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Short communication

The Reactivity of Antiserum Raised against Native Glucose-6-phosphate Dehydrogenase with Denatured Glucose-6-phosphate Dehydrogenase in Competitive ELISA

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We have previously reported that anti-glucose-6-phosphate dehydrogenase (G6PD) serum raised against native G6PD (nG6PD) enzyme recognized nG6PD antigen poorly in competitive enzyme-linked immunosorbent assay (ELISA) (Kim, 1997). In the present study, we investigated whether anti-G6PD serum raised against nG6PD can react with denatured G6PD effectively in competitive ELISA. We used partially active G6PD (paG6PD) by repeated freeze-thawing or SDS-denatured G6PD (SDS-G6PD) as both immobilized and soluble antigens, and anti-G6PD serum raised against nG6PD for competitive ELISA. The polystyrene cuvettes coated with either paG6PD or SDS-G6PD were challenged with a mixture of a limiting amount of anti-G6PD serum and various doses of paG6PD or SDS-G6PD as competitors, followed by incubation with alkaline phosphatase-anti-IgG conjugate. The competitive ELISA with paG6PD or SDS-G6PD antigen exhibited the sigmoidal dose-response curve characteristic of competition immunoassays. Furthermore, Triton-denatured G6PD (Triton-G6PD) was used in competitive ELISA. The paG6PD, SDS-G6PD, or Triton-G6PD used as competitors increased the inhibition of antibody binding to immobilized either of nG6PD or denatured G6PD compared with nG6PD competitor. The inhibition by denatured G6PD competitors was more pronounced at high competitor concentrations than at low counterparts. We conclude that anti-G6PD serum raised against nG6PD can effectively react with denatured G6PD in competitive ELISA and that our anti-G6PD serum recognizes denatured enzymes better than active enzymes.

Keywords: Competitive, Denatured, ELISA, G6PD, Native.

Introduction

Enzyme-linked immunosorbent assay (ELISA) is an accurate, convenient method for detecting low levels of antigen and antibody (Kemeny and Chantler, 1988; Gosling, 1990). A vast amount of information has been accumulated about the recognition of native antigen by antibodies in ELISA. However, the recognition of denatured antigen by antibodies to native antigen in ELISA has only been studied to a limited degree. Recently, a few investigators reported conflicting results about the reactivity of denatured proteins with antibodies raised against native proteins (Burks *et al.*, 1992; Vashishtha and Fischetti, 1993; Johnstone *et al.*, 1994; Chang *et al.*, 1995; Ksiezak-Reding *et al.*, 1995; Koch *et al.*, 1996; Li and Ownby, 1996; Mechin *et al.*, 1996;). Burks *et al.* (1992) found that heat denaturation of peanut and soybean proteins did not affect binding of antibodies raised against native proteins in ELISA. 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.

We previously attempted to set up a competitive ELISA with native glucose-6-phosphate dehydrogenase (nG6PD) as immobilized and soluble antigens, and anti-G6PD serum raised against nG6PD, in order to study the regulation of

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hepatic G6PD. However, we were unable to establish the competitive ELISA with nG6PD due to the ineffective competition for antibody binding between immobilized and soluble G6PD antigens (Kim, 1997). In the present study, we investigated whether antiserum raised against nG6PD can recognize denatured G6PD effectively in competitive ELISA, thus increasing the sensitivity of the competition assay.

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). Rabbit anti-rat G6PD serum was prepared as previously described (Winberry and Holten, 1977).

Denaturation of G6PD Partially active G6PD (paG6PD) was prepared by repeated freezing and thawing. The remaining activity of paG6PD was 20% of that before treatment. Detergents such as 1% SDS and 10% Triton were used to denature G6PD enzyme. These concentrations of detergents did not affect the competitive ELISA under our conditions.

Competitive ELISA The optimal conditions for the competitive ELISA were previously determined (Kim, 1997). The polystyrene cuvettes were washed with 0.2% glutaraldehyde and coated with 2 ng of nG6PD or denatured G6PD in 0.3 ml of coating buffer overnight at 4°C. The coated cuvettes were challenged for 2 h at RT with 0.3 ml of PBS-Tween-BSA buffer containing anti-G6PD serum diluted 1:1600 and 0.05–1000 ng of nG6PD or denatured G6PD. After an additional 2 h incubation at RT 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

We previously found that the competitive ELISA with nG6PD as both immobilized and soluble antigens, and antiserum raised against nG6PD, did not exhibit the typical sigmoidal dose-response curve characteristic of competition immunoassays, due to the poor competition between soluble and immobilized antigens for antibody binding (Kim, 1997). To investigate whether physically or chemically denatured G6PD can be recognized effectively by anti-G6PD antibodies raised against nG6PD, we used paG6PD or SDS-G6PD as immobilized and soluble antigens. The optimal concentration of SDS used to denature G6PD was chosen to be 1% and this concentration did not affect the competitive ELISA under our conditions (data not shown). The competitive ELISA was carried out under the optimal concentrations of antigen and antibody, 2 ng of immobilized nG6PD per cuvette, 1:1600 dilution of anti-G6PD serum, and various doses ranging from 0.05–500 ng of soluble nG6PD as competitors (Kim, 1997). The competitive ELISA with both paG6PD and SDS-G6PD antigens exhibited the typical sigmoidal dose-response curve (Fig. 1). Notably, high doses of soluble paG6PD or SDS-G6PD used as competitors effectively inhibited the binding of antibodies to the immobilized either of paG6PD or SDS-G6PD. With 500 ng of soluble paG6PD or SDS-G6PD, which is 250 times the amount of immobilized G6PD, there was 89% or 77% inhibition of antibody binding to the immobilized G6PD, respectively, whereas with 500 ng of soluble nG6PD, there was less than 50% inhibition (Fig. 2). The concentration of paG6PD competitor, which demonstrated 50% competition, was similar to that of the SDS-G6PD competitor, suggesting a similar affinity for antibody binding. Furthermore, we compared the competition

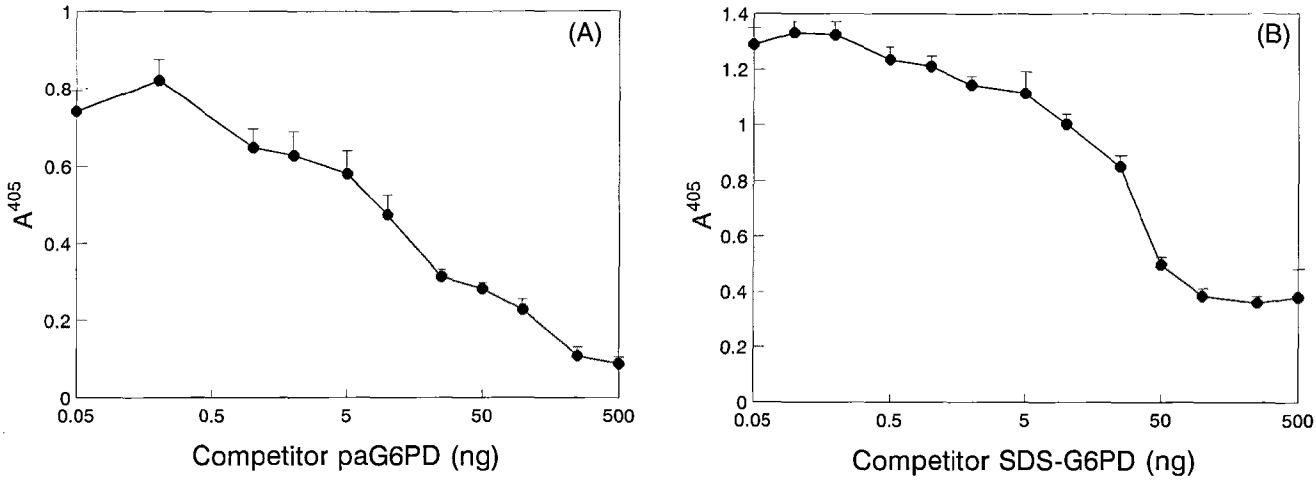

Fig. 1. Competitive ELISA with paG6PD or SDS-G6PD and anti-G6PD serum raised against nG6PD. The competitive ELISA was carried out as described in "Materials and Methods". The polystyrene cuvettes were coated with 2 ng of paG6PD (A) or SDS-G6PD (B) and challenged with 0.05–500 ng of paG6PD and anti-G6PD serum diluted 1:1600. The alkaline phosphatase-anti-IgG conjugate was diluted 1:400. Each value represents the mean $A_{405} \pm SE$ of quadruplicate tests.

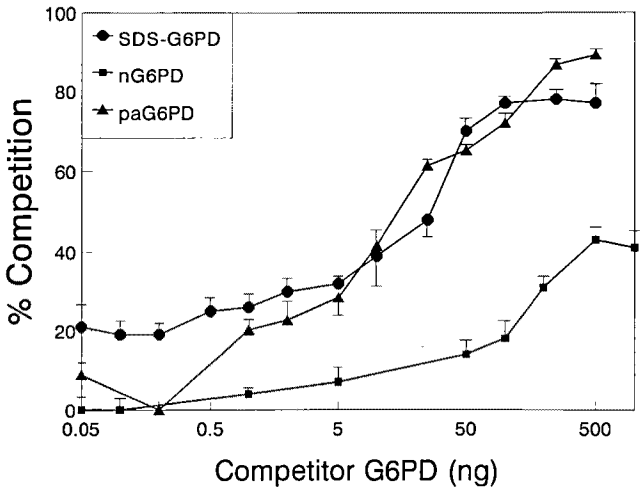

Fig. 2. Comparison of competitive ELISA of variously-treated G6PD antigens. The competitive ELISA was carried out as described in "Materials and Methods". The polystyrene cuvettes were coated with 2 ng of nG6PD, paG6PD, or SDS-G6PD and challenged with 0.05–1000 ng of nG6PD, paG6PD or SDS-G6PD, respectively, and anti-G6PD serum diluted 1:1600. The alkaline phosphatase-anti-IgG conjugate was diluted 1:400. Percentage of competition is expressed as the ratio of A_{405} difference between zero ng and various concentrations of competitor to A_{405} without competitor. Each value represents the mean percentage ± SE of quadruplicate tests.

between variously-treated G6PD antigens at 5 or 100 ng of competitor concentration. Ten percent of Triton was used to denature G6PD and this concentration of detergent did not affect the competition assay (data not shown). When nG6PD was coated onto the cuvettes, the effective competition (68–80%) was observed with 100 ng of paG6PD, SDS-G6PD, and Triton-G6PD competitors, whereas 28% competition was observed with the nG6PD competitor (Fig. 3A). However, 5 ng of paG6PD and SDS-G6PD competitors competed ~2-fold better than that of nG6PD and Triton-G6PD competitors (Fig. 3A). In a similar pattern to competition with immobilized nG6PD, when paG6PD was coated onto the cuvettes, 75–85% competition was observed with 100 ng of paG6PD, SDS-G6PD, and Triton-G6PD competitors and 9% competition with 100 ng of nG6PD competitor (Fig. 3B). No competition was observed with 5 ng of nG6PD competitors while there was 9–29% competition with 5 ng of paG6PD, SDS-G6PD, and Triton-G6PD competitors in paG6PD-coated cuvettes (Fig. 3B). When SDS-G6PD was coated onto the cuvettes, the effective competition (67–94%) was observed with 100 ng of paG6PD, SDS-G6PD, and Triton-G6PD competitors while there was almost no competition with 100 ng of nG6PD competitor (Fig. 3C). From comparison of the competition at 100 ng of competitor, it became clear that antiserum raised against nG6PD recognized denatured G6PD better than native G6PD.

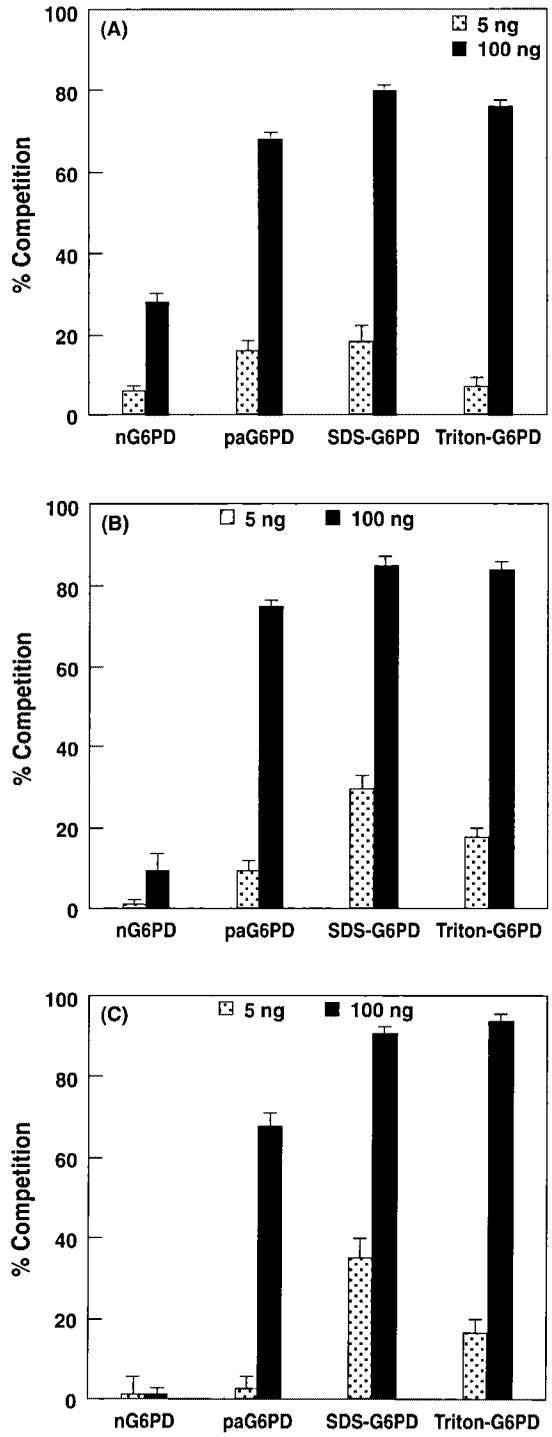

Fig. 3. Comparison of competition between immobilized G6PD and various G6PD competitors for antibody binding. The polystyrene cuvettes were coated with 2 ng of nG6PD (A), paG6PD (B), or SDS-G6PD (C) and challenged with 5 or 100 ng of nG6PD, paG6PD, SDS-G6PD, or Triton-G6PD, and anti-G6PD serum diluted 1:1600. The alkaline phosphatase-anti-IgG conjugate was diluted 1:400. Percentage of competition is expressed as the ratio of A_{405} difference between zero ng and 5 or 100 ng of competitor concentration to A_{405} without competitor. Each value represents the mean percentage ± SE of triplicate tests.

Discussion

This study shows that antibodies to nG6PD can effectively recognize denatured G6PD in competitive ELISA. We found that the competitive ELISA with paG6PD or SDS-G6PD exhibited the typical sigmoidal dose-response curve under the optimal concentrations of antigen and antibody. The physically and chemically denatured G6PD used as competitors increased the inhibition of antibody binding to immobilized G6PD compared with active G6PD competitor. The inhibition was more pronounced at high concentrations of denatured competitor than at low counterparts. We previously observed that nG6PD failed to fully compete for antibody binding sites at concentrations 500-fold higher than at that of immobilized G6PD. The present study suggests that our anti-G6PD serum raised against nG6PD has high cross-reactivity with denatured G6PD in competitive ELISA. Our results imply that denatured G6PD by freeze-thawing has similar epitope density and conformation to SDS- or Triton-G6PD. Furthermore, SDS- and Triton-G6PD antigens appear to have similar conformation as demonstrated by its equally high affinity for antibody binding. In contrast to our finding, Burks *et al.* (1992) found that thermal denaturation of peanut and soybean proteins did not affect antibody binding in ELISA. Koch *et al.* (1996) observed that antiserum raised against native ovalbumin had poor cross-reactivity with denatured ovalbumin in ELISA. The reason for the high cross-reactivity of our antiserum with denatured G6PD is not clear yet. However, it is possible that since the Freund's incomplete adjuvant contains oil, which may denature proteins, the treatment of antigen with the adjuvant for the immunization might have caused exposure of hydrophobic sites on the antigen, resulting in more antigenic sites on treated antigen than on native antigen, so that antiserum raised against the treated antigen could recognize both native and denatured antigens and react to treated antigen better than to native ones in competitive ELISA. Our antibody to G6PD is very specific, 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 in liver homogenates of rats under different dietary conditions with high specificity (Morikawa *et al.*, 1984; Tomlinson *et al.*, 1988; Kim *et al.*, 1990; Kim *et al.*, 1991). Those results provide the evidence that the anti-G6PD serum has high specificity for G6PD. The ineffective competition between immobilized and soluble nG6PD antigens for antibody binding in ELISA, observed in our previous and present studies, might have been in part due to the conformational changes and denaturation of immobilized G6PD 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; Butler *et al.*, 1997). Attempts have been made to overcome the denaturation tendency by many investigators (Smith and Wilson, 1986; Jemmerson, 1987; Kenett, 1988; Schwab and Bosshard, 1992; Houen and Koch,1997). We conclude that physically or chemically denatured G6PD can be recognized effectively by anti-G6PD antibodies raised against nG6PD and that the treatment of G6PD with detergent can increase the sensitivity of competitive ELISA under the present assay conditions. It may be inferred, from these results, that the adjuvant added to native antigen to elicit antibodies somewhat exposes hydrophobic sites, leading to more antigenic sites on treated antigen than on native ones, so that the antiserum produced recognizes denatured antigen better than native ones.

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