Glycative Stress Research

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Original article

Evaluation *in vitro* of AGE-crosslinks breaking ability of rosmarinic acid

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Abstract

***Objective*:** Although numerous works were carried out to evaluate the ability of various molecules to break AGE, none of them, to our best knowledge, were done to evaluate the ability of these molecules to break protein crosslinks considered globally. The objective of this work was to show clearly the albumin polymerization elicited by reaction with ribose and the ability of a polyphenolic acid extracted from rosemary (*Rosmarinus officinalis*), rosmarinic acid, to break preformed albumin polymers.
***Methods*:** Albumin was glycated by incubation with ribose and the resulting albumin polymers were evaluated by the size exclusion Chromatography (SEC) and fluorimetry. After elimination of ribose by dialysis, the proteins were treated by rosmarinic acid, aminoguanidine, carnosine, and Alagebrium (ALT-711; Alteon) as a positive control. Reversion of glycation was measured by the ratio between polymerized and native albumin before and after treatment by the reversing molecules.
***Results*:** Rosmarinic acid was shown to be an as good crosslinks-breaker as Alagebrium. At the opposite, aminoguanidine and carnosine, the glycation reaction inhibitors do not produce a significant reversion of albumin polymerization.
***Discussion*:** Albumin polymerization elicited by glycation by ribose can be measured by SEC and fluorimetry of the resulting polymers. We have shown that rosmarinic acid is able to reverse this polymerization to a similar extent to Alagebrium.
***Conclusion*:** Rosmarinic acid seems a promising molecule, known as safe and able *in vitro* to break protein AGE-related crosslinks approximately as efficiently as Alagebrium, considered until now as the reference molecule in this field. It could be a promising treatment of diabetics, skin aging, and of elderly impaired vessels-related diseases as nephropathy, neuropathy and retinopathy.

***KEY WORDS*:** advanced glycation end products (AGEs), rosmarinic acid, crosslink, AGE-breaker.

Introduction

Proteins nitrogen radicals and reducing sugars are involved in the so-called Maillard reaction, whose mechanism was first described in 1953 by Hodge [1)], to produce condensed products. Advanced glycation end-products (AGEs) are the final products of complex chemical non enzymatic reactions leading to the formation of intra and inter-molecular cross-links between adjacent proteins, impairing their functionalities and modifying their physical properties [2,3)]. Crosslinking of proteins is a pathophysiological mechanism that has been recognized as a causative factor in diabetic complications and age-related diseases [4)] like nephropathy, retinopathy, vascular functionality impairments and skin loss of elasticity. In healthy individuals, loss of functionality of proteins increases slowly with age [5)]. In diabetic patients, AGE accumulation and protein crosslinking are accelerated due to high glucose concentration [6)].

Therapeutic approaches include prevention of AGE-formation with better control of blood sugar in diabetic individuals and/or use of AGE-inhibitors and use of AGE-breakers, molecules able to break protein AGE-related crosslinks. Today, numerous molecules are known to be able to inhibit the glycation process *in vivo* and *in vitro* [7)] at one step or the other, leading to a slow reduction of glycated proteins, mostly proteins with medium or fast turnover. Inhibition is far less efficient on slow turnover proteins like collagen in skin, joints or elastin in aorta wall. Discovering safe molecules able to break AGE-protein crosslinks would be a major progress toward the improvement of conditions related to aging and diabetes.

One of the major issues in finding such molecules is that glycation protein crosslinks are based on numerous molecules among them pentosidine, glucosepane and methylglyoxal-lysine dimer (MOLD), to cite a few [8)]. Thus, finding a universal AGE crosslink-breaker, able to cleave all the glycation crosslinks is unlikely. In 1996, a thiazolium derivative, Alagebrium or ALT-711 (Alteon Inc., Montvale, NJ, USA) was reported as a potent AGE-derived crosslinks breaker [9)], without describing the type of crosslink being broken by the molecule. Later in 2013, it was shown that it was able to break alpha-

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dicarbonyl groups present in Amadori products before formation of crosslinks [10].

Inter-molecular crosslinking of proteins leads to protein polymerization [11]. The extent of polymerization after reaction of proteins with reducing sugar can be considered as a global marker of AGE-related crosslinking. In this work, we describe a method *in vitro* to evaluate the rate of AGE-crosslinking in albumin and we show that rosmarinic acid (***Fig. 1***) presents glycated proteins crosslinks breaking ability.

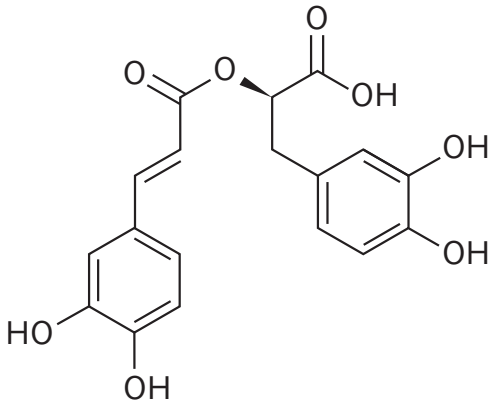

Fig 1. **Structure of rosmarinic acid.**
Molecular formula, C18H16O8; molecular weight, 360.3.

Materials and methods

Preparation of glycated crosslinked albumin

Reagents and materials used were as follows: bovine serum albumin (BSA), Fraction V ≥ 96 % (Sigma A9647, St. Louis, MO, USA); D (-) ribose, min. 99.0 % (Sigma R7500) ; acetic acid, 99.8 % (Acros ref 222140010, Acros Organics, Geel, Belgium); ammonium hydroxide (A.C.S. Sigma-Aldrich 22,122-8); dialysis tubing, benzoylated, Avg. Flat width 32 mm (1.27 inches) (Sigma D7884); Sephadex G-50 (Sigma G-50-80).

Glycation of albumin

Solutions were prepared in following conditions; BSA, 24 % (w/w) in ammonium acetate buffer at pH 7.4 and ribose solution, 30 % (w/w) in ammonium acetate at pH 7.4. Then, 250 g of BSA solution were mixed with 50 g of ribose solution, sterilized by filtration on a 0.2 µm membrane and left for 6 days at 37°C. The glycated BSA solution is dialysed against water for 4 days at room temperature to eliminate ribose and stop the glycation reaction.

Size Exclusion Chromatography (SEC)

Dialysed BSA solution is purified by SEC on Sephadex G50 in ammonium acetate buffer pH 7.4. The orange-brown fraction is collected.

Determination of albumin crosslink rate

Reagents and equipment for high-performance liquid chromatography (HPLC) were as follows; Water Chromasolv Plus for HPLC (Sigma-Aldrich 34877); HPLC Waters Integrity System with HP fluorescence detector (Hewlett Packard, Palo Alto, CA, USA); Low-Temperature Evaporative Light-Scattering Detectors (ELSD) (Sedex 55; SEDERE, Alfortville, France); HPLC column (TSK Gel Super SW3000; Tosoh Bioscience, Tokyo, Japan). Analysis conditions were; flow rate, 0.3 mL/min ; column temperature, 25 °C ; isocratic 24 min; ammonium acetate buffer, pH = 6.0; acetic acid, 0.5 mL; water, qs 100 mL; ammonium hydroxide, qs pH= 6.0; detection wave length; fluorescence (excitation 335 nm; emission 385 nm); injection, 10 µL of water diluted samples 1/2.5 (V/V).

Calculation of BSA crosslink rate and of crosslink breaking ability of molecules

Glycation of albumin by ribose elicits polymerization of albumin. The multiple polymers are then separated and evaluated globally from the total surface of the peaks corresponding to the different polymers. Relative concentration of BSA polymers is obtained by calculating their chromatogram areas ratio.

At_t, At_e = Total area of proteins before and after treatment by potential crosslinks breaker.

Ag_t, AG_e = Total area of protein polymers (crosslinked BSA) before and after treatment by potential crosslinks breaker.

Tg = crosslink ratio of glycated BSA before treatment by potential crosslinks breaker ($Tg = Ag_t/At_t$,).

Eg = crosslink ratio of glycated BSA after treatment by potential crosslinks breaker ($Eg = Ag_e/At_e$).

Percent crosslinks breaking ability of potential crosslinks breaker is given by the final formula:

$Dg = ((Tg/Eg) \times 100))$.

AGE-breaking ability of molecules

Test samples used were; Alagebrium (Alteon); L-carnosine (Sigma-Aldrich ref C9625); aminoguanidine bicarbonate (Sigma-Aldrich ref 109266); rosmarinic acid (Sigma-Aldrich ref R4033).

Results

Crosslinking breaking ability of tested molecules are shown in ***Table 1*** and diagram in ***Fig. 2***.

Discussion

After reaction with ribose, albumin is polymerized as shown by SEC. Percentage (%) of the monomer is converted into polymers. This polymerization is stable in time and can be reverted by Alagebrium and rosmarinic acid, both recovering approximately 50% of the monomer involved in polymerization. Rosmarinic acid can be extracted and purified from rosemary (*Rosmarinus officinalis*).

This results shows that beyond the Alagebrium ability to break alpha-dicarbonyl AGE radicals, it is also able to break preformed AGE-related albumin crosslinks. Rosmarinic acid produces approximately the same results, showing that a natural molecule with a long and in depth documented safety is able to reverse at least *in vitro* protein crosslinks which are at the origin of mechanical structure-based glycated protein property and functionality impairment.

Table 1. Crosslinks breaking ability.

Tested molecules	Crosslinks breaking ability	P values
Control	3.18%	
Alagebrium	46.38%	< 0.001
Carnosine	-2.73%	0.345
Aminoguanidine bicarbonate	9.12%	0.072
Rosmarinic acid	52.66%	< 0.001

P values, probability values compared with control, by Student t test, n = 3.

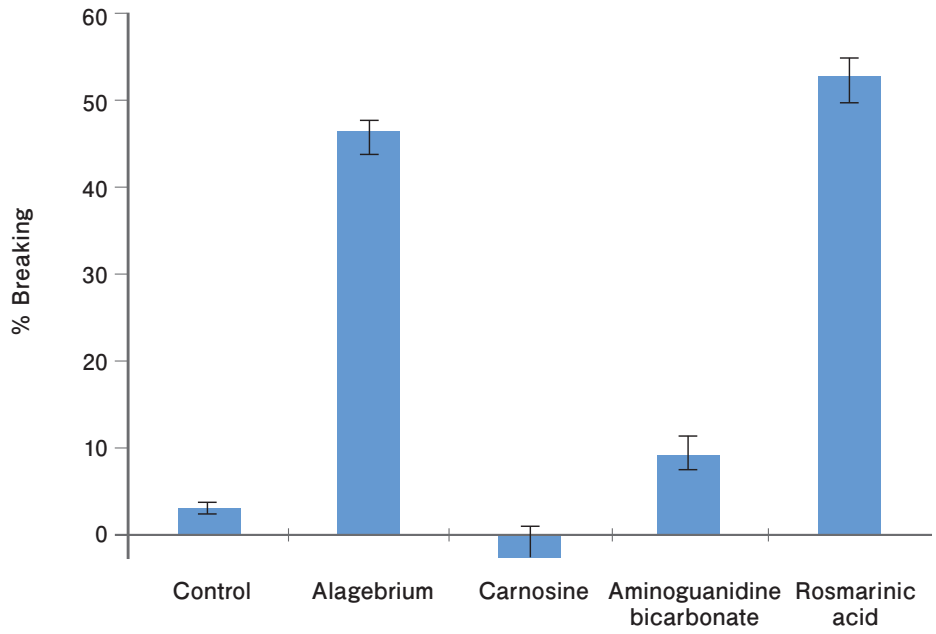

Fig 2. **Crosslink breaking ability.**
Bars indicate standard deviation. Number of measurement; n = 3.

Conclusion

Rosmarinic acid is a natural product well known for its safety. We have shown that it is possible to reverse AGE-related crosslinks and that it could be a promising treatment of diabetics, skin aging, and of elderly impaired vessels-related diseases as nephropathy, neuropathy and retinopathy to name a few.

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Conflicts of interest statement

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