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ORIGINAL ARTICLE

Glycation promotes the formation of genotoxic aggregates in glucose oxidase

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Abstract This study investigates the effect of pentose sugars (ribose and arabinose) on the structural and chemical modifications in glucose oxidase (GOD) as well as genotoxic potential of this modified form. An intermediate state of GOD was observed on day 12 of incubation having CD minima peaks at 222 and 208 nm, characteristic of α-helix and a few tertiary contacts with altered tryptophan environment and high ANS binding. All these features indicate the existence of molten globule state of the GOD with ribose and arabinose on day 12. GOD on day 15 of incubation forms β structures as revealed by CD and FTIR which may be due to its aggregation. Furthermore, GOD on day 15 showed a remarkable increase in Thioflavin T fluorescence at 485 nm. Comet assay of lymphocytes and plasmid nicking assay in presence of glycated GOD show DNA damage which confirmed the genotoxicity of advance glycated end products. Hence, our study suggests that glycated GOD results in the formation of aggregates and the advanced glycated end products, which are genotoxic in nature.

Keywords AGEs · Aggregation · CD · Comet assay · Fluorescence · Molten globule · Plasmid nicking assay

Introduction

Over the past few years, there has been a growing interest on the effect of reducing sugars on protein structural properties (Corzo-Martinez et al. 2010). The non-enzymatic interaction between reducing sugars with amino groups of the lysine residues in proteins is generally known as Maillard reaction and it is considered to be extremely important in food science (Myung-Chan et al. 2010). Protein crosslinking by glycation results in the formation of detergent insoluble and protease-resistant aggregates (Usha et al. 2010). The interaction comprises a complex network of reactions that results into the formation of both large protein aggregates and low-molecular-weight products that are believed to impart the various flavour, aroma and colour characteristics found in foods. Globule-like protein aggregations (pro-amyloid fibrils) have been documented to be toxic to neurons (Sanghera et al. 2008). Aggregation and/or glycation may seriously affect protein structure, function and stability.

The formation of molten globule-like state has been reported during progression of glycation reaction in vitro (Chen et al. 2010). The final step consists of crosslink formation between products in which heterogeneous structures called advanced glycation end products (AGEs) (Brouwers et al. 2011) are formed. Subsequent reactions (e.g., dehydration, oxidation, condensation) result in the irreversible formation of a heterogenous group of compound (Schleicher et al. 2001). AGEs were found to induce DNA damage in pig kidney cells (Stopper et al. 2003). Also report on the non-enzymatic reaction of glucose in Alzheimer's disease, to form AGEs on long-lived protein deposits, which induce oxidative stress and subsequently disturb glucose metabolism has been established (Munch et al. 1998). Glycation alters the biological activity of proteins and their degradation processes. However, the characteristics and cytotoxicity of molten globule-like protein states induced by glycation have not been clarified yet. Hence, we are the first one to report that glycation of

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Glucose oxidase (GOD) with ribose and arabinose can promote protein misfolding and aggregation.

GOD (β-D-glucose: oxygen-1-oxidoreductase, EC 1.1.3.4) is a homodimeric enzyme (Kriechbaum et al. 1989). It is a hydrogen peroxide-generating flavoprotein, catalysing the oxidation of β-D-glucose to D-glucono-1, 5-lactone. It is used in the food industry for the removal of glucose from powdered eggs, as a source of hydrogen peroxide in food preservation, for gluconic acid production, and in the production of beer and soft drinks in which its reaction serves an antioxidant function (Grassino et al. 2011). It is also used extensively for the quantitative determination of D-glucose in blood, food, fermentation products and tears (Huanfen et al. 2011).

For better understanding of the protein–sugar interactions, we have attempted to study the relationship between the enzyme in its native state and the structural properties of the whole GOD molecule in presence of sugars. Glycation of protein by pentoses also leads to the formation of AGEs and cross-linking products (Lee et al. 1999). In our present study, we have investigated the pentose sugars namely, ribose and arabinose as they are 10–100 times more reactive with protein than glucose, so that their lower concentrations can cause glycation. Ribose is a naturally occurring sugar in human body and arabinose is needed from the plants. A great deal of work has been carried out on glycation of proteins with glucose but few research groups have attempted to monitor the effect of pentose sugars on the structure of GOD and its genotoxicity assessment. Therefore, to specify the contribution of structure, our investigations used infrared absorption spectroscopy, tryptophan fluorescence and CD of GOD.

Materials and methods

Chemicals

GOD isolated from *Aspergillus niger* was purchased from Sigma Aldrich chemicals Co. (USA). Reagents used for electrophoresis and other chemicals including ribose and arabinose were obtained from SRL Chemicals (India). Other chemicals and reagents used in the study were of analytical grade.

Glycation of GOD

AGEs were prepared by incubating 2 mg/ml GOD in sodium azide (0.02%), 20 mM phosphate buffer (pH 7.2) using ribose (0.2 M) and arabinose (0.2 M) as modifiers for (3–15 days) at 37°C. GOD without any sugar was incubated under the similar conditions and was used as control. Buffers (20 mM phosphate buffer, 0.1 M sodium tetra borate buffer) were used as such and all glassware were autoclaved (121°C at 15 psi for 15 min) prior to use to inactivate proteases. All solutions were filtered by 0.2 µm sterile filters under aseptic conditions before their use. After incubation, AGEs and their control were extensively dialysed against phosphate buffer saline at 4°C that were later stored in aliquot at −20°C till further analysis. Protein concentration was determined by Lowry et al. (1951).

Determination of GOD activity

The activity of glycated GOD was measured according to Worthington method (1988). The reaction kinetics was determined by the increase in absorbance at 460 nm resulting from the oxidation of *o*-dianisidine through a peroxidase coupled system. The increase in absorbance at 460 nm was recorded and GOD activity was expressed in units of enzyme per ml.

Fluorescence studies

Fluorescence data were recorded with a RF-1501 spectrofluorophotometer (Shimadzu Co. Japan). Fluorescence spectra were recorded in a 10-mm pathlength quartz cell. Sample excitation was performed at 280 nm, while its emission spectrum was recorded in the range 300–400 nm. Protein concentration used for fluorescence studies was 0.625 µM. Fluorescence of maldondialdehyde-modified protein and pentosidine-like-fluorescence were also monitored by exciting the samples at 370 and 335 nm with emission at 440 and 400 nm, respectively (Hand et al. 2007; Yamamoto et al. 2002).

Circular dichroism (CD) studies

CD was measured with a JASCO J-815 spectropolarimeter calibrated with ammonium D-10-camphorsulfonate. Cells of path lengths 0.1 and 1 cm were used for scanning between 250–190 and 300–250 nm, respectively. Each spectrum was the average of three scans. CD analysis was carried out on GOD samples withdrawn from the reaction mixture under incubation for 3–15 days kept at 37°C. Protein concentrations used were 1.875 µM for far-UV and 3 µM for near-UV CD analysis in the presence of 20 mM phosphate buffer (pH 7.2).

Fourier transform infra red spectroscopy (FTIR)

FTIR spectra were recorded with an Interspec-2020 Fourier transform spectrometer in D_2O. Each spectrum was the average of six scans. Protein concentrations of the samples were typically 30 µM. The scanning wavenumber was chosen from 1500 to 1700 cm^{-1}.

Determination of free amino groups

The free amino groups present in treated GOD were measured by trinitrobenzene sulphonate (TNBS) method (Haynes et al. 1967). The absorbance was taken at 420 nm against a blank devoid of any glycated protein.

8-Anilino-1-naphthalene-sulphonic acid (ANS) fluorescence measurements

ANS binding was measured by fluorescence emission spectra with excitation at 380 nm and emission in the range of 400–600 nm. ANS concentration was taken 100 molar excess of protein concentration while protein concentration was taken in the vicinity of 4.5 μM (Matulis et al. 1999).

Thioflavin T (Th T) assay

Fluorescence spectra were recorded with a Shimadzu RF 540 spectrofluorophotometer in a 10-mm pathlength quartz cell. The excitation wavelength was 440 nm and the emission was recorded from 460 to 600 nm. Final concentration of protein in the sample was 2 μM while the concentration of Th T was 10 μM.

Plasmid nicking assay of glycated GOD

To assess the genotoxic effect, pUC19 DNA plasmid was incubated with glycated GOD and the extent of DNA damage was observed. Reaction mixture (30 μL) containing Tris–HCl buffer (pH 7.5, 10 mM), pUC19 DNA plasmid (0.5 μg) and 5 μL of glycated GOD was incubated for 3 h at 37°C. 10 μL of a solution containing 40 mM EDTA, 0.05% Bromophenol blue (tracking dye) and 50% (v/v) glycerol was added to the treated plasmid after incubation and was then subjected to electrophoresis in a submerged 1% agarose gel. Ethidium bromide stained gel was then viewed and photographed on a UV-transilluminator.

Comet assay (single cell gel electrophoresis) of the glycated GOD

Isolated lymphocytes were exposed to glycated GOD in a total reaction volume of 1.0 ml. Incubation was performed at 37°C for 1 h. After incubation, the reaction mixture was centrifuged at 716.8 g, the supernatant was discarded and pelleted lymphocytes were resuspended in 100 μL of PBS and processed further for Comet assay. Comet assay of glycated GOD was performed under alkaline conditions by the procedure of Singh et al. (1988) with slight modifications. Fully frosted microscopic slides pre-coated with 1.0% normal melting agarose (dissolved in Ca^{2+} and Mg^{2+} free PBS) were used at 50°C. Around 10,000 cells were mixed with 80 μL of 1.0% low melting point agarose to form a cell suspension and pipetted over the first layer and covered immediately by a cover slip. The slides were placed on a flat tray and kept on ice for 10 min to solidify the agarose. The cover slips were removed and a third layer of 0.5% low melting point agarose (80 μL) was kept. Cover slips were placed over it which was then allowed to solidify on ice for 5 min. The cover slips were removed and the slides were immersed in cold lysis buffer containing 2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10. 1% Triton X-100 was added before use for a minimum of 1 h at 4°C. After lysis, DNA was allowed to unwind for 30 min in alkaline electrophoretic solution (pH > 13) consisting of 300 mM NaOH and 1 mM EDTA. Electrophoresis was performed at 4°C in field strength of 0.7 V/cm and 300 mA current. The slides were then neutralized with cold 0.4 M Tris, pH 7.5, stained with 75 ml ethidium bromide (20 mg/ml) and covered with a cover slip. They were then placed in a humidified chamber to prevent drying of the gel and analysed on the next day. Slides were scored using an image analysis system (Comet 5.5; Kinetic Imaging, Liverpool, UK) attached to an Olympus (CX41) fluorescent microscope (Olympus Optical Co., Tokyo, Japan) and a COHU 4910-integrated CC camera (equipped with 510–560 nm excitation and 590 nm barrier filters) (COHU, San Diego, CA, USA). Comets were scored at 100X magnification. Images from 50 cells (25 from each replicate slide) were analyzed. The parameter taken to assess lymphocytes DNA damage was tail length (migration of DNA from their nucleus, μm) and was automatically generated by the Comet 5.5 image analysis system.

Statistics

Data have been expressed as mean ± standard deviation ($n = 3$). Statistical analysis was performed with one-way ANOVA software. Data were considered significant at $p < 0.05$.

Results

Effect of glycation on the activity of GOD

When GOD (2 mg/ml) was incubated with 200 mM ribose and 200 mM arabinose, separately, in 20 mM phosphate buffer with 0.02% sodium azide, pH 7.2 and 37°C for 15 days, there was a progressive decline in enzymatic activity (Fig. 1). After 15 days, the decrease in the activity from baseline for native GOD was ~30%, for 200 mM arabinose glycated GOD was 60.44% and for 200 mM ribose glycated GOD, it was 71.74%. Ribose glycated

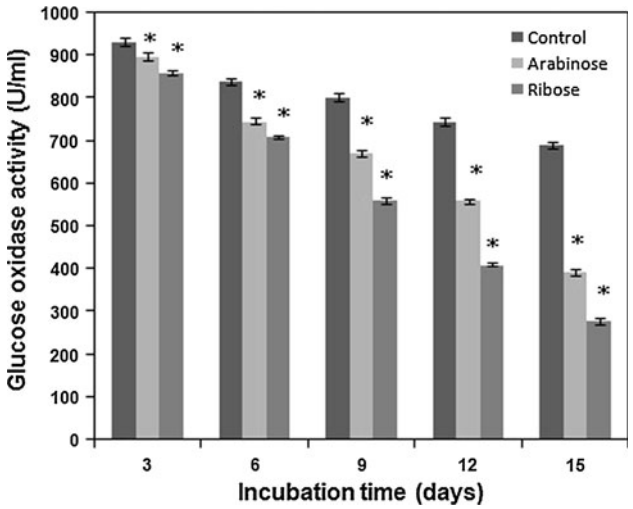

Fig. 1 *Bar graph* represents the activity of native GOD, arabinose and ribose glycated GOD. *Error bars* indicate the mean ± SD ($n = 3$). *Significance $p < 0.05$ with respect to control

GOD shows more decrement in the activity as compared with arabinose glycated GOD.

Fluorescence analysis

Figure 2a shows the relative fluorescence of GOD as the function of varying incubation time of GOD with arabinose and ribose. When 2 mg/ml GOD was incubated with 200 mM ribose and arabinose each separately in 20 mM phosphate buffer with 0.02% sodium azide, pH 7.2 and 37°C for 15 days, there was a progressive decrease in fluorescence intensity. The maximum decline was observed on day 15. The decrease in fluorescence was more prominent in ribose-incubated GOD. Fig. S1a and S1b (online resource) demonstrate changes in the microenvironment of aromatic amino acid residues of GOD on incubation with arabinose and ribose, respectively. In Fig. S1a, emission maximum at 340 nm was observed in native GOD (curve 1), but when GOD was incubated with arabinose for 3–15 days (curves 2–6) a decline in fluorescence intensity was observed. In comparison with day 3, there was a red shift of 10 nm on days 12 and 15 (curve 5 and 6). Fig. S1b also represents drop in fluorescence intensity in GOD incubated with ribose, from day 3 to day 15 (curve 2–6) in comparison with native (curve 1) accompanied by a red shift of 10 nm for days 12 and 15. Minimal fluorescence in both the cases was detected on day 15, indicating that there may be formation of aggregates. Ribose being a reactive sugar induces faster changes as compared with arabinose.

Figure 2b shows the bar graph at an excitation wavelength of 335 nm for pentosidine-like fluorescence whereas Fig. 3 represents the same for malondialdehyde-modified protein-like fluorescence at 370 nm. Both the graphs show an increase in fluorescence intensity in the presence of arabinose and ribose as compared with control. Their formation was observed from day 3 followed by days 6, 9, 12 and 15. With progression of incubation time, formation of pentosidine-like AGEs and malondialdehyde-modified protein was found to increase. GOD incubated with arabinose and ribose for 6 days showed similar formation of malondialdehyde-modified protein-like fluorescence, with further increase in fluorescence from days 6 to 15.

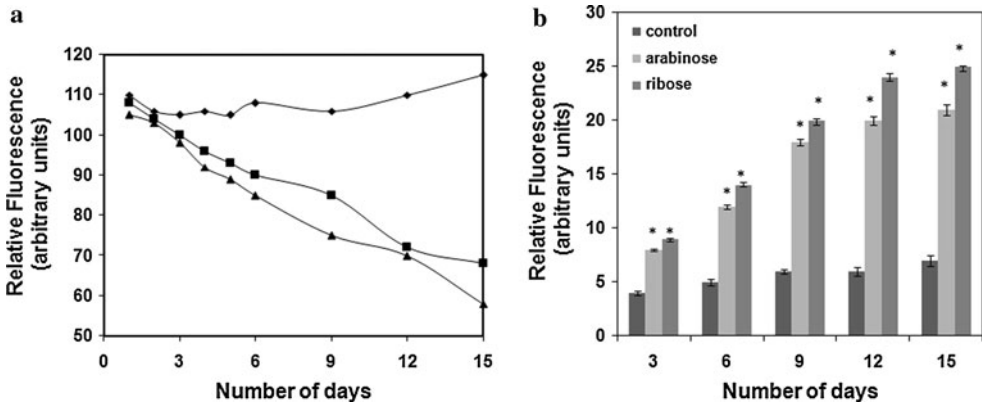

Fig. 2 **a** Relative fluorescence of native GOD [*filled diamond*], arabinose incubated GOD [*filled square*], and ribose incubated GOD [*filled triangle*]. The GOD concentration was 0.625 μM and the path length was 1 cm. The fluorescence was monitored at an excitation wavelength of 280 nm (Peaks achieved on respective days are plotted) with a slit width of 5 nm. All the data have been expressed in the mean ± SD ($n = 3$). *Significance $p < 0.05$ with respect to control. **b** *Bar graph* represents fluorescence intensity of pentosidine-like AGEs formation during the incubation of GOD with arabinose and ribose. The GOD concentration was 0.625 μM and the pathlength was 1 cm. The fluorescence was monitored at an excitation wavelength of 335 nm (Peaks achieved at respective days are plotted) with a slit width of 5 nm. *Error bars* indicate the mean ± SD ($n = 3$). *Significance $p < 0.05$ with respect to control

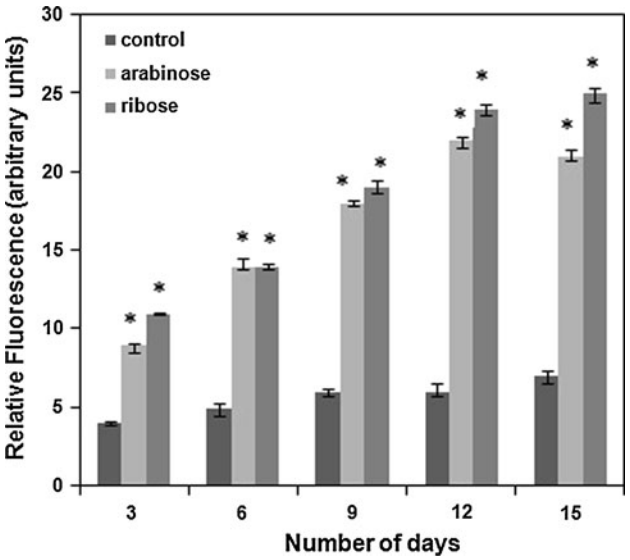

Fig. 3 *Bar graph* represents malondialdehyde-modified protein-like fluorescence formation during the incubation of GOD with arabinose and ribose. The GOD concentration was 0.625 μM and the path length was 1 cm. The fluorescence was monitored at an excitation wavelength of 370 nm (Peaks achieved on respective days are plotted) with a slit width of 5 nm. *Error bars* indicate the mean ± SD ($n = 3$). *Significance $p < 0.05$ with respect to control

CD studies

To monitor the changes in secondary structure of GOD (2 mg/ml) in the presence of 200 mM arabinose in 20 mM phosphate buffer with 0.02% sodium azide, pH 7.2 and 37°C, far-UV studies were carried out (Fig. 4a). In the far-UV region, the CD spectrum of native GOD illustrates the presence of substantial α-helical conformation (curve 1). There is a significant decline in negative elipticity on days 3, 6 and 9 (curve 2, 3 and 4) corresponding to native (curve 1). The glycation-induced transition monitored by elipticity at 208 and 222 nm showed the presence of the residual helical structure even on day 12 (curve 5), indicating the stability of the protein secondary structure. This type of change observed in the CD spectra on day 12 upon modification of GOD by arabinose suggests that there may be formation of molten globule like structures. With further increase in incubation time up to 15 days (curve 6), there is loss in α-helix structure and the appearance of minima peak at 217 nm which is indicative of formation of β-sheet conformation.

Figure 4b shows the far-UV CD spectra of native GOD (curve 1) and ribose (200 mM) incubated states of GOD (curves 2–6) in 20 mM phosphate buffer with 0.02% sodium azide, pH 7.2 and 37°C for different time period. GOD incubated for 3, 6 and 9 days (curve 2, 3 and 4) shows the presence of α-helix structure. Spectra of ribose glycated GOD on day 12 (curve 5) demonstrate the occurrence of residual helical structure; it may propose the molten globule state of GOD. Appearance of a prominent minimum at 217 nm for day 15 (curve 6) with the loss of the minima at 208 and 222 nm suggests the formation of β-sheet conformation in GOD.

Near-UV CD spectra of the native state of GOD (curve 1) showed a prominent positive CD band at 274 nm (Fig. 4c). There is a decrease in positive elipticity of GOD incubated with arabinose for different time period. GOD incubated for 6 (curve 2) and 12 days (curve 3) shows reduction in elipticity relative to curve 1. Interestingly, the near-UV CD spectra shows a similar pattern in intensity but less in magnitude as native state on day 12, suggesting regain of its tertiary contacts. In day 15 sample (curve 4), band at 274 nm vanishes indicating disruption of the native tertiary structure. Figure 4d shows the near-UV CD spectrum of ribose incubated GOD, displaying a prominent band at 274 nm that indicate asymmetric environment of its constituent aromatic amino acid residues. In case of GOD incubated for 12 days (curve 3), appearance of tertiary interactions might be due to mimicking of hydrophobic surfaces brought about by ribose. Loss of signals occurs in day 15 (curve 4) samples which may be due to disorder tertiary structure of GOD.

FTIR analysis

To detect the changes in secondary structure of GOD (2 mg/ml) in the presence of 200 mM arabinose in 20 mM phosphate buffer with 0.02% sodium azide, pH 7.2 and 37°C, FTIR analysis was performed. As depicted in Fig. 5a, wavenumber analyses between 1,500 and 1,700 cm^{-1} revealed a peak corresponding to the region of amide band protein (NH). The amide I region of the infrared spectrum of native GOD shows a maximum at about 1,617 cm^{-1} and a very intense shoulder around 1,530 cm^{-1} (curve 1). These two spectral features indicate the presence of both α-helical and β-type conformations (Susi et al. 1967). As the time of incubation of GOD with arabinose increases, peaks shift to lower frequencies on days 12 (curve 3) and 15 (curve 4). Loss in peak at 1,530 cm^{-1} and shift in peak at 1,617 cm^{-1} in 15 days (curve 4) incubated sample may be due to conversion of α-helix to β-sheet. Presence of extensive β-sheet structure indicates that there may be formation of aggregated species (Amani and Naeem 2011). Strikingly, this type of infrared spectrum has been shown to be characteristic of amyloid fibrils (Chiti et al. 1999).

Figures 5b shows the FTIR of native GOD (2 mg/ml) and GOD incubated with ribose (200 mM) for different days. The shift in peak wavenumber starts from day 12 (curve 3) of incubation. It suggests that the presence of ribose alters the enzyme's conformation. Loss in the peak at 1,530 takes place on day 12 (curve 3) confirming loss of α-helical structure. Sample incubated for 15 days (curve 4)

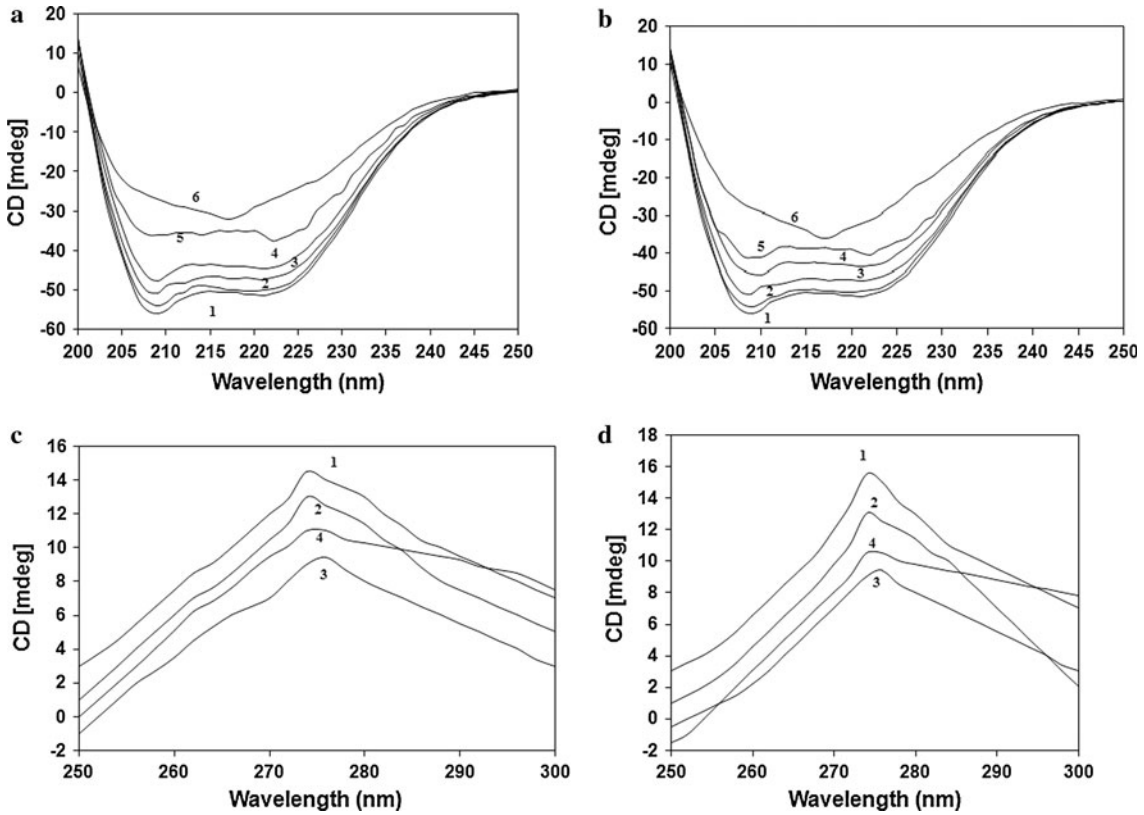

Fig. 4 **a** Far-UV CD spectra of native GOD (curve 1) and arabinose glycated GOD for 3 days (curve 2), 6 days (curve 3), 9 days (curve 4), 12 days (curve 5), 15 days (curve 6) showing changes in secondary structure during the progress of glycation reaction. GOD concentration was 1.875 μM and path length was 0.1 cm. **b** Far-UV CD spectra of native GOD (curve 1) and ribose glycated GOD for 3 days (curve 2), 6 days (curve 3), 9 days (curve 4), 12 days (curve 5), 15 days (curve 6) showing changes in secondary structure during the progress of glycation reaction. GOD concentration was 1.875 μM and path length was 0.1 cm. **c** Near-UV CD spectra of native GOD (curve 1) and arabinose glycated GOD for 6 days (curve 2), 12 days (curve 3) and 15 days (curve 4). GOD concentration was 3 μM and path length was 0.1 cm. **d** Near-UV CD spectra of native GOD (curve 1) and ribose glycated GOD for 6 days (curve 2), 12 days (curve 3) and 15 days (curve 4). GOD concentration was 3 μM and path length was 0.1 cm

shows shift of peak from 1,617 to 1,610 cm^{-1}, the most prominent feature in the amide I region of the infrared spectrum of the GOD (Zolda et al. 2004). Thus, GOD contains a relative proportion of β-sheet structure on day 15. This shift may be due to aggregation of GOD in the presence of ribose. This component, of the amide I band, can be assigned unambiguously to protein segments of the β-sheet conformation (Susi et al. 1967; Susi and Byler 1986).

Detection of free amino groups in glycated GOD

At an early stage of Maillard reaction, the protein containing free amino groups such as ε-NH_2 groups of lysine reacts with the carbonyl groups leading to the loss of free amino groups. GOD when incubated with arabinose and ribose (200 mM each) in 20 mM phosphate buffer with 0.02% sodium azide, pH 7.2 and 37°C for 3–15 days shows loss in free amino groups in its glycated form. For this, GOD was subjected to TNBS method. The result demonstrates that the content of free amino groups in glycated GOD significantly decreases from day 3 (Fig. 6). Results also show that ribose is more active sugar than arabinose as loss in free amino group is more in case of ribose as compared with arabinose. Nagy et al. (2009) have also reported decrease in free amino groups upon glycation.

ANS fluorescence

Hydrophobic probe ANS is widely used for the characterization of molten globule state of proteins (Matulis et al. 1999). A continuous increase in ANS fluorescence was found from day 3 to day 12 (Fig. 7) in GOD (2 mg/ml) incubated with ribose (200 mM) as well as with arabinose (200 mM) in 20 mM phosphate buffer with 0.02% sodium azide, pH 7.2 and 37°C.

Th T fluorescence

Th T fluorescence intensity was recorded for GOD samples (2 mg/ml) incubated with and without reducing sugars at

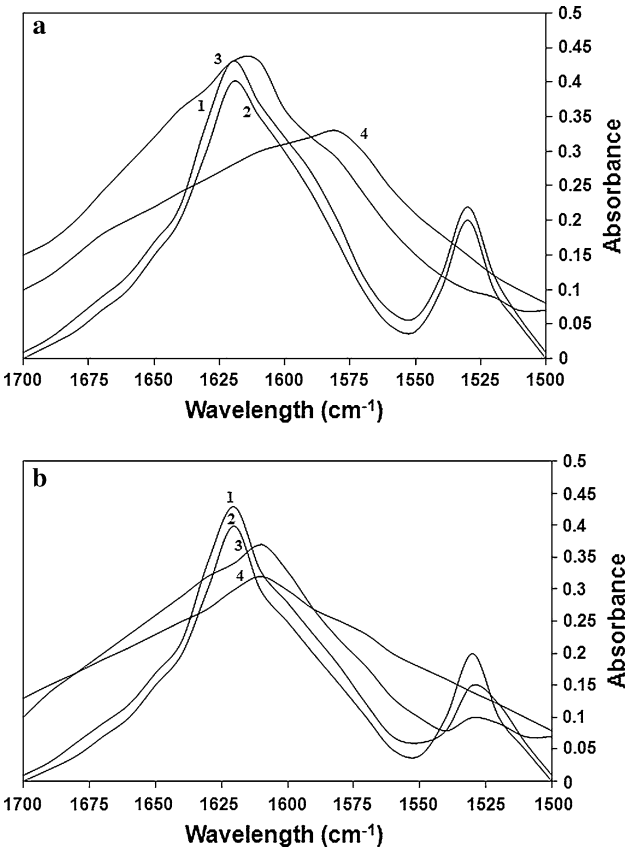

Fig. 5 **a** FTIR spectra of native GOD (curve 1) and arabinose glycated GOD for 6 days (curve 2), 12 days (curve 3) and 15 days (curve 4). GOD concentration used was 30 μM. **b** FTIR spectra of native GOD (curve 1) and ribose glycated GOD for 6 days (curve 2), 12 days (curve 3) and 15 days. GOD concentration used was 30 μM

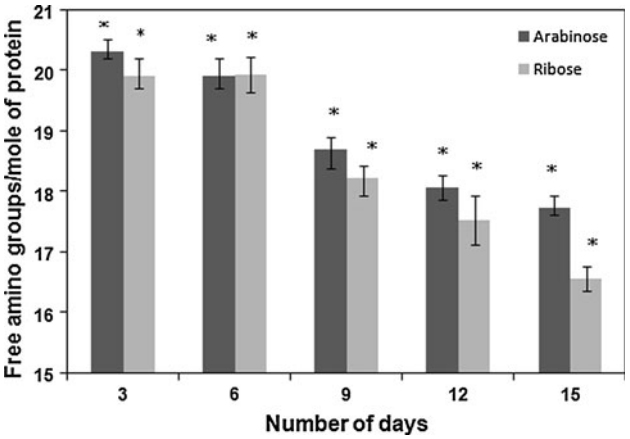

Fig. 6 *Bar graph* represents free amino groups present per mole of protein verses time of incubation of GOD in days. Amino group is determined by using TNBS which binds to free amino groups of GOD and gives absorbance at 420 nm. *Error bars* indicate the mean ± SD ($n = 3$). *Significance $p < 0.05$ with respect to control

482 nm (Fig. 8). Figure shows that the extent of formation of GOD aggregates is more in the presence of ribose (200 mM) followed by arabinose (200 mM). Negligible aggregates were detected in native. There is an increase in Th T fluorescence with increase in incubation time maximum attaining on day15. It can be noted here that ribose incubated GOD shows more Th T fluorescence relative to arabinose incubated GOD. As can be seen from figure, there is more aggregate formation at day 15 in ribose incubated GOD compared with arabinose.

Treatment of plasmid pUC19 DNA with glycated GOD

The damaging effect of a ribose and arabinose glycated GOD on the plasmid DNA was observed. Figure 9a shows that there is a difference in the mobility of plasmid treated by GOD incubated with arabinose and ribose for 15 days (lane 2 and 3) in 20 mM phosphate buffer with 0.02% sodium azide, pH 7.2 and 37°C compared with the negative control (lane 1). Formation of open circular form of plasmid DNA was observed when it was treated with arabinose and ribose glycated GOD. Lane 4 represents positive control of methyl methane sulfonate (25 μg/ml) treated plasmid which results in conversion of supercoiled plasmid into open circular and linear form. As the nicking of plasmid has taken place it can be concluded that glycated products have genotoxic potential. Figure 9b shows the mobility changes in glycated samples in relation to negative and positive control.

Effect of 15-day glycated GOD sample on DNA breakage in lymphocyte

Figure 9c demonstrates the effect of different treatments on lymphocytes. Figure 9ci represents the negative control

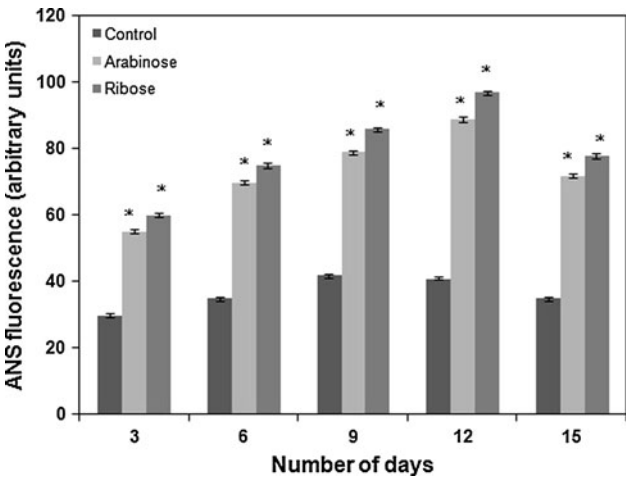

Fig. 7 ANS fluorescence of native GOD, arabinose and ribose glycated GOD versus time of incubation. The GOD concentration was 4.5 μM and the path length was 1 cm. The fluorescence was monitored at an excitation wavelength of 380 nm (Peaks achieved on respective days are plotted) with a slit width of 5 nm. *Error bars* indicate the mean ± SD ($n = 3$). *Significance $p < 0.05$ with respect to control

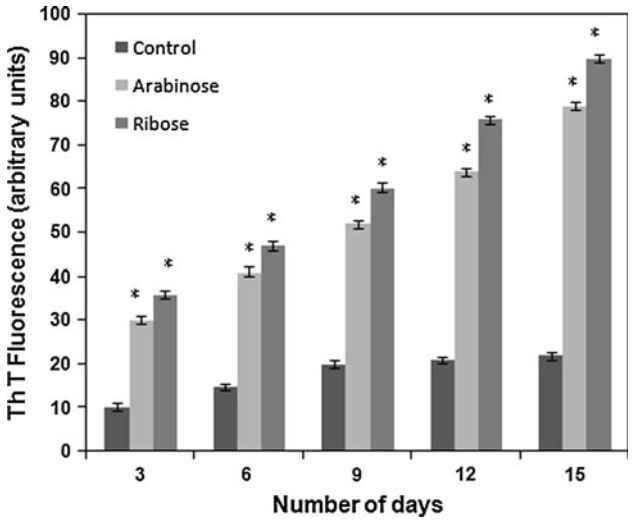

Fig. 8 Th T fluorescence of native GOD, arabinose and ribose glycated GOD versus time of incubation. The GOD concentration was 2 μM while Th T concentration was 10 μM and the path length was 1 cm. The fluorescence was monitored at an excitation wavelength of 440 nm (Peaks achieved on respective days are plotted) with a slit width of 5 nm. *Error bars* indicate the mean ± SD ($n = 3$). * Significance $p < 0.05$ with respect to control

(without any treatment) image of lymphocyte. Nuclear DNA damage in lymphocytes by arabinose and ribose glycated GOD has been depicted in Fig. 9cii and 9ciii. These images clearly demonstrate that glycated GOD cause a significant damage and have a genotoxic effect on lymphocytes in vitro. Figure 9civ represents effect of methyl methane sulfonate as positive control on nuclei of lymphocyte. When arabinose and ribose glycated GOD were taken in a concentration of 2 mg/ml, they caused nuclear DNA breakage of about 10 and 12 μm tail length, respectively. On the other hand, 20-μm-long tails are seen in the nuclei of lymphocytes due to the damaging effect of positive control (Fig. 9d). DNA damage in case of glycated GOD-treated lymphocyte may be attributed to the fact that the nuclear pore complex is permeable to small molecules such as AGEs (Husain and Naseem 2008).

Discussion

Catalytic activity of GOD clearly demonstrated functional changes in the enzyme. Modification of lysine and arginine residues by arabinose/ribose may bring about alteration at the active site of the GOD which results in decrease in activity of glycated GOD. Here, there is more loss in the activity of ribose glycated GOD compared with arabinose glycated one. Thus, we can confirm that ribose is more active than arabinose.

The intrinsic fluorophore tryptophan is an excellent parameter to monitor the polarity of tryptophan environment in the protein and is highly sensitive to the surrounding environment (Jha and Kishore 2011). Of the ten Trp residues in each monomer, four are located in the flavinic coenzyme activity domain (Haouz et al. 1998). The dip in fluorescence may be due to the energy transfer from Trp to flavin groups or may be due to internalization of a single exposed residue at position 133 (Zolda et al. 2004), upon incubation with these sugars. Curves of arabinose and ribose incubated GOD showed a single-step, two-state, cooperative transition. Fluorescence is widely used for the detection of AGEs such as pentosidine-like AGEs and malondialdehyde-modified protein. Increase in fluorescence intensity with time, upon incubation of GOD with arabinose/ribose indicated AGEs formation. Ribose appeared more reactive than arabinose as judged by formation of a higher level of pentosidine-like AGE fluorescence and malondialdehyde-modified protein-like fluorescence.

Physical changes in the modified GOD has been analysed by CD and FTIR studies. In the native state, GOD possesses a relatively strong far-UV and near-UV CD signal. The CD spectrum of native GOD in the far-UV region is typical for a protein containing α-helices and β-sheet, confirming the native conformation of GOD (Mossavarali et al. 2006). The transition to the molten globule state has been shown to be accompanied by loss of tertiary interactions, whereas most of the secondary structure is preserved. Also, there is disappearance of the CD bands in the near-UV region manifesting that there is loss in tertiary structure in the molten globule state of protein. The conformational changes in GOD by incubating it with arabinose and ribose for different time period was monitored in the amide I band region of FTIR spectra. Molten globule state attained on day 12 of incubation with arabinose and ribose give their spectral peaks at, both, 1,530 and 1,617 cm^{-1} in FTIR spectra similar to the native, confirming that there is no loss in secondary structure. Shift in wavenumber demonstrates that the amide groups of the glycated enzyme experience a wider range of microenvironments with respect to the enzyme molecule in solution. Disappearance of peak at 1,530 cm^{-1} and appearance only at 1,617 cm^{-1} in state attained on day 15 of incubation of GOD in the FTIR analysis confirm that there is loss in α-helix and gain in β-sheet conformation. As it is a well-known fact that aggregates have extensive amount of β-sheet structure, it can be concluded that state on day 15 may be aggregated state. These findings provide the evidence of α-helix to β-sheet conformational transition of protein (Sundd et al. 2000). In both the cases no signals were detected after 15 days' incubation indicating that protein precipitation might have taken place after aggregation.

Binding of arabinose and ribose to amino groups present in GOD resulted in the decrease of its free amino groups. In this situation, free amino groups present per mole of

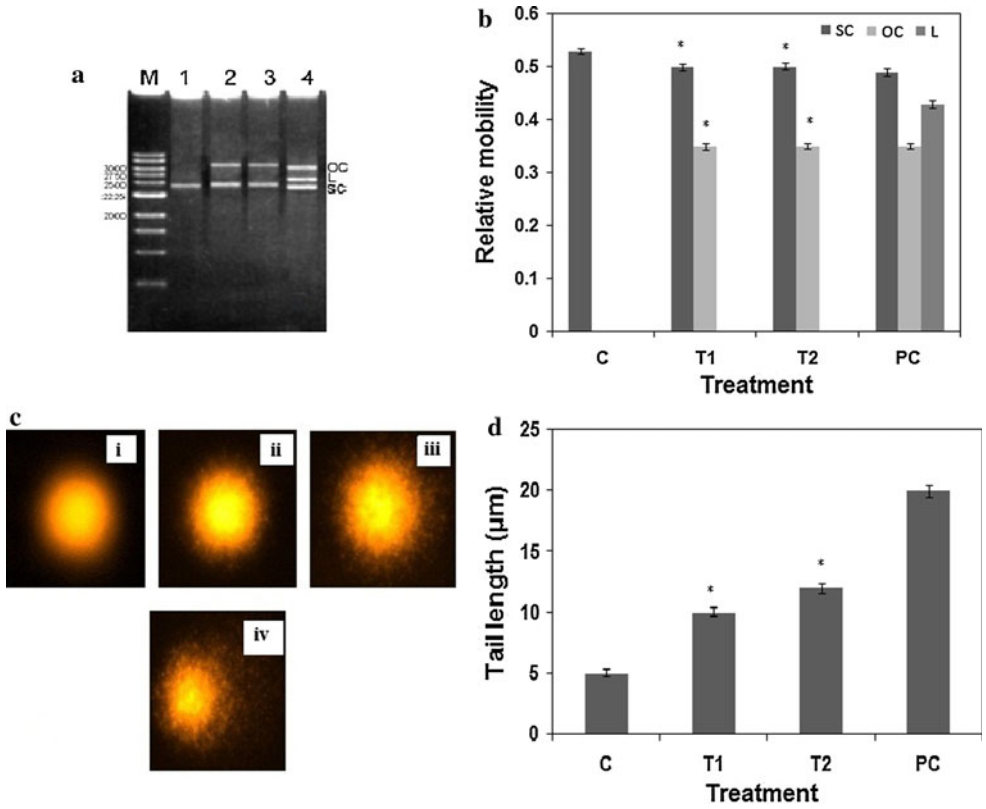

Fig. 9 **a** Plasmid DNA breakage in negative control (1), treated by day 15 sample of arabinose glycated GOD (2), treated by day 15 sample of ribose glycated GOD (3) and positive control of plasmid treated with 3 μl of methyl methane sulfonate (25 μg/ml) (4). OC denotes open circular, *L* denotes linear and SC denotes supercoiled form of plasmid DNA. Molecular markers are run in lane 'M'. **b** Relative mobility plot of plasmid DNA breakage in negative control (C), treated by day 15 sample of arabinose glycated GOD (T1), treated by day 15 sample of ribose glycated GOD (T2), treated by 3 μl of methyl methane sulfonate (25 μg/ml) as positive control (PC). *Error bars* indicate the mean ± SD ($n = 3$). *Significance $p < 0.05$ with respect to control bar ''C''. **c** Images of lymphocyte nuclei damage (i) negative Control (ii) treated by day 15 sample of arabinose glycated GOD (iii) treated by day 15 sample of ribose glycated GOD (iv) treated by 3 μl of methyl methane sulfonate (25 μg/ml) as positive control. **d** Lymphocyte DNA breakage in negative control (C), treated by day 15 sample of arabinose glycated GOD (T1), treated by day 15 sample of ribose glycated GOD (T2), treated by 3 μl of methyl methane sulfonate (25 μg/ml) as positive control (PC). *Error bars* indicate the mean ± SD ($n = 3$). *Significance $p < 0.05$ with respect to control bar ''C''

arabinose modified GOD are more as compared with ribose modified GOD, further confirming that ribose is more active. Glycation of GOD exposes more hydrophobic regions of the protein to the surface making them available for ANS binding (Amani and Naeem 2011). It can be seen that binding of ANS at 12-day incubated GOD produced a prominent increment in fluorescence intensity relative to 15-day incubated sample in both the cases. As hydrophobic cluster are loosely pack resulting in large surface area and high ANS binding are the characteristic of molten globule state, hence this state is characterized as molten globule state of GOD at day 12. With further increase in incubation time, decrease in ANS fluorescence intensity on day 15 may be attributed to the unfolding of ANS binding sites or burial of the binding sites due to aggregation of arabinose/ribose glycated GOD. Comparing the data of both the sugars it can be said that owing to more unfolding of GOD there is more ANS intensity in case of ribose compared with arabinose. This finding also confirms that ribose is more active.

Partial denaturation of proteins caused by arabinose/ribose often results in formation of folding intermediates that contain almost the same level of the secondary structure as the native protein, but decreased number of the tertiary contacts (Ptitsyn 1992), unpaired domains (Horowitz and Criscimagna 1990), or incorrectly formed disulfides bonds (Ewbank and Creighton 1991). As a result, such intermediates tend to be highly hydrophobic and consequently can easily precipitate, forming large aggregates. In addition to their role in protein folding, molten globules may have other biological functions also. For example, intrinsically unstructured or partially structured proteins existing under physiological conditions may serve as a conformational switch or target for gene regulation (Wright and Dyson 1999). From the far-UV CD, near-UV CD, tryptophan fluorescence, ANS binding and Th T

binding, we propose that glycation induces a molten globule state of GOD on day 12 of incubation with arabinose and ribose and it tends to aggregate with further increase in incubation time which has the characteristics of native state.

Various compounds, like methylglyoxal, are present in human body that can act as glycating agents and can readily react with amino groups of proteins to produce AGEs, which have a role in the pathophysiology of ageing and diabetic complications (Thornalley 2008). Glucose, a key cellular fuel in the body, can also cause damage to the body by glycating the proteins resulting to loss their shape and ultimately making them insoluble or unstable. A good example of this is cataracts which occur when protein glycation takes place. The process of glycation lowers the level of solubility of the lens proteins which in turn leads to a loss of transparency in the lens. DNA damage has also been reported in specific diabetic cells when DNA interacts with glycated products. These glycated products produce both base modification and apurinic/apyrimidinic sites in DNA, in addition to the strand breaks (Mullokandov et al. 1994). The inactivation of catalase and SOD in diabetic patients is a consequence of glycation, where sugar combining with catalase and SOD could lead to their inactivation and thus to accumulating peroxide and superoxide, which might contribute to the overall complications of diabetes and aging. (Yan and Harding 1997). Cytotoxic effect of AGEs have also been reported in diabetic patients (Boyanova and Huppertz 2009).

We have performed our glycation studies with GOD in the presence of pentose sugars (ribose and arabinose) and found out that glycated GOD and other AGEs formed are genotoxic in nature. Plasmid nicking assay and comet assay were performed to check the toxicity of glycated GOD to DNA or on whole cell and we got significant damage in both the cases. Open circular form of plasmid was observed after incubation with GOD glycated by means of arabinose/ribose. Control and glycated GOD treated cells induce the radial diffusion from the nucleus of single-stranded DNA fragments and generates, upon incubation with ethidium bromide, a fluorescent image similar to a halo concentric to the nuclear remnants. The greater the level of DNA fragmentation the bigger the area of the halo is, thus allowing a quantitative determination of the nuclear injury. The nature of the nuclear DNA injury revealed in lymphocytic cells by the alkaline-comet assay is consistent with the plasmid nicking assay. One of the most important involvement of glycated GOD and other AGEs may be the generation of free radicals via innate immune system. AGE modified proteins elicit an acute phase response in microglial cells or macrophages, which in turn while degrading these modified proteins can release cytokines and oxygen radicals ultimately leading to direct toxicity (oxidative stress, Ca-influx etc.) or indirect toxicity (acute phase reactions etc.). A diagrammatic representation of this mechanism is demonstrated in Fig. 10.

Conclusion

In the present paper we have investigated the effect of arabinose and ribose on protein glycation and aggregation, taking GOD as target protein. From our studies, it can be concluded that arabinose and ribose glycated GOD can induce the formation of molten globule state on day 12 of incubation as is revealed by far-UV, near-UV CD studies and ANS fluorescence. Further, our studies also establish that in comparison with arabinose, ribose was found to cause rapid glycation of GOD. Both ribose and arabinose were shown to induce aggregation in GOD upon incubation for 15 days as evident by far-UV CD, near-UV CD, FTIR, ANS and Th t fluorescence analysis. Carrying our studies to higher level, we have examined the glycated product for its genotoxic effects and we got significant results. Our work will facilitate a further understanding of the genotoxicity of glycated protein product, conformational changes and aggregation of proteins in the presence of sugars that is very similar to the hyperglycemic conditions in the diabetic patients.

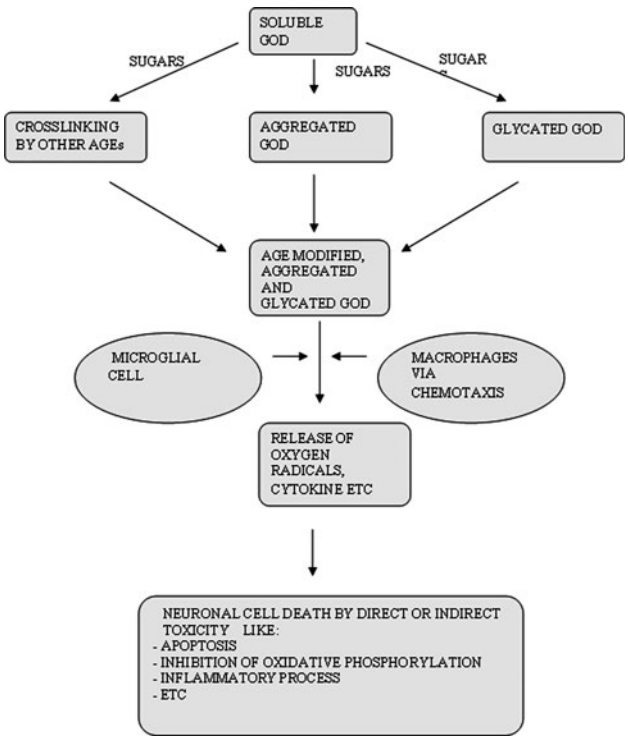

Fig. 10 Diagrammatic representation of toxicity induced by modified GOD

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Conflict of interest The authors declare that there are no conflicts of interest.

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