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Inhibition of Ascorbic Acid-induced Modifications in Lens Proteins by Peptides

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Abstract: The effects of three dipeptides L-phenylalanyl-glycine, glycyl-L-phenylalanine, and aspartame (L-aspartyl-L-phenylalanine, methyl ester) as inhibitors of the ascorbic acid-induced modifications in lens proteins were studied. Their efficiency was compared to that of two known inhibitors — aminoguanidine and carnosine. The tested dipeptides diminished protein carbonyl content by 32–58% and most moderated the formation of chromophores, as measured by the absorbency at 325 nm of the glycated proteins. The appearance of non-tryptophan fluorescence (excitation 340 nm/emission 410 nm) was observed for proteins glycated with ascorbic acid. All of the dipeptides examined, as well as aminoguanidine, decreased this glycation-related fluorescence. The potential inhibitors prevented the intensive formation of very high molecular weight aggregates. A competitive mechanism of their inhibitory effect was proposed, based on the reactivity of individual substances toward ascorbic acid. These findings indicate that they have a potential for use as alternatives for aminoguanidine as an anti-glycation agent. Copyright © 2003 European Peptide Society and John Wiley & Sons, Ltd.

Keywords: glycation; cataract; ascorbic acid; crystallins; inhibitors; dipeptides; aspartame; carnosine

INTRODUCTION

Glycation of lens crystallins is generally thought to contribute to the development of senile and diabetic cataracts. The interaction of protein amino groups with reducing sugars results in the production of early glycation products (Schiff bases and Amadori products) and eventually leads to conformational changes and the oxidation of thiol groups. In the case of long-lived proteins, such as lens crystallins, further reactions of early glycation products lead to the formation of cross-linked structures, chromophores and fluorophores, which are referred to as advanced glycation end products (AGEs).

Glucose and ascorbic acid (ASA) levels in the lens are similar — 1–2 mM (Paterson and Delamere, 1992; Taylor *et al.*, 1997), but ascorbic acid is much more reactive than glucose at equivalent concentration (Lee *et al.*, 1998). Although the reduced form of ASA does not participate in the glycation of lens proteins (Slight *et al.*, 1990), a decrease in antioxidant defensive system of the lens with aging and cataract development can give rise to oxidation products, derived from ASA, which readily react with lens proteins (Nagaraj *et al.*, 1991). For this reason, the nonenzymatic reactions of lens crystallins with degradation products of ascorbic acid have emerged as a significant contribution to changes observed in senile and diabetic cataract lenses. Supporting

Abbreviations: AG, aminoguanidine; AGE, advanced glycation end products; ASA, ascorbic acid; DNPH, dinitrophenylhydrazine; DTPA, diethylenetriaminepentaacetic acid.

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that possibility is the finding of elevated levels of fluorophore LM-1 in lenses from poorly controlled diabetic dogs (Nagaraj *et al.*, 1996) and human diabetic lenses (Tessier *et al.*, 1999). *In vitro* studies have shown that LM-1 can arise anaerobically from dehydroascorbic acid and its degradation products, including pentoses, but not from glucose or fructose (Nagaraj *et al.*, 1992).

In recent years, strong efforts have been undertaken to develop physiologically active substances which delay or prevent protein modifications caused by glycation. Several potential drug candidates have been examined *in vitro* or in diabetic animal models. Among the pharmacological agents tested, aminoguanidine (AG) has been reported to be one of the most effective inhibitors of AGE formation in lens proteins (Lewis *et al.*, 1990; Matsumoto *et al.*, 1997). Other guanidine derivatives, arginine (Menzel *et al.*, 1991) and metformin (Beisswenger *et al.*, 1999) have shown similar effects on protein glycation. Aspirin (Swamy *et al.*, 1989), paracetamol, and ibuprofen (Blakytny and Harding, 1992) have been intensively studied as possible anti-cataract drugs, but the mechanism of their action remains uncertain. It has been found that free amino acids (e.g. lysine, glycine, glutamic acid and aspartic acid) reduce the incorporation of radioactive labeled glucose (Ramakrishnan *et al.*, 1996) and galactose (Ramakrishnan *et al.*, 1997) into lens proteins. Potential beneficial effects on the prevention of protein glycation have been reported for vitamin B_1 and vitamin B_6 (Booth *et al.*, 1996), carnosine (Hipkiss *et al.*, 1998), and taurine (Devamanoharan *et al.*, 1997).

A competitive mechanism of action of these drug candidates is most often proposed. The inhibitors are generally strong nucleophiles capable of reacting with reducing monosaccharides and other carbonyl metabolites and, hence, preventing modifications of protein amino groups. Recently, we reported that phenylalanine-containing dipeptides have a higher reactivity towards glucose and fructose compared with other dipeptides (Argirova *et al.*, 1999) and showed that the presence of a benzene ring in the dipeptide molecules enhances their glycating potency. In the present work, we report on a test involving the dipeptides glycyl-L-phenylalanine (Gly–Phe), L-phenylalanyl-glycine (Phe–Gly), and aspartame (L-aspartyl-L-phenylalanine, methyl ester), as possible inhibitors of lens protein glycation. The ability of these compounds to retard protein modifications caused by ASA is compared with that of some known inhibitors, namely, aminoguanidine and carnosine (β-alanyl-L-histidine).

MATERIALS AND METHODS

Materials

L-Ascorbic acid, carnosine, aspartame, Gly-Phe, Phe-Gly, aminoguanidine hydrochloride, guanidine hydrochloride and diethylenetriaminepentaacetic acid (DTPA) were purchased from Sigma (St Louis, MO, USA). 2,4-dinitrophenylhydrazine was obtained from Merck (Darmstadt, Germany). Total water-soluble bovine lens protein was isolated as previously described (Ahrend and Bours, 1997). Absorption spectra of protein solutions (1 mg/ml in 50 mM sodium phosphate buffer pH 6.9) in the region 200–500 nm were recorded on a Cary-1 (Varian, Australia) spectrophotometer. Fluorescent readings of protein solutions (0.25 mg/ml in 50 mM phosphate buffer pH 6.9) were obtained using a Perkin Elmer LS-3B fluorescence spectrometer. SDS–PAGE was carried out on 12.5% gels, run on a Biometra (Göttingen, Germany) electrophoreses unit according to the method of Laemmli (1970).

In vitro Glycation of Lens Proteins with Ascorbic Acid

Total water-soluble bovine lens protein was dissolved in 100 mM sodium phosphate buffer pH 6.9, containing 1 mM DTPA. In order to maintain a low oxygen concentration and to avoid extensive oxidation of ASA, the protein solution was degassed under reduced pressure for 30 min prior dissolving the ASA. The potential inhibitors were applied as aliquots from concentrated stock solutions in the same buffer. Reaction mixtures were filtered through 0.22 μm Millipore membrane filters into sterile plastic capped vials of an appropriate volume in order to avoid access to air. Incubations were carried out in triplicate with 4 mg/ml lens proteins, 5 mM ASA, and potential inhibitors (0.5 or 1 mM). The solution, containing protein and ASA, is referred to as the positive control. The negative control contained the same protein in buffer alone.

Solutions were incubated at 37.0 °C for 4 weeks. At the end of incubation period the low molecular weight compounds were removed by extensive dialysis against 50 mM sodium phosphate buffer pH 6.9 at 4 °C. Dialyzed solutions were transferred into vials and stored at −20 °C prior analyses.

Small aliquots of the reaction mixtures were filtered through 3 kDa pore size Whatman VectraSpin micro tube filters (Whatman, UK) and the UV spectra of the filtrates, which contained the low molecular weight products, were scanned.

Assay of Oxidative Damage to Lens Proteins

The method described by Reznick and Packer (1994) was used to measure carbonyl groups on proteins. Briefly, protein solutions at 2 mg/0.5 ml were mixed with equal volumes of 10 mM dinitrophenylhydrazine (DNPH) in 2.5 M HCl and the mixtures were incubated at room temperature for 1 h with constant shaking. Thereafter trichloroacetic acid was added to a final concentration of 10% (w/v) and precipitated proteins were washed three times with 1 ml of ethanol–ethylacetate (1 : 1, v/v) to remove excess DNPH. The pellets were dried and dissolved in 1 ml of 6 M guanidine hydrochloride in 20 mM sodium phosphate buffer pH 6.5. Control samples were treated identically in 2.5 M HCl but without DNPH. The absorbance of both DNPH and HCl incubated proteins was measured at 370 nm and the difference in OD_{370} readings between each DNPH treated sample vs the HCl control was obtained. Protein content of the final protein solutions was measured using the Bradford (1976) method, with purified bovine lens protein as standard.

The results are expressed as nmol of carbonyl groups per mg protein based on an absorption coefficient of 22 000 M^{-1} cm^{-1} for most aliphatic hydrazones. Each sample was assayed in duplicate.

Statistical Analysis

The paired or unpaired Student's *t*-test, as appropriate, was used to evaluate the effect of inhibitors on modification of the lens proteins. Differences were regarded as significant at $p < 0.05$.

RESULTS

All methods applied clearly demonstrated that there were no statistically significant differences ($p > 0.1$, paired *t*-test) between the values obtained for the starting non-incubated protein and proteins incubated in buffer alone (negative control). All values obtained for the positive control differed significantly from the negative control ($p < 0.0002$, unpaired *t*-test).

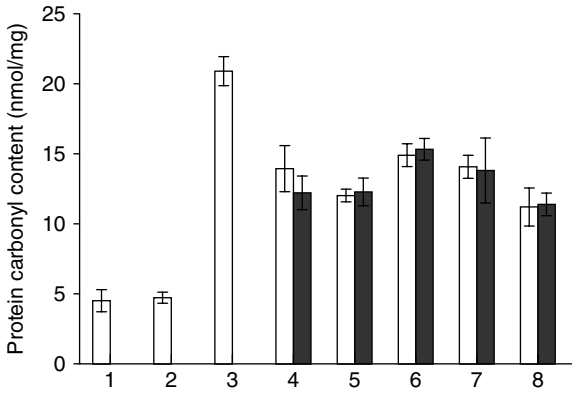

Figure 1 The effects of tested inhibitors on the ascorbic acid-induced oxidation of lens proteins measured by protein carbonyl content. **1**, non-incubated protein; **2**, negative control (no ascorbic acid); **3**, positive control (with 5 mM ascorbic acid), **4–8**, proteins incubated with 5 mM ascorbic acid and aminoguanidine, carnosine, aspartame, Gly–Phe and Phe-Gly, respectively. (□) 0.5 mM concentration of inhibitors; (■) 1 mM concentration of inhibitors. Each column represents the mean ± SD of three replicate experiments.

Changes in Protein Carbonyl Content

Protein carbonyl content was used to assess the oxidative damage to lens proteins as the result of glycation. The incubation of protein with 5 mM ASA resulted in a considerable increase in protein carbonyl content, reaching values as high as 21.12 nmol/mg (Fig. 1), while the starting protein contained 4.55 nmol carbonyl groups per mg protein. All of the potential inhibitors led to an appreciable ($p < 0.005$, unpaired *t*-test) decrease in the formation of carbonyl groups compared with the positive control. No differences between the effects shown by the two tested concentrations of inhibitors were observed. Phe–Gly and carnosine showed the strongest effects. The increase in protein carbonyl content in the presence of these dipeptides was 41.4 and 46.6%, respectively, compared with that of positive control (100%).

Changes in the UV Spectra of Glycated Proteins

The UV absorption of the proteins above 300 nm increased after incubation with ASA. No new absorption maximum appeared, but a shoulder at about 325 nm developed in the spectra (Fig. 2). The

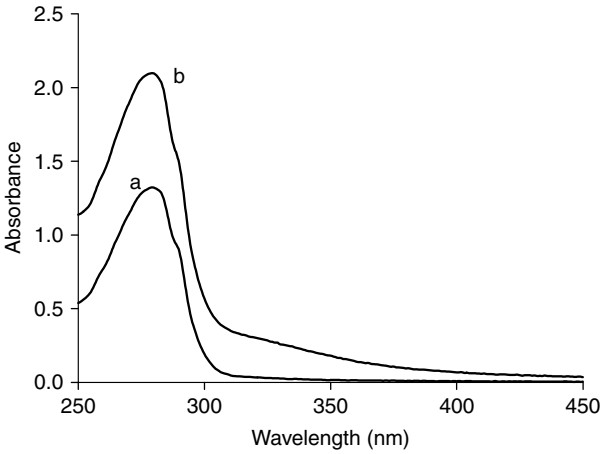

Figure 2 UV/vis spectra of (a) the negative control, and (b) the positive control. Protein concentration of both solutions is 1 mg/ml.

optical density at 325 nm was used to estimate the formation of chromophores as a result of glycation (Table 1). All inhibitors tested caused diminution in protein absorption at 325 nm, except for Gly–Phe. Carnosine and Phe–Gly were effective in both concentrations. Statistically significant effects of potential inhibitors were found for AG (1.0 mM) and aspartame (0.5 mM).

Appearance of Non-tryptophan Fluorescence

Specific non-tryptophan fluorescence at 410 nm (excitation at 340 nm) was found for ASA-modified protein in scanning the region 300–400 nm (excitation) and 350–450 nm (emission). The intensity of the emission at the same wavelength was measured for solutions that had been incubated with ASA and the potential inhibitors and the obtained values are shown in Table 1. Only carnosine had no affect on the formation of fluorophores, when used at a concentration of 0.5 mM, while the others caused a considerable decrease in glycation-related fluorescence.

Cross-linking of Lens Proteins During Reaction with ASA

All the potential inhibitors were tested for their ability to diminish protein cross-linking caused by ASA. As shown in lane 2 of Fig. 3, the starting total water soluble lens protein consists of five monomers with molecular weight between 21.5 and 28 kDa. Incubation of the same protein without ASA resulted in no change in this electrophoretic profile (lane 3). Incubation with ASA led to appearance of a non-disulfide dimer with a molecular weight of approximately 43 kDa in both positive control and protiens incubated with inhibitors. A large amount

Table 1 Formation of chromophores and fluorophores as a result of incubation of lens protein (4 mg/ml) with ascorbic acid (ASA) and potential inhibitors

Ascorbic acid concentration (mM)	Inhibitor	Inhibitor concentration (mM)	Absorbance at 325 nm (absorption units/mg)	Intensity of fluorescence, excitation 340 nm/ emission 410 nm (arbitrary units/mg)
0	—	—	0.039 ± 0.004	1.1 ± 0.3
5	—	—	0.302 ± 0.018	22.9 ± 0.4
5	AG	0.5	0.256 ± 0.035	$14.3 \pm 0.3^{*}$
5		1	$0.239 \pm 0.026^{*}$	$12.5 \pm 0.3^{*,\dagger}$
5	Carnosine	0.5	$0.254 \pm 0.016^{*}$	22.0 ± 0.1
5		1	$0.218 \pm 0.013^{**,\dagger}$	$19.5 \pm 0.3^{*,\ddagger}$
5	Aspartame	0.5	$0.255 \pm 0.014^{*}$	$18.7 \pm 0.3^{*}$
5		1	0.283 ± 0.017	$18.6 \pm 0.6^{*}$
5	Gly–Phe	0.5	0.282 ± 0.026	$18.0 \pm 0.5^{*}$
5		1	0.279 ± 0.027	$15.1 \pm 0.2^{**}$
5	Phe–Gly	0.5	$0.224 \pm 0.011^{**}$	$14.9 \pm 0.1^{**}$
5		1	$0.226 \pm 0.009^{**}$	$12.8 \pm 0.2^{**}$

Values are means ± SD of three experiments.
* $p < 0.05$ vs positive control; ** $p < 0.001$ vs positive control; † $p < 0.005$ vs the other tested concentration; ‡ $p < 0.001$ vs the other tested concentration.

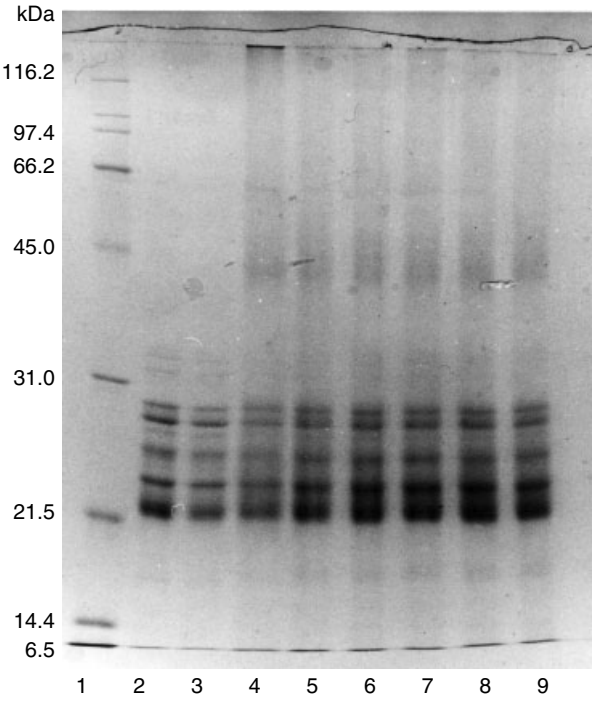

Figure 3 SDS–PAGE showing the effects of tested inhibitors on cross-linking of ascorbic acid-treated lens crystallins (10 μg). Lane 1, molecular weight standards (Bio-Rad, Germany); lane 2, initial lens protein; lane 3, negative control (no ascorbic acid); lane 4, positive control (with 5 mM ascorbic acid); lanes 5–9, lens proteins incubated with 5 mM ascorbic acid and 1 mM aminoguanidine, carnosine, aspartame, Gly–Phe and Phe–Gly, respectively.

of high molecular weight aggregates (molecular weight above 200 kDa) that did not enter the separating gel was observed in the positive control (lane 4, top of the gel). The presence of potential inhibitors (1 mM) suppressed the formation of these highly cross-linked polymers (lanes 5–9). Analogous results were observed for proteins incubated in the presence 0.5 mM inhibitors.

DISCUSSION

In vivo dual effects of ASA provoke considerable scientific interest (Podmore *et al.*, 1998; Carr and Frei, 1999), particularly with respect of development of human senile and diabetic cataract (Garland, 1991). It is generally assumed that the function of ascorbic acid in the lens is to act as an antioxidant, scavenging oxygen radicals delivered from photosensitizers. Recent epidemiologic studies (Leske *et al.*, 1991; Mares-Perlman *et al.*, 2000) have shown that increased dietary intake of vitamin C can decrease cataract risk.

On the other hand, considerable experimental evidence has accumulated in support of the assumption that ASA oxidation products can be involved in formation of senile cataract. Oxidized ASA (dehydroascorbic acid) is regenerated through the glutathione redox cycle. However, high glucose levels within the lens may impair ascorbate regeneration and hence increase pro-oxidant properties of ASA.

The experiments presented here were designed to test the issue of whether dipeptides, other than carnosine, are able to protect lens proteins against ASA-induced modifications. A variety of methods were applied to evaluate the extent of the changes accompanying protein glycation *in vivo* and *in vitro*, since a diversity of chemical structures is formed. The accumulation of protein carbonyl derivatives on serum and tissue proteins has been observed during glycoxidation reactions with ascorbic acid (Miyata *et al.*, 1998). These active carbonyls can be generated either via the formation of AGEs and their precursors, which contain aldehyde or ketone groups, or by polypeptide chain fragmentation. Since protein fragmentation was insignificant, as evidenced by SDS–PAGE profiles of glycated proteins (Fig. 3), the first mechanism seems to be the most important one.

Many of the known AGEs absorb UV light above 300 nm; some are fluorescent, although the structure of the fluorophore(s), responsible for the typical AGE fluorescence at 440 nm (excitation 370 nm), has not yet been ascertained. Since some AGE structures include two amino acid side chains, they could serve as cross-linkers of protein molecules. These three methods have been widely used as general characteristics of glycated proteins.

Our results demonstrate that all tested dipeptides are capable of lowering the extent of modification of lens proteins *in vitro*. The formation of chromophores, fluorophores and cross-linkers on lens crystallins was suppressed by the presence of these substances. The effects of Gly–Phe, Phe–Gly and aspartame, in some cases, were even stronger than that of carnosine, depending on the type of modification. Since Gly–Phe, Phe–Gly and aspartame possess neither an imidazole ring nor a β-amino acid residue, we conclude that these structural features are not important in terms of the inhibitory effect observed. The only common reactive center in the molecules tested is the primary amino group. This leads us to assume that the inhibition of protein glycation could be a general property of the

low molecular weight peptides, but that their specific structure could affect the reactivity of the target amino group, making it a preferable glycation site.

We assume a competitive mechanism of action is operative for these dipeptides considering the presence of a reactive primary amino group in the molecule. When the UV spectra of filtrates, obtained by ultrafiltration of reaction mixtures, were scanned, their spectra matched those obtained from model systems containing inhibitors and ASA without protein (data not shown). This indicates that the low molecular weight compounds were intensively glycated when incubated with protein and ASA. However, dipeptides as well as amino acids and proteins are polyfunctional molecules and other mechanisms cannot be excluded. Hydroxylated phenylalanines (tyrosine isomers) have been detected in model glycation studies (Fu *et al.*, 1998). This confirms the conclusion that the benzene ring in the phenylalanine molecule is capable of scavenging hydroxyl radicals, produced during free radical mediated glycation (glycoxidation). The peptide bond along with carboxyl and amino groups, possess good chelating capacity. It is feasible that dipeptides bind transition metal ions, thus blocking the oxidative degradation of ascorbic acid whereby they prevent the formation of more reactive carbonyl intermediates.

A clear relationship between the concentrations of the inhibitors and the effects observed does not exist. In some cases we found a stronger effect at 1.0 mM concentration and, in some, significant difference between the two concentrations tested was not observed. Considering the diversity of intermediates and the complexity of the glycation cascade, the mechanism of their action remains unsettled and further investigations are needed. Recently, it has been reported that carnosine reacts with protein carbonyl groups, which are induced by glycation thus preventing the formation of crosslinkers which are thought to result from reactions between these carbonyls and protein amino groups (Brownson and Hipkiss, 2000). It is evident from this finding that the competitive mechanism could be operative at different stages of glycation process.

One can argue that the dipeptides might have too short lifetimes to demonstrate their protective effect *in vivo* because of their degradation by dipeptidases. It should be pointed out that the activity of proteolytic enzymes in the lens is extremely low, the proteins in the lens nucleus are as old as the organism is. Thus, an appropriate local application could provide a sufficient lifetime for dipeptides to scavenge ASA degradation products.

The major finding from this study is that the inhibiting potency of the dipeptides studied is similar or higher than that of AG. This suggests that possible alternatives to aminoguanidine are potentially available as anti-glycation drugs particularly in view of recent reports concerning its side effects (Skamarauskas *et al.*, 1996; Okada and Ayabe, 1995). Since some dipeptides are physiological metabolites (carnosine) or have been proven to be nontoxic (aspartame), this class of inhibitors becomes more attractive.

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