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Note

Separation of two forms of glutathione peroxidase from human erythrocytes by hydrophobic chromatography

RACHEL FORWARD and RAMI ALMOG*
Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201 (U.S.A.)
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The seleno enzyme glutathione peroxidase (EC 1.11.1.9) may prevent red blood cell damage due to membrane peroxidation[1–3]. The enzyme is believed to protect the cell by its role in peroxide decomposition, including peroxides of unsaturated membrane lipids[2,3]. However, it it still not clear how a water soluble enzyme can associate with lipids in the hydrophobic region of the cell membrane[4]. Hydrophobic chromatography on Phenyl-Sepharose has been used in this study as a tool in exploring surface hydrophobicity of glutathione peroxidase, and in developing a procedure for isolating different forms of the enzyme from human erythrocytes. This method has been used recently to determine protein hydrophobicity, to develop new purification procedures, and to separate hydrophobic and hydrophilic forms of other enzymes[5–7].

MATERIALS AND METHODS

Chemicals

CM-Sephadex, Phenyl-Sepharose CL-4B, DEAE-Sephacel, Sephacryl S-300, and Sephadex G-25 gels were purchased from Pharmacia, reduced glutathione (GSH), oxidized glutathione (GSSG), nicotinamide adenine dinucleotide phosphate (NADPH) type x, and glutathione reductase, type III from Sigma, Coomassie Brilliant Blue G-25 from Polysciences. Packed red blood cells (one day old) were obtained from American Red Cross, Northeastern New York Region. All other chemicals were reagent grade.

Chromatography was performed using a Uvicord ultraviolet monitor, chart recorder and ultrarack fraction collector from LKB-Productor. A Perkin-Elmer 320 spectrophotometer was used for absorption measurements. Unless otherwise mentioned all phosphate buffers include 1 m*M* GSH and 0.5 m*M* EDTA.

Assays

Glutathione peroxidase activity was measured by a coupled assay system in which oxidation of GSH was coupled to NADPH oxidation catalyzed by glutathione reductase[8]. The reaction mixture consisted of 1 unit/ml glutathione reductase, 1 m*M* GSH, 0.25 m*M* NADPH, 3 m*M* sodium azide, 3 m*M* EDTA, 0.1 ml sample and 100 m*M* phosphate, pH 7.0. The mixture was incubated for 10 min, at 37°C and then

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was made 1 m*M* in hydrogen peroxide to start the reaction. The rate of the reaction was measured by disappearance of NADPH, as expressed by a change in absorbance per min at 340 min. The change in absorbance is directly proportional to enzyme units. Blank values, obtained without addition of samples, were subtracted from assay values. When activity was measured prior to the removal of all hemoglobin, the enzyme solution was diluted in Drabkin's reagent (1.2 m*M* potassium ferricyanide and 15 m*M* in potassium cyanide) to prevent interference by hemoglobin[9]. Proteins were determined by a Coomassie Brilliant Blue dye assay[10].

Gel preparation

Gels were equilibrated as follows; CM-Sephadex with 1 m*M* phosphate, pH 6.0; Phenyl-Sepharose in 700 m*M* phosphate, pH 7.0; DEAE-Sephacel in 1 m*M* phosphate, pH 7.0. These suspensions were filtered before use. Sephacryl S-300 was equilibrated in 1 m*M* phosphate, 10% ethanol at pH 7.0 before packed in a column. Chromatography was performed at room temperature (22°C) and enzyme fractions and solutions were kept at 4°C.

Hemolysate preparation

A unit of fresh packed human red blood cells was divided into ten centrifuge bottles (approximately 25 ml per bottle). The cells were washed twice with 150 ml isotonic saline solution [0.145 *M* sodium chloride–0.1 *M* phosphate pH 7.0 (9:1)]. They were hemolyzed by adding four volumes of 1 m*M* phosphate, pH 7.0 and then frozen and allowed to thaw once. The mixture was centrifuged for 30 min at 16 000 *g* to remove stroma.

Preparation of hemoglobin-free glutathione peroxidase solution

Hemoglobin was removed from the hemolysate by batch adsorption on CM-Sephadex. Pre-equilibrated resin was added to the hemolysate (at pH 6.0) until most of the hemoglobin was absorbed to the gel, as monitored by the absence of red color in a small filtered aliquot. The resultant suspension was stirred for 1 h, filtered, and the gel was washed with equilibrating buffer to remove unbound glutathione peroxidase. The filtrate and wash were pooled and assayed for glutathione peroxidase activity and protein concentration.

Separation of two forms of glutathione peroxidase

The hemoglobin-free enzyme solution was brought to 85% saturation in ammonium sulfate by adding solid salt at pH 7.0. The mixture was allowed to stand for 1 h prior to centrifugation at 16 000 *g* for 10 min. The resultant pellet was redissolved in 50 ml of 700 m*M* phosphate, pH 7.0. Binding of the enzyme to Phenyl-Sepharose was achieved by batch adsorption and elution was achieved through a column. This two-step procedure, called hereafter in this paper batch adsorption chromatography is very rapid and can be scaled up for treating larger volumes of material. Pre-equilibrated Phenyl-Sepharose was added to the enzyme solution until no glutathione peroxidase activity was detected in small filtered aliquots. Essentially all the enzyme activity was bound to the resin. The suspension was stirred for 1 h and then poured into a column. The enzyme was eluted by a step phosphate gradient of 700 m*M*, 100 m*M*, 10 m*M* and 1 m*M* phosphate, at pH 7.0. Fractions with enzyme activity were

pooled and desalted by passage through a Sephadex G-25 column (22 × 2.5 cm I.D.).

Ion-exchange and size-exclusion chromatography

Pre-equilibrated DEAE-Sephacel was added to the desalted pool until no enzyme activity was detected in small filtered aliquots. The mixture was stirred for 1 h and then poured into a column. The enzyme was eluted by a 350-ml linear salt gradient of 350 m*M* sodium chloride, in 1 m*M* phosphate at pH 7.0. The fractions with enzyme activity were pooled and were brought to 85% ammonium sulfate saturation by adding solid salt to the solution at pH 7.0. The mixture was allowed to stand for 1 h prior to centrifugation at 16 000 *g* for 10 min. The pellet was redissolved in 1 m*M* phosphate, 10% ethanol, pH 7.0 and chromatographed on a Sephacryl S-300 column (35 × 4.0 cm I.D.).

RESULTS AND DISCUSSION

Hydrophobic gel chromatography is a relatively new technique for protein purification. Its use is growing rapidly since proteins can be separated on the basis of their hydrophobic surface properties which are not directly used in the more common separation methods based on differences in charge and size[5–7].

The hydrophobic surface properties of glutathione peroxidase were exploited in this study, to develop a procedure for separating two forms of the enzyme from human erythrocytes. The stepwise elution of the enzyme from hydrophobic resin such as Phenyl-Sepharose showed two enzyme activity peaks (Fig. 1). The first peak was eluted with 700 m*M* phosphate and a second peak was desorbed by 100 m*M* phosphate at pH 7.0. The two activity peaks, when pooled, could not be separated by chromatography on ion-exchange resin (DEAE-Sephacel) or on size-exclusion gel (Sephacryl S-300). Two overlapping activity peaks were observed in each case (Figs.

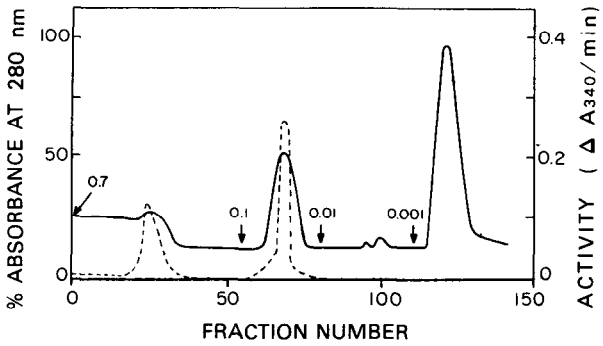

Fig. 1. Resolution of glutathione peroxidase into two forms by hydrophobic batch adsorption chromatography on Phenyl-Sepharose. After adsorbing glutathione peroxidase on Phenyl-Sepharose in suspension at 0.7 *M* phosphate, pH 7.0, the resultant slurry was packed in a column (30 × 2.5 cm I.D.) and eluted by a step phosphate gradient of 0.7 *M*, 0.1 *M*, 0.01 *M* and 0.001 *M* phosphate buffers at pH 7.0 (indicated by arrows). Fractions of 4 ml were collected at a flow-rate of 40 ml/h. (——) Percent absorbace at 280 nm; (– – –) glutathione peroxidase activity. Fraction with activity larger than 0.06 were pooled from both peaks for further use.

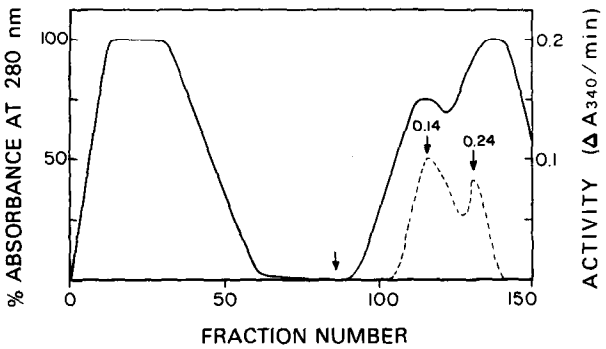

Fig. 2. DEAE-Sephacel batch adsorption chromatography. The pooled eluate from Phenyl-Sepharose chromatography was adsorbed on DEAE-Sephacel in 1 m*M* phosphate pH 7.0. The resultant slurry was packed in a column (30 × 2.5 cm I.D.) and eluted with linear 0.35 *M* sodium chloride gradient in 1 m*M* phosphate pH 7.0. Fraction of 4 ml were collected at a flow-rate of 38 ml/H. (——) Percent absorbance at 280 mm; (---) glutathione peroxidase activity. The fraction at which the gradient was applied is indicated by an arrow as well as sodium chloride concentration at which maximum enzyme activity was observed.

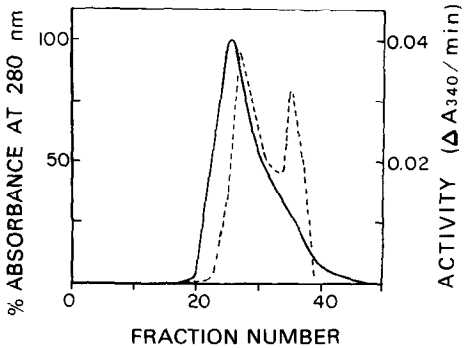

Fig. 3. Sephacryl S-300 column chromatography. The pooled enzyme fraction from DEAE-Sephacel chromatography was applied to Sephacryl S-300 column (30 × 4.0 cm I.D.) and eluted with 1 m*M* phosphate buffer, pH 7.0, containing 10% ethanol. Fractions of 4 ml were collected at a flow-rate of 25 ml/h. (——) Percent absorbance at 280 nm; (---) glutathione peroxidase activity.

2 and 3). A similar pattern was also observed by Awasthi *et al.*[11] during purification of the enzyme.

The sample treatment preceding the Phenyl-Sepharose batch adsorption chromatography can affect the phosphate concentration needed for removal of the two forms from the column. For example, the two forms were eluted from Phenyl-Sepharose column chromatography at pH 8.0 with 100 m*M* and 1 m*M* phosphate respectively when isolated from hemolysate by chromatography on a DEAE anion-exchange column, pH 7.2, followed by 20 to 30% ammonium sulfate fractionation, and CM-Sephadex column chromatography.

The complete separation of the two forms by hydrophobic interaction chromatography suggests that the hydrophobic surface properties of these forms may be significantly different. The form which was eluted in the second activity peak (at

lower phosphate concentration) must have larger hydrophobic surface area[12]. It may be capable therefore of association with lipids in the hydrophobic core of membranes and can play an important role in prevention of membrane peroxidation.

Hydrophobic chromatography on Phenyl-Sepharose was shown to be capable of separating two forms of human erythrocytes glutathione peroxidase. A major effort is currently underway to obtain these forms in high purity using this method.

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