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Simple and sensitive high-performance liquid chromatographic method for the investigation of dynamic changes in the redox state of rat serum albumin

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Abstract

Serum albumin is a mixture of mercaptalbumin (reduced form) and non-mercaptalbumin (oxidized form), i.e. a protein redox couple in serum. To investigate dynamic changes in the redox state of rat serum albumin (RSA), we developed a simple and sensitive high-performance liquid chromatographic (HPLC) system using an ion-exchange column with a linear gradient of ethanol concentration. Furthermore, we applied this HPLC system to examine dynamic changes in the redox state of RSA caused by severe oxidative stress such as exhaustive physical exercise. Using this system, we successfully separated RSA to rat mercaptalbumin (MA_r) and rat non-mercaptalbumin (NA_r), and also found the best conditions for the clear separation of RSA. In the experiments with exhaustive exercise, mean values for the MA_r fraction in control and exercise groups were 76.2±1.8 and 69.0±3.5%, respectively. The MA_r in the exercise group was significantly oxidized compared with that of the control group ($P<0.01$). These results suggested that RSA might act as one of the major scavengers in extracellular fluids under severe oxidative stress.

Keywords: Redox state; Rat serum albumin

1. Introduction

Serum albumin is the most abundant protein in the circulatory system and has a number of functions. One of the most important features of serum albumin is that it has one free sulfhydryl group in position 34 (Cys-34). This free sulfhydryl group is thought to be functionally important because Cys-34 is conserved in the serum albumin of most mammals [1]. Serum albumin is a mixture of mercaptalbumin (reduced form) and non-mercaptalbumin (oxidized form) in extracellular fluids such as serum and interstitial fluid [2,3]. The former has one free sulfhydryl group in Cys-34, while the latter has a ligand bound to the sulfhydryl group in Cys-34. Non-mercaptalbumin is composed of at least three kinds of compounds. The major component is mixed disulfide with cystine or glutathione [2,3]. Non-mercaptalbumin, moreover, has oxidation product higher than mixed disulfide, such as sulfenic (–SOH), sulfinic ($–SO_2H$) or sulfonic ($–SO_3H$) states, as very minor component in extracellular fluids. The minor one has been tenta-

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tively designated as non-mercaptalbumin(Oxi) [4]. Sogami et al. [2,3] developed a convenient high-performance liquid chromatographic (HPLC) system using an Asahipak GS-520H column, which is able to separate human serum albumin (HSA) to human mercaptalbumin (HMA) and human non-mercaptalbumin (HNA). Furthermore, Era et al. [4] developed an HPLC system using an Asahipak ES-502N column, which is able to separate HSA to HMA, [HNA containing mixed disulfide with cystine or glutathione (HNA(Cys) or HNA(Glut))] and HNA(Oxi). The fraction of HMA (f(HMA)) has been shown to be markedly decreased in various diseases and some physiological conditions, compared with that in healthy young male subjects [2–8]. The mechanisms of such oxidation of mercaptalbumin, or more precisely the mechanisms of mercapt→non-mercapt conversion, are not well understood. However, in our previous study using an ES-502N column [9], albumin in aqueous humor was shown to be extremely oxidized in patients with senile cataract, suggesting that oxidative stress is an initiating factor for the development of maturity onset cataract and hydrogen peroxide (H_2O_2) is the major oxidant [10]. To clarify the fundamental mechanisms of mercapt→non-mercapt conversion of albumin under various pathophysiological states, it is important to study the relationship between oxidative stress and the dynamic changes in redox state of serum albumin. In such studies, experimental conditions for oxidative stress should be as consistent as possible among subjects. Therefore, to precisely control oxidative stress, it would of course be best not to use human subjects but experimental animals. Nishimura et al. [11] developed an HPLC system using an *N*-methylpyridinium polymer-based column to examine the redox state of commercial albumin samples from human and animals, and could separate human, bovine and rabbit serum albumins to each mercapt- and non-mercaptalbumins. However, they could not succeed the separation of rat serum albumin (RSA).

In this study, we investigated whether an ES-502N column could be used to separate RSA to rat mercaptalbumin (MA_r) and rat non-mercaptalbumin (NA_r), and optimized the experimental conditions to obtain clear resolution. Furthermore, we examined dynamic changes in the redox state of RSA caused by severe oxidative stress such as exhaustive physical exercise.

2. Experimental

2.1. Samples for optimization of HPLC conditions

Male Wistar rats weighing 240–260 g were obtained from Japan SLC (Shizuoka, Japan). The rats were anesthetized and blood samples were collected from the heart. Sera were immediately obtained by centrifugation and then filtered through a Dismic-13cp filter unit (0.45μm, Advantec Toyo, Tokyo, Japan). The sera were immediately stored at −80 °C until analysis. Reduced glutathione (GSH, Sigma, MO, USA), oxidized glutathione (GSSG, Sigma, MO, USA) and H_2O_2 (Santoku Chemical Industry, Tokyo, Japan) were used for identification of albumin peaks. To identify albumin peaks, rat serum treated with a 10-fold concentration of GSH, GSSG or H_2O_2 against RSA were incubated for 1 h at 25°C, 3 h at 25 °C or 1 h at 25°C, respectively.

2.2. Samples and experimental conditions for physical exercise

Male Wistar rats weighing 240–260 g were obtained from Japan SLC (Shizuoka, Japan). The rats were randomly divided into exercise and control groups. Rats of both groups were housed in separate cages under controlled temperature (24°C) on a 12:12 h light–dark cycle and received food and tap water ad libitum. All rats were familiarized with running on a motor-driven treadmill for 15 min once a day for practice, for 2–3 days during the 1 week before experiment. The experimental time course for exercise and control groups is as follows: The rats in the exercise group were exercised on the treadmill for 120 min. The speed and incline of the treadmill were set at 10–11 m/min and 5°, respectively. As several rats could not run for exhaustion until end of this period, these conditions were taken to provide exhaustive exercise to rats. We obtained blood samples from rats that were able to run throughout the exercise period ($n=7$). In the control group, rats were fasted during this 120 min period ($n=7$).

Both groups were immediately anesthetized after

the exercise or fasting period, and blood samples were immediately collected from the heart. Sera were obtained by centrifugation and then filtered through a Dismic-13cp filter unit (0.45 μm, Advantec Toyo, Tokyo, Japan). The sera were immediately stored at −80 °C until analysis.

2.3. HPLC system

We used two HPLC systems for ion-exchange chromatography and for gel filtration. The former system was used to examine the redox state of RSA and the latter was used to measure the molecular mass of the protein.

The HPLC system for ion-exchange consisted of a Model AS-8010 autosampler (injection volume of 2 μl), a Model CCPM double-plunger pump and a Model FS-8000 fluorescence detector (excitation wavelength, 280 nm; emission wavelength, 340 nm) in conjunction with a Model SC-8020 system controller, all from Tosoh Co., Tokyo, Japan. A Shodex-Asahipak ES-502N (10×0.76 cm I.D., previous name was Asahipak ES-502N, DEAE-form for ion-exchange HPLC, Showa Denko Co., Tokyo, Japan) ion-exchange column was used. Column temperature was 37±0.5°C. Measurements were carried out by solvent gradient elution with increasing ethanol concentration from 0 to 10% in 0.05 *M* sodium acetate–0.40 *M* sodium sulfate (pH 4.85) at a flow-rate of 1.0 ml/min. To optimize the elution profile, several ethanol concentration gradients were tested. All data were transferred to personal computer (Fujitsu, Tokyo, Japan) for data analysis.

The HPLC profiles obtained using the ion-exchange HPLC system were subjected to numerical curve fitting with a simulation software (Peakfit, version 4, SPSS Science, IL, USA); each peak shape was approximated by a Gaussian function. The values for fractions of MA_r ($f(MA_r)$) and NA_r ($f(NA_r)$) were obtained by the following equations:

$$f(MA_r)(\%) = [MA_r/(MA_r + NA_r)] \times 100,$$

$$f(NA_r)(\%) = [NA_r/(MA_r + NA_r)] \times 100$$

The HPLC system for gel filtration consisted of a Model AS-80 autosampler (injection volume of 100 μl), a Model CCPM double-plunger pump and a Model UV-8000 UV detector (operated at 280 nm) in conjunction with a Model SC-8020 system controller, all from Tosoh Co., Tokyo, Japan. A TSK-GEL G3000SWXL column (30×0.78 cm I.D., Tosoh Co., Tokyo, Japan) for gel filtration was used at room temperature. Eluent buffer was 0.05 *M* sodium acetate–0.40 *M* sodium sulfate (pH 4.85) at a flow-rate of 0.5 ml/min. All data were transferred to a personal computer (Fujitsu, Tokyo, Japan). A commercial HSA preparation (0.001% HSA in 0.05 *M* sodium phosphate–0.15 *M* sodium chloride (pH 6.99), Calbiochem Behring Co., CA, USA) was used for comparison with RSA with respect to molecular mass.

On both HPLC systems, deaeration of buffer solution was carried out by bubbling with helium gas. All chemicals and reagents were of analytical grade. All solvents and solutions were filtered through a Sterivex-GS filter unit (0.22 μm, Millipore, MA, USA) before use.

Mann–Whitney's *U*-test was used to evaluate the significance of differences in the redox state of RSA between control and exercise groups. Values are expressed as means±SD.

3. Results and discussion

3.1. Separation of RSA to MA_r and NA_r, and optimization of experimental conditions

In a previous study [9], we have successfully obtained clear separation of HSA to HMA and HNAs using an ES-502N column (an ion-exchange column) with an increasing ethanol concentration in acetate–sulfate buffer (pH 4.85). The organic solvent such as ethanol can be used to lower hydrophobic interactions, as in the case of reverse phase supports. With ion-exchangers, an increasing ethanol concentration usually increases electrostatic interaction. Therefore, from the results on elution conditions of HSA separation [9], it is possible to say that the major difference in physicochemical properties between HMA and HNA might be the hydrophobic and/or electrostatic interactions between albumin–resin supports.

We examined whether it was possible to separate RSA to MA_r and NA_r, using an ES-502N column,

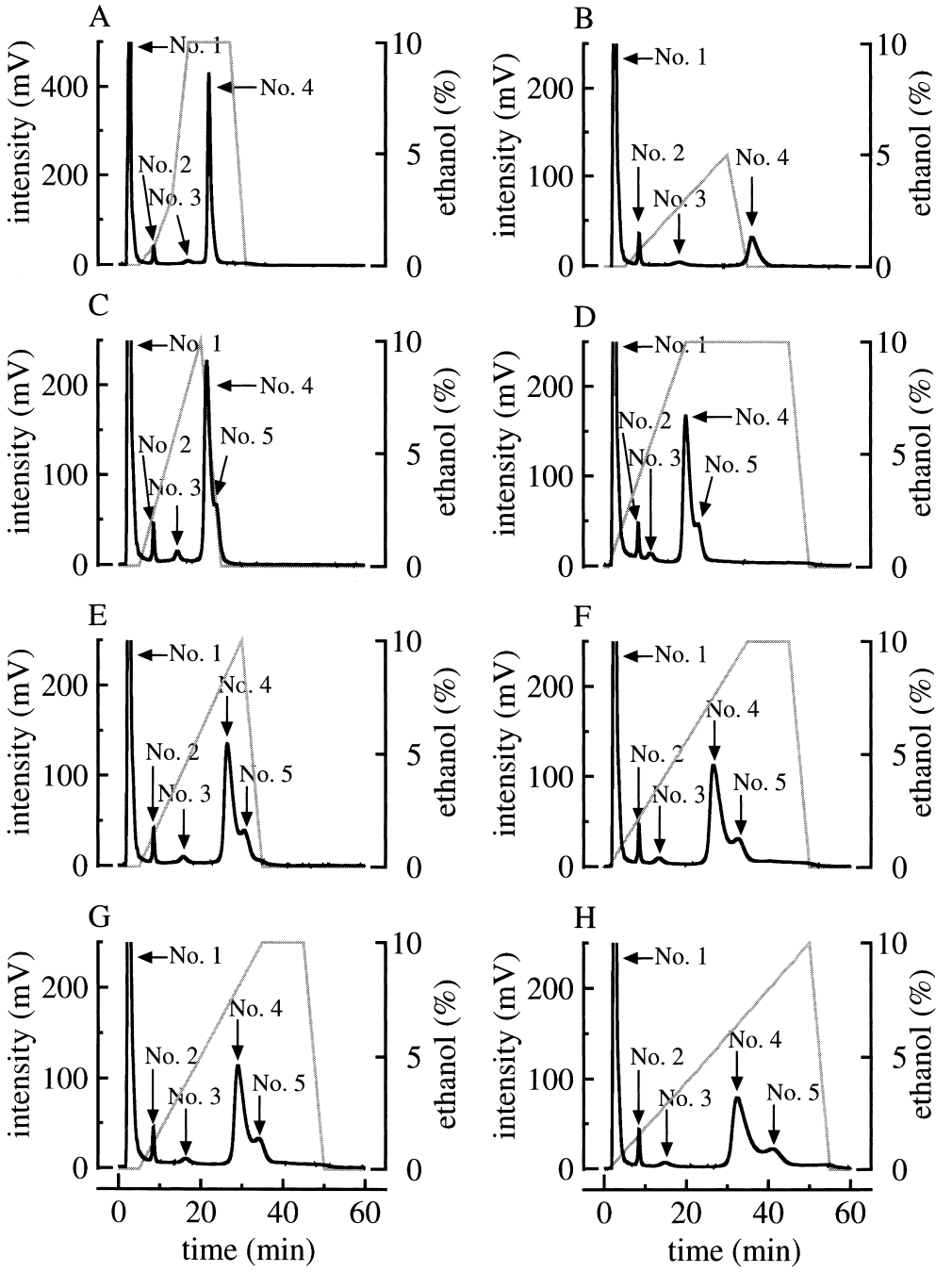

Fig. 1. HPLC profiles of a rat serum eluted using an ES-502N column with different ethanol concentration gradient conditions. All profiles were obtained under the same elution conditions except for ethanol concentration gradient. Buffer solution was 0.05 *M* sodium acetate–0.40 *M* sodium sulfate (pH 4.85). Flow rate was 1.0 ml/min. Injection volume was 2 μl. Column temperature was 37±0.5°C. Numbers indicate each fraction eluted from serum. Dark solid lines indicate gradient conditions in each HPLC profile.

and also examined the optimal ethanol concentrations that could yield clear fractions of RSA. Fig. 1 shows eight HPLC profiles of rat serum obtained using different ethanol concentration gradient conditions. We obtained four fractions using each gradient condition shown in Fig. 1A and B, and then obtained five fractions using each gradient condition shown in Fig. 1C–H. In the case of HSA separation using the same column, HSA fractions were eluted in the latter half of the HPLC profile [9]. Therefore, we felt that RSA might be contained in two fractions in the latter half of the HPLC profiles of rat serum, i.e. fraction Nos. 4 and 5 (Fig. 1C–H).

To identify these two fractions, we used a gel filtration column (G3000SWXL) and the HPLC profiles of three samples obtained using a G3000SWXL column are shown in Fig. 2. A commercial HSA preparation (0.001% HSA in 0.05 *M* sodium phosphate–0.15 *M* sodium chloride (pH 6.99), Calbiochem Behring Co.; Fig. 2C) was used for comparison with fraction Nos. 4 (Fig. 2A) and 5 (Fig. 2B) with respect to molecular mass. Retention times of fraction Nos. 4 and 5 shown in Fig. 2 were practically identical to those of commercial HSA. Molecular masses of HSA and RSA were 66.4 and 65.8 kDa, respectively, which were calculated from the primary structure of each protein [1]. As the molecular mass of RSA was almost identical to that of HSA, fraction Nos. 4 and 5 shown in Fig. 1 were identified as RSA.

Two ethanol concentration gradient conditions for the ES-502N column, which were well suited for separation of HSA to HMA and HNAs, have been reported [4,9]. To separate RSA, we first used these two gradient conditions for HPLC profile as shown in Fig. 1A and B. However, these gradient conditions were unable to separate RSA. As shown in Fig. 1C–H, we found other gradient conditions that were

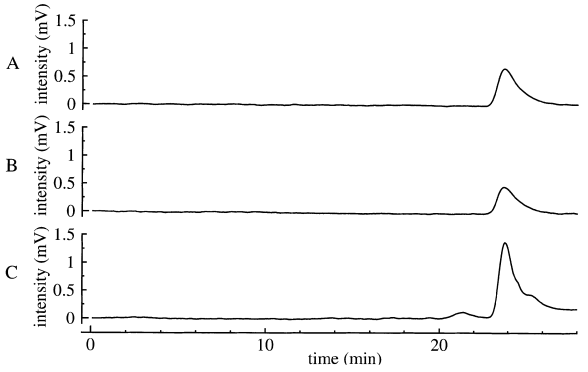

Fig. 2. HPLC profiles (280 nm) eluted with a gel filtration column (G3000SWXL). Fractions No. 4 (A) and No. 5 (B) of rat serum were manually collected by an ion-exchange column (ES-502N) with the gradient conditions used in Fig. 1H. A commercial HSA preparation (C) was used for comparison with fraction Nos. 4 and 5. Eluent buffer of 0.05 *M* sodium acetate–0.40 *M* sodium sulfate (pH 4.85) was used at a flow-rate of 0.5 ml/min at room temperature.

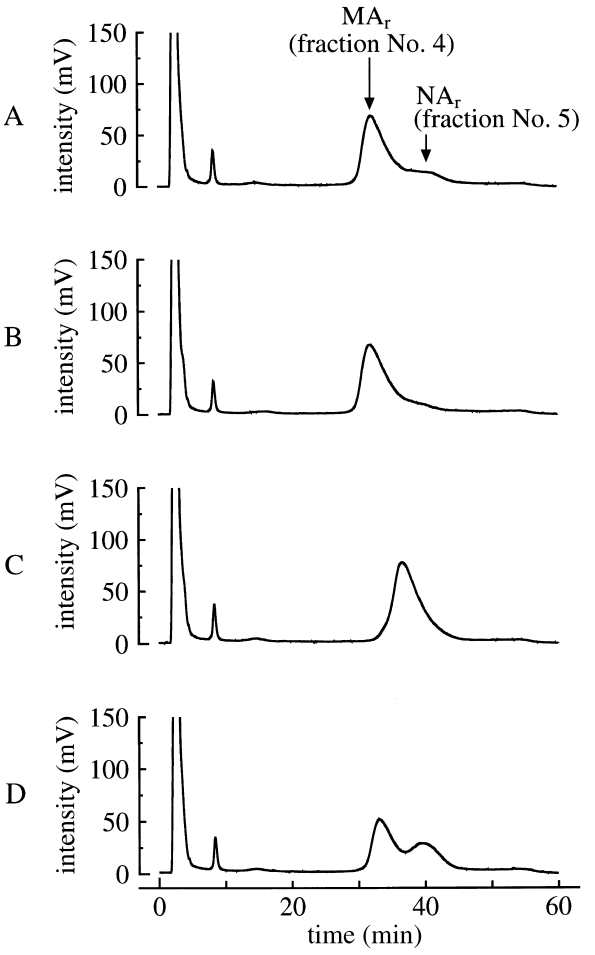

Fig. 3. HPLC profiles of a rat serum eluted using an ES-502N column with the gradient conditions used in Fig. 1H. Rat serum was directly injected (A). Rat sera were incubated with a 10-fold concentration of GSH for 1 h at 25 °C (B), GSSG for 3 h at 25 °C (C) or H_2O_2 for 1 h at 25 °C (D). MA_r and NA_r fractions were identified from these profiles.

able to separate RSA to two peaks. The gradient condition shown in Fig. 1H was best suited for separation of RSA to MA_r and NA_r.

GSH reduces non-mercaptalbumin containing mixed disulfide with glutathione [4,12]. GSSG oxidizes free sulfhydryl group in mercaptalbumin, and forms a mixed disulfide with albumin [4]. H_2O_2 oxidizes free sulfhydryl group in mercaptalbumin, and forms sulfenic, sulfinic or sulfonic acid [13]. Therefore, we identified RSA peaks using GSH, GSSG and H_2O_2, as shown in Fig. 3. It was clear that fraction Nos. 4 and 5 were MA_r and NA_r, respectively.

3.2. Observation of dynamic changes in the redox state of RSA by exhaustive physical exercise

Recently, the role of reactive oxygen species (ROS) in the pathogenesis of certain human diseases such as atherosclerosis, cancer, inflammatory joint diseases and diabetes has been recognized [14,15]. In addition, various antioxidants to scavenge ROS in both intra- and extracellular fluids have attracted increasing attention [16]. Extracellular fluids contain many low molecular mass antioxidants such as tocopherol, ascorbic acid, β-carotene, glutathione and uric acid [17]. In a recent paper, Halliwell [18] noted that albumin has antioxidant capability in extracellular fluid. Albumin is the most abundant protein in the circulatory system, and has various important properties. One of these properties is its ability to bind reversibly to an incredible variety of ligands such as transition metal ions, fatty acids, bilirubin, cysteine and glutathione [19]. Some studies have suggested that albumin scavenges ROS indirectly caused by binding with redox-active transition metal ions that are capable of catalyzing reactions with ROS [18,20]. However, there is also evidence that albumin may act more directly as a ROS scavenger or as a participant in scavenging reactions [7,9,21,22]. Furthermore, there is strong evidence that the reaction center is the free sulfhydryl group on Cys-34 of albumin [7,9,21,22].

To determine the role of Cys-34 in the action of albumin as a scavenger in detail, it is necessary to investigate the relationship between mercapt⇔non-mercapt conversion and ROS in living systems. To investigate this relationship, oxidative stress to induce ROS should be controlled between experimental subjects. However, it is difficult to control the oxidative stress between human subjects. Therefore, it would probably be best to use experimental animals under uniform conditions. As already described above, the HPLC system using an ES-502N column can be used to examine the redox state of RSA. Therefore, we can investigate the relationship between mercapt⇔non-mercapt conversion of albumin and ROS in rat serum using our HPLC system under various conditions. It is necessary to choose a model in which it is possible to induce severe oxidative stress under simple conditions; we chose

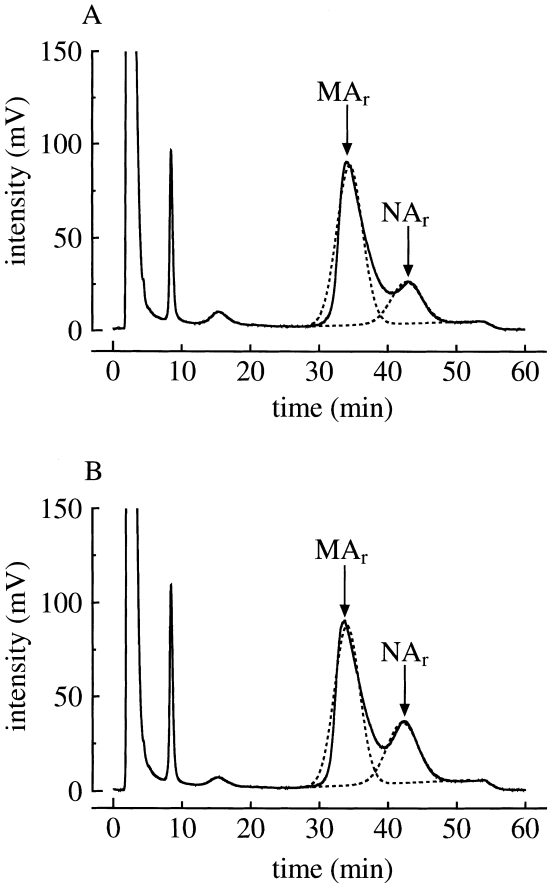

Fig. 4. Typical HPLC profiles of rat serum for control (A) and exercise (B) groups eluted using an ES-502N column. The elution conditions were same as those in Fig. 1H. The profiles were subjected to numerical curve fitting (dashed line). The f(MA_r) values in control and exercise groups were 76.4 and 68.3%, respectively.

an exhaustive physical exercise model of rats using a treadmill. It was reported that exhaustive physical exercise may increase ROS production, and oxidative damage occurs in the muscles, liver, blood and perhaps other tissues in rats [23,24].

Fig. 4A and B show typical HPLC profiles of rat serum for control and exercise groups, respectively. MA_r and NA_r were eluted in the latter half of the HPLC profiles. Both profiles were essentially identical except for the proportion of MA_r and NA_r fractions. As shown in Table 1, the mean values ($\pm$SD) of f(MA_r) for control ($n=7$) and exercise ($n=7$) groups were 76.2 ± 1.8 and $69.0\pm3.5\%$, respectively. The $\bar{f}(MA_r)$ in the exercise group was significantly decreased as compared with that of the control group ($P<0.01$). These results indicated that exercise in this experiment induced significant oxidation of MA_r.

GSH is one of the major intracellular antioxidants against oxidative damage caused by ROS. Orrenius et al. [25] reviewed secretion of GSSG from intracellular fluid under oxidative stress. Glutathione peroxidase scavenges H_2O_2 and hydroperoxide, and glutathione-*S*-transferase scavenges hydroperoxide in intracellular fluid, resulting in oxidation from GSH to GSSG. To maintain GSSG at a low level, NADPH-linked glutathione reductase primarily catalyzes reduction from GSSG to GSH. However, under conditions of oxidative stress such as exercise, the capacity of this system does not always appear to be high enough to handle the GSSG formed. Therefore, under conditions of oxidative stress, the cells actively secrete excess GSSG into the extracellular fluid [25]. These phenomena were observed in erythrocytes, perfused liver preparations and isolated hepatocytes [25]. Sastre et al. [26] demonstrated that exercise caused an increase in blood GSSG levels in both humans and rats. Lew et al. [27] showed that plasma GSSG levels were significantly increased after exhaustive exercise in the rat. Cystine and GSSG in extracellular fluid react with sulfhydryl groups of protein such as albumin [28] according to the following reaction:

$$\text{ALBUMIN-SH} + \text{RS-SR} \rightleftarrows \text{ALBUMIN-S-SR} + \text{RSH}$$

where RS-SR is cystine or GSSG, and RSH is cysteine or GSH.

In this experiment, it was suggested that excess GSSG might be secreted from the cell into the extracellular fluid during exercise, and the GSSG might react with MA_r. Therefore, most of the NA_r produced during exercise might be mixed with GSSG.

Recently, Inayama et al. [29] reported that protein-bound sulfhydryl groups in human plasma were

Table 1
The mean values for the fraction of MA_r ($\bar{f}(MA_r)$) and NA_r ($\bar{f}(NA_r)$) in control and exercise groups

	control group		exercise group
n	7		7
$\bar{f}(MA_r)$	76.2 ± 1.8%	**	69.0 ± 3.5%
$\bar{f}(NA_r)$	23.8 ± 1.8%	**	31.0 ± 3.5%

**, $P<0.01$ (Mann–Whitney's *U*-test).
Data listed as mean±SD.

significantly decreased after a full marathon race, compared with that before the race, using 5, 5′-dithiobis 2-nitrobenzoic acid (DTNB) method. However, it is necessary that the change in sulfhydryl of HSA should be directly investigated in order to understand a functional feature of HSA as a scavenger under oxidative stress. Here we demonstrated the mercapt⇔non-mercapt conversion of RSA directly by exhaustive exercise under controlled conditions. Therefore, using our HPLC system, the dynamic change in the redox state of HSA could be examined under the exhaustive exercise such as a full marathon race.

The role of ROS in certain human diseases is attracting increasing attention [14,15]. In such diseases, it is important to investigate the mechanism of mercapt⇔non-mercapt conversion of albumin. For this purpose, it is necessary to control experimental conditions related to oxidative stress. In the present study, we showed that the HPLC system using an ES-502N column and ethanol concentration gradient is a convenient tool with which to examine dynamic changes in the redox state of RSA associated with severe oxidative stress.

4. Abbreviations

GSH	Reduced glutathione
GSSG	Oxidized glutathione
H_2O_2	Hydrogen peroxide
HMA	Human mercaptalbumin
HNA	Human non-mercaptalbumin
HPLC	High-performance liquid chromatography
HSA	Human serum albumin
MA_r	Rat mercaptalbumin
NA_r	Rat non-mercaptalbumin
ROS	Reactive oxygen species
RSA	Rat serum albumin

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