DIFFERENTIAL DETERMINATION OF CATIONIC AND ANIONIC GLUTATHIONE S-TRANSFERASES BY ENZYME IMMUNOASSAY*

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Specific enzyme immunoassays for cationic and anionic glutathione S-transferases were established using the specific antibodies which were purified by antigen-bound adsorbent column chromatography. The enzyme immunoassay for cationic glutathione S-transferase had high specificity to cationic enzyme, but showed no cross reactivity with anionic one, and *vice versa*. The recovery of cationic glutathione S-transferase by the enzyme immunoassay was 94.7%, and coefficient of variation for within day and day-to-day precision were 7.8–10.4% and 8.5–12.5%, respectively. The enzyme immunoassay for anionic glutathione S-transferase also had a good recovery and precision.

Using these enzyme immunoassays for glutathione S-transferases, sera of various patients were analyzed. Serum cationic glutathione S-transferase was increased in patients with hepatitis and hepatoma, and anionic glutathione S-transferase in serum was increased in patients with liver cirrhosis.

Keywords—cationic glutathione S-transferase; anionic glutathione S-transferase; enzyme immunoassay; liver disease

INTRODUCTION

Glutathione S-transferases (EC 2.5.1.18, GS-T) are important bilirubin binding protein in liver cell supernatant fraction of various mammals including humans.¹⁾ GS-T was not selenium-dependent and had quite different physical and enzymatic characteristics from the glutathione peroxidase.²⁾ There are two groups in GS-T.^{3,4)} Cationic GS-T, which is focused in the pH range from 7 to 9, are divided into 6 or more species.³⁾ Anionic GS-T, which has pI of 5 is found in many organs.⁴⁾ Only cationic GS-T isoenzymes have an additional glutathione peroxidase activity,⁵⁾ and ligandin is one of cationic isoenzymes.⁶⁾

As far as we know, lipid hydroperoxides are hydrolyzed by glutathione peroxidase which is selenium-dependent and the cationic GS-T, and the two enzymes are responsible for the reduction

of hydroperoxides in tissues.⁷⁾ From these reasons, the differential determination of cationic and anionic GS-T in sera or in some tissues will be important to investigate the physiological role of GS-T and also will be useful for clinical investigations. In particular, lipid hydroperoxides have a high cell toxicity. Though methods for the determination of GS-T in sera of various patients had been described, there were many problems.^{6,8)} It was hard to determine the GS-T activity in serum even if the most reactive substrate was chosen, because the concentration of this enzyme in serum was very low. Moreover, immunological cationic and anionic GS-Ts were not differentiated by use of the ordinary antibody.⁴⁾

In this paper, we describe the enzyme immunoassays (EIA) for cationic and anionic GS-Ts using specific antibody to each enzyme, and

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serum levels of these enzymes in various patients are investigated.

MATERIALS AND METHODS

Materials — Human liver GS-T was purified according to the report of Awasthi *et al.*,⁴⁾ and separated into cationic GS-Ts, GS-T α , β , γ , δ , ϵ ,³⁾ and anionic GS-T, that was GS-T ω , which had different isoelectric points. Polystyrene beads were purchased from Ichiko Co. (Japan). *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) was obtained from Pierce Co. (U.S.A.) β -Galactosidase was obtained from Boehringer Mannheim GmbH. (West Germany). Other reagents used were at least the reagent grade and purchased locally.

Preparation of Antibody — Female rabbits were immunized with the purified enzyme (100 μ g) emulsified in Freund's complete adjuvant and were injected into each food pad. The immunization procedure was repeated subcutaneously at intervals of two weeks. Approximately eight weeks after the first injection, the animals were bled and the crude IgG fraction were prepared by fractionation with 33% saturated $(\text{NH}_4)_2\text{SO}_4$, followed by passage through a column DEAE-cellulose.⁹⁾ The IgG fraction raised against cationic GS-T β was further passed through an immobilized GS-T β column (by use of activated CH-Sepharose 4B) equilibrated with 50 mM phosphate buffer (pH 7.5) containing 0.5 M NaCl. The column was washed with the same buffer and subsequently the bound antibody was eluted with 0.2 M Na_2CO_3 solution containing 0.5 M NaCl. This specific antibody against cationic GS-T was dialyzed against distilled water and stored at 4°C. Anti-anionic GS-T specific antibody was purified by using an immobilized GS-T ω column.

Preparation of Anti-GS-T Antibody Coated Polystyrene Beads — Polystyrene beads were washed with 10% detergent (Scat 20-X, Nakarai Chemicals Co.) and then immersed in 0.25 M sodium phosphate buffer (pH 7.0) containing anti-cationic or anionic GS-T specific antibody (0.1 mg/ml) at 37°C for 2 h and allowed to stand at 4°C overnight. Then, the beads were immersed

in 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM MgCl_2 , 1% bovine serum albumin (BSA) and 0.1% NaN_3 at 37°C for 2 h, and thereafter washed with 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM MgCl_2 , 0.1% BSA and 0.1% NaN_3 (buffer A) and stored in buffer A at 4°C until use.

Preparation of the Antibody- β -galactosidase Conjugate — The antibody- β -galactosidase conjugate was prepared using MBS as a crosslinking reagents by the method of O'Sullivan *et al.*¹⁰⁾ Anti-cationic or anionic GS-T specific antibody was dialyzed against 0.1 M phosphate buffer (pH 7.0) (1 mg/ml). 40 μ l of MBS solution (2 mg of MBS was dissolved in 0.1 ml dioxane) was added to 1 ml of the antibody solution, mixed and kept at 30°C for 1 h. The solution was applied to a Sephadex G-25 column (0.9 $\times$ 15 cm) equilibrated with 10 mM phosphate buffer (pH 7.0) containing 10 mM MgCl_2 , and 50 mM NaCl and eluted with the same buffer. 1.5 mg of β -galactosidase was mixed with the antibody eluted from the column and maintained at 30°C for 1 h. The reaction was terminated by the addition of 1 M 2-mercaptoethanol to give a final concentration of 10 mM. The conjugate solution was applied to a Sepharose 6B column (0.9 $\times$ 30 cm) equilibrated with buffer A and eluted with the same buffer. Measurement of β -galactosidase activity was carried out according to the method of Craven *et al.*¹¹⁾ using *o*-nitrophenyl- β -D-galactoside as a substrate. The enzyme activity with immunologically active antibody was determined by the standard enzyme immunoassay procedure, and fractions with this activity was used as an antibody- β -galactosidase conjugate in this enzyme immunoassay.

Standard Procedure for Enzyme Immunoassay — The antibody-coated polystyrene bead and sample (10 μ l) of serum were added in 140 μ l of 10 mM sodium phosphate buffer (pH 7.0) containing 0.1 M NaCl, 1 mM MgCl_2 , 0.1% NaN_3 , 1% BSA, and 0.05% Tween 20 (buffer B) and shaken at 37°C for 2 h. The bead was washed twice with 1.5 ml of 10 mM sodium phosphate

buffer (pH 7.4) containing 0.15 M NaCl, and 0.05% Tween 20 (buffer C), and incubated with 5 μ l of antibody- β -galactosidase conjugate in 150 μ l of buffer B and shaken at 37°C for 5 h. The bead was washed twice with 1.5 ml of buffer C and then β -galactosidase bound to the polystyrene bead was assayed using 4-methylumbelliferyl- β -D-galactoside as a substrate.¹²⁾

Determination of Serum Lipid Peroxide — The assay of lipid peroxide was performed according to the method of Ohkawa *et al.*¹³⁾ A fifth ml of serum was added 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH, and 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA). The mixture was made up to 4 ml with distilled water, and then heated in an oil bath at 95°C for 60 min using a glass ball as

a condenser. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of the mixture of *n*-butanol and pyridine (15:1, v/v) were added and shaken vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was taken and its absorbance at 532 nm was measured. The level of lipid peroxides was expressed as nmol of malondialdehyde.

RESULTS

Specificities of Anti-cationic and Anionic GS-T Antibodies

Anti-cationic GS-T antibody reacted with GS-T α - ϵ and showed one fused precipitin line on double immunodiffusion as shown in Fig. 1 (a), but this antibody revealed no precipitin line with anionic GS-T as shown in Fig. 1 (b). In addition, the cross-reactivity of this antibody against

FIG. 1. *Specificity of Anti-cationic Glutathione S-Transferase Antibody*

A, double immunodiffusion of cationic glutathione S-transferase; center well: anti-cationic glutathione S-transferase specific antibody; peripheral wells: cationic glutathione S-transferases α - ϵ ; B, immunoelectrophoresis of cationic and anionic glutathione S-transferases; upper well: cationic glutathione S-transferase; lower well: anionic glutathione S-transferase; sulcus: anti-cationic glutathione S-transferase specific antibody; C, immunotitration of glutathione S-transferase using anti-cationic S-transferase specific anti-antibody; ○: cationic glutathione S-transferase; ●: anionic glutathione S-transferase. Immunoelectrophoresis was performed at 4°C and 10 V/cm with 1% agarose gel in 30 mM Veronal buffer (pH 8.6) for 60 min.

cationic and anionic GS-Ts were investigated by standard enzyme immunoassay. Figure 1 (c) shows that anti-cationic GS-T antibody is specific to cationic enzyme rather than anionic GS-T. Also, anti-anionic antibody was investigated by immunoelectrophoresis and by standard enzyme immunoassay. This antibody show no precipitin line against cationic GS-T (Fig. 2 (a)) and the cross-reactivity of this antibody to cationic GS-T is 10^{-3} compared to anionic one (Fig. 2 (b)).

Fundamental Investigation for EIA

The time required for the first incubation of anti-cationic or anionic GS-T antibody coated polystyrene bead in the presence of 500 pg of cationic GS-T or 100 ng of anionic GS-T, at 37°C was investigated under the constant conditions of the second immunoreaction (37°C, 6 h). On the other hand, the second immunoreaction time at

37°C was examined under the optimum conditions of first immunoreaction (37°C, 2 h). The first immunoreaction almost reached a plateau value within 1 h. When the second immunoreaction was performed at 37°C, the enzyme activity was increased with the second incubation time, and reached the equilibrium in 5 h. Under the above conditions, the enzyme immunoassays were performed using purified cationic and anionic GS-Ts as standard. The calibration curves for cationic and anionic GS-T are shown in Fig. 3 and Fig. 4, respectively. These enzyme immunoassays could detect from 5.0 to 250 pg of cationic GS-T and from 1.0 to 50 ng of anionic GS-T, respectively.

The within-day and day-to-day precisions were studied using serum samples. The coefficient of variation (C.V.) for within-day was 10.4% (13

FIG. 2. *Specificity of Anti-anionic Glutathione S-Transferase Antibody*

A, immunoelectrophoresis of anionic and cationic glutathione S-transferases; upper well: anionic glutathione S-transferase; lower well: cationic glutathione S-transferase; sulcus: anti-anionic glutathione S-transferase specific antibody; B, immunotitration of glutathione S-transferase using anti-anionic glutathione S-transferase specific antibody; ● : anionic glutathione S-transferase; ○ : cationic glutathione S-transferase. Immunoelectrophoresis was performed at 4°C and 10 V/cm with 1% agarose gel in 30 mM Veronal buffer (pH 8.6) for 60 min.

pg/tube, $n=10$), 7.8% (41 pg/tube, $n=10$) and 9.8% (71 pg/tube, $n=10$) for the assay of cationic GS-T and 10.7% (6.1 ng/tube, $n=10$), 8.6% (11 ng/tube, $n=10$) and 10.1% (32 ng/tube, $n=10$) for the assay of anionic GS-T, respectively. The

day-to-day precision (C.V.) was 11.4% (13 pg/tube, $n=10$), 8.5% (41 pg/tube, $n=10$) and 12.5% (71 pg/tube, $n=10$) for cationic GS-T and 10.3% (6.1 ng/tube, $n=10$), 9.2% (11 ng/tube, $n=10$) and 11.6% (32 ng/tube, $n=10$) for anionic GS-T, respectively. The average recovery was 94.7% for the determination of cationic GS-T, and 101.4% for the assay of anionic GS-T.

Serum Cationic and Anionic GS-T Concentration in Various Diseases

Figure 5 shows serum cationic GS-T concentration in normal subjects and in patients with various diseases. The normal range of serum cationic GS-T concentration was 1.06 ± 0.64 ng/ml (mean $\pm$ SD). Serum cationic GS-T levels were high in liver diseases, with elevations being especially remarkable both in only 2 patients with fatty liver. The normal range of anionic GS-T in serum was 0.57 ± 0.45 g/ml, as shown in Fig. 6. Patients with hepatocirrhosis had high serum anionic GS-T concentration. However, the concentrations of cationic GS-T or anionic GS-T in serum had no correlation with serum aspartate aminotransferase (cationic GS-T-AST, $r=0.246$; anionic GS-T-AST, $r=0.414$), alanine aminotransferase (cationic GS-T-ALT, $r=0.035$; anionic GS-T-ALT, $r=0.092$) and alkaline phosphatase (cationic GS-T-AIP, $r=0.053$; anionic GS-T-AIP, $r=0.058$).

Moreover, in order to investigate the correlation between the serum hydroperoxide levels and the GS-Ts concentration, TBA reacting substances were determined. Consequently, there was no correlation between TBA reacting substances and cationic GS-T ($r=-0.276$) or anionic GS-T ($r=-0.201$).

DISCUSSION

There are two different types of GS-Ts, and cationic and anionic GS-T have different substrate specificity.¹⁴⁾ Therefore, two enzymes may have different physiological roles and differential determination of cationic or anionic GS-T must be performed in order to investigate the relationship between its behavior and diseases. It has been reported that GS-Ts were immunologically simi-

FIG. 3. Calibration Curve for Cationic Glutathione S-Transferase

The fluorescence intensity of resulting solution in the absence of antigen was about 1.2.

FIG. 4. Calibration Curve for Anionic Glutathione S-Transferase

The fluorescence intensity of resulting solution in the absence of antigen was about 5.5.

lar not only among cationic GS-Ts³⁾ but also between cationic and anionic species.⁴⁾ In this paper, we have prepared a specific antibody for GS-T

α - ϵ or GS-T ω , respectively, which is the representative of cationic or anionic GS-Ts.⁴⁾ Immunological difference of two GS-T groups

FIG. 5. Cationic Glutathione S-Transferase Concentration in Sera of Normal Subjects and Patients with Various Diseases

Bars indicate the mean value of cationic glutathione S-transferase concentration, and figures in parentheses show the number of patients.

FIG. 6. Anionic Glutathione S-Transferase Concentration in Sera of Normal Subjects and Patients with Various Diseases

Bars indicate the mean value of anionic glutathione S-transferase concentration, and figures in parentheses show the number of patients.

was confirmed by the experiments of double immunodiffusion and the enzyme immunoassay. Cationic GS-Ts, such as GS-T α , β , γ , δ , ϵ , showed fused one precipitin line against anti-cationic GS-T specific antibody, and this antibody showed no precipitin line against anionic GS-T. On the other hand, anti-GS-T specific antibody showed no precipitin line against cationic GS-T. Using these antibodies, which were specific to cationic and anionic GS-Ts, enzyme immunoassays for cationic and anionic GS-T were developed. These assays were the ordinary sandwich enzyme immunoassay using polystyrene beads as solid phase and β -galactosidase as a labeled enzyme. The detection range, precisions and recoveries of these assays were good enough to measure the serum level of GS-Ts. Even if the same amount of the specific antibody was used to coat the polystyrene beads, the difference of detection range for cationic and anionic GS-Ts was observed. This was probably due to quality of the antibody, but not due to quantity.

Serum level of anionic GS-T was about 500 times higher than that of cationic GS-T. Serum cationic GS-T may derive from liver, because of the increase of it in the patients with liver diseases. Anionic GS-T increased in the patients with liver cirrhosis. The above results imply that the level of cationic GS-T is not determined by the conventional methods for measuring the GS-T activity,⁶⁾ and these enzyme immunoassays may offer the clinical significance of serum GS-Ts.

Recently, it has been reported that the lipid peroxide concentration in serum was increased in patients with fulminant hepatitis, liver cirrhosis (decompensated) and fatty liver,¹⁵⁾ and GS-Ts were comparatively specific enzyme in the liver.¹⁶⁾ In the present results, there is no correlation between serum GS-T concentration and serum lipid hydroperoxide level. It is reported that GS-Ts have a short half-life rather than ALT and AST.¹⁷⁾ Therefore, taking into the above considerations, serum GS-Ts concentrations may reflect the liver disorders, and provides a new and useful tool for the diagnosis of liver diseases.

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