Fibrinogen as a Ferritin-Binding Protein in Horse Plasma

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ABSTRACT. Lower apparent concentrations of ferritin were observed in horse plasma than in serum using the enzyme-linked immunosorbent assay (ELISA). However, the ferritin concentrations in plasma and serum were increased to the same level on heating the samples at 75°C for 15 min. These results suggest that horse plasma has specific ferritin-binding protein(s) which inhibit(s) the ferritin assay. The apparent ferritin concentrations in horse serum were markedly decreased by adding horse fibrinogen to the serum. It was also found that fibrinogen bound to spleen ferritin and inhibited the immunoassay of this protein. From these results, it was concluded that horse fibrinogen is one of the ferritin-binding proteins which inhibit the immunoassay of horse ferritin.—KEY WORDS: ferritin, ferritin-binding protein, fibrinogen, horse, plasma.

Ferritin is a large protein which has a molecular weight of about 450,000 and a core including up to 4,500 iron atoms [8]. The iron core is surrounded by 24 subunits [8]. Tissue ferritins consist of variable proportion of H (heavy, Mr≈21,000) and L (light, Mr≈19,000) subunits [8]; whereas serum ferritin contains L and G (glycosylated, Mr≈23,000) subunits and binds to concanavalin A [3, 10].

Yamada and Gabuzda [12] and Covell et al. [2] have shown the interactions between tissue (or serum) ferritins and plasma (or serum) proteins. Orino et al. [7] previously observed that the enzyme immunoassay of horse spleen ferritin and serum ferritin was inhibited by serum components (possibly ferritin-binding proteins), of which the inhibitory activity disappeared by heat treatment at 75°C for 15 min. Niitsu et al. [6] also showed that the apparent concentrations of human serum ferritin determined by radioimmunoassay were increased by treatment of the serum with sodium dodecyl sulfate (SDS) or heat. From these findings, the ferritin-binding proteins in serum are considered to affect the immunoassay of ferritin by concealing the epitopes of ferritin. However, no direct evidence for the inhibition of ferritin assay by ferritin-binders has ever been obtained.

Although Bellotti et al. [1] identified alpha-2-macroglobulin, complement proteins C3 and C4, and immunoglobulins as ferritin-binding proteins in human serum, they did not examine whether these proteins inhibited the immunoassay of ferritin.

In the present study, we observed the existence of ferritin-binding protein(s) specific for plasma, based on the difference in apparent ferritin concentrations between horse serum and plasma, and confirmed that fibrinogen is one of the ferritin-binding proteins having an inhibitory effect on the immunoassay of ferritin.

MATERIALS AND METHODS

Materials: Horse spleen ferritin, horse fibrinogen (clottable protein 94%), and horse and bovine serum albumin were obtained from Sigma Chemical Co., U.S.A. Immunu Plate Maxisorp F96 microtiter plates were from Nunc, Denmark. Ferritin monomer was purified from a commercial preparation of spleen ferritin as described previously [7]. Horse serum transferrin was purified as before [9].

Protein determination: Protein was determined by the method of Lowry et al. [5] with bovine serum albumin as a standard.

Antibodies to horse spleen ferritin: Rabbit antisera to horse spleen ferritin were prepared, and antibodies were purified from the antisera by affinity chromatography as described previously [7].

Horse serum and plasma: Blood samples were collected from thoroughbreds which were bred at Kitasato University. Plasma was obtained from the heparinized blood. The serum and plasma samples were stored at 4°C in the presence of 0.05% sodium azide.

ELISA for horse ferritin: The ferritin concentrations were measured by sandwich ELISA (System A) developed in the previous method [7]. Horse serum and plasma diluted 1:21 with ELISA buffer (20 mM sodium phosphate, 150 mM NaCl, 0.1% bovine serum albumin, 0.1% Tween 20 and 0.02% sodium azide, pH 7.0) were or were not heated at 75°C for 15 min, and then centrifuged at 24,000 × g for 15 min at 4°C. The resulting supernatants were applied to ELISA to determine the ferritin.

Assay of ferritin-binding activity of horse plasma protein: One hundred microliters of 10 μg/ml or 100 μg/ml horse plasma protein in phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.0) were added to each well of the Immuno Plate Maxisorp F96 microtiter plates, and the plates were kept overnight at 4°C. The plates were washed three times (each for 20 min) with ELISA buffer, and 100 μl aliquots of 10 μg/ml horse spleen ferritin in ELISA buffer were added. The plates were incubated at 37°C for 2 hr. After washing with ELISA buffer, 100 μl aliquots of 0.25 μg/ml alkaline phosphatase-labeled antibody to ferritin prepared as described previously [7] were added to the wells, and the plates were incubated at 37°C for 2 hr. After washing, 200
μl of the substrate solution (3 mM p-nitrophenyl phosphate, 100 mM glycine/NaOH, 1 mM ZnSO₄, 1 mM MgCl₂, pH 10.0) were added to each well. The absorbance at 405 nm of p-nitrophenol released during incubation at 37°C for an appropriate time was determined with a Titertek Multiskan MCC/340 (Flow Laboratories Inc., U.S.A.).

RESULTS

The difference in apparent ferritin concentration between serum and plasma: The apparent concentrations of ferritin in plasma were found to be lower than those in serum. However, the ferritin concentrations in serum and plasma were increased to almost the same level on heating the samples at 75°C for 15 min beforehand (Table 1). These results suggest that plasma has specific ferritin-binding protein(s) which inhibit(s) the immunoassay of horse ferritin and is (are) inactivated by heat treatment.

Inhibition of the ferritin assay by fibrinogen: Increasing amounts of horse fibrinogen were added to horse sera, and the ferritin concentrations were then determined to know whether this protein had the inhibitory activity in the immunoassay of serum ferritin. Figure 1 shows that the addition of fibrinogen decreased the apparent ferritin concentrations in a dose-dependent manner. Fibrinogen was then added to the solutions of horse spleen ferritin. The measured concentrations of spleen ferritin were also decreased dose-dependently by adding fibrinogen (Fig. 2).

Binding of fibrinogen to ferritin: Figure 3 shows that horse fibrinogen had markedly high ferritin-binding activ-

<table>
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<th>Horse No.</th>
<th>Serum (ng/ml)</th>
<th>Plasma (ng/ml)</th>
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<td>5</td>
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<td>139</td>
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a) Serum and plasma were obtained from the same blood samples.

Fig. 1. The effect of fibrinogen on the immunoassay of serum ferritin. Various amounts of horse fibrinogen were added to three horse sera (○, ● and □) diluted 21-fold with ELISA buffer containing 10 mM EDTA, and the ferritin concentrations of the mixtures were determined.

Fig. 2. Inhibition of the immunoassay of spleen ferritin by fibrinogen. Various amounts of horse fibrinogen were added to 12.5 ng/ml (○) or 25 ng/ml (●) horse spleen ferritin in ELISA buffer, and the ferritin concentrations of the mixtures were determined.

Fig. 3. Ferritin-binding activity of fibrinogen. Horse fibrinogen (A), serum transferrin (B), or serum albumin (C) was added to each well of the microtiter plates (each 10 μg/well or 1 μg/well) and their ferritin-binding activities were determined as described in the text.
FIBRINOGEN AS A FERRITIN-BINDING PROTEIN

787

ity; whereas the activities of serum transferrin and serum albumin were negligible.

The mixture of fibrinogen and spleen ferritin was applied to a Sepharose CL-6B column (2×100 cm) equilibrated with PBS. However, the complex formation between these proteins was not detected by the gel filtration method (data not shown).

DISCUSSION

We demonstrated here that horse fibrinogen is a plasma-specific ferritin-binding protein which inhibits the immunoassay of horse serum and spleen ferritins. The concentration of fibrinogen in the 21-fold diluted plasma is calculated to be about 0.05–0.2 mg/ml from a normal fibrinogen level in horse plasma (1.0–4.0 mg/ml) [4], and this range of fibrinogen did decrease the apparent serum ferritin level (Fig. 2). Therefore, the differences in apparent ferritin concentrations between horse serum and plasma (Table 1) are considered to be partly (if not entirely) due to fibrinogen. Besides fibrinogen, blood coagulation factors II (prothrombin), V and VIII are also present in plasma but not in serum. It may be necessary to examine whether these factors are involved in the inhibition of plasma ferritin assay.

When blood coagulates, serum ferritin is considered not to bind to fibrin converted from fibrinogen because the serum and the plasma showed the same ferritin concentrations after heat treatment. If the serum ferritin still binds to fibrin after blood coagulation, the ferritin concentrations in heat-treated serum should be lower than those in heat-treated plasma.

Human ferritin binders greatly interact with human tissue ferritins, whereas they show little interaction with human plasma ferritin [2]. This explains the rapid clearance of human tissue ferritins from the circulation and the slow clearance of human serum ferritin [11]. The questions as to whether horse H-subunit-rich heart ferritin binds to ferritin-binders and whether there are differences in clearance rate between horse plasma ferritin and tissue ferritins in the circulation remain to be clarified.

REFERENCES


