic interaction, and gel permeation chromatography (15). However, we used a faster and more simple and efficient method based on the use of Blue Sepharose for purifying rHSA. The structural characteristics, stability, and ligand-binding properties of the protein were elucidated. Furthermore, preclinical studies were performed using the rat as a model. The experimental results clearly indicate that the rHSA is correctly processed and purified because it has a tertiary structure and *in vitro* and *in vivo* behaviors comparable to those of HSA isolated from serum.

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MATERIALS AND METHODS

Materials

HSA was donated by Chemo-Sera-Therapeutic Research Institute (Kumamoto, Japan) and was defatted using charcoal treatment as described by Chen (16). *N*-acetyl-L-tryptophan was purchased from Nacalai Tesque (Kyoto, Japan). Sodium caprylate and dansylsarcosine were purchased from Sigma Chemical Co. (St Louis, MO). Ibuprofen was obtained from Kaken Pharmaceutical Co. (Osaka, Japan). Potassium warfarin was donated by Eisai Co. (Tokyo, Japan). Pranoprofen was donated by Yoshitomi Pharmaceutical Industries (Fukuoka, Japan). Phenprocouman was donated by Hoffman–LaRoche (Basel, Switzerland). Restriction enzymes, T4 polynucleotide kinase, calf intestinal alkaline phosphatase, a DNA ligation kit, and TaKaRa EX *Taq* DNA polymerase were obtained from Takara Shuzo Co. Ltd. (Kyoto, Japan). A DNA sequence kit was obtained from Perkin–Elmer Applied Biosystems (Tokyo, Japan). *Pichia* Expression kit was purchased from Invitrogen Co. (San Diego, CA). Blue Sepharose CL-6B was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). $Na^{125}I$ was a product of NEN™ Life Science Products, Inc. Other chemicals used were obtained from commercial suppliers.

Synthesis and Purification of rHSA

Construction of Expression Vector

A chimaera plasmid (pJDB-ADH-L10-HSA-A) having cDNA for the mature form of HSA along with an L10 leader sequence (coding for EFMKLLLLLLLLLSSSSSSSS) was a gift from Tonen Co. (Tokyo, Japan). The L10-HSA coding region was amplified by polymerase chain reaction with a forward and a reverse primer carrying a 5′-terminal *Eco*RI site and cloned into the *Eco*RI-digested methanol-inducible pHIL-D2 vector (Invitrogen) to construct HSA expression vector pHIL-D2-HSA as detailed in Figure 1. All of the cloning procedures were conducted in accordance with the guidelines for research involving recombinant DNA molecules issued by the Ministry of Education, Science, Sports and Culture of Japan.

Expression of rHSA

The pHIL-D2-HSA expression vector was introduced into the yeast species *Pichia pastoris* (strain GS115) by the lithium chloride method following the protocol given by the manufacturer (Invitrogen). A yeast clone that contained the expression cassette stably integrated into the chromosomal DNA was isolated. In the growth phase, the cells were grown to confluence in 100 mL of BMGY [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 1% glycerol] in 1-L flasks at 30°C with shaking. The cells were grown to an attenuance of 2 at 600 nm after 30 h of incubation. In the induction phase, the growth-phase cells were harvested by centrifugation (1500 × g, 10 min, 4°C), and cell pellets were resuspended with 1 L of BMMY [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base with ammonium sulfate without amino acids, 4×10^{-5}% biotin, 1% methanol] in 3-L flasks and returned to the 30°C shaker. The cells were grown for an additional 96 h. Methanol (100%) was added to give a final concentration of 0.5% methanol every 24 h to maintain induction.

Purification of rHSA

The growth medium was separated from the yeasts by centrifugation (6,000 × g, 10 min, 4°C), and the secreted rHSA was isolated from the growth medium as follows (Fig. 2A). The medium was brought to 60% saturation with ammonium sulfate at room temperature. The temperature was then lowered to 4°C, and the pH was adjusted to 4.4, the isoelectric point of HSA (17). The precipitated protein was collected by centrifugation (10,000 × g, 20 min, 4°C) and re-

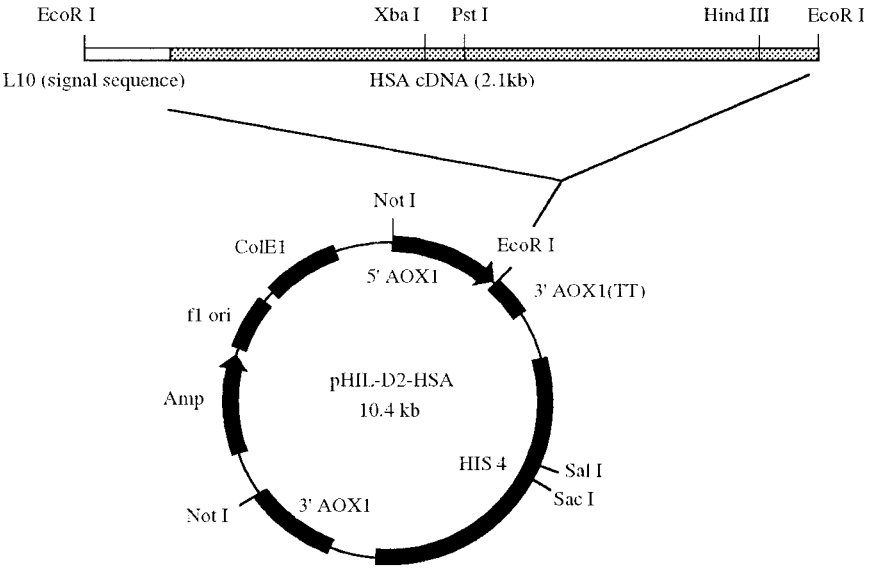

Fig. 1. Expression construct for the recombinant production of rHSA in *Pichia Pastoris.* The hatched box indicates cDNA coding for the mature form of rHSA, and the white box indicates a L10 signal sequence.

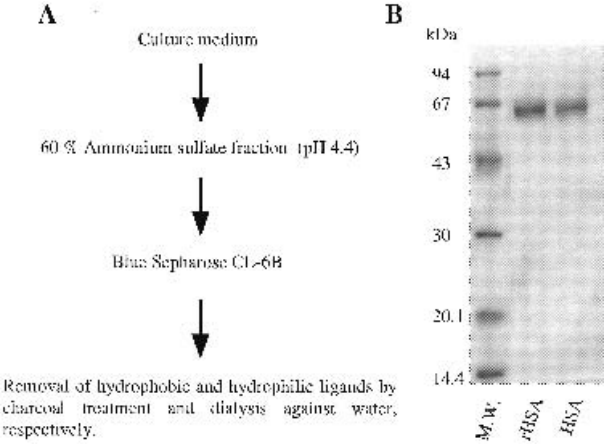

Fig. 2. (A) Flow chart for the purification of rHSA. (B) Sodium polyacrylamide gel electrophoresis of rHSA and HSA. M.W., molecular weight markers.

suspended in distilled water. Dialysis was performed for 48 h at 4°C against 100 volumes of distilled water, followed by a further 24 h against 100 volumes of 200 mM sodium acetate buffer at pH 5.5. Afterwards, the solution was loaded on to a column of Blue Sepharose CL-6B. The column was washed with about 5 bed volumes of 200 mM sodium acetate buffer at pH 5.5, and then the rHSA was eluted with 3 M sodium chloride. The eluted rHSA was deionized, defatted using charcoal treatment as described by Chen (16), freeze-dried, and then stored at –20 °C until use. A small portion (0.5 mg) of the resulting protein was boiled in 0.025 M Tris buffer, pH 6.8, containing 100 mM dithiothreitol and immediately analyzed by sodium polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2B). The recombinant protein exhibited a single band and migrated to the same position as HSA. Sample purity was estimated by density analysis of Coomassie Brilliant Blue-stained protein bands on the SDS-PAGE gels. Each protein sample was more than 97% pure. N-terminal amino acid sequence of the protein was determined by a Perkin–Elmer ABI 477A protein sequencer.

Intrinsic CD Spectra Analysis

Measurements were made with a Jasco J-720 type spectropolarimeter (Tokyo, Japan) at 25°C. Far-ultraviolet (UV) and near-UV spectra were recorded from 200 to 250 nm and from 250 to 350 nm using protein concentrations of 1.5 μM and 15 μM, respectively, in 67 mM sodium phosphate buffer (pH 7.4). For calculation of the mean residue ellipticity [θ], the molecular mass of albumin was taken as 66.5 kDa.

Determination of HMA and HNA

The HPLC system was comprized of a Shimadzu LC-4A pump (Tokyo, Japan) equipped with a gradient programmer and a Shimadzu SPD-2AS UV monitor. The column packing material, *N*-methylpyridinium polymer cross-linked with ethylene glycol dimethacrylate, was prepared as described previously (18,19). Albumin was eluted with a 30-min linear gradient from 0 to 0.5 M sodium chloride in 0.05 M Tris-acetate buffer (pH 6.5) at a flow rate of 0.5 mL/min at 25 °C with detection at 280 nm.

Solvent-Induced Denaturation of rHSA and HSA Using Guanidine Hydrochloride (GdnCl)

Samples of 1.5 mg/mL of rHSA or HSA in 67 mM sodium phosphate buffer (pH 7.4) with different concentrations of GdnCl were incubated at 25°C for 24 h prior to analysis to ensure that equilibrium had been achieved. After equilibrium had been attained, circular dichroism measurements were performed with a Jasco J-720 type spectropolarimeter. Ellipticity at 222 nm was used. Assuming a two-state behavior, the equilibrium denaturation profiles were analyzed using a linear extrapolation method as follows: The equilibrium constant for unfolding, K_D, in the presence of a denaturant can be calculated from Equation 1:

$$K_D = \frac{f_N - f_{obs}}{f_{obs} - f_D} \quad (1)$$

where f_{obs} is the observed ellipticities, and f_N and f_D, calculated from the respective baselines before and after denaturation, are the ellipticity of native and denaturant form of albumin, respectively. The values of K_D can be used to calculate ΔG (Eq. 2):

$$\Delta G = -RTlnK_D \quad (2)$$

In this equation, R is the gas constant, and T is the absolute temperature. Now, because it has been found that the free energy of unfolding of proteins, ΔG, in the presence of GdnCl, is linearly related to the concentration of guanidine hydrochloride ([*GdnCl*]), ΔG_{H2O} and m can be calculated by plotting ΔG vs. [*GdnCl*] as indicated in Equation 3:

$$\Delta G = \Delta G_{H2O} - m[GdnCl] \quad (3)$$

where ΔG_{H2O} is the apparent free energy of unfolding in the absence of denaturant, and m is the slope of the linear plots of ΔG vs. [*GdnCl*]. This denaturation process induced by GdnCl was confirmed to be a reversible process, because removal of GdnCl by dialysis from the samples showed similar intensities of CD as for the native forms of rHSA and the HSA.

Differential Scanning Calorimetry (DSC)

DSC was performed on a MicroCal MC-2 ultrasensitive DSC (MicroCal Inc., Northampton, MA) using heating rates of 1 K/min and a sample concentration of 0.1 mM in 67 mM sodium phosphate buffer (pH 7.4). The calorimetric reversibility of the thermally induced transition was checked by reheating the protein solution in the calorimetric cell after cooling from the first run, and it was observed that heating to above 85 °C caused irreversible denaturation of the proteins. The data obtained from DSC and the temperature dependence of excess molar heat capacity, C_p, were applied to nonlinear fitting algorithms to calculate the thermodynamic parameters, thermal denaturation temperature (T_m), and calorimetric enthalpy (ΔH_{cal}), by employing Using Origin™ scientific software.

Ligand-Binding Experiments

To study ligand binding, we added one of the racemic compounds (warfarin, phenprocoumon, pranoprofen, and ibuprofen) or dansylsarcosine to 10 μM rHSA or HSA solu-

tions, respectively, in 67 mM sodium phosphate buffer (pH 7.4 for stereoselective ligand-binding experiments, and pH 6, 7, 8, or 9 for the effect of the N-B transition on ligand-binding) to give a final ligand concentration of 5 μM. The unbound ligand fractions were separated using the Amicon MPS-1 micropartition system with YMT ultrafiltration membranes by centrifugation (2000 × g, 25 °C, 40 min). Adsorption of warfarin, phenprocoumon, pranoprofen, ibuprofen, or dansylsarcosine onto the filtration membranes and apparatus were negligible. Unbound ligand concentrations were determined by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump equipped with a fluorescence detector (Hitachi F-1000) and a Hitachi D-2500 chromatointegrator. For the stereoselective ligand-binding experiments, the assay was performed on a Chiralcel OJ column (25 cm × 0.64 cm i.d., Daicel Chemical Industries, Ltd., Tokyo, Japan) with a mobile phase composed of *n*-hexane:2-propanol:acetic acid (87.5:12:0.5, v/v) for warfarin, phenprocoumon, pranoprofen, and ibuprofen at a flow rate of 1.0 mL/min. For the effect of the N-B transition on the racemic warfarin and dansylsarcosine binding, an Inertsil ODS-2 column (5 μm, 4.6 × 150 mm) was used as the stationary phase. The mobile phase consisted of 0.1 M acetate buffer (pH 4.5):acetonitrile (40:60, v/v) for warfarin and dansylsarcosine. The flow rate was 1.0 mL/min. The excitation/emission wavelengths were 310 nm/390 nm, 340 nm/382 nm, 250 nm/382 nm, 263 nm/293 nm, and 350 nm/482 nm for warfarin, phenprocoumon, pranoprofen, ibuprofen, and dansylsarcosine, respectively. Unbound fraction (%) was calculated according to the following equation:

$$\text{Unbound fraction (\%)} = [\text{ligand concentration in filtered fraction/total ligand concentration (before ultrafiltration)}] \times 100$$

Z-values, which is an indicator for the hydrophobicity of binding sites, were estimated according to the procedure described by Turner and Brand (20).

In Vivo Studies

Iodinated albumin was prepared by the method of Hunter and Greenwood (21), and the labeled protein was separated from the iodination reaction mixture on Bio-Gel PD-10. The recovered [^{125}I]-albumin had a specific activity of 15×10^6 cpm/mg. Labeled proteins in 0.9 % NaCl were injected intracardially into rats (200–255 g, male, Sprague–Dawley); the kinetics of protein clearance from the circulation were measured in samples of blood obtained from lateral tail veins. Urine and feces were collected at 24-h intervals from animals kept in metabolic cages. Acid precipitability of recovered radioactivity was determined by addition of 1 mL 20 % trichloroacetic acid to 25 μL of plasma or urine both diluted to 100 μL with water, followed by centrifugation in an Eppendorf Microfuge. Radioactivity was measured in both supernatant and pellet. In the case of feces and the different tissues, radioactivity was determined by direct counting of the material.

RESULTS AND DISCUSSION

Structural Characterization

N-terminal amino acid sequence analysis for 20 cycles revealed that about half of the recombinant protein has two additional serine residues derived from the L10 leader sequence, and that the remaining has zero, one, or three extra serine residues as compared with HSA isolated from serum. This N-terminal heterogeneity could be due to the substrate specificity of the processing enzymes within the yeast and/or caused by the presence of extracellular aminopeptidase(s). SDS-PAGE analysis revealed that the molecular mass of rHSA is identical to that of HSA (Fig. 2B). Taken together these findings exclude the possibility of large truncations or extensions of the N- and C-terminal ends.

To obtain information about secondary and tertiary protein structures, CD measurements were performed in the far-UV and near-UV regions. rHSA and HSA display the same spectra in these regions (data not shown). These results suggest identical tertiary structures of rHSA and HSA.

Human serum albumin is a heterogenous mixture of HMA and HNA (8,9). In serum, HMA is in equilibrium with HNA, and previous reports have shown that the HMA fraction decreases with aging and in various diseases, such as chronic renal failure, nephrotic syndrome, various type of hepatitis, and liver cirrhosis (22,23). HNA is a general term that includes, for example, HSA-cysteine disulfide and oxidized HSA, and in this work these forms are called HNA_{Cys} and HNA_{oxy}, respectively. Sugii and coworkers have developed an *N*-methylpyridinium polymer-based (4VP-Me) column that is able to separate HMA from the different forms of HNA (18). The results of running rHSA and HSA samples on such a column are given in Table I. As shown in Table I, approximately 60% of rHSA existed as HMA, a value that is nearly the same as that found for serum from normal subjects (2). However, the HMA fraction of our commercial preparation of HSA was approximately 40%. This relatively low value is caused by an increased amount of HSA_{Cys} (Table I). On the other hand, there was no difference in the fraction of HSA_{oxy} in the two albumin preparations. Cys-34 is one of the major ligand binding sites for thiol-containing compounds, such as cysteine, glutathione, and captopril (19), as well as for various metal ions, such as Cd^{2+}, Au^{3+}, Hg^{2+}, and Ag^{2+}, which bind to this residue in a reversible manner (2). Recently, Meyer and coworkers reported that Cys-34 is also capable of binding superoxide and nitric oxide (24). In the present experiment, we found a higher content of HMA in the rHSA preparation than in the HSA preparation. This finding indicates that the ligand-binding properties of Cys-34 are not impaired in our rHSA.

Effect of Fatty Acids and *N*-Acetyl-L-Tryptophan on Structural Stability

A major problem in the usage of serum albumin in clinical applications is viral contamination, e.g., hepatitis, HIV, and herpes virus. At present, pasteurization (60°C, 10 h) is

Table I. Fractions (%) of Different Albumin Forms in Samples of rHSA and HSA

Albumin preparation	Fraction (%)			
	HMA	HNA_{Cys}	HNA_{oxy}	Non-identified form (s)
rHSA (fatty acid free)	63.3	14.7	15.2	6.8
HSA (fatty acid free)	38.9	40.5	13.7	6.9

generally used to eliminate such contamination. Sodium caprylate and *N*-acetyl-L-tryptophan are widely used to stabilize HSA during the heat treatment. Thus, it is important to elucidate the protein stability and to estimate the effects of the stabilizers on the stability of albumin. Therefore, the structural properties of rHSA were investigated by studying its thermal denaturation in the absence and presence of fatty acids and *N*-acetyl-L-tryptophan. In an attempt to do a more complete study, the GdnCl-induced denaturation was also studied.

The unfolding of rHSA and HSA by GdnCl was followed by changes in their far-UV CD spectra (ellipticities at 222 nm). The transition curve of both albumins exhibited an apparent two-state denaturation behavior (data not shown). Using these data, thermodynamic parameters on GdnCl denaturation were estimated. Table II shows the effect of fatty acids, *N*-acetyl-L-tryptophan, and pasteurization on those parameters. The value of m is generally accepted as an index of the extent of exposure of non-polar surfaces, and ΔG_{H2O} represents the apparent free energy of secondary structure in the absence of GdnCl. Adding palmitate or oleate to the albumins led in both cases to an increace in m and ΔG_{H2O} from approx. 2.5 kcal/mol/M to approx. 3.0 kcal/mol/M, and from approx. 5.0 kcal/mol to approx. 6.6 kcal/mol, respectively. However, addition of sodium caprylate with *N*-acetyl-L-tryptophan resulted in almost a doubling of both m and ΔG_{H2O} as compared with the albumins without additives (control). If the albumins first had been pasteurized (60 °C, 10 h) without additives, they completely lost their cooperative denaturation behavior. However, if the proteins first had been pasteurized in the presence of sodium caprylate and *N*-acetyl-L-tryptophan, then their stabilities (e.g., m and ΔG_{H2O} values) were very much improved and comparable to that observed without pasteurization (Table II).

The thermal denaturation was estimated by DSC (Table III). rHSA and HSA with or without fatty acid showed single sharp endotherms (data not shown). Significant increases in thermodynamic parameters (T_m, ΔH_{cal}) were observed by adding palmitate and oleate. Smaller, but still significant, increments were observed after addition of sodium caprylate with *N*-acetyl-L-tryptophan. After pasteurization without additives none of the albumins had an endotherm (data not shown). However, for both proteins the situation was very much improved by adding sodium caprylate with *N*-acetyl-L-tryptophan before pasteurization (Table III).

Recent crystallographic analyzes showed that albumin has three homologous domains that are composed of two subdomains connected by a flexible region. The analyzes also showed that interdomain and intersubdomain interactions contribute significantly to the stability of the albumin molecule (5–7). Therefore, the similar structural stability observed here indicates that there are no major differences in the secondary and tertiary structures of rHSA and HSA.

Ligand Binding

Albumin has two major ligand binding sites, namely the warfarin site (site I) and the indole and benzodiazepine site (site II). By using warfarin (site I-ligand) and dansylsarcosine (site II-ligand) as fluorescence probes (12), we have confirmed that the rHSA used in this study has the two binding sites just mentioned (data not shown).

To characterize and compare the microenvionment of the ligand binding sites on the two albumins, Z values, which are measures of the hydrophobicity in the binding sites, were determined using the solvent-dependent emission maximum wavelength (λ_{max}) of probes. Turner and Brand (20) estimated the Z values for 1-anilinonaphthalene-7-sulfonate binding sites on 20 proteins and constructed a hydrophobicity scale. We also adopted Z values as a measure for hydrophobicity in this study. The λ_{max} of warfarin and dansylsarcosine was plotted as a function of Z values. There were good correlations between λ_{max} and the Z value in both systems (data not shown). As seen in Table IV, the Z values for the warfarin and dansylsarcosine binding sites estimated for the two albumins were almost the same, indicating that the ligand binding sites of rHSA have hydrophobicities similar to those of HSA.

Many chiral compounds, which are commercially available, are clinically used as racemates. However, it has been demonstrated that the pharmacologic activity and pharmacokinetic behavior can be different for optical isomers, and plasma protein binding is stereoselective for many chiral drugs. Therefore, stereoselective ligand binding (warfarin, phenprocoumon, pranoprofen, and ibuprofen) to rHSA and

Table II. Effect of Fatty Acids, *N*-Acetyl-L-Tryptophan and Pasteurization on the Thermodynamic Parameters Estimated by CD (222 nm) for the Guanidine Hydrochloride Denaturation of rHSA and HSA[a]

	rHSA		HSA	
Additives	m (kcal/mol/M)	ΔG_{H2O} (kcal/mol)	m (kcal/mol/M)	ΔG_{H2O} (kcal/mol)
Control concentration without additives	2.47 ± 0.09	5.24 ± 0.26	2.63 ± 0.12	5.03 ± 0.19
Palmitate	3.01 ± 0.08[c]	6.90 ± 0.16[c]	3.06 ± 0.26[c]	6.63 ± 0.28[c]
Oleate	2.82 ± 0.02[c]	6.52 ± 0.15[c]	2.91 ± 0.15[c]	6.80 ± 0.16[c]
Sodium caprylate				
N-acetyl-L-tryptophan	4.43 ± 0.76[d]	8.97 ± 1.03[d]	5.08 ± 0.21[d]	8.93 ± 0.67[d]
Pasteurization without additives[b]	ND[e]	ND[e]	ND[e]	ND[e]
Pasteurization with additives[b]	4.75 ± 0.90[d]	8.69 ± 0.58[d]	4.98 ± 0.35[d]	8.80 ± 0.36[d]

[a] The following concentrations were used; [rHSA or HSA] = 1.0×10^{-4} M, [additives] = 5.0×10^{-4} M. Data presented as means ± standard deviation; n = 3.

[b] Pasteurization (60°C, 10 h) was performed with or without sodium caprylate and *N*-acetyl-L-tryptophan.

[c,d] Statistically significant difference compared with control concentration; $P < 0.05$ and $P < 0.01$, respectively.

[e] Not detectable because of no cooperativity.

Table III. Effect of Fatty Acids, *N*-Acetyl-L-Tryptophan and Pasteurization on the Thermodynamic Parameters Estimated by Differential Scanning Calorimetry for the Thermal Denaturation of rHSA and HSA[a]

Additives	rHSA		HSA	
	T_m (°C)	ΔH_{cal} (kcal/mol)	T_m (kcal/mol)	ΔH_{cal} (kcal/mol)
Control concentration without additives	59.7 ± 0.1	146.3 ± 1.7	59.7 ± 0.1	150.5 ± 1.0
Palmitate	79.9 ± 0.2[c]	267.5 ± 1.2[c]	79.5 ± 0.1[c]	270.5 ± 3.7[c]
Oleate	79.1 ± 0.1[c]	271.2 ± 3.4[c]	79.5 ± 0.1[c]	280.0 ± 2.4[c]
Sodium caprylate				
N-acetyl-L-tryptophan	71.1 ± 0.1[c]	239.5 ± 3.5[c]	71.1 ± 0.1[c]	240.0 ± 12.7[c]
Pasteurization without additives[b]	ND[d]	ND[d]	ND[d]	ND[d]
Pasteurization with additives[b]	71.7 ± 0.1[c]	235.3 ± 4.2[c]	71.6 ± 0.2[c]	245.0 ± 5.0[c]

[a] The following concentrations were used; [rHSA or HSA] = 1.0×10^{-4} M, [additives] = 5.0×10^{-4} M. Data presented as means ± standard deviation; n = 3.
[b] Pasteurization (60°C, 10 h) was performed with or without sodium caprylate and N-acetyl-L-tryptophan.
[c] Statistically significant difference compared with control; $P < 0.01$.
[d] Not detectable because of no cooperativity.

HSA was investigated by using an ultrafiltration method (Fig. 3). (R)-isomers and (S)-isomers were separated by a chiral-stationary phase HPLC column. Figure 3 shows comparable results for rHSA and HSA, namely that the (S)-isomer has a greater affinity than the (R)-isomer in the case of warfarin, phenprocoumon, and pranoprofen. By contrast, (R)-ibuprofen has a greater affinity for albumin than (S)-ibuprofen. These results are in accordance with previous reports (25–27).

It is well known that the primary binding sites of warfarin and phenprocoumon are within site I, and those of pranoprofen and ibuprofen are within site II. As shown in Figure 3, rHSA and HSA have similar stereoselective ligand-binding properties. Thus, it seems that these ligand-binding sites (site I and site II) of rHSA are identical to those of HSA.

It is generally accepted that HSA undergoes a structural transition in the neutral pH region 6–9 (2). It has been found that binding of several ligands depends on the state of the N-B equilibrium, due to differences in the affinity for HSA in the N and B conformation (13,14). The effect of the N-B transition on ligand binding was monitored by studing high-affinity binding of warfarin (site I-ligand) and dansylsarcosine (site II-ligand). For warfarin binding, the order of the bound fraction is pH 9 > pH 8 > pH 7 > pH 6, but for dansylsarcosine binding, the order of the bound fraction is pH 6 > pH 7 > pH 8 > pH 9 (Fig. 4). These tendencies were found for both albumins and, furthermore, there is a good correlation between the results obtained with rHSA and those found for HSA.

In Vivo Studies

rHSA and HSA labeled with ^{125}I was given intravenously to rats, and no differences were found for the blood half-lives of the two proteins; 29.2 ± 3.7 h for rHSA and 31.0 ± 3.9 h for HSA. The urinary and fecal excretion of radioactivity after 96 h were also similar in the two cases; 64.5 ± 13.6% (urine) and 3.6 ± 1.4% (feces) of injected radioactivity in the case of rHSA, and 68.8 ± 3.2% (urine) and 4.5 ± 1.9% (feces) of total radioactivity in the case of HSA. All the numbers given here represent average values ± standard deviation of three determinations. Finally, the tissue distribution of the two albumins was comparable with the skin, liver, kidneys, and spleen taking up the most radioactivity.

Table IV. Estimated Z Values of Binding Sites from Emission Maxima of Bound Probes

Probe	Z Value	
	rHSA	HSA
Warfarin	71.0 ± 0.1	71.0 ± 0.1
Dansylsarcosine	66.3 ± 0.3	65.8 ± 0.4

CONCLUSIONS

The present way of isolating rHSA makes use of the fact that albumin and Cibacron Blue interact strongly but reversibly (2). Therefore, in stead of purifying the protein by a combination of ion exchange, hydrophobic interaction and/or gel permeation chromatography (15), we made use of a column of Blue Sepharose, which is Cibacron Blue 3G coupled to Sepharose. We tried to run the eluant from the Blue Sepharose column on an anion exchange column. However, adding this step to the procedure did not improve purification. Use of

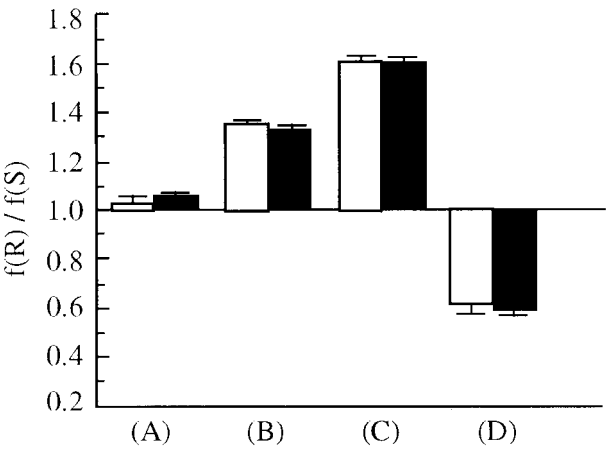

Fig. 3. Enantiometric free fraction ratios [f(R) / f(S)] of warfarin (A), phenprocoumon (B), pranoprofen (C), and ibuprofen (D) in the presence of rHSA (open columns) and HSA (filled columns). All values are means ± standard deviation of at least three experiments.

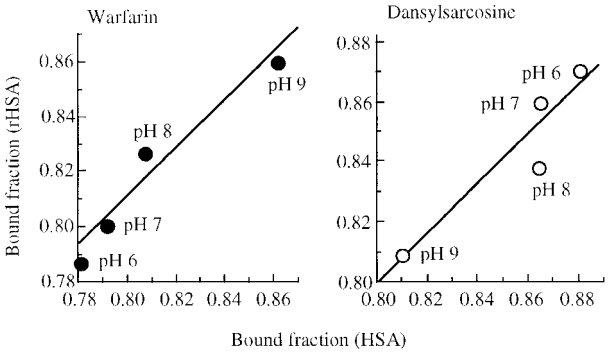

Fig. 4. Correlation between bound fraction of warfarin or dansylsarcosine to rHSA or HSA and pH.

the present approach results in fairly rapid purification (about 3 days) of rHSA with a good recovery (about 80%).

For the *in vitro* and *in vivo* studies, rHSA was purified further, namely by removing the hydrophobic ligands that might be bound to the protein. For this purpose, we decided to treat rHSA (and HSA) with charcoal following the procedure of Chen (16) and not by the widely used method of passing the protein through a column of Lipidex-1000, because the efficiency of the latter method to remove hydrophobic ligands from albumin has been seriously questioned (28).

The present tests showed that the rHSA overexpressed and secreted from the yeast species *Pichia pastoris* and purified according to the present procedure has very similar structural characteristics, stability, ligand-binding properties, and metabolism as HSA isolated from serum. However, toxicologic studies, including antigenicity tests, should be done for clinical applications of the rHSA produced in this work, and they are currently under way at this laboratory.

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