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GLYCATION OF COLLAGEN: THE INFLUENCE OF AMINOGUANIDINE AND CU^{2+} IN THE FORMATION OF FLUORESCENT AGEs

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ABSTRACT. In diabetes and aging long-lived proteins such as collagen are non-enzymatically modified by reducing sugars. The major initial product is a fructose-lysine compound, which results from glycation of ε-amino groups on lysine residues. In the subsequent Maillard reactions, products known as advanced glycation end products (AGEs) are formed and accumulates with age. Glycation may also be modulated by sugar oxidation through reactive dicarbonyl compounds and transitional cathions may accelerate this process. A hydrazine compound, aminoguanidine has been shown to inhibit the characteristic changes in diabetes and in the same time reduce the formation of AGEs. In our work we revealed an activation by Cu^{2+} and an inhibition by aminoguanidine on fluorescent AGEs formation following collagen glycation with ribose.

KEY WORDS. collagen, glycosylation, AGEs, aminoguanidine

INTRODUCTION

The Maillard reactions or "nonenzymatic browning" are a complex series of reactions between reducing carbohydrates with lysine side chains or amino terminal groups of proteins [1]. In the first step of this process rather labile Schiff bases are formed which generally rearrange into more stable Amadori products. These Amadori compounds are slowly degraded in complex reaction pathways via

dicarbonyl intermediates to a large group of compounds subsumed summarily under the term of "advanced glycation end products" (AGEs); this overall reaction sequence proceeds both *in vitro* and *in vivo* [2].

Glycation may also be modulated by glucose oxidation. Through sequential reduction of a transitional metal cathions and molecular oxygen glucose auto-oxidizes to highly reactive dicarbonyl compounds that can bind to proteins and also generates free radicals [3, 4].

In long live tissue proteins, such as collagen and lens crystallines, these chemical modifications accumulate with age and may contribute to pathophysiologies associated with aging and long term complications of diabetes and atherosclerosis [5]. With age, collagen becomes less soluble, more crosslinked, more glycosylated and it accumulates yellow and fluorescent compounds. In diabetes, several of these changes occur at an earlier age, suggesting an apparent acceleration of the aging process [6]. Compared with age-matched nondiabetics, collagen from diabetics is less soluble, is more resistant to digestions by collagenase, has more pepsin-releasable high molecular weight peptides and is more nonenzymatically glycosylated [7].

A hydrazine compound, aminoguanidine has been shown to inhibit the characteristic changes in diabetes and in the same time reduce the AGEs [8]. The mechanism of action is still controversial. Originally it was believed that aminoguanidine reacts only with the aldehyde groups on Amadori products [9] but it has recently been suggested that it reacts with the released oxidation products, such as 3-deoxyglucosone [10].

The aim of this work is to reveal the influence of aminoguanidine and Cu^{2+} on fluorescent AGEs formation in the glycation of collagen with ribose.

MATERIAL AND METHODS

Preparation of collagen samples

Achilles collagen type I (Sigma) was suspended in 0.5 mol/l acetic acid with 1mg/ml pepsin and digested over 20 hours at 37 ˚C. The samples were centrifuged at 3400 rpm for 20 minutes. After centrifugation the supernatant-pepsin-soluble fraction (P-fraction) was adjusted to pH 8 with cold 5M NaOH to neutralize the pepsin and dialysed at 4^{o}C against 0.01M PBS pH 7.4. Collagen was estimated by measuring the amount of hydroxiproline according to the method of Stegemann and Stalder [11]. Hydroxiproline was assumed to make up 14 % of the collagen by weight.

In vitro glycation

Aliquots of solubilized collagen (24 mg/ml) were incubated with 200 mM ribose in 0.01M PBS, pH 7.4 containing 3 mM NaN_3, with or without 10 mM aminoguanidine for 6 weeks at 37° C and for ten days at 37° C in the presence of 100 µM Cu^{2+} with or without 10 mM aminoguanidine. Free sugar was removed by dialysis against MilliQ water.

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 separation of glycated collagen. Samples (313 µg collagen/100 µl injection volume in 0.01M PBS

pH 7.4) were injected and eluted in 5% acetonitrile in 0.01M PBS pH 7.4, at a constant flow rate of 0.8 ml/min. Fractions of 1ml were collected for fluorescence assay. The absorption was automatically recorded at 280 nm.

Collagen associated fluorescence

Fluorescence emission spectra were recorded at 335 and 370nm excitation wavelengths for each collected fractions (JASCO FP 750 spectrofluorometer).

RESULTS AND DISCUSSION

Chromatographic profile (Fig.1) of 6 weeks ribose glycated collagen shows one peak with the retention volume slightly decreased but with a major increase in absorbance at 280nm compared to the corresponding peak from unglycated collagen (20.8 mAu vs. 1.6 mAu). For this peak (maxima in fraction 10 both in glycated and unglycated collagen chromatographic profile) we obtained a significant increase in AGEs related fluorescence for glycated collagen (Fig. 1, 2, Table 1). Modifications in UV absorbance and fluorescence could be explained by formation of heterocyclic compounds from Lys and Arg residues and form ribose derived deoxyglucosones. From these compounds only pentosidine with $\lambda_{ex}335/\lambda_{em}385$ nm fluorescence parameters is well characterized [12]. A broad fluorescence spectra center at $\lambda_{ex}370/\lambda_{em}440$ nm was obtained, due to many others possible compounds such as pyrroles, pyridines, furans, pyrazines, imidazoles with structures not well defined. The UV absorbance of fraction 10 of glycated collagen in the presence of aminoguanidine is approximately half (8.4 mAu) of the corresponding value in the absence of aminoguanidine (Fig. 1). Pentosidine linked fluorescence remains high, as in collagen incubated only with ribose but $\lambda_{ex}370/\lambda_{em}440$ nm fluorescence dropped to values comparable to unglycated collagen (Fig. 2, Table 1). Aminoguanidine may form substituted triazynes with Amadori products with fluorescence spectra similar to pentosidine, blocking the pathways to fluorescent and nonfluorescent AGEs.

Modifications in chromatographic patterns and fluorescence spectra obtained for collagen incubated only 10 days with ribose and Cu^{2+} resembles those obtained for 6 weeks incubation of collagen with ribose (Fig. 3, 4). In the presence of oxygen and transitional metals cathions, ribose can rapidly form deoxyglucosones (in hours) through autoxidation. Further, the highly reactive deoxyglucosones reacts with amino groups of proteins to form fluorescent and non fluorescent AGEs [7]. On the other hand formation of the deoxyglucosones through the Amadori pathway is a much slower process; after the initial molecular rearrangement of the Schiff base into an Amadori product this adduct undergo degradations, dehidratations and other complex rearrangements which finally form very reactive intermediates such as 1,3 and 4-deoxygluocosones [13]. These could explain the higher pentosidine related fluorescence observed (Table 2) after 10 days of incubation of collagen in the presence of Cu^{2+} than after 6 weeks incubation only with ribose.

In the presence of aminoguanidine the formation of AGEs related fluorescence at $\lambda_{ex}370/\lambda_{em}440$ nm was inhibited to approximately 88% of the corresponding value of 10 days incubation of collagen with Cu^{2+}. The decrease in $\lambda_{ex}335/\lambda_{em}385$ nm

fluorescence in the presence of aminoguanidine (Fig. 4, Table 2) was greater than in the corresponding 6 weeks incubation, indicating that less substituted triazynes were formed on the Amadori pathway.

CONCLUSION

The initial steps in collagen glycation are complex processes involving oxidation of a reducing sugar and formation of Amadori products. Both pathways generate highly reactive deoxyglucosone which in turn react with amino groups of proteins to form fluorescent and non fluorescent AGEs. Transitional metal cathions such as Cu^{2+} appear to accelerate the formation of deoxyglucosones, shortening the time necessary for AGEs development. Aminoguanidine inhibit the pathways to fluorescent and nonfluorescent AGEs through the formation of substituted triazynes instead of reactive pentosidine. Also, it is possible that aminoguanidine reacts with free deoxyglucosones formed by Cu^{2+} activated autoxidation of ribose, blocking them to further react with the amino groups on collagen.

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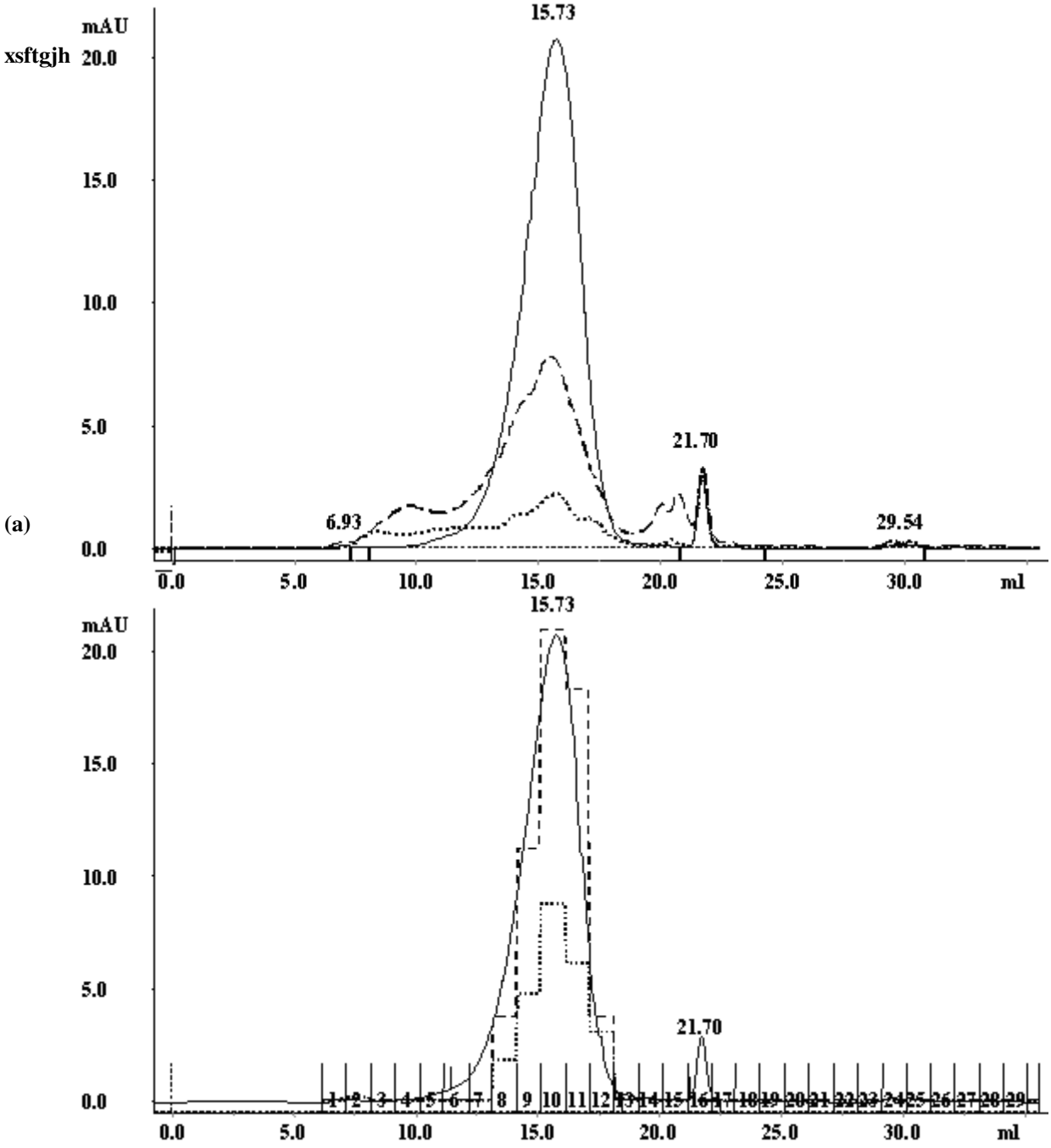

Figure 1. Influence of aminoguanidine (AG) on collagen glycation: 6 weeks incubation at 37^0C in 10mM PBS pH 7.4, 313 μg collagen/100 μl injection volume

a) FPLC separation of collagen samples on Superdex 200 HR 10/30 column: collagen 24mg/ml + 200mM Rib (—); collagen + 200 mM Rib and 10 mM AG (- -); unglycated collagen (…)

b) Fluorescence of glycated collagen (200 mM Rib) fractions; (- -) 335/385nm, (…) 370/440nm

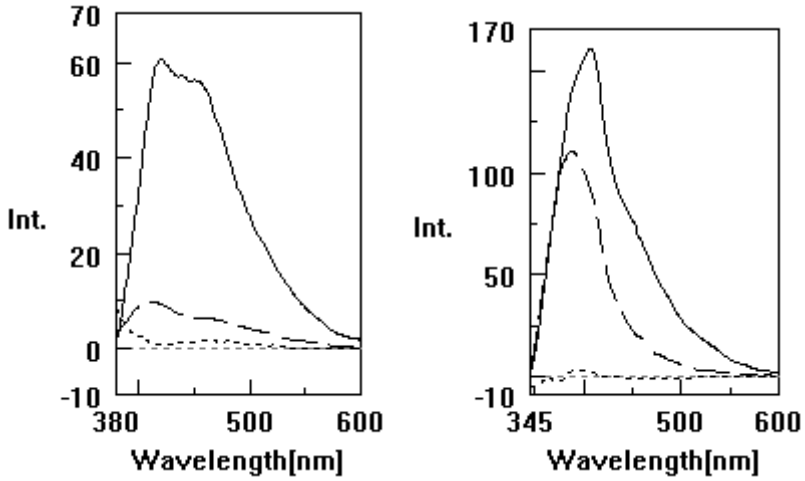

Figure 2. Fluorescence emission spectra of collagen fractions; 6 weeks incubation at 37^{0}C in PBS 10mM pH 7.4; collagen 24mg/ml + 200mM Rib (—); collagen + 200 mM Rib and 10 mM aminoguanidine (- -); unglycated collagen (…)

a) Fluorescence emission spectra of fractions 10 of collagen samples at 335 nm excitation

b) Fluorescence emission spectra of fractions 10 of collagen samples at 370nm excitation

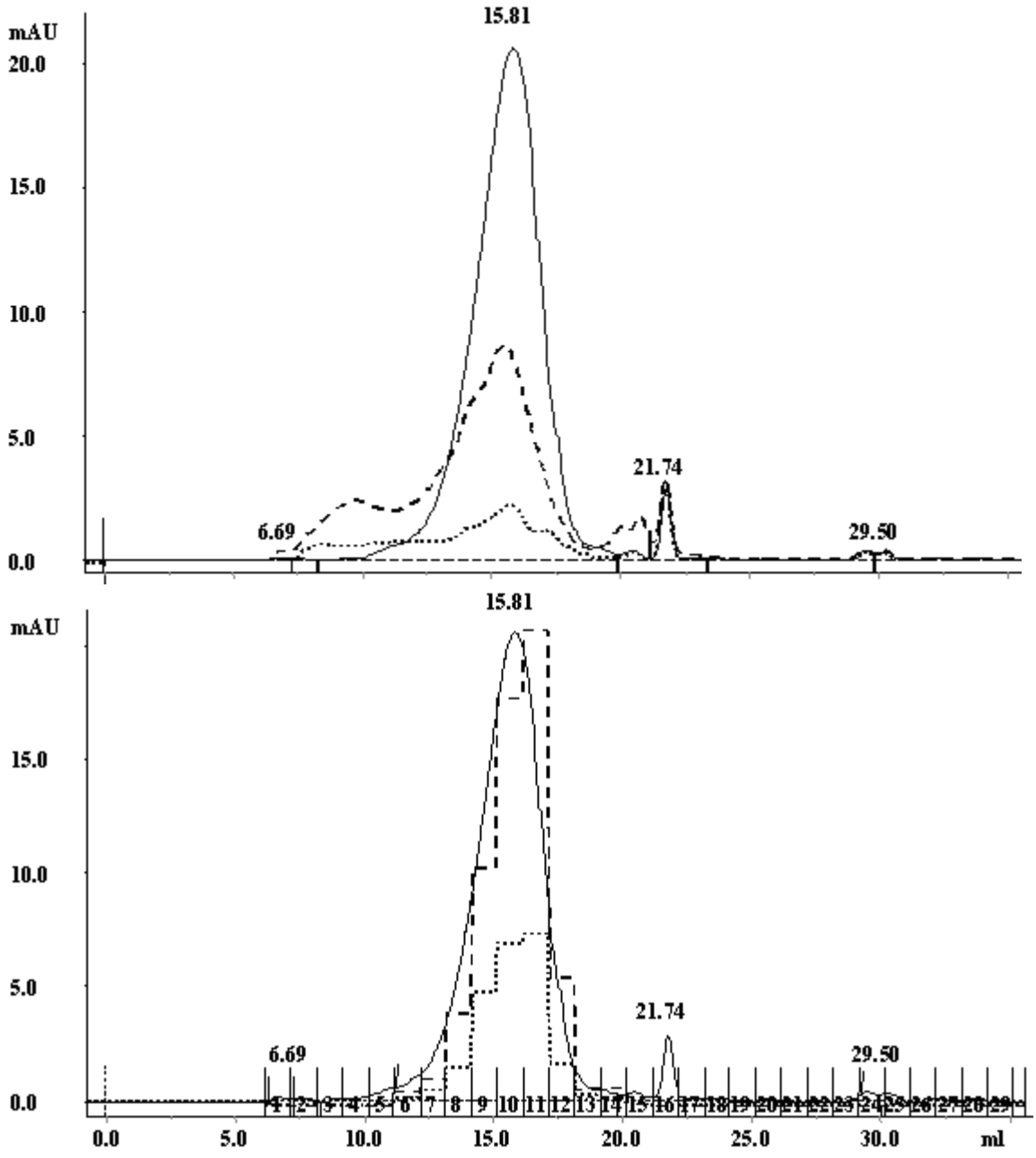

Figure 3. Influence of aminoguanidine (AG) and Cu^{2+} on collagen glycation: 10 days incubations at 37^0C in 10mM PBS pH 7.4, 313 μg collagen/100 μl injection volume

a) FPLC separation of collagen samples on Superdex 200 HR 10/30 column: collagen 24mg/ml + 200mM Rib + Cu^{2+} 100 μM (—); collagen + 200mM Rib + Cu^{2+} 100 μM and 10 mM AG (- -); unglycated collagen (…)

b) Fluorescence of glycated collagen (200 mM Rib) fractions; (- -) 335/385nm, (…) 370/440nm

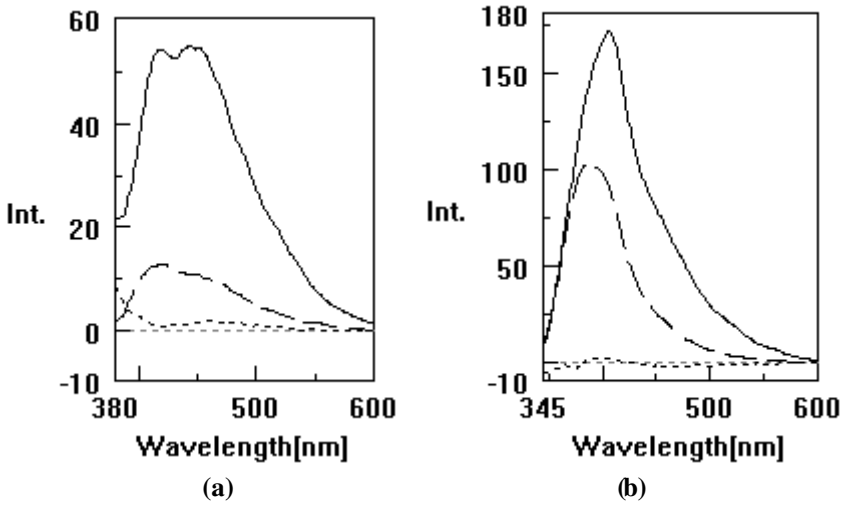

Figure 4. Fluorescence emission spectra of collagen fractions; 10 days incubation at 37^{0}C in PBS 10mM pH 7.4; collagen 24mg/ml + 200mM Rib + Cu^{2+} 100 μM (—); collagen + 200mM Rib + Cu^{2+} 100 μM and 10 mM AG (- -); unglycated collagen (…)

a) Fluorescence emission spectra of fractions 10 of collagen samples at 335 nm excitation

b) Fluorescence emission spectra of fractions 10 of collagen samples at 370nm excitation

Table 1. Fluorescence emission maxima of glycated collagen fractions 10 separated by FPLC (6 weeks incubation)

Sample	**λ_{ex}335/ λ_{em}385nm (RFU)**	**λ_{ex}370/ λ_{em}440 nm (RFU)**
Collagen, unglycated	3.7	1.4
Glycated collagen: 200mM Rib	135	57
Glycated collagen: 200mM Rib, 10mM aminoguanidine	107	6.2

Table 2. Fluorescence emission maxima of glycated collagen fractions 10 separated by FPLC (10 days incubation)

Sample	**λ_{ex}335/ λ_{em}385 nm (RFU)**	**λ_{ex}370/ λ_{em}440 nm (RFU)**
Unglycated collagen	3.7	1.4
Glycated collagen: 200mM Rib, Cu^{2+} 100μM,	161	58
Glycated collagen: 200mM Rib, Cu^{2+} 100μM, 10mM aminoguanidine	57	6.9