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Research Article

Aged and Unaged Garlic Exerts Differential Effects on Cellular Aging via Modulation of Tissue Degradative and Antioxidant Enzymes Activity – A Cell-Free in Vitro Study

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Abstract

The effects of aged and unaged garlic on cell aging processes via enzymatic and oxidative pathways were examined in this cell-free *in vitro* study. Aged and unaged garlic, quercetin, and S-allyl cysteine inhibited collagenase and elastase dose-dependently. Quercetin and unaged garlic showed stronger collagenase inhibition and weaker elastase inhibition than S-allyl cysteine and aged garlic. Quercetin and aged garlic scavenged radicals more effectively than unaged garlic and S-allyl cysteine. Superoxide dismutase activity was significantly augmented by quercetin and unaged garlic when compared to aged garlic and S-allyl cysteine. Aged garlic contained higher amounts of S-allyl cysteine, total flavonoid and polyphenols, and lower quercetin content when compared to unaged garlic. Aged and unaged garlic exerted different effects on cellular aging by modulating collagenase, elastase, and superoxide dismutase activities. The different effects can potentially be attributed to different organosulfur and phenolic compositions.

Highlights

- Aged and unaged garlic inhibit collagernase and elastase activities differently.
- Aged and unaged garlic increases superoxide dismutase activity differently.
- Aged and unaged garlic have different organosulfur and phenolic compositions

Keywords: Aged Garlic; Unaged Garlic; Collagenase; Elastase; Superoxide Dismutase; Radical Scavenging Activity.

Introduction

Aged garlic is produced by exposing whole bulbs of raw garlic to high temperature (70°C) under controlled humidity (90% RH) [1,2] for more than one month [3,4]. The unique sweet and syrupy balsamic taste of aged garlic with its smooth, soft texture and black appearance, are the results of a series of transformations, including decomposition, acid reduction, and hydrolysis during aging [1,3].

The formation of free radicals in cellular processes, such as mitochondrial energy synthesis, electron transport, and peroxisomal fatty acid metabolism, are thought to stimulate cell aging [5]. These radicals damage cellular proteins and DNA irreversibly, resulting in cellular aging [6]. Ingestion of diets rich in phytochemicals or functional food products with antioxidant properties can potentially help alleviate such radical-mediated oxidative stress and aging 6. Phytochemicals naturally present in aged and unaged garlic may exert significant radical scavenging activity and delay the process of aging in vivo. At present, various studies have reported the antioxidant properties of unaged garlic [7,8], but there is limited data on the antioxidant effects of aged garlic.

Collagen and elastin are essential structural proteins for maintaining the physiological cellular structure and structural integrity of major tissues and organs, degradation of these proteins by enzymes such as collagenase and elastase is an integral part of the aging process [9,10]. Inhibitions of collagenase and elastase are thought to delay the aging processes [9,10]. To date, data on the effects of aged and unaged garlic on the activities of collagenase and elastase is limited.

Garlic, aged or unaged, is hypothesized to inhibit cellular aging by modulating aging-associated enzyme activities and alleviating oxidative damage. S-allyl cysteine (SAC) and phenolic compounds, like quercetin (Q) in aged and unaged garlic contribute significantly to their anti-aging activities. The study aimed to examine the inhibitory effects of aged and unaged garlic, Q, and SAC on cellular aging enzymes, collagenase and elastase. It also examined their radical scavenging activities and ability to modulate superoxide dismutase (SOD) activity.

Methods

Chemicals and Materials

N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide, human, leukocyte elastase,HEPES,pH,7.5,N-[3-(2 furyl)acryloyl]-Leu-Gly-Pro-Ala(FALGPA), tris(hydroxymethyl)-methyl-2-aminoethane sulfonate, calcium chloride dihydrate, ethylenediaminetetraacetic acid, ninhydrin, citric acid, Folin-Ciocalteu's reagent, sodium carbonate, glucose, gallic acid, concentrated sulfuric acid, hydrochloric acid, sodium hydroxide, 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), quercetin (Q), S-allyl cysteine (SAC), and ammonia were purchased from

Merck (Bayswater, VIC, Australia); Phorbol 12-myristat 13-acetate (PMA), phosphate-buffered saline (PBS) and diethylenetriamine penta-acetic acid from Sigma-Aldrich (St. Louis, MO, USA); aluminium(III) chloride, potassium acetate from Merck (NJ,USA); acetonitrile, ethanol and methanol were from Tri-Tech Medical (Avon, OH, USA); ficoll-paque was from GE Healthcare (Uppsala, Sweden); Hanks' balanced salt solution (HBSS) and Roswell Park Memorial Institute (RPMI) 1640 were from Gibco Invitrogen (Carlsbad, CA, USA); and dextran 500 was from Amersham Biosciences (Uppsala, Sweden).

The aged and unaged garlic powders were prepared and encapsulated by Defu Foodstuff Pte Ltd. (Singapore). The aged garlic was prepared by incubating fresh garlic cloves at 70°C and 75% humidity for 40 days. The raw and aged garlic cloves were dried, powdered and packed into individual capsules.

Aqueous extracts of the aged and unaged garlic (AGE and UGE, respectively) were prepared by extracting the respective garlic powders (0.5 g) in 50 mL deionised water (50% v/v) in an ultrasonic bath at room temperature for 20 minutes.

Collagenase and elastase enzyme activity

The capacity of Q, SAC, AGE, and UGE to inhibit collagenase enzyme was determined using a colorimetric assay [11]. Briefly, Q, SAC, AGE or UGE (final concentrations, 10, 50, 100, and 200 μg L^{-1}) were incubated with collagenase (final concentration, 0.035 U mL^{-1}) at 37 °C for 15 minutes. At the end of incubation, collagenase substrate (FALGPA; final concentration, 0.01 μmol mL^{-1}) was mixed with the enzyme. Absorbance was immediately measured at 345nm in kinetic mode every 2 minutes, for 15 minutes. Control experiments were conducted without the treatment compounds. Blank measurements were taken without collagenase. The rate of collagenase reaction was calculated as the negative change in absorbance at 345 nm over 15 minutes. The change in collagenase activity was expressed as the percentage change in absorbance relative to the control after blank adjustment. The IC_{50} value was estimated using the plotted concentration-response graph.

The effects of Q, SAC, AGE, and UGE on elastase activity were measured using a fluorometric assay 12. Briefly, Q, SAC, AGE or UGE (final concentrations, 10, 50, 100, and 200 μg L^{-1}) were incubated with neutrophil elastase (final concentration, 0.156 mU mL^{-1}) at 37°C for 15 minutes. At the end of incubation, elastase substrate (N-Methoxysuccinyl-Ala-Ala-Pro-Val-p-nitroanilide) was mixed with the enzyme and incubated for 1.5 hours. Control experiments were carried out without the treatment compounds and blanks were measured without elastase enzyme. The fluorescence activity was read at an excitation wavelength of 485nm and an emission wavelength of 525nm. The change in elastase activity was expressed as the percentage change in fluorescence activity relative to the control. The IC50 value was estimated using the plotted concentration-response graph.

Superoxide dismutase activity

The effects of Q, SAC, AGE, and UGE on SOD activity were measured using a commercially available SOD activity assay kit (Abcam, MA, USA). Briefly, Q, SAC, AGE or UGE (final concentrations, 10, 20, 50, 100, and 200 μg L^{-1}) were incubated with the provided SOD enzyme solution (20 μL) and WST substrate (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfo-phenyl)-2H-tetrazolium, monosodium salt) solution (200 μL) at 37°C for 20 minutes. At the end of incubation, the absorbance was read at a wavelength of 450 nm. The treatment compounds were added using PBS as vehicle. Control experiments were carried out

without the treatment compounds and blank measurements were taken without SOD enzyme. The SOD activity (change%) was expressed as the percentage change in absorbance relative to the control after blank adjustment.

Radical scavenging capacity

The radical scavenging activity of Q, SAC, AGE, and UGE was determined using the 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) method [13]. The ethanolic DPPH solution (48 mg L^{-1}) was freshly prepared. Q, SAC, AGE or UGE was mixed into the freshly prepared DPPH solution (1:9 volume ratios) and the change in absorbance at 517 nm was measured using a spectrophotometer. A calibration curve was prepared with Trolox (0-5 mmol L^{-1}). The radical scavenging results were expressed in mmol L-1 trolox.

Total phenolic and flavonoid assays

The total phenolic contents were determined using a modified Folin-Ciocalteu's method 14. The total phenolic content was expressed as gallic acid equivalents. The analyses were performed in triplicate.
The total flavonoid contents of the AGE and UGE were measured by a modified Dowd colorimetric method 15. The total flavonoid content was expressed as the quercetin-equivalence in 1 g garlic.

Quercetin and S-allyl cysteine determination

The amounts of Q present in the AGE and UGE were measured using HPLC coupled with a diode array detector. Briefly, AGE or UGE (10 mL) was hydrolysed in methanolic potassium hydroxide under nitrogen gas at 60°C for 3 hours. The supernatant was filtered through a 0.45 μm FILTREX cellulose acetate syringe filter before HPLC injection. The filtered supernatant (20 μL) was chromatographed isocratically on a reverse phase C18 column (Merck Purospher ® Star RP-18, 5 μm particle size, 8 A° pore size, 100 mm x 4.60 mm) at a flow rate of 1.0 mL/min using a Waters HPLC series 2695 with a mobile phase methanol, acetonitrile and water (v/v, 60:20:20). Q was measured at 262 nm and the quantity was determined by comparing the Q integrated peak area with a predetermined calibration curve. Triplicate experiments were performed.

The amount of SAC present in the AGE and UGE were measured using a modified, high-performance liquid chromatography (HPLC)-UV method 16. Briefly, the supernatant of AGE or UGE (10 mL) was filtered through a 0.45 μm FILTREX cellulose acetate syringe filter before HPLC injection. The filtered supernatant (20 μL) was chromatographed on a reverse phase C18 column (Merck Purospher ® Star RP-18, 5 μm particle size, 8 A° pore size, 100 mm x 4.60 mm) at a flow rate of 1.0 mL/min using a Waters series 2695 HPLC. Gradient elution was performed with mobile phase methanol, acetonitrile, and water (v/v, 60:20:20). SAC was measured at 208 nm. The quantity of SAC was determined by comparing the SAC integrated peak area with a predetermined calibration curve. Triplicate experiments were performed.

Statistical analysis

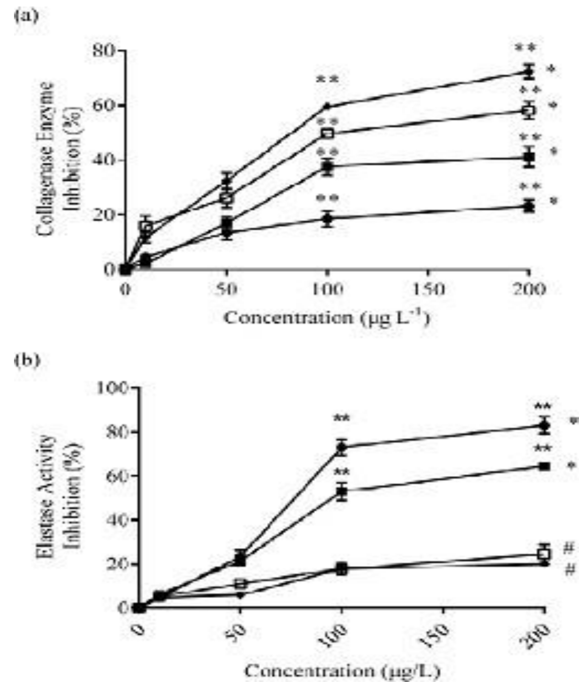
Statistical analysis was conducted using SPSS version 23 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± standard deviation of five experiments. One-way ANOVA with post hoc Bonferroni multiple comparisons were performed on specific concentration points as well as the areas under the curves in concentration-response results. Between-group differences for two groups were analysed using unpaired t-tests. Statistical difference was marked significant when $p<0.05$.

Results

Collagenase and elastase enzyme activity

AGE, Q, SAC & UGE showed dose-dependent collagenase inhibition responses (Figure 1a). All four treatments inhibited collagenase activity to significantly different extents, with inhibitory activity increasing in the order: SAC < AGE < UGE and Q (Figure 1a). Q showed the greatest potency against collagenase activity (IC50=80 μg L^{-1}), whereas SAC exerted negligible collagenase inhibitory activity (Figure 1a). All four treatments did not differ in their inhibitory activities on collagenase at the lower concentrations (<50 μg L^{-1}) (Figure 1a). UGE inhibited collagenase activity to a significantly greater extent than AGE at 100 and 200 μg L^{-1} (Figure 1a).
AGE, Q, SAC, and UGE inhibited elastase in a dose-dependent manner up to 200 μg L-1 (Figure 1b). AGE and SAC exhibited significantly higher elastase inhibition than Q and UGE (Figure 1b). SAC was the most potent elastase inhibitor (IC_{50}=75μgL^{-1}) exhibiting significantly greater inhibition than the other three treatments (Figure 1b). At lower concentrations (<50 μg L^{-1}), all four treatments did not differ in their inhibitory activities on elastase (Figure 1b). AGE inhibited elastase activity to a significantly greater extent than UGE at the higher (100-200 μg L^{-1}) concentrations (Figure 1b).

Figure (1): The inhibition of **(a)** collagenase and **(b)** elastase enzyme activity (%) of aged garlic extract (■), quercetin (♦), S-allyl cysteine (●), and unaged garlic extract (□) at different treatment concentrations (10 to 200 μg L^{-1}) (n=5). *p<0.05 *vs*. other treatments using ANOVA with posthoc Bonferroni multiple comparisons of the area under the curve. $^{\#}$p<0.05 *vs*. AGE and SAC using ANOVA with posthoc Bonferroni multiple comparisons of area under the curve.**p< 0.05 *vs*. other treatments using ANOVA with posthoc Bonferroni multiple comparisons at the specified concentration.

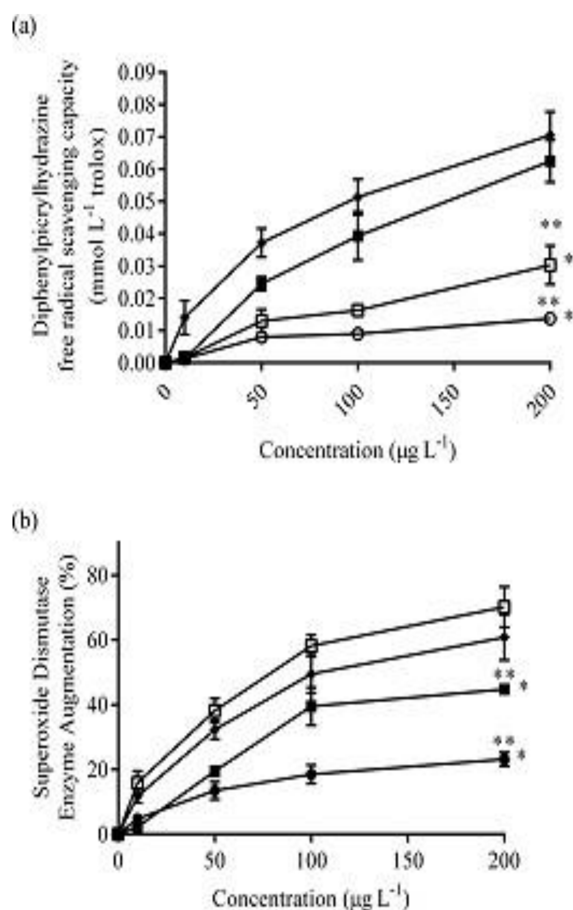


Radical scavenging capacity and superoxide dismutase activity

AGE and Q exerted significantly greater DPPH radical scavenging activity compared to UGE and SAC at tested concentrations (200 μg L^{-1}) (Figure 2a). AGE and Q did not differ significantly in their DPPH radical scavenging activity (Figure 2a).

Q and UGE augmented SOD to a significantly greater extent compared to AGE and SAC and at a concentration of 200 μg L-1 (Figure 2b). SAC increased SOD activity to a lower extent compared to AGE at concentrations of 100 and 200 μg L^{-1} (Figure 2b). Significant difference in SOD activity was not observed between Q and UGE (Figure 2b).

Figure (2): The capacities to **(a)** scavenge diphenylpicrylhydrazine free radical and **(b)** augment superoxide dismutase enzyme activity (%) by aged garlic extract (■), quercetin (♦), S-allylcysteine (●), and unaged garlic extract (□) at different treatment concentrations (10 to 200 μg L^{-1}) (n=5). *p<0.05 *vs*. other treatments using ANOVA with posthoc Bonferroni multiple comparisons of area under the curve. **p<0.05 *vs*. other treatments using ANOVA with posthoc Bonferroni multiple comparisons at the specified concentration.



Quercetin, S-allyl cysteine, total flavonoid and total polyphenol contents

The amount of quercetin in AGE (0.184 ± 0.053 μg g-1) was significantly less than UGE (0.398 ± 0.035 μg g^{-1}).

AGE contained significantly higher amounts of SAC (0.373 ± 0.031 mg g^{-1}) compared to UGE (0.211 ± 0.017 mg g^{-1}).

AGE contained significantly higher total flavonoid (1.27 ± 0.03 μg mL-1) compared to UGE (0.29 ± 0.01 μg mL^{-1}). The amount of polyphenols in AGE (6.39 ± 0.27 mg gallic acid equivalent g^{-1}) was significantly higher than UGE (0.22 ± 0.06 mg gallic acid equivalent g-1).

Discussion

Aged and unaged garlic extracts inhibited collagenase and elastase activities which are involved in cellular aging/degenerative processes [9,10]. AGE and UGE were also shown to exert significant radical scavenging activities and augmented SOD enzyme activity. The aged and unaged garlic preparations can potentially delay the aging processes by collagenase and elastase [9,10], and antioxidant activity [6].

During aging of garlic, unstable compounds like alliin are altered to form stable, water-soluble compounds, such as S-allyl cysteine (SAC) [1,3]. Aged garlic has been reported to contain significantly higher SAC and polyphenol contents compared to unaged garlic [17-20]. Consistent with previous studies, the AGE used in this study contained significantly higher SAC, total phenolic, and flavonoid contents compared to the UGE. Quercetin (Q) is one of the better-known polyphenols present in garlic [21]. While the total flavonoid and phenolic contents were increased, the amount of Q in the garlic was significantly reduced after the aging processes. Quercetin is potentially degraded or metabolised by enzymatic reactions, and further studies are required to elucidate the mechanisms of these reactions. Further

studies are also required to investigate the effects of aging on garlic's phenolic compositions.

AGE and UGE exerted different influences on the collagenase and elastase activities in correlation with the differences in SAC and Q contents between the two garlic preparations. Q inhibited collagenase to a significantly greater extent compared to SAC, which was consistent with the observed greater collagenase inhibiting effects of UGE compared to AGE. The opposite trend was observed for elastase. Previous studies have examined the anti-collagenase activity of Q and SAC, but their activities were not compared within the same study [22-26]. Caffeic acid and SAC isolated from garlic significantly inhibited the degradation of type I procollagen and expression of matrix metalloproteins in vivo and also attenuated the histological collagen fiber disorder in vivo [22]. Quercetin is reported to be a potent inhibitor of collagenase, with IC50 values of 286 μmol L^{-1} [23] and 40 μmol L^{-1} [24]. The higher IC_{50} values reported may be due to the different assays, and higher collagenase concentrations used in the respective assays. Quercetin does not influence the expression of collagenase in chondrocytes [25].

Various studies have reported the antioxidant actions of aged and unaged garlic, but few compared the antioxidant activities of aged and unaged garlic preparations [7,20], [27-29]. Aged garlic is reported to be a more effective antioxidant than its unaged counterpart [20]. Contrary to previous reports that cite SAC as being an effective antioxidant [7,27,30], the data from this study indicated that SAC had no significant radical scavenging activity. The results suggested that the radical scavenging activity of AGE and UGE can potentially attributed to their phenolic compositions, such as Q. The results agreed with previous studies which suggest that phenolic compounds present in the AGE and UGE scavenged free radicals, neutralised H2O, and inhibited lipid peroxidation [28, 29]. reported significant positive correlations between the phenolic contents in garlic and its free radical scavenging activity [29].

The results of this study suggest that garlic preparation alleviated oxidative damage by augmenting the activities of antioxidant enzymes, such as SOD. Aged garlic preparation was more effective in increasing SOD activity than the unaged. Garlic intake increased tissue SOD and catalase activities in ultraviolet-treated Shk-1 hairless mice [31]. Ingestion of garlic extract did not change the SOD enzyme activity in the blood plasma of patients with atherosclerosis, although it significantly lowered blood plasma and erythrocyte malondialdehyde levels [32]. Similar results were observed in patients with high cholesterol [33]. Caffeic acid and SAC alleviate oxidative stress by modulating the NF-κB activities [22]. Oral administration of AGE (600 mg/kg daily for eight weeks) significantly increased the total antioxidant levels and catalase activity in diabetic rats [34]. The associations of AGE, UGE, and Q on SOD enzyme activity suggest that phenolic compounds and flavonoids, such as quercetin, in the garlic preparations contributed significantly to the modulation of SOD activity [18]. Reported the differential effects of AGE and UGE on SOD activity, with UGE increasing SOD activity to a greater extent. The experiments in the study were performed using physiologically relevant concentrations and the results are more likely to be transferable to in vivo observation.

A complete profiling of the chemical constituents of the aged and unaged garlic would assist in explaining the difference in biological activities. Various chemical constituents in garlic preparations, including sulfur and phenolic compounds, may contribute to their biological activities [7, 28]. The findings are limited to the cell-free in vitro model. Further *in vitro* cell or *in vivo* studies are required to confirm their biological efficacy.

Conclusion

Aged and unaged garlic exerted different anti- aging effects via modulation of collagenase, elastase, and SOD activities. The differential effects can potentially be attributed to different organosulfur and phenolic composition.

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