Inhibitory Effects of Horse Serum on Immunoassay of Horse Ferritin

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ABSTRACT. The effects of horse serum on the immunoassay of horse ferritin were investigated using two sandwich enzyme-linked immunosorbent assay (ELISA) systems. In System A, affinity-purified antibody to horse spleen ferritin and its conjugate with alkaline phosphatase were used as the first and second antibodies, respectively. In System B, whole antiserum and its conjugate with the enzyme were used. The recoveries of horse spleen ferritin added to horse sera were very low in either system (50–71% in System A; 42–79% in System B). However, heat treatment of the sera at 75°C for 15 min improved the recoveries (90–96%) in System A, whereas the recoveries in System B were not sufficiently improved by the same treatment (75–83%). The apparent concentrations of ferritin in adult and newborn horse sera increased after heat treatment of the samples. From these results, it is concluded that horse serum contains the heat-unstable substance(s) which inhibits the immunoassay of horse ferritin.—KEY WORDS: ELISA, ferritin-binding protein, horse, serum ferritin, spleen ferritin.


Ferritin is an iron storage protein with a molecular weight of about 450,000 and is made up of 24 subunits [16]. Three types of subunits have been identified; L (light, Mr=19,000), H (heavy, Mr=21,000), and G (glycosylated, Mr=23,000) [5, 13, 16]. Tissue ferritins consist of a variable proportion of H and L subunits, whereas serum ferritin, which is probably secreted by cells, contains almost only L and G subunits. The G subunit is responsible for the binding of serum ferritin to concanavalin A.

Many investigators developed the immunoassay for serum ferritin, and a high degree of correlation between the serum ferritin concentration and the amount of iron stores has been established in domestic animals as well as in humans [1, 9, 14, 15, 17]. Therefore, the assay of serum ferritin is a useful clinical index. On the other hand, it is known that serum or plasma contains ferritin-binding proteins [3, 4, 18]. If these proteins conceal the epitopes of serum ferritin, the serum ferritin concentrations measured by immunological methods are considered to be underestimated. Niiitsu et al. [10] found that the treatment of human serum with sodium dodecyl sulfate (SDS) or heat increased the apparent concentrations of human serum ferritin determined by radioimmunoassay, and speculated that these treatments denatured the putative ferritin-binding proteins which probably interfered with the binding of anti-ferritin antibodies to serum ferritin.

Although Smith et al. [15] developed the enzyme immunoassay of horse ferritin, they did not show the effects of horse serum on the assay. In the present study, we therefore, reinvestigated the effects of horse serum on sandwich ELISA of horse ferritin.

MATERIALS AND METHODS

Horse spleen ferritin: Because a commercial preparation of horse spleen ferritin (Sigma Chemical Co., U.S.A.) contained ferritin oligomers as well as ferritin monomer [11], it was passed through a column (2×100 cm) of Sepharose CL-6B (Pharmacia, Sweden) equilibrated with phosphate-buffered saline (PBS: 20 mM sodium phosphate, 150 mM NaCl, pH 7.0) at a flow rate of 12.5 ml/hr. The fractions (each 5 ml) containing ferritin monomer were collected and pooled.

Purified ferritin monomer showed a single band on 5% polyacrylamide gel disc electrophoresis [6] (data not shown) and a single precipitin line was formed between the ferritin and rabbit antiserum to the ferritin in immunelectrophoresis [12] (Fig. 1). These results indicate that the purified ferritin monomer is homogeneous. The ferritin monomer was used in the following experiments.

Protein was determined by the method of Lowry et al. [8] using bovine serum albumin (Boehringer Mannheim, F.R.G.) as a protein standard.

Horse serum: Blood samples were collected from thoroughbreds which were bred at Kitasato University and Taihei Stud Farm, Inc. (Aomori, Japan). Serum samples were stored at −20°C until use.

Preparation of antisera to horse spleen ferritin and purification of antibodies: Two female rabbits (Clea
samples in ELISA buffer were then added. The plates were incubated at 37°C for 2 hr. After washing with ELISA buffer, 100 μl aliquots of alkaline phosphatase-labeled anti-ferritin antibodies (System A) or enzyme-labeled antiserum to ferritin (System B), which was prepared essentially by the method of Avrameas [2] and was appropriately diluted with ELISA buffer, were added 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) was 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.).

Treatment of sera for ELISA: Horse sera diluted appropriately with ELISA buffer 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 used for ELISA to determine ferritin. In the recovery experiment, known amounts of horse spleen ferritin were added to the diluted sera and the samples were treated as already described.

RESULTS

The recoveries of horse spleen ferritin added to horse sera: Figure 2 shows typical standard curves for horse spleen ferritin obtained by ELISA Systems A and B. The assay limit was 1 ng/ml for either system.

![Standard curves for horse spleen ferritin in ELISA Systems A (●) and B (○). Each point represents the mean of triplicate determinations.](image)
Table 1. The effect of heat treatment on the recovery of horse spleen ferritin added to horse serum in ELISA Systems A and B

<table>
<thead>
<tr>
<th>Serum No.</th>
<th>Added ferritin (ng/ml)</th>
<th>Untreated serum</th>
<th>Heat-treated serum</th>
<th>System B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Found ferritin (ng/ml)</td>
<td>Recovery (%)</td>
<td>Found ferritin (ng/ml)</td>
<td>Recovery (%)</td>
</tr>
<tr>
<td>1</td>
<td>4.8</td>
<td>7.8</td>
<td>11.0</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>10.5</td>
<td>15.3</td>
<td>13.5</td>
</tr>
<tr>
<td>2</td>
<td>4.8</td>
<td>6.8</td>
<td>11.0</td>
<td>9.8</td>
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<td></td>
<td>9.5</td>
<td>8.5</td>
<td>15.0</td>
<td>13.5</td>
</tr>
<tr>
<td>3</td>
<td>4.8</td>
<td>12.5</td>
<td>19.0</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>9.5</td>
<td>14.5</td>
<td>23.0</td>
<td>22.0</td>
</tr>
</tbody>
</table>

Known quantities of horse spleen ferritin were added to three horse sera diluted 21-fold with ELISA buffer, and the concentrations were determined by the two ELISA systems. However, the recoveries were very low in either system (50–71% in System A; 42–79% in System B) (Table 1). This low recovery was considered to be due to ferritin-binding proteins in serum [10]. The antigenicity of horse spleen ferritin is heat-stable [7]. If the putative ferritin-binders were heat-labile, the inhibition of the ferritin assay by them was expected to be eliminated by heat treatment. We therefore heated the sera at 75°C for 15 min. After the treatment, almost all ferritin amounts added were recovered in System A (90–96%), whereas the recoveries were still low in System B (75–83%) (Table 1). Heating the sera at 50°C or 60°C for 15 min did not sufficiently improve the recovery. Addition of EDTA (10 mM) had no effect on the recovery.

Horse spleen ferritin was added to normal rabbit sera diluted 21-fold with ELISA buffer, and the ferritin concentrations were assayed by System A without heating the samples. The recoveries ranged from 87 to 89%, indicating that the rabbit serum has only a weak inhibitory effect on horse ferritin immunoassay.

**Effects of heat treatment on immunoassay of horse serum ferritin:** We used ELISA System A for serum ferritin assay, because the recoveries were still low in System B even after heat treatment (Table 1).

A horse serum diluted with various amounts of ELISA buffer was heated or left untreated, and then assayed for ferritin concentration to examine the effect of serum dilution on the serum ferritin assay. There were linear relationships ($y = 0.999$) between serum dilution and determined ferritin from 1:11 dilution to 1:51 dilution in both heated and untreated sera (Fig. 3).

Ferritin concentrations of heat-treated sera from newborn and adult horses were measured significantly higher than those of untreated sera (Table 2).

The intra-assay coefficient of variation from four measures ($n=4$) of the same serum was 7.6%. The inter-assay coefficient of variation ($n=14$) was 6.1%.

![Fig. 3](image_url)
Table 2. The effect of heat treatment on serum ferritin concentrations

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Serum ferritin (ng/ml)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adult horse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Untreated</td>
<td>Heat-treated</td>
</tr>
<tr>
<td>1</td>
<td>66</td>
<td>110</td>
<td>20</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
<td>140</td>
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<tr>
<td>3</td>
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</tr>
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<td>4</td>
<td>66</td>
<td>108</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
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<td>8</td>
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<td>159</td>
<td>93</td>
</tr>
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<td>72</td>
</tr>
<tr>
<td>10</td>
<td>76</td>
<td>112</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Newborn horse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Untreated</td>
<td>Heat-treated</td>
</tr>
<tr>
<td></td>
<td></td>
<td>46±7.9</td>
<td>57±9.0</td>
</tr>
</tbody>
</table>

Mean±S.E. 94±9.1 143±10.7<sup>c</sup> 46±7.9 57±9.0

<sup>a</sup> Three to 14 years of age.<br>
<sup>b</sup> One to 3 days of age.<br>
<sup>c</sup> Significantly different from untreated serum (p<0.05) by Student’s t-test.

DISCUSSION

Niiitsu <i>et al.</i> [10] first observed an increase in the serum ferritin concentrations when the human serum samples were treated with 1% SDS or heated at 75°C for 10 min, and considered that these treatments denatured the putative ferritin-binding proteins concealing the epitopes of serum ferritin. We also found here an inhibition of the immunoassay of horse ferritin by serum. Dilutions of serum up to 1:51 had no effect on this inhibition (Fig. 3). Heat treatment of serum at 75°C for 15 min was effective in eliminating the serum inhibition. Therefore, we considered that horse serum contains some components (probably ferritin-binding proteins) which interfere with the antigen-antibody reaction, and that heat treatment denatures them, leaving intact ferritin in solution because ferritin is heat-stable [7]. We were not able to use SDS to denature the putative ferritin-binders in the horse serum because SDS inhibited our sandwich ELISA systems even at a concentration of 0.1%. SDS probably denatures not only antibodies in the system but also ferritin.

The serum inhibition of the ferritin assay was also found in newborn horse sera. This suggests that there are ferritin-binding proteins in the horse serum in the early period after birth.

Smith <i>et al.</i> [15] recovered almost all horse ferritin added to horse serum using the sandwich ELISA system consisting of anti-ferritin whole serum and its conjugate with horseradish peroxidase, and did not detect the inhibitory effects of serum on ferritin assay. However, the inhibition of horse ferritin assay by horse serum was detected by ELISA Systems A and B which were developed in the present study. System B is similar to the system of Smith <i>et al.</i> The differences in antigen or antiserum used may be the reason why the serum inhibition was not found in their ELISA system.

Because the recoveries of horse ferritin added to horse sera were still low in ELISA System B even after heat treatment, System A using affinity-purified antibody is superior to System B using whole antiserum.

From the assay range of ferritin in our ELISA system (Fig. 2), the ferritin level in horse serum (Table 2) and the linearity of serially diluted serum in the ferritin assay (Fig. 3), it is considered that horse sera are appropriately diluted in the range from 1:11 to 1:51 and then can be routinely applied to ferritin assay.

Alpha-2-macroglobulin, complement proteins C<sub>3</sub> and C<sub>4</sub>, and immunoglobulins were identified as ferritin-binding proteins in human serum by Bellotti <i>et al.</i> [3]. We detected the binding of horse immunoglobulins (IgA, IgG, IgM) to horse spleen ferritin using the system which consisted of ferritin-coated microtiter plates, rabbit antisera specific for horse immunoglobulin H-chain (α, γ, μ), and alkaline phosphatase-labeled goat antibodies to rabbit IgG (to be published elsewhere). In the present investigation, we did not prove that the ferritin-binding proteins inhibited the horse ferritin.
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assay. It is necessary to elucidate whether the ferritin-binders are really responsible for the serum inhibition of the ferritin assay.

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REFERENCES