

# Ability of Carnosine and Other Skeletal Muscle Components To Quench Unsaturated Aldehydic Lipid Oxidation Products

Shengying Zhou and Eric A. Decker\*

Department of Food Science, Chenoweth Laboratory, University of Massachusetts, Amherst, Massachusetts 01003

Breakdown of lipid peroxides results in the formation of aldehydic compounds which are toxic to biological systems and deleterious to food quality. To determine the potential of skeletal muscle compounds to protect biomolecules from lipid oxidation products, the ability of carnosine and various other related compounds to quench monounsaturated and polyunsaturated aldehydes was investigated. Carnosine, the most abundant dipeptide in skeletal muscle, is capable of quenching  $\alpha,\beta$ -monounsaturated aldehydes and 4-hydroxy-2-*trans*-nonenal (HNE) more effectively than its constituent amino acid. Carnosine (5 mM) reduced 44% of headspace *trans*-2-hexenal (0.5 mM) after 1 h incubation at 40 °C and pH 7.4. Other histidine-containing dipeptides and the amine compounds, spermine and spermidine, had similar or slightly lower quenching activity than carnosine. Glutathione and thiocetic acid had superior quenching ability than carnosine, but their overall contribution to aldehyde quenching compared to carnosine is limited due to their lower concentration in skeletal muscle. The results suggest that carnosine could be important for decreasing the toxicity of lipid oxidation products in biological systems and for minimizing rancidity in muscle foods.

**Keywords:** Carnosine; antioxidant; aldehydes; 4-hydroxy-2-*trans*-nonenal; lipid oxidation

## INTRODUCTION

Carnosine ( $\beta$ -alanyl-L-histidine) was first detected in beef extract by Gulewitsch and Amiradzibi (1900) at the beginning of the century. Since then, carnosine and the related dipeptides, anserine, ophidine, and homocarnosine, have been found at concentrations of up to 50 mM in the skeletal, cardiac, and nervous tissues of vertebrates (Davey, 1960; Crush, 1970; Harris et al., 1990; O'Dowd et al., 1990). Although several roles of carnosine have been postulated, its precise functions in biological systems are still obscure. Carnosine was reported to serve as a buffer in muscle to offset the production of lactic acid during exercise (Bate-Smith, 1938; Harris et al., 1990). Carnosine also chelates copper (Brown, 1981; Decker et al., 1992), cobalt (Brown and Antholine, 1979), and zinc (Yoshikawa et al., 1991). Researchers have found that carnosine can act as neurotransmitter in the brain (Snyder, 1980) and carnosine activates various enzymes including muscle calpain II (Johnson and Hammer, 1989), myofibrillar-APase (Parkers and Ring, 1970), and phosphorylase b (Johnson et al., 1982).

Autoxidation of polyunsaturated fatty acids in both biological and food systems is known to be accompanied by the formation of a complex mixture of secondary breakdown products of lipid peroxides including aldehydic compounds (Poli et al., 1985; St. Angelo et al., 1987). These compounds can further react with biomolecules and cause a number of adverse effects including loss of enzyme activity (Szweda et al., 1993), mutagenicity and toxicity to mammalian cells (Yau, 1979), and modification of DNA (Reiss et al., 1972) and low-density

lipoprotein (Sternberg et al., 1989; Alaiz et al., 1994). Lipid oxidation products also cause alterations in flavor, color, functional properties, and nutritive value of foods (St. Angelo et al., 1987; Decker et al., 1997).

The role of various antioxidants found in biological and food systems is to interfere or delay the production of primary lipid oxidation products. However, little is known about the protection of biomolecules from the deleterious effects of secondary lipid oxidation products if the antioxidant defense line is penetrated. Of these secondary lipid oxidations,  $\alpha,\beta$ -monounsaturated aldehydes are one of the most reactive.  $\alpha,\beta$ -Monounsaturated aldehydes react with nucleophilic compounds including amines and sulfhydryls. The objective of this study was to investigate the ability of carnosine and other amines and sulfhydryls found in skeletal muscle to interact with various unsaturated aldehydes which typically arise from the breakdown of lipid peroxides. If carnosine is capable of effectively quenching aldehydic lipid oxidation products, this could help explain its function and behavior in both biological systems and foods.

## MATERIALS AND METHODS

**Materials.** *trans*-2-Hexenal, *trans*-4-decenal, *trans*-2-nonenal, *trans,trans*-2,4-hexadienal, *trans,trans*-2,4-decadienal, glutathione (reduced), and spermidine were purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI). Leucine,  $\beta$ -alanyl- $\beta$ -alanine, leucylglycine, valylhistidine, leucylhistidine, and isoleucylhistidine were purchased from BACHEM Bioscience Inc. (King of Prussia, PA). Methylene chloride and acetonitrile (HPLC grade) were from Fisher Scientific (Fair Lawn, NJ). The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): L-carnosine (>99%, Lot 27H0883), homocarnosine, glycylhistidine, alanylhistidine, DL-6,8-thiocetic acid (reduced form), 4-hydroxy-2-*trans*-nonenal (HNE), spermine, L-alanine,  $\beta$ -alanine, L-valine, glycine, DL-lysine,  $\gamma$ -aminobutyric acid, and imidazole.

\* To whom correspondence should be addressed [telephone (413) 545-1026; fax (413) 545-1262; e-mail edecker@foodsci.umass.edu].

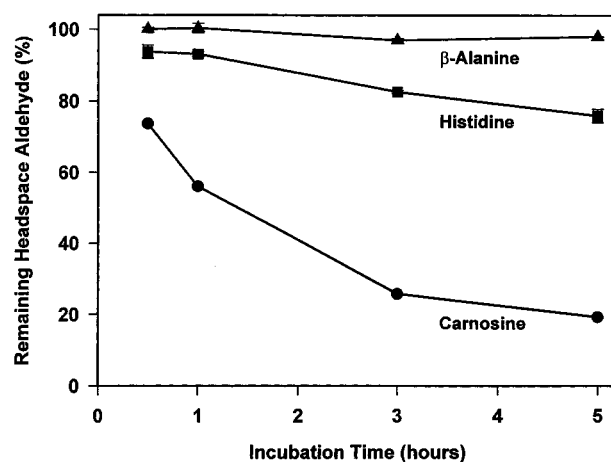
**Removal of Hydrazine from Carnosine.** Amino acids and dipeptides were examined for hydrazine concentration as described by Zhou et al. (1998). Hydrazine was not detected in all amino acids and dipeptides except for carnosine, which was contaminated at a level of 0.01% (wt). The removal of hydrazine from carnosine is based on the fact that hydrazine readily reacts with hexanal, while little interaction occurs between carnosine and hexanal under the same conditions (Zhou et al., 1998). To 80 mL of 0.12 M KCl, 5 mM phosphate buffer containing 50 mM carnosine (pH 7.4) was added 5  $\mu$ L of hexanal, and the mixture was vigorously shaken for 2 min to facilitate the dispersion of hexanal into the buffer solution. The flask was capped, sealed with Parafilm, and incubated at 40 °C for 20 min. The carnosine solution was then extracted in a separatory funnel three times with 30 mL of methylene chloride to remove the unreacted hexanal and hydrazone. The top carnosine solution was separated from the bottom methylene chloride layer, and the remaining methylene chloride in the carnosine solution was removed at 40 °C under a flow of nitrogen with constant stirring. The exact concentration of carnosine was determined by high-performance liquid chromatography (HPLC) as described in the section of HPLC analysis of carnosine. After purification, hydrazine concentrations in carnosine were reduced to  $2 \times 10^{-4}\%$  (wt). The purified carnosine stock solution was used for all subsequent studies.

**Interaction of Carnosine, Amino Acids, Related Dipeptides, Amine Compounds, Glutathione, and Thiocetic Acid with Aldehydes.** Carnosine, amino acids, related dipeptides, amine compounds, glutathione, and thiocetic acid (0.1–10 mM) in 0.12 M KCl, 5 mM phosphate buffer (pH 7.4) were mixed with individual aldehydes (0.5 mM). The mixed solution (1 mL) was sealed in headspace vials preflushed with nitrogen and incubated at 40 °C in the carousel of a Hewlett-Packard (HP) 19395A headspace sampler (Avondale, PA). Headspace aldehydes were automatically sampled and injected into the gas–liquid chromatograph (GLC) column by the headspace sampler. Calculation of headspace aldehyde concentrations was based on differences on flame ionization detector (FID) responses between the buffer containing aldehydes only and the buffer containing carnosine or related compounds plus aldehydes.

**Headspace Sampler and Gas–Liquid Chromatograph (GLC) Conditions.** Headspace analyzer conditions were the following: oil sample bath temperature, 40 °C; sample loop and transfer line temperature, 100 °C; pressurization, 10 s; venting, 10 s; injection, 1 min. GLC analyses were conducted with a HP 5890 gas chromatograph coupled with a HP 3392A integrator. Aldehydes were separated on a HP methyl silicone (DB-1) fused silica capillary column (50 m, 0.31 mm i.d., 1.03  $\mu$ m film thickness). The oven temperature was maintained at 110 °C. Injector temperature was 200 °C, and eluted compounds were detected with a FID set at 250 °C.

**HPLC Measurement of Carnosine and Its Reaction Products with *trans*-2-Hexenal.** Carnosine (5 mM) and *trans*-2-hexenal (0.5 mM) were mixed in 0.12 M KCl, 5 mM phosphate buffer (pH 7.4). The mixture was incubated at 40 °C, and carnosine concentration was analyzed after 0.5, 1, 3, and 5 h. The HPLC system consisted of a Waters Model 510 pump, a Waters 740 integrator (Milford, MA), and a Hitachi L-4200 UV–vis detector operating at 210 nm (Hitachi Instruments, Inc.). Separation of carnosine was achieved using a HYPERSIL ODS (C18) column (5  $\mu$ m; 4.6  $\times$  250 mm; Alltech Associates Inc., Deerfield, IL). The mobile phase ran at 1.5 mL/min and consisted of 0.1 M phosphate buffer (pH 7.4) and methanol (85:15, v/v).

**HPLC Measurement of 4-Hydroxy-2-*trans*-nonenal (HNE) Concentration during Its Interaction with Carnosine and Leucylhistidine.** Carnosine and leucylhistidine (5 mM) were incubated with HNE (0.5 mM) in 0.12 M KCl, 5 mM phosphate buffer (pH 7.4) at 40 °C for 3 h. Changes in HNE concentrations during incubation were monitored by HPLC. The HPLC conditions were the same as described above, except that the UV–vis detector was set at 220 nm and



**Figure 1.** Changes in headspace *trans*-2-hexenal concentrations during 5 h of incubation (40 °C) in a model consisting of 0.12 M KCl, 5 mM phosphate buffer (pH 7.4), 0.5 mM *trans*-2-hexenal and carnosine, histidine, or  $\beta$ -alanine (5 mM). Data represent means  $\pm$  standard deviation of triplicate analyses.

**Table 1.** Decrease in Headspace *trans*-2-Hexenal Concentration after 1 h of Incubation of Various Dipeptides and Amino Acids (5 mM) with *trans*-2-Hexenal (0.5 mM) in 0.12 M KCl, 5 mM Phosphate Buffer at 40 °C and pH 7.4<sup>a</sup>

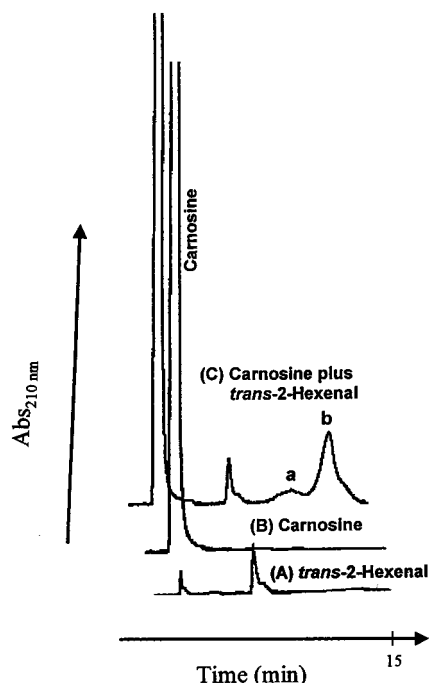
peptide or amino acid	remaining headspace <i>trans</i> -2-hexenal (%)
L-alanine	99.1 $\pm$ 1.6
$\beta$ -alanine	100.4 $\pm$ 1.0
L-valine	98.6 $\pm$ 1.9
glycine	99.1 $\pm$ 2.0
leucine	96.2 $\pm$ 0.2
$\gamma$ -aminobutyric acid	99.0 $\pm$ 1.0
DL-lysine	92.7 $\pm$ 0.2
$\beta$ -alanyl- $\beta$ -alanine	95.1 $\pm$ 0.9
alanylglycine	96.5 $\pm$ 1.0
glycylalanine	94.5 $\pm$ 1.6
leucylglycine	88.9 $\pm$ 0.2
imidazole	93.5 $\pm$ 3.6
L-histidine	91.7 $\pm$ 0.7
$\beta$ -alanylhistidine (carnosine)	56.0 $\pm$ 0.4
( $\gamma$ -aminobutyryl)histidine (homocarnosine)	77.5 $\pm$ 2.0
glycylhistidine	75.5 $\pm$ 0.6
alanylhistidine	77.0 $\pm$ 0.2
valylhistidine	59.0 $\pm$ 0.4
leucylhistidine	62.1 $\pm$ 1.1
isoleucylhistidine	54.4 $\pm$ 0.6

<sup>a</sup> Data represent means  $\pm$  standard deviation of triplicate analyses.

the mobile phase consisted of 60% double distilled water and 40% acetonitrile (Lang et al., 1985).

## RESULTS AND DISCUSSION

In a comparison of the ability to quench *trans*-2-hexenal, carnosine was much more reactive than its constituent amino acids (Figure 1). After 1 h of incubation, carnosine decreased 44% of headspace *trans*-2-hexenal, compared to 8% for histidine.  $\beta$ -Alanine did not reduce headspace *trans*-2-hexenal during the 5 h incubation period. In addition to carnosine, skeletal muscle contains other compounds which can potentially interact with unsaturated aldehydes including amino acids, peptides, polyamines, and sulfhydryls. Table 1 shows the ability of various amino acids and dipeptides to quench *trans*-2-hexenal at 40 °C and pH 7.4. Non-histidine amino acids (except for lysine) had little

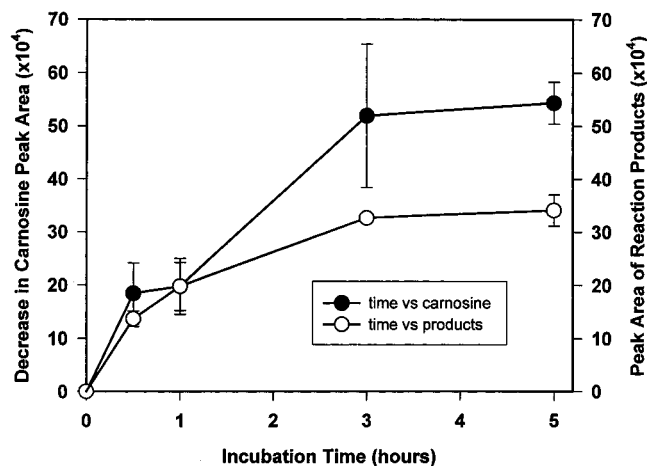


**Figure 2.** High-performance liquid chromatograms of (a) *trans*-2-hexenal (0.5 mM), (b) carnosine (5 mM), and (c) their mixture after 5 h of incubation in 0.12 M KCl, 5 mM phosphate buffer (pH 7.4) at 40 °C.

interaction with *trans*-2-hexenal ( $\leq 4\%$  reduction) suggesting that the  $\alpha$ -amino group of amino acids do not react strongly with the carbonyl group of  $\alpha,\beta$ -unsaturated aldehydes under the conditions of this model system. Lysine decreased headspace *trans*-2-hexenal 7% suggesting that  $\epsilon$ -amino groups are more reactive than  $\alpha$ -amino group (Table 1). Dipeptides containing non-histidine amino acids quenched *trans*-2-hexenal more effectively than their individual amino acids suggesting that the peptide bond increased the reactivity of the  $\alpha$ -amino group (Table 1). Leucylglycine was the most effective quencher of the non-histidine dipeptides tested.

Histidine and imidazole had similar *trans*-2-hexenal quenching activity (8% and 7% reduction, respectively) (Table 1). This suggests that the imidazole nitrogen groups and not the  $\alpha$ -amino group was the major reaction site in histidine. The histidine-based dipeptides tested were over 2.5-fold more effective than histidine at quenching *trans*-2-hexenal (Table 1). This observed increase could be due to increased reactivity of the imidazole group. However, the higher quenching activity of histidine based dipeptides could also be due to increased reactivity of the  $\alpha$ -amino group since non-histidine dipeptides also show increased reactivity compared to individual amino acids. Increased aldehyde quenching by carnosine may also be due to the specific steric configuration of the molecule (Rozhkova et al., 1996). Isoleucylhistidine and carnosine were the most effective histidine-based dipeptides at quenching *trans*-2-hexenal at pH 7.4 (46% and 44% reduction respectively). In a comparison of histidine-containing dipeptides with amino acids with  $\alpha$ -amino groups, quenching activity increased as the size of the hydrophobic alkyl side groups adjacent to the  $\alpha$ -amino group increased. Homocarnosine did not fit this trend which may be due to the unique configuration or chemical properties of the  $\gamma$ -aminobutyryl group.

Figure 2 shows the HPLC chromatograms of *trans*-2-hexenal (A), carnosine (B), and their mixture (C) after



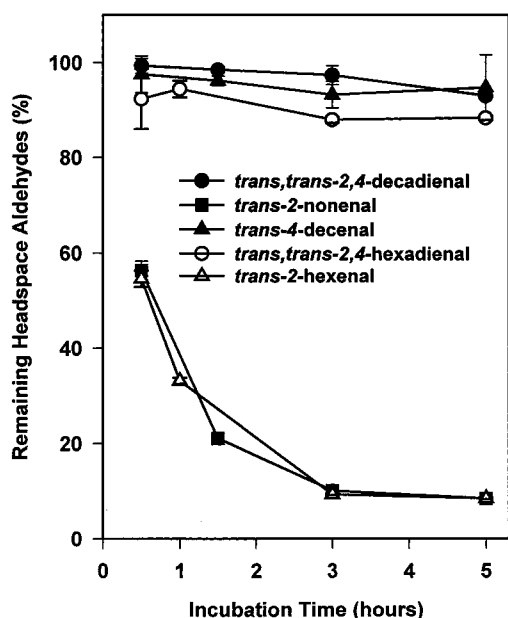
**Figure 3.** Changes in peak area of carnosine and total peak area of reaction products during 5 h of incubation of carnosine (5 mM) with *trans*-2-hexenal (0.5 mM) in 0.12 M KCl, 5 mM phosphate buffer (pH 7.4) at 40 °C. Data represent means  $\pm$  standard deviation of triplicate analyses.

5 h of incubation at 40 °C and pH 7.4. At least two reaction products were produced in the mixture of *trans*-2-hexenal plus carnosine (C), while they were not detected in *trans*-2-hexenal (A) or carnosine (B) alone. The increase in total peak area of the two products correlated well with the decrease in carnosine peak area (Figure 3). After 1 and 3 h of incubation with 0.5 mM of *trans*-2-hexenal, carnosine concentration was reduced from 5.0 to 4.86 and 4.59 mM, respectively. Decrease in carnosine concentrations during its reaction with *trans*-2-hexenal indicates that the aldehyde quenching was primarily due to chemical reactions between carnosine and *trans*-2-hexenal and not to physical factors which would alter headspace aldehyde concentrations. The different reaction products observed by HPLC could represent the formation of adducts at different sites (e.g. imidazole- and  $\alpha$ -amine groups) or through different mechanisms (e.g. Schiff base condensation or Michael-type addition).

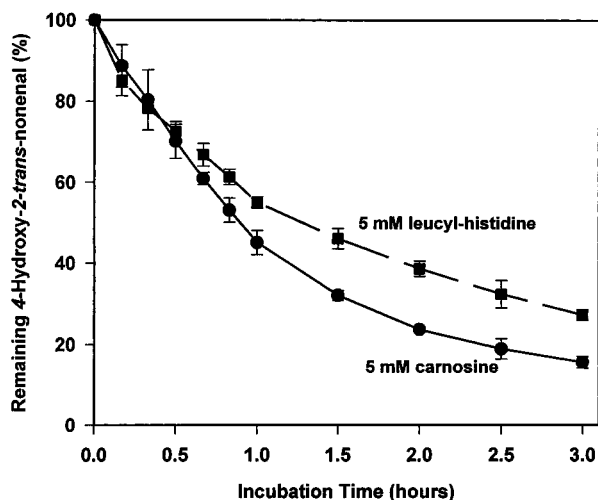
Similar reduction in headspace aldehydes was observed when another  $\alpha,\beta$ -monounsaturated aldehyde, *trans*-2-nonenal (0.5 mM), was incubated with carnosine (10 mM) (Figure 4). However, carnosine reacted weakly with the polyunsaturated aldehydes, *trans,trans*-2,4-decadienal and *trans,trans*-2,4-hexadienal and with *trans*-4-decenal, a nonconjugated monounsaturated aldehyde. After 5 h of incubation, only 12, 7, and 5% reduction in headspace *trans,trans*-2,4-hexadienal, *trans,trans*-2,4-decadienal, and *trans*-4-decenal were observed, respectively. The inability of carnosine to react strongly with the polyunsaturated aldehydes and the nonconjugated monounsaturated aldehyde suggests that carnosine is interacting with  $\alpha,\beta$ -monounsaturated aldehydes through the formation of Michael type addition products. It has been reported that *trans*-2-octenal reacts with histidine model compounds, *N*-(carbobenzoyloxy)-L-histidine and poly(L-histidine), through a Michael-type addition of the imidazole nitrogen of histidine to the  $\alpha,\beta$ -unsaturated bond of *trans*-2-octenal (Alaiz and Girón, 1994a,b).

HNE is a toxic lipid oxidation product which can react with proteins, phospholipids, and nucleic acids resulting in modification and cross-linking of protein or peptide molecules (Uchida and Stadtman, 1992; Nadkarni and Sayre, 1995). Carnosine is capable of quenching HNE in a manner similar to the other  $\alpha,\beta$ -monounsaturated





**Figure 4.** Changes in headspace concentrations of unsaturated aldehydes after 1 h of incubation of aldehydes (0.5 mM) with carnosine (10 mM) in 0.12 M KCl, 5 mM phosphate buffer (pH 7.4) at 40 °C. Data represent means  $\pm$  standard deviation of triplicate analyses.



**Figure 5.** Changes in 4-hydroxy-2-trans-nonenal (HNE, 0.5 mM) concentrations during 3 h of incubation with carnosine or leucylhistidine (5 mM) in 0.12 M KCl, 5 mM phosphate buffer (pH 7.4) at 40 °C. Data represent means  $\pm$  standard deviation of triplicate analyses.

aldehydes (Figure 5). After 1 and 3 h of incubation at 40 °C and pH 7.4, carnosine decreased 55% and 84% of headspace HNE in phosphate buffer. Leucylhistidine was also able to quench HNE but slightly less effective than carnosine. Tsai and Sokoloski (1995) reported that HNE could react with *N*<sub>α</sub>-acetyl-L-histidine, a model compound through a Michael-type addition of the imidazole nitrogen atom of histidine with the  $\alpha,\beta$ -unsaturated bond of HNE.

In addition to amino acids and peptides, other nucleophilic compounds exist in skeletal muscle which could quench  $\alpha,\beta$ -monounsaturated aldehydes including the polyamines spermine and spermidine and the sulfhydryls glutathione and thiocetic acid (Table 2). Spermine and spermidine decreased headspace *trans*-2-hexenal 20 and 14%, respectively. The increased quenching activity of the polyamines compared to lysine

**Table 2.** Decrease in Headspace *trans*-2-Hexenal Concentration after 1 h of Incubation of Amine and Sulfhydryl Compounds with *trans*-2-Hexenal (0.5 mM) in 0.12 M KCl, 5 mM Phosphate Buffer at 40 °C and pH 7.4<sup>a</sup>

compd	concn (mM)	remaining headspace aldehydes (%)	
		<i>trans</i> -2-hexenal	<i>trans,trans</i> -hexadienal
spermine	5.0	80.4 $\pm$ 0.7	
spermidine	5.0	86.2 $\pm$ 0.5	
glutathione	0.1	89.5 $\pm$ 1.8	96.9 $\pm$ 1.3
	0.5	72.1 $\pm$ 0.8	86.3 $\pm$ 1.3
	1.0	52.7 $\pm$ 0.6	79.3 $\pm$ 2.7
	5.0	5.6 $\pm$ 0.3	19.0 $\pm$ 1.1
thiocetic acid	0.1	92.2 $\pm$ 0.2	96.1 $\pm$ 4.0
	0.5	51.1 $\pm$ 3.0	91.9 $\pm$ 2.6
	1.0	24.5 $\pm$ 0.6	68.0 $\pm$ 4.5
	5.0	2.8 $\pm$ 0.1	15.9 $\pm$ 0.8

<sup>a</sup> Data represent means  $\pm$  standard deviation of triplicate analyses.

(Table 1) is likely due to the fact that they contain two  $\epsilon$ -amino groups. Table 2 also shows the ability of thiocetic acid and glutathione to quench *trans*-2-hexenal and *trans,trans*-2,4-hexadienal at concentrations of 0.1–5 mM. Both thiocetic acid and glutathione were more effective at reducing headspace *trans*-2-hexenal and *trans,trans*-2,4-hexadienal concentrations than carnosine and related dipeptides (Table 1). After 1 h incubation of 1 mM glutathione with 0.5 mM *trans*-2-hexenal at 40 °C, 47 and 21% of headspace *trans*-2-hexenal and *trans,trans*-2,4-hexadienal were reduced, respectively, compared to 76 and 32% for thiocetic acid. The higher quenching activity of thiocetic acid could be attributed to its additional sulfhydryl group. Overall on a molar basis, the order of *trans*-2-hexenal quenching was spermidine < spermine < carnosine < glutathione < thiocetic acid (Tables 1 and 2). However, typical concentrations in skeletal muscle are in the order of spermidine (3.0 mg/kg of pork, Hernández-Jover et al., 1996) < spermine (34 mg/kg of pork, Hernández-Jover et al., 1996) < glutathione (69 mg/kg of turkey, Lee et al., 1996) < carnosine (2800 mg/kg of pork, Crush, 1970). When the reactivities of glutathione and carnosine are compared on the basis of their muscle concentrations (approximately 0.3 mM for glutathione and 10 mM for carnosine), carnosine decreased headspace *trans*-2-hexenal 67% (Figure 1) compared to 19% for glutathione (Table 2). Even though sulfhydryls are much stronger quenchers of  $\alpha,\beta$ -monounsaturated aldehydes than carnosine, when comparisons are made on the basis of muscle concentrations, it becomes evident that carnosine would likely be the most important aldehyde quencher.

## CONCLUSION

Carnosine quenched  $\alpha,\beta$ -monounsaturated aldehydes more effectively than its constituent amino acids, resulting in the formation of carnosine–aldehyde adducts. Aldehyde–peptide adduct formation likely occurs on the imidazole nitrogens through Michael-type addition. In addition to carnosine, several other amine (spermine and spermidine) and sulfhydryl compounds (thiocetic acid and glutathione) exist in skeletal muscle which can quench  $\alpha,\beta$ -monounsaturated aldehyde. The sulfhydryl compounds have superior quenching activity compared to carnosine; however, their lower concentrations in skeletal muscle suggest that carnosine would be more important *in situ*. Other histidine-containing dipeptides such as leucylhistidine, isoleucylhistidine, and valyl-

histidine had similar activity to carnosine to quench  $\alpha,\beta$ -monounsaturated aldehydes. Better understanding of the mechanisms by which carnosine and related dipeptides quench  $\alpha,\beta$ -monounsaturated aldehydes could lead to the design of peptides which could quench aldehydes more effectively than carnosine.

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