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Competitive Enzyme-Linked Immunosorbent Assay for Glucose-6-Phosphate Dehydrogenase

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Abstract : To construct a competitive ELISA standard curve for the detection of glucose-6-phosphate dehydrogenase (G6PD), we used highly purified native G6PD (nG6PD) as both immobilized and soluble antigens and anti-G6PD serum raised against nG6PD as antibody. The polystyrene cuvettes coated wit h nG6PD were challenged with a mixture of a limiting amount of anti-G6PD serum and various doses of nG6PD as competitors followed by incubation with alkaline phosphatase-anti-IgG conjugate. The competitive ELISA did not exhibit the typical sigmoidal dose-response curve characteristic of competition immunoassays under the optimal concentrations of antigen and antibody. The soluble nG6PD used as competitor failed to effectively inhibit the binding of antibodies to the immobilized nG6PD. The addition of NADP, a cofactor of G6PD enzyme, to coating buffer used for immobilizing nG6PD to the cuvettes and PBS-Tween-BSA buffer for diluting competitors did not improve the inhibition of antibody binding to immobilized nG6PD by soluble n/G6PD. The addition of BSA to coating buffer did not increase inhibition, either. Surprisingly, when partially active G6PD (paG6PD), obtained by repeated freeze-thawing, was used as competitor, the antibody binding to either immobilized nG6PD or immobilized paG6PD was inhibited 49~58%. We conclude that an effective competitive ELISA system with nG6PD enzyme and anti-G6PD serum for the detection of G6PD may not be established due to the poor inhibition of antibody binding to immobilized nG6PD by soluble nG6PD under the present assay conditions and that the inhibition may be improved by using an inactivated enzyme as competitor regardless of the type of immobilized antigen used. These results imply that the immobilized nG6PD may undergo denaturation upon binding to the polystyrene cuvettes and that our anti-G6PD serum may recognize denatured enzyme better than active enzyme.

Key words : competition, enzyme linked immunosorbent assay, glucose-6-phosphate dehydrogenase, native antigen, denaturation.

Rat liver G6PD is a dimeric enzyme which catalyzes the rate-limiting step of the pentose-phosphate pathway. G6PD uses NADP as a cofactor and produces NADPH which is utilized for biosynthetic pathways. G6PD is regulated by diets and hormones. Previous studies showed that the G6PD activity increased by a high carbohydrate diet or insulin whereas the activity decreased by fasting, fat diet or glucagon (Morikawa *et al.*, 1984; Manos *et al.*, 1986; Tomlinson *et al.*, 1988; Kim *et al.*, 1990; Kim and Holten, 1991; Boll *et al.*, 1996; Brooks and Lampi, 1996; Kabir and Ide, 1996; Maeda *et al.*, 1996; Stabile *et al.*, 1996). In addition to diets and hormones, oxidative stress and metal ions have been shown to regulate G6PD activity (Berg *et al.*, 1995; Cramer *et al.*, 1995; Maier *et al.*, 1996; Stangl and Kirchgessner, 1996). Furthermore, some of these changes in activity have been shown to be caused by changes in G6PD mRNA levels suggesting pretranslational regulation (Kletzien *et al.*, 1985; Katsurada *et al.*, 1986; Tomlinson *et al.*, 1988; Kim *et al.*, 1990; Berg *et al.*, 1995; Cramer *et al.*, 1995; Stabile *et al.*, 1996). In contrast to the pretranslational regulation of G6PD, Dao *et al.* (1982) suggested that the low activity of G6PD by high fat diet observed by using monoclonal antibody in a solid phase radioimmunoassay might be due to an inactivation of preexisting enzyme and concluded that the regulation of G6PD by high fat diet occurred at the posttranslational level.

ELISA is an accurate, convenient method for detecting low levels of protein and antibody (Kemeny and Chantler, 1988; Gosling, 1990). ELISA can be competitive or noncompetitive depending on the presence of competitor. There are a few variants of competitive ELISA. Among the variants, the indirect procedure is more

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sensitive and has the advantage of the use of only one type of enzyme-labeled antibody conjugate. The indirect assay for the measurement of antigen involves the inhibition of specific antibody binding to antigen coated on the solid phase by a test antigen. The amount of antibody binding assessed by the binding of a secondary antibody conjugate directed to a species of the primary antibody is inversely proportional to the amount of antigen present in the sample.

In order to study the regulation of hepatic G6PD, we attempted to set up a competitive ELISA with purified active G6PD as immobilized and soluble antigens and anti-G6PD serum raised against purified active G6PD.

Materials and Methods

Purification of rat liver G6PD and preparation of rabbit anti-rat G6PD serum

The rat liver G6PD was purified according to Winberry and Holten (1977). Partially active G6PD was prepared by repeated freezing and thawing. The remaining activity of paG6PD was 20% of that before treatment. Rabbit anti-rat G6PD serum was prepared as previously described (Winberry and Holten. 1977).

Titration of the antigen concentration used for coating and of antibody concentration

In order to maximize the sensitivity of the assay, the amount of G6PD coated onto the cuvettes and the concentration of anti-G6PD serum were determined. The polystyrene cuvettes were washed with 0.2% glutaraldehyde and coated with 0.05 to 4 ng of purified nG6PD in 0.3 ml of coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, 0.003 M NaN_3, pH 9.6) overnight at 4℃. The coated cuvettes were challenged for 2 h at room temperature with 0.3 ml of PBS-Tween 20 (0.05%)-BSA (100 μg/ml) buffer (pH 7.4) containing anti-G6PD serum diluted 1:200 to 1:6400. After an additional 2 h incubation at room temperature with alkaline phosphatase-anti-IgG conjugate diluted 1:400 in PBS-Tween-BSA buffer, the p-nitrophenyl phosphate substrate was applied in diethanolamine buffer (pH 9.8). Color development was terminated by the addition of 0.1 ml of 1.5 N NaOH. Absorbances at 405 nm were read on a Gilford EIA automatic analyzer.

Competitive ELISA

The competitive ELISA was performed in Gilford EIA cuvettes as described by Wright *et al.* (1983). The polystyrene cuvettes were washed with 0.2% glutaraldehyde and coated with 2 ng of purified nG6PD in 0.3 ml of coating buffer overnight at 4℃. The coated cuvettes were challenged for 2 h at room temperature with 0.3 ml of PBS-Tween-BSA buffer containing anti-G6PD serum diluted 1:1600 and 0.05 to 1000 ng of nG6PD. After an additional 2 h incubation at room temperature with alkaline phosphatase-anti-IgG conjugate diluted 1:400 in PBS-Tween-BSA buffer, the p-nitrophenyl phosphate substrate was applied in diethanolamine buffer. Color development was terminated by the addition of 0.1 ml of 1.5 N NaOH. Absorbances at 405 nm were read on a Gilford EIA automatic analyzer.

Results

To maximize the sensitivity of ELISA, the optimal concentration of nG6PD antigen coated onto the cuvettes and dilution rate of anti-G6PD serum were determined (Fig. 1 & 2). Absorbances at 405 nm showed linearity between 1 and 4 ng of immobilized nG6PD per cuvette at all anti-G6PD serum dilutions tested (Fig. 1). We chose 2

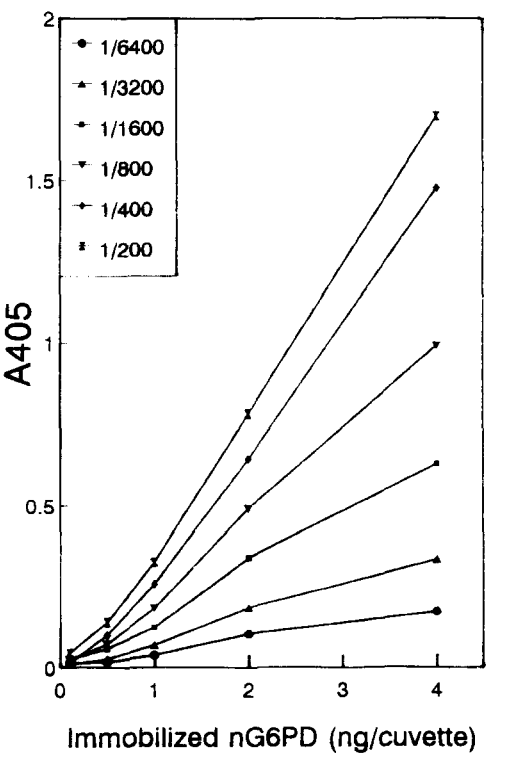

Fig. 1. Titration of G6PD concentration coated onto the cuvettes and of anti-G6PD serum concentration in ELISA. The polystyrene cuvettes were coated with 0.1 to 4 ng of purified nG6PD in 0.3 ml of coating buffer overnight at 4℃. The coated cuvettes were incubated for 2 h with 0.3 ml of PBS-Tween-BSA buffer containing anti-G6PD serum diluted 1:200 to 1:6400. After an additional 2 h incubation with alkaline phosphatase-anti-IgG conjugate diluted 1:400 in PBS-Tween-BSA buffer, the p-nitrophenyl phosphate substrate was applied in diethanolamine buffer. Color development was terminated by the addition of 0.1 ml of 1.5 N NaOH. Absorbances at 405 nm were read on a Gilford EIA reader. Each value represents the mean A_{405} of quadruplicate tests.

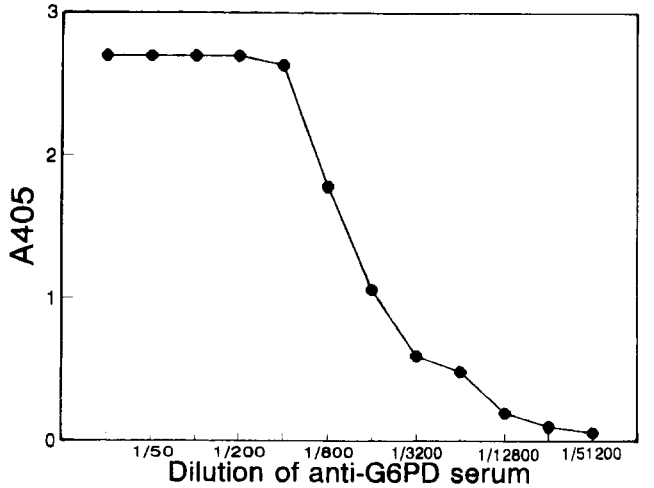

Fig. 2. Dilution curve of anti-G6PD serum in ELISA. The cuvettes were coated with 2 ng of nG6PD and incubated with anti-G6PD serum diluted 1:25 to 1:51200. Each value represents the mean A_{405} of quadruplicate tests.

ng of immobilized nG6PD per cuvette in all of the assays. Further, anti-G6PD serum dilution curve was constructed using 2 ng of immobilized nG6PD (Fig. 2). The curve was sigmoidal and antibody became limiting below 1:400 dilution. To insure a limiting antibody in the competitive ELISA, 1:1600 dilution of anti-G6PD serum was routinely used in the assays. The competitive ELISA was carried out to construct a standard curve with 2 ng of immobilized nG6PD, various doses ranging from 0.05 to 1000 ng of soluble nG6PD as competitors, and 1:1600 dilution of anti-G6PD serum. The ELISA did not exhibit the typical sigmoidal dose-response curve characteristic of all competition immunoassays (Fig. 3). Notably, high doses of soluble nG6PD used as competitor did not effectively inhibit the binding of antibodies to the immobilized nG6PD. With 500 ng of soluble nG6PD which is 250 times the amount of immobilized nG6PD, there was less than 50% inhibition of antibody binding to the immobilized nG6PD.

Attempts were made to improve the competition between immobilized and soluble nG6PD for binding to antibodies. BSA was included in the coating buffer to try to prevent denaturation of diluted nG6PD antigen. The addition of BSA to coating buffer failed to increase the inhibition of antibody binding to immobilized nG6PD by soluble nG6PD as demonstrated by the similar ratios (0.90 vs. 0.92) of A_{405} at 100 ng of soluble nG6PD to A_{405} at 5 ng of soluble nG6PD (the ratio 100:5) (Fig. 4). However, the values of A_{405} in the presence of BSA were 37-39% those in the absence of BSA. A solution of NADP, a cofactor of G6PD, was added to both coating buffer and PBS-Tween-BSA buffer used for diluting soluble G6PD in order to keep G6PD enzyme in a stable conformation. As was the case with BSA added to coating buffer, the addition of NADP did not improve the inhibition (Fig. 5). Since the immobilized nG6PD might undergo denaturation to some extent upon adsorption to the solid phase, it was possible that competition between soluble nG6PD competitor and immobilized nG6PD for antibody binding was not effective. Thus, we used 20% active G6PD (paG6PD), which we accidentally had by repeated freeze-thawing, as soluble antigen. When nG6PD was coated onto the cuvettes and

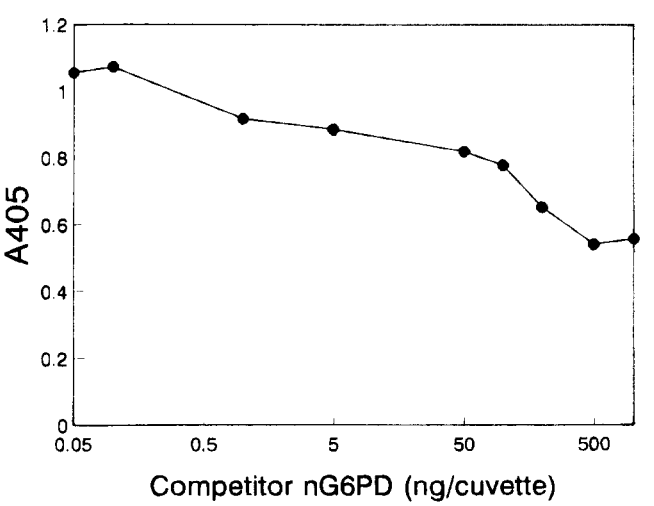

Fig. 3. The competitive ELISA with nG6PD and anti-G6PD serum. The competitve ELISA was carried out as described in "Materials and Methods". The polystyrene cuvettes were coated with 2 ng of nG6PD and challenged with 0.05 to 1000 ng of nG6PD competitor and anti-G6PD serum diluted 1:1600. The alkaline phosphatase-anti-IgG conjugate was diluted 1:400. Each value represents the mean A_{405} of quadruplicate tests.

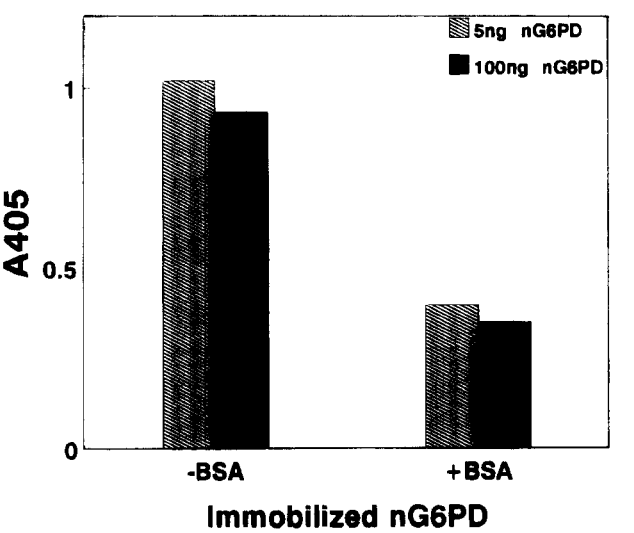

Fig. 4. Effect of BSA in immobilized nG6PD on competitive ELISA. The polystyrene cuvettes were coated with 2 ng of nG6PD containing 56 ng/ml of BSA and challenged with 5 or 100 ng of nG6PD competitor and anti-G6PD serum diluted 1:1600. The alkaline phosphatase-anti-IgG conjugate was diluted 1:400. Each value represents the mean A_{405} of triplicate tests.

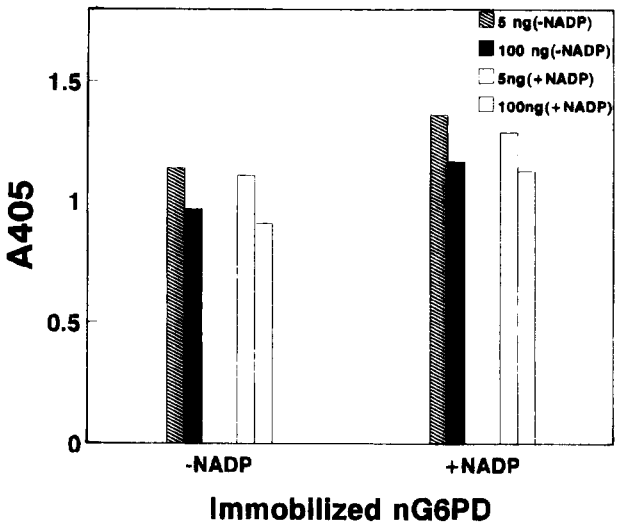

Fig. 5. Effect of NADP in immobilized and soluble nG6PD on competitive ELISA. The competitive ELISA was carried out as described in Fig. 4 legend. The NADP in a final concentration of 0.1 mM was added to the coating buffer and PBS-Tween-BSA buffer used for diluting competitors and anti-G6PD serum. Each value represents the mean A_{405} of triplicate tests.

paG6PD was used as competitor, the ratios 100:5 in the absence and presence of NADP were markedly reduced to 0.40 and 0.44, respectively (Fig. 6) compared to those (0.85 and 0.86) with nG6PD competitors as shown in Fig. 5. These reductions in the ratios 100:5 by paG6PD competitors represent 49% and 53% inhibition of antibody binding to immobilized nG6PD compared to those by nG6PD competitors. However, when

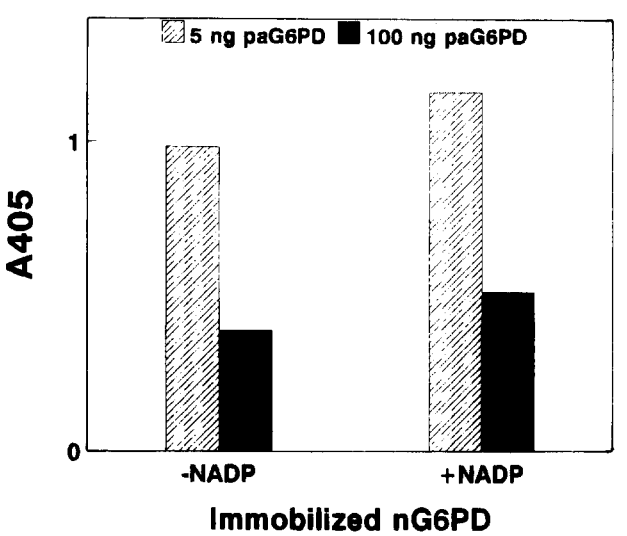

Fig. 6. Effect of paG6PD used as competitors on competitive ELISA. The polystyrene cuvettes were coated with 2 ng of nG6PD and challenged with 5 or 100 ng of paG6PD competitor and anti-G6PD serum diluted 1:1600. The alkaline phosphatase-anti-IgG conjugate was diluted 1:400. Each value represents the mean A405 of triplicate tests.

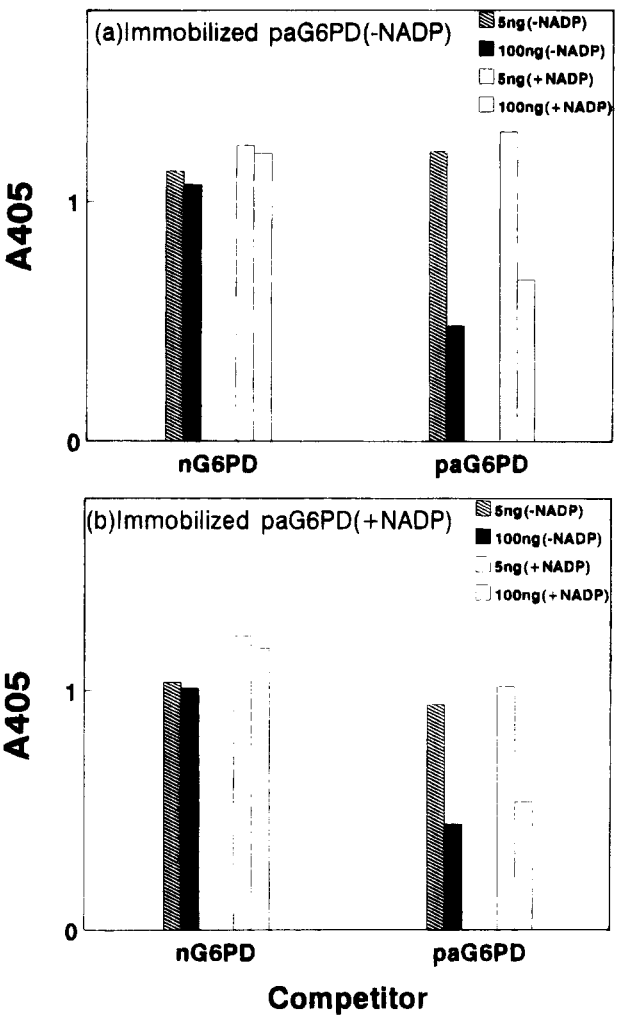

Fig. 7. Comparison of competition between immobilized paG6PD and either nG6PD or paG6PD competitor for antibody binding. The polystyrene cuvettes were coated with 2 ng of paG6PD and incubated with 5 or 100 ng of either nG6PD or paG6PD competitor in the presence (a) or absence (b) of NADP in a final concentration of 0.1 mM and anti-G6PD serum diluted 1:1600. The alkaline phosphatase-anti-IgG conjugate was diluted 1:400. Each value represents the mean A_{405} of triplicate tests.

paG6PD was used as both immobilized and soluble antigen, a further inhibition was not observed (52%~58%) (Fig. 7). Moreover, the addition of NADP to coating buffer and PBS-Tween-BSA buffer was not able to improve the competition. When paG6PD was coated onto the cuvettes and competed with soluble nG6PD, the competition did not occur as demonstrated by the ratio 100:5 of 0.95~0.98 (Fig. 7). Although the ratios 100:5 with immobilized paG6PD (Fig. 7) was similar to those with immobilized nG6PD (Fig. 4 & 5), the incubation time with p-nitrophenyl phosphate substrate to obtain the same A_{405} was approximately 6 times shorter with immobilized paG6PD (Fig. 7) than with immobilized nG6PD (Fig. 5).

Discussion

This study shows that a competitive ELISA system with nG6PD enzyme and anti-G6PD serum for the detection of G6PD may not be established due to ineffective competition between immobilized .and soluble n-G6PD for antibody binding under the present assay conditions. We found that the competitive ELISA for n-G6PD did not exhibit the typical sigmoidal dose-response curve characteristic of competition immunoassays under the optimal concentrations of antigen and antibody. Especially, high concentrations of competitors did not effectively inhibit the antibody binding to the immobilized nG6PD. We also used different titers of anti-G6PD serum in competitive ELISA and obtained similar results to the present study (data not shown). Since the G6PD enzyme uses NADP as a cofactor and has a NADP binding site, we assumed that the final concentration of NADP in nG6PD preparations used as immobilized and soluble antigens might not be enough to keep the conformation of G6PD in a stable condition. However, the addition of NADP to coating buffer and PBS-Tween buffer did not increase the competition. In contrast to nG6PD competitors, paG6PD competitors inhibited antibody binding to either immobilized nG6PD or immobilized paG6PD effectively although the color development with p-nitrophenyl phosphate substrate was faster with immobilized paG6PD than with immobilized nG6PD, suggesting that both nG6PD and paG6PD, when bound to the plastic surface, can compete with soluble paG6PD for binding to antibodies. It is possible that both repeated freeze-thawing and adsorption onto the plastic surface of nG6PD might have denatured G6PD, resulting in the increase of immunological activity. These results suggest that our anti-G6PD serum raised against native G6PD has high cross-reactivity with denatured G6PD in competitive ELISA. Furthermore, our results imply that the exposed antigenic sites of immobilized paG6PD may be more than those of immobilized nG6PD and the conformation of exposed antigenic sites of immobilized nG6PD may be similar to that of paG6PD. In contrast, Koch *et al.* (1996) reported that antiserum raised against native ovalbumin had low cross-reactivity with denatured ovalbumin as demonstrated by ELISA and immunoaffinity chromatography and concluded that native ovalbumin had different immunogenic epitopes from denatured ovalbumin.

Our anti-G6PD serum is very specific for G6PD as demonstrated by the presence of a single band on Western immunoblot (data not shown). Previously, the same anti-G6PD serum was used to immunoprecipitate G6PD from liver homogenates and hepatocytes of rats under different dietary conditions (Morikawa *et al.*, 1984; Tomlinson *et al.*, 1988; Kim *et al.*, 1990; Kim and Holten, 1991). The poor competition observed in our competitive ELISA might have been due to the conformational changes and denaturation of immobilized nG6PD upon binding to the polystyrene cuvettes. The tendency of bound proteins to denaturation in ELISA has been pointed out previously in other studies (Soderquist and Walton, 1980; Hollander and Katchalski-Katzir, 1986; Shields *et al.*, 1991; Schwab and Bosshard, 1992). Attempts have been made to overcome the denaturation tendency by many investigators (Smith and Wilson, 1986; Jemmerson, 1987; Kenett, 1988; Schwab and Bosshard, 1992). Recently, however, Houen and Koch (1997) reported an ELISA system which prevented denaturation of immobilized proteins by adsorbing the protein to aluminum hydroxide gel before coating the plastic surface. In conclusion, the present study shows that an effective competitive ELISA with purified nG6PD enzyme and our anti-G6PD serum for the detection of G6PD could not be established due to the poor competition between immobilized nG6PD and soluble nG6PD for antibody binding and that the competition may be improved with the inactivated enzyme as competitor irrespective of the type of bound antigen. These findings imply that the immobilized nG6PD may undergo denaturation upon adsorption to the plastic surface and our anti-G6PD antibodies may recognize denatured enzyme better than active enzyme. Further study is needed to characterize the specificity of anti-G6PD serum for denatured G6PD.

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