In Vitro Antiglycation Activity of Arbutin

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Abstract

Non-enzymatic glycosylation (glycation) between reducing sugar and protein results in formation of advanced glycation endproducts (AGEs), which is believed to play important roles in pathogenesis of diabetic and aging complications. Thus, agents that inhibit the formation of AGEs are purported to have therapeutic potentials in patients with diabetes or age-related diseases. The present study was conducted to determine glycation inhibitory activity of arbutin, a naturally occurring compound with an antioxidative property, by using an *in vitro* glucose-bovine serum albumin (BSA) assay. Glucose and BSA were coincubated at 60^{o}C in the presence or absence of arbutin or aminoguanidine, a known inhibitor of glycation reaction. Following a 24-hour incubation period, the glycated BSA product was precipitated with trichloroacetic acid (TCA) and redissolved in alkaline phosphate buffered saline (PBS). The formation of glycated BSA was relatively quantitated by measuring fluorescence intensity. Excitation and emission wavelength were at 370 nm and 440 nm, respectively. The result of this study demonstrated that arbutin inhibited glycation of BSA by glucose in a dose-dependent manner. In this assay, the 50% inhibition was observed at 5 and <1 mM concentration of arbutin and aminoguanidine, respectively. Its inhibitory activity was further confirmed when the glycation reaction was allowed to proceed at lower temperature (37^{o}C) for 14 days with similar findings. Taken together, these data indicated that arbutin possessed an *in vitro* antiglycation activity; however, its activity was relatively less than that of aminoguanidine when tested by glucose-BSA assay under the condition used in this study.

Keywords: arbutin, glycation, antiglycation, advanced glycation endproducts, glucose-BSA assay

Introduction

Non-enzymatic reaction between reducing sugar and free amino group of proteins, also known as Maillard reaction, leads to the formation of glycated protein termed Amadori product. Further rearrangement, oxidation and reduction of the Amadori product result in the formation of several advanced glycation endproducts (AGEs) such as pentosidine, carboxymethyllysine, crossline and pyralline. Some of these products can react with a free amino group nearby and form crosslinking between proteins (Ulrich and Cerami, 2001). The crosslinked protein, e.g. crosslinked collagen, are postulated to confer pathological conditions found in patients with diabetes and aging, such as arterial stiffness and decreased myocardial compliance, resulting from the loss of collagen elasticity (Singh et al., 2001; Aronson, 2003). Thus, agents that inhibit the formation of AGEs are purported to have therapeutic potentials in patients with diabetes and age-related diseases.

The oxidation process is believed to play an important role in AGEs formation. Further oxidation of Amadori product leads to the formation of intermediate carbonyl compounds that can react with the nearby lysine or arginine residues to form protein crosslink and AGEs. The reactive carbonyl compounds may also be generated from the metal ion-catalyzed autooxidation of glucose (Rahbar and Figarola, 2003; Voziyan et al., 2003). Therefore, agents with antioxidative or metal-chelating property may retard the process of AGEs formation by preventing further oxidation of Amadori product and metal-catalyzed glucose oxidation. In this regard, several natural compounds known to possess antioxidative property, such as curcumin, rutin, garcinol and flavonoid-rich extracts, have been shown to prevent AGEs formation *in vitro* and *in vivo* (Sajithal et al., 1998; Yamaguchi et al., 2000; Kim and Kim, 2003; Kiho et al., 2004).

Arbutin (hydroquinone-β-D-glucopyranoside) is a naturally occuring compound found in various plant species of diverse family such as Ericaceae (*Arctostphylos* spp.), Betulaceae (*Betula alba*) and Rosaceae (*Pyrus communis* L.) (Petkou et al., 2002). It was developed for used as a skin-whitening agent in cosmetics due to its inhibitory effect on tyrosinase activity. Arbutin has been shown to possess antioxidative and free radical scavenging properties (Ioku et al., 1992; Petkou et al, 2002; Myagmar et al., 2004). The present study was conducted to determine whether arbutin also possesses an antiglycation activity when tested *in vitro* by the glucose-BSA assay.

Materials and Methods

Materials

BSA (fraction V), arbutin, glucose and TCA were purchased from Sigma-Aldrich (St. Louis, USA).

Antiglycation assay

Antiglycation assay was performed according to the methods reported by Matsuura and colleagues with slight modification (Matsuura et al., 2002). In all experiments, the final reaction volume was 1 ml and was performed in 1.5-ml Eppendorf tube. Albumin (1 mg/ml final concentration) was incubated with glucose (500 mM final concentration) in the presence of arbutin, aminoguanidine or PBS as control buffer at specified concentration. The reaction was allowed to proceed at 60^{o}C for 24 hours, and thereafter, stopped by adding 10 µL of 100% (w/v) trichloroacetic acid (TCA). The TCA-added mixture was kept at 4^{o}C for 10 minutes before subject to centrifugation at 10000 g. The precipitate was redissolved with alkaline PBS (pH 10) and immediately quantitated for the relative amount of glycated BSA based on fluorescence intensity by spectrofluorometer F-4500 (Hitachi, Japan). The excitation and emission wavelengths used were at 370 nm, and 440 nm, respectively. In time-course studies, the reaction was allowed to proceed to different time-points as indicated before the reaction was stopped by TCA and relative fluorescent intensity was measured. In some studies to determine the effects of temperature, the reaction mixture was incubated at 37^{o}C for 14 days, followed by TCA precipitation and fluorescent intensity measurement in a similar manner as stated previously.

Statistical analysis

Results are presented as means $\pm$ SD from three to five experiments as indicated in each figure legend. Statistical differences among three or more groups were determined by one-way analysis of variance (ANOVA) followed by a Dunnett's post hoc test of all groups versus the respective control group. The percentage inhibitions of arbutin and aminoguanidine on glycation reaction performed at each concentration were compared using unpaired t test with two-tailed test. In each analysis, a *p* value of less than 0.05 was considered statistically significant. All tests were performed with GraphPad Prism, version 3.0 for Windows (GraphPad Software, Sandiego, CA).

Results

Initially, antiglycation activity of arbutin was tested for its ability to inhibit the formation of glycated BSA at 60^{o}C without TCA precipitation. As shown in Figure 1, in contrast to aminoguanidine, it was not possible to detect any inhibition due to arbutin's fluorescent property which made detection of any changes in fluorescent intensity unsuccessful. To overcome this problem, the method of Matsuura and associates was employed (Matsuura et al., 2002). TCA was used to precipitate glycated BSA and allow removal of any interference from the reaction mixture. Using this method, the time course of glycated BSA formation based on fluorescent intensity was

compared. In the absence of arbutin and aminoguanidine, the fluorescent intensity increased over time and reached maximum after 24 hours. However, in the presence of arbutin or aminoguanidine, the fluorescent intensity was significantly decreased at all time-points studied, indicating less formation of glycated BSA (Figure 2).

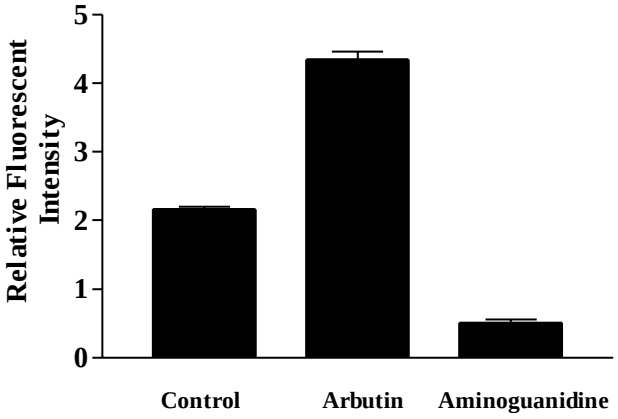

Figure 1 Test of arbutin and aminoguanidine activity on inhibition of glycation reaction without TCA precipitation. BSA (1 mg/ml) was incubated with glucose (500 mM) in the presence of arbutin (5 mM) or aminoguanidine (5 mM) or PBS as control buffer at 60^{o}C for 24 hours. The formation of glycated products was measured by relative fluorescent intensity (excitation wavelength 370 nm and emission wavelength 440 nm). Data shown are means $\pm$ SD from three experiments performed in duplicate.

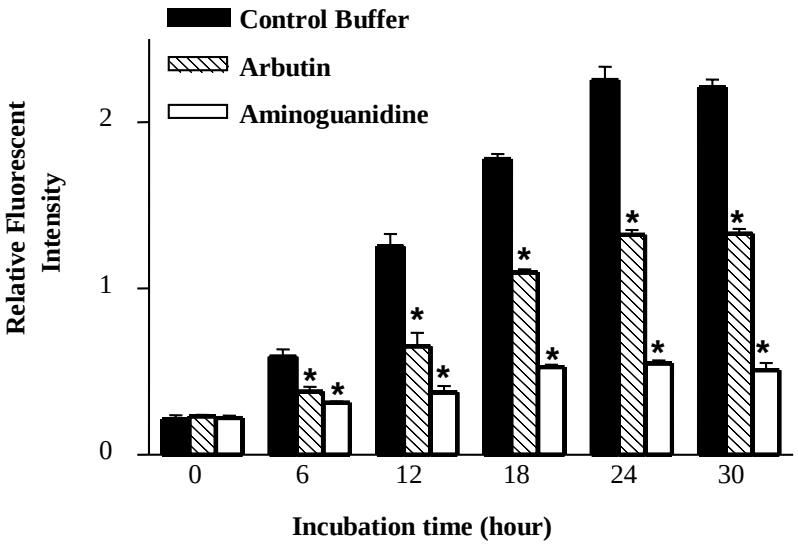

Figure 2 Time course inhibition of arbutin and aminoguanidine on glycation reaction based on measurement of fluoresecent intensity. BSA (1 mg/ml) was incubated with glucose (500 mM) in the presence of arbutin (5 mM) or aminoguanidine (5 mM) or PBS as control buffer at 60^{o}C for 24 hours. Following a TCA precipitation, the precipitate was redissolved and measured for relative fluorescent intensity (excitation wavelength 370 nm and emission wavelength 440 nm). Data shown are means $\pm$ SD from five experiments performed in duplicate. Statistical difference was determined by ANOVA followed by a Dunnett's post hoc test. * indicates significant difference ($p < 0.01$) compared with the respective control group.

The inhibitory activity of arbutin on BSA glycation by glucose was further confirmed in the dose-dependent studies. In these experiments, arbutin or aminoguanidine at various concentrations was incubated with BSA and glucose at 60°C for 24 hours. Thereafter, the reaction was stopped by TCA followed by measurement of fluorescent intensity of the redissolved precipitates. As shown in Figure 3, both arbutin and aminoguanidine inhibited the formation of glycated BSA dose-dependently in a similar manner. However, the inhibitory activity of arbutin on glycation reaction observed is less than that of aminoguanidine. Under the condition studied, aminoguanidine inhibited more than half of the reaction at concentration less than 1 mM while arbutin inhibited approximately 50% at about 5 mM. Thus, arbutin exerts a weaker inhibitory effect on BSA glycation by glucose than that exhibited by aminoguanidine.

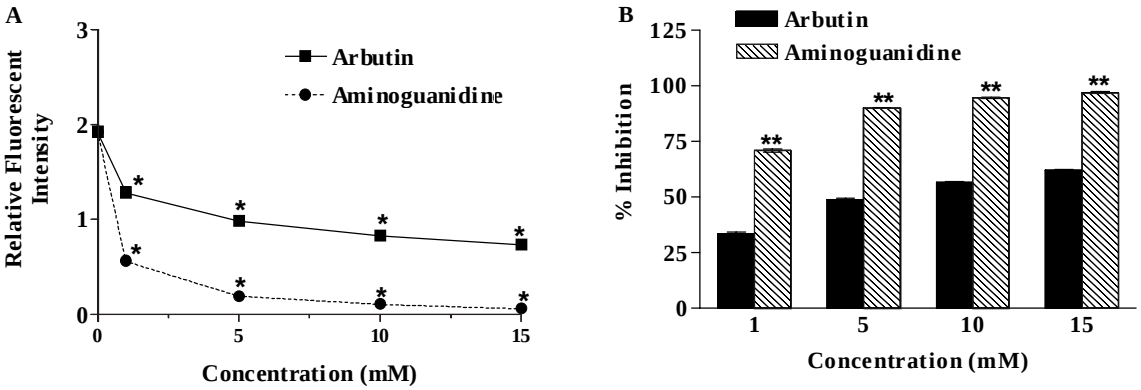

Figure 3 Dose-dependent inhibition of arbutin and aminoguanidine on BSA glycation by glucose. BSA (1 mg/ml) was incubated with glucose (500 mM) in the presence of arbutin or aminoguanidine at indicated concentrations or PBS as control buffer at 60°C for 24 hours. Following a TCA precipitation, the precipitate was redissolved and measured for relative fluorescent intensity (excitation wavelength 370 nm and emission wavelength 440 nm). Data shown are means ± SD from three experiments performed in duplicate. In A (relative fluorescent intensity), statistical difference was determined by ANOVA followed by a Dunnett's post hoc test. * indicates significant difference ($p < 0.01$) compared with the control group (0 mM). In B (percentage inhibition), statistical difference was compared with unpaired t test. ** indicates significant difference ($p < 0.0001$) between arbutin and aminoguanidine group.

Upon questioning the effects of temperature on antiglycation activity, glucose-BSA assay was performed at 37°C. The glycation reaction at 37°C proceeded at much slower rate than at 60°C, thus the incubation period was prolonged to 14 days in the presence of 0.02% sodium azide to prevent bacterial growth in reaction mixture. As shown in Figure 4, the result was very similar to that observed at 60°C, suggesting that temperature did not affect arbutin and aminoguanidine activity in this study.

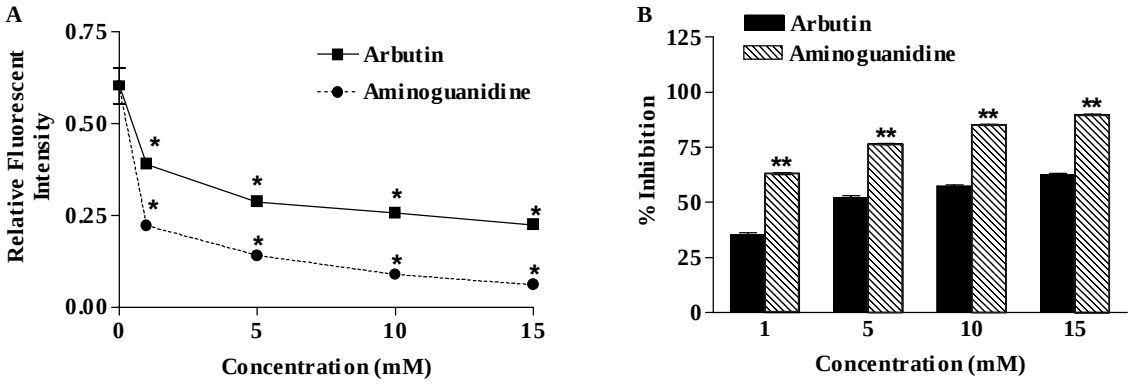

Figure 4 Dose-dependent inhibition of arbutin and aminoguanidine on BSA glycation by glucose performed at 37°C. BSA (1 mg/ml) was incubated with glucose (500 mM) in the presence of arbutin or aminoguandine at indicated concentrations or PBS as control buffer at 37°C for 14 days. Following a TCA precipitation, the precipitate was redissolved and measured for relative fluorescent intensity (excitation wavelength 370 nm and emission wavelength 440 nm). Data shown are means ± SD from three experiments performed in duplicate. In A (relative fluorescent intensity), statistical difference was determined by ANOVA followed by a Dunnett's post hoc test. * indicates significant difference ($p < 0.01$) compared with the control group (0 mM). In B (percentage inhibition), statistical difference was compared with unpaired t test. ** indicates significant difference ($p < 0.0001$) between arbutin and aminoguanidine group.

Discussion

In the present study, arbutin was demonstrated to possess antiglycation activity when tested by glucose-BSA assay. The antiglycation activity of arbutin was dose-dependent. However, arbutin activity was relatively less than that of aminoguanidine when compared on an equimolar basis. In this study, the glucose-BSA assay employed to evaluate the antiglycation activity was the method of Matsuura and colleagues (Matsuura et al., 2002). This assay was developed as a screening tool for searching of glycation inhibitors from natural product extracts that often contain interfering substances such as fluorescence and quencher materials. Use of TCA enables the removal of any soluble interfering substances prior to the measurement of fluorescent intensity and allows relative quantitation of the amount of glycated BSA. The assay was validated and found to correlate well with other methods for antiglycation assay (Matsuura et al., 2002). By this technique, the problem of arbutin autofluorescence causing the measurement of fluorescent intensity impossible was eliminated.

The mechanism of arbutin antiglycation activity is currently unknown and was not explored in the present study. Previous studies have demonstrated antioxidative and free radical scavenging property of arbutin (Petkou et al., 2002; Myagmar et al., 2004). Thus, like other natural compounds that have been shown to possess antiglycation activity, it is very likely that arbutin may exert its inhibitory effect on glycation by impeding further oxidation of glycated proteins and metal-catalyzed oxidation of glucose that leads to the formation of AGEs. Arbutin is a glycosylated hydroquinone and recently other quinone compounds have also been reported to exhibit antiglycation activity (Jung et al., 2005). These quinone compounds including derivatives of benzoquinone and naphthoquinone also possess antioxidative activity as shown by their inhibitory effects on lipid peroxidation. However, the antiglycation potency seems to correlate only partially with the antioxidative property. This finding leads the authors to conclude that other mechanisms must be involved in the antiglycation activity of these quinone compounds. The mechanism of arbutin antiglycation activity will be the subject of future studies.

Arbutin has been commonly used in cosmetics as a skin-whitening agent mainly due to its antityrosinase activity (Petkou et al., 2002). The finding that arbutin possesses antiglycation activity implies that arbutin applied topically may improve skin elasticity by its inhibition of the glycation of skin collagen. Crosslinking of skin collagen following AGEs formation is considered to contribute to age-related skin stiffening from the loss of collagen elasticity. In support of this postulation, ALT-711, an antiglycation agent has been reported to improve skin hydration and elasticity when applied topically to the skin of 24-month-old rat for 3 days (Vasan et al., 2003). Whether topical application of arbutin will have the same effect on skin elasticity remains to be studied in both animal and human.

Conclusion

Arbutin, a naturally occuring compound with antioxidative and radical scavenging activity, was demonstrated to possess an *in vitro* antiglycation activity based on glucose-BSA assay. Its antiglycation activity was relatively less than that of aminoguanidine in this assay. The mechanism of arbutin antiglycation activity and its application as a therapeutic agent need further investigation.

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