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Complexing of Glucose Oxidase with Anti-Glucose Oxidase Antibodies or the $F(ab)'_2$/F(ab)′ Fragments Derived Therefrom Protects Both the Enzyme and Antibody/Antibody Fragments against Glycation

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Abstract—Incubation of *Aspergillus niger* glucose oxidase with glucose, fructose, or ribose results in remarkable inactivation of the enzyme. Glucose oxidase incubated with the sugars migrated as a diffuse band of low intensity and silver stained poorly after SDS-PAGE. Purified anti-glucose oxidase antibodies and $F(ab)'_2$ or F(ab)′ derived therefrom were effective in restricting the inactivation of the enzyme induced by the sugars, providing up to 90% protection. The sugars also caused remarkable changes in the electrophoretic behavior of anti-glucose oxidase antibodies and the fragments, but complexing with glucose oxidase restricted the changes both in the enzyme and the antibody/antibody fragments.

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It is now well recognized that long-term exposure of proteins to sugars results in a sequence of non-enzymatic transformations collectively described as glycation [1]. The reaction involves attachment of the aldehyde function of non-cyclic sugars to protein amino groups to form a reversible Schiff base, which undergoes rearrangement to form relatively stable Amadori products. The Amadori products, through a series of oxidation and dehydration reactions, are transformed into a variety of carbonyl compounds that act as propagators of the reaction and further react with the free amino groups of proteins to yield advanced glycation end-products (AGEs) [2]. The variables that regulate protein glycation *in vivo* are the concentrations of sugars and protein, the half-life of the protein, its reactivity in terms of accessible amino groups, and cellular permeability to the sugars [3, 4].

Glycation is a major *in vivo* source of reactive oxygen and carbonyl species [5]. The cytotoxicity of glycation is mainly the result of modification of specific properties of proteins and induction of cross-linkage, aggregation, and precipitation. The glycation-induced alterations interfere with normal functioning of some proteins, especially of those with long half-lives, and manifest themselves in a variety of progressive diseases of aging. These include vascular disease, kidney disease, stiffness of joints, and Alzheimer's dementia, and the complications are exacerbated in uncontrolled diabetes [6]. Proteins like hemoglobin, albumin, collagen, etc. have been identified as major targets of AGE modification [6]. Increasingly being recognized is the glycation of immunoglobulins [7-12] resulting in their functional modification such as decreased protein A binding and complement fixation [9] as well as decreased antigen binding [10-12]. Some enzymes also undergo glycation-induced loss of biological activity. These include malate dehydrogenase [13], glutathione reductase [14], and glyceraldehyde-3-phosphate dehydrogenase [15]. Exposure of Cu,Zn-SOD (superoxide dismutase) to sugars as well as methylglyoxal results in its covalent crosslinking, fragmentation, loss of enzymatic activity, and release of copper ions [16].

It is now accepted that glycation and the resulting AGE formation play an important role in pathology of a variety of diabetes-related complications, and restriction

Abbreviations: AGEs) advanced glycation end-products; DEAE cellulose) diethylaminoethyl cellulose; DNPH) dinitrophenylhydrazine; IgG) immunoglobulin G; TCA) trichloroacetic acid.

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of glycation seems to alleviate the resulting damage [17]. A number of strategies for protection against glycation are being currently explored with varying degrees of success, including the use of inhibitors of glycation, protein crosslinking, and AGE formation, as well as crosslink breakers [18, 19]. Inhibitors of glycation like aspirin modify amino groups of proteins and prevent attachment of sugars [20, 21]. Some like aminoguanidine react with sugars, diverting them from Maillard reactions on proteins [22]. The crosslink breakers, on the other hand, reverse the connections between protein molecules [13].

The protective role of some chaperones against glycation-induced inactivation and loss of antigenicity of some enzymes has also been reported [14, 23]. Molecular chaperones are known not only to assist protein folding, but also stabilize proteins against denaturation and prevent aggregation [24, 25]. Some antibodies have also been shown to act like chaperones facilitating folding and preventing aggregation of protein antigens [26]. More recently we have demonstrated that anti-SOD antibodies and monomeric/dimeric F(ab)$'_2$/F(ab)′ derived therefrom provide remarkable protection against inactivation of the enzyme induced by various reducing sugars [27] and more reactive α-oxoaldehydes, glyoxal [28] and methylglyoxal [29].

Since antibodies can be raised against any protein, it was envisaged that they could be used as potential anti-glycation agents. Antibodies themselves undergo various glycation-induced alterations including loss of ability to bind to the antigen [12]; it was therefore considered of interest to explore the susceptibility of antibodies complexed with antigenic enzyme to glycation-induced alterations. The objective of the present study was to evaluate the possible protective role of anti-enzyme antibodies and antibody fragments derived therefrom against sugar-induced inactivation of glucose oxidase. It has been shown that intact anti-glucose oxidase antibodies, F(ab)$'_2$, and F(ab)′ restrict the enzyme inactivation as well as other glycation induced alterations in glucose oxidase and the antibody molecules.

MATERIALS AND METHODS

Glucose oxidase isolated from *Aspergillus niger*, DEAE-cellulose, guanidine-HCl, and dinitrophenylhydrazine (DNPH) were purchased from Sigma (USA). Reagents used for electrophoresis and other chemicals including glucose, fructose, ribose, and trichloroacetic acid (TCA) were obtained from SRL Chemicals (India). Other chemicals and reagents used in the study were of analytical grade.

Immunization of rabbits and IgG purification. Anti-glucose oxidase antibodies were raised in rabbits following the standard protocol using Freund's complete/incomplete adjuvants. The animals were immunized by injecting subcutaneously a glucose oxidase preparation (200 μg) in 20 mM sodium-phosphate buffer emulsified with Freund's adjuvant. After resting the animals for 21 days, they were boosted with weekly injections of 100 μg of glucose oxidase along with the Freund's incomplete adjuvant for three consecutive weeks. The animals were finally bled through a marginal ear vein; the serum was separated and preserved at −20°C. Formation of antibodies was monitored by Ouchterlony double immunodiffusion and direct ELISA [30, 31]. IgGs were purified by ammonium sulfate precipitation followed by ion-exchange chromatography on DEAE cellulose [32].

Preparation and purification of F(ab)$'_2$. IgG purified from antiserum was digested with pepsin to obtain F(ab)$'_2$ fragments. Pepsin (2 mg) was dissolved in 1 ml of 20 mM sodium-acetate buffer, pH 4.5. IgG dialyzed against the same buffer was then mixed with the enzyme solution to give an enzyme/IgG ratio of 1 : 100, and the mixture was incubated at 37°C for 6 h. The reaction was terminated by the addition of solid Tris salt that increased the pH of the reaction mixture to 8.0. The reaction mixture was then dialyzed against 50 mM Tris-HCl, 100 mM NaCl, pH 7.5, buffer. IDA-Sepharose was washed with 50 mM EDTA and 500 mM NaCl, pH 8.0. Copper chloride solution (20 mM) was used to load the matrix with copper ions. The matrix was then washed with the operating buffer (50 mM Tris-HCl, 100 mM NaCl, pH 7.5) and mixed with the dialyzed extract. After incubation for 1 h at room temperature, the unbound F(ab)$'_2$ was collected by centrifugation at 1000*g* [33]. The fragments were then characterized by SDS-PAGE.

Preparation and purification of F(ab)′. F(ab)′ monomers were prepared using a kit supplied by Pierce Co. (USA) following the manufacturer's protocol. IgG (10 mg/ml) purified from the antiserum was dialyzed against 100 mM sodium phosphate buffer, pH 7.0. Cysteine-HCl (1 mg/100 mg IgG), EDTA (0.5 mg/10 mg), and papain (1 mg/10 mg IgG) were added to the dialyzed IgG and the mixture incubated for 4 h at 37°C. The digest was rapidly frozen to inhibit further digestion and was later dialyzed overnight at 4°C against 10 mM sodium phosphate buffer, pH 8.0. DEAE-cellulose was regenerated and equilibrated with 10 mM sodium phosphate buffer, pH 8.0. The dialyzed digest was applied to the column and a gradient of phosphate buffer was run from 0.01-0.3 M at pH 8.0. The first peak to emerge was predominantly that of F(ab)′ followed by the peak containing the Fc fragment. The F(ab)′ was then characterized by SDS-PAGE.

Incubation of glucose oxidase with sugars. To investigate the effects of exposure to the sugars, glucose oxidase (1 mg/ml) was incubated with 100 mM glucose, fructose, or ribose in 20 mM sodium phosphate buffer, pH 7.2. Where indicated 10-fold molar excess (relative to the enzyme) of anti-glucose oxidase-IgG, F(ab)′, or F(ab)$'_2$ was included. All the solutions in these experiments were prefiltered through a 0.2-μm pore-size Millipore (USA) filter, and the incubations were performed in autoclaved

tubes under septic conditions. No bacterial growth was detectable during the period of incubation. Glucose oxidase incubated without either sugar under the same conditions served as control [34]. Glucose oxidase activity was determined spectrophotometrically using the peroxidase–*o*-dianisidine hydrochloride system [35].

SDS-PAGE. SDS-PAGE of the samples was carried out in a slab gel electrophoresis apparatus manufactured by Biotech (India) as described earlier [36]. A concentrated stock solution of 30% acrylamide and 0.8% bisacrylamide in 1 M Tris-HCl, pH 8.8, with 10% SDS was used (10% resolving gel). The gels were silver stained for visualization of protein bands [37].

Determination of protein bound carbonyl groups. Glucose oxidase-bound carbonyl groups were estimated as follows; briefly, a 200 µl aliquot (containing 0.1 mg of protein) was mixed with 400 µl of 7 mM DNPH in 2 M HCl. The mixtures were run in duplicate, and the control protein samples were devoid of DNPH. After incubation for 1 h at room temperature, the DNPH-hydrazones were precipitated by adding 500 µl of TCA (4% w/v) and centrifuged for 5 min at 14,000*g*, and the pellet was dispersed in ethanol–ethyl acetate (1 : 1 v/v) to remove unreacted DNPH and centrifuged. After four such washes, the pellet was resuspended in 0.6 ml of 6 M guanidine-HCl solution in 20 mM phosphate buffer, already adjusted to pH 2.3 with trifluoroacetic acid. The hydrazones were dissolved completely only by freezing overnight at –20°C and thawing. From the solution, a 200-µl aliquot was taken into a microplate, and the absorbance was measured at 379 nm by a microplate reader. The results are expressed as nanomoles of carbonyl per mg of sample protein using a $\varepsilon_{379\ nm} = 22{,}000\ M^{-1}\cdot cm^{-1}$ [38].

RESULTS AND DISCUSSION

***In vitro* glycation of glucose oxidase.** *Electrophoretic analysis*. *Aspergillus niger* glucose oxidase was incubated with glucose, fructose, or ribose for up to 8 days and analyzed by SDS-PAGE in the presence of the thiol reductant β-mercaptoethanol as detailed in the text. Glucose oxidase not exposed to the sugars migrated as a single band (180 kD), but on incubation with the sugars a clear alteration in electrophoretic behavior was evident. This included a time-dependent broadening of the protein band and decrease in band intensity. The sugar-induced alterations, however, appeared most prominent when the enzyme was incubated with ribose followed by fructose and glucose (Fig. 1). Glycation of proteins has been shown earlier to result in chemical modification, degradation, and/or crosslinking of protein [39]. The observed decrease in the band intensity apparently results from such effects of sugars [40, 41]. Small peptides were not seen in the lanes containing enzyme incubated with the sugars. A decrease in protein mobility along with the presence of material that failed to enter the gel and could be silver stained on top of the gel was observed. The alterations were prominent in ribose treated samples, followed by those exposed to fructose and glucose, apparently due to their relative reactivity in glycation reactions [16, 42, 43].

Carbonyl group content of glucose oxidase incubated with various reducing sugars

Sugar	Carbonyl groups, nmol/mg*
Control (without sugar)	2.2 ± 0.09
Glucose	12.2 ± 0.11
Fructose	22.6 ± 0.10
Ribose	28.8 ± 0.17

* All the values are mean ± S.D. for three experiments performed in duplicate.

Protein bound carbonyl groups. The carbonyl content of glucose oxidase incubated with ribose, fructose, or glucose is shown in the table. As evident, ribose and fructose caused the formation of far more carbonyl groups in the enzyme as compared to glucose. As observed earlier with other proteins [44], glucose oxidase incubated with neither sugar contains a very small amount of carbonyl groups. Carbonyl content is considered a reliable indica-

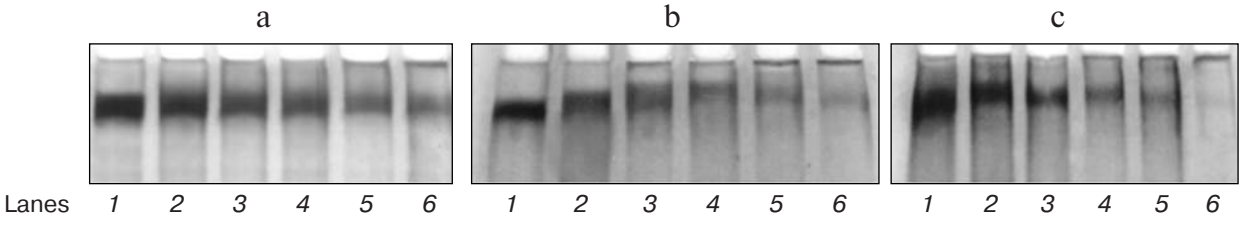

Fig. 1. SDS-PAGE of glucose oxidase with reducing sugars. Glucose oxidase (1 mg/ml) was separately incubated with 100 mM of various sugars for up to 8 days in 20 mM phosphate buffer, pH 7.2, at 37°C. Lanes: *1*) glucose oxidase incubated without sugar for 8 days; *2-6*) the enzyme incubated for 1, 3, 5, 6, and 8 days, respectively, with glucose (a), fructose (b), or ribose (c). The samples were subjected to SDS-PAGE in the presence of β-mercaptoethanol. Samples containing 12 µg protein were loaded in each lane, and the gels were silver-stained for visualization of protein bands.

tor of and is by far the most commonly used marker of protein oxidation and glycation, but the yield of carbonyl group formation varies from protein to protein depending on the number/location of free amino groups present in the respective protein [45, 46]. The relative reactivities of ribose, fructose, and glucose in generating carbonyls substantiate the effects described in Fig. 1.

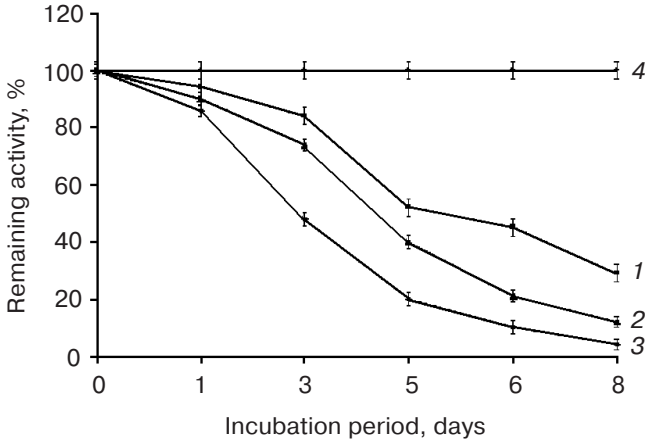

Fig. 2. Enzyme activity of glucose oxidase incubated with reducing sugars. Glucose oxidase (1 mg/ml) was incubated with 100 mM glucose (*1*), fructose (*2*), or ribose (*3*) separately for 8 days in 20 mM phosphate buffer, pH 7.2, at 37°C. The control sample (*4*) contained glucose oxidase incubated without sugar up to 8 days. Glucose oxidase activity was determined as described in the text. Each value represents the average for three independent experiments performed in duplicate.

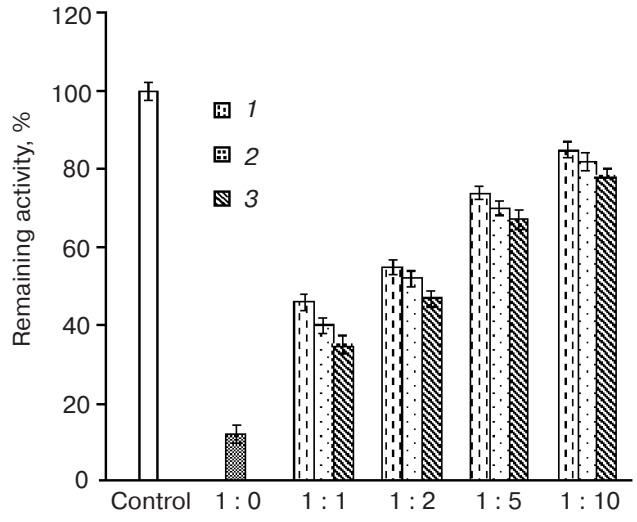

Fig. 3. Protection by anti-glucose oxidase IgG as well as F(ab)′ and F(ab)′$_2$ derived therefrom against fructose-induced inactivation of glucose oxidase. Anti-glucose oxidase IgG (*1*), F(ab)′$_2$ (*2*), and F(ab)′ (*3*) in molar ratio between 1 : 1 and 1 : 10 were included in incubation mixtures containing glucose oxidase in 20 mM phosphate buffer, pH 7.2, with 100 mM fructose for up to 8 days at 37°C. The control sample (white column) contained glucose oxidase incubated alone (without any sugar or antibody/antibody fragments). Glucose oxidase activity was determined as described in the text. Each value represents the average for three independent experiments performed in duplicate.

Effect of incubation on the activity of glucose oxidase. The effect of incubation with sugars on the activity of glucose oxidase is summarized in Fig. 2. In agreement with previous observations [16, 47], incubation with the sugars at 37°C resulted in marked decrease in the activity of the enzyme. Ribose caused maximum inactivation, followed by fructose, and glucose was least reactive in this regard. Loss of enzyme activity at the end of day 8 was 96, 88, and 71%, respectively, in the samples incubated with ribose, fructose, and glucose. Glucose oxidase incubated in the absence of the sugar retained essentially complete activity and exhibited little alteration in electrophoretic behavior under the conditions employed. Several enzymes including superoxide dismutase and malate dehydrogenase undergo glycation-induced loss of biological activity [13, 26, 48] but rates of inactivation of various enzymes differ significantly.

Effect of anti-glucose oxidase IgG, F(ab)′$_2$, and F(ab)′ on the sugar-induced inactivation of glucose oxidase. Figure 3 shows that the concentration of antibodies and antibody fragments in the incubation mixture containing fructose causes a proportionate increase in the protection; when the antibody/enzyme ratio was increased to 10, the enzyme retained about 85% of its activity. Therefore, for the investigation of the protective effect of antibodies and the fragments against glycation-induced alterations, glucose oxidase was incubated with 10-fold molar excess of anti-glucose oxidase IgG, F(ab)′$_2$, and F(ab)′ in the absence and presence of ribose, fructose, and glucose. As shown in Fig. 4, glucose oxidase incubated with sugars in the absence of antibodies or antibody fragments showed a marked decrease in activity. Ribose was most inhibitory, resulting in 96% loss in activity, while fructose and glucose caused a loss of 88 and 71% activity, respectively. Anti-glucose oxidase antibodies and the F(ab)′/F(ab)′$_2$ exerted remarkable protective effect against enzyme inactivation induced by the sugars. Under the conditions used, protection afforded by antibodies, F(ab)′$_2$, and F(ab)′, respectively, was 90, 87, and 80% for glucose, 85, 82, and 78% for fructose, and 80, 75, and 70% for ribose. The IgGs used in the present study were obtained by purification on a DEAE-cellulose column and hence comprise the entire IgG class. Although the titer of the antibodies in the immune serum was very high, it could not be ascertained as to what percentage of the IgGs was glucose oxidase-specific. Protection against the sugar-induced alterations was specific since nonspecific IgGs were not protective (result not shown).

SDS-PAGE of glucose oxidase and antibody/antibody fragment complexes incubated with sugars. Reports from other workers [10, 11] and our own studies [12] suggest that antibodies undergo sugar-induced alterations including loss of ability to bind to antigens. Experiments were therefore performed to investigate whether antibody com-

plexed with glucose oxidase also undergoes such modifications. As evident from Fig. 5, the SDS-PAGE of antibody–glucose oxidase complex did not change significantly on incubation with glucose, fructose, and ribose (Fig. 5a, lanes *5* and *6*). In contrast, glucose oxidase incubated with the sugars showed marked variations in electrophoretic behavior described earlier. Remarkable alterations were also observed in the behavior of the antibody. As shown in the figure, a clear decrease was observed in the intensity of low molecular weight and high molecular weight bands, with the former almost disappearing in samples incubated with fructose and ribose. Formation of aggregated material could be stained at or close to the top of the gel. Protection against the sugar-induced alterations was also observed when glucose oxidase was complexed with F(ab)′ or F(ab)′$_2$ and incubated with sugars (Fig. 5, b and c). Both F(ab)′ and F(ab)′$_2$ migrated as a band corresponding to molecular weight of 50,000, close to that of the low molecular weight band of the antibody. Incubation of the fragments with fructose and ribose resulted in remarkable decrease in intensity, diffusion of the band, and formation of aggregate especially on treatment with ribose (Fig. 5, b and c, lanes *4*). A comparison of lanes *5*

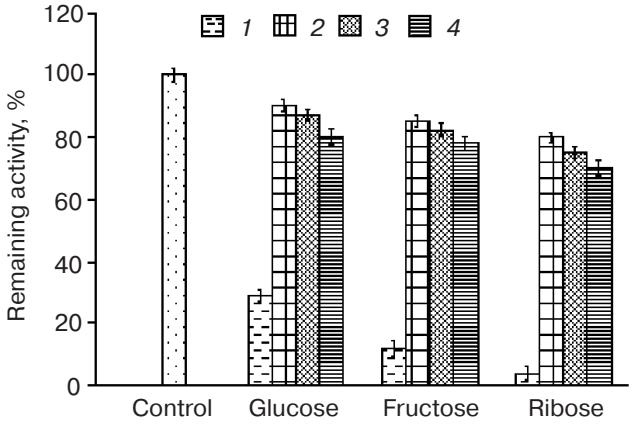

Fig. 4. Protection by anti-glucose oxidase IgG as well as F(ab)′$_2$ and F(ab)′ derived therefrom against the loss of enzyme activity induced by reducing sugars. Glucose oxidase was incubated with 0.1 M of reducing sugars (*1*) and a 10-fold molar excess of IgG (*2*), F(ab)′$_2$ (*3*), or F(ab)′ (*4*) for 8 days at 37°C, and glucose oxidase activity was determined. The control sample contained glucose oxidase incubated alone (without any sugar or antibody/antibody fragments). Glucose oxidase activity was determined as described in the text. Each value represents the average for three independent experiments performed in duplicate.

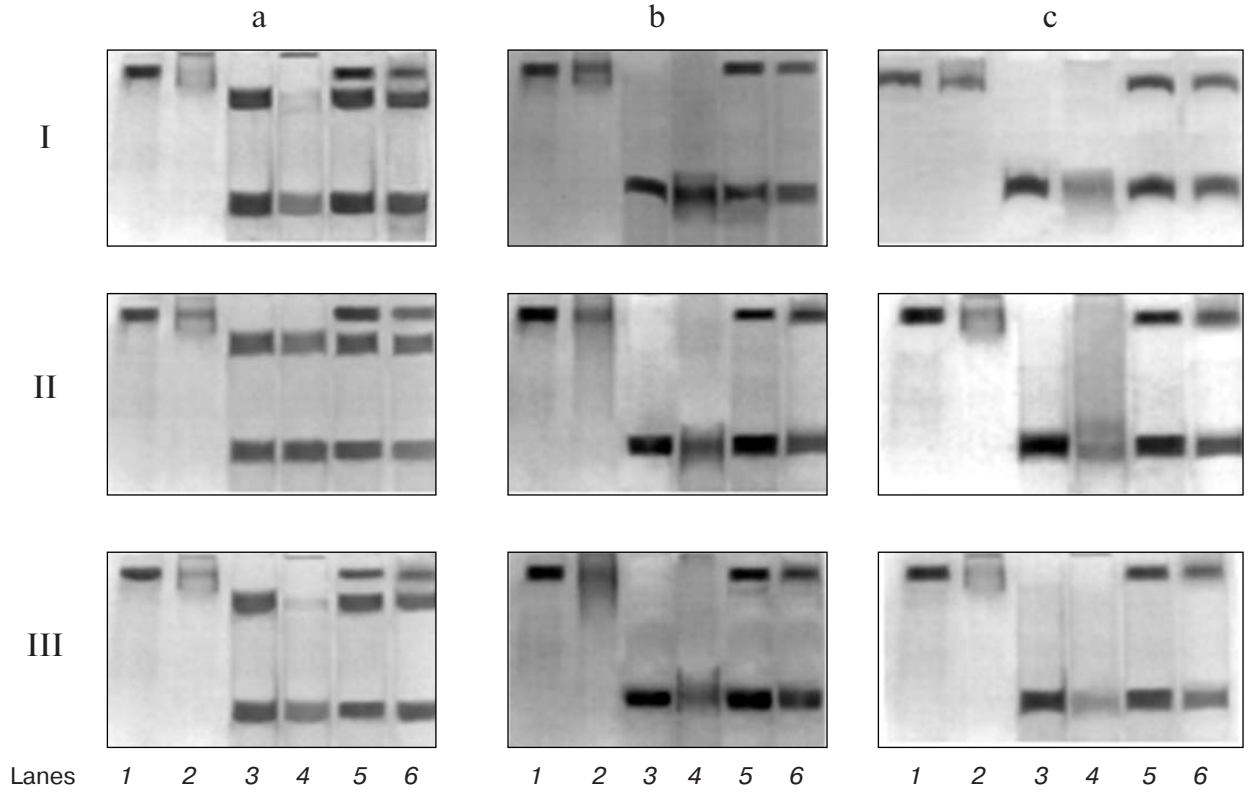

Fig. 5. Protection by anti-glucose oxidase IgG (a) as well as F(ab)′$_2$ (b) or F(ab)′ (c) derived therefrom against inactivation of glucose oxidase induced by reducing sugars. Glucose oxidase was incubated under conditions described in the legend to Fig. 1 with 10-fold molar excess of antibody/antibody fragments and subjected to SDS-PAGE in the presence of β-mercaptoethanol. a) Lanes *1*, *3*, and *5* contain glucose oxidase, anti-glucose oxidase IgG, or the mixture of glucose oxidase and anti-glucose oxidase IgG in the absence of sugars; lanes *2*, *4*, and *6* contain the above listed incubated with glucose (aI), fructose (aII), or ribose (aIII). b, c) Samples were loaded in gel lanes similarly to that in panel (a) except that F(ab)′$_2$ and F(ab)′ fragments replaced the IgG, respectively.

and *6* (in Fig. 5, b and c) suggests that both the fragments and antibody resisted the sugar-induced alterations.

Figure 5 shows electrophoresis of the samples on SDS-PAGE in the presence of the thiol reductant β-mercaptoethanol. The band intensity of the sugar-exposed glucose oxidase–antibody complex or that with F(ab)′ or $F(ab)'_2$ were found to be comparable with those of untreated samples. Glucose oxidase and its antibody or fragments separately incubated with sugar showed appreciable decrease in band intensity along with the presence of material that failed to enter the gels. Intermolecular crosslinking of the enzyme, the antibody, or the antibody fragments can result in the formation of aggregates, which may be too large to enter the gel pores and hence may not be detected on the gels. In these experiments, also ribose appeared most reactive compared to other sugars. Control samples, i.e. samples incubated in the absence of the sugar, exhibited little or no fragmentation under the conditions employed. However, it should be noted that the level of protection with intact antibodies, $F(ab)'_2$, and F(ab)′ appeared comparable.

It is now recognized that glycation contributes remarkably to the complications associated with diabetes and related disorders. A number of agents that prevent or restrict glycation reactions are therefore being evaluated for therapy against such disorders. These include those that compete with amino groups of proteins for reaction with the sugars, bind to protein and prevent access to amino groups, remove the open chain form of the sugar, or restrict their further transformation into AGEs [19]. α-Crystalline occurring in the eye lens and various other tissues [49] has also been shown to protect various enzymes against heat-induced inactivation/aggregation [50] and more recently against glycation-induced alterations [51-53], suggesting that strengthening of the crystalline-mediated stabilization of protein could be an effective strategy in combating glycation-related complications.

Several antibodies have been shown to also act as chaperones and facilitate the refolding and/or aggregation of proteins and enzymes [26]. It has been shown from this laboratory that anti-Cu,Zn-superoxide antibodies protect the enzyme against inactivation induced by some sugars [27]. The antibodies also protected the enzyme from undergoing inactivation and aggregation induced by glyoxal [26] and methylglyoxal [28]. Antibodies may have an advantage as anti-glycation agents since specific antibodies can be readily raised against any enzyme. Also, the high association constant binding of antibodies to protein antigen results in marked lowering of the free energy and stabilization of the antigen. Hydrogen exchange rate investigations on lysozyme and cytochrome *c* amide group suggested that in the complex the exchange rates were remarkably low not only at the point of contact between the antigen and the antibody, but also in regions far removed from the epitope recognized by the antibody [54, 55]. Several enzymes complexed with monoclonal/polyclonal antibodies exhibit enhanced stability against various forms of inactivation [56]. Our earlier studies have shown that antibodies raised against the liable region of RNase were more protective than those raised against the N-terminal peptide [57]. The labile region-specific antibodies also improved the thermal stability of a mutant RNase in which the labile region contained an altered amino acid [58]. Studies with anti-lysozyme antibodies, however, suggest that binding of a monoclonal antibody raised against the native enzyme improves the thermal stability of the enzyme antigen even if they do not recognize epitopes located close to the mutation site. The D67H mutation of human lysozyme related to systemic amylodosis undergoes partial unfolding and aggregation with a melting temperature 10°C lower than that of the native enzyme [59]. Binding of c-Ab-HUL6 increased the melting temperature of the enzyme by 15°C, although the residue whose mutation leads to the destabilization and aggregation does not make any contact with the antibody [60]. It therefore may not be essential to identify the specific epitope of the target protein for raising antibodies, although such a selection might improve the effectiveness of the antibody.

Bivalent antibodies have the inherent risk of crosslinking and aggregation the protein and may not be suitable for immunotherapy directed at the soluble proteins. The protection afforded by the monomeric F(ab)′ fragment suggests bivalent antibodies are not essential for protection against glycation (Figs. 4 and 5). In an earlier study we demonstrated that binding of F(ab)′ monomer to bromelain improves the stability of the enzyme against denaturation [61].

Protection observed against the glycation-induced alterations in the antibody [12] and antibody fragment when these are present as a complex with the enzyme is interesting (Fig. 5). α-Crystalline also undergoes glycation-mediated loss of chaperone activity and ability to protect enzymes against glycation [62]. Unlike the antibodies, the binding of the chaperones to target protein is readily reversible, increasing the susceptibility of both the chaperone and the target protein.

Immunotherapy for the prevention of aggregation of β-amyloid peptide in Alzheimer's disease [63] and prion protein has been envisaged [64]. This study indicates the potential of immunotherapy against glycation-induced alterations in proteins.

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