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Bousova, Iva; Vukasovic, Danka; Juretic, Dubravka; Palicka, Vladimir; Drsata, Jaroslav

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Enzyme activity and AGE formation in a model of AST glycoxidation by D-fructose *in vitro*

IVA BOUŠOVÁ[1]
DANKA VUKASOVIĆ[2]
DUBRAVKA JURETIĆ[2]
VLADIMÍR PALIČKA[3]
JAROSLAV DRŠATA[1]*

[1]*Department of Biochemical Sciences*
Charles University in Prague
Faculty of Pharmacy, Hradec Králové
Czech Republic

[2]*Department of Medical Biochemistry and Haematology*
Faculty of Pharmacy and Biochemistry
University of Zagreb, Croatia

[3]*Medical Faculty Hospital,*
Charles University in Prague
Medical Faculty, Hradec Králové
Czech Republic

Non-enzymatic glycation as the chain reaction between reducing sugars and free amino groups of proteins has been shown to correlate with physiological ageing and severity of diabetes. The process involves oxidative steps (glycoxidation). In this paper, the effect of D-fructose as a reactive sugar on aspartate aminotransferase (AST) as a model protein was monitored by measurements of the enzyme activity and formation of fluorescent advanced glycation end products (AGEs). Change in the AST activity was considered as a measure of the overall protein damage caused by glycation, and total AGEs and pentosidine represent, at least partly, the formation of glycoxidation products. Catalytic activity of AST in an incubation mixture containing D-fructose (50 mmol L^{-1}), decreased compared to control values to 42% ($p < 0.05$) and to 11% ($p < 0.05$) on the 5th and on 21st day of incubation, respectively. In the presence of fructose, total fluorescent AGEs concentration was significantly higher since 5th day of incubation (110%, $p < 0.05$) and the fluorescent pentosidine concentration from 15th day of incubation (117%, $p < 0.05$) compared to control values, respectively. Catalytic activity of AST clearly and quantitatively demonstrated functional changes in the enzyme molecule caused by structural modifications initiated by fructose, while the evaluation of AGE formation and especially that of pentosidine by fluorescence measurement was less reliable.

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Reducing sugars (*e.g.*, glucose, fructose, galactose, mannose, ribose) and certain other carbohydrate relatives (*e.g.*, ascorbic acid) are inherently reactive toward nucleophiles (Maillard reaction) (1). Glucose can react with a free amino group (nucleophilic nitrogen base), such as an ε-amino group of protein lysine residue, to form an adduct commonly referred to a Schiff base. Formation of the Schiff base from sugar and amine is a fast and highly reversible reaction. Formation of an Amadori product (AP) from the Schiff base is

* Correspondence, e-mail: drsata@faf.cuni.cz

slow but it is much faster than the reverse reaction, so the Amadori glycation tends to accumulate in proteins. The later stage, complex pigments and crosslinks have come to be known as advanced glycation end products (AGEs). AGEs constitute a heterogenous group of irreversible adducts that were originally characterized by their yellow-brown colour. It is known that these irreversible products include many protein-protein crosslinking structures as well as various non-crosslinked structures. Many, but not all of these products incorporate ultraviolet-visible and/or fluorescent chromophores (2).

There are three routes to producing AGEs: (*i*) via the autooxidative pathway (sugar gives reactive products by autooxidation) (3), (*ii*) via conventional Amadori rearrangement (4), and (*iii*) from the Schiff base (5). Reactive oxygen species: $O_2^{\bullet -}$, H_2O_2, $^{\bullet}OH$ (ROS) may contribute to these reactions, which require trace levels of catalytic redox-active transition metal ions. The process includes also oxidative steps and is therefore called glycoxidation (6).

Both glycation and oxidation reactions lead to the formation of AGEs, including the subclass of AGEs known as glycoxidation products (GOPs): pentosidine and N^{ε}-(carboxymethyl)-lysine (CML). The production of AGEs is accelerated by oxidative stress without the presence of sugar. There is evidence for a rise of pentosidine and CML from ascorbic acid (9).

Glycoxidation is considered to cause gradual deterioration in the structure and function of tissue proteins and to contribute to the pathophysiology of normal ageing. This post-translational modification of proteins and other macromolecules with amino groups is accelerated by hyperglycemia in diabetes, by hyperlipidemia in atherosclerosis, and by oxidative stress in other chronic diseases including Alzheimer's and Parkinson's disease (8–10). Furthermore, in diabetic patients with impaired renal function, food-derived AGEs with crosslinking activity can accumulate in serum to a significant degree, implying that normal kidney function is important for protection against dietary AGEs (11, 12). Another source of orally uptaken AGEs seems to be smoking, since significantly increased serum AGE levels have been observed in diabetic smokers compared to diabetic non-smokers (13).

It means that the level of AGE proteins reflects the kinetic balance of two opposite processes: the rate of AGE compounds formation and the rate of their degradation and elimination (14). This fact indicates the possibility of therapeutic intervention against AGEs by stimulation of their degradation or by inhibition of their formation (15). Further possibilities of therapy come from the knowledge of the character of protein modifications caused by glycoxidation (changes in their physico-chemical properties, such as solubility, isoelectric point, charge, ability of crosslinking, resistance to denaturation, *etc.*) (2).

Giardino *et al.* (16) demonstrated by *in vitro* experiments that one-week incubation of endothelial cells in the presence of a high glucose concentration resulted in a 13.8-fold increase in the intracellular AGE-content. In parallel, the mitogenic activity of endothelial cells cultivated at a high glucose concentration decreased markedly. The observed loss in mitogenic activity was due to the post-translational modification of the basic fibroblast growth factor (bFGF) by AGE, the major AGE-modification in endothelial cells.

In our laboratory, a model of aspartate aminotransferase or alanine aminotransferase as a protein and D-fructose as the glycating agent was developed and applied in several glycation studies (17, 18), in which enzyme activity served as the criterion of protein

modification. The objective of this study is to show the possible applicability of this model for monitoring the oxidation part of the glycation process in the presence of fructose through measurements of fluorescent AGEs and pentosidine.

EXPERIMENTAL

Chemicals

In vitro glycation was studied with aspartate aminotransferase (AST, EC 2.6.1.1), cytosolic enzyme from porcine heart, suspension in ammonium sulphate (SERVA Electrophoresis GmbH, Germany and Roche Diagnostics GmbH, Germany). Fructose was used as the glycating agent (Sigma-Aldrich Co. Ltd., USA). Other chemicals used, such as $NaH_2PO_4 \times 2H_2O$, $Na_2HPO_4 \times 12H_2O$, NaN_3, and uric acid, were of analytical grade. The reagent kit for enzyme activity measurements was purchased from Roche Diagnostics GmbH, Germany. Albumin, Bovine Fraction V, 96–99%, was used as the reference substance for fluorescence measurements (Sigma Chemical Company, USA).

Buffer, enzyme and fructose preparation

Phosphate buffer (0.1 mol L^{-1}, pH 7.4) with 0.05% sodium azide was used for the preparation of incubation mixtures.

Suspension of AST was centrifuged for 20 min at 5000 rpm (at 4 °C), the supernatant was removed and the protein pellet was reconstituted in 0.1 mol L^{-1} phosphate buffer pH 7.4 to yield the stock solution with the enzyme concentration of 2.66 mg mL^{-1}.

100 mmol L^{-1} stock solution of fructose was prepared in 0.1 mol L^{-1} phosphate buffer pH 7.4 with 0.05% sodium azide.

Preparation of incubation mixtures

Incubation mixtures for investigation of the catalytic activity of AST and for the study of AGEs and pentosidine formation were prepared according to Table I.

Final concentrations of substances in the incubation mixture for activity of AST were as follows: 1.33 mg mL^{-1} AST and 50 mmol L^{-1} fructose. All samples were incubated for

Table I. Incubation mixtures

Incubation mixture	Enzyme (mL)	Buffer (mL)	Fructose (mL)	Final volume (mL)
Activity of AST				
AST	0.208	0.208	–	0.416
AST + fructose	0.208	–	0.208	0.416
AGEs and pentosidine formation				
AST	1.56	1.56	–	3.12
AST + fructose	1.56	–	1.56	3.12

up to 21 days at 37 °C, and enzyme activity was measured in triplicate on days: 0, 1, 5, 8, 12, 15, and 21. Control values represent the catalytic activity of AST incubated without fructose for the same time.

Final concentrations of substances in the incubation mixture for AGEs and pentosidine formation were as follows: 1.33 mg mL^{-1} AST and 50 mmol L^{-1} fructose. All samples were incubated for up to 15 days at 37 °C. The amount of 600 μL of each sample was taken on days: 0, 1, 3, 5, and 15 and stored in a freezer at –80 °C. Fluorescence was measured in triplicate at the end of the experiment.

Catalytic activity measurement

AST catalytic activity was measured by the IFCC-recommended method (19), on an automated clinical chemistry analyzer Hitachi 917 (Japan).

Fluorescence measurements

The effects of glycation and glycoxidation were determined from the specific fluorescence intensity resulting from pentosidine and total AGEs level. Fluorescence measurements of glycated AST samples were monitored using a luminescence spectrometer Perkin-Elmer LS 50B (UK) at excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 335/385 nm (optimum for AGE biomarker pentosidine) and 370/440 nm for the total AGEs level. Bovine serum albumin (BSA) was used as the reference substance in a concentration of 1 mg mL^{-1}. Relative fluorescence of pentosidine or total AGEs was expressed in arbitrary units per mg of protein (1 AU corresponding to the fluorescence of 1 mg $mL^{-1}$of BSA).

Statistics

Differences between means were assessed using the Mann-Whitney rank sum-test. The values $p < 0.05$ were considered statistically significant. SigmaStat program for Windows, version 2.0, Jandel Corporation (USA) was used for statistical analysis.

RESULTS AND DISCUSSION

In vitro glycation and glycoxidation were studied with AST. The enzyme was chosen as a model protein for its availability in a highly purified stable form (cytosolic porcine heart enzyme) and because of the presence of a few lysine residues in the molecule, which can undergo the glycoxidation process. Furthermore, Okada *et al.* (20) considered glycation to be one of the possible mechanisms for the existing variants of this enzyme in animal cells.

Effect of glycation on catalytic activity of AST

Effect of 50 mmol L^{-1} fructose as the glycating agent on the catalytic activity of AST was monitored for 21 days at 37 °C. Effect of glycation on lowering the enzyme activity was evident from the beginning of the incubation with fructose. The most rapid decrease

occurred from the 1st to the 5th day. Catalytic activity of AST decreased to 84% on the first day and to 42% ($p < 0.05$) on the fifth day compared to the control value, representing catalytic activity of AST incubated without fructose for the same time. Enzyme activity continued to decrease to 23% on the 12th day, and to 11% on the 21st (Table II).

Table II. Catalytic activity of AST in incubation mixture with and without fructose

Incubation mixture[a]	AST (μkat L^{-1})[b]						
	Time (day)						
	0	1	5	8	12	15	21
AST	6.09 ± 0.38	5.29 ± 0.34	4.33 ± 0.17	6.59 ± 0.03	5.47 ± 0.06	6.83 ± 0.15	5.23 ± 0.58
AST + 50 mmol L^{-1} fructose	5.94 ± 0.23	4.43 ± 0.34	1.82 ± 0.41[c]	2.55 ± 0.13[c]	1.26 ± 0.06[c]	1.43 ± 0.08[c]	0.58 ± 0.02[c]

[a] Enzyme concentration: 1.33 mg mL^{-1}.
[b] Average activity ± *SD*, $n = 3$.
[c] Significantly different from the samples without fructose measured on the same day ($p < 0.05$).

In this study, the authors' intention was to monitor glycation and glycoxidation processes through the changes in catalytic activity of AST as well as by detection of its final products (total AGEs and pentosidine). Due to previous results, which indicated that glucose or glucose-6-phosphate *in vitro* showed weak or no significant effects on the catalytic activity of AST and that the Heyns product derived from fructose (ketoses) was considerably more reactive than the Amadori product, fructose was used as the glycating agent (18, 21, 22). Protein glycation by fructose (fructation) was also suggested to a major determinant in diabetes complications, especially in cells with a hyperactive sorbitol pathway (23). Another advantage of the use of fructose as the glycating agent in this study was that the process of fructation is probably connected with a higher rate of AGEs production than the process of protein modification by glucose. Although fluorescence is a useful marker for detection of AGE formation, the fluorescent AGE crosslinks (represented by pentosidine) are thought to account for only one percent or less of the total crosslinking structures formed under physiological conditions (24). Thus, the major AGE structures responsible for protein-protein crosslinking *in vivo* are the non-fluorescent structures that have not yet been conclusively identified.

Effect of fructose on the production of AGEs

Table III shows the effect of fructose on the total AGEs formation during fifteen days of enzyme incubation at 37 °C. Compared to the control values, the fluorescence measured at λ_{exc} 370 nm/λ_{em} 440 nm was significantly higher in samples with fructose on the 5th (110%, $p < 0.05$) and the 15th day (145%, $p < 0.05$).

Table IV shows the effect of fructose on pentosidine formation during fifteen days of enzyme incubation at 37 °C. Compared to the control values, the fluorescence measured at λ_{exc} 335 nm/λ_{em} 385 nm was significantly higher in samples with fructose on the 15th day (117%, $p < 0.05$).

Table III. Fluorescence of glycated AST expressed as total AGEs (λ_{exc} 370 nm/λ_{em} 440 nm)

Incubation mixture[a]	Fluorescence (AU per mg of protein)[b,c]				
	Time (day)				
	0	1	3	5	15
AST	2.153 ± 0.075	2.231 ± 0.015	2.304 ± 0.034	2.186 ± 0.051	2.410 ± 0.030
AST + 50 mmol L^{-1} fructose	2.128 ± 0.015	2.212 ± 0.052	2.387 ± 0.011	2.465 ± 0.045[d]	3.511 ± 0.071[d]

[a] Enzyme concentration: 1.33 mg mL^{-1}.
[b] 1 AU corresponding to the fluorescence of 1 mg BSA mL^{-1}.
[c] Average fluorescence ± *SD*, $n = 3$.
[d] Significantly different from the samples without fructose measured on the same day ($p < 0.05$).

Table IV. Fluorescence of glycated AST expressed as pentosidine (λ_{exc} 335 nm/λ_{em}385 nm)

Incubation mixture[a]	Fluorescence (AU/mg per mg of protein)[b,c]				
	Time (day)				
	0	1	3	5	15
AST	2.472 ± 0.313	2.404 ± 0.073	2.513 ± 0.120	3.315 ± 0.092	3.733 ± 0.127
AST + 50 mmol L^{-1} fructose	2.323 ± 0.080	2.325 ± 0.074	2.459 ± 0.036	3.484 ± 0.247[d]	4.483 ± 0.337[d]

[a] Enzyme concentration: 1.33 mg mL^{-1}.
[b] 1 AU corresponding to the fluorescence of 1 mg BSA mL^{-1}.
[c] Average fluorescence ± *SD*, $n = 3$.
[d] Significantly different from the samples without fructose measured on the same day ($p < 0.05$).

According to the results, fructose induced a decrease in the catalytic activity of AST throughout the study period. A statistically significant decrease was observed even on the fifth day of incubation with fructose, while a substantial increase in fluorescence as the measure of total AGEs and pentosine formation was observed after 15 days.

CONCLUSIONS

This study presents the results of the measurements of fluorescent AGEs as representatives of the complex glycoxidating process. We can conclude that the proposed model is efficient enough for the investigation of AST modification by fructose using the measurement of the catalytic activity of the enzyme. In our future experiments with natural antioxidants, we are going to improve the AST-fructose model by introducing the transition metal ions (25, 26), which should support the oxidation part of the glycation process.

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S A Ž E T A K

Katalitička aktivnost i stvaranje AGE-produkata u modelu glikoksidacije AST D-fruktozom *in vitro*

IVA BOUŠOVÁ, DANKA VUKASOVIĆ, DUBRAVKA JURETIĆ, VLADIMÍR PALIČKA i JAROSLAV DRŠATA

Neenzimska glikacija je proces kojim se reducirajući šećeri kovalentnim vezama povezuju s amino skupinama proteina, bez posredovanja enzima. Taj proces dijelom je i oksidativan pa se često naziva glikooksidacija. Konačni produkti glikacije (AGE) nastaju polimerizacijom mnogih intermedijarnih produkata. U ovom radu, praćen je u *in vitro* uvjetima, mjerenjem katalitičke aktivnosti enzima i fluorescencije nastalih ukupnih AGE produkata i pentozidina, utjecaj D-fruktoze kao reaktivnog šećera na aspartat aminotransferazu (EC 2.6.1.1.) kao modelni protein. Promjena katalitičke aktivnosti enzima pokazatelj je promijenjene funkcije proteina glikacijom dok je intenzitet fluorescencije ukupnih AGE produkata i pentozidina samo djelomičan pokazatelj glikoksidacijskih promjena. Katalitička aktivnost AST izmjerena IFCC preporučenom metodom u inkubacijskoj smjesi koja je sadržavala 50 mmol L^{-1} fruktoze, smanjena je na 42% ($p < 0.05$) petog dana, te na 11% ($p < 0.05$) dvadeset i prvog dana. Statistički su značajne razlike u katalitičkoj aktivnosti enzima inkubiranog sa i bez fruktoze već nakon petog dana. Stvaranje ukupnih AGE produkata i pentozidina statistički je značajno nakon pet, odnosno 15 dana inkubacije enzima s fruktozom. Katalitička aktivnost enzima jasno ukazuje na funkcionalne promjene uzrokovane glikiranjem, dok je evaluacija stvaranja ukupnih AGE produkata, a pogotovo pentozidina, mjerenjem fluorescencije manje pouzdan pokazatelj promjena.

Ključne riječi: neenzimska glikacija, glikoksidacija, aspartat aminotransferaza, krajnji produkti glikacije, pentozidin

Faculty of Pharmacy and Medical Faculty, Hradec Králové, Czech Republic

Farmaceutsko-biokemijski fakultet, Zagreb