A Simplified Method for the Estimation of Glutathione Peroxidase Activity and Selenium Concentration in Bovine Blood

CHIKAKO KINOSHITA, KOH-ICHI SAZE, SHYOJI KUMATA, TAKAYOSHI MASTUKI, and SOTA HOMMA
Central Research Laboratories, Nippon Zenyaku Kogyo Co., Ltd., Sasagawa-Asaka, Koriyama, Fukushima, 963-01 Japan

ABSTRACT

Twelve hybridoma clones were established that produced mouse monoclonal antibodies to bovine erythrocyte glutathione peroxidase. In these monoclonal antibodies, GPF-1, GPI-2, and GPJ-1, which showed marked reaction to this enzyme, were examined for reactivity to erythrocyte lysates from 12 different species of animals and from humans and for the inhibition of glutathione peroxidase activity. GPF-1 and GPJ-1 reacted markedly with glutathione peroxidase in erythrocyte lysates from ruminants and pigs and inhibited enzymatic activity. Conversely, GPI-2 showed positive reaction to hemolysate from all mammals used, except for mice, and did not inhibit enzymatic activity. To determine the concentration of bovine glutathione peroxidase in erythrocyte lysates, a sandwich ELISA was developed using GPJ-1, both as a coated antibody and as a peroxidase-labeled antibody. This ELISA system was a sensitive procedure with a detection limit of 6 ng/ml for glutathione peroxidase protein. Using blood samples from 121 cows, optical density by this ELISA was well correlated with the glutathione peroxidase activity and with the selenium concentration in bovine whole blood. The sandwich ELISA using GPJ-1 is a rapid and simple screening method to estimate glutathione peroxidase activity in bovine whole blood.

INTRODUCTION

To determine the activity of glutathione peroxidase (GSH-Px; EC 1.11.1.9) Paglia and Valentine (13) developed the procedure based on the enzyme reaction between NADPH₂ and peroxides. This procedure has been variously modified by several investigators (1, 7, 9, 14), but the modifications require use of specialized laboratory equipment. Board and Peter (3) and Backall and Scholz (2) simplified the method so that enzyme activity could be analyzed based on the rate of defluorescence on filter paper under long-wave ultraviolet rays. Such a test for clinical use is needed to screen for GSH-Px deficiency. The ELISA system is available to estimate GSH-Px activity simply and sensitively, and many samples may be assayed simultaneously using the ELISA system. Suemizu et al. (18) reported results obtained by ELISA for GSH-Px detection using the monoclonal antibody (MAb) to human GSH-Px. In the present study, we produced MAb against bovine erythrocyte GSH-Px and developed an ELISA system using one of these MAb to estimate GSH-Px activity in bovine whole blood.

MATERIALS AND METHODS

GSH-Px and Production of MAb

The GSH-Px used, which was purified from bovine erythrocytes, had a molecular mass of 84,000 Da and was obtained from Funakoshi Co. (Tokyo, Japan).

The MAb to GSH-Px were produced according to the method of Köhler and Milstein (6). Five-week-old BALB/c mice were injected intraperitoneally four times at intervals of 2 wk with 100 μl of 0.15 M PBS containing GSH-Px at the concentration of 1 mg/ml and 100 μl of Freund's incomplete adjuvant (Difco Laboratories, Detroit, MI). Three days after the last injection, these immunized mice were exsanguinated under ether anesthesia, and their spleen cells were fused with P3-X63-Ag8-6.5.3 mouse myeloma cells. A screening test by ELISA, using the coated antigen of bovine erythrocyte GSH-Px, was applied to the culture supernatants. Colonies that produced specific MAb to GSH-Px were subcloned three times by the limiting dilution method. Among 12 strains of the
established hybridomas, 3 strains producing MAb that were strongly reactive to GSH-Px were transplanted into the peritoneal cavity of BALB/c mice. Each MAb in the ascitic fluid was highly purified on a Protein A Sepharose column (Pharmacia, Uppsala, Sweden). The isotype of MAb obtained was determined with the Mouse Typer™ kit (Bio-Rad Co., Richmond, CA).

**Inhibition Test of Enzyme Activity**

The inhibition of GSH-Px activity by 3 different MAb, GPF-1, GPI-2, and GPJ-1, was examined. H155-2, used as a negative control, was a MAb to *Actinobacillus pleuropneumoniae* serotype 5a and was the same subclass as the 3 MAb described previously. Each MAb, in amounts of 2.5, 1.3, 0.63, 0.31, and 0.16 µg, was mixed with 0.55 µg of bovine erythrocyte GSH-Px protein and incubated for 1 h at 37°C; then, enzyme activities in the mixed solutions were determined. These tests were run parallel to those for GSH-Px without MAb under the same conditions. The protein concentrations of GSH-Px and MAb were measured by Coomassie protein assay reagent (Pierce, Rockford, IL).

**Preparation of MAb Labeled with Peroxidase**

Three MAb, GPF-1, GPI-2, and GPJ-1, were labeled with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO), according to the method of Nakane and Kawaoi (11). The MAb were purified through the gelfiltration column of Superose 12 prep grade (Pharmacia).

**Sandwich ELISA for Quantification of GSH-Px**

GPF-1 (100 µl) was added to wells of immunoplates (Nunc Co., Roskilde, Denmark) at the concentration of 250 ng per well and incubated for 1 h at 37°C; thereafter, the wells were incubated with 100 µl of 1% BSA in 0.15 M PBS for 1 h at 37°C. The wells were washed once with PBS containing 0.05% Tween-20, and then 50 µl of GSH-Px diluted with PBS at the optimal concentration or cell lysate was added. Cell lysate was diluted with hemolytic Gey's balanced salt solution (10) at 1:20 (vol/vol) and with PBS at 1:16 (vol/vol). Following incubation of the wells for 1 h at 37°C and five washings with PBS containing 0.05% Tween-20, 50 µl of peroxidase-labeled GPF-1 diluted at 1:200 (vol/vol) with PBS were added to each well and incubated for 1 h at 37°C. Again, the wells were washed five times with the same buffer, and 50 µl of substrate solution [0.0675% (wt/vol) 2,2'-azino-bis (3-ethylbenzthiazone-6-sulfonic acid) diammmonium salt and 0.025% (vol/vol) H₂O₂ in 100 mM citrate buffer solution; pH 4.0] were added. After incubation for 10 min at 37°C, the reaction was stopped by the addition of 50 µl of 0.32% NaF solution, and the optical density at 414 nm was measured by a microplate reader (model 2550; Bio-Rad Co.). The sandwich ELISA system using GPF-1 or GPI-2 mostly followed the same protocol as GPJ-1, except that the concentration of coated antigen was 40 or 200 ng per well and the peroxidase-labeled MAb was diluted at 1:200 (vol/vol) or 1:70 (vol/vol) in PBS, respectively.

**Reactivity of 3 MAb to Erythrocyte Lysates from Several Species of Animals**

Reactivity of GPF-1, GPI-2, and GPJ-1 to erythrocyte lysates from humans and 12 species of animals (cows, goats, sheep, pigs, horses, dogs, cats, rabbits, guinea pigs, mice, chickens, and geese) under apparently normal conditions was examined by the sandwich ELISA. Erythrocyte lysates were prepared by dilution of whole blood with hemolytic Gey's balanced salt solution at 1:20 (vol/vol). Phosphate-buffered saline was used as a negative control.

**Bovine Blood Samples**

Bovine blood samples were obtained from 121 cows (62 Holstein-Friesian and 59 Japanese black) ranging in age from 1 wk to 9 yr. Cows were reared in Fukushima prefecture, and parity ranged from 0 to 6. Blood samples were collected from the cervical vein into heparinized vacutainers and were used for the sandwich ELISA to determine GSH-Px activity within 12 h. The remaining samples were maintained at 4°C until used for the determination of selenium concentration.

**Determination of GSH-Px Activity and Selenium Concentration**

The GSH-Px activity of all samples was assayed by the method of Paglia and Valentine (13) as modified by Scholz and Hutchinson (14), and the selenium concentration of all blood samples was determined by HPLC with fluorescence detection by Kumata and Homma (8).

**Statistical Analysis**

One-way ANOVA was used to determine significant differences among optical densities, indicating the responses of three MAb against erythrocyte ly-
sates from each animal and from humans (5). For the analysis of correlation among optical densities by ELISA, GSH-Px activities, and selenium concentrations of whole blood, linear regression was used (5).

RESULTS

Characterization of MAb to Bovine Erythrocyte GSH-Px

Twelve hybridomas producing MAb to bovine erythrocyte GSH-Px were established in four different subclasses of Ig (3 IgM, 6 IgG1, 2 IgG2b, and 1 IgG3). Three of the hybridomas, GPF-1 (IgGl), GPI-2 (IgGl), and GPJ-1 (IgGl), which showed marked reaction to GSH-Px, were examined for reaction against the erythrocyte lysates from 12 species of animals and from humans. Data were expressed as means (±SE) of optical density values at 414 nm for three individual samples (Table 1). GPJ-1 showed a marked reaction to the erythrocyte lysates from cows, goats, sheep, and pigs and a weak reaction to those from horses, dogs, cats, and guinea pigs, but no reaction to the others. Conversely, GPI-2 showed positive reactions to the erythrocyte lysates from humans and animals, except for mice, chickens, and geese; however, the reactions to erythrocyte lysates from rabbits and guinea pigs were weaker than the reactions to the other positive lysates. GPF-1 reacted positively to the erythrocyte lysates from ruminants, pigs, and guinea pigs, but reaction to the sample prepared from guinea pigs was comparatively weak.

Inhibition of Enzyme Activity by MAb

Figure 1 shows the results of inhibition of GSH-Px activity by four MAb; H155-2, used as a negative control, and GPI-2 could not inhibit GSH-Px activity.

<table>
<thead>
<tr>
<th>Erythrocyte lysate</th>
<th>MAb</th>
<th>GPF-1</th>
<th>GPI-2</th>
<th>GPJ-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>X</td>
<td>SE</td>
<td>X</td>
<td>SE</td>
</tr>
<tr>
<td>Cow</td>
<td>1.751</td>
<td>0.006</td>
<td>1.641</td>
<td>0.014</td>
</tr>
<tr>
<td>Goat</td>
<td>1.854</td>
<td>0.002</td>
<td>1.676</td>
<td>0.008</td>
</tr>
<tr>
<td>Sheep</td>
<td>1.538</td>
<td>0.018</td>
<td>1.675</td>
<td>0.007</td>
</tr>
<tr>
<td>Pig</td>
<td>1.036</td>
<td>0.012</td>
<td>1.531</td>
<td>0.005</td>
</tr>
<tr>
<td>Horse</td>
<td>0.239</td>
<td>0.007</td>
<td>0.854</td>
<td>0.077</td>
</tr>
<tr>
<td>Dog</td>
<td>0.322</td>
<td>0.006</td>
<td>0.816</td>
<td>0.041</td>
</tr>
<tr>
<td>Cat</td>
<td>0.210</td>
<td>0.022</td>
<td>0.937</td>
<td>0.029</td>
</tr>
<tr>
<td>Rabbit</td>
<td>0.238</td>
<td>0.008</td>
<td>0.502</td>
<td>0.019</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>0.364</td>
<td>0.066</td>
<td>0.738</td>
<td>0.093</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.221</td>
<td>0.002</td>
<td>0.212</td>
<td>0.007</td>
</tr>
<tr>
<td>Chicken</td>
<td>0.188</td>
<td>0.001</td>
<td>0.209</td>
<td>0.009</td>
</tr>
<tr>
<td>Goose</td>
<td>0.229</td>
<td>0.011</td>
<td>0.206</td>
<td>0.020</td>
</tr>
<tr>
<td>Human</td>
<td>0.223</td>
<td>0.011</td>
<td>1.576</td>
<td>0.088</td>
</tr>
<tr>
<td>Control2</td>
<td>0.237</td>
<td>0.010</td>
<td>0.242</td>
<td>0.009</td>
</tr>
</tbody>
</table>

1Values represent means and standard errors of optical densities at 414 nm of three individual samples.

2Values of PBS as a negative control.

**Significantly different from control (P < 0.01).
However, GPF-1 and GPJ-1 inhibited the enzyme activity depending on the concentration of MAb. Approximately 90% of enzymatic activity of 0.55 μg of GSH-Px was inhibited by 1.3 μg of GPJ-1.

Sandwich ELISA

The result of sandwich ELISA using GPJ-1 and a commercial bovine GSH-Px is shown in Figure 2. The optical densities obtained by the ELISA showed nearly linear increases from 0.5 to 1.5 at GSH-Px concentrations ranging from approximately 50 to 450 ng/ml. The optical density in PBS used as a background was 0.2; therefore, the detection limit of GSH-Px protein in this system was estimated at 6 ng/ml. Using whole blood samples from 121 cows, the optical densities by ELISA with GPJ-1 and GSH-Px activities were well correlated (Y = 7.05 X + 1.77; r = 0.961; Figure 3). In the same samples, the selenium concentrations of bovine whole blood were also highly correlated with the GSH-Px activities (Y = 20.94 X - 6.04; r = 0.954; Figure 4) and with the optical densities by ELISA (Y = 149.7 X + 29.09; r = 0.930; Figure 5). There were no differences in these correlations among Holstein-Friesian and Japanese black cows.

DISCUSSION

GPI-2, one of 12 MAb, responded to erythrocyte GSH-Px from nine species of animals and from humans. Conversely, GPF-1 and GPJ-1 reacted strongly to erythrocyte GSH-Px from ruminants and pigs, but reacted weakly to those of guinea pigs. Although the optical densities by ELISA with GPJ-1 in the samples from horses, dogs, and cats were significantly higher than that of the control, these differences were thought to be negligible because values were <0.2.

These results suggest the presence of species differences in the structure of erythrocyte GSH-Px. In the inhibition test of GPF-1, GPI-2, and GPJ-1 against GSH-Px activity, GPJ-1 was the most active. Thus, GPJ-1 is superior to the other 2 MAb in the recognition of active sites of this enzyme, which is why GPJ-1 was selected for use in the ELISA system.

Hemolytic Gey's balanced salt solution, which was used in this system to simplify the freeze-thawing process for preparing the hemolysate, caused the lysis of erythrocytes only. Accordingly, the GSH-Px concentration determined in the sample, excluding leuko-
cytes and thrombocytes, might have been slightly low compared with that in the sample used in the freeze-thawing method. However, Scholz and Hutchinson (14) reported that 98% of GSH-Px activity in bovine whole blood was contained within erythrocytes, and the correlation coefficient between optical density by ELISA with GPJ-1 and GSH-Px activity in bovine whole blood was very high (r = 0.961) in this experiment. Therefore, the approximate estimation of the enzyme activity in bovine whole blood is thought to be possible using this ELISA system.

Scholz and Hutchinson (14), Scholz et al. (15), and other investigators (1, 2, 4, 12, 17, 19, 20) reported that GSH-Px activity and selenium concentration in bovine or sheep blood were well correlated. In this experiment, a correlation coefficient between the two factors was high (r = 0.954), and the optical density by ELISA and selenium concentration in bovine whole blood also were well correlated (r = 0.930). These results indicate that the estimation of GSH-Px activity and selenium concentration is indirectly possible by the ELISA system developed in this experiment.

Suemizu et al. (18) examined the relationship between optical density by ELISA using MAbs to human GSH-Px and enzyme activity of human erythrocyte lysates and found that the correlation coefficient was 0.72. In comparison, the correlation coefficient between ELISA using GPJ-1 and GSH-Px activity of bovine blood samples was 0.961 in this experiment. Although the cause of the difference in these two studies was not clear, the specificity of MAbs to GSH-Px might have been an important factor.

On the basis of the standard curve of the GSH-Px concentration detected by ELISA with GPJ-1, the determination of GSH-Px concentration would be most reliable in the optical density range of 0.5 to 1.5 in this ELISA system. Smith et al. (16) reported that 70 ng of selenium/ml of plasma fell at the lower end of the normal range in bovine blood and translated to about 200 ng of selenium/ml of whole blood. This value corresponds with the optical density of 1.142 that was obtained from the sandwich ELISA. Accordingly, the lower end of the normal range can be estimated using the sandwich ELISA, which indicates that the sandwich ELISA is available as a screening test for the detection of deficiencies in GSH-Px and selenium.

The ELISA system developed in this experiment might be useful for simple and rapid screening of the selenium status of a large number of dairy or beef cattle.

ACKNOWLEDGMENTS

The authors thank the director and researchers of Fukushima Animal Husbandry Experiment Station and its Numajiri branch for assistance in sample collection and Kazunori Hashimoto, Nippon Zenyaku Kogyo Co., Ltd., for useful comments and suggestions.

REFERENCES