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Non-Enzymatic Glycation and Formation of Advanced Glycation End-Products Alters the Activity and Related Kinetic Properties of Aldose Reductase

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Aldose reductase was incubated with and without either fructose or glucose for 42 days to initiate the glycation process. The concentrations of fructosamine were measured on every 7th day using the standard nitroblue tetrazolium reagent assay. Carboxymethyllysine formed was determined using enzyme linked immunosorbent assay-based methods, fluorescent end-products were measured using spectrofluorometric methods. Activities were assayed by measuring the absorbance of co-factor nicotinamide adenine dinucleotide phosphate hydrogen at 340 nm. Fructosamine, carboxymethyllysine protein adducts and fluorescent end-products were significantly higher ($p < 0.001$) when aldose reductase was incubated with fructose or glucose than without. Although the glycation of aldose reductase did not result in the alteration of both the optimum pH and temperature of the enzyme, both the activity and V_{max} were increased, whereas K_m was decreased. Non-enzymatic glycation of aldose reductase increases both its activity and V_{max}, while decreasing its K_m. Additionally, glycation did not affect the pH of enzyme and temperature optima.

Keywords: **Non-enzymatic glycation, Aldose reductase, Polyol pathway, Advanced glycation end-products, Kinetic parameters.**

INTRODUCTION

Aldose reductase (AR) [EC 1.1.1.21] is a nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) dependent enzyme that catalyzes the reduction of a wide variety of aldehydes, including glucose to their corresponding alcohols [1,2]. It is a cytosolic, 316 amino acid residues monomeric enzyme expressed in most mammalian cells and has a molecular weight of 35.9 kDa [1,3-6]. In hyperglycaemic conditions such as observed in diabetes mellitus, aldose reductase converts glucose into sorbitol *via* the polyol pathway. The resultant sorbitol is then converted into fructose by the action of enzyme sorbitol dehydrogenase [3]. The combined action of aldose reductase and sorbitol dehydrogenase depletes the cofactors, nicotinamide adenine dinucleotide phosphate (NADPH) and nicotinamide adenine dinucleotide (NAD) leading to a redox imbalance. This imbalance resulted in increased production of reactive oxygen species. Reactive oxygen species (ROS), which accumulate in tissues such as the vasculature, lens, retina, peripheral nerves and renal glomerulus [7-9].

Advanced glycation end-products (AGEs) are products of non-enzymatic glycation of proteins, lipids and nucleic acid with reducing sugars like glucose, fructose or ribose [10]. They are formed progressively with aging on long-lived proteins like collagen but accumulate at an accelerated rate in conditions associated with hyperglycaemia or oxidative stress such as diabetes mellitus and renal failure [11]. Moreover, AGEs and the AR-polyol catalyzed pathway are implicated in the pathogenesis of both microvascular (nephropathy, retinopathy, neuropathy) and macrovascular atherosclerotic diseases (*e.g.* stroke and myocardial infarction) [12,13]. There is evidence of a cross talk between the polyol AR-catalyzed pathway and the formation of AGEs in that the ROS produced in the polyol pathway promote the production of α-dicarbonyl compounds (*e.g.*

glyoxal, methyl glyoxal and 3-deoxyglucosone) which react further with free amino groups of lysine and arginine on proteins to produce AGEs [14].

Many studies [15,16] have shown that the activity of AR is markedly increased in hyperglycaemic states. Therefore, it is possible that this increase in activity of AR could be due to its modification through non-enzymatic glycation. There are two possible ways in which non-enzymatic glycation could influence the activity of AR. Firstly, ROS-derived α-dicarbonyl compounds may non-enzymatically glycate AR, thereby forming AR-AGEs which may alter the structure and activity of AR [17]. Secondly, the formation AGEs on AR may decrease its susceptibility to inhibition by AR inhibitors [18].

While there are several studies that have investigated and reported on the role of the AR catalyzed polyol pathway in the formation of AGEs, there is little information in the literature regarding the effect of AGEs on the activity and kinetic properties of AR. Furthermore, since the process of non-enzymatic glycation result in the formation of different types or classes of AGEs, It is not known which type or class of AGEs affect the activity of AR. Thus, the aim of the current study was to investigate *in vitro* the effect of different types of AGEs on the activity and related kinetic properties of AR.

EXPERIMENTAL

Commercial enzyme AR (Cat. no. A6338; 335 U), NBT colorimetric reagent (Cat. no. N6876), SDS-PAGE and reagents were obtained from Lehlabile Scientific (Sigma-Aldrich). Materials for total immunogenic AGEs and CML-protein adducts include ELISA kits obtained from CellBiolabs. A microplate reader model Ascent Thermoscan-Multiscan. All the chemicals used in the study were of analytical grade.

***in vitro* Preparation of AGE modified AR enzyme:** The AGE modified AR enzyme was prepared by the method as described by Cortizo & Gagliardino [19] with some slight modifications. In brief, the commercial enzyme AR (33.5 U/mL) was incubated in 10 mL phosphate buffered saline pH 7.4 at 37 ºC with and without either 100 mM fructose or glucose for 42 days (to ensure non-enzymatic glycation as is uncontrolled hyperglycaemia) under aerobic conditions in the presence of 3 mM NaN_3 (Merck) to prevent bacterial growth [20]. On every 7th day of incubation periods, aliquots from the incubation mix-tures were withdrawn and concentrated in Amicon microcentri-fuge filters to remove unbound sugars (fructose or glucose) and low molecular weight compounds. Thereafter the mixtures were tested for fructosamine content. At the end of incubation, aliquots from the incubation mixtures were transferred into 2 mL Eppendorf tubes and stored at –20 ºC for use in subsequent investigations.

Measurement of fructosamine: Fructosamine content, a marker of early reversible glycation was measured using standard nitroblue tetrazolium (NBT) chromogenic method as described by Johnson *et al*. [21]. All fructosamine assays measurements were made in duplicates in a 96-well microplate as described by Baker *et al*. [22]. The enzyme samples (50 µL) incubated with and without fructose or glucose were added to 100 µL of 100 mM sodium carbonate buffer, pH 10.8. To start the reaction, 50 µL of 0.25 mM NBT (freshly prepared) was added into each well including the blanks. The microplates were incu-bated for 15 min at 37 ºC. The subsequent reductions of NBT to formazan were measured spectrophotometrically at 560 nm against the controls. The absorbance readings obtained were converted to concentrations using a standard curve. Standards and NBT reagent were made fresh every day and stored at -20 ºC and 4 ºC, respectively.

Measurement of total immunogenic AGEs and CML-protein adducts: Total immunogenic AGEs and CML-protein adducts carboxymethyllysine (CML) were assayed by means of ELISA Kit (Cell Biolabs). The AGE-BSA, CML-BSA standards and enzyme samples (10 µL/mL) were adsorbed onto a 96-well protein binding plate for 2 h at 37 ºC. The total immunogenic AGEs, CML-protein adducts present in the sample or standard were probed with an anti-AGE-protein and anti-CML-protein adduct antibodies respectively, followed by Horse raddish peroxidase (HRP) conjugated secondary antibody. The total AGE-protein or CML-protein adduct content in the enzyme samples were determined by comparing with a standard curve prepared from AGE-BSA and CML-BSA standards, respectively. The assays were performed in duplicates and in accordance with the manufacturer's instructions. The results obtained were compared with that of AGE-BSA and CML-BSA standard curve to quantify the amount of total AGE-proteins and CML-protein adducts respectively derived from AR incubated with fructose or glucose.

Measurement of fluorescent AGEs: Fluorescent AGEs were measured in diluted samples (1:5) at an excitation wavelength 370 nm and an emission wavelength 440 nm. The presence of fluorescent AGEs was quantified as arbitrary units and measured in triplicates using a SpectraMax M2 plate reader as described earlier.

Measurement of AR enzyme activity: The AR activity was determined by the method described by Kashimura *et al*. [23]. The amount of NADPH used were measured in a 500 µL reaction medium containing 100 µL of 100 mM HEPES-KOH buffer (pH 8.0), 100 µL of 5 mM glucose substrate, 50 µL NADPH coenzyme and 100 µL of AR enzyme. The standard solutions (2, 5, 50 and 100 µM NADPH) were prepared in a final volume of 350 µL. The decrease in the amounts of NADPH was monitored at 340 nm with a microplate reader. The concentration of NADPH was calculated from the standard curve using the absorbance of respective standard solutions. From the Lineweaver-Burk double reciprocal plot K_m and V_{max} were determined from the slope, *x*-intercept and *y*-intercept, respectively. The Michaelis-Menten equation was the used to calculate the rate of reaction and enzyme activities.

RESULTS AND DISCUSSION

Present study investigated the effect of non-enzymatic glycation on the activity and kinetic properties of aldose reductase (AR) *in vitro*. To identify the nature and types of AGEs formed as a result of non-enzymatic glycation, total immunogenic AGEs, CML adducts and fluorescent AGEs were also measured.

Fructosamine: Fig. 1 represents the graphs fructosamine concentrations of aldose reductase (AR) plotted against incubation time for 42 days with and without either fructose or glucose. Controls were AR incubated under identical conditions without either fructose or glucose.

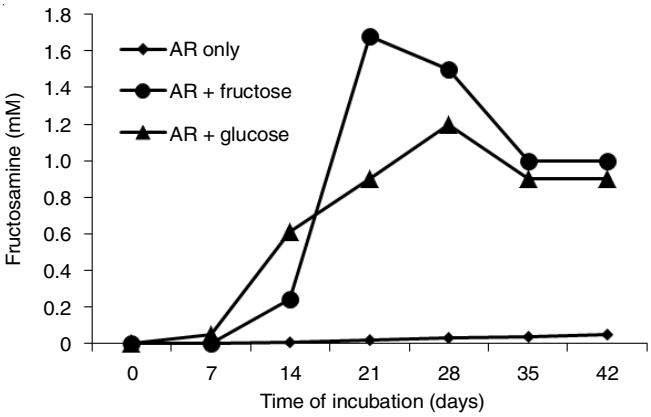

Fig. 1. Graph of fructosamine concentration (mM) plotted against days of incubation. The enzyme aldose reductase (AR) was incubated with and without either 100 mM fructose or glucose for 42 days. Data represents means ± SD. $p < 0.005$ *versus* controls. AR only represents aldose reductase incubated without fructose or glucose and serve as control

With regard to total immunogenic AGEs, the results show a significant difference in total immunogenic AGEs between AR incubated with fructose or glucose than AR incubated without fructose or glucose (Fig. 1).

A significant difference in fructosamine levels was observed between AR mixtures incubated with and without either fructose or glucose. After 7 days, AR incubated with AR + fructose resulted in fructosamine concentration of 0.00142 ± 0.003 mM, reaching a maximum concentration of 2.046 ± 0.001 mM after 28 days. On the other hand, incubation of AR with AR + glucose led to fructosamine concentration of 0.05 ± 0.002 mM, with a maximum concentration of 1.523 ± 0.018 mM after 21 days. AR incubated without fructose or glucose led to no significant change in fructosamine concentration (0.009 ± 0.0013 mM) after 7 and 28 days (0.003 ± 0.0010 mM). The rate of glycation was rather slow for AR + fructose in the first 14 days and drastically increasing between 14 to 28 days. This observation suggests that fructose is a significantly more effective glycating agent than glucose in the initiation of non-enzymatic glycation. Therefore, we can confirm that in this study, non-enzymatic glycation was achieved and fructosamine concentration of AR incubated with fructose or glucose increased with time of incubation as well as the glycating potential of the reducing sugar. Since, the rate of fructosamine formation for fructose was higher than that of glucose. It was not surprising as glucose is one of the least reactive sugars in relation to glycation among all the natural monosaccharides though its concentration dominates in living systems. This property might have been the reason for the evolutionary choice of glucose as the universal carbohydrate energy carrier. These findings were consistent with a study conducted by Smith *et al.* [24].

Total immunogenic AGEs: The total immunogenic AGEs were measured by means of ELISA techniques. The immunogenic AGEs of AR derived after incubation with and without either fructose or glucose is shown in Fig. 2. A significant difference was observed between AR incubated with fructose or glucose and AR incubated without fructose or glucose. Data represent means ±S.D. $p < 0.001$ versus controls (AR enzyme incubated without either fructose or glucose).

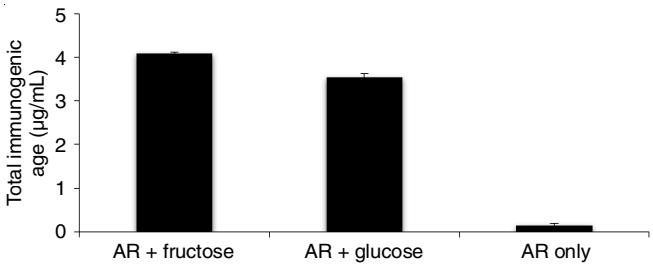

Fig. 2. Total immunogenic AGEs levels (µg/mL) of AR obtained after incubation with and without either fructose or glucose for 42 days. Aldose reductase (AR). Data represents means ± SD. $p < 0.005$ *versus* controls. AR only represents AR incubated without fructose or glucose and serve as control

CML-protein adducts: CML-protein adducts were measured by means of ELISA technique as described elsewhere. The CML-protein content of the enzyme AR derived from incubation with and without 100 mM of either fructose or glucose are shown in Fig. 3.

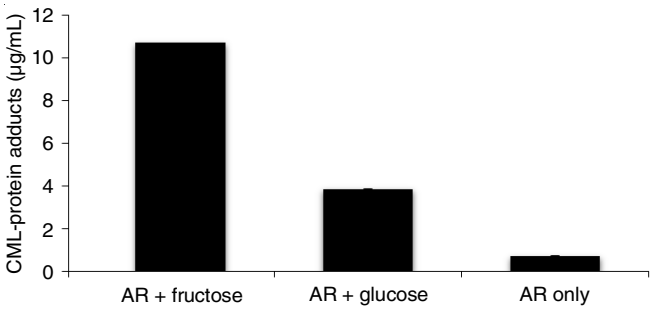

Fig. 3. CML-protein content (µg/mL) obtained from enzymes incubation with and without fructose or glucose for 42 days. Aldose reductase (AR). Data represents means ± SD. $p < 0.005$ *versus* controls. AR only represents aldose reductase incubated without fructose or glucose and serve as control

Fluorescent AGEs: The appearance of fluorescent AGEs on AR was measured by the characteristic fluorescence emission maximum at 440 nm upon excitation at 366 nm. For this assay, the protein concentrations of all samples were adjusted to 1 mg/mL and fluorescence measurements were expressed as arbitrary units (a.u), percentage relative fluorescence compared to that of a 1 µg/mL quinine standard, as previously described [20]. Fig. 4 shows the amount of fluorescence AGEs of AR derived from incubation with and without either 100 mM fructose or glucose. The controls were AR incubated without fructose or glucose.

With regard to total immunogenic AGEs, the results show a significant difference in total immunogenic AGEs between AR incubated with fructose or glucose than AR incubated with-

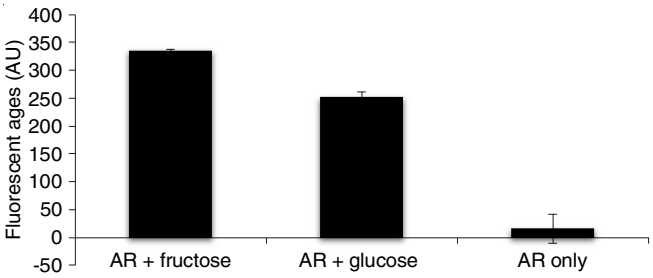

Fig. 4. Amount of fluorescent AGEs obtained from the enzyme AR after incubation for 42 days with and without either fructose or glucose. Data is represented as means ±SD. $p < 0.001$ *versus* control. AR only, aldose reductase incubated without either fructose or glucose. Fluorescence is represented in arbitrary units (AU)

out fructose or glucose (Fig. 1). The AR + fructose and AR + glucose measured 3.36 ± 0.03 µg/mL and 3.538 ± 0.049 µg/mL, respectively while AR only measured 0.143 ± 0.049 µg/mL. Similarly, CML-protein adducts for AR + fructose and AR + glucose measured 5.342 ± 0.006 µg/mL and 3.825 ± 0.0219 µg/mL, respectively while AR only measured 1.252 ± 0.016 µg/mL. Furthermore, fluorescent AGEs on AR + fructose and AR + glucose were 335.67 ± 1.53 AU and 251.277 ± 9.20 a.u, respectively whereas AR only measured 16.151 ± 2.614 a.u. A significant difference ($p < 0.001$) in the nature and types of AGEs formed is observed between AR incubated with either fructose or glucose compared with AR incubated without either fructose or glucose (Figs. 1-3). This observation clearly indicated that non-enzymatic glycation was achieved as supported by the presence of total immunogenic AGEs, CML adducts and fluorescent AGEs in incubation mixtures.

Aldose reductase enzyme activity: Results obtained were used to plot the Lineweaver-Burk double reciprocal plot relationship 1/V (min/µmol) against 1/([S]) (μM^{-1}) to determine the enzyme activity, K_m and V_{max} of AR. Fig. 5 compares the enzyme activity, K_m and V_{max} of AR incubated with and without either fructose or glucose. Table-1 show the enzyme activity, K_m and V_{max} of the AR. Enzyme activity or enzyme unit is defined as the amount of enzyme catalysing the conversion of 1 µmol of glucose substrate per minute time at pH 8.0 at 37 ºC and substrate concentration of 5 mM. An enzyme unit is the amount of a particular and active enzyme. One unit is the amount of enzyme that produces a certain amount of enzyme activity. The units of enzyme activity are enzyme units (U).

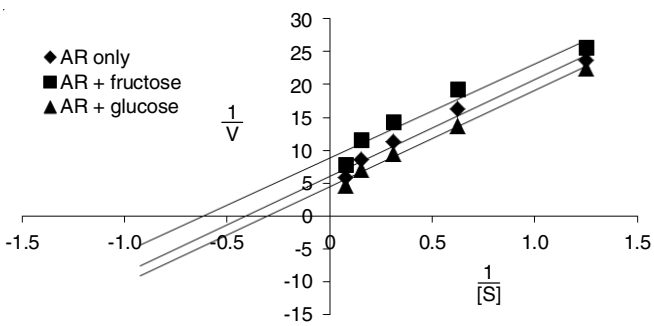

Fig. 5. Lineweaver-Burk double reciprocal plot 1/V (min µmol^{-1}) against 1/[S] (μM^{-1}) to determine the enzyme activity of AR incubated with and without either fructose or glucose. Data represents means ± SD. $p < 0.001$ *versus* controls. AR only represents aldose reductase incubated without fructose or glucose and serve as control

TABLE-1
ENZYME ACTIVITY, K_m AND V_{max} VALUES OF AR (ALDOSE REDUCTASE) INCUBATED WITH OR WITHOUT FRUCTOSE OR GLUCOSE. DATA REPRESENTS MEANS ± SD. $p < 0.005$ *versus* CONTROLS. AR ONLY REPRESENTS ALDOSE REDUCTASE INCUBATED WITHOUT FRUCTOSE OR GLUCOSE AND SERVE AS CONTROL

Enzyme	K_m (µM)	V_{max} (µM/min)	Enzyme activity (U)
AR only	0.411 ± 0.0012	0.165 ± 0.0012	0.6 ± 0.012
AR + fructose	0.615 ± 0.0010	0.113 ± 0.0010	0.4 ± 0.001
AR + glucose	0.299 ± 0.0013	0.227 ± 0.0013	0.8 ± 0.013

The effect of non-enzymatic glycation on enzyme activity and kinetic parameters K_m and V_{max} were also investigated. Fig. 5 shows the Lineweaver-Burk double reciprocal relationship (1/V (min µmol^{-1}) against 1/([S]) (μM^{-1}). The graphs show an increased enzyme activity of AR + glucose with 0.832 ± 0.134 U, while a slight decrease in activity of AR + fructose with 0.426 ± 0.45 U compared with control AR only with activity of 0.623 ± 0.12 U. Through extrapolation of Lineweaver-Burk double reciprocal relationship we determined the K_m and V_{max}. The K_m of AR + glucose was found to be decreased whereas that of AR + fructose was found to be increased with 0.299 ± 0.0013 µM and 0.615 ± 0.001 µM respectively compared with AR only with 0.411±0.0012 µM. From this observation, it is suggested that glycation of AR with glucose increases the affinity of the enzyme AR to substrate glucose whereas glycation with fructose decreases the affinity of AR to glucose. While taking into account that glucose concentration is elevated in diabetes mellitus, fructose utilization is increased as a consequence. In this context, AR catalyzed polyol pathway which generate fructose contributes to the formation of AGEs by promoting the formation of reactive oxygen species (ROS) through depletion of cofactors NADPH and NAD^+ rather than dependent on the glucose substrate concentration. On the other hand, glycation with glucose increased AR catalytic activity through high substrate concentration dependent mechanism. Findings are in correlation with the increased activity of AR observed in uncontrolled chronic hyperglycaemia such as diabetes mellitus where the concentration of circulating glucose is abnormally high [25].

Effect of pH and temperature on AR activity: The AR activities incubated with and without fructose or glucose were measured at different pH values (pH 3, 5, 8, 11 and 13) and different temperatures (20, 30, 37, 45 and 55 ºC). Fig. 6a represents the graph of enzyme activity plotted against buffered pH for AR incubated with and without fructose or glucose to determine the effect of non-enzymatic glycation on the optimum pH of AR incubated with fructose and glucose. Fig. 6b shows a graph of enzyme activity plotted against incubation temperature for AR incubated with and without fructose or glucose to determine the effect of non-enzymatic glycation on optimum temperature of AR.

Both AR + fructose and AR + glucose have higher activity compared with control (AR only). The AR + fructose showed relatively higher activity than AR + glucose. A maximum enzyme activity was observed at pH 8.0 for all AR enzyme incubation

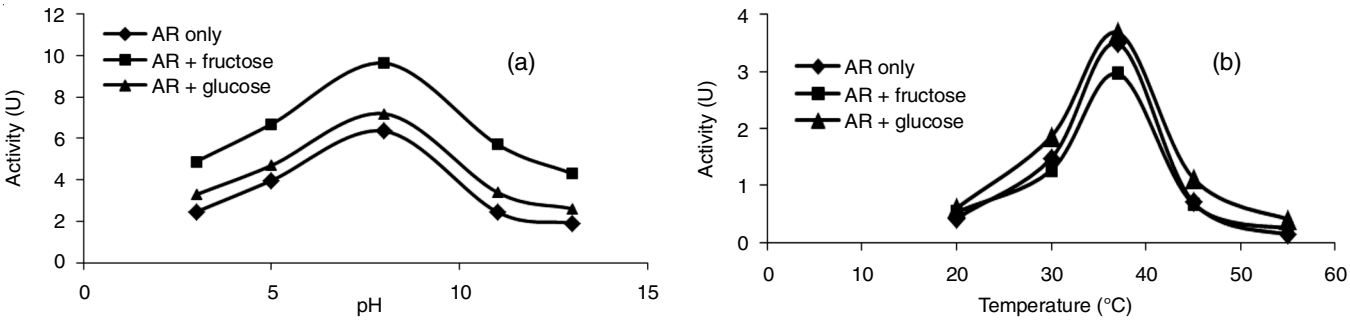

Fig. 6. Graph of enzyme activity plotted against (a) buffer pH and (b) incubation temperature for AR incubated with and without fructose or glucose. Data represents means ± SD. $p < 0.005$ *versus* controls. AR only represents aldose reductase incubated without fructose or glucose and serve as control

mixtures. The activity of AR + glucose was found to be higher with 0.82 ± 0.05 U while AR + fructose was lower with 0.465 ± 0.018 U compared with AR only with 0.636 ± 0.044 U. Similarly, a steady decline in activity was observed as pH increases and little or no activity was observed above pH 13.0. Although there were slight variations in enzyme activities between the AR mixtures, the observation suggested that the non-enzymatic glycation and formation of AGEs did not affect the optimum pH for AR enzyme activity as both AR incubated with and without fructose or glucose had optimum pH 8.0 consistent with the supplier's specification.

For effect of temperature, the AR + glucose led to slightly higher activity whereas AR + fructose showed a lower activity that control (AR only). In all AR incubation mixtures, there was a steady increase in activity between 20 ºC and 30 ºC followed by a sharp increase in activity between 30 ºC and 37 ºC. However, a sharp decline in activity was observed after 37 ºC and no activity beyond 55 ºC. Similarly, non-enzymatic glycation did not have a profound effect on the optimum temperature of AR. While no significant effect observed, there were variations in enzyme activities between AR enzymes incubated with and without either fructose or glucose where AR + glucose activity was higher with 3.68 ± 0.186 U and AR + fructose lower with 2.96 ± 0.22 U compared with AR only with 3.496 ± 0.123 U at optimum temperature. These findings are in consistent with study conducted by Ramasamy & Goldberg [3]. Furthermore, results showed that the enzyme AR is sensitive to changes in temperature than they are to pH as shown by a broad and narrow-bell shaped graphs for pH and temperature, respectively.

Conclusion

Aldose reductase (AR) was incubated with and without fructose or glucose to investigate the effects of non-enzymatic glycation on enzyme activity (V_{max}) and kinetic parameters (K_m). Initiation and progress of non-enzymatic glycation was observed when concentrations of fructosamine increased with incubation time. These findings demonstrated that non-enzymatic glycation is dependent on incubation time. Furthermore, data suggest that different types of non-enzymatic glycation end-products were formed that is fluorescent, non-fluorescent and crosslinking advanced glycation end-products (AGEs). In addition, results indicated that non-enzymatic glycation altered th enzyme activity (K_m and V_{max}). Therefore, non-enzymatic glycation and formation of AGEs play a role in the pathogenesis of such health disorders related to hyperglycaemia through different yet complicated cellular mechanisms resulting in prolonged enzyme dysfunction due to modification of structure.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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