Recombinant Human Serum Albumin Containing 3 Copies of Domain I, Has Significant *in Vitro* Antioxidative Capacity Compared to the Wild-Type

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Human serum albumin (HSA), the most abundant protein in serum, functions as carrier of drugs and contributes to maintaining serum colloid osmotic pressure. We report herein on the preparation of a genetic recombinant HSA, in which domains II and III were changed to domain I (triple domain I; TDI). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) results indicated that the purity of the TDI was equivalent to that of the wild type (WT). Both far- and near-UV circular dichroism (CD) spectra of the TDI showed that its structural characteristics were similar to the WT. Ligand binding capacity was examined by an ultrafiltration method using 3-carboxy-4-methyl-5-propyl-2-furanpropanoic acid (CMPF) and ketoprofen as markers for site I and site II, respectively. The binding capacity of TDI for both ligands was lower than that for the wild type. TDI significantly suppressed the oxidation of dihydrorhodamine 123 (DRD) by H2O2 compared to the WT. Our current results suggest that TDI has great potential for further development as HSA a product having antioxidantive functions.

Key words human serum albumin; antioxidative property; domain I; ligand-binding

HSA is the most abundant protein in serum (60% of total plasma protein), and is considered to be involved in maintaining normal colloid osmotic pressure and drug-binding carrier. HSA is a single, non-glycosylated polypeptide, that is comprised of about 67% α-helix but no β-sheet structures in its heart-shaped structure.1–5 HSA consists of three homologous domains (I, II and III), each of which contains two subdomains, A and B. Among its thirty-five cysteine residues (Cys), thirty-four are involved in disulfide bonds and one, the Cys residue at 34th (Cys34), has been described above. In a previous study, we reported that the Cys34 residue was oxidized to a significant extent in renal failure patients compared to normal human subjects.4) This finding indicates that HSA directly functions as a ROS scavenger, and that the Cys34 residue plays an important role in this process.

In the current study, in an effort to obtain a more functional recombinant albumin, we attempted to produce a new type of recombinant albumin, triple domain I (TDI), from *Pichia pastoris*, in which both domains II and III were replaced with domain I. The structural properties, and ligand-binding and antioxidative capacities of TDI were compared to wild type (WT). Based on these comparisons, we conclude that TDI can be useful and beneficial for clinical use.

**MATERIALS AND METHODS**

**Materials** A chimeric plasmid (pJDB-ADH-L10-HSA-A) containing cDNA for the mature form of HSA along with an L10 leader sequence was a gift from the Tonen Co. (Tokyo, Japan).
Japan). The restriction enzymes and Escherichia coli JM109 were purchased from TaKaRa Bio Inc. (Otsu, Japan). The DNA sequence kit was purchased from Perkin-Elmer Applied Biosystems (Foster City, CA, U.S.A.). The Pichia Expression Kit was purchased from Invitrogen Corp. (Carlsbad, CA, U.S.A.). Dihydrorhodamine 123 (DRD) was purchased from Sigma (St. Louis, MO, U.S.A.). Radiolabeled 3-carboxy-4-methyl-5-propyl-2-furanpropionate ([3H]CMPF) was synthesized and purified by PerkinElmer, Inc. Life Sciences (Boston, MA, U.S.A.). [14C]Ketoprofen (12.95 µCi/mmol) was obtained from Hisamitsu Pharmaceutical Co., Inc., Tosu Laboratories (Saga, Japan). Other chemicals used were purchased from commercial suppliers.

Expression and Purification of Recombinant HSA, WT and TDI

The protocol used to express WT and TDI were described in a previous report, with minor modifications.15) Three kinds of cDNA for domain I, which each has restriction enzyme sites, HindIII, PstI and EcoRI was amplified using the appropriate primers (Fig. 1). Sequences of the primers used for TDI construction are as follows; Domain I-1, sense: 5′-GGT ACC TCG CGA CTC GAG AAA AGA GAT CAT GAG GTT GC-3′, antisense: 5′-GAC GTC GTT CTC GTG ACA CTT TTG GCC-3′, Domain I-2, sense: 5′-GAC GTC AAG CTT ACA AGA TGC CAT GTG ACA CAC AAA GAG TGA GTT GC-3′, antisense: 5′-GAC GTC CTC GAG ACT GCC ACA CTT GAG-3′, Domain I-3, sense: 5′-GAC GTC CTG CAG GAT AGA TGG ACA CTC CAG-3′, antisense: 5′-CAG GTG CAG CTT GAA TTC TTA TTC GAG ACT GCC ACA CTT GAG TC-3′, TDI, sense: 5′-GCT ACC TCG CGA CTC GAG AAA AG-3′, antisense: 5′-CAG GTG CAG GCT GG AAT TTC-3′. Each cDNA for coding domain I was ligated, and then amplified by PCR. The amplified cDNA coding TDI was inserted into pPIC9 (TDI-pPIC9). WT-pPIC9 and TDI-pPIC9 were transformed into Pichia pastoris (strain GS115) by electroporation respectively. The cells were resuspended in a buffered methanol complex medium for 4d at 30°C. The secreted recombinant was purified on a Blue Sepharose CL-6B column (Amersham Biosciences).

Circular Dichroism (CD) Spectra Measurements CD spectra were obtained using a JASCO J-720 spectropolarimeter (JASCO, Tokyo, Japan) at 25°C. Far- and near-UV CD spectra were recorded at protein concentrations of 5 µM of both WT and TDI in 67 mM phosphate buffer (pH 7.4).

Ligand-Binding Capacity of TDI The binding capacity of TDI and WT were investigated using [3H]CMPF and [14C]-ketoprofen as a sites I and II marker, respectively. TDI or WT (2 µM) and drug (1 µM) were mixed in 67 mM phosphate buffer at 25°C. Ultrafiltration tube set with a mixed solution was centrifuged at 2000rpm for 40 min at 25°C. Radioactivity of a mixed solution and the filtrate were measured by liquid scintillation counting.

Suppressive Effect of TDI on the Oxidation of DRD by H2O2 Oxidation of DRD by H2O2 generates rhodamine 123 (RD). A peak for the fluorescence intensity of DRD was observed at 536 nm when excited at 500 nm. The DRD and H2O2 concentrations were 5 µM and 12.5 mM in 67 mM phosphate buffer (pH 7.4). The progression of the reactions was spectrophotometrically monitored by RD fluorescence intensities at 25°C. Controls were performed without additives. The quenching of the control was 0%.

Statistical Analysis All data are presented as the mean ± standard deviation (S.D.). Statistical analyses of differences were performed by one-way ANOVA followed by a modified Fisher’s least square difference method.

RESULTS

Expression and Purification of TDI It is generally

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**Fig. 1. pPIC9 Expression Vector Encoding Triple Domain I (TDI)-Human Serum Albumin**
thought that domain I of HSA is comprised of from 193 to 197 amino acid residues. In this study, we constructed domain I of TDI to contain 204 amino acid residues. In this case, the Cys34 and Cys200 residues become free in TDI, whereas, in the WT molecule, Cys200 in involved in a disulfide bond with the Cys246 residue. Therefore, TDI theoretically would contain six free Cys residues, three sets of Cys34 and Cys200 per molecule. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicate that TDI was expressed and was obtained in pure form (Fig. 2).

**Structural Property of TDI** To compare the structural properties of TDI with the WT, we collected far- and near-UV CD spectra (Fig. 3). The peak intensity in the far-UV CD spectra at 209 nm and 222 nm reflect the content of α-helix structure. The result for far-UV CD spectra indicated that the α-helix content of TDI was less than that of the WT. The near-UV CD spectra also showed slight differences in tertiary structure between the WT and TDI. This result indicates that the secondary and tertiary structure was slightly unfolded compared to the WT or different from the WT.

**Ligand-Binding Capacity of TDI** The ligand-binding capacity of TDI was evaluated using CMPF and ketoprofen as site I and site II markers (Table 1). No significant binding of CMPF to TDI was observed. On the other hand, ketoprofen bound to TDI, even though it is a site II marker. This result suggests that, in TDI, the structure of binding site I is not maintained and that it contains a newly formed site II-like structure.

**Antioxidative Property of TDI** We previously reported that HSA efficiently suppressed the reaction from DRD to DR by H₂O₂. To evaluate the antioxidative properties of TDI, we investigated the extent with which TDI suppresses this reaction through inhibiting the oxidation of DRD (Fig. 4). The TDI significantly suppressed the decrease in the fluorescence intensity of DRD by H₂O₂ compared to WT (EC₅₀; WT: 23.45±9.00, TDI: 3.87±1.51, p<0.05). This result indicates that TDI has a greater antioxidative capacity than the WT.

<table>
<thead>
<tr>
<th>Protein</th>
<th>[³H]CMPF (%)</th>
<th>[¹⁴C]Ketoprofen (%)</th>
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<tbody>
<tr>
<td>WT</td>
<td>34.97±0.65</td>
<td>72.77±1.39</td>
</tr>
<tr>
<td>TDI</td>
<td>1.78±1.70</td>
<td>19.61±1.68</td>
</tr>
</tbody>
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The sample solution contained 1µM of [³H]CMPF or [¹⁴C]ketoprofen and 2µM WT or TDI in 67mM sodium phosphate buffer (pH 7.4 and 25°C). All values are means±S.D. calculated from the results of four independent experiments. Symbol, #, represents the statistic difference, p<0.001.
there was a slight difference. This suggests that TDI has basically same structural properties as the WT.

In our current study, we constructed a recombinant HSA (TDI), in which all three domains were the same, i.e., only domain I, and successfully obtained purified TDI, the molecular weight of which was equivalent to that for the WT (Fig. 2).

CD spectra indicated that the α-helix content of TDI was less than that for the WT (Fig. 3). This difference may be due to the number of disulfide bonds in the molecule. Native HSA and WT contain 17 disulfide bonds. Domains I, II and III in native HSA and the WT have five, six and six disulfide bonds respectively, whereas TDI is comprised of three versions of domain I, including five disulfide bonds, resulting in a total of 15 disulfide bonds. However, the far- and near CD spectral patterns for the TDI and WT were very similar even though there was a slight difference. This suggests that TDI has basically same structural properties as the WT.

In ligand-binding experiments, CMPF used as site I marker did not bind to TDI (Table 1). Site I is located between subdomains IB and IIA. Trp214, Arg218, Leu219, Arg222 and His242 in subdomain IIA are involved in ligand-binding at site I. We previously produced recombinants of each domain I, II and III, and investigated their ligand-binding capacities. We found that warfarin and 5-dihydrotestosterone and n-butyl p-aminobenzoate, markers of subsite IA, IB and IC respectively, did not show significant binding to domain I. Taking these collective findings into consideration, it appears that domain I may not be able to form a ligand-binding site, such as site I, without domain II or domain III being present. Interestingly, ketoprofen showed a slight level of binding to TDI even though it is generally considered to be a site II marker. In a previous study, we found that fatty acid-induced conformational changes in HSA resulted in an increased binding affinity of ketoprofen to subdomain IA, whereas binding to subdomain IIA was decreased. Therefore, TDI may form a new binding site for ketoprofen as observed in our previous study. Details of the mechanism responsible for this might be clarified by a X-ray crystallographic study.

It is known that HSA in patients with diabetic nephropathy or chronic renal failure is in a highly oxidized form. Imai et al. reported that strenuous exercise induced the oxidation of HSA. We found that Cys residues of HSA are oxidized in hemodialysis patients. This finding suggests that the oxidation of Cys residues of HSA represent a potential marker for recognizing the degree of diseased state and that HSA functions as an antioxidant against oxidative damage. Our current results suggest that the higher antioxidative capacity of TDI is due to the presence of six free Cys residues. We measured the numbers of free Cys residues of TDI treated with dithiothreitol using Ellman’s reagent (5,5′-dithiobis-2-nitrobenzoic acid (DTNB)), and found it were 5.5±0.3 protein. This result indicates that TDI has six Cys residues as expected. However, in order to know the accurate number of free Cys residues of TDI in non-reduced condition, it is necessary to get the X-ray crystal structure of TDI. Therefore, we will implement the X-ray crystal structure analysis in near future. Moreover, clinical use of TDI may be useful as more multifunctional albumin product with function not only for maintaining osmotic pressure, but also for arresting ROS generated during inflammation such as burn. In addition, it may be able to prepare the recombinant proteins which have antioxidant property by genetically fusing with TDI. There are several reports that nitrous oxide (NO) can oxidize Cys residues in HSA, and that this nitrosylated HSA functions as a carrier of NO. We previously reported that nitrosylated HSA has an antibiotic effect on bacteria and antitumor effects in human cancer cells. Therefore, TDI has the potential for serving as a useful carrier of NO compared to the WT molecule.

**Conflict of Interest** The authors declare no conflict of interest.

**REFERENCES**


