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Antioxidant effects of fructosyl arginine, a Maillard reaction product in aged garlic extract

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The amino-carbonyl (Maillard) reaction of amino acids with sugars is a nonenzymatic browning reaction that takes place during the processing, cooking, and storage of foods. Maillard reaction products (MRPs) have been shown to possess interesting chemical and biological properties including antimutagenic and antioxidant activity. In this study, we determined the antioxidant effects of fructosyl arginine (Fru-Arg), a MRP in aged garlic extract. Low density lipoprotein (LDL) was incubated with Cu^{2+} at 37°C and 5% CO_2 for 24 hours, which resulted in an increase of thiobarbituric acid reactive substances (TBARS) indicating lipid peroxidation. Coincubation of Cu^{2+} with Fru-Arg and LDL resulted in a significant inhibition of TBARS formation. Pulmonary artery endothelial cells (PAEC) were exposed to 0.1 mg/mL oxidized LDL (Ox-LDL) at 37°C and 5% CO_2 for 24 hours. Lactate dehydrogenase (LDH) release, as an index of cell membrane damage, and TBARS were measured. Ox-LDL caused an increase of LDH release and TBARS formation. Pretreatment of PAEC with Fru-Arg inhibited these changes. Murine macrophages were incubated with Ox-LDL, and the release of peroxides was measured using a fluorometric assay. Ox-LDL caused an increased release of peroxides. Coincubation of macrophages with Fru-Arg and Ox-LDL inhibited the release of peroxides dose-dependently. In a cell free system, Fru-Arg was shown to scavenge hydrogen peroxide. These data suggest that Fru-Arg is a potent antioxidant, and thus may be useful for the prevention of atherosclerosis and other disorders associated with oxidative stress. (J. Nutr. Biochem. 10:372–376, 1999) © *Elsevier Science Inc. 1999. All rights reserved.*

Keywords: aged garlic extract; fructosyl arginine; Maillard reaction product; antioxidant; low density lipoprotein; atherosclerosis

Introduction

The amino-carbonyl reaction, or nonenzymatic browning, refers to the interaction between amino and carbonyl compounds, especially reducing sugars, which leads to complex changes in biological and food systems. It also is called the Maillard reaction in honor of Louis-Camille Maillard who first described it in 1912.[1] In foodstuffs, the Maillard reaction is responsible for changes in the flavor, color, nutritive value, and the formation of stabilizing and antimutagenic compounds.[2–6] The antioxidant effect of Maillard reaction products (MRPs) of glycine-glucose first was reported in 1954.[7] Since that time, MRPs have been shown to retard lipid peroxidation and have generated considerable interest among food specialists and nutritionists.

Aged garlic extract (AGE) is a commercial garlic product prepared by a special aging process.[8] Our laboratory has reported that AGE and one of its main constituents, *S*-allylcysteine (SAC), protected endothelial cells from hydrogen peroxide (H_2O_2)-induced oxidant injury.[9] We also have reported that AGE and several of its sulfur compounds inhibited Cu^{2+}-induced low density lipoprotein (LDL) oxidation[10] and oxidized LDL (Ox-LDL)-induced cell injury.[11,12]

In the present study, the antioxidant effects of fructosyl

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arginine [*N*-α-(1-deoxy-D-fructos-1-yl)-L-arginine; Fru-Arg], which is a MRP isolated from AGE, were determined using several in vitro assay systems. We now report that Fru-Arg can inhibit LDL oxidation, Ox-LDL-induced injury in endothelial cells, and release of reactive oxygen species in macrophages, indicating that Fru-Arg is also an antioxidant compound in AGE.

Methods and materials

Chemicals

Fru-Arg, SAC, and allicin were provided by Wakunaga of America Co., Ltd. (Mission Viejo, CA USA). Fru-Arg was isolated from AGE and synthesized according to the method of Matsuura et al.[13] by dissolving 0.88 g arginine and 1.8 g glucose in 10 mL glacial acetic acid with constant stirring at 80°C for 2 hours. The reaction mixture was concentrated and purified by chromatographic techniques. The purity of the Amadori compound was examined by thin layer chromatography on silica gel 60 F_{254} (Merck, Darmstadt, Germany). Human LDL, Hanks' balanced salt solution (HBSS), H_2O_2, ethylenediamine tetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), Triton X-100, trichloroacetic acid (TCA), and phosphate buffered saline (PBS) were purchased from Sigma Chemical Co. (St. Louis, MO USA). Pyridine, *n*-butanol, and acetic acid were purchased from Fisher Scientific Co. (Pittsburgh, PA USA). Horseradish peroxidase and 2,2′-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] (ABTS) were obtained from Boehringer Mannheim Co. (Indianapolis, IN USA). Cupric sulfate ($CuSO_4.5H_2O$) was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ USA). 2′7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes (Eugene, OR USA). CytoTox96TM Nonradioactive Cytotoxicity Assay Kit was supplied by Promega Co. (Madison, WI USA). Eagle's minimum essential medium (EMEM), Dulbecco's modification of Eagle's medium (DMEM), trypsin-EDTA solution, and penicillin-streptomycin solution were from Mediatech Co. (Washington, DC USA). Bovine calf serum (BCS) was obtained from HyClone Laboratories (Logan, UT USA).

Cell lines

Bovine pulmonary artery endothelial cells (PAEC) and murine macrophage cell line (J774) were obtained from the American Type Culture Collection (Rockville, MD USA). PAEC and J774 cells were grown in EMEM with 20% BCS and DMEM with 10% BCS, respectively. The media were supplemented with 200 U/mL penicillin and 0.2 mg/mL streptomycin. Cells were incubated at 37°C in a humidified 5% CO_2 atmosphere for 3 to 4 days before experimental use. Viability of cells used throughout the experiments was always greater than 95% as determined by trypan blue exclusion.

Preparation of Ox-LDL

LDL was dialyzed at 4°C for 48 hours against 500 volumes of PBS to remove EDTA. For preparation of Ox-LDL, LDL (5 mg/mL) was incubated with 20 μM $CuSO_4$ at 37°C for 24 hours and then dialyzed at 4°C for 48 hours against 500 volumes of PBS to remove Cu^{2+}.[14] Protein content was determined.[15] Presence of Ox-LDL was confirmed using agarose gel electrophoresis.[16]

LDL oxidation

This assay was performed as described previously.[11] Briefly, 0.1 mL of LDL (0.2 mg/mL) was added to 0.8 mL of 5 μM $CuSO_4$ and incubated at 37°C for 24 hours. The reaction was stopped by adding 0.1 mL of 10 mM EDTA. The extent of lipid peroxidation was determined by measuring thiobarbituic acid reactive substances (TBARS) at 532 nm in the Spectronic 2000 spectrophotometer (Bausch & Lomb, Rochester, NY USA). Tetraethoxypropane was used as a reference standard and the results were expressed in nmol/mg protein.

Lactate dehydrogenase release

PAEC (8×10^4 cells/well) in 24-well plates were preincubated with 0.1, 1, 10, and 20 mM of Fru-Arg for 24 hours, washed with HBSS, and then incubated with 0.1 mg/mL Ox-LDL in HBSS for 24 hours. The supernatant was collected from each well and stored at 4°C. Cell monolayers were treated with 0.2 mL of 0.8% Triton X-100 for 30 minutes at room temperature to disrupt cell membranes. The lysates were then collected. Lactate dehydrogenase (LDH) activity was measured in both the supernatant and the cell lysate fractions using the CytoTox96TM Nonradioactive Cytotoxicity Assay Kit following the manufacturer's instructions. This assay is based on the conversion of a tetrazolium salt to a red formazan product. The intensity of color is proportional to LDH activity. Absorbance was determined at 492 nm with a 96-well plate ELISA reader (400 AT EIA, Whittaker Bioproducts, Walkersville, MD USA). The percent of LDH released from the cells was determined using the formula:

Percent release = LDH activity in supernatant/

(LDH activity in supernatant + LDH activity in cell lysate).

Lipid peroxidation in endothelial cells

PAEC (8×10^4 cells/well) in 24-well plates were preincubated with different concentrations of Fru-Arg for 24 hours, washed with HBSS, and then incubated with 0.1 mg/mL Ox-LDL in HBSS for 24 hours. The supernatant in the wells was collected, and the extent of lipid peroxidation was determined by measuring TBARS as described previously.[17] TCA (12.5%, 0.2 mL) and then TBA reagent (0.4 mL of 0.6% TBA and 1 mM EDTA) were added to an aliquot of the supernatant. This reaction mixture was heated at 95°C for 20 minutes. After cooling with tap water, 3 mL of *n*-butanol were added, and the mixture was shaken vigorously for 30 seconds. After centrifugation at 2,000 rpm for 10 minutes, the *n*-butanol layer was removed and measured with excitation of 515 nm and emission of 553 nm, using LS-3 Fluorescence Spectrophotometer (Perkin-Elmer, Norwalk, CT USA). The value of fluorescence was calculated by comparing with standards prepared from tetraethoxypropane.

Peroxides released from macrophages treated with Ox-LDL

Peroxides were measured by a fluorometric assay using DCFH-DA as a probe.[18] DCFH-DA, a nonfluorescent compound, is de-acylated by viable cells to highly fluorescent 2′,7′-dichlorofluorescein (DCF) by H_2O_2 and lipid peroxides. Harvested J774 (2×10^5 cells/well) in 96-well plates were incubated for 2 hours to be attached to the well plates. After incubation, the media were removed, and cells were washed and incubated with 0.2 mL of 1, 5, 10, and 20 mM of Fru-Arg in HBSS, 0.1 mg/mL Ox-LDL, and 10 μL of 0.5 mM DCFH-DA. The fluorescence intensity (relative fluorescence unit) was measured at 485 nm excitation and 530 nm emission every 30 minutes for 3 hours, using the 7620 Microplate Fluorometer (Cambridge Technology, Watertown, MA USA).

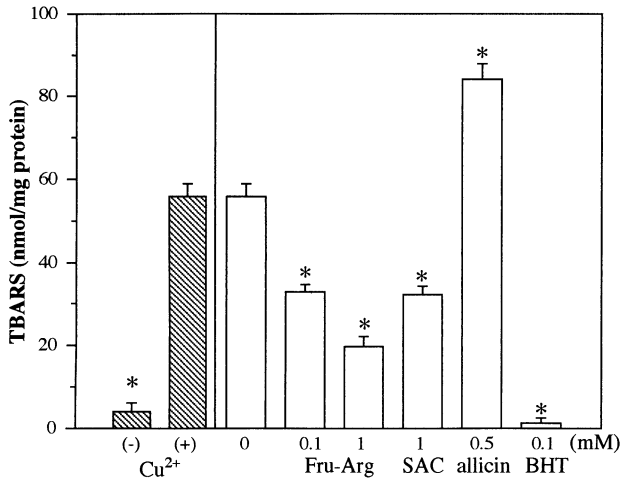

Figure 1 Effects of garlic compounds on Cu^{2+}-induced low density lipoprotein (LDL) oxidation. LDL (0.2 mg/mL) was incubated with each garlic compound and Cu^{2+} for 24 hours. Thiobarbituric acid reactive substances (TBARS), as an index of lipid peroxidation, were measured. Butylated hydroxytoluene (BHT), which is a known antioxidant, was used as a reference. Data represent means ±SE of triplicate samples. *Significant difference ($P < 0.05$) compared with control treated with Cu^{2+} but without garlic compounds. Fru-Arg, fructosyl arginine; SAC, *S*-allylcysteine.

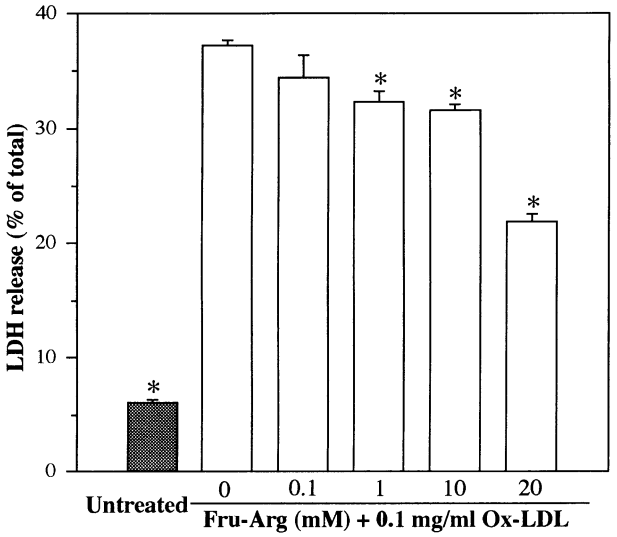

Figure 2 Effects of fructosyl arginine (Fru-Arg) on oxidized low density lipoprotein (Ox-LDL)-induced lactate dehydrogenase (LDH) release. Pulmonary arterial endothelial cells were preincubated with various concentrations of Fru-Arg at 37°C and 5% CO_2 for 24 hours. Cells were washed with Hanks' balanced salt solution, incubated with 0.1 mg/mL Ox-LDL for 24 hours, and then LDH release was determined as an index of cell membrane damage. Data represent means ±SE of triplicate samples. *Untreated* indicates that cells were not exposed to Ox-LDL. *Significant difference ($P < 0.05$) compared with control exposed to Ox-LDL but without pretreatment with Fru-Arg.

H_2O_2 Scavenging assay

The scavenging effect of Fru-Arg on H_2O_2 was determined according to the method of Okamoto et al.[19] One hundred microliters of 50 nM H_2O_2, 0.1 mL of different concentrations of Fru-Arg or HBSS, 0.6 mL of 10 U/mL peroxidase, and 0.6 mL of 0.1% ABTS were added to 1.8 mL of 0.1 M phosphate buffer (pH 6.0). The solution was then incubated at 37°C for 15 minutes. Absorbance at 414 nm was measured using a Spectronic 2000 spectrophotometer (Bausch & Lomb).

Statistical analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test for significant difference, and results were expressed as the mean ±SE. A *P*-value of less than 0.05 was considered significant. All statistical procedures were performed with Statgraphics software version 5.0 (STSC, Inc., Rockville, MD USA).

Results

Figure 1 shows the effects of Fru-Arg and two other garlic compounds on Cu^{2+}-induced LDL oxidation. Fru-Arg and SAC are water-soluble compounds in AGE, and allicin is an oil-soluble compound derived from the raw garlic. Incubation of LDL with Cu^{2+} resulted in a sevenfold increase of TBARS. Fru-Arg and SAC inhibited TBARS formation and the activity of Fru-Arg appeared to be tenfold stronger than SAC. Allicin, at half the dose of SAC, significantly increased LDL oxidation.

The effects of Fru-Arg on cell membrane injury induced by Ox-LDL are shown in *Figure 2*. Incubation of PAEC with Ox-LDL for 24 hours caused a 30% increase in LDH release. Pretreatment of PAEC with Fru-Arg minimized the LDH release. *Figure 3* shows the effects of Fru-Arg on Ox-LDL-induced lipid peroxidation in endothelial cells. Incubation of PAEC with Ox-LDL for 24 hours resulted in an increase of TBARS. Pretreatment of PAEC with Fru-Arg reduced TBARS formation.

The effects of Fru-Arg on peroxides released from macrophages are shown in *Figure 4*. Incubation of macrophages with Ox-LDL caused an increase of fluorescence, indicating release of peroxides. Fru-Arg inhibited this release in a dose-dependent manner.

The direct scavenging effect of Fru-Arg on H_2O_2 was performed in a cell free system. A dose-dependent decrease of H_2O_2 reflecting scavenging by Fru-Arg was noted, with significant activity observed in all dosages used (*Table 1*).

Discussion

The amino-carbonyl (Maillard) reaction of amino acids and sugars is a nonenzymatic browning reaction that takes place during the processing, cooking, and storage of foods. This reaction affects the development of color and flavoring and has become a significant focus of attention in the food industry. During the past 75 years, numerous studies have been published dealing with Maillard reactions such as chemistry,[2] flavor development,[3] nutrition, toxicology,[4] antimutagenicity,[5,6] and antioxidant properties.[7,19–21] The antioxidant effect of glycine-glucose, which is a MRP, was first reported in 1954.[7] Since that time, antioxidant activities of MRPs have been studied extensively using amino acids-sugars model systems.[20–23] MRPs from basic amino acids such as arginine, histidine, or lysine with reducing sugars have been shown to have very strong activities in vitro.[21]

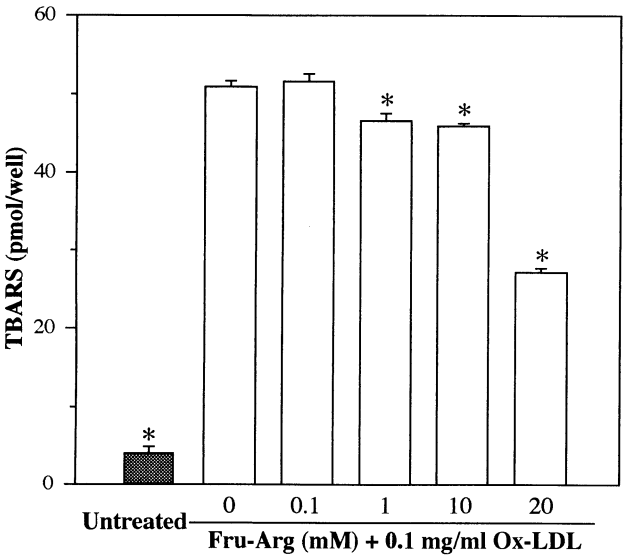

Figure 3 Effects of fructosyl arginine (Fru-Arg) on oxidized low density lipoprotein (Ox-LDL)-induced lipid peroxidation. Pulmonary arterial endothelial cells were preincubated with various concentrations of Fru-Arg at 37°C and 5% CO_2 for 24 hours. Cells were washed with Hanks' balanced salt solution, incubated with 0.1 mg/mL Ox-LDL for 24 hours, and then thiobarbituric acid reactive substances (TBARS) indicating lipid peroxidation were determined. Data represent means ±SE of triplicate samples. *Significant difference ($P < 0.05$) compared with control exposed to Ox-LDL but without pretreatment with Fru-Arg.

More recently, the antioxidant effect of MRPs was shown in an in vivo study.[24] Feeding rats MRPs from histidine, lysine, arginine, or glycine and sugars significantly inhibited TBARS formation in the liver. It is believed that the

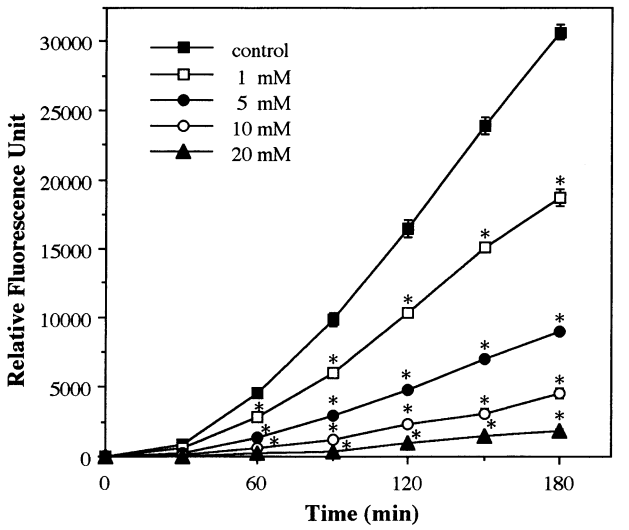

Figure 4 Effects of fructosyl arginine (Fru-Arg) on release of peroxides in macrophages. J774 cells were incubated with various concentrations of Fru-Arg and 0.1 mg/mL oxidized low density lipoprotein (Ox-LDL). 2′,7′-Dichlorofluorescein fluorescence indicating peroxides was monitored every 30 minutes for 3 hours. Data represent means ±SE of triplicate samples. *Significant difference ($P < 0.05$) compared with control exposed to Ox-LDL but without pretreatment with Fru-Arg.

Table 1 Scavenging effect of Fru-Arg on hydrogen peroxide

Sample		H_2O_2 (nmol ± SE)	Scavenging (%)
Control		5.00	—
Fru-Arg	5 μM	3.99 ± 0.08*	20.3
Fru-Arg	10 μM	3.52 ± 0.09*	29.5
Fru-Arg	50 μM	1.07 ± 0.02*	78.5
Fru-Arg	100 μM	0.77 ± 0.06*	84.6
Ascorbic acid	1 μM	4.65 ± 0.08*	7.1
Ascorbic acid	5 μM	0.55 ± 0.09*	89.0

Data represent means ± SE of triplicate samples.
*Significant difference compared with control ($P < 0.05$).
H_2O_2–hydrogen peroxide. Fru-Arg–fructosyl arginine.

observed effects were mainly derived from melanoidins—brown nitrogenous polymers with high molecular weights.[25,26] However, the Maillard reaction also produces low molecular weight compounds.[27–29] Fru-Arg, a low molecular weight MRP isolated from the non-saponin fraction of Korean red ginseng,[13] was shown to lower blood pressure and to improve microcirculation in rabbits.[30]

In the present study, Fru-Arg, a MRP isolated from AGE, was used to determine its antioxidant activity against oxidation of LDL. We demonstrated that Fru-Arg inhibited Cu^{2+}-induced LDL oxidation. SAC, a water-soluble compound in AGE, also inhibited it. We also tested the effect of allicin, which is the main compound derived from raw garlic. We were surprised to find that allicin did not inhibit Cu^{2+}-induced LDL oxidation. On the contrary, it actually enhanced LDL oxidation. Allicin is an antimicrobial compound in raw garlic. Allicin has been reported to induce stomach injury in animals[31] and oxidize the iron of hemoglobin in red blood cells with methemoglobin formation.[32]

We also determined the effects of Fru-Arg on Ox-LDL-induced injury in vascular endothelial cells by measuring LDH release. LDH is an intracellular enzyme released into the medium upon cell membrane damage. Ox-LDL caused a significant increase in LDH release. Fru-Arg inhibited this release. To elucidate the mechanisms of cell injury, TBARS indicating lipid peroxidation were measured. Ox-LDL resulted in a tenfold increase of TBARS formation in PAEC. Pretreatment with Fru-Arg inhibited the increase of TBARS. These data suggest that Fru-Arg can protect endothelial cells from Ox-LDL-induced injury.

Macrophages undergo an oxidative burst in response to phagocytic or membrane stimuli, with production and release of various reactive oxygen metabolites such as superoxide anion, H_2O_2, hydroxyl radical, and nitric oxide.[33] These metabolites modify the production and release of reactive oxygen species and several kinds of inflammatory cytokines in endothelial cells.[34–36] Such events may accelerate the formation of atherogenic lesions and cell death. In this study, we showed that Ox-LDL caused an increase of peroxides released from macrophages. Fru-Arg inhibited the release of peroxides dose-dependently. Moreover, we observed the direct scavenging effect of Fru-Arg on H_2O_2 in a cell free system. Based on the work of O'Brien and Morrissey,[37] it is likely that Fru-Arg will have both metal complexing and reducing properties, both of which might

contribute to the antioxidant activity observed in the present study.

We previously reported that SAC is a major antioxidant compound in AGE.[9–12] The present study indicates that Fru-Arg, a MRP of AGE, also can contribute to the antioxidant activity of this garlic preparation.

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