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THE RELATIONSHIP BETWEEN AGEs, Cu^{2+} AND CROSSLINKING OF COLLAGEN

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The propagation of the glycation process in the presence and absence of 0.1 mM Cu^{2+} on pepsin soluble collagen (PSC) by *in vitro* incubation with glycated BSA (AGE-BSA), for 10 days and 6 weeks was investigated. Our experiments have shown the progression of crosslinking of PSC in the presence of soluble AGE-BSA but in the absence of sugar. The Cu^{2+}ions initially induced protein fragmentation, possibly due to reactive oxygen species (ROS) generation, and the increase of fluorescence corresponding to low molecular peptides, probably through the progression of pentosidine formation. After 6 weeks of incubation, the developed molecular aggregates had lower molecular weights, UV absorbance and fluorescence in comparison with that obtained in the absence of Cu^{2+}. It seems that the progression of glycation was possible through the degradation of existing early and advanced glycation products in the presence of 0.1 mM Cu^{2+} that generated ROS and other more reactive products that could attack the unglycated proteins.

INTRODUCTION

Collagen, an important constituent of most of the tissues that are damaged in aging and diabetes, undergoes extensive biochemical modifications that result in decreased solubility and susceptibility to enzymes, accelerated cross-linking which generates the formation of supramolecular aggregates and increased browning and fluorescence.[1-4] It seems that these modifications are due to the Maillard reactions or glycation. Nonenzymatic protein glycation by reducing sugars begins with the reversible formation of a Schiff base aldimine adduct that undergoes rearrangement to a ketoamine, the Amadori products. This Amadori pathway has been considered one of the primary routes for the formation of AGE products *in vivo*.[5,6] The specific mechanisms for the late Maillard reaction, *i.e.* conversion of the initial Amadori adducts to irreversible formation of AGEs, involving complex sequential and parallel reactions, such as condensations, rearrangements, fragmentations and oxidations, that probably form a variety of very reactive dicarbonyl compounds are not completely understood.[7, 8] The oxidative conditions and trace amounts of transition metal ions play a role during *in vivo* and *in vitro* AGEs formation process named glycoxidation.[9] The transition metal ions like Cu^{2+} are required to catalyze electron transfer from ene-diol intermediates resulted from autoxidation of reducing sugars or fragmentation of Schiff base and Amadori compounds, which lead to the generation of ROS and dicarbonyl compounds.[10-12] In these reactions, Cu^{2+} is probably reduced to Cu^{+} and O_2 is oxidized to superoxide anion via peroxide hydrogen. The Cu^{+} ion oxidizes to Cu^{2+} and can catalyze the decomposition of peroxide hydrogen to hydroxyl radical[13]. Some AGEs such as N^{ε}-(carboxymethyl)lysine (CML)[14-16] and pentosidine fluorescent crosslinking products[17,18] have become highly useful biomarkers of glycoxidative damage. A direct role for AGEs in the complications of diabetes was proposed when it was found that their infusion into normal rats induces glomerular sclerosis and albuminuria.[19] Recent data have confirmed that two-thirds of orally absorbed dietary AGEs are retained in tissues in bioreactive forms.[20]

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In the present work, the influence of Cu^{2+}ions in the propagation of the *in vitro* collagen glycation in the presence of soluble glycated bovine serum albumin (AGE-BSA) in aerobic conditions was investigated.

RESULTS

We have recently reported[21] that the FPLC elution pattern of BSA control sample showed only one peak corresponding to a 67.96 kDa molecular weight (retention volume 13.20 mL) while the glycated BSA presented two peaks of 83.29 kDa (retention volume 12.76 mL) and 161.46 kDa (retention volume 10.81 mL), which contains the dimer of the glycated BSA monomer of 83.29 kDa (Fig. 1A). The formation of AGE-BSA was also proved by measuring the fluorescence level at 385nm emission wavelength after a 335 nm excitation wavelength. The fluorescence level was 3.77 fluorescence units (RFU) for BSA and 74.9 RFU for AGE-BSA at 1 mg/mL protein concentration. At 440 nm, emission after a 370 nm excitation was 3.7 RFU for BSA (1mg/mL) and 65.7 RFU for AGE-BSA (1 mg/mL). The SDS-PAGE analysis were in accordance with gel filtration pattern and has shown the formation of an AGE-BSA monomer of 77.049 kDa and a 155.84 kDa dimer whereas the monomer of control BSA was of 68.66 kDa.[21]

Propagation of glycation process in the absence and presence of 0.1 mMCu^{2+}

The pepsin-soluble collagen (PSC) was separated by gel filtration and the elution pattern revealed four peaks with molecular weights of: 34.67, 48.32, 70.0 and 111.71 kDa, corresponding to retention volumes of: 15.70, 14.18, 13.14 and 12.01 mL, respectively (Fig. 1 B).

The gel filtration elution pattern of control sample (mixture AGE-BSA + PSC, 0 day incubation) in the absence or presence of 0.1 mM Cu^{2+} showed three important peaks corresponding to the 161.46 kDa AGE-BSA dimer (retention volume 10.81 mL), 83.29 kDa AGE-BSA monomer (retention volume 12.76 mL) (Fig. 1A and C) and 34.6 kDa collagen peptides (15.70 mL retention volume) (Fig. 1B and C).

In the absence of 0.1 mM Cu^{2+} after 10 days of the mixture incubation, the chromatographic pattern revealed a slight modification which consisted in a 1.8 fold increase of UV absorbance of 34.6kDa collagen peptides peak and of AGE-BSA dimer one and a slow decrease in their retention volumes to 15.67 and 10.79 mL respectively, that corresponds to a slight increase in the molecular weight (Fig. 2). The AGE-BSA monomer peak remained unchanged and a 405.08 kDa peak with a higher molecular weight appeared (retention volume 7.60 mL). After 6 weeks of incubation in the absence of 0.1 mM Cu^{2+}, large cross-linking compounds with molecular weights between 45.59 and 405.08 kDa (retention volume between 14.65 and 7.60 mL) with a maximum level UV absorbance associated to the 111 kDa (12.03 mL) product appeared. The collagen peptides peak with retention volume of 15.57 mL (35.62 kDa) had a lower increase in molecular weight compared to the control but it presented a higher increase in UV absorbance (Fig. 2).

The pattern of the relative fluorescence of proteins mixture fractions collected by gel filtration recorded at λ_{ex}335nm /λ_{em}385nm, showed after 10 days incubation in the absence of Cu^{2+} an increase of fluorescence level with a maximum at fractions 9-10 that correspond to collagen peptides (Fig. 3A). At λ_{ex}370nm/λ_{em}440nm the maximum level of fluorescence was reached at fraction 5 that corresponds to the AGE-BSA dimer (Fig. 3B). After 6 week mixture incubation in the absence of Cu^{2+} the fluorescence at λ_{ex}335nm /λ_{em}385nm revealed a 9 fold increase at fractions 5–6, that corresponds to AGE-BSA dimer and a 4.7 fold increase at fractions 9–10, which correspond to 35.6 kDa collagen peptides peak, compared to control. At 370 nm excitation wavelength the maximum increase of florescence level was maintained at fraction 5 and 9, of 12 and 4.5 times respectively (Fig. 3).

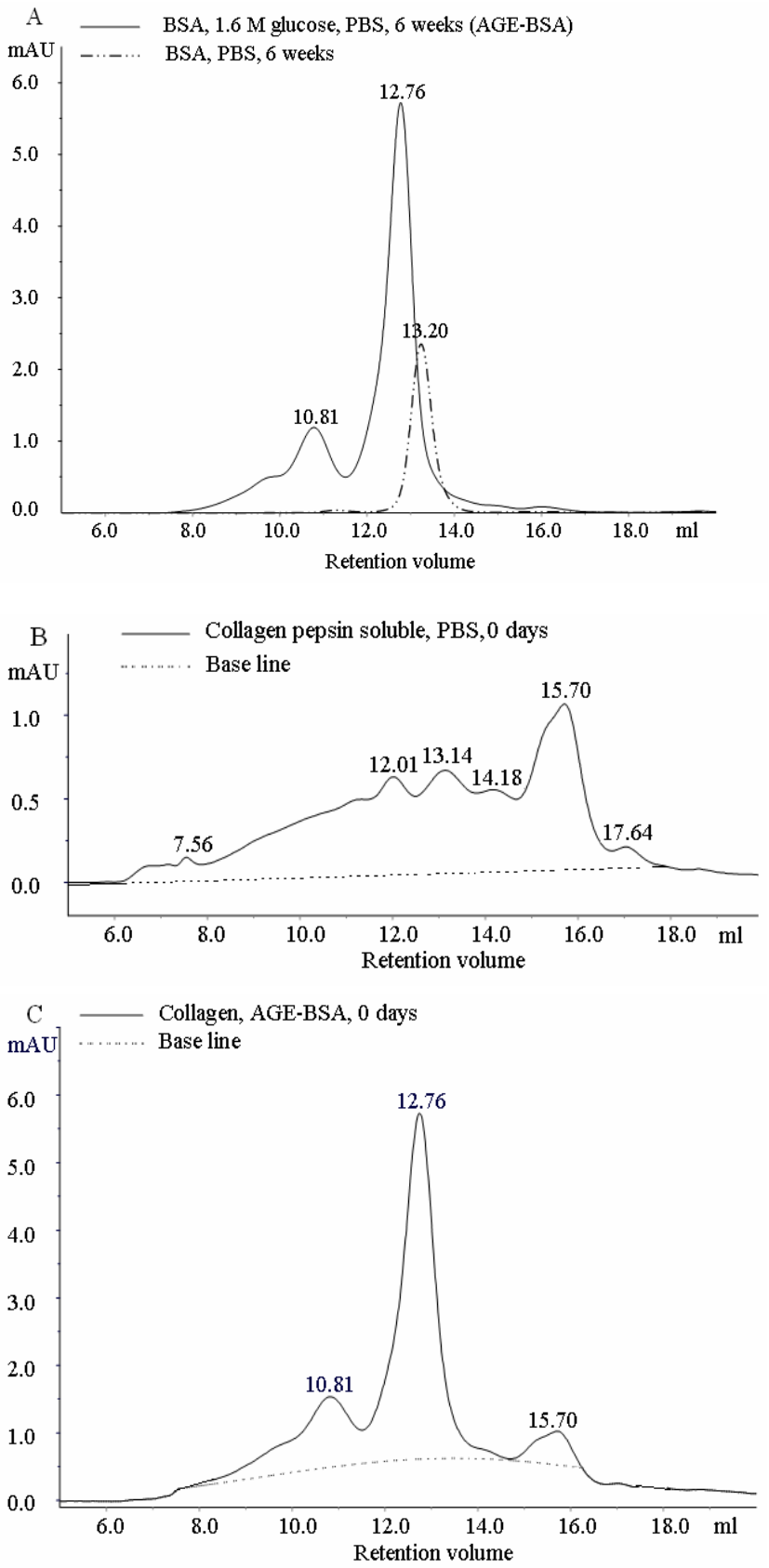


Fig. 1 – FPLC separation on Superdex 200 HR 10/30 column of : A) 2.5 µg AGE-BSA/ 100 µL injection volume (BSA glycated with 1.6 M D-glucose, 6 weeks at 37˚C in 10 mM PBS, pH 7.4) and 2.5 µg BSA/ 100 µL injection volume (6 weeks incubation of BSA at at 37˚C in 10 mM PBS, pH 7.4 – unglycated BSA); B) 150 µg PSC/100 µL injection volume and C) 150 µg PSC +2.5 µg AGE-BSA/ 100 µL injection volume.

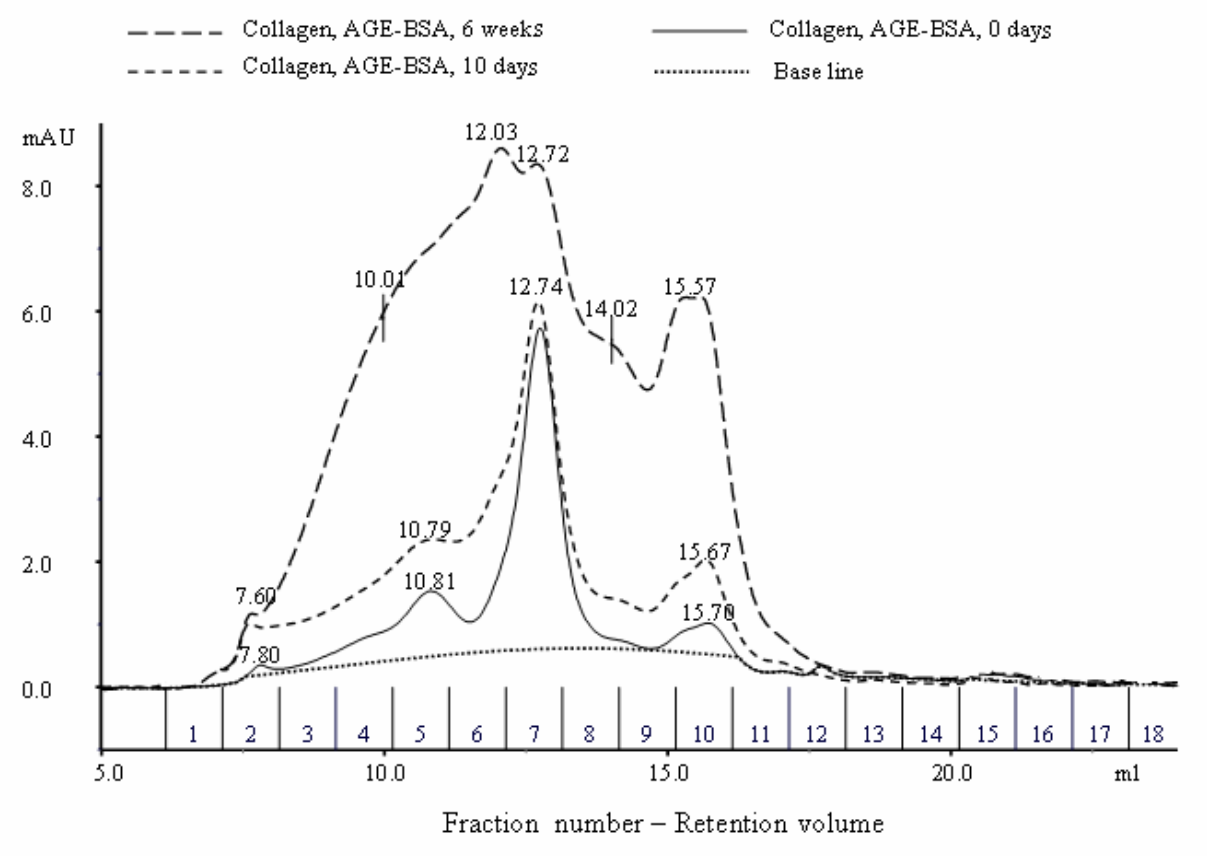
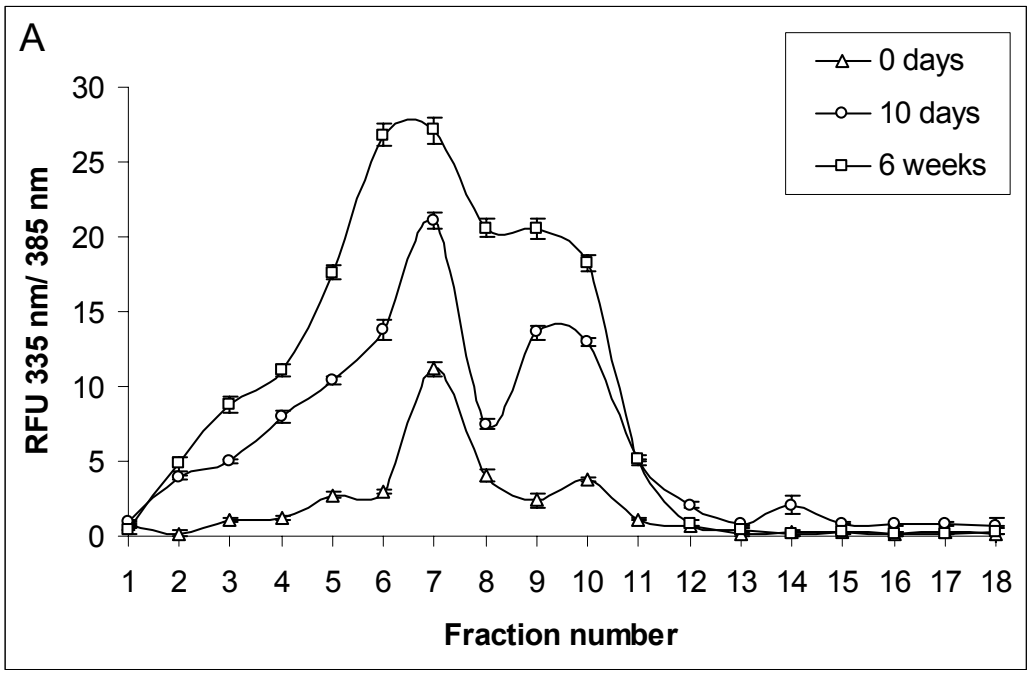


Fig. 2 – FPLC elution pattern of collagen peptides glycated with AGE-BSA in the absence of 0.1 mM Cu^{2+}.



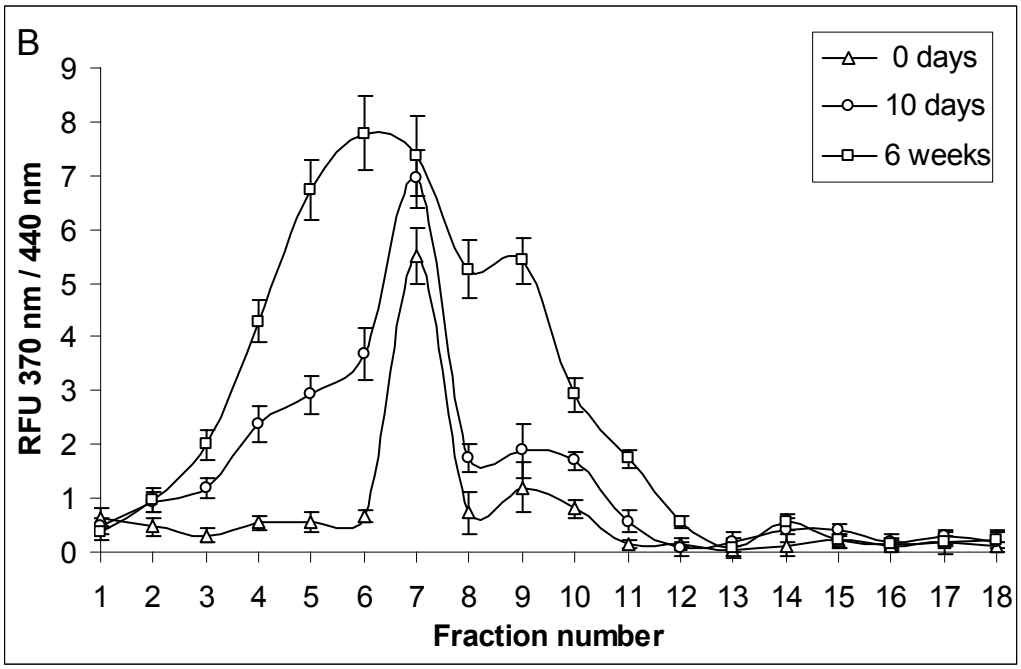


Fig. 3 – Relative fluorescence recorded at A) λ_{ex}335nm /λ_{em}385nm and B) λ_{ex}370nm /λ_{em}440nm of collagen peptides glycated with AGE-BSA in the absence of 0.1 mM Cu^{2+} fractions separated by gel filtration. The data are shown as the mean ± SD for three independent experiments run in triplicate each time.

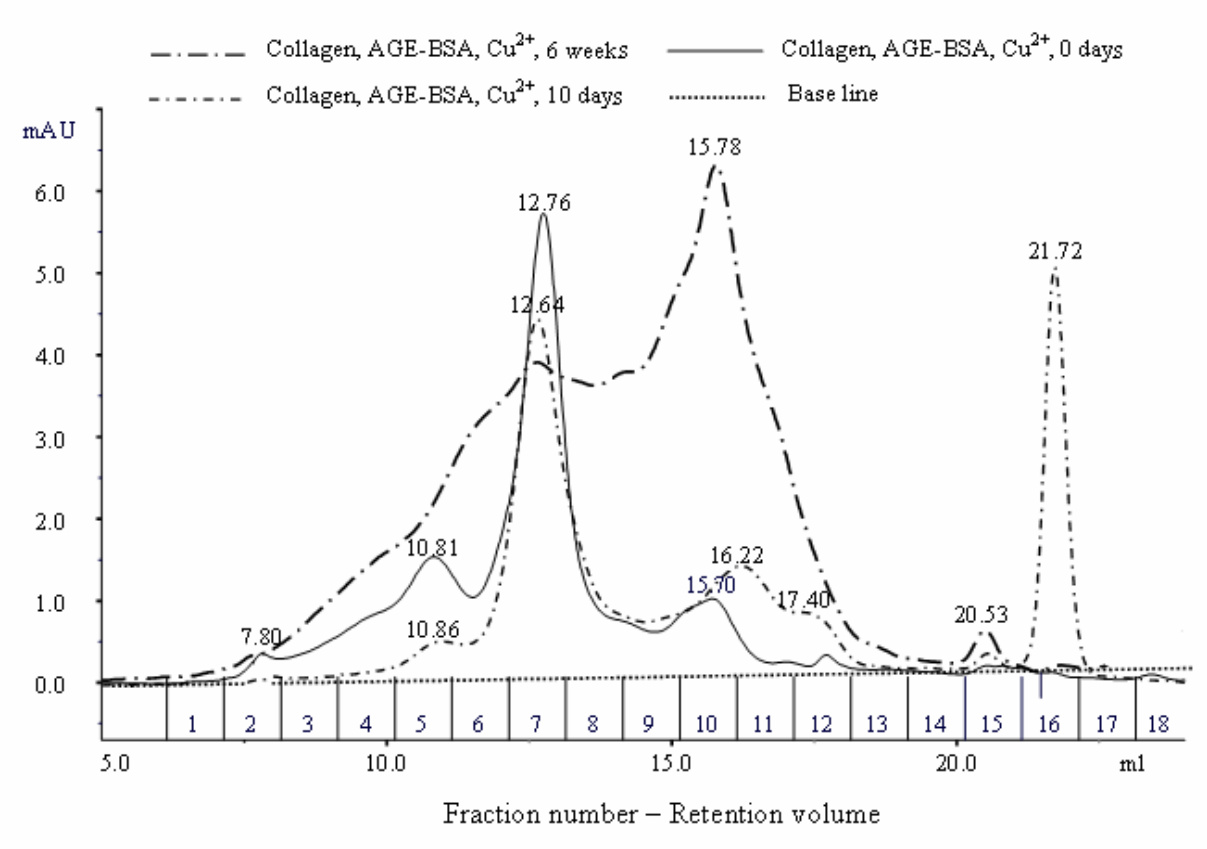


Fig. 4 – FPLC elution pattern of collagen peptides glycated with AGE-BSA in the presence of 0.1 mM Cu^{2+}.

The elution pattern of the collagen peptides glycated for 10 days with AGE-BSA in the presence of 0.1 mM Cu^{2+} differs significantly from that obtained in the absence of Cu^{2+} (Fig. 4). Thus, it showed the appearance of new peaks with retention volumes of 17.40, 20.53 and 21.72 mL, that correspond to lower molecular weights of 27.63, 23.10 and 21.50 kDa, the decrease of the molecular weight of collagen peptides of 34.63 kDa (15.70 mL) until 30.6 kDa (16.22 mL), and significant decrease in UV absorbance of AGE-BSA dimmer was noticed. The extension of incubation time of collagen peptides with AGE-BSA and 0.1 mM Cu^{2+} until 6 weeks had generated the disappearance of low molecular weight peptides peaks and the formation of a molecular aggregates with molecular weight between 34.02–375.21 kDa that correspond to a retention volume between 15.78 and 7.80 mL with a maximum UV absorbance for the 34.02 kDa product (Fig. 4). The new formed peaks after 10 days of incubation in the presence of 0.1 mM Cu^{2+} presented a higher fluorescence level at λ_{ex}335nm /λ_{em}385nm compared with the one recorded at λ_{ex}370nm/λ_{em}440nm. After 6 weeks incubation in the presence of Cu^{2+} at both excitation wavelengths the maximum fluorescence recorded corresponded to a 34.02 kDa product (retention volume 15.78 mL) (Fig. 5).

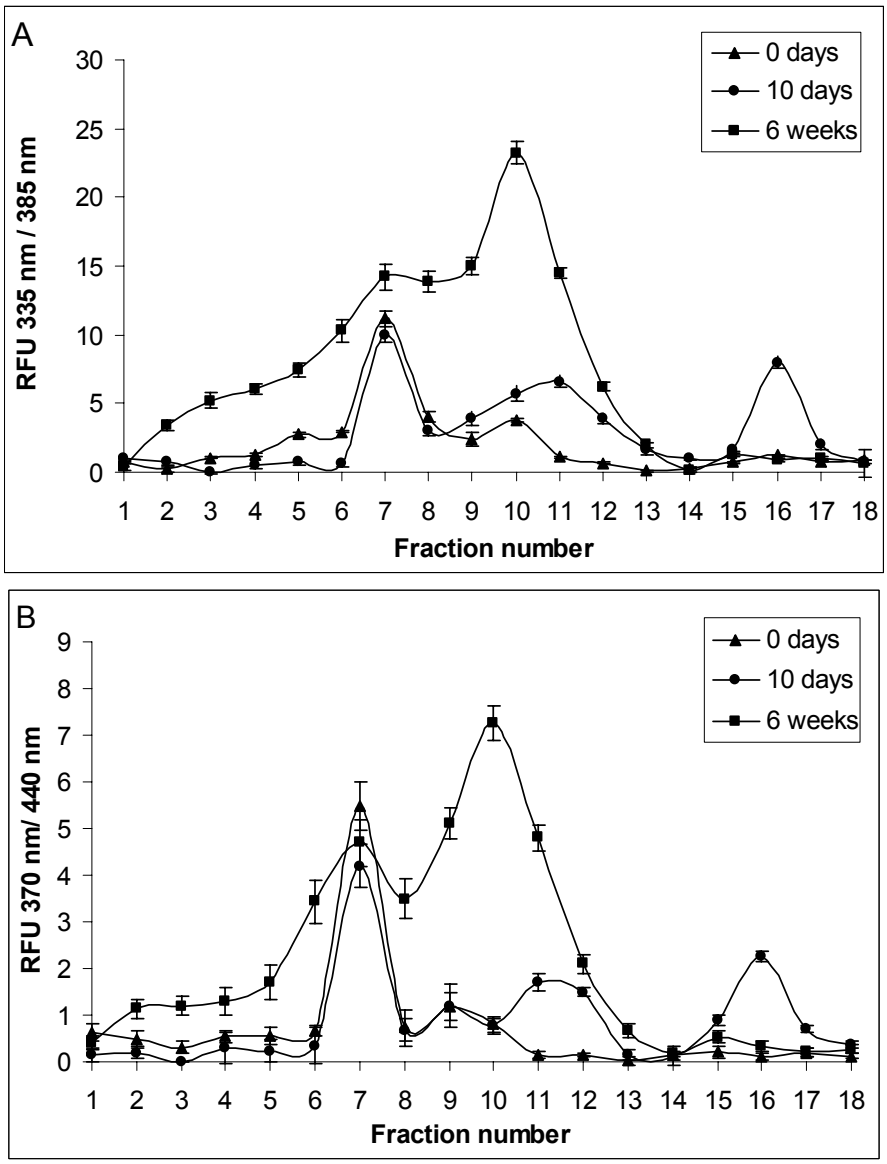


Fig. 5 – Relative fluorescence recorded at A) λ_{ex}335nm /λ_{em}385nm and B) λ_{ex}370nm /λ_{em}440nm of collagen peptides glycated with AGE-BSA in the presence of 0.1 mM Cu^{2+} fractions separated by gel filtration. The data are shown as the mean ± SD for three independent experiments run in triplicate.

DISCUSSION

Our work studied the effects of Cu^{2+} on the cascade of *in vitro* propagation of the glycation protein process in the absence of reducing sugars and in the presence of glycated BSA. The glycation of protein with reducing sugars in the presence or in the absence of transitional metal was intensively studied [13, 22-25] but the possibility that soluble AGEs can attack unmodified protein, and the role of small amounts of transitional metals in this process have not been rigorously explored.

The chromatographic pattern of collagen peptides incubated 10 days with AGE-BSA in the absence of 0.1 mM Cu^{2+} showed that the cross-linking process was slow; the molecular weight of peaks increased with few hundred Da, but the increase of fluorescence level of collagen peptides peaks and AGE-BSA dimmer ones was considerable. This may be due to the existing early glycation products (Schiff base and Amadori product) of glycated BSA which can undergo sequential reactions such as rearrangements, oxidation and fragmentation that probably form reactive dicarbonyl compounds like glyoxal, methylglyoxal and 3-deoxyglucosone and short chain sugars.[26, 27] These very reactive products can attack lysine and arginine residues of peptides and finally form intra- or intermolecular fluorescent AGEs like pentosidine.[28] The 405.08 kDa compound had associated a lower fluorescence, probably because glucosepan, a non fluorescent cross-linking compound is the major product formed from Amadori products derivates from glucose. Recently, Biemel [29] demonstrated that the dideoxyosone *N*6-(2,3-dihydroxy-5,6-dioxohexyl)-L-lysinate is not only the pivot in the formation of glucosepane but also it is expected to be the source for numerous other Maillard products. The increase of λ_{ex}370nm/λ_{em}440nm fluorescence level of fraction 5 that corresponds to AGE-BSA dimer could be explained by the formation of "crossline-like compound".

In our opinion, the chromatographic and fluorescence profiles of collagen peptides incubated for 6 weeks with AGE-BSA in the absence of 0.1 mM Cu^{2+} revealed the continuation of AGEs formation and cross-linking process and demonstrated that soluble AGEs could be the cause of collagen cross-linking noticed in diabetes, even in the absence of free glucose.

The differences between the chromatographic pattern of the collagen peptides glycated with AGE-BSA in the presence and absence of 0.1 mM Cu^{2+} could be explained by the fragmentation and conformational alteration of the peptides, which occur in the presence of the transitional metal. We have noticed a similar fragmentation of collagen peptides glycated with reducing sugars in the presence of Cu^{2+} after 10 days of incubation.[13] These modifications could implicate Schiff bases and Amadori products bound to AGE-BSA in the formation of hydroxyl radical, via hydrogen peroxide, and α-oxoaldehyde by metal-catalyzed oxidation of ene-diols. Thus, hydrogen peroxide could be formed in the oxidation of glycoaldehyde to glyoxal. This compound can arise from several steps: the retroaldol condensation of Schiff base adduct probably form glycoaldehyde, that can enolize to 1,2-ene-diol, and an oxidative Cu^{2+}-catalyzed process by which this compound is transformed to glyoxal with the formation of superoxid anions. These anion radicals can then dismutate to hydrogen peroxide and the redox chemistry of copper ion may catalyze the formation of hydroxyl radical. Amadori products can, also, form H_2O_2 via two pathways. One is the 1,2-enolization pathway which can lead to glucosone and H_2O_2. The other one is the 2,3-enolization pathway which, under oxidative conditions, is thought to generate H_2O_2 and CML by Bayer-Villiger oxidation.[30] CML, a product of glycoxidation of proteins can be formed from glyoxal, too.[31] This compound has a negative charge at physiological pH and may be implicated in the conformational change of glycated collagen peptides after 10 days incubation in the presence of 0.1 mM Cu^{2+} with AGE-BSA. Furthermore, copper ions can be complexated by Nε-fructoselysine and CML.[16]

The increase of relative fluorescence at λ_{ex}335nm /λ_{em}385nm of glycated collagen peptides during 10 days in the presence of AGE-BSA and Cu^{2+} was probably due to the accelerated formation of pentosidine in this redox system.

After six weeks of peptides collagen incubation with AGE-BSA and 0.1 mM Cu^{2+}, the chromatographic patterns showed that the crosslinking process was in progress, the peptides with lower molecular weight disappeared, compared to the 10 days glycation in the same condition, characterized by the fragmentation process. It seems that Cu^{2+} complicated the cascades of glycation reactions of collagen by soluble AGEs and delayed the formation of molecular aggregates with high molecular weight. This situation was due, probably, to the increased oxidative process in the presence of copper ions

that has generated hydroxyl radicals which induced protein fragmentation in the early step of glycation. At the same time, Cu^{2+} could favour the formation of CML from Amadori products or from glyoxal and diminish the formation of crosslink AGEs by Amadori pathway.

The effect of soluble AGEs on collagen cross-linking is independent of the presence or absence of Cu^{2+} and once AGEs are formed, it could induce cross-linking of collagen even in the absence of both glucose and Cu^{2+}.

This *in vitro* experiment has proven the progression of protein cross-linking in the presence of glycated protein even in the absence of sugar and this phenomenon could explain why in diabetes certain complications progress during periods of good glycemic control.

MATERIALS AND METHODS

MiliQ water and all buffers used for incubation were passed through a Chelex-100 resin column (Bio-Rad) to remove any trace metal ions and to reduce at maximum their undesirable influence in the glycation process.

Preparation of AGE-BSA. Soluble glycated bovine serum albumin (AGE-BSA) was synthesized in *vitro*, according to the methods previously described by us.[21] Solutions of 1.6 M D-glucose (Sigma-Aldrich) and 100 mg/mL bovine serum albumin (BSA Sigma, RIA grade, fraction V) have been co-incubated in sterile 10 mM phosphate buffer saline (PBS), pH 7.4 (Invitrogen), for 6 weeks under aerobic conditions at 37˚C, in the presence of 3mM NaN_3 (Merck KGaA) to prevent bacterial growth. Controls with BSA solution only were simultaneously kept under the same conditions. After 6 weeks the free sugar was removed by dialysis against 10 mM PBS, pH 7.4 for 48 h. The protein concentration was determined through the Bradford method.[32] The formation of the AGE-BSA complex was analyzed by Fast Protein Liquid Chromatography (FPLC), sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fluorescence spectroscopy.

Glycation of collagen. The limited pepsin digestion was conducted by treating about 100 mg of type I collagen from bovine Achilles tendon (Sigma-Aldrich) with 1mg/mL pepsin (Sigma-Aldrich) for 20 hours in 0.5 mol/L acetic acid (Sigma-Aldrich) at 37 ˚C in a shaking incubator, as previously described.[13, 33] The resulting suspension was centrifuged at 12000xg for 30 minutes at 10 ˚C. The extracted collagen into the supernatant was referred to as the pepsin-soluble collagen (PSC). The PSC was successively dialyzed against 0.01 M acetic acid, MilliQ water and 10 mM PBS pH 7.4 (Invitrogen). The PSC concentration was estimated by measuring the amount of hydroxiproline according to the method of Stegemann and Stalder.[34] Hydroxiproline was assumed to make up to 14% of the collagen by weight. Aliquots of PSC (24 mg/mL) were incubated with 0.5 mg/mL AGE-BSA in 10 mM PBS, pH 7.4 containing 3 mM NaN_3 (Merck KGaA), in the presence or absence of 0.1 mM Cu^{2+} for ten days and 6 weeks respectively, under aerobic conditions at 37°C. The samples were kept in dark, in conical flasks, in a shaker incubator Certomat BS-T Beckman Coulter at 50 rpm. Individual samples were removed at desired times and frozen at - 20°C until analysis was performed. The cross-linking of collagen induced by AGE-BSA and the development of fluorescent related products were assessed via FPLC and fluorescence spectroscopy of FPLC collected fractions.

Fast protein liquid chromatography. A FPLC automated system (ÄKTA FPLC- Amersham Pharmacia Biotech) with a size exclusion column (Superdex 200 HR 10/30) was used for the separation of glycated and unglycated proteins. Samples of 100 µL, with a concentration of: 2.5 µg BSA, 2.5 µg AGE-BSA, 150 µg collagen and mixtures of 150 µg collagen + 2.5 µg AGE-BSA prepared in 10 mM PBS pH 7.4 were loaded on the column and eluted with 5% acetonitrile and 10 mM PBS pH 7.4 mixture, at a constant flow rate of 0.8 mL/min. The protein absorption was automatically recorded at 280 nm and fractions of 1 mL were collected.

Fluorescence assays. The fluorescence detection of Maillard compounds and pentosidine-like products was spectrofluorometrically recorded at λ_{ex}370nm/λ_{em}440nm and at λ_{ex}335nm /λ_{em}385nm respectively against 10 mM PBS, pH 7.4 as blank [25] for every protein mixture (collagen + AGE-BSA) collected fraction. For the characterization of AGE-BSA, the fluorescence emission spectra between 380 and 600 nm (370 nm excitation) and between 345 and 600 nm (335 nm excitation) were scanned using a JASCO FP 750 spectrofluorometer, as previously described.[21]

Statistical analysis. All our experiments were done in triplicates. The samples were run in triplicate each time and the results are expressed as mean ± SD.

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