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Antioxidant Properties of Carnosine Re-evaluated in a Ferrylmyoglobin Model System and in Cooked Pork Patties

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The antioxidative effect of purified carnosine (i.e., separated from the common contaminant hydrazine) has been evaluated in two systems: (i) Carnosine was found to possess poor reducing properties toward the prooxidant ferrylmyoglobin; at pH ∼5 the presence of carnosine did not increase the rate of reduction of MbFe(IV)═O compared to autoreduction, whereas at pH 7.4 the rate constant for reduction by carnosine was 0.010 ± 0.002 $M^{-1}\cdot s^{-1}$ (I = 0.16; 25.0 °C). (ii) In cooked pork patties prepared from meat (longissimus dorsi and masseter) with purified or nonpurified carnosine added, the effect of purified carnosine was insignificant when compared to control patties, whereas patties with carnosine contaminated with hydrazine had a lower oxidation level than patties with purified carnosine. Carnosine is concluded not to deactivate the prooxidant ferrylmyoglobin and not to have any antioxidative effect in cooked pork.

KEYWORDS: Carnosine; ferrylmyoglobin; meat; antioxidant

INTRODUCTION

Carnosine (β-alanyl-L-histidine) is a dipeptide found in muscle in rather high concentrations, especially in glycolytic muscle (*1*). It is believed to be an endogenous water-soluble antioxidant, because several studies have shown an antioxidative effect of carnosine in model systems and in meat preparations (*2*). Proposed antioxidative mechanisms include interference with metal ions, which catalyze breakdown of hydroperoxides (*3*), a simple pH effect due to the buffering capacity of carnosine (*3*), scavenging of singlet oxygen (*4*), scavenging of hydroxyl radicals and peroxyl radicals (*5*), and quenching of secondary lipid oxidation products (*6*). Carnosine has also been reported to react with ferrylmyoglobin, a strongly oxidizing compound and initiator of radical processes. MbFe(IV)═O is formed when myoglobin reacts with hydrogen peroxide, and carnosine was found to reduce MbFe(IV)═O to MbFe(III) and further to reduce MbFe(III) to MbFe(II)O_2 (*7*).

In 1998, Zhou et al. (*8*) reported that commercial preparations of carnosine were contaminated with hydrazine, a reducing compound which also is known to react with aldehydes. It was furthermore found that both hydrazine and carnosine interfered with detection of lipid oxidation by the TBARS method, mainly by reaction with secondary lipid oxidation products. The presence of hydrazine in a sample together with carnosine offers an alternative explanation to the antioxidative activity ascribed to carnosine (*8*), and the role of carnosine needs to be re-evaluated in this light.

Few studies have been reported in which carnosine is known to be free of hydrazine. Gopalakrishnan et al. (9) found a significant but weak antioxidative activity of carnosine-containing extracts of mechanically separated pork, which were tested in a phospholipid liposome system with iron/ascorbate as an initiator of oxidation and in ground salted pork stored at −15 °C for 12 weeks. The ability of carnosine to inhibit oxidation in phospholipid liposomes catalyzed by metal ions and ascorbate was confirmed in a study using purified synthetic carnosine (*10*). Purified carnosine is further able to react with aldehydes formed as secondary products in lipid oxidation, primarily by reaction with α,β-unsaturated aldehydes but also with saturated and polyunsaturated aldehydes (*6*, *10*). Zhou and Decker (*6*) and Decker et al. (*10*) suggested that this effect of carnosine could be important for removal of toxic products of lipid oxidation in vivo. Finally, Decker et al. (*10*) reported that purified carnosine is capable of scavenging peroxyl radicals generated by 2,2′-azobis(2-amidopropane) (AAPH) and ascribed this activity to the histidine part of the molecule.

In the present study the effect of purified carnosine on oxidation is further investigated. The electron-donating properties of carnosine are tested in a myoglobin system, and antioxidative activity is further tested in a meat system. Moreover, in the latter system pH is standardized in all samples in order to be able to differentiate between a primary antioxidative effect of carnosine and a buffering effect of the dipeptide.

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MATERIALS AND METHODS

Chemicals. L-Carnosine (>99%, lot 89H1240), hexanal, hydrazine sulfate, and myoglobin (horse heart, type III) were obtained from Sigma Chemical Co. (St. Louis, MO). Analytical grade H_2O_2 (Perhydrol 30%) and all other chemicals (analytical grade) were obtained from Merck (Darmstadt, Germany). Water was purified through a Millipore Q-Plus purification train (Millipore Corp., Bedford, MA).

Purification of Carnosine. Hydrazine in stock solutions of carnosine was removed according to the procedure given by Zhou and Decker (*6*) with minor modifications. Eighty milliliters of 0.2 M carnosine in 0.12 M KCl and 5 mM phosphate buffer (pH 7.4) was mixed with 20 μL of hexanal and shaken for 2 min. The mixture was then incubated at 40 °C for 20 min before it was extracted three times in a separatory funnel with 30 mL of methylene chloride. The procedure was repeated three times, and finally the remaining methylene chloride in the carnosine solution was removed at 40 °C under a flow of nitrogen. The concentration of carnosine was determined spectrophotometrically ($\epsilon_{210} = 7900\ M^{-1}\cdot cm^{-1}$) (as determined from a dilution series).

Determination of Hydrazine in Carnosine Stock Solutions. Hydrazine concentrations were determined according to a standard method (*11*). Depending on hydrazine concentrations, the carnosine stock solutions were diluted with water before 2.5 mL of carnosine solution was mixed with 50 μL of concentrated HCl, 5.45 mL of water, and 1.0 mL of *p*-dimethylaminobenzaldehyde solution (20 mg/mL methanol). Absorbances in the resulting reaction samples were recorded at 458 nm after 10 min using an HP8453 UV−vis diode array spectrophotometer (Hewlett-Packard Co., Palo Alto, CA). A hydrazine standard curve was used to calculate the hydrazine concentrations.

Synthesis of Ferrylmyoglobin. MbFe(III) dissolved in 5.0 mM phosphate buffer (ionic strength 0.16 adjusted with NaCl) was purified on a Sephadex G25 column (40 × 25 cm, Pharmacia Biotech AB, Uppsala, Sweden). The eluted MbFe(III) was diluted with the phosphate buffer to yield a ~0.1 M solution as determined spectrophotometrically at 525 nm, $\epsilon_{525} = 7700\ M^{-1}\cdot cm^{-1}$ (*12*). Ferrylmyoglobin was formed by the reaction of MbFe(III) with excess H_2O_2 corresponding to a factor of 1.3, and the solution was left at 25.0 °C for 10 min to allow decay of perferrylmyoglobin to ferrylmyoglobin before subsequent reaction with carnosine.

Kinetic Experiments. Buffered carnosine solutions were made by appropriate dilution of the purified stock solution with phosphate buffer and adjustment to a final ionic strength of 0.16 with NaCl. A carnosine solution of 1.40 mL was thermostated in the cell holder of an HP8453 UV−vis diode array spectrophotometer before the addition of 1.40 mL of prethermostated MbFe(IV)=O solution, and recording of absorption spectra as a function of time was started after mixing. Pseudo-first-order rate constants for the reactions were calculated by nonlinear regression analysis, using the decay in ferrylmyoglobin concentration, which was calculated according to the following expression (*13*):

$$[\text{MbFe(IV)=O}]\ (\mu M) = -62(A_{490} - A_{700}) + 242(A_{560} - A_{700}) - 123(A_{580} - A_{700}) \quad (1)$$

Carnosine was in excess relative to MbFe(IV)=O by at least a factor of 40 in the reaction mixtures; the buffer concentration was 20 mM and the ionic strength 0.16 ± 0.01 adjusted with NaCl. pH in the reaction mixtures (thermostated) was measured after recording of the absorption spectra had been completed.

Experiments with the reduction of MbFe(IV)=O by hydrazine were carried out as described above except that buffered hydrazine solutions ($I = 0.16$) were used instead of carnosine solutions.

Preparation of Meat Samples for the Storage Experiment. Fresh meat (masseter from the jaw structure and longissimus dorsi) from slaughtered pigs was obtained from a slaughterhouse (Slagteriskolen, Roskilde, Denmark). The two types of meat were ground by use of a Fleischwolf X70 meat grinder (Scharfen, Witten, Germany). Each type was divided into three parts and amended: (i) 0.2 M purified carnosine in 0.12 M KCl, 5 mM KH_2PO_4; (ii) 0.2 M unpurified carnosine in 0.12 M KCl, 5 mM KH_2PO_4; or (iii) a control solution (0.12 M KCl, 5 mM KH_2PO_4), resulting in final amounts of 506 mg of carnosine per 100 g of meat being added to longissimus dorsi samples and 619 mg of carnosine per 100 g of meat being added to masseter samples. The final content of KCl in all samples was 0.1%. Before addition, pH in the carnosine/KCl solutions was adjusted to 5.8 with 1.0 M HCl. The samples were allowed to equilibrate for 24 h at 2.0 °C before they were transferred as patties of 150 g to hermetically sealed aluminum cans [inner sealing, aluminum pigmented epoxy phenol 7 g/m^2; weld protection, white polyester sealing; approved for food contact (U.S. Food and Drug Administration, §175−300)]. Four cans were used for each treatment, and they were stored at −22 °C until use.

Thiobarbuturic Acid Reactive Substances (TBARS). At the first day of the storage experiment, the patties in the cans were heated for 1 h at 95 °C in a water bath. This heat treatment has earlier been shown to maximize oxidation in similar samples (*14*). After the heat treatment, the patties were transferred to plastic trays, and a fourth of each patty was cut for TBARS analysis before the trays were stored at 4 °C in sealed polyamide/polyethylene bags (20 μm/70 μm). Formation of TBARS in the patties was analyzed by using the extraction method of Vyncke (*15*, *16*) with the modifications suggested by Sørensen and Jørgensen (*17*). The analyses were performed at days 0, 2, 5, and 8 after the heat treatment, and the results were expressed as micromoles of malonaldehyde per kilogram of meat.

Measurement of Meat pH. The pH in thawed meat patties was measured before heat treatment with a Metrohm 6.0226.100 electrode (Metrohm Ltd., Herisau, Switzerland).

Statistical Analysis. The development in TBARS for the two types of meat was obviously very different, and the ranges of measured TBARS in the two groups do not overlap after 2 days of storage. Hence, the statistical analyses were carried out separately for each type of meat. The measured TBARS were transformed with the natural logarithm to obtain homogeneous variance. The development in log-transformed TBARS over storage time was analyzed by analysis of variance where days of storage, treatment, and the interaction between days of storage and treatment were included as fixed effects, whereas patty was included as a random effect. Within this model contrasts between the treatment groups were tested by pairwise comparison via *t* test. On the basis of the stastitical analysis one TBA value (of quadropole determination) was concluded to be an outlier and was omitted from the analysis of the statistical data (purified carnosine, day 2, one patty), leaving three instead of four measurements.

RESULTS

The hydrazine content of the commercial carnosine preparation was determined to be 0.092% (wt), and it was purified to contain 1.6×10^{-4}% (wt), corresponding to a 575-fold decrease in hydrazine content.

Carnosine (in excess) was found to reduce MbFe(IV)=O by a pseudo-first-order reaction. The myoglobin reaction product was MbFe(III), not MbFe(II)O_2, as indicated by the absorption spectra (**Figure 1**). In contrast, hydrazine was shown to reduce both MbFe(III) and MbFe(IV)=O [either directly or via MbFe(III)] to MbFe(II)O_2 (data not shown).

Reduction of MbFe(IV)=O by carnosine proceeded at a rate comparable to that of the autoreduction of MbFe(IV)=O, which accordingly had to be taken into account when reaction rate constants were calculated. In some of the kinetic experiments at pH 7.3—primarily in mixtures with low concentrations of carnosine—the conversion of MbFe(IV)=O to MbFe(III) was not strictly of first order, as judged by poor fits of the expression

$$[\text{MbFe(IV)=O}]_t = [\text{MbFe(IV)=O}]_0 \exp(-k_{obs}t) \quad (2)$$

to the experimental data. This was most likely caused by the autoreduction of MbFe(IV)=O, the rate of which deviates from first-order kinetics at pH >~7 (*18*). For the remaining data, to which a satisfactory fit of eq 2 could be obtained, the observed first-order rate constant (k_{obs}) increased linearly with increasing

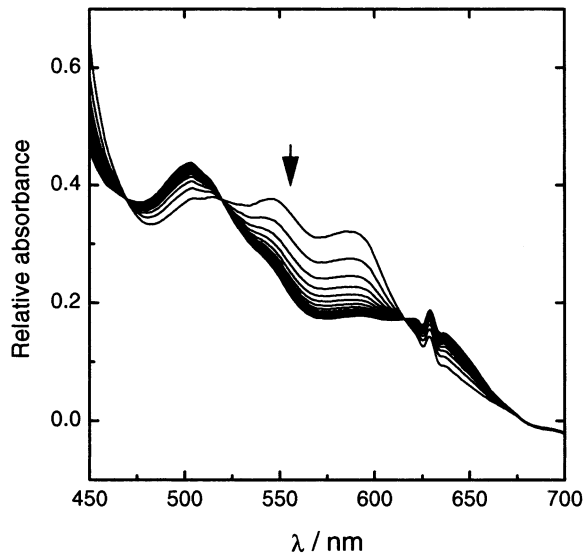

Figure 1. Reaction between 49 μM activated myoglobin [essentially MbFe(IV)═O] and 6.00 mM purified carnosine at pH 7.30 (0.02 M phosphate buffer) and 25 °C at an ionic strength of 0.16 (NaCl). Absorption spectra were recorded with intervals of 720 s and 148.5 min elapsed from initiation of the reaction to recording of the final spectrum. The direction of spectral changes in relation to the initial spectrum is indicated by an arrow.

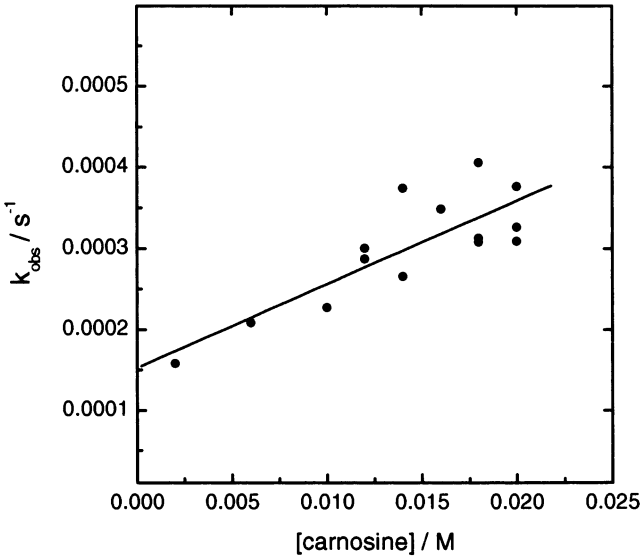

Figure 2. Observed pseudo-first-order rate constants k_{obs} for reduction of MbFe(IV)═O by purified carnosine at 25 °C, pH 7.30, $I = 0.16$. The second-order rate constant $k_2 = 0.010 \pm 0.002$ $M^{-1}\cdot s^{-1}$ was obtained by linear regression according to the expression $k_{obs} = k_{auto} + k_2$[carnosine] (concentration mol·L^{-1}), where k_{auto} is the rate constant for autoreduction of MbFe(IV)═O.

concentrations of carnosine (**Figure 2**) in agreement with the expression

$$k_{obs} = k_{auto} + k_2[\text{carnosine}] \quad (3)$$

where k_2 is the second-order rate constant for the reduction of MbFe(IV)═O by carnosine and k_{auto} is the rate constant for the parallel autoreduction of MbFe(IV)═O (assumed to follow first-order kinetics). The rate constant for the latter reaction was determined by linear regression to $k_{auto} = 1.5 \times 10^{-4} \pm 0.3 \times 10^{-4}$ s^{-1}, in agreement with the value of $\sim 2 \times 10^{-4}$ s^{-1} previously reported (*19*) (pH 7.4, 25 °C). The second-order rate constant for the reduction of MbFe(IV)═O by carnosine was found to be 0.010 ± 0.002 $M^{-1}\cdot s^{-1}$, which is low compared to data reported for other compounds of relevance as antioxidants in meat products (*20*).

An attempt was also made to measure the rate of reduction of MbFe(IV)═O by carnosine at pH 5.0. However, at this pH the overall conversion of MbFe(IV)═O to MbFe(III) was dominated by the autoreduction of MbFe(IV)═O, and the rate of reduction in the presence and absence of carnosine was very similar.

For the storage experiment with minced meat patties, two muscles with different endogenous carnosine contents were chosen: longissimus dorsi, a glycolytic muscle with 313 ± 35 mg of carnosine/100 g of muscle, and masseter, an oxidative muscle with 21.06 ± 1.47 mg of carnosine/100 g of muscle, as reported by Aristoy and Toldrá (*1*). According to this, the final amount of carnosine in the patties corresponded to 2.6 times the original content in the longissimus dorsi samples and to 30 times the original content in the masseter samples. No pH difference between different samples was detected in thawed meat patties before heat treatment and storage (pH 5.8 in longissimus dorsi samples and pH 5.7 in masseter samples), and thus any significant buffering effect of carnosine solutions versus control solution had been eliminated.

The development in TBARS in cooked patties during 8 days of refrigerated storage is shown in **Figure 3**. The oxidation levels reflected by the TBARS value differ for the two muscles, with masseter patties oxidizing more quickly and reaching higher TBARS values than longissimus dorsi patties.

The analysis of variance of the development in TBARS over time of the longissimus dorsi patties showed a significant effect of treatment ($p = 0.03$) and a significant effect of storage days ($p < 0.0001$). The TBARS were higher in patties containing purified carnosine compared to patties subject to the control treatment, which again had higher TBARS than patties containing unpurified carnosine. However, a pairwise comparison via *t* test showed a significant difference only between the two different carnosine treatments, whereas the control treatment was not significantly different from any of the carnosine-treated groups. In light of the small size of the study, an effect of purified carnosine, reflecting a slight pro-oxidative effect, cannot be excluded.

The analysis of variance of the development in TBARS over time of the masseter patties also showed an effect of treatment, but here the effect varies over time corresponding to a significant interaction between treatment and storage days ($p = 0.009$). Pairwise comparisons of the treatment groups showed that at the first day of analysis (day 0), patties treated with unpurified carnosine had significantly lower TBARS than the other treatment groups ($p < 0.0003$). After 2 days, the patties treated with unpurified carnosine had TBARS values significantly different from those of patties treated with purified carnosine ($p = 0.0004$), whereas the control patties were not significantly different from either of the two treatments. Pairwise comparison did not show significant differences between TBARS at the last 2 days of analysis (days 5 and 8).

DISCUSSION

The slow reduction of MbFe(IV)═O by carnosine observed in the kinetic experiments with pH 7.3 and the inability of carnosine to reduce MbFe(III) to MbFe(II)O_2 clearly indicate that the antioxidative activity of carnosine cannot be due to reducing properties. The inability of carnosine to reduce MbFe(III) to MbFe(II)O_2 in our study suggests that the slow conversion of MbFe(III) to MbFe(II)O_2 previously observed (*7*)

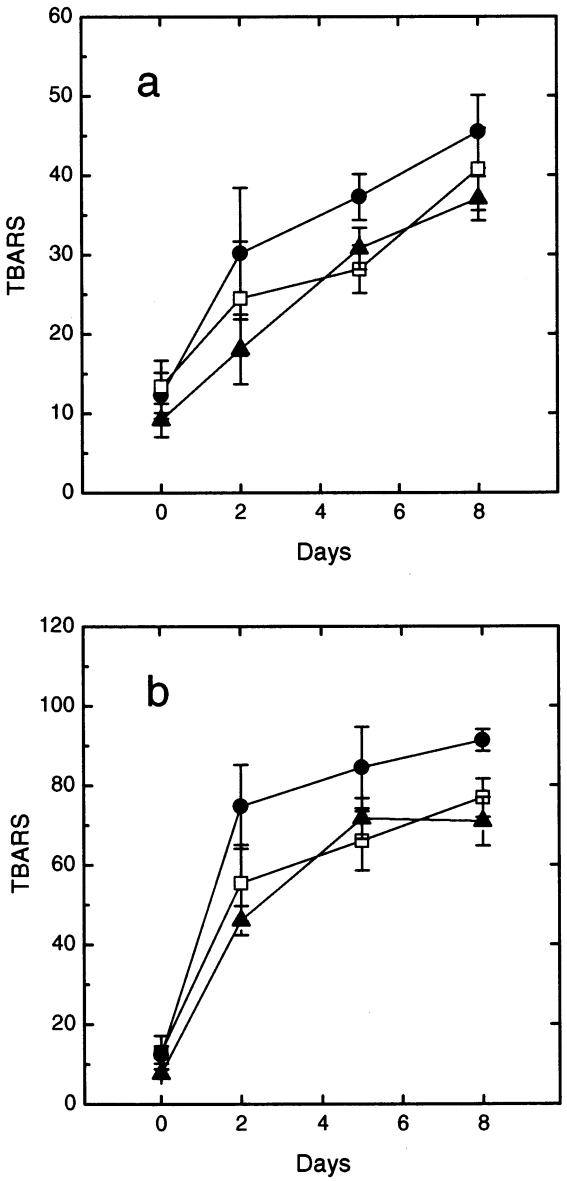

Figure 3. Development of TBARS (determined as μmol of malonaldehyde/kg of meat) in cooked samples of minced pork during storage at 4 °C. Standard deviations are shown for each experimental point ($n = 4$). (a) Longissimus dorsi patties: ●, 506 mg of purified carnosine added per 100 g of meat; ▲, 506 mg of carnosine contaminated with hydrazine added per 100 g of meat; □, control. (b) Masseter patties: ●, 619 mg of purified carnosine added per 100 g of meat; ▲, 619 mg of carnosine contaminated with hydrazine added per 100 g of meat; □, control.

is caused by hydrazine, because the use of a preparation of carnosine with a content of, for example, 0.1% hydrazine results in a hydrazine concentration between 2 and 8 times the concentration of MbFe(IV)═O under the actual experimental conditions.

At the pH of relevance to meat, the rate of autoreduction of MbFe(IV)═O exceeds the rate of reduction by carnosine. This finding agrees well with the fact that the autoreduction of MbFe(IV)═O is subject to specific acid catalysis (*19*), whereas the increased reactivity always observed for MbFe(IV)═O at low pH is caused by protonization of a group with p$K_a \sim 5$ (*21*). The rate of reduction of MbFe(IV)═O by an external electron donor will accordingly increase less at decreasing pH compared to the rate of autoreduction of MbFe(IV)═O, which will increase by a factor of 10 when the pH is lowered 1 unit. Although the role of hypervalent myoglobin in meat systems is not fully understood, any protective role of carnosine toward hypervalent myoglobin in meat is highly unlikely, because of the slow rate of reaction compared to autoreduction.

In the experiment with cooked pork patties, the different oxidation behaviors of longissimus dorsi and masseter patties are in agreement with the general observation that oxidative muscles have lower oxidative stability than glycolytic muscle. This difference has among other factors been linked to the difference in carnosine content (*1*), a suggestion that is not supported by the present data showing insignificant differences between control patties and patties with an admixture of purified carnosine for both longissimus dorsi and masseter patties.

When patties containing hydrazine-contaminated carnosine were compared with patties containing purified carnosine, significantly lower oxidation levels were measured in hydrazine-contaminated patties throughout the storage period for longissimus dorsi samples and in the first part of the storage period for masseter samples (i.e., the difference is significant in samples of TBARS of $<\sim 45$ μmol/kg, regardless of muscle type; cf. **Figure 3**). This finding suggests an antioxidative effect of the contaminating hydrazine. Furthermore, values for TBARS of control patties are generally between TBARS values for patties containing purified carnosine and patties containing hydrazine-contaminated carnosine. The differences in log-transformed TBARS values are small, making it impossible to discriminate between a prooxidative effect of pure carnosine and an antioxidative effect of hydrazine-contaminated carnosine. In a study with cooked, ground salted and unsalted pork (Boston butts), Decker and Crum (*22*) observed a significant antioxidative effect of (unpurified) carnosine in samples with a concentration of 0.5% carnosine in unsalted pork and a concentration of 1.5% in salted pork (2% NaCl). In comparison, the concentration of KCl in the present study was 0.1%, and the added amounts of carnosine were 0.5% in longissimus dorsi patties and 0.6% in masseter patties. However, because the effect of (unpurified) carnosine most probably is due mainly to the presence of hydrazine, the use of carnosine preparations with different levels of contamination might account for the less pronounced antioxidative effect of unpurified carnosine found in our investigation.

The lack of antioxidative effect of purified carnosine in cooked pork patties is in accordance with the view that at least some of the earlier reported antioxidative activity of carnosine is due to its buffering capacity (*3*), because this effect was eliminated in our study by adjusting the pH of all solutions to 5.8 prior to addition to the ground meat. Heat treatment in the meat matrix may also have influenced the activity of carnosine in this study, although heat treatment of (unpurified) carnosine alone had no effect on its antioxidative activity (*23*).

As for the role as a primary antioxidant, the activity of carnosine as a peroxyl radical scavenger (*24*) does not seem to be important in cooked meat. Finally, the ability of carnosine to quench secondary lipid oxidation products (*6, 8*) does not seem to have affected the level of TBARS developed in cooked pork patties during 8 days of storage, because no significant difference was found between the treatment with (purified) carnosine and the control. Nevertheless, the removal of aldehydes may still be an important feature of carnosine in vivo, because the amounts of secondary oxidation products in vivo would be much lower than in the cooked meat in this study.

In conclusion, purification of carnosine preparations to remove the strong reductant hydrazine has been shown to alter significantly the antioxidative activity both in a model system

and in a real meat product. Thus, results from at least some former studies are most likely influenced by the presence of hydrazine, as has already been discussed (*8*, *10*, *24*). The present results further confirm that the antioxidative activity of carnosine is more limited than usually expected. In addition, the buffering effect of carnosine may be of importance for the slower oxidation processes seen in some carnosine experiments, stressing that rigorous control of pH is essential.

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