Enzyme Immunoassay for Human Ferritin

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We describe an enzyme immunoassay with use of β-D-galactosidase for quantitation of ferritin in human serum. The minimum detectable ferritin concentration is 0.25 μg/L of serum, which is comparable to results obtained by radioimmunoassay. The correlation coefficient between values determined by enzyme immunoassay and radioimmunoassay was 0.95 (n - 1 = 85, p <0.001).

Enzyme immunoassays are now being used to measure humoral constituents (1). In these assays, measurements of radioactivity, as in radioimmunoassay, are replaced by determination of enzyme activity. The reagents are more stable, and simpler equipment is usually required for the analysis. Here, we describe a method of enzyme immunoassay for human ferritin in which we use a solid-phase antiserum, together with an enzyme-labeled antibody as indicator of the reaction.

Materials and Methods

Analytical Procedures

Preparation of ferritin and antibody. Human liver ferritin was prepared by methods described previously (2). In brief, the tissue was homogenized with four parts of distilled water and the homogenate was heated at 75 °C for 10 min. After centrifugation at 10,000 X g for 30 min, the supernatant fluid was passed through a Millipore filter (pore size, 0.45 μm). Ferritin was precipitated from the filtrate with half-saturated ammonium sulfate (final concentration) and the precipitated protein was dissolved in a small volume of phosphate buffer (20 mmol/L, pH 7.0). The solution was then subjected to gel filtration, first on a Sephadex G-200 column and then on a column of Sepharose 6B. Preparative-scale gel electrophoresis was used at the final step of purification. Ferritin preparations thus obtained were electrophoretically homogeneous. Concentrations of purified ferritin were estimated by the method of Lowry et al. (3). The preparation of rabbit antiserum against human liver ferritin was based on the method of Marcus and Zinberg (4). Antiferritin sera thus obtained did not cross react with other serum proteins or with cellular components on examination by the double-immunodiffusion technique.

The IgG fraction from the antiserum was obtained by fractionation with NaN₂SO₄ (5), followed by passage through a column of diethylaminoethyl-cellulose (6). Concentrations of IgG solutions were estimated from their absorptivity (assuming α = 15 at 280 nm).

Rabbit IgG coupled to silicone-rubber strings. IgG-loaded silicone rubber strings were prepared essentially by previously described methods (7).

Silicone rubber strings (3 mm in diameter; Sanko Plastic Co. Ltd., Osaka, Japan) were cut into pieces 4 mm long, carefully washed with detergent (Scat 20-X; Nakarai Chemicals Ltd., Kyoto, Japan), extensively rinsed with water, incubated with rabbit IgG fraction (130 μg/mL) in sodium phosphate buffer (0.25 mol/L, pH 7.5) for 30 min at room temperature in a beaker, and stored overnight at 4 °C in the same solution.

The IgG-coupled silicone rubber strings were washed three times with the pH 7.5 sodium phosphate buffer, then three times with 10 mmol/L sodium phosphate buffer, pH 7.0, containing, per liter, 0.1 mol of NaCl, 1 mmol of MgCl₂, 1 g of NaN₃, and 1 g of bovine serum albumin (buffer A). They were stored in the buffer A at 4 °C for at least a week before use. Under these conditions they were stable for more than six months.

Antibody–Fab'-β-D-galactosidase (EC 3.2.1.23) complexes. On the basis of the previous observation (8) that immunoassay with Fab'-enzyme complex is more sensitive than that with IgG–enzyme complex, the Fab' fragment was prepared from antiferritin rabbit IgG fraction by the methods of Nisonoff and Rivers (9).

About 20 mg of the IgG was dialyzed overnight vs. sodium acetate buffer (0.1 mol/L, pH 4.5) at 4 °C, then incubated at 37 °C overnight with 0.4 mg of pepsin (EC 3.4.4.1) in a final volume of 1.5 mL. The digestion was terminated by adjusting the pH to 8.0 with 1 mol/L NaOH. The reaction mixture was subsequently chromatographed on a column of Sephadex G-150, in sodium borate buffer (0.1 mol/L, pH 8.0), and fractions represented by the main peak were collected.

The preparations of Fab'(ab')₂ fragments thus obtained were dialyzed vs. sodium acetate buffer (0.1 mol/L, pH 5.0) and stored at 4 °C until use. Concentrations of Fab' were calculated by using an absorptivity (a) value of 14.8 at 280 nm.

Conjugation of the Fab'(ab')₂ fragment with β-D-galactosidase consists essentially of two steps: treatment of Fab'(ab')₂ fragments containing sulfhydryl groups with excess N,N'-o-phenylenediamine, to introduce maleimide residue into the fragment, then incubation of the dimaleimide-treated Fab' fragments with β-D-galactosidase, which also contains sulfhydryl groups.

Firstly, 10 mg of Fab'(ab')₂ fragments was incubated with 10 mmol/L 2-mercaptoethylamine in a final volume of 2 mL of 0.1 mol/L sodium acetate buffer (pH 5.0, 37 °C, 90 min), then chromatographed on a 1.0 X 40 cm column of Sephadex G-25 in the same buffer.

As the second step, we introduced maleimide residues into the Fab' fragments by adding the Fab' solutions (1.5–2 mg) dropwise to 1.0 mL of a saturated (0.75 mmol/L) solution of N,N'-o-phenylenediamine in the pH 5.0 sodium acetate buffer at 0 °C, and incubated the mixture at 30 °C for 20 min. Compounds of low relative molecular mass, including excess N,N'-o-phenylenediamine, were removed on a 1.0 X 40 cm column of Sephadex G-25 in sodium acetate buffer (0.1 mol/L, pH 5.0).

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The dimaleimide-treated Fab' fragments (0.4–0.7 mg) were then incubated with 20 μL of β-D-galactosidase (5 g/L solution) at 30 °C for 20 min in a final volume of 1 mL of sodium acetate buffer, pH 5.0. No decrease in enzyme activity was detected during the incubation.

The incubation mixtures were adjusted to pH 7.0 with 1 mol/L NaOH, and then 2 μL each of bovine serum albumin solution (50 g/L) and 1 mol/L MgCl₂ solution were added, to stabilize the enzyme activity. Each incubation mixture was kept at 4 °C for 24 h to 72 h, and then applied to a 1.5 × 40 cm column of Sepharose 6B that had been equilibrated with sodium phosphate buffer (10 mmol/L, pH 7.0) containing, per liter, 0.1 mol of NaCl, 1 mol of MgCl₂, 1 g of Na₃, and 0.1 g of bovine serum albumin. Fractions containing the major enzymatic activities (6–10 mL) were collected (Figure 1).

The amount of Fab'–β-D-galactosidase complexes were expressed as units of enzyme activity, defined as described previously (8).

To estimate the immunological reactivity of this preparation, we used affinity chromatography on a ferritin-coupled Sepharose 4B column. The immobilization of human liver ferritin to BrCN-activated Sepharose 4B (Pharmacia Fine Chemicals, Sweden) was essentially as described elsewhere (10).

Fab'–enzyme complex, 13 688 units in 0.1 mL, was applied to the ferritin-coupled Sepharose column (3.5 × 100 mm); the same amount of enzyme activity was applied to a bovine serum albumin-coupled Sepharose column as a control. The enzyme activities directly passed through the columns were 10 750 units and 12 625 units for these test and control experiments, respectively. In other words, the percentages enzyme activity adsorbed to the columns were, respectively, 21.46 and 7.77%. Thus we concluded that 13.6% of the Fab'–β-D-galactosidase complex in this preparation was actually immunologically reactive to ferritin.

Solid-phase enzyme immunoassays. The silicone-rubber strings loaded with rabbit IgG were incubated with 50 μL of the patient’s serum or various amounts of standard ferritin, in a final volume of 0.15 mL of buffer A. After shaking them at 37 °C for 4 h, we allowed the incubation mixtures to stand at 4 °C overnight. The silicone-rubber strings were then washed twice with 1.0 mL of buffer A and incubated with 1970 micro-units of rabbit antibody Fab'–β-D-galactosidase complex in a final volume of 0.15 mL of buffer A, with shaking, at 37 °C for 6 h.

The silicone rubber strings were then washed twice with 1.0 mL of buffer A and transferred into another test tube, to eliminate nonspecific binding of the complex to the wall of the test tubes, and the enzyme activities bound to the silicone rubber strings were determined. The silicone rubber strings were preincubated in 0.1 mL of buffer A at 30 °C for 5 min and the enzyme reaction was started by adding 50 μL of 3 × 10⁻⁴ mol/L 4-methylumbelliferyl-β-D-galactoside (Sigma Chemical Co., St. Louis, MO 63178). After 10 min of incubation at 30 °C with shaking, the amounts of 4-methylumbellifere formed were determined fluorometrically (Hitachi Spectrofluorometer, Model 204). The wavelengths used were 360 nm (excitation) and 480 nm (emission).

**Immunoradiometric assay for ferritin.** Immunoradiometric assays of serum ferritin, with paper discs as the solid phase, were performed as detailed elsewhere (10). Filter-paper discs with a diameter of 5 mm (Toyo filter No. 51) were activated with BrCN according to the method of Ceska and Lundkvist (5). Sodium sulfate-precipitated immunoglobulin fractions of rabbit antiserum prepared against purified ferritin was applied to affinity chromatography of BrCN-activated Sepharose to which human liver ferritin was previously immobilized. After sufficient washing with phosphate-buffered isosonic saline, antiferritin antibody fraction was eluted with 3 mol/L KSCN in phosphate-buffered isosonic saline and dialyzed against phosphate-buffered isosonic saline overnight. The antibody solution thus obtained (1 g/L) was diluted with 200 mL of 0.1 mol/L NaHCO₃ containing 0.5 mol of NaCl per liter. The solution was then incubated with 5 g of activated paper discs for 3 h at 4 °C and the discs were washed twice with 500 mL of the same buffer for 10 min. The remaining reactive groups were blocked by adding 200 mL of 50 mmol/L ethanolamine in 0.1 mol/L NaHCO₃ containing 0.5 mol/L NaCl for 3 h at room temperature. The discs were again washed twice with 500 mL of 0.5 mol/L NaHCO₃ and twice with 0.1 mol/L acetic acid buffer, for 30 min each time. Finally, the discs were washed twice with 500 mL of a mixture of bovine serum albumin (1 g/L), normal rabbit serum (1 mL/L), and sodium azide (20 mg/L). We iodinated the purified antiferritin antibody as described elsewhere (11). The assay procedure was based essentially on the method of Ceska and Lundkvist (5), modified by using the above mixture as the incubation buffer. Radioactivity was counted with an LKB Wallac Autogamma scintillation counter. Standard curves for this assay system showed satisfactory linearity over a range of 0.25 to 100 μg/L.

**Serum Samples**

Serum samples were collected from 11 normal subjects and 75 patients with various diseases, including iron-deficiency...
anemia, hemochromatosis, acute and chronic myelogenous leukemias, gastric carcinoma, pancreatic carcinoma, Hodgkin’s disease, acute and chronic hepatitis, systemic lupus erythematosus, idiopathic portal hypertension, and peptic ulcer.

Before assay, the sera were suitably diluted with incubation buffer A.

Results

Figure 2 shows a typical standard curve for the enzyme immunoassay for human ferritin. The smallest concentration detectable by this method is 0.25 μg/L, a sensitivity fully comparable to that for the radioimmunoassay for human ferritin described previously (10).

Ferritin concentrations in sera from 75 patients and 11 normal subjects were determined by both enzyme immunoassay and radioimmunoassay, and Figure 3 shows the resulting correlation. The correlation coefficient was 0.95 (n = 85, p < 0.001).

Discussion

The clinical implications of serum ferritin have been recently explored by many investigators. Addison et al. (12) first demonstrated that the serum ferritin concentration was closely related to the amount of storage iron in normal subjects, patients with iron deficiency, and patients with iron overload. Several later studies have confirmed this relation and have concluded that measurement of serum ferritin is an accurate index to the status of body iron (13–16). Clinically, therefore, measurement of serum ferritin provides a useful tool for distinguishing between anemia caused by iron depletion and anemia attributable to other causes (e.g., chronic inflammation and malignancy). Patients with true iron-deficiency anemia exhibit extremely low ferritin values, ranging from a few micrograms to 20 μg per liter, as compared to normal values of 20 to 250 μg/L. Recently, an enzyme immunoassay for serum ferritin was reported by Thériault and Pagé (17), although its sensitivity is somewhat less than that for immunoradiometric assays. The sensitivity of our assay (detection limit, 0.25 μg/L), almost equivalent to that of a radioimmunoassay (10), is such as to suffice for quantitation of such low concentrations of ferritin as are present in serum in iron-deficiency anemia. Another clinical application of serum ferritin measurement is sero-diagnosis of cancer. Abnormally increased values for serum ferritin, ranging from few hundred to several thousand micrograms per liter (10), which are not related to body-iron stores, are generally observed in patients with cancer. In the present investigation, serum ferritin concentrations in various diseases, which mainly included neoplastic disease, were measured by enzyme immunoassay and radioimmunometric assay. An excellent correlation between the two methods (r = 0.95, n = 85, p < 0.001) was evident.

Enzyme immunoassay obviously avoids radiation hazard, requires relatively minimal equipment and stable and relatively inexpensive reagents, and is much easier to perform. Therefore, such assays should find wide application for determining serum ferritin concentration in clinical laboratories and research.

References