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Effects of Carnosine and Related Compounds on Monosaccharide Autoxidation and H_2O_2 Formation

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The effects of carnosine and related compounds (CRCs) including anserine, homocarnosine, histidine, and β-alanine on monosaccharide autoxidation and H_2O_2 formation were investigated. The incubation of CRCs with D-glucose, D-glucosamine, and D, L-glyceraldehyde at 37°C increased the absorption maxima at 285 nm, 273 nm, and 290~330 nm, respectively. D, L-glyceraldehyde was the most reactive sugar with CRCs. The presence of copper strongly stimulated the reaction of carnosine and anserine with D-glucose or D-glucosamine. Carnosine and anserine stimulated H_2O_2 formation from D-glucose autoxidation in a dose-dependent manner in the presence of 10 μM Cu (II). The presence of human serum albumin (HSA) decreased their effect on H_2O_2 formation. Carnosine and anserine has a biphasic effect on α-ketoaldehyde formation from glucose autoxidation. CRCs inhibited glycation of HSA as determined by hydroxymethyl furfural, lysine residue with free ε-amino group, and fructosamine assay. These results suggest that CRCs may be protective against diabetic complications by reacting with sugars and protecting glycation of protein.

Key Words: Carnosine and related compounds, Glucose oxidation, α-ketoaldehyde, Free radicals, Glycation, Antioxidant

INTRODUCTION

Histidine-containing dipeptides such as carnosine, anserine, and homocarnosine are present in considerable amounts in several tissues of vertebrates including skeletal muscle, eye, olfactory system, and brain (Crush, 1970; Flancbaum et al, 1990). Several physiological functions of these dipeptides have been postulated. They function as neurotransmitters in the olfactory bulbs, as a physiological activator for myosin ATPase, and as regulators of the other enzymes (Parker & Ring, 1970; Ikeda et al, 1980). At physiological concentrations, these dipeptides also act as antioxidants by chelating transition metals and/or scavenging oxygen free radicals (Brown, 1981; Kohen et al, 1988; Babizhayev et al, 1994; Chan et al, 1994; Lee et al, 1999a). Recently carnosine has been reported to have an antiglycating activity on α-crystallin, ovoalbumin and actin (Hipkiss et al, 1995; Kuleva & Kovalenoko, 1997; Hipkiss & Chana, 1998). The effect of carnosine may be due to its antioxidant activity.

The oxidation of glucose is catalyzed by trace amounts of transition metals, generating free radicals, hydrogen peroxide, and reactive ketoaldehydes (Wolff & Dean, 1986, 1987; Hunt et al, 1988; Jiang et al, 1990; Lee et al, 1999b). The *in vitro* exposure of macromolecules to concentrations of glucose representative of hyperglycemia is widely considered a relevant model for the functional degeneration occurring in diabetes mellitus and aging. Proteins such as low density lipoprotein, albumin, lens crystalline, collagen, and hemoglobin, for example, undergo struc-

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tural alterations in the presence of glucose by the process of 'glycosylation', the nonenzymatic attachment of glucose to amino acid groups of the proteins (Kennedy et al, 1982; Hunt et al, 1988; Lyons et al, 1991). Protein glycosylation increases in diabetes and is a possible contributing factor to tissue damage (Goldstein et al, 1982).

A metal chelating agent such as diethylenetriamine-pentaacetic acid (DEPAEAC) or ethylenediamine-tetraacetic acid (EDTA) inhibits the glycosylation of bovine serum albumin and protein browning induced by glucose *in vitro* (Wolff & Dean, 1987). There is also evidence that metal-catalyzed glucose oxidation generates hydroxyl radicals (Hunt et al, 1988). The hydroxyl radical scavenger, sorbitol, inhibits glucose-mediated fragmentation and benzoate hydroxylation. H_2O_2 is presumably the precursor of the proximal protein oxidant since catalase inhibits glucose-stimulated protein fragmentation. Metal ions may play an important role in the increase of oxidative stress associated with diabetic complications (Mateo et al, 1978; Noto et al, 1983). Experimental glycation studies may provide further evidence for potential agents that can inhibit biological autoxidative processes and their resultant consequences in non-malignant diseases associated with diabetes mellitus and aging.

The manifold positive effects of carnosine can be employed in clinical practice for the treatment of many pathological conditions such as breast cancer, atherosclerosis, gastritis, ischemia-reperfusion injury, and cataracts (Babizhayev, 1989; Cho et al, 1991; Borgadus et al, 1993; Boissonneault et al, 1994). Although carnosine is known to protect against protein glycation due to its antioxidant activity, there is little information about the reactivity of carnosine with sugars and no comparison of carnosine and related compounds (CRCs) on antiglycating action. In this study, the reactivity of CRCs with monosaccharide including D-glucose, D-glucosamine, and D, L-glyceraldehyde and CRCs' effects on the formation of hydrogen peroxide and α-ketoaldehyde were investigated.

METHODS

Materials

D-glucose, D-glucosamine, D, L-glyceraldehyde, trichloroacetic acid (TCA), thiobarbituric acid (TBA), Cu, Zn-SOD, catalase, glyoxal, L-carnosine, homocarnosine, L-anserine, L-histidine, β-alanine, glutathione, and human serum albumin (HSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All solutions were prepared in chelax-treated phosphate buffer (pH 7.4).

UV spectroscopy

The reaction mixtures prepared in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM D-glucose, D-glucosamine, or D, L-glyceraldehyde and 5 mM CRC in the absence/presence of 10 μM Cu (II) were incubated for various time periods at 37°C. An ultraviolet absorption of the reaction mixtures was scanned using a spectrophotometer (Model UV 2100 U, Shimadzu Co., Kyoto, Japan).

Determination of hydrogen peroxide

The reaction mixtures in 10 mM sodium phosphate buffered saline (pH 7.4) containing 40 mM D-glucose, 10 μM Cu (II), and various concentrations of CRCs were incubated at 37°C for various time periods, and 0.1 ml of the reaction mixture was then mixed with 0.9 ml of the color reagent (0.1 mM xylenol orange, 0.25 mM Fe (II), and 0.1 M sorbitol in 25 mM H_2SO_4). After standing for 30 min at room temperature, the absorbance at 560 nm was measured, and the concentration of hydrogen peroxide was calculated using a known molar absorption coefficient ($1.5 \times 10^4\ M^{-1}cm^{-1}$) (Wolff, 1994). Addition of catalase (140 I.U.) in the incubation mixture completely abolished hydrogen peroxide accumulation.

Determination of α-ketoaldehyde

The reaction mixtures in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM D-glucose, 10 μM Cu (II), and CRC solutions were incubated for 3~5 days at 37°C. An aliquot (0.3 ml) of the above mixture was further incubated with 0.1 ml of 0.5 N sodium formate (pH 2.9) and 0.1 ml of 0.1 N Girard T-reagent for 10 min at 30°C, after which 0.5 ml of 0.1 N sodium formate (pH 2.9) was added to the mixtures. The absorbance at 295 nm was read against appropriate blanks [test solution+Cu (II)], and the concentrations of α-ketoaldehyde were determined using glyoxal as a standard solution (Mitchel

& Birnboim, 1977).

Cuprous ion measurement

The reduction of Cu (II) was assessed by following the appearance of bathocuproine-reactive Cu (I) (Williams et al, 1977). The reaction mixtures in 0.1 M sodium phosphate buffered saline (pH 7.4) included 25 μM Cu (II), 25 mM D-glucose, and 5 mM CRCs were incubated for 3 h at 37°C, and then 0.2 mM bathocuproine sulfonic acid was added to the reaction mixtures. The absorbance at 485 nm was measured. The concentration of Cu (I) was calculated using the standard of 10 μM Cu (I) reduced by the addition of 0.5 mM ascorbic acid as a reductant for Cu (II).

Thiobarbituric acid reactive substance (TBARS) determination

The reaction mixtures (1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM glucose, 25 μM cupric chloride, and 5 mM CRCs were incubated for 3 h at 37°C, and then 0.8 ml of the incubated mixtures was used for TBARS determination after adding 0.1 ml of 100 mM deoxyribose, 50 μl of 0.5 mM Cu (II) and 50 μl of 2 mM ascorbic acid, followed by subsequent incubation for 1 h at 37°C. One ml of TBA stock solution [1% (w/v) TBA in 50 mM NaOH+2.8% (w/v) TCA] was added to the final incubation mixture, heated in a boiling waterbath for 10 min, and the absorbance at 532 nm was then measured (Halliwell & Gutteridge, 1981).

Glycation of HSA

Hydroxymethyl furfural (HMF): Reaction mixtures (vol. 1 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 mg HSA, 25 mM D, L-glyceraldehyde, and 5 mM CRCs were incubated for 7 days at 37°C. The incubation mixtures (0.7 ml) were diluted with 3 ml of deionized distilled water (DDW) after which 0.5 ml of 40% (w/v) TCA was added to the diluted reaction mixtures. After centrifugation at 1,000×g for 10 min at 5°C, the supernatants were discarded, and 2 ml of DDW and 1 ml of 1 M oxalic acid were added to the pellet and vortexed for 20 sec. The protein solutions were hydrolyzed for 4 h in an oven (110°C) after which 0.5 ml of 40% TCA was added to the hydrolysate, followed by centrifuging at 1,000×g for 10 min. The supernatant (1.0 ml) was mixed with 0.5 ml of 0.05 M TBA and incubated for 30 min at 40°C. The absorbance was measured at 443 nm, and the concentration of HMF was determined using a HMF standard curve (Ma et al, 1981)

Determination of lysine with free ε-amino group: Sodium bicarbonate buffer [4% (w/v), pH 8.5, 0.8 ml] was mixed with the 0.2 ml of the above incubated mixture and pre-incubated for 10 min at 40°C after which 1 ml of freshly prepared 0.1% (w/v) trinitrobenzenesulfonic acid (TNBS) was added. After incubation of the mixture for 2 hrs at 40°C, 3 ml of concentrated HCl was added to the test tubes, and they were then heated at 110°C for 100 min. The hydrolysate (5 ml) was diluted with 3 ml DDW and washed twice with 20 ml of diethyl ether to remove N-amino-TNP (trinitrophenol) complex. The residual diethyl ether was removed from the bottom layer by evaporation in a hot water-bath. The absorbance at 346 nm was read, and the concentrations were calculated using a molar absorption coefficient, 1.5×10^4 $M^{-1}cm^{-1}$ for ε-TNP-L-lysine (Kakade & Leiner, 1969).

Fructosamine assay: This method relies on the ability of ketoamines (fructosamine) to act as reducing agents in alkaline solution. The remaining 0.1 ml of the incubation mixtures was used for determining capability of reducing nitro blue tetrazolium (NBT). Sodium carbonate (0.9 ml, 0.1 M, pH 10.8) and 50 l of 0.2% NBT (dissolved in 20% ethanol) were added to the incubation mixtures, then incubated for 10 min at 37°C. The absorbance (dark blue) at 530 nm was read (Johnson et al, 1982).

RESULTS

Non-enzymatic reaction of proteins, peptides, and amino acids with sugars has been studied extensively. The pathway initially involves the production of Schiff's base, followed by an Amadori rearrangement and, eventually, the formation of advanced glycation end products (AGEs). Incubation of 25 mM D-glucose with 5 mM carnosine or anserine for 2 days at 37°C showed a typical UV absorption maximum at 285 nm, regardless of the presence of 10 μM Cu (II) (Fig. 1a, b). The increase in the absorbance at 285 nm was dose- and time-dependent (data not shown). The presence of 10 μM Cu (II) stimulated

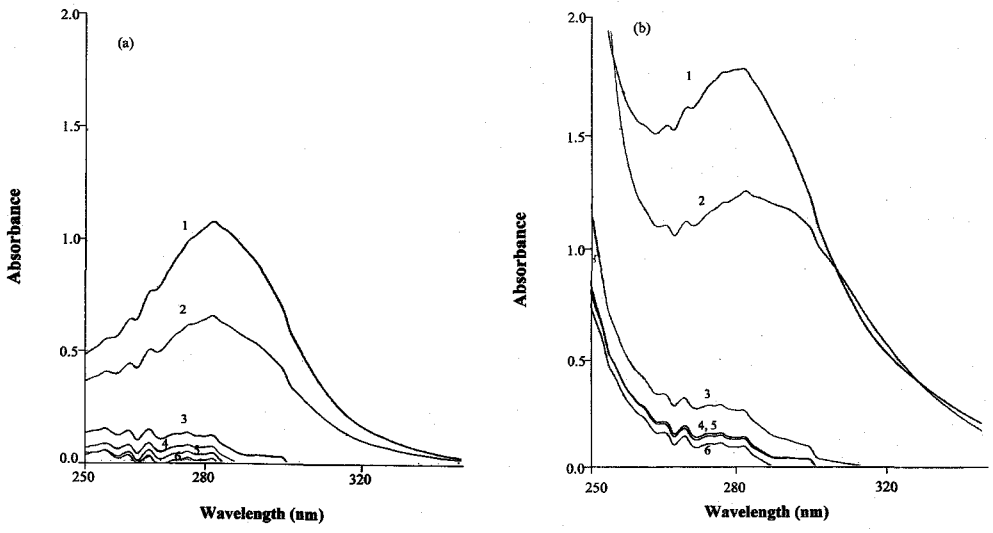

Fig. 1. Spectra of reaction mixtures in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM D-glucose and 5 mM CRCs in the absence (a)/presence (b) of 10 μM Cu (II) after incubation for 2 days at 37°C. 1: carnosine, 2: anserine, 3: homocarnosine, 4: histidine, 5: β-alanine, 6: buffer only.

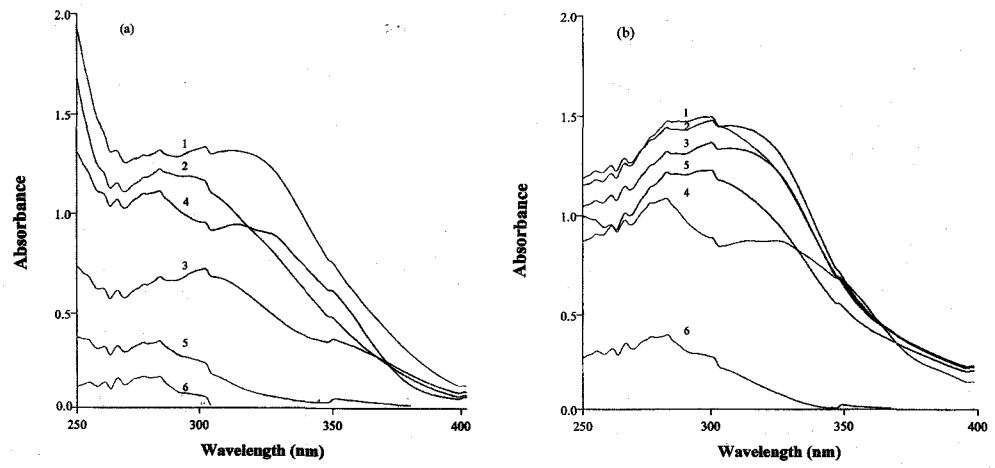

Fig. 2. Spectra of reaction mixtures in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM D, L-glyceraldehyde and 5 mM CRCs in the absence (a) of 10 μM Cu (II) after incubation for 60 min at 37°C and in the presence of 1 mg HSA (b) after incubation for 18 h at 37°C ($\times$10 dilution), 1: carnosine, 2: anserine, 3: homocarnosine, 4: histidine, 5: β-alanine, 6: buffer only.

the reaction of D-glucose with carnosine and anserine (Fig. 1b). Homocarnosine, histidine, and β-alanine did not show the absorption maxima at 285 nm, when incubated with D-glucose for 2 days at 37°C (Fig. 1). Incubation of D,L-glyceraldehyde with CRCs for 60 min at 37°C shifted the absorption maxima from 285 nm to 290~330 nm (Fig. 2a). When D,L-glyceraldehyde was incubated with CRCs for 18 h in the presence of 1 mg HSA, the spectra was similar to that without HSA, except that β-alanine showed more reactivity than histidine (Fig. 2b). The reactivity of D,L-glyceraldehyde with CRCs was much stronger than D-glucose and D-glucosamine (Fig. 1, 2 and 3). Incubation of D-glucosamine for 2 h at 37°C showed the absorption maxima at 273 nm (Fig 3a, b). The presence of 10 μM copper stimulated the reaction of D-glucosamine (Fig. 3b). Carnosine and anserine strongly stimulated the reaction in the absence of 10

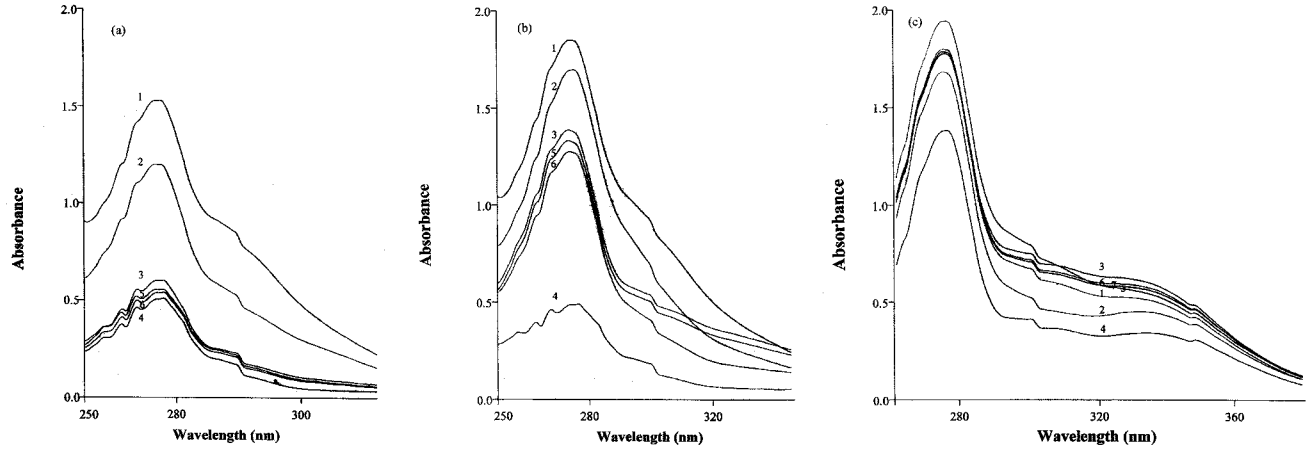

Fig. 3. Spectra of reaction mixtures in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM D-glucosamine and 5 mM CRCs in the absence (a)/presence (b) of 10 μM Cu (II) after incubation for 2 h at 37°C, and 50 mM D-glucosamine and 5 mM CRCs (c) after incubation for 20 h at 37°C. 1: carnosine, 2: anserine, 3: homocarnosine, 4: histidine, 5: β-alanine, 6: buffer only, 7: lysine.

μM Cu (II). Histidine strongly lowered the absorption maxima at 273 nm in the presence of 10 μM Cu (II) (Fig. 3b). Another absorption maxima at 340 nm appeared as increasing the incubation time of D-glucosamine at 37°C (Fig. 3c). Carnosine, anserine, and histidine actually lowered the absorption maxima at 340 nm after incubation for 20 h at 37°C (Fig. 3c).

The presence of metal ions such as iron and copper catalyzes glucose autoxidation, resulting in the generation of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals. In the absence of Cu (II), a small amount of H_2O_2 was produced from D-glucose autoxidation in 0.1 M phosphate buffer (pH 7.4) and Cu, Zn-SOD slightly stimulated the H_2O_2 formation. In the presence of 10 μM Cu (II), however, incubation of D-glucose increased the production of H_2O_2, which was dependent on D-glucose concentrations (Fig. 4). Cu, Zn-SOD (290 units) stimulated the production of H_2O_2 from D-glucose autoxidation in the presence of 10 μM Cu (II), but catalase (280 units) abolished the accumulation of H_2O_2 (Table 1). These results indicate that the autoxidation of D-glucose was involved in the formation of superoxide anions. Among CRCs, only carnosine and anserine increased the production of H_2O_2 in the presence of 10 μM Cu (II), compared to the control, whereas L-histidine actually inhibited the production of H_2O_2. The effect of carnosine on the production of H_2O_2 was greater than that of anserine (Fig. 5). In the absence of Cu

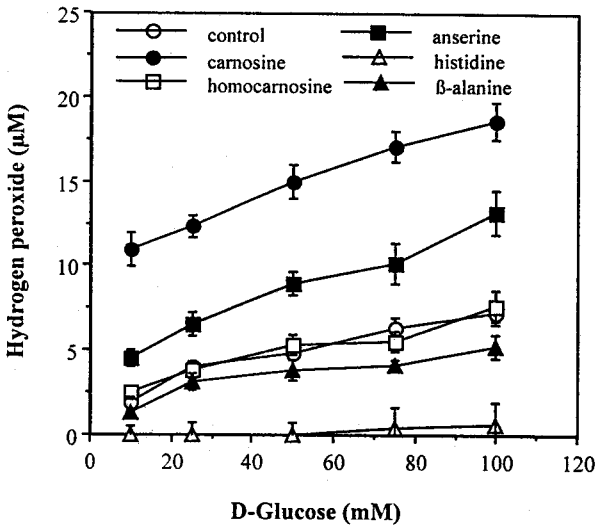

Fig. 4. Effect of 5 mM CRCs on formation of hydrogen peroxide from glucose autoxidation in the presence of copper. Reaction mixtures (vol, 1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing various concentrations of D-glucose, 5 mM CRCs, and 10 μM Cu (II) were incubated for 3 h at 37°C. Catalase (280 units) abolished the production of hydrogen peroxide. Data represent mean±SD of three determinations.

(II), the effect of these compounds on the production of H_2O_2 was negligible (Fig. 5). This result indicates that the SOD-like activity of carnosine and anserine requires the presence of Cu (II). The production of H_2O_2 by carnosine and anserine from D-glucose au-

Table 1. Effect of 5 mM carnosine and related compounds on production of hydrogen peroxide and reduction of Cu (II)

Test compounds	Hydrogen peroxide (μM)	Cu (I) (μM)	TBARS (nmole/ml)
Buffer	7.0±1.0	1.4±0.3	4.1±0.5
Carnosine	14.9±0.5*	14.8±1.2*	1.9±0.3*
Homocarnosine	8.2±1.1	2.1±0.5	2.5±0.5*
Anserine	13.5±1.3*	15.4±1.9*	2.1±0.2*
Histidine	2.4±0.5*	0.3±0.2*	2.7±0.6*
β-Alanine	6.8±0.6	1.6±0.3	3.9±0.7
Catalase (280 units)	1.1±0.4*	7.0±1.1*	0.8±0.2*
SOD (290 units)	12.1±1.3*	2.8±0.5*	5.7±0.6*

Reaction mixtures (vol, 1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 25 mM glucose, 25 μM Cu (II), and 5 mM test solutions were incubated for 3 h at 37°C, and 100 μl of the incubated mixtures was used for determination of hydroperoxide and the remaining 900 μl was used for cuprous ion determination after adding 100 μl of 2 mM bathocuproine. Data represent mean±SD of three determinations. *Significant difference ($p<0.05$) from control (Buffer).

toxidation was increased dose-dependently in the presence of 10 μM Cu (II) (Fig. 5a, b). The presence of 1 mg HSA decreased the effect of carnosine and anserine on H_2O_2 formation, probably by chelating the added Cu (II).

In the presence of metal ions, H_2O_2 produced from glucose autoxidation may break down to hydroxyl radicals via the Fenton reaction. In the presence of glucose and Cu (II), carnosine, homocarnosine, anserine, and histidine inhibited deoxyribose degradation by 54, 39, 49, and 34%, perhaps indicating that they have a hydroxyl radical scavenging activity or prevent the Fenton reaction by chelating Cu (II) (Table 1). Catalase also inhibited the reaction by 80%, but β-alanine did not. These results indicate that although carnosine and anserine increase production of H_2O_2 in the presence of Cu (II), they may also protect against cellular or molecular damage by hydroxyl radicals. In addition, carnosine and anserine stimulated reduction of 25 μM Cu (II) by 59% and 62%, respectively, in the presence of 25 mM D-glucose (Table 1). Histidine, however, actually inhibited the reduction of Cu (II).

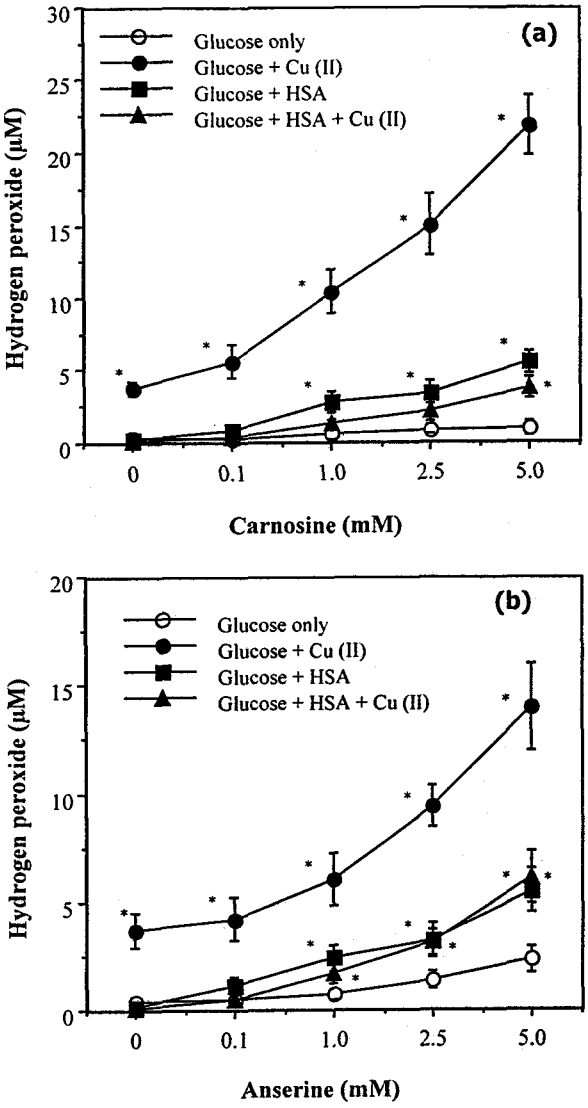

Fig. 5. Effect of (a) carnosine and (b) anserine on formation of hydrogen peroxide from glucose autoxidation. Reaction mixtures (vol, 1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing various concentrations of test solutions and 25 mM D-glucose and/or 1 mg of human serum albumin (HSA) and/or 10 μM Cu (II) were incubated for 3 h at 37°C. Catalase (280 units) abolished the production of hydrogen peroxide. Data represent mean±SD of three determinations. *Significantly different ($p<0.05$) from the glucose only group by student's t-test.

Metal ions can catalyze formation of α-ketoaldehyde from glucose autoxidation. The effect of CRCs on the formation of α-ketoaldehyde was studied in the presence of copper. The formation of α-ketoaldehyde increased with increasing incubation time and glucose

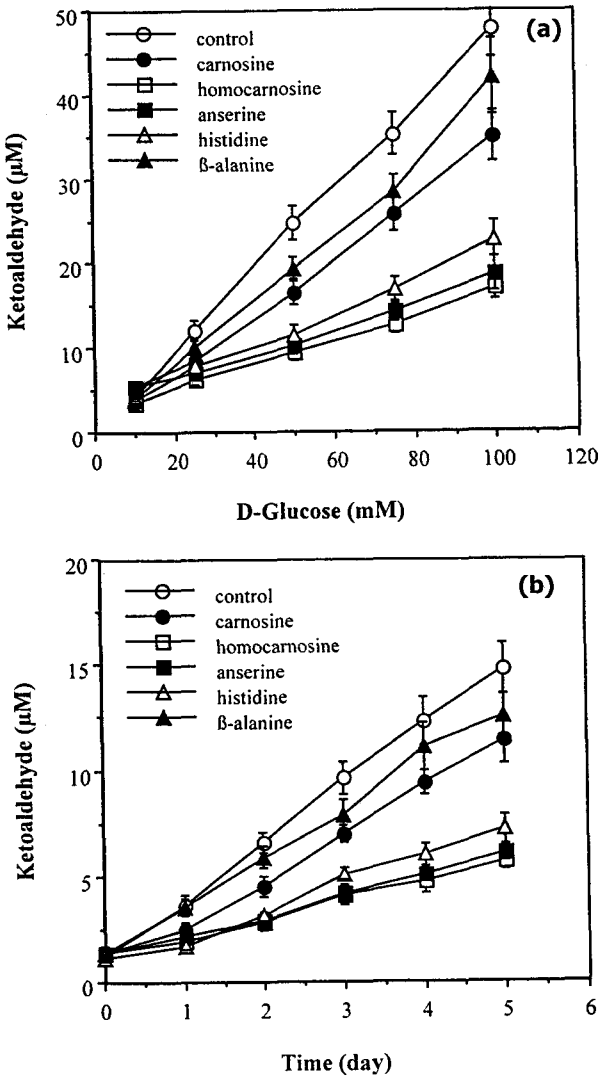

Fig. 6. Effect of 1 mM CRCs on (a) D-glucose-dependent or (b) incubation time-dependent α-ketoaldehyde formation from glucose autoxidation. Reaction mixtures (vol, 1.0 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM CRCs and 10 μM Cu (II) and were incubated at 37°C. Data represent mean±SD of three determinations. *Significantly different ($p<0.05$) from the control (buffer) by student's t-test.

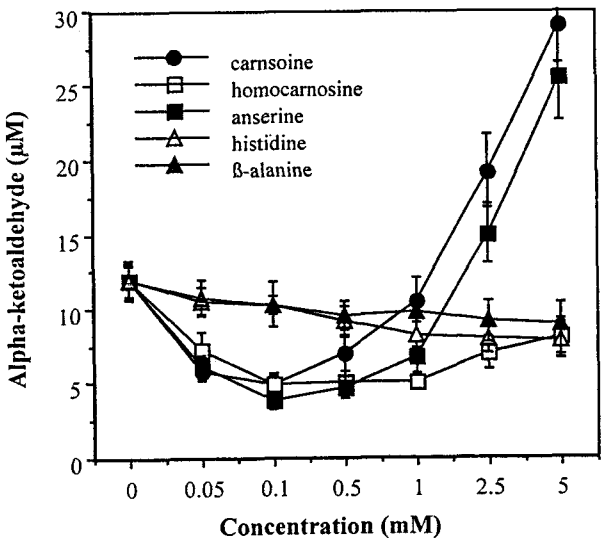

Fig. 7. Effect of CRCs on α-ketoaldehyde formation from glucose autoxidation. Reaction mixtures (vol, 1.0 ml) in 0.1 μM potassium phosphate buffer (pH 7.4) containing 25 mM D-glucose, 10 M Cu (II), and various concentration of CRCs were incubated for 6 days at 37°C. Data represent mean±SD of three determinations.

Table 2. Effect of 5 mM carnosine and related compounds on glycation of human serum albumin by D, L-glyceraldehyde

Compounds	HMF (M)/ HAS (M)	Free Lys (M)/ HSA (M)	Furctosamine (A_{530})
HSA only	0.35±0.05	54.5±3.8	0.07±0.01
Control (buffer)	2.12±0.11	27.6±2.9	1.38±0.06
Carnosine	1.64±0.18*	39.2±3.4*	0.64±0.02*
Homocarnosine	1.67±0.12*	38.0±4.1*	0.66±0.03*
Anserine	1.79±0.10*	38.3±4.4*	0.71±0.02*
L-Histidine	1.79±0.15*	40.2±3.6*	0.97±0.02*
β-Alanine	1.75±0.17*	34.2±2.6*	0.88±0.01*

Reaction mixtures (vol. 1 ml) in 0.1 M potassium phosphate buffer (pH 7.4) containing 10 mg human serum albumin (HSA), 25 mM D, L-glyceraldehyde, and 5 mM test solutions were incubated for 7 days at 37°C. Data represent the mean±SD of three determinations. *Significantly different from the control (buffer) ($p<0.05$).

concentration (Fig. 6a, b). In the presence of 10 μM Cu (II), carnosine and anserine have a biphasic effect on α-ketoaldehyde formation (Fig. 7). At the concentrations of less than 1 mM, carnosine and anserine inhibited the α-ketoaldehyde formation. Histidine significantly inhibited α-ketoaldehyde formation at the concentrations of more than 2 mM (Fig. 7).

As glycated proteins can release HMF by acid hydrolysis, HMF has been used as a marker for protein glycation or diabetes. Lysine residues of proteins are very reactive with sugars, especially with D, L-glyceraldehyde. The effect of CRC on glycation of

HSA was studied by determining the release of hydroxymethyl furfural, the disappearance of the ε-amino group of lysine, and fructosamine (ketoamine) formation in the presence of 25 mM D, L-glyceraldehyde and 10 mg/ml HSA (Table 2). CRCs significantly inhibited the release of HMF from glycated HSA, indicating that CRCs protect against the glycation by sugars. CRCs also abolished a significant decrease in free lysine residues of HSA by glycosylation. In addition, CRCs inhibited the formation of fructosamine, which has known to be very reactive with proteins (Table 2). The protective effects of CRCs might be due to the reaction of the CRCs with D, L-glyceraldehyde (shown in Fig. 2b), the competition with lysine residues as an amino sink, inhibition of metal-catalyzed autoxidative glycation, and/or scavenging α-ketoaldehyde, thereby resulting in inhibition of further reaction with proteins.

DISCUSSION

There is considerable evidence suggesting that oxidative stress contributes to development of the diabetic complications (Wolff et al, 1991). The levels of antioxidants such as ascorbic acid, vitamin E, uric acid, and glutathione (GSH) are all decreased in diabetes (Oberley, 1988). There are also increased levels of plasma lipid peroxidation products, measured as TBARS. Although biological systems are replete with antioxidants such as vitamin E, there is some evidence for abnormality in levels of catalytic transition metals in individuals with diabetes mellitus (Noto et al, 1983). Together with hyperglycemia these could contribute to oxidative stress which is associated with diabetic complications such as cataract and atherosclerosis (Oberley, 1988; Wolff et al, 1991). In diabetic complications, copper may play a role since total plasma copper concentration is higher in diabetes than in normal subjects (Mateo et al, 1978; Noto et al, 1983). Free copper ions are also detected in human atherosclerotic lesions (Smith et al, 1992). These observations suggest that the copper ion-mediated oxidative damage to protein may be an important process *in vivo*. In our *in vitro* study, copper was used as a catalyst for the production of α-ketoaldehyde and oxygen reactive species such as H_2O_2 and hydroxyl radicals.

The nonenzymatic modification of glucosamine are known to involve at least two processes (Horowitz, 1991). The first probably consists of an autocondensation, followed by subsequent expulsion of a proton, giving rise to pyrazine species that absorbs at 273 nm. The second, a slower process, gives rise to species absorbance at 320~360 nm. The latter process, but not the former, was inhibited by DETAPAC, indicating that the generation of the 320~360 nm absorbing species occurs at least in part via a metal-catalyzed oxidation (Horowitz, 1991). Our study confirmed the absorption maxima at 273 nm during early reaction and another absorption maxima at 340 nm during later reaction. Carnosine and anserine stimulated the early reaction but inhibited the later metal-catalyzed reaction. Considerable loss of D-glucosamine during incubation in phosphate buffer occurred with the generation of novel unexpected products (Candiano et al. 1988). The one of the products was 2,5-bis (tetrahydroxybutyl) pyrazine. Carnosine and anserine are expected to increase the pyrazine species during incubation at physiological conditions.

Glyceraldehyde and other simple monosaccharides autoxidize under physiological conditions generating α-ketoaldehyde (dicarbonyl) and intermediates of dioxygen reduction: superoxides, hydrogen peroxides, and hydroxyl radicals (Thornalley et al, 1984). Fig. 8 showed a schematic flow of monosaccharide autoxidation in the presence of transition metal ions. Enolization of monosaccharides is prerequisite to the oxidative process. Transition metal ions can catalyze ene-diol oxidation but metal chelators can retard the ene-diol oxidation.

The production of superoxide during monosaccharide oxidation has been probed by ESR technique using the spin-trapping agent, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) (Thornalley et al, 1984). SOD increased DMPO-R and DMPO-OH production from oxidizing glyceraldehyde/DMPO incubations, resulting from enhancement of H_2O_2 formation. Complexes of copper : carnosine and copper : anserine can dismute superoxide radicals (Kohen et al, 1991). This action of carnosine and anserine might contribute to the increase in H_2O_2 formation from the glucose autoxidation in the presence of Cu (II). However, CRC protected against deoxyribose degradation, presumably by scavenging hydroxyl radicals produced from H_2O_2 via the Fenton reaction. CRCs can quench 50~95% of hydroxyl radicals produced by Fe (II) and H_2O_2, using the EPR technique of spin trapping (Chan et al, 1994). The hydroxyl radical scavenging

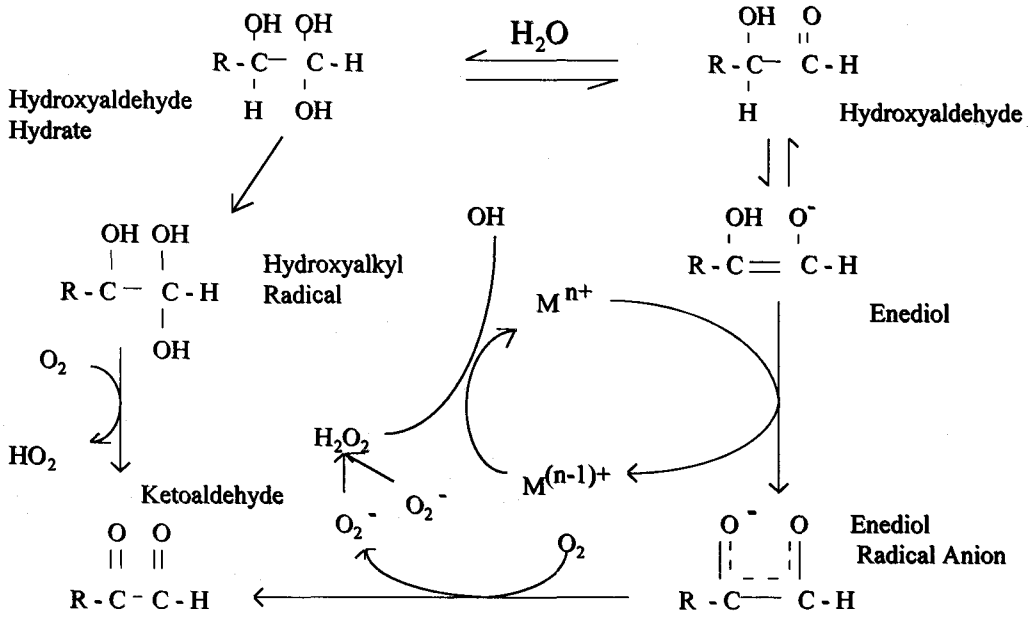

Fig. 8. A schematic flow of monosaccharide autoxidation in the presence of transition metal ions.

property is related to the presence of the peptide bond and the amino acid composition of dipeptides.

α-Ketoaldehydes are produced from monosaccharide autoxidation and by degradation of the Amadori products which are formed from rearrangement of a Schiff base (Wolff et al, 1991). These secondary products are more protein-reactive than the parent monosaccharides and can react with protein to form cross-links as well as AGEs (Njorge et al, 1986). Many proteins are modified adversely when incubated with glucose *in vitro*. When exposed to glucose *in vitro*, albumin shows diminished ligand binding capacity due to conformational alterations, SOD loses activity, and lens crystallins aggregate due to glycation (Shaklia et al, 1984; Arai et al, 1987; Monnier et al, 1992). Higher levels of glycated erythrocyte SOD are found in aged erythrocyte and in diabetes (Arai et al, 1987). In our study, although carnosine and anserine at high concentrations increased the formation of α-ketoaldehyde from glucose autoxidation, they may scavenge effectively the dicarbonyl compounds, thereby preventing proteins from further reaction with ε-group of lysine residues. Aminoguanidine also increases the generation of H_2O_2 and dicarbonyl compounds by glycosylated proteins (Skamarauskas et al, 1996). It is, however, known to inhibit the accumulation of fluorescent products, typically associated with the formation of AGEs. The antiglycating effect of aminoguanidine may be due to its interaction with reactive dicarbonyls or amadori products (Skamarauskas et al, 1996).

Given a total of 59 lys/HSA molecules, it was concluded that the reacted Lys residues represented about 8% of those on the surface of the HSA molecule. Incubation of HSA with 25 mM D,L-glyceraldehyde increased the nonenzymatic attachment of the sugar to Lys residues of HSA, representing about 53% of Lys residues reacted with D,L-glyceraldehyde. In our study, CRCs decreased significantly the nonenzymatic glycation of HSA in the presence of 25 mM D,L-glyceraldehyde, as measured by release of HMF, free lysine groups of HSA and fructosamine assay. As shown in both Fig. 2b and Table 2, the protectve effects of CRCs on HSA glycation was strongly associated with the reactivity of CRCs with D,L-glyceraldehyde. Specially, β-alanine was more effective on the protection of HSA glycation than histidine. Amino acids would be primary targets of Maillard reactants, thereby protecting the protein from damage (Monnier et al, 1991). Our results indicate that amino acids such as histidine and β-alanine as well as dipeptides are protective against diabetic complications. In addition, glycated carnosine and alanine are known to be non-mutagenic as determined by the Ames test; whereas, glycated lysine is mutagenic (Hipkiss et al, 1995).

The results from our *in vitro* studies suggest that carnosine and related compounds, which can be endo-

genously synthesized or supplied by diet, may be physiological antioxidants capable of effectively protecting against free radical-mediated damage of biomolecules thereby preserving their biochemical and physiological functions in biological systems. These compounds also protect against sugar-induced glycation of proteins, suggesting a possibility of use as therapeutic agents to minimize diabetic complications. This *in vitro* study documents another property of carnosine and related compounds, whereby they act as an amino sink for glycation. *In vivo* studies are further required to illustrate the anti-glycating activity of these compounds in detail.

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