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A SIMULTANEOUS VISUALIZATION OF THE ANTIOXIDANT ENZYMES GLUTATHIONE PEROXIDASE AND CATALASE ON POLYACRYLAMIDE GELS

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A simple and sensitive method for the simultaneous visualization of glutathione peroxidase and catalase on polyacrylamide gels is described. The procedure included: (1) running samples on a 7.5% polyacrylamide gel, (2) soaking the gel in a certain concentration of reduced glutathione (0.25–2.0 mM), (3) soaking the gel in GSH plus H_2O_2 or cumene hydroperoxide, (4) finally staining with a 1% ferric chloride 1% potassium ferricyanide solution. The best concentration of glutathione for simultaneous visualization of glutathione peroxidase and catalase was 0.25 mM; 1.5 mM glutathione was the best concentration for visualization of glutathione peroxidase alone. The method is sensitive enough to detect catalase and glutathione peroxidase in mouse liver homogenates and also it is specific for glutathione peroxidase since other peroxidases such as lactoperoxidase, horseradish peroxidase and glutathione S-transferase cannot be visualized. Using this method, it was found that unlike catalase, glutathione peroxidase is heat resistant (68°C, 1 min), but sensitive to 10 mM sodium iodoacetate.

KEY WORDS: Catalase, glutathione peroxidase, staining.

INTRODUCTION

Catalase (E.C.1.11.1.6) and glutathione peroxidase (E.C.1.11.1.9) are two of the primary antioxidant enzymes. Both enzymes can reduce H_2O_2 to H_2O.[1,2] Catalase (CAT) can be quantitated by enzymatic assay and polyacrylamide gel staining.[3–6] Glutathione peroxidase (GPX) can also be measured by biochemical assays.[2] However, there is no staining system to visualize the enzyme on polyacrylamide gels. We have modified the staining methods for CAT to visualize GPX.

MATERIALS AND METHODS

Chemicals

Mouse liver catalase, human and bovine erythrocyte glutathione peroxidase, human placental glutathione S-transferase, horseradish peroxidase, bovine milk lactoperoxidase, bovine intestine mucosal glutathione reductase, glutathione (reduced form), β-NADPH, diaminobenzidine, and cumene hydroperoxide were purchased

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from Sigma Chemical Co. Hydrogen peroxide, potassium ferricyanide, and ferric chloride were products of Fisher Scientific Co. Sodium iodoacetate was from Aldrich Chemical Company, Inc. Triton X-100 was from Mallinckrodt, Inc. Acrylamide was from Schwarz/Mann Biotech. N,N'-methylene bisacrylamide was from Eastman Kodak Co. Molecular weight markers were obtained from Bio-Rad.

Tissue Preparation

Mice (Swiss strain) were killed by cervical dislocation. The liver was quickly removed, washed with 50 mM phosphate buffer (pH. 7.0) and homogenized on ice in a Tissuemizer (Tekmar Company) at high speed for three 20 seconds bursts. The homogenate was centrifuged at 50,000 × g for 1 hour. The supernatant was saved for CAT, GPX, and protein assay and for gel electrophoresis.

Staining Methods

Two methods of staining were used after electrophoresis was performed on native 7.5% polyacrylamide slab gels with 5% stacking gels. The first one was the ferricyanide method as described by Woodbury *et al.*[4] with our modification to allow it to detect both CAT and GPX. Gels were soaked in three changes of reduced glutathione (GSH, 0.25–2.0 mM) for a total of 45 min, then soaked in 0.003% hydrogen peroxide or 0.008% cumene hydroperoxide plus GSH for 10 min. The gels were next rinsed twice with distilled water. They were then stained with 1% ferric chloride/1% potassium ferricyanide solution which was made fresh and mixed immediately before use from equal volumes of 2% stock solution of each. The gels were stained until they became dark green with yellow activity bands. The staining solution was then poured off, the gels briefly rinsed with water, and then wrapped in Saran Wrap plus aluminum foil and stored at 4°C. Ferricyanide is a strong oxidizing agent, and it is necessary to wear gloves during staining. The second method utilized diaminobenzidine and previously published procedures[5,6] with slight modification. All solutions were made in 50 mM potassium phosphate at pH 7.0. Bands of catalase activity were localized by soaking the gel first with horseradish peroxidase (10 μg/ml), GSH (0.25–2.0 mM), β-NADPH (0.2 mM), and glutathione reductase (1 unit/ml) for 45 min and then with horseradish peroxidase plus H_2O_2 (5.0 mM) or cumene hydroperoxide (15 mM) and GSH, β-NADPH and glutathione reductase for another 10 min. followed by rinsing and subsequent soaking in 0.5 mg/ml diaminobenzidine until the gels become brown with achromatic catalase bands. In some experiments, GSH, NADPH, or glutathione reductase were omitted from the staining procedure. The gels were stored as described above. Diaminobenzidine is a possible carcinogen; therefore, gloves should be worn during staining.

Catalase and Glutathione Peroxidase Assay

Catalase was measured as described by Beers and Sizer[1] and Cohen *et al.*[3] Enzyme activity was described in terms of k units per mg protein. Glutathione peroxidase activity in mouse liver was measured by the method of Lawrence *et al.*[2] Results are expressed as μ moles NADPH oxidized per min per mg protein.

Protein Assay

Protein was determined by the method of Lowry *et al.*[7] with the use of defatted bovine serum albumin as the standard.

RESULTS

Since we used commercially prepared CAT and GPX in this study, we thought it necessary to determine the purity of the enzymes. Figure 1a shows a native acrylamide gel (7.5%) stained for protein with Coomassie blue. Lane 1 contained mouse liver CAT. Multiple bands were observed. There were two main groups of bands, both of which showed multiple bands. We will later show that CAT activity occurred in both of these groups. The fastest migrating band shown in lane 1 is apparently not CAT as no staining occurs there. In contrast, lanes 2 and 3, which contained bovine and human GPX, respectively, had only one large dark band. There lanes were grossly overloaded to look for impurities. There were multiple bands in these darkly staining regions, and more than one of them stained for GPX activity, as will be shown later.

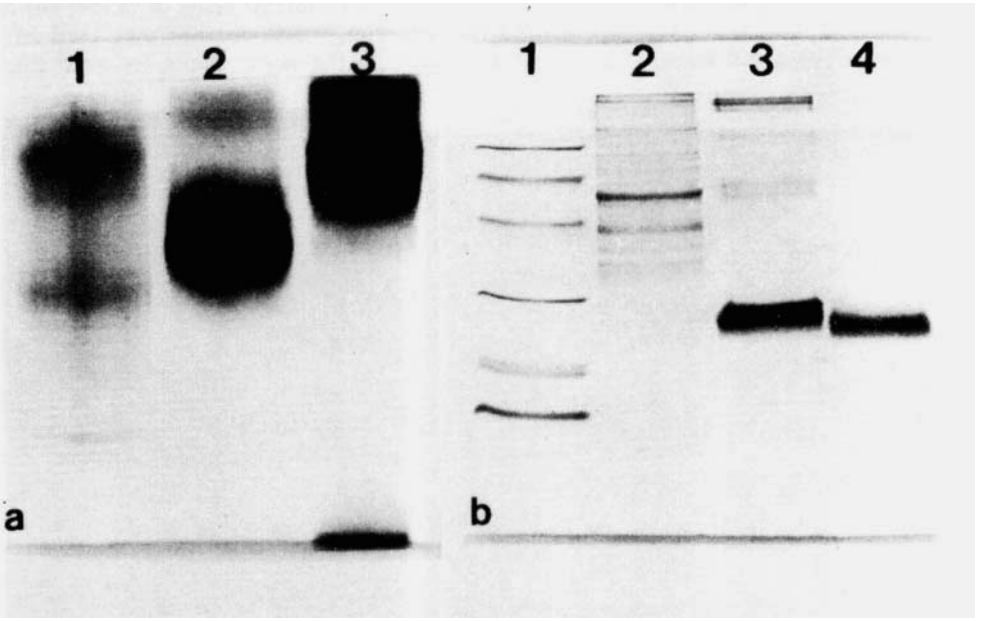

FIGURE 1 (a) Native gel (7.5%) of CAT and GPX stained with Coomassie blue. Lane 1 contains 100 μg of mouse liver CAT. Lane 2 contains 125 μg of bovine GPX. Lane 3 contains 75 μg of human GPX. (b) 12.5% SDS gel of CAT and GPX stained with Coomassie blue. Lane 1 contains molecular weight markers (Bio-Rad). These markers are (from top to bottom): phosphorylase B (97,400), bovine serum albumin (66,200), ovalbumin (42,699), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,400), and lysozyme (14,400). Lane 2 contains 20 μg of Sigma mouse liver CAT. Lane 3 contains 15 μg of bovine GPX. Lane 4 contains 5 μg of human GPX.

When these samples were electrophoresed on 12.5% SDS-dissociating gels and stained for protein with Coomassie blue, the results shown in Figure 1b were obtained. Lane 1 contained molecular weight markers. Lane 2 contained mouse liver CAT. It appeared that multiple impurities were present. Lane 3 contained bovine GPX. A few minor slow-migrating impurities appeared to be present but the preparation was relatively pure. In contrast human, GPX, as shown in lane 4, appeared to be very pure, since it exhibited only one band on both native and SDS gels.

The two GPX preparations appeared to be relatively pure, whereas the mouse liver CAT preparation contained many impurities. Moreover, in all the preparations, there appeared to be multiple forms of the active protein. Heterogeneity in CAT preparations has been observed many times before and has been attributed to many factors.[8]

Figure 2a illustrates the appearance of gels stained by the ferricyanide method after electrophoresis of mouse liver CAT and human erythrocyte GPX. The staining procedure used H_2O_2 as a substrate. Lane 1 contained 20 units of CAT (as measured by Sigma), lane 2 contained 50 mU of pure bovine GPX, and lane 3 contained 20 mU of pure human GPX (as measured by Sigma). Pure CAT showed one strong, slowly migrating band and two weak, fast-migrating bands (impossible to see on the photograph), but GPX did not stain. The two faster migrating bands are seen only weakly in fresh samples. With storage and/or heat, these two bands increase in strength. The sample used in lane 1 had been exposed to 37° for 72 hours to bring out these bands. Both CAT and GPX did not stain when cumene hydroperoxide was used as a substrate (data not shown). As shown in Figure 2b the same results occurred using

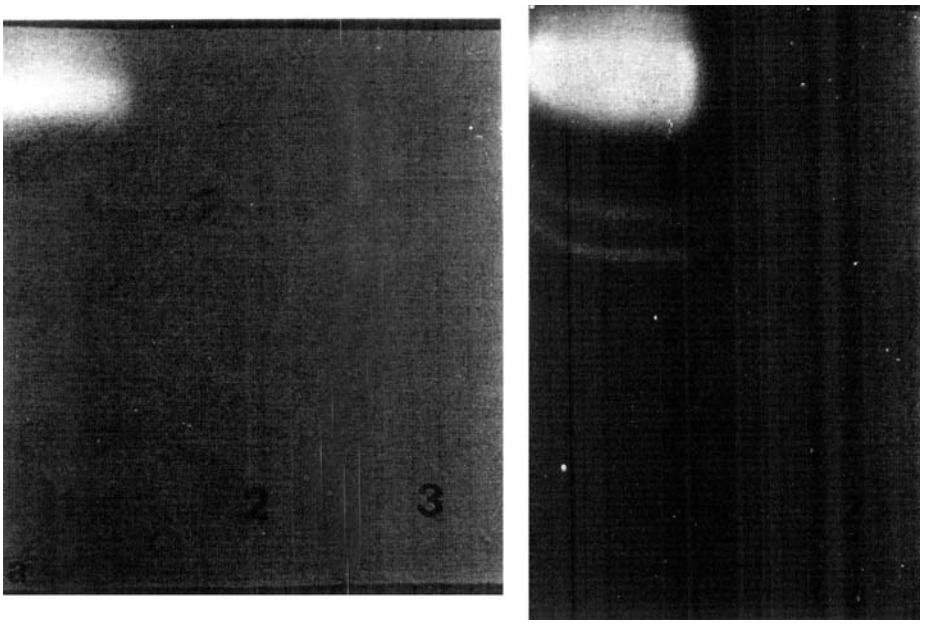

FIGURE 2 (a) Native gel stained with ferricyanide method. Lane 1 contains 20 U of mouse liver CAT. Lane 2 contains 50 mU of pure bovine GPX. Lane 3 contains 20 mU of pure human GPX. (b) Native gel stained by DAB method. Lane 1 contains 20 U of mouse liver CAT. Lane 2 contains 12.5 mU of human GPX.

the diaminobenzidine (DAB) staining method; lane 1 contained mouse liver CAT, while lane 2 contained human GPX. As with the ferricyanide procedure, CAT showed activity but GPX did not. CAT produced three bands; the fast-migrating, weakly-staining bands were seen more easily with the DAB method than the ferricyanide method in this particular experiment. Once again no bands appeared when cumene hydroperoxide was used as a substrate (data not shown).

Figure 3a and 3b show gels stained using the ferricyanide method with H_2O_2 as substrate and with reduced glutathione added during staining. In each gel, lane 1 contained CAT and lane 2 human GPX. For the experiment shown in Figure 3a, 0.25 mM GSH was used. Three bands of CAT (lane 1) and 2 bands of GPX (lane 2) were visualized. Thus both enzymes can be seen when this concentration of GSH is utilized. In Figure 3b, 1.5 mM GSH was used in staining. Now CAT was inhibited, while GPX still shows 2 strong bands. Thus this concentration of GSH can be used to visualize only GPX. At 2.0 mM GSH, GPX was also inhibited (data not shown).

Figure 3c shows the gels obtained when cumene hydroperoxide was used as substrate instead of H_2O_2. 0.5 mM GSH was used in the staining procedure. Only the two lower faint bands of CAT stain, showing the upper band can only use H_2O_2 as a substrate. GPX stained much brighter using cumene hydroperoxide than with H_2O_2. With the addition of 2 mM GSH, however, GPX was also inhibited even when using cumene hydroperoxide (data not shown).

Thus, GSH was shown to have a large effect on the proteins stained by the ferricyanide method. We wondered whether it would effect the DAB staining procedure. With H_2O_2 as substrate and using 1.0 mM GSH in the staining procedure,

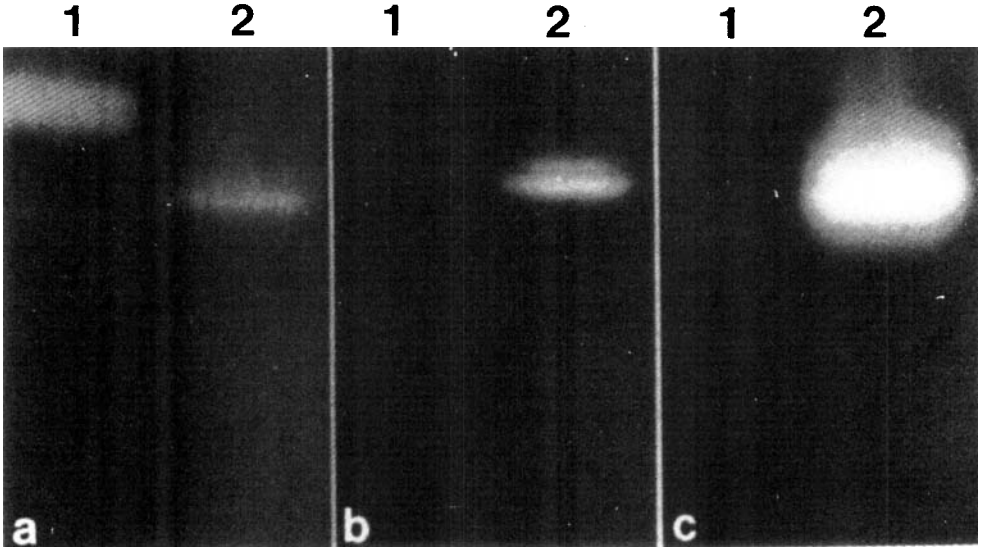

FIGURE 3 (a) Native gel stained with ferricyanide method in the presence of 0.25 mM GSH. Lane 1 contains 20 U of mouse liver CAT. Lane 2 contains 12.5 mU of human GPX. (b) Native gel stained by ferricyanide method in the presence of 1.5 mM GSH. Lane 1 contains 20 U of mouse liver CAT. Lane 2 contains 25 mU of human GPX. (c) Native gel stained by ferricyanide method using 0.5 mM GSH. 0.008% cumene hydroperoxide rather than H_2O_2 was used as substrate. Lane 1 contains 20 U of mouse liver CAT. Lane 2 contains 12.5 mU of human GPX.

we found that no difference was seen from gels stained without GSH. That is, CAT was visualized (no inhibition), but GPX was not (data not shown). We wondered whether the GSH was being rapidly oxidized in this system, so we added a reducing system to the staining solution – i.e., 0.2 mM NADPH and 50 U of glutathione reductase. Once again, GPX did not show banding, but the three CAT bands were much stronger (data not shown). This is probably because NADPH protects CAT against inactivation by H_2O_2.[9-10]

Our data also indicates that the GSH-ferricyanide method is specific for glutathione peroxidase and not other peroxidases. Thus, GPX was visualized, but horseradish peroxidase, lactoperoxidase (data not shown), or glutathione-S-transferase (Figure 5, lane 3) were not visualized.

The GSH-ferricyanide method can also visualize GPX and CAT in cell homogenates, as shown in Figure 4. Lane 1 contained mouse liver CAT, lane 2 human GPX, and lane 3 contained 100 μg of mouse liver homogenate. (149 mU CAT and 105 mU GPX, according to our assay.) One can see two bands in mouse liver, with the upper diffuse band migrating like CAT and the lower sharper band like GPX.

In order to verify our assay, we have looked for other ways to distinguish between CAT and GPX. Catalase is sensitive to heat,[11] as shown in Figure 5. If samples (either pure enzyme or liver homogenate) were heated to 68°C for 1 minute before loading on the gel, the CAT bands (lane 1) were inhibited, but not the GPX bands (lane 2).

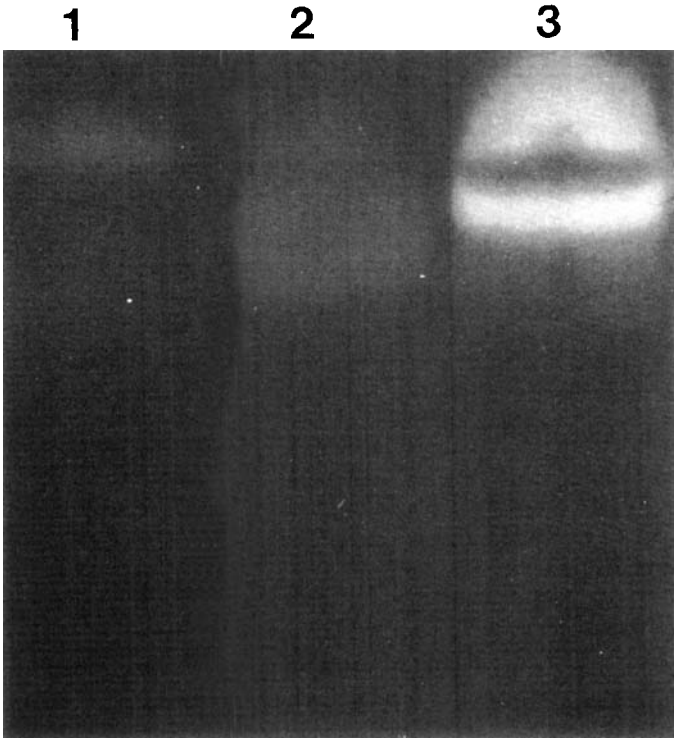

FIGURE 4 Native gel stained by ferricyanide method in the presence of 0.25 mM GSH. Lane 1 contains 0.5 U of pure mouse liver CAT. Lane 2 contains 5 mU of human GPX. Lane 3 contains 100 μg of mouse liver homogenate.

Lane 3 in Figure 5 contained human glutathione-S-transferase and did not show any activity; unheated samples of this enzyme also showed no activity (data not shown). Lane 4 in Figure 5 contained mouse liver homogenates and exhibited only a GPX band after heating. An inhibitor of GPX, iodacetate, has recently been reported.[12] In our study, shown in Figure 6, 10 mM sodium iodoacetate inhibited pure human GPX (lane 2) and bovine GPX (lane 3) and also the enzyme in mouse liver homogenate (lane 4). It also decreased the inhibitory effect of GSH on pure catalase staining, leading to a more intense CAT band (lane 1).

DISCUSSION

We have shown that the ferricyanide method for staining catalase can be modified to stain for GPX simply by adding GSH to the staining solution. A low amount of GSH allowed both CAT and GPX to be visualized while high levels of GSH inhibited CAT. The reason for this inhibition is unknown, but we have preliminary evidence showing that GSH can inhibit CAT.

Another peculiar observation was that GSH had no effect on the staining of CAT when using the DAB method – i.e. GPX was not visualized nor was CAT inhibited. It has been reported that horseradish peroxidase catalyzed oxidation of GSH to form glutathionyl radical GS., glutathione sulfonate and GSSG.[13,14] This may explain why

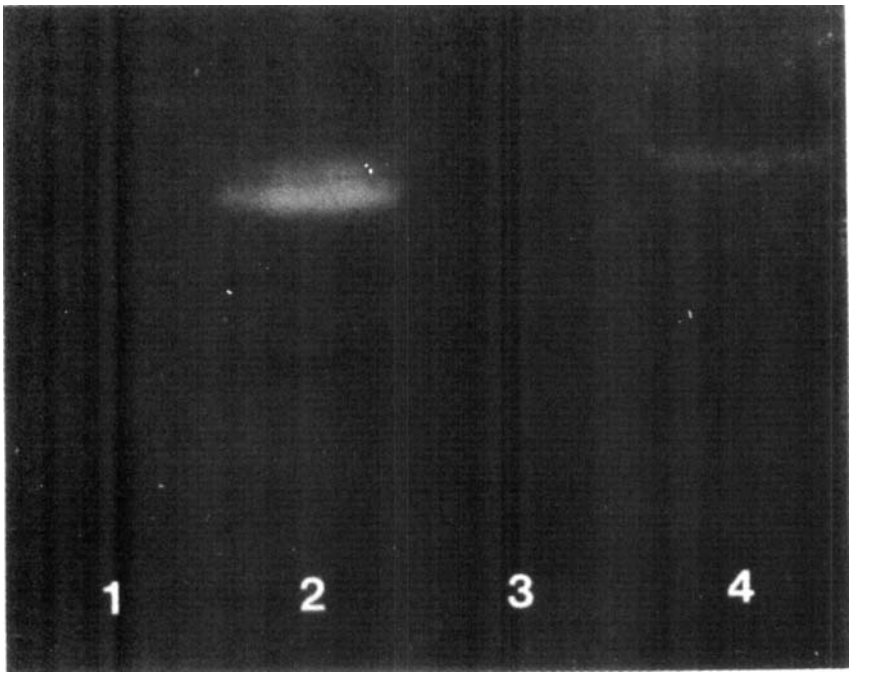

FIGURE 5 Native gel stained by ferricyanide method in the presence of 0.25 mM GSH. All samples were heated at 68°C for 1 minute prior to electrophoresis; activity values were measured before heating. Lane 1 contains 10 U of mouse liver CAT. Lane 2 contains 12.5 mU of human GPX. Lane 3 contains 2.5 U of human glutathione-S-transferase. Lane 4 contains 370 μg of mouse liver homogenate (containing 550 mU of CAT and 390 mU of GPX).

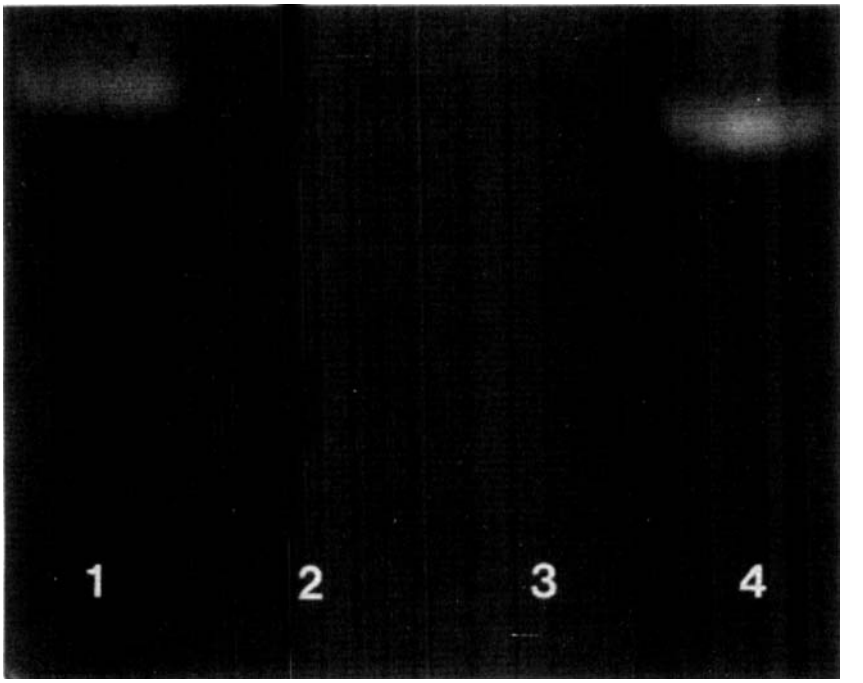

FIGURE 6 Native gel stained by ferricyanide method in the presence of 0.25 mM GSH. All samples were treated with 10 mM sodium iodoacetate during staining; activity values were measured before adding iodoacetate. Lane 1 contains 1 U of mouse liver CAT. Lane 2 contains 10 mU of human GPX. Lane 3 contains 20 mU of bovine GPX. Lane 4 contains 5 μg of mouse liver homogenate (containing 7.4 mU of CAT and 5.3 mU of GPX).

the DAB method does not work since the method involves using horseradish peroxidase in the presence of GSH; GSH may have been used by horseradish peroxidase, and was thus not available for GPX. In the presence of glutathione reductase, some GSH may regenerate by the reduction of GSSG, but the amount of GSH may be too small. The inhibitory effect of GSH on catalase may be also eliminated by NADPH, which is also present in the staining solution.[15]

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