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Comparative Study of Antioxidant Activity between Carnosine and Its Amino Acid Constituents

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Abstract

A study was conducted to compare and analyze the antioxidant mechanism of carnosine to its constituent amino acids (β-alanine and L-histidine) and to imidazole. Concentrations ranging from 5 to 100 mM of carnosine, β-alanine, L-histidine and imidazole were prepared and antioxidant activity assays (TBARS inhibition, metal chelating activity, free radical scavenging activity) were conducted. The TBARS inhibition of carnosine was due to the imidazole ring present in the histidine and with no inhibition contributed to β-alanine. Metal chelating properties of carnosine was also due to the imidazole ring and not to histidine or β-alanine, while free radical scavenging activity of carnosine was attributed to histidine and not due to imidazole or β-alanine. Overall, results demonstrate that β-alanine and the peptide bond between L- histidine and β-alanine do not play a role in the antioxidant activity of carnosine. Furthermore, results show that imidazole has antioxidant properties alone, and therefore, it could be used as an antioxidant in various foods and feed applications.

Keywords: antioxidant activity, β-alanine, carnosine, histidine, imidazole

Abbreviations: TBARS=Thiobarbituric acid reactive species; DPPH= 2,2-diphenyl-1-picryl-hydrazyl; TCA = Trichloroacetic acid; Ferrozine = 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"- disulfonic acid sodium salt

1. Introduction

Carnosine is a water-soluble antioxidant dipeptide composed of β-alanine and histidine that includes an imidazole ring (Figure 1a, 1b, 1c, 1d). Histidine is an essential amino acid for humans while β-alanine is non-essential amino acid synthesized in the liver as a final product of uracil and thymine degradation (Matthews & Traut, 1987). Carnosine acts as a buffer in muscle tissue (Davey, 1960; Skulachev, 2000; Smith, 1938), a potent antioxidant in skeletal muscles (Chan & Decker, 1994; Kohen et al., 1988), an aid in muscle contraction (Avena & Bowen, 1969; Severin et al., 1963) and a neurotransmitter in the brain (Trombley et. al, 2000; Tomonaga et al., 2004; Tomonaga et al., 2005). Furthermore, carnosine is a regulator of calcium, improving contraction in cardiac muscles (Roberts & Zaloga, 2000), and possesses anti-aging properties (Hipkiss, 1998; Hipkiss & Brownson, 2000; Reddy et al., 2005) along with serving as a chelating agent of metal ions (Baran, 2000; Chan & Decker., 1994; Kohen et al., 1988). Carnosine has exhibitedpharmacological and therapeutic effects in humans to treat diabetic vascular problems (Menini et al., 2020), lung disease (Tanaka & Kawahara, 2020), brain disorders (Schon et al. 2019) and kidney disease (Kartarzyna, 2020). Efficacy of carnosine against these degenerative conditions has been evaluated in several animal studies and has been linked with its ability to reduce oxidation and protein carbonylation (Chmielewska et al., 2020; Ghodsi & Kheirouri, 2018; Davies & Zhang, 2017). In fact, overexpression of genes responsible for production of carnosine synthase in mice prevented myocardial injury by controlling intracellular pH and preventing accumulation of lipid oxidation products (Zhao et al., 2020). In addition to its direct antioxidant activity, carnosine activates the nuclear factor erythroid 2, more commonly known as the Nrf2 transcription factor, that is a cellular regulator of antioxidant responses (Moi et al., 1994). Thus, the direct and indirect antioxidative impact of carnosine could have therapeutic applications for several oxidative stress diseases (Aldini et al., 2021); however, Aldini and coworkers suggested that before using carnosine for therapeutic applications, more research is needed to explain the molecular mechanisms of the antioxidant properties of carnosine (Aldini et al., 2021).

1.1 Food Applications

Food scientists have special interest in carnosine due to its antioxidant activity. Carnosine has been widely used byathletes as a dietary supplement, assisting with stimulation and contraction of muscle (Jukić et al., 2021). Furthermore, it has been reported that carnosine could play an important role as a functional food ingredient since it reduces the oxidative stress level and endothelial inflammation associated with cardiovascular disease (Jukić et al., 2021). Animal feeding studies have reported that carnosine supplementation reduced body weight, blood glucose levels, and blood pressure levels, while protecting kidney function and low-density lipoproteins from oxidation (Lee et al., 2005; Sauerhofer et al., 2007; Aldini et al., 2011; Mong et al., 2011; Nagai et al., 2012). Various mechanisms of carnosine antioxidant activity have been reported that make it attractive as a functional ingredient in human food, including metal chelation, reactive oxygen scavenging, free radical scavenging and peroxide decomposing (Baran, 2000; Chan & Decker., 1994; Kohen et al., 1988). The structure-function relationships of carnosine indicates that the imidazole moiety of carnosine and the peptide linkage between histidine and β-alanine are responsible for its overall antioxidant activity (Aruoma et al.,1989; Boldyrev et al., 1993; Boldyrev et al., 1997; Hartman et al., 1990; Kohen et al., 1988; Quinn et al., 1992); however, the mechanisms by which this occurs is not clear. Other reports have suggested that the individual amino acids in carnosine (histidine and β-alanine) have little to no antioxidant activity (Kohen et al., 1988) or antioxidant activity is inconsistent (Karel et al., 1966; Erickson et al., 1990). These reports suggest the need for additional research on the antioxidant processes of carnosine.

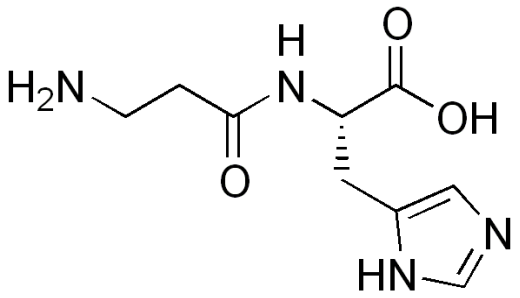

Figure 1a: Two dimensional structure of carnosine

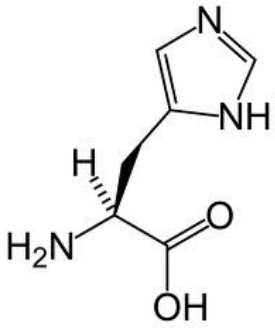

Figure 1 b: Two dimensional structure of histidine

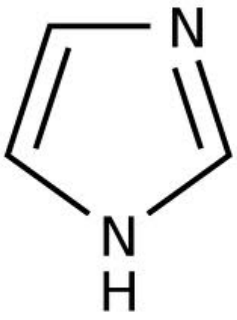

Figure 1c: Two dimensional structure of imidazole

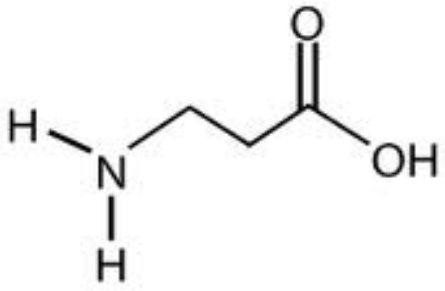

Figure 1d: Two dimensional structure of β-alanine

1.2 Objective

This study compared carnosine with its constituent amino acids, β-alanine and L-histidine as well as with imidazole to attempt to elucidate the antioxidant activity of carnosine and its constituent amino acids.

2. Methods

2.1 Materials

Ferrous chloride, ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"- disulfonic acid sodium salt], TCA [trichloroacetic acid], DPPH [2,2-diphenyl-1-picryl-hydrazyl], 2-thiobarbituric acid, L- carnosine, L-histidine, β-alanine, imidazole, L-α-phosphatidylcholine Type IV-S, and TEP [3,3,3-tetarethoxypropane] were all

purchased from Sigma Aldrich (Saint Louis, Missouri, USA). L-ascorbic acid sodium was purchased from Arcos Organics (New Jersey, USA) while BHT [butylated hydroxytoluene] was purchased from MP Biomedical, Inc (Solon, Ohio, USA). All reagents were ACS grades or purer.

2.2 Preparation of Reagents

Samples of different molar concentrations (5, 25, 50, 100 mM) of L- carnosine, L-histidine, β-alanine and imidazole were prepared using nano pure water just prior to experimentation. All reagents were prepared fresh on the day of testing.

2.3 TBARS Inhibition

Thiobarbituric acid reactive species (TBARS) inhibition was analyzed using a phosphatidyl choline emulsion system, as described by Kansci et al. (1997) and Gopalkrishnan et al. (1999). TBA/TCA reagent was prepared by a method described by Tarladgis et al. (1960). Two mg/ml phosphatidyl choline emulsion was prepared in 5mM phosphate buffer (pH 7.0) using Polytron® PT2100 homogenizer (Capitol Scientific Inc., Austin, Texas, USA). Phosphatidyl choline emulsion (1.8 ml) was mixed in 0.5 ml of sample and held for 5 min to allow interaction of carnosine or each of the constituents (i.e. histidine, β-alanine and imidazole) with the emulsion. After 5 min, the emulsion oxidation was initiated by adding catalyst $FeCl_2$ (50 µl) and sodium ascorbate (100 µl) achieving a 40 µM concentration of $FeCl_2$ and sodium ascorbate in emulsion system.

Polypropylene tubes (BD Falcon, Mississauga, ON, Canada) containing the emulsion were incubated at 37 ℃ in a water bath (Precision, Model#283, Thermo scientific, Winchester, VA, USA) and after 2 hours the reaction was terminated by adding 50 µl of 10% BHT solution. To each tube, 2.5ml of TBA/TCA solution was added and vortexed with Vortex Genie 2 ^{TM} (Model# G-560, Fisher Scientific, Bohemia, New York) followed by heating in water bath at 90 ℃ for 15 min. After heat treatment, the tubes were cooled under running tap water and then centrifuged at 5000g for 15 min at 4 ℃ in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilmington, DE, USA).

Absorbance was measured at 531nm using a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, New Jersey, USA) and water was used as a blank. Malondialdehyde (MDA) was calculated using a standard curve prepared from 1, 1, 3, 3,-tetraethpxypropane (TEP) at concentrations from 0 to 70 nM MDA. All reagents were prepared fresh daily prior to experimentation. Tubes containing reagents but without sample were used as a negative control.

Percent TBARS Inhibition was calculated using following formula (Gopalkrishnan et al., 1999):

{(MDA without extract - MDA with extract) / MDA without extract} x100

2.4 Free Radical Scavenging Assay

Free radical scavenging assay was performed as described by Yen et al. (2002). DPPH radical (0.2mM) was dissolved in absolute ethanol. Two ml of carnosine or histidine or β-alanine or imidazole was mixed with 2ml of DPPH and the mixture was allowed to stand for 30 min in dark and the absorbance of the resultant solution was measured at 517nm with spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, New Jersey, USA). Mixture without sample was used as negative control and absolute ethanol was used as a blank.

Percent free radical scavenging activity was calculated as:

{(Absorbance of control at 517 nm - Absorbance of sample at 517 nm)/Absorbance of control at 517 nm} x 100

2.5 Metal Chelating Activity

Chelating activity on Fe^{2+} was measured using the method described by Yen & Wu (1999). In this method, 1ml of carnosine or histidine or β-alanine or imidazole was added to 3.7ml of nanopure water and the mixture was reacted with 100 µl of 2mM $FeCl_2$ and 200 µl of 5mM ferrozine for 20 min kept at ambient temperature. After 20min absorbance was measured at 562nm using spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, New Jersey, USA). Nanopure water was used as a blank. Tubes with no sample was used as a negative control.

Percent chelation was calculated using formula:

{1- absorbance of the sample at 562nm/ absorbance of control at 562nm} x 100

2.6 Statistical Analysis

The experiment was replicated 4 times testing the antioxidant capacity using three assays (TBARS, free-radical scavenging and metal-chelating) of carnosine, histidine, β-alanine, and imadizole each at four concentrations (5,

24, 50 and 100mM). Treatments were the compounds and concentrations of the compounds. The main effects of compounds, concentration and the two-way interaction were tested for significance ($p \leq 0.05$) for effect on antioxidant activity results using an analysis of variance (ANOVA). To separate treatment means, the least significant difference (LSD) test was used. Both tests used a Type I Error probability of 0.05 and the SAS (Statistical Analysis Software Edition 9.2, SAS Institute Inc., 2023) was used to perform the statistical calculations.

3. Results and Discussion

3.1 TBARS Inhibition

The TBARS assay measures malondialdehyde content which is the most abundant product of lipid oxidation along with other secondary products of the reaction. Carnosine, histidine, and imidazole showed a positive trend of increased inhibition with an increase in molar concentration of each constituent (Table 1). Imidazole displayed the greatest inhibition followed by histidine and carnosine ($p \leq 0.05$) at each molar concentration. L-histidine showed higher TBARS inhibition values than carnosine at 50 and 100mM ($p \leq 0.05$) while lower TBARS values than carnosine at 25mM ($p \leq 0.05$). When a concentration of 5 mM was used, TBARS values for carnosine and L-histidine did not differ ($p \geq 0.05$). β-alanine did not exhibit TBARS inhibition at any molar concentrations tested. Results indicated that TBARS inhibition of carnosine was due to the imidazole ring present in the histidine. Erickson and Hultin (1988 and 1992) studied lipid oxidation in fish and found that histidine inhibits lipid peroxidation in a system catalyzed by ferrous and ascorbate (Erickson and Hultin, 1988; 1992). Kohen et al. (1988) reported that the antioxidant activity of histidine in muscle and brain was due largely to imidazole, and they reported that imidazole alone contributed to a 39% reduction in oxidation in soybean phosphatidyl-choline liposomes. Wade and Tucker (1998) studied the antioxidant characteristics of L-histidine and reported it to be the most active amino acid at scavenging singlet oxygen at a rate of two and three times higher than tryptophan or methionine, respectively.

Table 1. Antioxidant activities of carnosine and its constituents in different assays

	Conc.	Carnosine	Histidine	β-alanine	Imidazole
TBARS Inhibition (%)	**100mM**	12.83 ±1.15$^{e, f}$	18.80 ±0.48^{c}	0 ±0.00^{I}	32.84 ±1.14^{a}
	50mM	11.21 ±0.43$^{g, f}$	14.70 ±1.58$^{d, e}$	0±0.00^{I}	24.53±2.04^{b}
	25mM	8.32 ±0.92^{g}	4.48±0.41^{h}	0.05±0.02^{I}	16.28±1.50$^{c, d}$
	5mM	0.42 ±0.41^{iI}	0±0.00^{I}	0 ±0.00^{I}	4.62 ±0.92^{h}
Metal Chelating Activity (%)	**100mM**	96.72 ±1.31$^{a, b}$	0 ±0.00^{d}	0.36 ±0.11^{d}	98.83 ±0.37^{a}
	50mM	96.44 ±1.54$^{a, b}$	0 ±0.00^{d}	0.58 ±.20^{d}	98.94 ±0.40^{a}
	25mM	92.74 ±3.38$^{a, b}$	0 ±0.00^{d}	0.82 ±0.21^{d}	98.79 ±0.37^{a}
	5mM	86.56 ±5.99^{b}	0 ±0.00^{d}	0.83 ±0.38^{d}	96.92 ±0.97$^{a, b}$
Free Radical Scavenging Activity (%)	**100mM**	41.13 ±12.40^{a}	18.44 ±0.38^{c}	0.95±0.45^{h}	0.38±0.14$^{h, g}$
	50mM	25.16 ±7.58^{b}	13.51 ±0.23^{d}	1.00±0.46^{h}	0.25±0.09^{h}
	25mM	11.75 ±3.54^{d}	10.42 ±0.23^{e}	0.59 ±0.20^{h,g}	0±0.00^{h}
	5mM	1.86 ±0.58^{g}	5.90 ±0.22^{f}	0.71 ±0.29^{h}	0 ±0.00^{h}

1) *Percent TBARS Inhibition= {(MDA without extract-MDA with extract) / MDA without extract} x100*
2) *Percent Metal chelating activity= {1- absorbance of the sample at 562nm/ absorbance of control at 562nm} x 100*
3) *Percent Free radical scavenging = {(Absorbance of control- Absorbance of sample)/Absorbance of control } x 100*
4) *All values are Mean ±SEM (N=4);*
5) ^{aIi} *means with different superscripts for each assay are significantly different ($p \leq 0.05$).*

3.2 Metal Chelating Activity

Metal chelating activity of carnosine was similar to imidazole with no significant differences ($p \geq 0.05$) at any concentrations tested (Table 1). Histidine did not exhibit metal chelating activity while β-alanine exhibited detectable metal chelation at all concentrations, however, these levels did not differ between concentrations ($p \geq 0.05$). Data revealed that carnosine exhibited metal chelation associated with its imidazole ring and not due to

histidine, β-alanine or the presence of a peptide bond.

Carnosine acts as a metal chelator to retard oxidation reactions forming complexes with copper (Cu) ions, zinc (Zn), vanadium (V), nickel (Ni) and magnesium (Mn) ions (Baran, 2000). The majority of the studies on carnosine chelating activities have focused on copper and zinc because copper promotes oxidation of low-density lipoproteins and zinc is an important cofactor in enzymes and metalloproteins (Trombley et al., 2000). Trombley et al. (2002) also suggested that carnosine, copper and zinc play important roles in neuromodulation and neuroprotection in mammalian central nervous systems. The protonated nitrogen (N^3) in the imidazole ring interacts with Cu II ions and zinc (Zn II) and thus makes a stable metal complex (Chan and Decker., 1994), but it does not form a complex with iron (Fe III) (Decker et al., 1992). However, the results of the current study verify those of Kohen et al, (1988) that carnosine forms a complex with Fe^{2+} in the same manner it does Cu^{2+}. Thus, carnosine might stabilize feedstuffs against oxidation by chelating Fe^{2+} and also as a carrier in diet to facilitate absorption.

3.3 Free Radical Scavenging Activity

Free radical scavenging activity increased with concentration of carnosine and histidine ($p \leq 0.05$) (Table 1), while β-alanine and imidazole showed slight to no scavenging of free radicals ($p \geq 0.05$). Carnosine exhibited free radical scavenging activity primarily due to histidine and not due to imidazole ring present in its structure. These results are in agreement with those reported by Wu et al. (2003) in their study of antioxidant properties of carnosine, anserine, free amino acids and combinations. Kohen et al. (1988) suggested that the hydrogens on the methylene carbon next to the imidazole ring are the likely proton donor terminating the oxidation reaction caused by free radicals. In our study, data for free radical scavenging activity of histidine (at 25mM exhibited 4.48 % scavenging) was slightly lower than Wu et al. (2003) (at 20mM exhibited 7.4% scavenging). This difference was likely due to lower concentration of DPPH (0.1mM) used by Wu et al. (2003) compared to the present study.

Oxygen radical scavenging capacity of carnosine, homocarnosine and anserine against peroxyl radicals was studied by Kohen et al. (1988) by using voltametric measurements in AMVN {2, 2-azobis (2, 4-dimethylvaleronitrile)} and AAPH {2, 2'-azobis (2-amidino-propane dihydrochloride)} systems under physiological conditions. They also compared different structures such as carnosine, anserine, GABA {γ-amino butyric acid}, imidazole, L-alanine, β-alanine and found that inhibition of peroxyl radicals by carnosine was 53%, histidine 42%, imidazole 39%, and anserine 39% while GABA, L-alanine and β-alanine showed no inhibition. Inhibition against peroxyl radicals is due to donation of a hydrogen atom to peroxyl radicals. Carnosine, histidine, and β-alanine results from Kohen et al (1988) were similar to those found in the current study while results for imidazole differed. The un-availability of electrons in the imidazole ring to donate to the DPPH radical in the current study verses the availability of hydrogen atom to scavenge peroxyl radicals used by Kohen et al (1988) may be the reason for differing results for the imidazole.

In their study, Wu et al. (2003) measured antioxidant activity of carnosine, anserine, histidine, β-alanine, 1-methyl histidine and combinations at different concentrations using a ferric thiocyanate method to measure lipid autoxidation, DPPH radical scavenging assay and Cu^{2+} chelating ability. These researchers concluded that the peptide linkage in carnosine is involved in its antioxidant activity (Wu et al., 2003). Similar conclusions were drawn by Chen and Decker (1994) and supported that the antioxidant properties of carnosine could also be attributed to the peptide bond present between β-alanine and histidine and not to histidine and β-alanine alone. Similar conclusions were also drawn by Yen et al. (2002), furthermore, these results agree with the findings of Kansci et al., (1997) who reported that carnosine antioxidant activity is multifunctional as it chelates metal ions, decreases free radicals and reacts with some secondary oxidation products.

4. Conclusions

Based on the present study, the peptide bond may not contribute significantly to antioxidant activity since carnosine, histidine and imidazole exhibited antioxidant ability in all tests while β-alanine did not show strong antioxidant activity in any test conducted. Therefore, it can be concluded that histidine and imidazole ring of histidine are the more important constituents contributing to antioxidant activity and the peptide bond to be less of a contributing factor.

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