J. Clin. Chem. Clin. Biochem.
Vol. 17, 1979, pp. 767–769

Separation of Glutathione Reductase in Human Serum by Gradient Gel Electrophoresis

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(Received June 19/September 6, 1979)

In commemoration of Professor Dr. Gábor Szász

Summary: A system of high resolution polyacrylamide gel electrophoresis is introduced. It allows the rapid separation of proteins from serum and tissue homogenates under non-denaturating conditions, and proves especially suited for the comparative analysis of enzymes. The enzyme glutathione reductase is identified on the gradient gels by contact printing with agar substrate gels, and the multiple forms of glutathione reductase in serum are demonstrated.

Trennung von Glutathionreductase im Serum des Menschen durch Gradientengelelektrophorese

Zusammenfassung: Ein Verfahren der hochauflösenden Polyacrylamidgelelektrophorese wird vorgestellt. Es erlaubt die schnelle Trennung von Proteinen aus Serum und Gewebshomogenaten unter nicht-denaturierenden Bedingungen und zeigt sich als besonders geeignet für die vergleichende Analyse von Enzymen. Das Enzym Glutathionreductase wird in den Gradientengelen durch Sandwich-Technik mit Agar-Substrat-Gelen nachgewiesen. Die multiplen Formen der Glutathionreductase im Serum werden demonstriert.

Introduction

The introduction of polyacrylamide gel electrophoresis to the analysis of proteins in plasma and serum several years ago (1, 2) greatly enhanced the resolution of these very complex protein mixtures. Many different techniques of polyacrylamide gel electrophoresis have so far been applied for the separation of serum proteins, including gradient gel electrophoresis (3,4) and two-dimensional methods (5). Unfortunately, the use of gel cylinders (4) does not allow satisfactory comparative investigations of enzymes on gels, and the high-resolution two-dimensional technique of *Anderson* (5) includes denaturating conditions and thus cannot be used for the analysis of enzymes.

We are involved in the study of enzymes in serum, looking for the possible clinical relevance of certain enzymes and their multiple forms. We therefore adapted out recently developed system of gradient gel electrophoresis (6) to the rapid separation of enzymes in serum. We combined this electrophoretic system with a contact printing technique using agar substrate gels for a convenient localization of enzymes on polyacrylamide gels.

Materials and Methods

Electrophoresis

Serum of healthy donors and of patients with enhanced activity of glutathione reductase was used for electrophoresis. The catalytic concentration of glutathione reductase was determined by the method of *Weidemann* (7). Polyacrylamide gradient gel electrophoresis was performed essentially according to *Anselstetter* (6). Exponential gradient slab gels contained: total concentration of monomers (T) = 37.5 to 210 g/l, cross-linking concentration (C) = 15 to 30 g/l; 0.4 mol/l Tris(hydroxymethyl) aminomethane (Tris)-HCl, pH 8.9; 3 to 1.5 mmol/l N,N,N',N'-tetramethylethylene diamine; 2 mmol/l ammonium persulfate. Electrophoresis buffer was 0.1 mol/l Tris – 0.1 mol/l borate, pH 8.17 and was precooled to 4 °C before use. For electrophoresis 1 volume of serum was in most cases diluted with 4 volumes of 200 ml/l glycerol slightly coloured with bromophenol blue. 5–25 µl of diluted serum were applied per lane. Electrophoresis was started with constant current for 20 minutes (10 min at 10 mA and a further 10 min at 20 mA) and continued with constant power of 3 watts (for approx 150 min) until the front of the albumin band reached a mark 10 mm above the lower end of the gel.

Contact printing

Substrate gels were prepared according to *Weidemann & Anselstetter* (8) and were carefully pressed onto the polyacrylamide gels, to avoid trapping any airbubbles. Each pair of gels was left at room temperature for 15 minutes and then was divided. Viewed on a transilluminating UV-lamp (366 nm, Model C-62,

0340-076X/79/0017-0767$2.00

Ultra Violet Products, INC. San Gabriel), the enzyme appears as a dark band on the brightly fluorescent gradient gel. Using this UV lamp and an UV absorption filter (Schott Nr. GG 10, 2 mm thick) the gels were photographed as previously described (8).

Glutathione reductase isolation

Glutathione reductase was isolated from human serum by the following batch procedure using ADP-Sepharose (Pharmacia): 100 mg ADP-Sepharose was washed with phosphate buffer (0.1 mol/l, pH 7.0) and after centrifugation the supernatant was discarded. 1 ml serum was added to the washed ADP-Sepharose. After approx. 60 minutes (swirled gently at room temperature) the suspension was centrifuged and the supernatant discarded. The ADP-Sepharose was then washed with phosphate buffer (10 mmol/l, pH 7.0) and the glutathione reductase eluted with 0.5 ml NADPH solution (12 mmol/l).

Results and Discussion

In the past we have tested different forms of linear and exponential gradient slab gels for the separation of serum proteins. The exponential gradient gel system described in this paper brings about a rapid and superior separation of serum proteins: e.g. bovine and human serum albumin with molecular weights of 67,000 and 69,000 daltons, respectively, are clearly separated (see fig. 2). The method facilitates also the high resolution analysis of enzymes. The use of precooled electrophoresis buffer is sufficient to keep the enzyme fully active during the short separation time (3 h). Enzyme bands produced by contact printing are sharp due to the short substrate diffusion time The localization of glutathione reductase on the gradient gels is completed within approximately 30 minutes.

The resulting pattern of serum proteins and of glutathione reductase in serum is shown in figure 1. The location of the two enzyme bands within the protein pattern is indicated by arrows. The glutathione reductase bands cannot be localized with Coomassie Blue on this gel since the protein mass of the enzyme lies below the detection limit of this stain (i.e. less than 10 ng of protein). Following the enzymatic reaction, however, as little as 50 U/l serum of catalytic concentration of glutathione reductase can be detected on the gels using UV-transillumination.

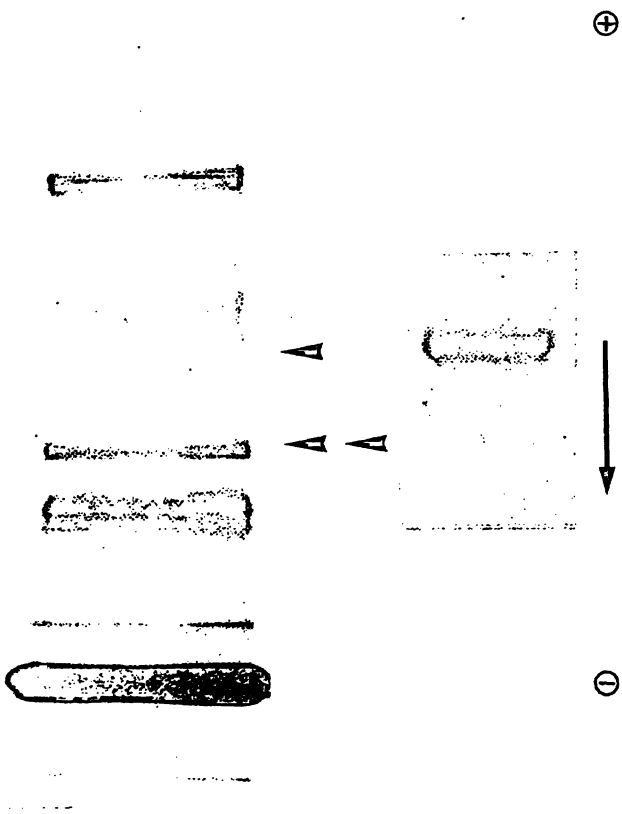

Fig. 1. Gradient gel electrophoresis of proteins and glutathione reductase in human serum. Left: Coomassie blue staining pattern of serum proteins (magnification 2×). 3 µl of a serum with enhanced catalytic concentration of glutathione reductase (384 U/l serum) were separated under the conditions given in Materials and Methods. The location of the two glutathione reductase bands within the protein staining pattern is indicated by arrows. The upper and lower ends of the gel are not shown in this magnification. Right: Visualization of glutathione reductase bands by UV-transillumination.

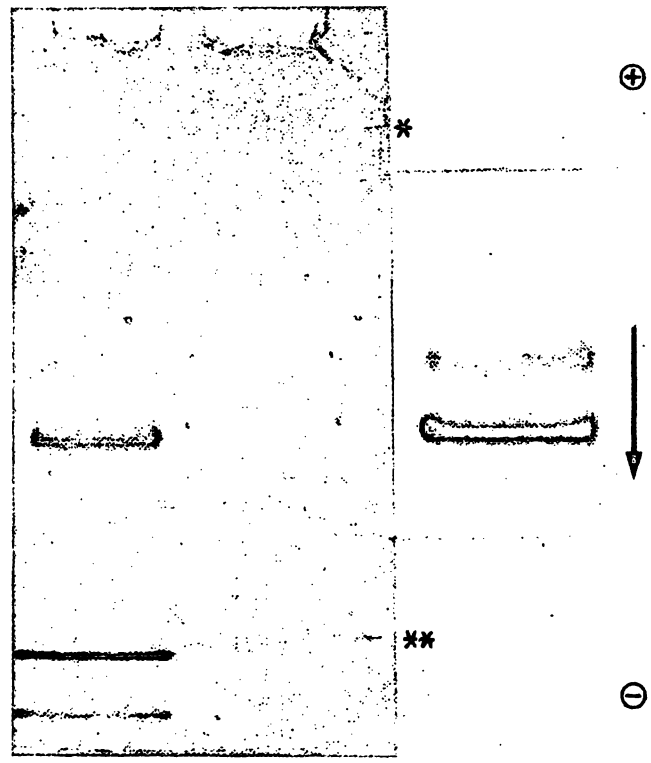

Fig. 2. Gradient gel electrophoresis of glutathione reductase in human serum after isolation with ADP-Sepharose. Left: Coomassie blue staining pattern. 5 µl of the isolate have been separated in the right lane of the gel under the conditions given in Materials and Methods. The dots on this lane indicate the location of the glutathione reductase bands obtained with UV-transillumination. Only traces of high molecular weight proteins (*) and albumin (**) are visible on the gel. For the sake of comparison approx. 2 µg each of three proteins of known molecular weight – from top to bottom: catalase (240,000), bovine serum albumin (67,000) and ovalbumin (45,000) – have been separated in the left lane of the gel. Right: Visualization of the glutathione reductase bands corresponding to the dots on the Coomassie pattern by UV-transillumination.

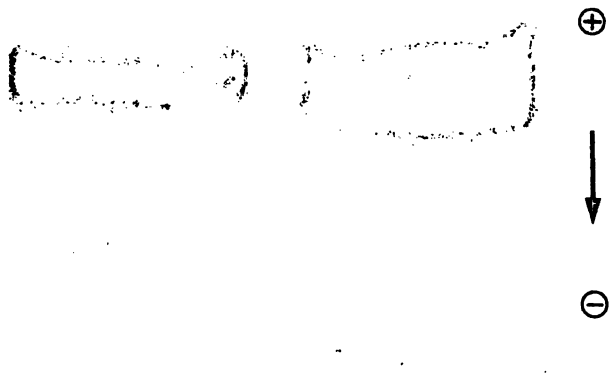

Fig. 3. Gradient gel electrophoresis of multiple forms of glutathione reductase in human serum, UV-transillumination. 5 µl of a serum with enhanced catalytic concentration of glutathione reductase (450 U/l serum) have been separated in each lane. To the sample in the right lane 2-mercaptoethanol has been added to a final concentration of 12.8 mmol/l. Conditions of electrophoresis were as given under Materials and Methods. See text for further explanation.

The great sensitivity of this electrophoretic system has been further revealed during the analysis of glutathione reductase in homogenates from fine needle biopsies of various tissues (in preparation). In a typical experiment, brief homogenization and centrifugation of only 3 mg of wet tissue yields supernatant material sufficient for 10–20 electrophoretic analyses. Aliquots of the untreated supernatant can be applied directly to the gels without causing deterioration of the banding pattern. This facilitates the comparative analysis of enzymes from serum with those from tissues of special interest.

Glutathione reductase has been purified from erythrocytes and spinach leaves by column chromatography with ADP-Sepharose (9 10, 11). We found a batch procedure using ADP-Sepharose more convenient for the isolation of glutathione reductase from human sera. Glutathione reductase isolated by this approach was almost free from contaminating protein (fig. 2). It behaved identically in gradient electrophoresis to the glutathione reductase in the unfractionated serum. This shows that the enzyme migrates in the same way whether it be isolated or in close vicinity to co-migrating proteins during electrophoresis.

Glutathione reductase is known to appear in multiple forms in serum (8), depending e.g. on the concentration of 2-mercaptoethanol. In our earlier studies of the multiple forms of glutathione reductase using agar gel electrophoresis and homogeneous polyacrylamide gel cylinders only one slowly moving band remained upon incubation of the sample with 2-mercaptoethanol in all sera tested. However, exponential gradient gel electrophoresis of some sera from patients with enhanced catalytic concentration of glutathione reductase revealed a complex of three clearly distinguishable slowly moving bands following incubation with 2 mercaptoethanol (fig. 3). Similar triplet glutathione reductase bands were obtained with homogenates from liver (but not e.g. from kidney) which have not been pretreated with 2-mercaptoethanol.

The high resolution of this gradient gel system could help to elucidate whether these multiple forms of glutathione reductase in serum might be of any clinical relevance. We assume that enzymes other than glutathione reductase can be investigated in a similar way by using this technique.

Acknowledgement

This work was supported by the Deutsche Forschungsgemeinschaft (V.A.).

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