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Antioxidant Activity of Carnosine and Phytate: Application as Meat Preservatives

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ABSTRACT

Antioxidant Activity of Carnosine and Phytate: Application as Meat Preservatives

by

Beom Jun Lee, Doctor of Philosophy
Utah State University, 1998

Major Professor: Dr. Deloy G. Hendricks
Department: Nutrition and Food Sciences

The antioxidant activity of carnosine and phytic acid was investigated using several model systems. Carnosine and phytic acid alone inhibited metal ion-catalyzed deoxyribose degradation. Carnosine strongly inhibited metal ion-catalyzed lipid peroxidation in liposomes and in ground beef homogenates. Phytic acid facilitated oxidation of Fe (II) to Fe (III), and it inhibited hemeprotein + H₂O₂-catalyzed lipid peroxidation in linoleic acid micelles.

Antioxidant and color stabilizing effects of carnosine and phytate were investigated in a beef model system. Both compounds increased the rate of pH decline in pre-rigor beef muscle and stabilized fresh meat color by inhibiting metmyoglobin formation and lipid peroxidation in raw samples during storage at 4°C. Both compounds inhibited heme degradation and lipid peroxidation in cooked beef during storage at 4°C. Iron released from heme was strongly related to lipid peroxidation in cooked beef.

Ascorbic acid inhibited metmyoglobin formation on the surface of ground beef patties but not in the bulk of the product. In contrast, carnosine inhibited metmyoglobin
formation and brown color development throughout the product. Carnosine increased cook yield and salt-soluble protein, but ascorbic acid had no effect on cook yield and decreased salt-soluble protein. Carnosine was more effective on inhibition of lipid peroxidation than was ascorbic acid.

Phytate greatly enhanced water-holding capacity of raw and cooked meat in a dilute beef model system. Effects of 0.5% sodium phytate, sodium pyrophosphate, and sodium tripolyphosphate, along with 1% NaCl, on physicochemical properties of restructured raw and cooked beef were compared. In raw beef, the treatments with sodium phytate, sodium pyrophosphate, and sodium tripolyphosphate increased meat pH and salt-soluble protein level, and inhibited metmyoglobin formation and lipid peroxidation, compared to the control. In cooked beef, the treatments with sodium phytate, sodium pyrophosphate, and sodium tripolyphosphate increased bind strength, cooked yield, moisture level, and meat pH, and inhibited lipid peroxidation. The treatments with sodium pyrophosphate and sodium tripolyphosphate increased inorganic orthophosphate level in both raw and cooked beef, compared to sodium phytate and the control.

These results indicate that carnosine and phytate can be used as meat preservatives for extending shelf-life and enhancing water-holding capacity of meat and meat products.
ACKNOWLEDGMENTS

I would like to thank Dr. Deloy G. Hendricks for making me finish a doctoral degree in nutrition and food sciences. To me it seemed to be very difficult to complete my doctoral degree because two previous major professors, Dr. Lawrence Piette and Dr. Arthur Mahoney, passed away when I was in the Toxicology Ph.D. program. After that, Dr. Deloy G. Hendricks accepted me as a student, and I continued to work on a doctoral degree in NFS under his guidance.

I would also like to thank the other members of my graduate committee, Dr. Daren P. Cornforth, Dr. Steve D. Aust, Dr. Charles E. Carpenter, and Dr. Von T. Mendenhall, for their helpful suggestions and advice in my research and the preparation of my dissertation.

Special gratitude is expressed to my parents and brothers for their support and encouragement and to my wife for helpful assistance. I could not have done it without all of you.

Beom Jun Lee
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CHAPTER 1
INTRODUCTION AND OBJECTIVES

INTRODUCTION

Lipid peroxidation is a major cause of quality deterioration in restructured or precooked meats (Akamittath et al., 1990; Pearson et al., 1983). The peroxidative deterioration of lipid changes the flavor, color, texture, and nutritive value of foods (Wilson et al., 1976). Many classes of lipid peroxidation products exert toxic effects in both whole animals and in cellular systems (Aust, 1989; Halliwell and Gutteridge, 1986; Pearson et al., 1983). Oxidation of muscle lipids involves the peroxidation of polyunsaturated fatty acids, which are located in the membranes of muscle foods (Keller and Kinsella, 1973). Various transition metals such as iron and copper and heme compounds play an important role in lipid peroxidation in skeletal muscle (Decker and Welch, 1990; Kanner et al., 1988). However, the role of heme proteins or nonheme iron in lipid peroxidation in muscle tissues is still controversial (Love, 1983; Monahan et al., 1993). Food processing such as grinding or cooking increases the degradation of heme compounds, thereby increasing the amount of free and low-molecular-weight iron compounds thought to be responsible for increased lipid peroxidation in muscle foods (Kanner et al., 1988; Schricker and Miller, 1983).

Lipid peroxidation products and free radicals are involved in the oxidation of oxymyoglobin to metmyoglobin and are associated with brown discoloration of meats (Renerre and Labas, 1987). Color in fresh meat and meat products is a strong indicator of meat quality. Several factors including packaging, oxygen tension, bacteria, pH, and temperature affect fresh meat color stability (Cornforth, 1994). In addition, non-meat
ingredients that have antioxidative and/or reducing activity can stabilize meat color, thus extending shelf-life of meat and meat products (Greene et al., 1971; Mitsumoto et al., 1991a, b; Trout and Dale, 1990). Ascorbate and vitamin E have received the most attention as antioxidants in meats, but metal chelators such as phosphates, and synthetic inhibitors of lipid peroxidation such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have also been shown to protect meat color (Decker and Crum, 1991; Greene et al., 1971; Mitsumoto et al., 1991a, b; Okayama et al., 1987; Sato and Hegarty, 1971; Smith et al., 1973; Young et al., 1987). In addition, the use of inorganic sodium phosphates in processed meat products has been increasing due to their beneficial effects in improving the functionality, palatability, and storage stability of meat (Knipe et al., 1985; Anjaneyulu et al., 1989; Bernthal et al., 1991; Moiseev and Cornforth, 1997).

The meat industry and many associated researchers have continuously looked for new ingredients to aid in extending shelf-life of meat products in addition to promoting health (Lee and Hendricks, 1995). In this respect, carnosine and phytic acid may be useful.

Carnosine (β-alanyl L-histidine) is an endogenous dipeptide found in the skeletal muscle of most vertebrates (Crush, 1970). Carnosine is known to inhibit lipid peroxidation in several systems (Boldyrev et al., 1987; Decker and Faraji, 1990; Decker and Crum, 1991; Decker et al., 1992; Kohen et al., 1988). The antioxidant properties of carnosine may result from its ability to chelate transition metals such as copper (Brown, 1981), its inactivation of peroxides or superoxide (Babizhayev et al., 1994; Kohen et al., 1991), and its influence on antioxidants such as α-tocopherol (Neifakh, 1966). These properties may enhance the antioxidant potential of muscle and may render carnosine useful as a natural food antioxidant (Decker and Faraji, 1990; Decker and Crum, 1991; Decker et al., 1992).
Phytic acid is a natural plant inositol hexaphosphate which makes up 1-5% of most cereals, nuts, legumes, oil seeds, pollen, and spores. Phytic acid can tightly bind to metal ions. The metal phytate complexes are highly insoluble over a wide pH range (Graf and Eaton, 1990). Although phytate has been reported to have antinutritional properties (generally only with fiber) with respect to mineral absorption (Harland and Morris, 1995), a few studies have suggested that phytate has no inhibitory effect (Hunter, 1981; Graf and Eaton, 1984) or may enhance mineral absorption in certain circumstances (Wettler et al., 1984). Phytate inhibits nonheme iron absorption but has no effect on heme iron absorption, which indicates that phytate can affect total iron absorption from meat (Carpenter and Mahoney, 1992). Kim et al. (1993) reported that meat enhanced nonheme iron absorption by iron-deficient rats only if the meals contained added phytate.

Phytic acid is a powerful inhibitor of iron-driven hydroxyl radical formation because it can form an iron chelate that is catalytically inactive (Graf et al., 1987).

The antioxidant properties of carnosine and phytic acid have been applied in clinical use as drugs or food supplements for free radical-mediated diseases such as cancer, atherosclerosis, diabetes, and ischemia perfusion injury (Boissonneault et al., 1994; Borgadus et al., 1993; Kaufman, 1986; Shamsuddin et al., 1988; Yoon et al., 1983; Yoshikawa et al., 1991a, b). Although the antioxidant activities of carnosine and phytic acid have been investigated in many studies, no data are available regarding their effects on color, metmyoglobin formation, and degradation of heme pigments in beef muscle. Also, the antioxidant effects of these compounds have not been compared to ascorbic acid or traditional phosphates such as sodium pyrophosphate and sodium tripolyphosphate, which have been used in the meat industry.
RATIONALE AND SIGNIFICANCE

Throughout history, the meat industry has utilized non-meat ingredients to extend the shelf-life of products. The classic use of salt and/or nitrites to control and limit microbiological growth and to provide flavor stability during storage has provided needed "insurance" for meat product shelf-life. Currently, consumers' concern for food safety continues to make methods of extending meat product shelf-life a high priority throughout the meat industry. As a result, the examination of new ingredients to aid in extending meat product shelf-life is an on-going, evolving area of research.

Color in fresh meat and meat products is a strong indicator of shelf-life. Non-meat ingredients that stabilize color or increase the time prior to meat color fading are viable aids in improving shelf-life of meat products. Ascorbic acid and citric acids have been shown to act as heavy metal chelators, and ascorbic acid acts as an oxygen absorber. It is through these mechanisms that ascorbic acid and citric acids have been shown to stabilize the heme-portion of muscle pigments.

The ingredients which stabilize flavor components of meat products during storage have been extensively investigated. A major issue in meat product flavor stability is off-flavor development, which results from the oxidation of lipids and deterioration of characteristic flavors during storage. Compounds that retard lipid peroxidation are classified as either chelators (compounds that bind metals, especially divalent metals such as iron and copper), free radical scavengers (compounds which react with free radicals that are formed during propagation), retarders (compounds which reduce hydroperoxides, but do not form additional free radicals), and singlet oxygen quenchers (compounds which quench singlet oxygen, therefore limiting initiation).
As reiterated in the literature review, phytic acid can chelate transition metals and stimulate oxidation of ferrous ion to ferric ion. Carnosine can inactivate free radicals and donate hydrogen ions. These actions of carnosine and phytic acid may render them useful as meat additives to improve meat product shelf-life by stabilizing meat color and preventing lipid peroxidation. In addition, carnosine and phytic acid provide beneficial health effects including prevention of cancer, atherosclerosis, diabetes, or cataracts. These effects may encourage their use as new ingredients in the meat industry.

**EXPERIMENTAL TITLES AND OBJECTIVES**

1. **Antioxidant Effects of L-carnosine on Liposomes and Beef Homogenates:** the objective was to evaluate antioxidant properties of L-carnosine using several model systems. The protective effects of carnosine against metal-catalyzed deoxyribose degradation and lipid peroxidation in liposomes and in ground beef homogenates were investigated.

2. **Metal-Catalyzed Oxidation of Ascorbic Acid, Deoxyribose, and Linoleic Acid as Affected by Phytic Acid in a Model System:** the objective was to investigate the effects of phytic acid in a model system of metal-involved oxidation reactions in the presence of ascorbic acid. We also investigated the effects of phytic acid on iron- or hemeprotein-catalyzed deoxyribose degradation.

3. **Antioxidant Effects of Carnosine and Phytic Acid in a Model Beef System:** the objectives were 1) to investigate the ability of carnosine and phytic acid to inhibit lipid peroxidation and metmyoglobin formation in raw beef muscle (treatment to prerigor beef muscle); 2) to evaluate effects of carnosine and phytic acid on lipid peroxidation and heme iron content in cooked beef (treatment to postrigor muscle). The relationships
between % metmyoglobin and meat color in raw beef and between lipid peroxidation and heme iron content in cooked beef were also evaluated.

4. A Comparison of Carnosine and Ascorbic Acid on Color, and Lipid Stability of Ground Beef: the objectives of this project were 1) to investigate the ability of carnosine, ascorbic acid, or the combination of carnosine and ascorbic acid to stabilize meat color and lipids in ground beef; 2) to investigate protective effect of carnosine against Cu (II)-catalyzed ascorbic acid oxidation in a model system.

5. Effect of Carnosine and Phytate on Water-Holding Capacity, Color, and TBA Values of a Dilute Beef Model System: the objective of this project was to investigate the effect of carnosine and sodium phytate on WHC, metmyoglobin formation, and lipid peroxidation using a dilute beef model system (50% beef + 50% water). To minimize the high pH effect of carnosine and phytate on WHC, metmyoglobin formation, and lipid peroxidation, the pH of carnosine and phytate solutions was adjusted to the same as the initial meat pH.

6. Effect of Sodium Phytate, Sodium Pyrophosphate, and Sodium Tripolyphosphate on Physicochemical Characteristics of Restructured Beef: the objective was to evaluate the effects of sodium phytate on physicochemical properties of meat and meat products, compared with traditional phosphates which have been widely used in the meat industry.

Thus, the main purpose of these studies was to 1) investigate the specific antioxidant properties of carnosine or phytate in meat and model systems and 2) establish the efficacy of using phytate in restructured meat products.
REFERENCES


Meat color is a primary criterion by which consumers evaluate meat quality and acceptability. Consumers prefer bright-red fresh meat, brown or gray-colored cooked meats, and pink cured meats (Cornforth, 1994). Myoglobin (Mb) is responsible for the color of fresh meat. On exposure to air the purple ferrous deoxygenated myoglobin (Mb) rapidly oxygenates on the surface to brighter red oxymyoglobin (MbO₂). During display myoglobin oxidizes to brownish red ferric metmyoglobin (MetMb) (MacDougall, 1982).

Myoglobin and hemoglobin are oxygen-binding heme proteins that are present in the skeletal muscle of most vertebrate species. The concentration of myoglobin is affected by species and muscle anatomical location, but hemoglobin concentration in meat depends on bleeding during slaughter. Myoglobin concentrations from 1.99 (semitendinosus muscle) to 3.64 mg/g (biceps femoris muscle) have been reported for different beef muscles (Rickansburd and Henrickson, 1967). Hemoglobin values in beef, expressed as a percentage of total pigments, comprised about 12-14% (Hunt and Hedrick, 1977). Total pigment values for beef ranged from 3.02-6.54 mg/g (Krzywicki, 1982).

The stability of fresh-meat color can be affected by several factors such as oxygen tension, bacterial growth, pH, MetMb reducing activity, retail lighting conditions, and exogenous reductants or antimicrobial agents (Cornforth, 1994). Extremely low or high oxygen tension extends fresh-beef color stability by preventing MetMb formation. High microbial growth promotes discoloration of fresh meat (George and Stratmann, 1952; Jensen, 1945). High ultimate pH (>6.0) causes dark-cutting beef. In ground beef,
· metmyoglobin reducing activity is very low because NADH is preferentially oxidized. Addition of various glycolytic or citric acid cycle intermediates increases the metmyoglobin reducing activity of the ground beef, presumably by serving as substrate for generation of NADH (Saleh and Watts, 1968).

Several non-enzymatic reductants and inhibitors of oxidation have been used to extend fresh meat color stability. Greene et al. (1971) reported that both pigment and lipid peroxidation of ground beef was more effectively inhibited by ascorbate plus butylated hydroxyanisole (BHA) or propyl gallate (PG) than by ascorbate alone. Govindarajan et al. (1977) reported improved color maintenance in ground beef with either added ascorbate, BHA, butylated hydroxytoluene (BHT), or PG. These compounds had no effect on the initial slow oxidation of myoglobin but extended by 2-4 days the time before rapid color deterioration began. Oxalate, an iron chelator when added at the 1% level, was found to inhibit color deterioration very effectively. The color-stabilizing effects of BHA, BHT, PG, and oxalate result from inhibition of lipid oxidation. Lipid peroxidation products or intermediates, including oxygen radicals, may either directly oxidize meat pigments or reduce the effectiveness of the pigment-reducing system (Greene et al., 1971).

**WATER-HOLDING CAPACITY OF MEAT**

Water-holding capacity (WHC) of meat is the ability of meat to hold all or part of its own and added water. The chemically bound portion of water in meat is rather small. The capillary force resulting from fibrillar protein structures, which are the intercellular structures of muscles, retard or prevent the movement of the water molecules. The main
water-retaining meat compounds are the myofibrillar proteins (Honikel and Hamm, 1994).

Although water is bound in both muscle and muscle products by capillary action, the microstructure that generates the capillary action is quite different. In muscle, the microstructure that produces the capillary suction is the pores located between the thick and thin filaments of the myofibrils which are approximately 10 nm in diameter under normal condition. In the presence of sodium chloride and/or sodium pyrophosphate (SPP), the pore size was 10-50% greater than this (Offer and Trinick, 1983). In processed muscle products, the combination of comminution and heating destroys much of the fine ultrastructure normally present in muscle. The protein rearrangement or aggregation causes the formation of larger pores (0.1-1.0 μM in diameter) which reduce the capillary suction force and, consequently, reduce the WHC (Trout, 1988).

The amount of water immobilization depends on the pH value which falls from about 7.0 in live muscle to around 5.5 in meat. The minimum WHC of meat exists around pH 5.3 since this is near the isoelectric point of the myofibrillar proteins (Honikel and Hamm, 1994). Thus, myofibrills can approach each other and decrease capillary space. Furthermore, the WHC depends on the muscle type and the species of animals as a result of their varying composition and structure (Honikel and Hamm, 1994).

Addition of sodium chloride increases WHC of meat because it decreases isoelectric point, thereby increasing capillary space. Prerigor beef homogenates containing 0.5 or 1.0% NaCl had higher WHC than similarly treated postrigor beef homogenates (Bernthal et al., 1989). The higher WHC of prerigor meat treated with salt may be due to a strong electrostatic repulsion between the dissociated myofibrillar
proteins myosin and actin caused by the combined influence of ATP, high pH, and increased ionic strength resulting from the salt addition.

In addition, the use of phosphates lowers the concentration of NaCl required to obtain beneficial functional characteristics of meat products. Because high sodium in the diet is known to contribute to development of hypertension, the replacement or reduction of NaCl can be beneficial for human health. Offer and Trinick (1983) showed that the presence of 10 mM SPP halved the concentration of NaCl needed to elicit swelling and water uptake in isolated myofibrils. The possible mechanisms of phosphates to enhance WHC of meat are 1) buffering (raising) the pH, 2) increasing the anionic electrolytes, 3) sequestering cations, 4) raising the ionic strength, and 5) dissociating actomyosin. Phosphates may also crosslink proteins or block reactive sites. Sodium pyrophosphate was the most effective phosphate for improving meat functional characteristics (Knipe et al., 1985). However, addition of 0.25% or 0.5% SPP to prerigor meat homogenates lowered pH values but did not enhance WHC for extended periods (Bernthal et al., 1991). Soy isolate, skim milk, and xanthan gums can also improve the WHC in frankfurter batties (Whiting, 1984).

**IRON AND ITS TOXICITY**

**Iron-catalyzed free radical formation**

The importance of iron in biological systems derives from its reduction/oxidation (redox) reactivity, as it mainly exists in two interchangeable forms: ferrous (Fe²⁺) and ferric (Fe³⁺) (Ramdath and Golden, 1989). The redox reactivity of iron is involved in the production of harmful molecules known as free radicals. Since free radicals may act to mediate tissue damage such as to initiate and propagate lipid peroxidation, they are
generally considered detrimental to health (Minotti and Aust, 1989). Species of free radicals found in oxygen-based biological systems such as the human body are highly reactive oxygen-centered types such as hydroxyl radical (HO') and superoxide anion (O$_2^-$) (Kehrre, 1993).

The transition metal iron is a primary culprit in the generation of reactive oxygen species (ROS). The variable oxidation states enable iron to accept and donate a single electron, and they are the basis for the formation and propagation of many radical reactions (Halliwell, 1991). One of the most important of these reactions is production of the extremely reactive and destructive hydroxyl radical. Hydroxyl radical is theoretically formed from superoxide anion and hydrogen peroxide via the Haber-Weiss Reaction (Minotti and Aust, 1987; 1989):

$$ O_2^- + H_2O_2 \rightarrow O_2 + OH^- + HO' $$

Yield of hydroxyl radical by this reaction is virtually nonexistent. To drive the reaction and generate appreciable hydroxyl radical, iron is needed to shuttle one electron to hydrogen peroxide as occurs in the Fenton Reaction (Minotti and Aust, 1987; 1989):

$$ H_2O_2 + Fe (II) \rightarrow Fe (III) + OH^- + HO' $$

These reactions have been sequenced in a pathway where superoxide anion generates both hydrogen peroxide and ferrous iron. This sequence of reactions exemplifies the role of iron in the production of hydroxyl radicals and is termed either the "iron-catalyzed Haber-Weiss reaction" or the "superoxide-driven Fenton reaction" (Minotti and Aust, 1987; 1989):
\[
\begin{align*}
  \text{O}_2^{-} & + \text{Fe (III)} \quad \longleftrightarrow \quad \text{O}_2 + \text{Fe (II)} \\
  \text{O}_2^{-} + \text{HO}_2^{-} + \text{H}^+ \quad \longrightarrow \quad \text{O}_2 + \text{H}_2\text{O}_2 \\
  \text{H}_2\text{O}_2 + \text{Fe (II)} \quad \longrightarrow \quad \text{Fe (III)} + \text{OH}^- + \text{HO}^- \quad \text{(the Fenton Reaction)}
\end{align*}
\]

\[\text{NET: } \text{O}_2^{-} + \text{H}_2\text{O}_2 \quad \longrightarrow \quad \text{O}_2 + \text{OH}^- + \text{HO}^- \quad \text{(Haber-Weiss Reaction)}\]

Clearly, a potentially harmful situation exists if iron in the body is able to participate in the reactions outlined above. Presumably because iron is potentially such a toxic oxidant, an elaborate system has been devised by the body to ensure that iron is not found in free ionic form. These substances, iron-binding proteins transferrin, ferritin, and hemosiderin, limit the availability of iron during transport and storage. Consequently, under normal physiological conditions, the concentration of iron capable of catalyzing free radical reactions should be low, if not negligible (Carpenter and Mahoney, 1992). However, much is still unknown about the relationship of iron absorption, transport, and storage, and how these interact to produce oxidative damage. \textit{In vitro} evidence indicates that radical-mediated mechanisms do release iron from ferritin, transferrin, hemosiderin, and even hemoglobin and myoglobin. This iron has been shown to then serve as a catalytic source for oxidative damage (Brieland and Fantone, 1991; Minotti et al., 1991; Reif, 1992; Sadrzadeh et al., 1984).

\textbf{Iron-catalyzed lipid peroxidation}

Lipid peroxidation is the oxidative deterioration of unsaturated lipids via a free radical chain reaction. In food systems, it causes rancidity of fats and oils and involves the direct reaction of oxygen with lipid to form potentially damaging free radicals and
unstable free radical precursors such as lipid peroxides (Gutteridge and Halliwell, 1990). Initiated \textit{in vivo} by environmental components inducing formation of either organic free radicals or ROS, the process begins with the abstraction of hydrogen from an unsaturated fatty acid. Lipid peroxidation then incites chemical and physical changes to unsaturated fatty acids present in cellular membranes and is thought to play a causative role in the pathogenesis of several acute and chronic conditions such as cancer, atherosclerosis, inflammation, shock, and liver injury (Halliwell, 1991). The free radical chain reaction process of lipid peroxidation is generally accepted as follows (Sevanian and Hochstein, 1985):

\begin{align*}
\text{Initiation} & : \text{RH (unsaturated fatty acid)} + \text{Initiator} \rightarrow R^\cdot \text{ (organic free radical)} \\
\text{Propagation} & : R^\cdot + O_2 \rightarrow ROO^\cdot \text{ (peroxyl radical)} \\
& \quad \quad \quad \quad \quad \rightarrow ROOH \text{ (lipid peroxide)} + R^\cdot \\
\text{Termination} & : ROO^\cdot + ROO^\cdot \rightarrow ROOR + O_2 \\
& \quad \quad \quad \quad \quad \rightarrow ROOR \\
& \quad \quad \quad \quad \quad \rightarrow RR \text{ (hydrocarbon)}
\end{align*}

Besides the direct production of membrane-damaging free radicals, as outlined above, lipid peroxidation generates lipid peroxides during propagation. The products can either directly damage other cellular membrane components or decompose into reactive peroxyl and/or alkoxy free radicals (Terao, 1990).
Iron is thought to play two important roles in lipid peroxidation (Miller et al., 1990; Minotti and Aust, 1987; 1989; 1992). As explained previously, iron has the ability to catalyze the generation of damaging hydroxyl radicals. Hydroxyl radicals can then participate in the initiation event of lipid peroxidation. Perhaps more important is iron's ability to propagate the lipid peroxidation chain reaction via metal-catalyzed decomposition of lipid peroxides into peroxyl and alkoxy radicals (Aikens and Dix, 1991; Halliwell, 1991):

\[
\text{ROOH} + \text{Fe (III)} \rightarrow \text{ROO}' (\text{peroxyl}) + \text{H}^+ + \text{Fe (II)}
\]

\[
\text{ROOH} + \text{Fe (II)} \rightarrow \text{RO}' (\text{alkoxyl}) + \text{OH}^- + \text{Fe (III)}
\]

Cellular damage secondary to iron-induced lipid peroxidation is thought to be the underlying pathogenic mechanism of the diffuse tissue injury seen in conditions of iron overload (hemochromatosis) and has been described in vivo and in vitro in animals (Bacon et al., 1986; Britton et al., 1987; Gordeuk et al., 1987). There are also significant associations between high storage and dietary levels of iron, and increased risk of cardiovascular disease. These associations possibly implicate "high-normal" iron levels in the pathogenesis of heart disease via a lipid peroxidation mechanism (Beard, 1993). Also, increased gastrointestinal formation of hydroxyl radicals has been documented in animal models utilizing high doses of oral iron (Kang et al., 1989; Slivka et al., 1986). The increased formation of hydroxyl radicals related to high iron intake may be involved in colon carcinogenesis (Nelson et al., 1989).
CARNOSINE

Localization and tissue concentration

Carnosine (β-alanyl-L-histidine) is one of the most abundant (1-20 mM) nitrogenous compounds present in the non-protein fraction of vertebrate skeletal muscle and certain other tissues (Crush, 1970). Carnosine can be endogenously synthesized by carnosine synthetase in muscle cells (Bauer and Schulz, 1994). Carnosine is a major imidazole in the skeletal muscle where fast glycolytic muscle has higher concentrations of carnosine than does slow oxidative muscle (O'Dowd et al., 1988). High levels of carnosine and histidine are also generally found in the central nervous system, heart, kidney, and spleen in mammals such as mice, rats, guinea pigs, and humans (Flancbaum et al., 1990).

Tissue carnosine concentrations can be influenced by histidine- or carnosine-supplemented diet. Carnosine can be absorbed by active transport in the brush border of the small intestine, and its absorption is regulated by dietary levels of amino acids, peptides, and/or proteins (Ferraris et al., 1988). In rats, orally administered carnosine is predominantly hydrolyzed in the small intestine and enters the blood in the form of its constituent amino acids, while in mice and pigs, most dietary carnosine enters the blood from the intestine and is then delivered to organs and tissues (Ferraris et al., 1988). Dietary histidine deficiency reduced skeletal muscle carnosine concentrations in rats, but high dietary histidine supplementation (5%) increased rat skeletal muscle carnosine concentrations (Tamaki et al., 1984). Supplementation with high concentrations of dietary carnosine (1.8-5.0%) also increased carnosine concentration in the skeletal muscle and the plasma of the rat (Tamaki et al., 1984). However, these dietary supplementations did not affect carnosinase activity in the kidney or the muscle of the rat. Dietary
supplementation with carnosine (0.0875%) and alpha-tocopherol (50 ppm) increased
carnosine concentrations 1.5-fold and alpha-tocopherol concentration 1.7-fold in rat liver
compared with appropriate control (Chan et al., 1994).

Carnosinase

The human body contains two dipeptidases which hydrolyze carnosine. One of
these is present in tissues, especially kidney, liver and brain, and should be referred to as
“non-specific dipeptidase” (Lenney, 1990). The other is serum carnosinase (Lenney et
al., 1982). Jackson et al. (1991) purified and characterized human serum carnosinase,
which is especially active in hydrolyzing carnosine and anserine, preferring dipeptides
with histidine in the C-terminal position. Primates have serum carnosinase, but non-
primates including dog, horse, calf, hog, rat, mouse, rabbit, and guinea pig do not have
serum carnosinase (Jackson et al., 1991). The level of carnosinase in the blood stream
increases with age in the human. In the first 10 months of life, no activity is detectable;
thereafter the concentration rises gradually, reaching adult levels at age 12-15 years
(Lenney et al., 1982). In a human study, large amounts of intact carnosine (up to 14% of
ingested dose) were recovered in the urine over 5 h after ingestion (Gardner et al., 1991).
The urinary excretion of carnosine was negatively related to the level of carnosinase in the
plasma. The highest carnosinase activity was also observed in subjects who regularly
underwent physical training.

Antioxidant activity

Chelation of metal ion. Carnosine can chelate transition metals such as copper,
zinc, cobalt, cadmium, and nickel. Zinc (II) and cadmium (II) ions produce weak
complexes with carnosine without changing the conformation of the dipeptide (Brown
and Antholine, 1979). These complexes are not affected by addition of histidine or cysteine. Copper (II) ions form three structurally different complexes with carnosine depending on the relative concentrations of copper (II) ion and carnosine at physiological pH and temperature. When either histidine, cysteine, or GSH is added to a solution of copper (II)-carnosine, a complex with a monomeric copper (II) ion is favored by formation of a mixed complex that contains both ligands. When the concentration of carnosine is 100-1000 times that of the copper (II) ion, a tetrameric complex is formed through the N-3 of the imidazole ring (Brown, 1981). Addition of a second ligand to this solution also produces mixed complexes in which the second ligand competes with the carnosine according to mass action considerations. Decker et al. (1992) reported that carnosine did not form a complex with iron as determined by $^1$H NMR spectra.

**Reaction with free radicals.** There is evidence that carnosine behaves like a multifunctional antioxidant when exposed to pulse radiolysis in water solutions in the presence of oxygen (Pavlov et al., 1993). Even at low concentrations, carnosine forms a charge-transfer complex with superoxide anion radicals which changes the reactivity of the radical. This may explain a role of carnosine as a catalyst like superoxide dismutase (SOD). The complex of copper:carnosine can dismute superoxide radicals released by activated neutrophils in an analogous mechanism to other amino acids and copper complexes (Kohen et al., 1991). Carnosine at physiological concentrations also showed a peroxyl radical trapping activity (Kohen et al., 1988).

**Inhibition of lipid peroxidation.** Even though carnosine is not capable of regenerating alpha-tocopherol radicals (Gorbunov and Erin, 1991), an indirect relationship between carnosine and alpha-tocopherol seems to exist *in vivo* as evidenced by alpha-tocopherol deficiency in rabbits resulted in reduced skeletal muscle carnosine
concentrations (McManus, 1960). Carnosine and anserine can not only inhibit the process of lipid peroxidation but can also lower the content of its products already accumulated (Dupin et al., 1987). Histidine and β-alanine did not exhibit an inhibiting effect on ascorbate-dependent lipid peroxidation in the sarcoplasmic reticulum of frog skeletal muscle (Dupin et al., 1987). However, Erickson and Hultin (1992) reported that increasing concentrations of histidine led to increased lipid peroxidation in enzymatic and nonenzymatic ascorbate and ferric ion-dependent systems in sarcoplasmic reticulum. Carnosine (25 mM) inhibited the catalysis of lipid peroxidation by iron, hemoglobin, lipoxidase, and singlet oxygen from 35 - 96%, suggesting that the antioxidant mechanism of carnosine is not only due to metal chelation (Decker and Faraji, 1990). Carnosine (0.5% and 1.5%) effectively inhibited lipid peroxidation in frozen salted ground pork during up to 6 months storage (Decker and Crum, 1991). The ability of carnosine (1.5%) to inhibit TBARS formation was stronger than other antioxidants such as alpha-tocopherol, BHT, and sodium triphosphate.

**Biological effects and clinical applications**

**Buffering and glycolytic activity.** There is a negative relationship between muscle work and carnosine content (Dupin and Stvolinskii, 1986). A decrease in carnosine concentration in the working muscle is not accompanied by its passage out into the surrounding solution or the appearance of β-alanine or L-histidine in the solution. The addition of carnosine to the Ringer solution surrounding the medium eliminated the fatigue of the muscle by increasing the intramuscular carnosine concentration on account of its uptake from the medium (Dupin and Stvolinskii, 1986). Possibly the muscle utilizes carnosine in some reactions associated with contraction in order to maintain a high level of work capacity.
At physiological pHs, both carnosine (pKa, 6.81) and anserine (pKa, 7.04) exhibit remarkable buffering activity by accepting protons. Buffering in muscle is important in offsetting the effects of an increased H⁺ load during intense exercise as a result of increased lactic acid production. The muscle buffering capacity of freeze-dried samples (d.m.) from the thoroughbred horse, greyhound dog, and man was investigated with the titration method (Harris et al., 1990). The buffering capacity was similar in horse and dog muscle and lowest in man. The higher buffering capacity of both horse and dog muscle could be explained mainly by the higher levels of histidine containing dipeptides in these two species (108.3 mmole carnosine/kg d.m. in horse and 33 mmole carnosine and 48.6 mmole anserine/kg d.m. in dog) compared with man (16 mmole/kg d.m.).

Carnosine and anserine are reported to have a very pronounced accelerating effect on a number of enzymatic reactions involved in anaerobic and aerobic metabolism of carbohydrates in muscle (Severin, 1964). Carnosine influences on phosphorylase b for glycogenolysis (Severin, 1964). The enzyme activity was pH-dependent. With a shift in pH from 6.5 to 7.0 carnosine inhibited phosphorylase b activity, whereas with acidification of the medium (from pH 6.5 to 6.0) carnosine increased the enzyme activity by 50%. Glycolysis activation can be due to either an increase in the pH and buffering capacity of the reaction mixture or to removal of possible heavy-metal inhibitors as chelated complexes on addition of the dipeptides to the reaction mixture (Davey, 1960).

**Wound healing effect.** Carnosine may serve as a reservoir for histidine to be used as a source of histamine in the trauma response of animals. Carnosine and anserine concentrations were lower in the muscle tissue of a wounded cock. Treatment with histidine and histamine, however, prevented the decrease in the concentration of carnosine (Fisher et al., 1978). Rats injected i.p. with 5 mg/kg BW of compound 48/80 (which is
known to stimulate histamine formation) showed 120% increase in muscle histidine decarboxylase activity and 100% increase in muscle histamine concentration (Greene et al., 1984). These results corresponded to an 80% increase in muscle carnosinase and 100% reduction of muscle carnosine concentration. Histidine concentration was not affected by the treatment. In rats treated with hydrocortisone to suppress healing, local treatment with carnosine increased tensile strength of the skin at the site of an incision wound (Nagai et al., 1986). Similar effects were found after the administration of β-alanine and histidine, but not of β-alanine alone. Fitzpatrick and Fisher (1982) reported that treatment with histidine and carnosine (1 mg/100 mg BW) increased tissue free-histidine concentration, skin-breaking strength, and collagen deposition in rats which were artificially wounded and fed a histidine-deficient diet for 7 days. The enhancement of wound healing by carnosine may be ascribed to stimulation of early effusion by histamine and of DNA and collagen biosynthesis by β-alanine (Nagai et al., 1986). Also, chronic infection of chicken and rats with Staphylococcus resulted in decreased tissue carnosine concentrations and increased free histidine concentrations (Fitzpatrick et al., 1980).

Clinical applications. The manifold positive effects of carnosine can be employed in clinical practice for the treatment of many pathological conditions which are mediated by free radical injury to tissue. Carnosine may have an antipromoting effect on mammary tumorigenesis. Dietary carnosine (0.825%, calculated as the equivalent of 25% beef diet) is capable of inhibiting 7,12-dimethylbenzo(a)anthracene-induced breast cancer in vitamin E-deficient rats (Boissonneault et al., 1994). Dietary carnosine extended the time to 50% incidence of palpable tumor from 12.7 weeks for the control to 18.9 weeks in the carnosine-supplemented group. Carnosine (2 mM) is capable of
inhibiting copper-catalyzed oxidation of LDL, suggesting that it could inhibit the development of atherosclerosis (Borgardus et al., 1993). Carnosine (1 mM) also reduced the rate of LDL oxidation by 2,2'-azobis (2-amidinopropane dihydrochloride), a peroxyl radical generator.

Zinc-carnosine complex (Z-103) can protect cold-resistant stress-induced hemorrhage lesions in the gastric glandular mucosa in rats. The protective action is likely to be mediated by its membrane-stabilizing action on mast cells and lysosomes in the gastric glandular mucosa (Cho et al., 1991). Also Z-103 was capable of inhibiting gastric damage and lipid peroxidation in gastric mucosa after ischemia-reperfusion injury (Yoshikawa et al., 1991a). The protective actions of Z-103 may be due to free radical scavenging activity (Yoshikawa et al., 1991b).

Carnosine inhibits cataract formation induced by lipid peroxidation products in rabbit eye (Babizhayev, 1989). The effectiveness of carnosine may be due to a direct interaction with lipid peroxidation products. Also, Boldyrev et al. (1987) reported that human eye lens contains considerable amounts of carnosine and GSH. During cataract development the level of both antioxidants decreases 7-10 fold. Incubation of rabbit eye lens with carnosine for 60 min led to the accumulation of carnosine in eye lens cells. Moreover, carnosine (0.5 -2.0 mM) effectively protected against the oxidation of GSH.

Carnosine in solution can be hydrolyzed or oxidized in the presence of a broad spectrum of transition metal ions, thus increasing the content of free amino acids, alanine and histidine (Kondrat'eva et al., 1993). The oxidation of carnosine also depends on pH and temperature. The higher the temperature and pH are, the greater the breakdown of carnosine. Addition of other antioxidants or chelates to a carnosine solution may result in useful preparations of carnosine for use as a drug.
Introduction

Plant foods such as cereals, legumes, and oilseed serve as a major source of nutrients for mankind. An important constituent of these foods is phytic acid. The salt form, phytate, commonly exists in cereals and legumes where it serves several physiological functions, especially during seed germination. Phytate is the major storage form of phosphorus and accounts for more than 80% of total phosphorus in cereals and legumes.

There is concern about the presence of phytate in cereal, legumes, and their derived foods since there is evidence that phytate decreases the bioavailability of essential minerals and proteins by forming complexes. However, the evidence is controversial because several studies showed that phytate has no effect or may even increase mineral absorption. On the other hand, phytate may have potentially beneficial applications for cereal and legume phytate based on their interaction properties with mineral or proteins. There is some evidence that phytate prevents colon and mammary cancer in animals, and it also decreases blood cholesterol level and kidney stones in humans. The chemical and biological actions of phytate are discussed.

Structure and chemical properties

The conformational structures of phytate have been derived from X-ray analysis, NMR and pH titrations. Johnson and Tate (1969) suggested that the phosphate at C-2 position is in the axial position, while the phosphate groups on C-1, C-3, C-4, C-5, and C-6 are equatorial (Fig. 2-1). On the other hand, Blank et al. (1971) concluded that the phosphate groups at C-1, C-2, C-3, C-4, C-5, and C-6 are axially disposed, while C-2
position is equatorial. Myoinositol hexaphosphate exists in either of two conformations in aqueous solution depending on pH: under acidic conditions 1-axial/5-equatorial conformation predominates, while under strong alkaline conditions, the inverted conformation, i.e., 5-axial/1-equatorial, prevails (Reddy et al., 1989). At pH 9.4 and 27°C, a 0.1 M sodium phytate solution contains equal amounts of the two conformations (Isbrant and Oertel, 1980).

The biosynthesis, accumulation, and dephosphorylation of phytate occur in electron-dense regions called globoids or aleurone particles of plant cells. Free myoinositol and glucose-6-phosphate play an important role in the formation of phytate. Glucose-6-phosphate is a precursor for phytate biosynthesis (Fig. 2-2) being catalyzed during seed development to 1L-myoinositol-1-phosphate by the NAD⁺-dependent enzyme, 1L-myoinositol-1-phosphate synthase (Loewus, 1983).
Photosynthesis or  
Carbohydrate storage products  

Glucose-6-phosphate  

1
Myoinositol-1-phosphate  

3

ATP / Mg^{++}  
Myoinositol

2

Myoinositol hexaphosphate  

Deposition in Globoids

Enzymes  1. 1L-myoinositol-1-phosphate synthase  
2. Myoinositol kinase  
3. Phosphoinositol kinase

Fig. 2-2—Schematic diagram for biosynthesis of phytate in seeds (Loewus, 1983).
Despite the tremendous potential energy inherent in six phosphoric ester linkages, phytic acid is inert and very stable. It can be stored as a solid for years, and in neutral or alkaline 50% aqueous solutions at 5 °C for several months before any decomposition products are generated. The release of 50% of the phosphorus requires acid hydrolysis in 5 N HCl at 100 °C for at least 6 h, while refluxing at 100 °C for 6 h at pH 12 releases no measurable phosphorus at all (Cosgrove, 1980). Both acid hydrolysis and phytase treatment result in a mixture of myo-inositol, inorganic phosphate and myo-inositol mono-, di-, tri-, tetra-, penta- and hexa-phosphate. Phytic acid has six strongly dissociated protons (pKa 1.1 to 2.1) and six weakly dissociated protons (pKa 4.6 to 10.0). Due to the dissociation of these ionizable protons, phytic acid has a tremendous potential for complexing positively charged proteins or multivalent cations in many foods.

**Occurrence, content, and dietary intake of phytate**

Phytic acid occurs widely in plant seeds, grains, roots, fruits, and vegetables as salts of mono- and divalent cations. It accumulates rapidly in seed and/or grains during ripening periods, and it constitutes 1-5% by weight of most cereals, nuts, legumes, and oil seeds (Table 2-1). Phytic acid makes up 0.14-2.22% of the weight of cereals, 0.05-3.29% of ready-to-eat cereal products, 0.03-2.41% of cereal-base foods, 0.22-9.15% of whole beans, and 0.05-5.20% of bean-based foods (Reddy et al., 1989). Cereal may be the major source of dietary phytate for American men consuming an omnivorous diet. The average American consumes about 750 mg phytate per day (Harland and Peterson, 1978). In general, vegetarians consume a higher amount of phytate than nonvegetarians. Ellis et al. (1987) reported that phytate intake from omnivorous self-selected diets varied
Table 2-1—Phytic acid content of various seeds

<table>
<thead>
<tr>
<th>Sample</th>
<th>Phytic acid (%, w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>1.1</td>
</tr>
<tr>
<td>Wheat bran</td>
<td>4.8</td>
</tr>
<tr>
<td>Corn</td>
<td>0.9</td>
</tr>
<tr>
<td>Corn germ</td>
<td>6.4</td>
</tr>
<tr>
<td>Soy beans</td>
<td>1.4</td>
</tr>
<tr>
<td>Sesame seeds</td>
<td>5.3</td>
</tr>
<tr>
<td>Lima beans</td>
<td>2.5</td>
</tr>
<tr>
<td>Barely</td>
<td>1.0</td>
</tr>
<tr>
<td>Oats</td>
<td>0.8</td>
</tr>
<tr>
<td>Wild rice</td>
<td>2.2</td>
</tr>
<tr>
<td>Peanuts</td>
<td>1.9</td>
</tr>
</tbody>
</table>

(Graf and Eaton, 1990)

from 585 mg/day in spring to 734 mg/day in winter for females and from 781 mg/day in spring to 762 mg/day in winter for males.

Interaction with protein

Phytic acid interacts with proteins at various pH values to form phytate-protein complexes (Cheryan, 1980). At an acidic pH, phytic acid has a strong negative charge; however, amino groups of many proteins would be positively charged since their pKa is near pH 9.0 to 10. This makes it possible for their compounds to interact with each other (Fig. 2-3). The basic amino acyl groups on protein include the amino terminal (if free), \( \varepsilon \)-amino group of lysine, imidazole group of histidine, and guanidyl group of arginine. In the intermediate pH range (6-8), both phytic acid and protein have a net negative charge, provided the isoelectric pKa of the protein under consideration is lower than the pH being.
considered. Under these conditions, interaction between two negatively charged protein and phytic acid molecules would not theoretically be possible. However, complexation occurs between phytic acid and proteins under these conditions (Cheryan, 1980).

Possible mechanisms include (1) a direct binding of phytic acid to $\alpha$-NH$_2$ terminal group and $\varepsilon$-amino group of lysine because they are still protonated and (2) a multivalent cation-mediated interaction (Fig. 2-4). Cation binding to protein molecules could be via binding to carboxyl or histidyl groups. Some binding may also occur without the mediation by metal ions because the lysyl and arginyl side chains may remain protonated in the intermediate pH range (Thompson, 1987). Direct binding between phytic acid and basic protein would also be possible under such conditions. At high pH values (>9.0), the nature of the interaction between phytic acid and protein is not clearly understood. There are indications that the interaction between phytic acid and protein is diminished when the pH is high.

By virtue of binding to protein, phytic acid may affect functional properties of proteins including protein solubility, charge, and structure (Thompson, 1987). Phytic acid inhibits enzyme activity such as polyphenol oxidase (Graf et al., 1987), $\alpha$-amylase
Fig. 2-4—Possible structure of phytic acid-protein complex at intermediate pH.

(Thompson, 1986), trypsin (Singh and Krikorian, 1982), and alcohol dehydrogenase (Altschuler and Schwartz, 1984), which indicate biological implications of phytic acid.

**Interaction with minerals**

The concern over phytate-mineral interactions arises due to the ability of phytate to form insoluble complexes with minerals at physiological pH values. Phytic acid exhibits a high affinity for all polyvalent cations in the following decreasing orders of stability: Cu$^{2+}$ > Zn$^{2+}$ > Ni$^{2+}$ > Co$^{2+}$ > Mn$^{2+}$ > Fe$^{3+}$ > Ca$^{2+}$ (Vohra et al., 1965). Copper and zinc appear to have a high affinity for phytate to form complexes. The solubility of the phytate-metal complexes depends on the pH and experimental conditions such as molar ratio of metal to phytate (Nolan and Duffin, 1987). Various structures are possible for these metal-phytate complexes (Fig. 2-5). The phosphate groups may chelate as in structure (I) to give 4-membered ring complexes (Anderson et al., 1977). Alternately two or more phosphate groups from the same or from different phytate molecules may complex to one metal cation giving structure (II) and the polymeric structure (III), respectively. Also a phosphate group may serve to bridge two metal ions as in structure (IV) (Jones et al., 1977). All of these complexes are probably coexistent and have different dissociation constants.
Grynspan and Cheryan (1983) investigated the effect of pH and molar ratio of calcium and phytic acid on the interaction between calcium and phytic acid. They concluded that 1) calcium-phytate complexes are soluble below pH 4.0 at all molar ratios, 2) above pH 4.0 the extent of solubility drop depends upon the calcium : phytic acid ratio, and 3) above pH 6.0 the solubility of the complexes decreases with increasing calcium : phytate ratio and complete precipitation occurs at a calcium : phytate ratio of 5 (Grynspan and Cheryan, 1983).

\[ (\text{PO}_3\text{H}^-)_5 \text{ - inositol - O-P- } \text{M}^{n+} \]

\[ \text{HO} \begin{array}{c} \text{O} \end{array} \begin{array}{c} \text{P} \end{array} \begin{array}{c} \text{O} \end{array} \begin{array}{c} \text{M}^{n+} \end{array} \begin{array}{c} \text{O} \end{array} \]

\[ \text{inositol - (PO}_3\text{H}^-)_4 \]

\[ (\text{III}) (\text{PO}_3\text{H}^-)_5 \text{ - inositol - O-P-O-M}^{n+} - O - P - O - \text{inositol - O-P-O-M}^{n+} - \]

\[ \text{OH} \begin{array}{c} \text{O} \end{array} \begin{array}{c} \text{OH} \end{array} \begin{array}{c} (\text{PO}_3\text{H}^-)_5 \end{array} \begin{array}{c} \text{OH} \end{array} \]

\[ (\text{IV}) (\text{PO}_3\text{H}^-)_5 \text{ - inositol - O-P- } \text{O-M}^{n+} \begin{array}{c} \text{O} \end{array} \begin{array}{c} \text{M}^{n+} \end{array} \]

Fig. 2-5—Possible structures of metal-phytate complexes.
Antioxidant properties of phytic acid

Iron-catalyzed reactions play a crucial role in the oxidative damage to biological materials. These reactions most often involve activation of oxygen and subsequent reaction with iron, generating even more reactive species such as hydroxyl radicals. All of the antioxidant properties of phytic acid likely derive from its relatively high binding affinity for iron. High concentrations of phytic acid solubilize iron in the form of Fe(III)$_1$-phytate, whereas low concentrations evidently precipitate iron as Fe(III)$_3$- and Fe(III)$_4$-phytate complexes (Graf and Eaton, 1990).

The six coordination sites of trivalent iron are occupied by water and hydroxide ions in aqueous solution. Most chelating agents displace five of these ligands and form a pentadentate chelate with H$_2$O occupying the sixth coordination site. EDTA forms a hexadentate chelate but due to its small size distorts the chelate and makes a seventh site available for H$_2$O. Phytic acid, however, is unique in occupying six coordination sites and displacing all of the coordination water in the Fe(III)$_1$-phytate complex (Graf et al., 1984). The addition of phytic acid causes a greatly accelerated rate of oxidation of Fe(II) to Fe(III) (Graf et al., 1987). These actions of phytic acid may prove its antioxidant activity and render it as a food additive to prevent warmed-over flavor (WOF) development in food systems. In cooked chicken, phytic acid substantially inhibited WOF during storage at 4 °C (Empson et al., 1991).

Biological effects of phytate

Although phytate has been reported to have antinutritional properties with respect to mineral absorption (Harland and Morris, 1995), a few studies have suggested that phytate has no inhibitory effect (Graf and Eaton, 1984; Hunter, 1981) or may even enhance mineral absorption in certain circumstances (Wettler et al., 1984). Furthermore, several animal and epidemiological studies indicate that phytate has an anticarcinogenic effect on mammary and colon carcinogenesis (Graf and Eaton, 1993; Nelson et al., 1989;
Pretlow et al., 1992; Shamsuddin et al., 1988; Vucenik et al., 1992). Phytate also decreases kidney stones, lowers blood cholesterol, and improves glycemic index in humans (Yoon et al., 1983). Phytate inhibits iron-driven hydroxyl radical formation and lipid peroxidation (Graf et al., 1984; Lee and Hendricks, 1995). Graf and Eaton (1985) proposed that inhibition of intracolonic hydroxyl radical generation via the chelation of reactive iron by phytate may help explain the suppression of colonic carcinogenesis and other inflammatory bowel diseases by diets rich in phytate.

Either the role of iron and/or phytate in colon cancer risk using dimethyhydrazine (DMH)-induced animal models was investigated (Nelson et al., 1989; Shamsuddin et al., 1988; Shamsuddin and Ullah, 1989; Siegers et al., 1988). Dietary iron supplements increased mitotic index and tumor incidence in the distal colon (Nelson et al. 1989; Siegers et al., 1988). Addition of phytate reversed the effect of iron overload on incidence of colon cancer and suppressed mitosis in the non-neoplastic epithelium of animals injected with DMH (Pretlow et al., 1992; Shamsuddin et al., 1988; Shamsuddin and Ullah, 1989). The antineoplastic action is more pronounced when phytate is administrated in combination with inositol. Also, inositol treatment alone has reduced the parameters of tumor inhibition (Shamsuddin et al., 1989).

In addition, the lower phosphorylated forms of inositol such as inositol tri- or tetraphosphate may play an important role in the signal transduction mechanism by regulating a variety of cellular processes including proliferation (Michell, 1986). Shamsuddin et al. (1989) proposed that inositol phosphates may be preventive for colon cancer and immunosuppressive disease by way of modulating cellular proliferation and differentiation. Phytase and phosphatase in foods and in the intestine dephosphorylate inositol hexaphosphate to the lower phosphorylated forms (Shamsuddin et al., 1988). Commercial phytate also contains lower phosphorylated inositol (Shamsuddin et al., 1988).
REFERENCES


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CHAPTER 3
ANTIOXIDANT EFFECTS OF L-CARNOSINE ON LIPOSOMES
AND BEEF HOMOGEMATES

ABSTRACT

The antioxidant activity of carnosine (β-alanylhistidine dipeptide) was investigated using several systems. In the presence of 100 μM ascorbic acid, carnosine inhibited metal ion-catalyzed deoxyribose degradation in a dose-dependent manner, indicating its hydroxyl radical-scavenging activity. Carnosine strongly inhibited metal ion-catalyzed liposomal lipid peroxidation in the presence of 100 μM ascorbic acid or 100 μM H₂O₂. Carnosine also inhibited thiobarbituric acid-reactive substances (TBARS) formation from ground beef homogenates. An increase in TBARS formation was caused by addition of 10 ppm Fe³⁺ to the homogenates. Carnosine presence prevented such increase and it may be useful as an adjunct against oxidative tissue damage or for increasing shelf-life.

INTRODUCTION

Several classes of oxidation products exert pathological effects in cellular and whole animal systems (Halliwell and Gutteridge, 1986). Lipid peroxidation, which is a major cause of quality deterioration in meats, is an important problem in restructured and precooked food products (Akamittath et al., 1990; Pearson et al., 1983). It changes the

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flavor, color, texture, and nutritive value (Wilson et al., 1976). Oxidation of muscle lipids involves the peroxidation of membrane polyunsaturated fatty acids (Keller and Kinsella, 1973). Transition metals such as Fe^{2+} and Cu^{2+} and heme compounds can catalyze this reaction and also are important in promoting damage of non-lipid molecules (Aust et al., 1985; Decker and Welch, 1990; Gutteridge, 1984; Kanner et al., 1988a). Refrigeration and cooking may increase degradation of heme compounds, thereby increasing the amount of free and low-molecular-weight iron compounds hypothesized to be responsible for increased lipid peroxidation (Kanner et al., 1988b; Schricker and Miller, 1983).

Carnosine (β-alanyl-L-histidine), an endogenous dipeptide found in the skeletal muscle of most vertebrates, is synthesized from β-alanine and L-histidine by carnosine synthetase (Bauer and Schulz, 1994; Crush, 1970; Flancbaum et al., 1990). Carnosine is known to inhibit lipid peroxidation in several systems (Boldyrev et al., 1987; Decker and Crum, 1991; Decker and Faraji, 1990; Decker et al., 1992; Kohen et al., 1988). The antioxidant mechanism may result from its chelation of transition metals, its enzyme-like activity (superoxide dismutase and lipid peroxidase) and/or its free radical scavenging ability (Babizhaye et al., 1994; Chan et al., 1994; Dahl et al., 1988; Decker et al., 1992; Kohen et al., 1988). These mechanisms may enhance the antioxidant potential of muscle and may be useful as a natural food antioxidant (Decker and Crum, 1991; Decker and Faraji, 1990; Decker et al., 1992).

Although carnosine is known to act as an antioxidant in several systems, there are a few reports that carnosine inhibits lipid peroxidation in food systems. It effectively inhibited oxidative rancidity in uncooked salted ground pork during frozen storage (Decker and Crum, 1991) and cooked ground pork during refrigerated storage (Decker and Crum, 1993). Our objective was to evaluate hydroxyl radical scavenging activity of carnosine using a metal-catalyzed deoxyribose system and we investigated protective properties of
carnosine against lipid peroxidation in liposomes and ground beef homogenates. Beef homogenate may be an effective medium to investigate the protective effect of watersoluble antioxidants, such as carnosine, against lipid peroxidation.

MATERIALS & METHODS

Materials

L-carnosine, phosphatidylcholine (from egg yolk), phytic acid, 2-thiobarbituric acid (TBA), Hepes (N-2-hydroxyethyl piperazine N'-2-2-ethanesulfonic acid), ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], tetraethoxypropane (TEP), and 2-deoxyribose were purchased from Sigma Chemical Company (St. Louis, MO). Trichloroacetic acid (TCA) and sodium hydroxide were obtained from EM Science (Cherry Hill, NJ). Hydrogen peroxide, sodium phosphate (dibasic anhydrous), nitric acid, ammonium acetate, ascorbic acid, and ethylenediaminetetraacetic acid disodium salt (EDTA) were obtained from Mallinckrodt Inc. (Paris, KY).

Methods

Homogenate preparation. Ground beef (25% fat) was homogenized (20% w/v) in 50 mM Hepes buffer using a polytron homogenizer (Omni 5000 International Co., Waterburg, CT) for 1 min at level 4. Buffered systems have been widely used to study oxidation reduction reactions in meat systems (Fischer and Deng, 1977; Kanner et al., 1991; Lee and Hendricks, 1995). The pH of the homogenate was adjusted with 0.1 N NaOH or 0.1 N HCl using a pH meter (Orion Research Incorporated Co., Boston, MA). The mixture containing 0.8 mL (taken with a 1-mL syringe) of the beef homogenate and either 0.2 mL distilled deionized water or 0.2 mL test solutions was incubated for 60 min at 37°C. After incubation, the mixture was tested for TBARS formation.
**Phosphatidylcholine liposome preparation.** Multilamellar liposomes were prepared by diluting 500 mg phosphatidylcholine (PC) in 10 mL chloroform. The chloroform solution was dried under vacuum and resuspended in 100 mL of degassed, argon-saturated 50 mM Hepes buffer (5 mg PC/mL). The suspension was sonicated with 10 pulses of 30 s under argon. The PC liposome preparations were stored at 4 °C under an argon atmosphere until used. The pH of the PC liposome was adjusted to 7.4 immediately prior to use.

**Chemical analysis.** The TBARS assay in phosphatidylcholine liposome and beef homogenates was performed using the method described by Buege and Aust (1978). After incubation of the reaction mixture (final volume, 1.0 mL) containing 3.5 mg PC liposome, 100 μM ascorbic acid or 100 μM hydrogen peroxide, 10 μM cupric, ferrous, or ferric chloride, and carnosine for 60 min at 37 °C, butylated hydroxytoluene (20 μL, 0.2%) was added to the mixture to stop the reaction and to prevent lipid peroxidation during heating. A 15% TCA/0.375% TBA/0.025 N HCl stock solution (2 mL) was added to 1 mL of the incubated liposome or beef homogenates. The mixtures were then heated for 10 min in a boiling water-bath (95 -100 °C) to develop pink color, cooled with tap water, and centrifuged for 20 min at 5,500 rpm. The supernatant containing the pink chromogen was quantified at 532 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). TBARS were calculated from a standard curve of MDA, a breakdown product of TEP.

Deoxyribose assay was used for detecting hydroxyl radicals (Aruoma, 1994). The deoxyribose degradation caused by hydroxyl radicals was determined by the formation of TBARS (Aruoma, 1994; Halliwell and Gutteridge, 1981). The reaction mixtures (pH 7.4, 1.0 mL) contained 0.1 M phosphate-buffered saline, 7 mM
deoxyribose, 100 μM ascorbic acid, 10 μM cupric, ferrous, or ferric ions, and carnosine solutions. The mixtures were incubated for 100 min at 37 °C. One mL stock solution [1% (w/v) TBA in 50 mM sodium hydroxide plus 2.8% (w/v) TCA] was added to the reaction mixture. It was then heated for 10 min in a boiling water-bath, cooled with tap water, and the absorbance of the pink chromogen was read at 532 nm against appropriate blanks.

The total iron concentration was determined in wet-ashed samples by using the ferrozine assay (Stookey, 1970). Each homogenized sample (1.0 mL) was digested with 5 mL concentrated nitric acid and 0.2 mL 30% hydrogen peroxide on a hot plate until a white ash formed. The ash was dissolved in 0.2 mL of 1.0 N HCl and diluted with 0.8 mL deionized water. One milliliter 1.0% ascorbic acid was added, and the tubes were vortexed. After 20 min, 1 mL 10% ammonium acetate buffer and 1 mL of 1 mM ferrozine color reagent were added, and the mixture was mixed well. The mixture was allowed to stand at room temperature for 45 min and the absorbance of each sample was determined at 562 nm.

The tissue nonheme iron was extracted with 1.0 mL 25% TCA and 1.0 mL 4% pyrophosphate (Foy et al., 1967), which were added to 1.0 mL 20% tissue homogenate and boiled in a water-bath for 20 min. The mixture was then centrifuged at 8,000 rpm for 10 min, and the supernatant was saved in a 10-mL volumetric flask. The extraction step was performed three times on the same sample. The collected supernatant was brought to a total volume of 10 mL with distilled deionized water. One mL supernatant was used for determining nonheme iron by the ferrozine method as described.
RESULTS & DISCUSSION

Inhibition of deoxyribose degradation

Addition of 10 μM iron salts or cupric salts in the presence of 100 μM ascorbic acid to deoxyribose caused degradation of the sugar into a compound which forms a chromogen with TBA. The iron salts were more effective catalysts for TBARS formation (Fig. 3-1). Carnosine effectively inhibited the metal-catalyzed degradation of deoxyribose. In the presence of 10 μM copper, even low concentrations of carnosine strongly inhibited deoxyribose degradation. Hydroxyl radicals are known to be responsible for the breakdown of deoxyribose (Gutteridge, 1987; Halliwell and Gutteridge, 1981). Therefore, these results indicate that carnosine has hydroxyl radical scavenging activity. However, our results do not exclude the formation of a catalytically unreactive complex with copper. The mechanism of deoxyribose degradation by hydroxyl radicals is summarized as (Aruoma, 1994; Janero, 1990):

\[
\begin{align*}
&\text{Fe}^{2+} \\
&\text{deoxyribose (DR)} \rightarrow \cdot\text{OH} + \text{DR} \rightarrow \text{fragments of DR} \rightarrow \text{MDA} \\
&\text{O}_2 \\
&\text{heat} \\
&\text{MDA} + 2 \text{TBA} \rightarrow \text{a chromogen (pink color, maximal absorbance at 532 nm)} \\
&\text{acid}
\end{align*}
\]

Fe\(^{2+}\) or Cu\(^{+}\) in aqueous solution slowly oxidizes to form \(\text{O}_2^{-}\), resulting in the rapid formation of \(\text{H}_2\text{O}_2\) by dismutation at neutral pH (Halliwell and Gutteridge, 1981). Ascorbic acid can also reduce \(\text{O}_2\) to \(\text{O}_2^{-}\). Fe\(^{2+}\) or Cu\(^{+}\) interact with \(\text{H}_2\text{O}_2\) to form \(\cdot\text{OH}\) via the Fenton reaction (Aust, 1989). Deoxyribose can also be degraded substantially by Fe\(^{3+}\) in the presence of a superoxide anion-generating system or a reducing agent (Gutteridge, 1984).
Carnosine forms a complex with copper (Brown, 1981) but not with iron (Decker et al., 1992). Carnosine quenches hydroxyl radicals produced by Fe$^{2+}$ and H$_2$O$_2$ as measured using the EPR technique of spin-trapping (Chan et al., 1994). In our results, carnosine strongly inhibited metal-catalyzed deoxyribose degradation regardless of the metal used as a catalyst. The inhibition of formation of TBARS was greatest at lower concentrations when copper was the transition metal used to generate free radicals. When iron was used the inhibition of TBARS formation occurred at greater concentrations of
carnosine indicating that carnosine was probably directly scavenging hydroxyl radicals rather than inhibiting hydroxyl radicals generation by the binding of iron.

**Inhibition of liposomal lipid peroxidation**

In the presence of 100 μM ascorbic acid, 10 μM Fe^{2+}, Fe^{3+}, or Cu^{2+} catalyzed lipid peroxidation in PC liposomes (Table 3-1). Among these Fe^{2+} stimulated liposomal lipid peroxidation the most. Carnosine inhibited TBARS formation from liposomes in a dose dependent relation in these systems, but the inhibitory effect of carnosine was related to the metal ions. Within the concentrations of 1-10 mM carnosine we used, inhibition rates of carnosine on lipid peroxidation were 89.1-95.0% in the Cu^{2+}-catalyzed system, 73.5-91.9% in the Fe^{2+}-catalyzed system, and 14.1-64.1% in the Fe^{3+}-catalyzed system.

**Table 3-1—Effect of L-carnosine on metal and ascorbic acid-dependent lipid peroxidation in PC liposomes**

<table>
<thead>
<tr>
<th>L-carnosine (mM)</th>
<th>Cu (II)</th>
<th>Fe (II)</th>
<th>Fe (III)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>4.41 ± 0.14</td>
<td>23.3 ± 0.14</td>
<td>0.92 ± 0.09</td>
</tr>
<tr>
<td>1</td>
<td>0.48 ± 0.01</td>
<td>6.18 ± 0.05</td>
<td>0.79 ± 0.09</td>
</tr>
<tr>
<td>5</td>
<td>0.44 ± 0.02</td>
<td>4.44 ± 0.11</td>
<td>0.72 ± 0.10</td>
</tr>
<tr>
<td>10</td>
<td>0.31 ± 0.03</td>
<td>4.00 ± 0.22</td>
<td>0.57 ± 0.05</td>
</tr>
<tr>
<td>15</td>
<td>0.28 ± 0.04</td>
<td>3.36 ± 0.01</td>
<td>0.48 ± 0.05</td>
</tr>
<tr>
<td>20</td>
<td>0.22 ± 0.02</td>
<td>1.88 ± 0.06</td>
<td>0.33 ± 0.06</td>
</tr>
</tbody>
</table>

*Data represent the mean ± SD of two determinations.
The addition of 10 μM Cu$^{2+}$ and Fe$^{2+}$ in the presence of 100 μM H$_2$O$_2$ also catalyzed lipid peroxidation of liposomes (Fig. 3-2). In both systems, carnosine effectively inhibited TBARS formation from PC liposomes. At concentrations of 1, 2.5, 5, 10, or 15 mM carnosine, inhibition rates were 57, 73, 80, 82, and 87% in the Cu$^{2+}$-catalyzed system, and 73, 76, 81, 83, and 87% in the Fe$^{2+}$-catalyzed system, respectively, compared to the controls.

![Graph](image_url)

Fig. 3-2—Effect of L-carnosine on metal and H$_2$O$_2$-dependent lipid peroxidation in PC liposomes. Data represent the mean ± SD of three determinations.
Most biological studies of lipid peroxidation involve transition metals, added to, or contaminating, the reaction mixtures. When Fe$^{2+}$, Cu$^+$, or certain chelates of these ions are added to liposomes, lipoproteins, or isolated membranes, lipid peroxidation occurs (Minotti and Aust, 1989). Although we used distilled deionized water, we could not absolutely eliminate trace contamination of transition metal ions to our systems. In our study, 100 μM H$_2$O$_2$ or 100 μM ascorbic acid without added metal ions stimulated liposomal lipid peroxidation, however, carnosine inhibited the reaction (not shown).

Mechanisms of transition metal-catalyzed lipid peroxidation also depend on the presence or absence of preformed lipid hydroperoxides (LOOH) (Minotti and Aust, 1992). Preformed LOOH are decomposed by Fe$^{2+}$ (Cu$^+$) to highly reactive lipid alkoxy radicals which, in turn, promote the formation of new LOOH. Fe$^{2+}$ can also decompose the preformed LOOH to lipid peroxyl radicals. The reaction by Fe$^{2+}$ is much faster than that by Fe$^{3+}$ (Halliwell and Chirico, 1993). Although we used fresh prepared liposomes, we expected preformed LOOH to be present, due to the higher amounts of TBARS formation by Fe$^{2+}$.

Potential antioxidant mechanisms of carnosine in the presence of transition metal ions include inactivation of free radicals and formation of an inactive carnosine-metal complex. In the sarcoplasmic reticulum of frog skeletal muscle, carnosine inhibited lipid peroxidation induced by exogenous Fe$^{2+}$+ascorbate and also diminished the amount of preformed lipid peroxidation products (Dupin et al., 1987). Carnosine (25 mM) also inhibits the catalysis of lipid peroxidation by iron, hemoglobin, lipoxidase, and singlet oxygen from 35 - 96%, suggesting that its antioxidant mechanism is not solely due to metal chelation (Decker and Faraji, 1990). Carnosine can scavenge superoxide anions, hydroxyl radicals, and peroxyl radicals (Boldyrev and Severin, 1990; Kohen et al., 1988).
In addition, it has a lipid peroxidase-like activity (Babizhayev et al., 1994). These actions of carnosine may have been involved in the inhibition of metal-catalyzed lipid peroxidation in our study. The formation of a carnosine-copper complex may have contributed to the strong inhibition of lipid peroxidation in the copper-catalyzed system. This strong inhibition at low concentrations was not observed in the iron-catalyzed system, indicating that carnosine was probably inhibiting oxidation by different mechanisms in the copper and iron-catalyzing systems.

**Antioxidant effect in beef homogenates**

In beef homogenates with 50 mM Hepes buffer (pH 7.4), carnosine (0 - 20 mM) effectively inhibited TBARS formation in a dose-dependent manner (Table 3-2). At 20 mM of carnosine, the inhibition was 76.2%, compared to the control.

**Table 3-2**—Effect of L-carnosine on formation of TBARS in beef homogenates

<table>
<thead>
<tr>
<th>Reagents</th>
<th>TBARS (nmole/mL homogenate)</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (D.D.W.)</td>
<td>8.4 ± 0.2</td>
<td>-</td>
</tr>
<tr>
<td>Carnosine, 20.0 mM</td>
<td>2.0 ± 0.2</td>
<td>76.2</td>
</tr>
<tr>
<td>10.0 mM</td>
<td>3.5 ± 0.1</td>
<td>58.3</td>
</tr>
<tr>
<td>2.0 mM</td>
<td>6.7 ± 0.1</td>
<td>22.6</td>
</tr>
<tr>
<td>0.2 mM</td>
<td>7.4 ± 0.1</td>
<td>11.9</td>
</tr>
<tr>
<td>0.02 mM</td>
<td>8.0 ± 0.1</td>
<td>4.8</td>
</tr>
<tr>
<td>Phytic acid, 2.0 mM</td>
<td>4.1 ± 0.1</td>
<td>51.2</td>
</tr>
<tr>
<td>EDTA, 2.0 mM</td>
<td>4.2 ± 0.3</td>
<td>50.0</td>
</tr>
</tbody>
</table>

*The reaction mixtures including 0.8 mL of 20% (w/v) ground beef homogenate in 50 mM Hepes buffer (pH 7.4) and 0.2 mL of L-carnosine, phytic acid, or EDTA were incubated for 60 min at 37°C and assigned to the TBA test. Determinations were triplicated and values are the mean ± SD.*
Decker and Crum (1991) reported that carnosine (0.5 and 1.5%) effectively inhibited TBARS formation in frozen pork stored up to 6 mo. Moreover, carnosine was more effective than sodium triphosphates and other lipid-soluble antioxidants such as alpha-tocopherol and BHT. Phytic acid (2 mM) and EDTA (2 mM) also inhibited TBARS formation in ground beef homogenates (Table 3-2). At the same concentration (2 mM) phytic acid and EDTA showed the stronger inhibitory effect than carnosine. Phytic acid and EDTA chelate iron and strongly facilitate oxidation of Fe$^{2+}$ to Fe$^{3+}$ in Heps buffer (pH 7.2) (Lee and Hendricks, 1997). Therefore, the strong inhibitory effect of phytic acid and EDTA may be due to chelation of iron or ferroxidase activity (Graf et al., 1987; Graf and Eaton, 1990). Lee and Hendricks (1995) reported that phytic acid inhibited lipid peroxidation effectively and dose-dependently in beef homogenates. Harel et al. (1988) reported that EDTA at the relatively low 20-25 μM inhibited redox-cycle membrane lipid peroxidation. Free metal ions increased lipid peroxidation in minced turkey muscle while EDTA inhibited lipid peroxidation in both raw and heated turkey muscle (Kanner et al., 1988b). The antioxidant effect of EDTA may be due to the inhibition of nonheme iron activity. EDTA does not, however, affect the oxidation catalyzed by hemoproteins such as hemoglobin or cytochromes.

**Iron-induced lipid peroxidation in beef homogenates**

Muscle tissue contains considerable iron bound to proteins, of which myoglobin is the most abundant. Total iron content in the ground beef we used was 18.9 μg/g wet weight, and nonheme iron content was 7.4 μg/g wet weight. Nonheme iron, usually expected to be chelated or bound, was 39.2% of the total iron. Addition of 10 ppm iron (FeCl$_3$) to the ground beef homogenates enhanced lipid peroxidation (Fig. 3-3).
However, the simultaneous addition of 0.2 mM carnosine prevented the increase in TBARS formation that had been caused by the addition of 10 ppm Fe$^{3+}$. Moreover, 10 mM carnosine markedly reduced TBARS production, regardless of addition of iron.

Free metal ion appears to be one of the most important catalysts of muscle lipid peroxidation. Food processes such as refrigeration, cooking, baking, and microwaving increase the amount of low-molecular-weight iron which can catalyze lipid peroxidation of meat, thereby promoting the development of rancidity and warmed-over flavor (Decker and Welch, 1990).

**Fig. 3-3—Effect of L-carnosine on formation of TBARS in ground beef homogenate with addition of ferric ion.** The reaction mixtures, containing 16% (w/v) ground beef homogenate in 50 mM Hepes buffer (pH 7.0), 10 ppm ferric chloride, and carnosine solutions, were incubated for 60 min at 37 °C and assigned to the TBA test. Data represent the mean ± SD of three determinations.
We added ferric ion which can produce lipid peroxyl radicals by decomposing the lipid hydroperoxides preformed in the muscle food or formed during homogenization, thereby stimulating lipid peroxidation (Schaich, 1992). Carnosine completely abolished the catalytic reaction of Fe$^{3+}$ added in beef homogenates, maybe due to its lipid peroxidase activity, resulting in reduction of lipid hydroperoxides (Babizhayev et al., 1994). In addition, free radical scavenging activity of carnosine may be involved in the inhibition of lipid peroxidation catalyzed by iron added to and already present in the beef homogenate.

**Influence on pH of homogenate**

The relation of carnosine inhibition of lipid peroxidation to pH level was determined at concentrations from 0.02-20 mM (Fig. 3-4). At pH 7.0 and 8.0, TBARS formation was 2- to 3-fold higher than at pH 5.0 and 6.0, perhaps because hemoprotein-catalyzed lipid peroxidation is most active at alkaline pH (Lee et al., 1975). At high pH, 20 mM carnosine strongly inhibited TBARS formation by 70-80%. At pH 6.0, the TBARS formation was lowest, and the inhibitory effect also was very low. At low pH, no matter how much carnosine was present, iron would still be available to participate in lipid peroxidation due to maintenance of the ferrous ion in acidic pH. However, over the pH range of 7.0 to 8.0, carnosine effectively inhibited TBARS formation from ground beef homogenates in a concentration-dependent manner. Decker and Faraji (1990) reported that 25 mM carnosine inhibited TBARS production by iron and ascorbate by 70-79% over the pH range of 5.1 to 7.0 in PC liposomes. In the hydrogen peroxide-activated hemoglobin system, carnosine inhibited TBARS formation by 60-100% over the pH range of 5.8-7.1 (Decker and Faraji, 1990). The rates of inhibition by carnosine were similar in the two studies.
Fig. 3-4—Effect of L-carnosine on formation of TBARS in ground beef homogenates at various pH levels. The reaction mixtures containing 0.8 mL 20% (w/v) ground beef homogenate in 50 mM Hepes buffer and 0.2 mL carnosine solution were incubated for 60 min at 37 °C and assigned to the TBA test. Data represent the mean ± SD of two determinations.

CONCLUSIONS

Carnosine, present in mammalian tissues, may act as an inherent antioxidant by mechanisms including its free radical scavenging and/or chelation of transition metals. Carnosine effectively inhibited lipid peroxidation in PC liposomes and ground beef
homogenates. Carnosine may be useful in meat processing as another naturally occurring antioxidant, helping to prevent off-flavor formation of meat and its products and increasing shelf-life.

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42: 779-781.
CHAPTER 4

METAL-CATALYZED OXIDATION OF ASCORBATE, DEOXYRIBOSE
AND LINOLEIC ACID AS AFFECTED BY PHYTIC ACID IN
A MODEL SYSTEM 1

ABSTRACT

Antioxidant activity of phytate was investigated in metal-catalyzed model systems. In a dose-dependent manner, phytate facilitated oxidation of Fe (II) to Fe (III) and inhibited formation of thiobarbituric acid-reactive substances (TBARS) from Fe (II)- or hemeprotein-catalyzed deoxyribose degradation. In the presence of 100 μM Fe (III), phytate inhibited reduction of Fe (III) to Fe (II) by 100 μM ascorbic acid and it consequently inhibited ascorbate oxidation. Phytate inhibited hemeprotein- and H₂O₂- catalyzed TBARS formation from linoleic acid micelles. Inhibition by phytate of iron + ascorbate-dependent lipid peroxidation depended on the concentration of ascorbate. These results indicate that phytate may be a useful antioxidant in the protection against oxidative deterioration of foods.

INTRODUCTION

Lipid peroxidation has been studied extensively in food science, nutrition, and clinical medicine because its products are related to food deterioration, cytotoxicity, and

many pathological reactions in degenerative diseases such as cancer (Halliwell and Chirico, 1993). Transition metals can catalyze the generation of reactive oxygen species such as the hydroxyl radical and superoxide anion. They can also propagate the lipid peroxidation chain reaction via metal-catalyzed decomposition of lipid peroxides into peroxyl and alkoxyl radicals (Halliwell and Chirico, 1993; Minotti and Aust, 1987, 1989). Both free iron and chelated iron may have important effects in the reaction. The interaction of hydrogen peroxide with heme proteins such as myoglobin, hemoglobin, and cytochrome releases free iron (Harel et al., 1988; Prasad et al., 1989). Moreover, the heme moiety, both free and protein-bound, initiates lipid peroxidation by producing reactive oxygen species and enhances the rate of lipid peroxidation by decomposing the preformed lipid peroxides (Grisham, 1985; Kanner and Harel, 1985a, b; Sadrzadeh et al., 1984).

Ascorbic acid is an important reductant in food and biological systems and may quantitatively be the most important enhancer of nonheme iron absorption in the diet. Ascorbic acid promotes iron reduction and solubilization, thereby preventing the formation of insoluble ferric hydroxides (Lynch and Cook, 1980). Ascorbic acid is used as a reductant in many model systems of lipid peroxidation (Fischer and Deng, 1977; Miller and Aust, 1989). Ascorbate can replace superoxide anion as the Fe (III) reductant in the superoxide-driven Haber-Weiss reaction (Khan and Martell, 1967). Ascorbate also autoxidizes, producing superoxide anion, which is dismutated to \( \text{H}_2\text{O}_2 \) and participates in the Fenton reaction (Khan and Martell, 1967).

Phytic acid is a natural plant inositol hexaphosphate constituting 1-5% of many cereals and legumes (Reddy et al., 1989). Cereal may be the major source of dietary phytate for Americans consuming a typical diet (750 mg phytate/day) (Harland, 1989;
Reddy et al., 1989). The majority of ingested phytate is undegraded during transit through the human gastrointestinal tract (Graf and Eaton, 1985). Metal phytate complexes are highly insoluble over a wide pH range (Graf et al., 1987). One phytate molecule can bind up to six divalent cations, and the metal could possibly bridge at least two phytate molecules, depending on the redox state (Graf and Eaton, 1990). Phytic acid is a powerful inhibitor of iron-driven hydroxyl radical formation because it forms a catalytically inactive iron chelate (Graf et al., 1984). These actions of phytic acid may contribute to its antioxidant activity on metal-catalyzed lipid peroxidation.

The antioxidant activity of phytic acid may be influenced by ascorbic acid, a strong reducing agent which can chelate with and change the redox potential of iron. Our objective was to investigate the effects of phytic acid in a model system on metal-involved oxidation reactions in the presence of ascorbic acid. We also investigated the effects of phytic acid on iron- or hemeprotein-catalyzed deoxyribose changes.

**MATERIALS & METHODS**

**Materials**

Phytic acid, deoxyribose, deferrooxamine methylate, 2-thiobarbituric acid (TBA), tetraethoxypropane, Hepes (N-2-hydroxyethyl piperazine N'-2-2-ethanesulfonic acid), 2,6-dichloroindophenol, butylated hydroxytoluene, and 2,2'-dipyridine were purchased from Sigma Chemical Company (St. Louis, MO). Trichloroacetic acid (TCA) and sodium hydroxide were obtained from EM Science (Cherry Hill, NJ). Hydrogen peroxide, sodium phosphate (dibasic anhydrous), ascorbic acid, and ethylenediaminetetraacetic acid disodium salt (EDTA) were obtained from Mallinckrodt Inc. (Paris, KY).
Linoleic acid micelle preparation

Linoleic acid (LA) micelles were prepared by diluting 500 mg linoleic acid in 20 mL chloroform. The chloroform solution was dried under vacuum and resuspended in degassed, argon-saturated 0.15 M NaCl (4 mg LA/mL) or 50 mM Hepes buffer (5 mg LA/mL). The suspension was sonicated (Branson Sonifier 250) with 10 pulses of 30 s under argon. The lipid preparations were stored at 4 °C under an argon atmosphere until used. The pH of the linoleic acid micelle solution was adjusted to 7.0 prior to use.

Chemical analysis

The TBARS assay in linoleic acid micelles was performed using the spectrophotometric method (Gutteridge and Quinlan, 1983). The reaction mixture (final volume, 1.0 mL) contained 0.7 mL linoleic acid micelles (pH 7.0), 0.1 mL of 1.0 mM H₂O₂, 0.1 mL of 0.3 mM Mb or 1.0 mM Fe (II) (0.1 mL of 0.5 mM Fe (II) + 0.1 mL of 0.5 mM Fe (III) without ascorbic acid and H₂O₂, or 0.1 mL of 1.0 mM Fe (III) + 0.1 mL ascorbic acid at various concentrations), and 0.1 mL test solutions (phytic acid, EDTA, and deferrioxamine). These mixtures were incubated for 60 (or 30) min at 37 °C; then 50 μL of 1% butylated hydroxytoluene in 95% ethanol was added to stop the reaction and to prevent lipid peroxidation during heating. Two mL of a TBA-TCA stock solution (1% TBA in 0.05 N NaOH plus 2.8% TCA in deionized water) was added to the reaction mixture. The mixture was then heated for 10 min in boiling water to develop the pink color and cooled with tap water. Two mL of 1-butanol was added to the mixture and vortexed thoroughly. The supernatant containing the pink chromogen was quantified at 532 nm with a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). TBARS were calculated from a standard curve of malondialdehyde, a breakdown product of tetraethoxypropane.
The deoxyribose degradation was determined by TBARS formation (Halliwell and Gutteridge, 1981). The reaction mixtures (final volume, 1.0 mL) containing 0.8 (or 0.7) mL of 10 mM deoxyribose in 0.1 M phosphate-buffered saline (pH, 7.0) and 0.2 mL of 0.1 mM Fe (II) chelates premixed and incubated for 20 min at 25 °C (or 0.1 mL of 0.3 mM hemeproteins + 0.1 mL of 3 mM H₂O₂ and 0.1 mL of test solutions) were incubated for 10 min at 37 °C. One mL of the stock solution (1% (w/v) TBA in 50 mM NaOH plus 2.8% (w/v) TCA) was added to the reaction mixture, which was heated for 10 min in a boiling water-bath and cooled with tap water. The absorbance of the pink chromogen was read at 532 nm.

The uptake of oxygen was measured with an oxygen electrode in Orion EA 940 expandable ion analyzer (Orion Research Inc., Boston, MA). Oxygen consumption rate was calculated by measuring change of dissolved oxygen concentration for 3 min at 25 °C. The reaction mixtures (10 mL) contained 7.0 mL of 10 mM deoxyribose in 0.1 M phosphate buffered-saline (pH 7.0), 1.0 mL of 0.3 mM hemoglobin or myoglobin, 1.0 mL of 3 mM H₂O₂, and 1.0 mL test solutions. The mixture was allowed to stand for 1 min at 25 °C. The dissolved oxygen concentration was then measured for 3 min using an oxygen electrode in an ion/pH meter.

Ferrous ion was assessed by following the disappearance of 2,2'-dipyridine (dipyridyl)-reactive Fe (II) (Drysdale and Munro, 1965). The reaction mixtures (1.0 mL) containing 50 mM Hepes buffer (pH 7.2), 100 μM Fe (II), and test solutions were allowed to stand for 5 min at 25 °C. After 0.1 mL of 5.0 mM 2,2'-bipyridine was added to the reaction mixtures, the absorbances were read at 520 nm.

Ascorbic acid concentrations were determined spectrophotometrically using 2,6-dichloroindophenol, reduced from a blue (red in acid condition) to colorless form by
ascorbic acid (Brewster and Turley, 1987). After the reaction mixtures including 35 mM Hepes buffer (pH 7.0), 100 μM Fe (III), 100 μM ascorbic acid, and various concentrations of phytic acid were allowed to stand for 20 min at 25 °C, 0.2 mL of 1 M metaphosphoric acid and 20 μL of 10 mM 2,6-dichloroindophenol were added, and the absorbance was read at 520 nm. The absorbance of ascorbic acid in the reaction mixtures including 100 μM ascorbic acid, 1 μM Cu (II), and phytic acid solutions was also monitored at 265 nm at timed intervals.

RESULTS

Phytic acid facilitated oxidation of Fe (II) to Fe (III) in 50 mM Hepes buffer (pH 7.2) as indicated by disappearance of bathocuproine-reactive Fe (II). The action of phytic acid was time- and concentration-dependent. In the presence of 100 μM Fe (II), 0.1 mM phytic acid catalyzed oxidation of Fe (II) by about 23, 70, 82, and 90% after standing for 1, 5, 10, and 15 min at 25 °C, respectively (unpublished data). With higher concentrations of phytic acid, higher amounts of Fe (III) were produced. Phytic acid at 0.1, 1.0, and 5.0 mM catalyzed oxidation of 100 μM Fe (II) by 71, 78, and 98%, respectively, after standing for 5 min at 25 °C (Table 4-1). Deferoxamine and EDTA also strongly catalyzed oxidation of Fe (II) to Fe (III). In the presence of 0.1 mM deferroxamine, 84% of 100 μM Fe (II) for 5 min at 25 °C was oxidized and in the presence of 0.1 mM EDTA, 100 μM Fe (II) was completely oxidized to Fe (III). The oxidation of Fe (II) to Fe (III) by chelates was closely related to their ability to inhibit formation of TBARS from deoxyribose degradation (Table 4-1). Phytic acid inhibited TBARS formation from deoxyribose in a dose-dependent manner. Compared to the
Table 4-1—Effect of chelates on oxidation of Fe (II) to Fe (III) and on degradation of deoxyribose in the presence of 100 μM Fe (II)*

<table>
<thead>
<tr>
<th>Chelates</th>
<th>Fe (II), μM</th>
<th>TBARS, nmole/10 mM deoxyribose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(% oxidation vs control)</td>
<td>(% inhibition vs control)</td>
</tr>
<tr>
<td>Control (buffer)</td>
<td>100</td>
<td>12.80 ± 1.23</td>
</tr>
<tr>
<td>Deferroxamine, 0.1 mM</td>
<td>13.6 ± 1.5 (86.4)</td>
<td>4.53 ± 0.43 (64.6)</td>
</tr>
<tr>
<td>EDTA, 0.1 mM</td>
<td>0.2 ± 0.2 (99.8)</td>
<td>1.39 ± 0.17 (89.1)</td>
</tr>
<tr>
<td>Phytic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>29.3 ± 1.9 (70.7)</td>
<td>3.50 ± 0.37 (78.8)</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>22.7 ± 1.8 (78.3)</td>
<td>2.40 ± 0.26 (81.2)</td>
</tr>
<tr>
<td>5.0 mM</td>
<td>2.1 ± 0.7 (97.9)</td>
<td>1.52 ± 0.27 (88.1)</td>
</tr>
</tbody>
</table>

* Data represent the mean ± SD of three determinations.

control, 0.1, 1.0, and 5.0 mM phytic acid inhibited deoxyribose degradation by 78, 81, and 88%, respectively. Deferroxamine and EDTA also strongly inhibited TBARS formation from deoxyribose degradation.

Phytic acid inhibited reduction of Fe (III) to Fe (II) by ascorbic acid, thereby consequently inhibiting Fe (III)-catalyzed ascorbic acid oxidation, as measured by the indophenol method (Fig. 4-1). In the presence 100 μM ascorbic acid and 100 μM Fe (III), the effect of phytic acid on the reduction of Fe (III) to Fe (II) correlated highly with the ability of phytic acid to inhibit the ascorbic acid oxidation (r = 0.99). At 0.1, 1.0, 5.0, and 10.0 mM phytic acid, 9.5, 4.5, 1.7, and 1.3 μM of Fe (III) were reduced by 100 μM ascorbic acid in 35 mM Hepes buffer (pH 7.0), corresponding to the oxidation of 10.9, 6.2, 2.9, and 2.1 μM ascorbic acid, respectively, after standing for 20 min at 25 °C.
Fig. 4-1—Effect of phytic acid on reduction of Fe (III) to Fe (II) by 100 μM ascorbic acid and the simultaneous oxidation of ascorbic acid by 100 μM Fe (III) in 35 mM Hepes buffer (pH 7.0) after standing for 20 min at 25°C. Data represent the means of two determinations.

Phytic acid also slowed Cu (II)-catalyzed ascorbic acid oxidation, as measured by UV absorbance at 265 nm (Fig. 4-2). In the presence of 1.0 μM Cu (II), 1.0 mM of phytic acid inhibited ascorbic acid oxidation by 35% after standing for 20 min at 25°C. In this system, the inhibitory effect of phytic acid was not dose-dependent; there was less inhibition with 5.0 mM phytic acid than with 1.0 mM phytic acid.
Fig. 4-2—Protection of phytic acid against Cu (II)-catalyzed ascorbic acid oxidation. The absorbances of the reaction mixtures including 50 mM Hepes buffer (pH 7.0), 1 μM Cu (II), 100 μM ascorbic acid, and phytic acid (PA) solution were monitored spectrophotometrically at 265 nm at time-intervals. Data represent means of four determinations.

In the presence of 0.3 mM H₂O₂, 30 μM Hb or 30 μM Mb stimulated oxygen uptake and TBARS formation from degradation of deoxyribose (Table 4-2). In a dose-dependent manner, phytic acid strongly inhibited oxygen uptake and TBARS formation from degradation of deoxyribose catalyzed by the hemeprotein and H₂O₂-activated system. The inhibition rate by phytic acid on TBARS formation was similar to its
**Table 4-2**—Effect of phytic acid on oxygen uptake rate and TBARS formation from the degradation of deoxyribose catalyzed by H$_2$O$_2$-activated hemeproteins

<table>
<thead>
<tr>
<th>Phytic acid (mM)</th>
<th>O$_2$ uptake (nmol/mL per min)</th>
<th>TBARS (nmole/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hb</td>
<td>Mb</td>
</tr>
<tr>
<td>0 (control)</td>
<td>1.90 ± 0.11</td>
<td>1.25 ± 0.11</td>
</tr>
<tr>
<td>0.1</td>
<td>1.20 ± 0.09</td>
<td>0.62 ± 0.07</td>
</tr>
<tr>
<td>1.0</td>
<td>0.84 ± 0.10</td>
<td>0.42 ± 0.03</td>
</tr>
<tr>
<td>10.0</td>
<td>0.60 ± 0.06</td>
<td>0.25 ± 0.04</td>
</tr>
</tbody>
</table>

Data represent mean ± SD of three determinations.

inhibition rate on oxygen uptake. The inhibitory effect of phytic acid was stronger in the Mb-catalyzed system than in the Hb-catalyzed system.

The effect of phytic acid on non-enzymatic iron-catalyzed lipid peroxidation in linoleic acid micelles was investigated using three systems including H$_2$O$_2$ + Fe (II), Fe (II) + Fe (III) (1:1), and H$_2$O$_2$ + Mb (Table 4-3). In the presence of 100 μM H$_2$O$_2$, 100 μM Fe (II) and 30 μM Mb catalyzed lipid peroxidation in linoleic acid micelles. In the absence of 100 μM H$_2$O$_2$, 50 μM Fe (II) +50 μM Fe (III) also catalyzed lipid peroxidation. Phytic acid effectively inhibited TBARS formation from linoleic acid micelles in the three systems. The ability of phytic acid to inhibit lipid peroxidation was dose-dependent. EDTA and deferoxamine also strongly inhibited the nonheme iron-catalyzed lipid peroxidation.
Table 4-3—Effect of phytic acid on iron- or Mb-catalyzed lipid peroxidation in linoleic acid micelles

<table>
<thead>
<tr>
<th>Chelators</th>
<th>Fe$^{2+}$ + H$_2$O$_2$</th>
<th>Fe$^{2+}$ + Fe$^{3+}$</th>
<th>Mb + H$_2$O$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (buffer)</td>
<td>4.28 ± 0.46</td>
<td>3.57 ± 0.12</td>
<td>11.5 ± 1.4</td>
</tr>
<tr>
<td>Phytic acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM</td>
<td>2.89 ± 0.33 (32.5)</td>
<td>1.92 ± 0.19 (46.2)</td>
<td>9.2 ± 1.1 (20.0)</td>
</tr>
<tr>
<td>1.0 mM</td>
<td>2.09 ± 0.18 (51.2)</td>
<td>1.39 ± 0.19 (61.1)</td>
<td>6.9 ± 0.8 (40.0)</td>
</tr>
<tr>
<td>10.0 mM</td>
<td>1.23 ± 0.15 (71.2)</td>
<td>0.82 ± 0.10 (77.0)</td>
<td>3.2 ± 0.6 (72.2)</td>
</tr>
<tr>
<td>EDTA, 0.1 mM</td>
<td>2.75 ± 0.20 (35.7)</td>
<td>1.37 ± 0.15 (61.6)</td>
<td>n.d.</td>
</tr>
<tr>
<td>Deferrooxamine, 0.1 mM</td>
<td>2.18 ± 0.16 (49.1)</td>
<td>0.98 ± 0.06 (72.5)</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Parenthesis indicates % inhibition versus control. Data represent the mean ± SD of three determinations.

We also investigated the effects of phytic acid on ascorbic acid-dependent lipid peroxidation in the presence of 100 μM Fe (III) (Fig. 4-3). In the absence of phytic acid, 0.1 mM ascorbic acid stimulated lipid peroxidation more than did 0.5 mM ascorbic acid. In the presence of 0.5 mM ascorbic acid and 100 μM Fe (III), 0.1, 1.0, 5.0, and 10.0 mM phytic acid inhibited lipid peroxidation by 25, 40, 50, and 55%, respectively, compared to the control (buffer only). In the presence of 0.1 mM ascorbic acid and 100 μM Fe (III), 0.1, 1.0, 5.0, and 10.0 mM phytic acid inhibited lipid peroxidation by 62, 71, 81, and 83%, respectively. The higher the concentration of phytic acid, the greater the inhibition of lipid peroxidation at both ascorbic acid concentrations.
**Fig. 4-3**—Effect of phytic acid on ascorbic acid-dependent lipid peroxidation in the presence of 100 μM Fe (III). Bar represents the mean ± SD of two determinations.

The concentration of ascorbic acid greatly influenced Fe (III)-catalyzed lipid peroxidation (Fig. 4-4). At 50 μM, ascorbic acid maximally catalyzed lipid peroxidation in the presence of 100 μM Fe (III). As concentrations of ascorbic acid increased, the TBARS formation from linoleic acid micelles decreased. The inhibitory effect of phytic acid on lipid peroxidation in the presence of 100 μM Fe (III) was influenced by the concentration of ascorbic acid. At 50 μM ascorbic acid, phytic acid most strongly inhibited TBARS formation. At lower than 1.0 mM ascorbic acid, 0.5 and 5.0 mM phytic acid effectively inhibited Fe (III)-catalyzed lipid peroxidation in the linoleic acid micelles,
while above 2.0 mM ascorbic acid, phytic acid seemed to slightly promote lipid peroxidation.

**DISCUSSION**

The interaction between phytic acid and ascorbic acid may have several nutritional implications. Although phytic acid reduces iron absorption in the small intestine (Fox and Tao, 1989; Moser and Bendich, 1991), the intake of ascorbic acid would effectively

![Graph](image)

**Fig. 4-4—Effect of phytic acid on lipid peroxidation of linoleic acid micelles in the presence of 100 μM Fe (III) and ascorbic acid at various concentrations.** Data represent the mean of three determinations.
counteract any inhibitory effects of phytate (Siegenberg et al., 1991). In the presence of a catalytic amount of metal ion, ascorbate is rapidly oxidized to dehydroascorbate through an electron transfer from ascorbate to metal, which is related to the generation of oxygen-derived free radicals (Khan and Martell, 1967; Samuni et al., 1983). In our results, phytic acid inhibited Fe (III) or Cu (II)-catalyzed ascorbic acid oxidation, perhaps by forming a catalytically ineffective iron-phytate complex for ascorbic oxidation and/or by stabilizing Fe\(^{3+}\) in the oxidized form (Graf and Eaton, 1990). The effects of phytic acid on inhibition of ascorbic acid oxidation and on oxidation of Fe (II) to Fe (III) may complicate bioavailability of nonheme iron from foods.

Hydroxyl radicals produced via the iron-catalyzed Fenton reaction can initiate lipid peroxidation and damage to deoxyribose in homogeneous reaction systems. Iron compounds can also increase the rate of lipid peroxidation by decomposing lipid hydroperoxides to alkoxy or peroxy radicals (Halliwell and Chirico, 1993; Minotti and Aust, 1987). In our results, the inhibitory effects of phytic acid on lipid peroxidation and deoxyribose degradation may be due to the formation of a tightly bound iron-phytic acid complex, which cannot participate in the Fenton reaction since phytic acid occupies all 6 co-ordination sites of Fe (III) (Graf and Eaton, 1990; Graf et al., 1984). The auto-oxidation of Fe (II) in solution occurs through the reduction of molecular oxygen and results in the formation of superoxide anion and Fe (III). This reaction is normally slow but is accelerated in the presence of chelators such as EDTA and phytic acid. A strong oxidant such as H\(_2\)O\(_2\) may also accelerate the oxidation of Fe (II) via the Fenton reaction, resulting in the formation of Fe (III) and \(^{\cdot}\)OH. Ferric salts are ineffective in production of hydroxyl radicals because, by themselves, they cannot produce superoxide anions in an aqueous solution (Halliwell and Gutteridge, 1981). Complexing ferrous ion with phytic
acid, deferroxamine, and EDTA rapidly and strongly facilitated the oxidation of Fe (II) to Fe (III). The ability of phytic acid, deferroxamine, and EDTA to facilitate oxidation of Fe (II) may also contribute to their ability to inhibit degradation of deoxyribose.

Minotti and Aust (1987) proposed a Fe$^{2+}$-O$_2$-Fe$^{3+}$ complex as another initiating species for lipid peroxidation. The rate of peroxidation appears to be maximal when the ratio of ferrous to ferric ion is one to one (Aust et al., 1985; Minotti and Aust, 1989). The ability of a chemical to shift the ratio of ferrous to ferric ion to 1:0 or 0:1 may reflect its antioxidant effect. The actions of ascorbic acid as an antioxidant or a prooxidant depend on its concentration. Low levels of ascorbic acid promoted warmed-over flavor (WOF) caused by lipid peroxidation, but high concentrations of ascorbic acid inhibit WOF as indicated by the TBA test (Fischer and Deng, 1977). At low concentrations of ascorbic acid, lipid peroxidation increases perhaps by facilitating the formation of a Fe$^{2+}$-O$_2$-Fe$^{3+}$ complex, which may initiate lipid peroxidation. However, when enough ascorbate is present to quickly reduce all of the ferric to ferrous ion, lipid peroxidation would be inhibited (Aust et al., 1985; Miller and Aust, 1989). In our study, the presence of ascorbic acid may complicate any inhibitory effect of phytic acid on iron-catalyzed lipid peroxidation because phytic acid and ascorbic acid have reverse actions on the reduction/oxidation of iron. In the presence of $< 1$ mM ascorbic acid, phytic acid effectively inhibited iron-catalyzed lipid peroxidation of linoleic acid micelles, perhaps due to its dominant ability to catalyze oxidation of Fe (II). However, the inhibitory effect of phytic acid was abolished in the presence of high concentrations of ascorbic acid. Deferroxamine is also a powerful inhibitor of lipid peroxidation and of hydroxyl radical formation dependent on the presence of iron salts (Halliwell, 1985). The binding of either Fe (II) or Fe (III) by deferroxamine may have various effects on the iron-catalyzed lipid
peroxidation, either via slow binding of Fe (III) or the rapid binding of Fe (II) with concomitant Fe (II) oxidation.

Several studies have suggested that hydrogen peroxide releases iron from hemoglobin and produces hydroxyl radicals (Grisham, 1985; Harel et al., 1988; Kanner and Harel, 1985a, b; Prasad et al., 1989; Sadrzadeh et al., 1984). Superoxide anions and hydroxyl radicals also release free iron from oxymyoglobin (Prasad et al., 1989). Heme-proteins appear to be highly susceptible to free radical attack, which may be another mechanism of free radical-mediated cellular injury (Prasad et al., 1989; Sadrzadeh et al., 1984). The interaction of H₂O₂ with MetMb produced an active species that initiated lipid peroxidation (Kanner and Harel, 1985a). A porphyrin cation radical (P⁺Fe⁺⁺⁺⁺⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻⁻) seemed to initiate lipid peroxidation, which was inhibited by several reducing compounds and antioxidants including EDTA and deferroxamine (Kanner and Harel, 1985b). However, several hydroxyl radical scavengers and catalase did not inhibit the reaction (Kanner and Harel, 1985b). Using electron spin resonance, Xu et al. (1990) reported that the free radicals from the reaction of MetMb with H₂O₂ may be a ferryl radical or an amino acid free radical originating from the Mb moiety. Phytic acid suppressed ferric ion- and myoglobin-t-butyl hydroperoxide-catalyzed lipid peroxidation in erythrocyte membranes (Ko and Godin, 1990, 1991). The inhibitory effect might be due to its chelating ability with iron, thus interfering with the interaction between heme-iron and hydrogen peroxide. Our results demonstrated the powerful oxidant ability of Hb and Mb activated by H₂O₂ to attack deoxyribose and to initiate lipid peroxidation in linoleic acid micelles. Phytic acid strongly inhibited the oxidative reaction of Hb and Mb.

The antioxidant or iron-chelating properties of phytic acid we found render this compound a unique and versatile food preservative. Therefore, phytic acid may be added
to foods high in lipid and iron, such as red meat, thereby preventing oxidative
deterioration of the food and increasing shelf-life. In addition, phytic acid may be useful
as a food supplement for ameliorating the extent of free radical-induced tissue damage.

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CHAPTER 5

ANTIOXIDANT EFFECTS OF CARNOSINE AND PHYTIC ACID IN A
MODEL BEEF SYSTEM

ABSTRACT

Antioxidant and color stabilizing effects of carnosine and phytate were compared in a fresh beef model system and in cooked beef. Both compounds increased the rate of pH decline in pre-rigor muscle. Phytate also increased the rate of post-mortem glycogen catabolism. Both antioxidants inhibited metmyoglobin formation in raw samples during storage. Phytate was more effective than carnosine for inhibition of lipid peroxidation. Heme iron content was negatively related to lipid peroxidation in cooked beef ($r = -0.92$). Phytate was also more effective for inhibition of iron release from heme during cooking. Phytate is recommended over carnosine as an effective antioxidant in cooked meats.

INTRODUCTION

Color in fresh meat and meat products is a strong indicator of quality. Several factors including packaging, oxygen tension, bacteria, pH, and temperature affect fresh meat color stability (Cornforth, 1994). Non-meat ingredients that have antioxidative and/or reducing activity can stabilize meat color thus extending shelf-life of meat and meat products (Greene et al., 1971; Mitsumoto et al., 1991a, b). Lipid peroxidation

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products and free radicals are involved in the oxidation of oxymyoglobin to metmyoglobin and associated with brown discoloration (Renerre and Labas, 1987). Ascorbate and vitamin E have been the most studied as antioxidants in meats, but metal chelators and synthetic inhibitors of lipid peroxidation may also protect meat color (Greene et al., 1971; Sato and Hegarty, 1971; Decker and Crum, 1991; Mitsumoto et al., 1991a, b).

Lipid peroxidation is a major cause of quality deterioration in restructured or precooked meats (Akamittath et al., 1990). It changes the flavor, color, texture, and nutritive value (Wilson et al., 1976). Warmed-over flavor (WOF) development can be retarded by many ingredients commonly used in meat products such as vitamin E, butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) (Sato and Hegarty, 1971; Decker and Crum, 1991). Such antioxidants, however, have limited applications in cooked intact muscle roasts because of low water solubility.

Carnosine (β-alanyl L-histidine) is an endogenous dipeptide found in the skeletal muscle of most vertebrates (Crush, 1970). Carnosine inhibits lipid peroxidation in several systems (Decker and Crum, 1991; Decker and Faraji, 1990; Decker et al., 1992; Kohen et al., 1988). Its antioxidant properties may result from its ability to chelate transition metals such as copper (Brown, 1981), its enzyme-like activity (Kohen et al., 1991), and its free radical scavenging activity (Chan et al., 1994; Lee and Hendricks, 1997a). These properties may increase the antioxidant potential of muscle and may render carnosine useful as a natural food antioxidant.

Phytic acid is a natural plant inositol hexaphosphate constituting 1-5% of many cereals and legumes (Reddy et al., 1989). Cereals are the major source of dietary phytate for Americans consuming a typical diet (750 mg phytate per day) (Harland, 1989). Metal
Phytate complexes are highly insoluble over a wide pH range (Graf et al., 1987). Phytic acid is also a powerful inhibitor of iron-related hydroxyl radical formation because it forms an inactive iron chelate (Graf et al., 1984; Graf and Eaton, 1990).

Carnosine and phytic acid have been investigated for their antioxidant activity in many studies, but no data are available for their effects on color, metmyoglobin formation, and degradation of heme pigments in beef muscle. Our objective was to study the ability of carnosine and phytic acid to inhibit lipid peroxidation and formation of metmyoglobin in raw beef muscle (treatment for pre-rigor beef muscle), thereby stabilizing meat color. We also evaluated their effects on lipid peroxidation and iron release from the heme moiety of cooked beef muscle (treatment for post-rigor muscle).

MATERIALS & METHODS

Materials

L-carnosine, phytic acid, 2-thiobarbituric acid (TBA), ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], ascorbic acid, α-amylglucosidase (E.C. 3.2.1), and tetraethoxypropane (TEP) were purchased from Sigma Chemical Co. (St. Louis, MO). Trichloroacetic acid (TCA) and sodium hydroxide were obtained from EM Science (Cherry Hill, NJ). Hydrogen peroxide, potassium phosphate (monobasic anhydrous), nitric acid, acetone, and ammonium acetate were obtained from Mallinckrodt Inc. (Paris, KY).

Sample preparation

Pre-rigor test. Beef (m. biceps femoris, 1 h post-mortem) was obtained from the Meat Laboratory at Utah State University. Visible fat and connective tissues were removed, and the muscle was chopped into small pieces with a stainless-steel knife.
Meat samples (315 g) were blended with 35 mL test solutions using a food processor (Braun Multipractic MC 100, Braun Co., Lynnfield, MA). Treatment groups included: 1) control (deionized water, DW), 2) 0.1 mM carnosine, 3) 1 mM carnosine, 4) 5 mM carnosine, 5) 0.1 mM phytic acid, 6) 1 mM phytic acid, and 7) 5 mM phytic acid. Test solutions were adjusted to pH 6.8 using dilute HCl or NaOH to correspond to the initial pH of the meat. Meat pH was directly measured using a glass pH electrode (Orion Research Incorporated Co., Boston, MA). Aliquots were stored covered in petri dish plates for 9 days at 4 °C. Sampling and weighing were performed in a cold room (4 °C).

Post-rigor test. Ground beef (m. biceps femoris, lean, 48 h post-mortem) was obtained from the Meat Laboratory at Utah State University. Meat samples (180 g) were blended with 20 mL test solutions using a blender (Sanyo SJ 3020 MB, Sanyo Electric Co., Chatsworth, CA). Treatment groups included: 1) control (deionized water), 2) 1 mM carnosine, 3) 5 mM carnosine, 4) 1 mM phytic acid, and 5) 5 mM phytic acid. Test solutions were adjusted to pH 5.8 using dilute HCl and NaOH. Aliquots were transferred into 50-mL polypropylene test tubes and stored for 6 h at 4 °C before cooking. The beef was cooked with 70 ± 2 °C (internal temperature) in a water-bath, then cooled in an ice-bath. The cooked beef was chopped into very fine pieces and stored in a Ziploc© storage bag (DowBrand, Indianapolis, IN) for 9 days at 4 °C. Sampling and weighing were performed in a cold room (4 °C).

Methods

Total iron. Total iron concentration was determined in wet-ashed samples using the ferrozine assay (Stookey, 1970). Beef muscle (0.2-0.3 g) in a test tube (16 x100 mm) was digested with concentrated nitric acid and 30% hydrogen peroxide on a hot plate until
a white ash was formed. The ash was dissolved in 0.2 mL of 1.0 N HCl and diluted with 0.8 mL deionized water. Ascorbic acid (1 mL, 1%) was added, and the tubes were vortexed. After 20 min, 1 mL 10% ammonium acetate buffer and 1 mL of 1 mM ferrozine color reagent were added, and the mixture was mixed well. The mixture was allowed to stand at room temperature for 45 min, then the absorbance was determined at 562 nm. The concentration of iron was determined from a standard curve made with iron standard solution (Sigma, St. Louis, Mo).

**Heme iron.** Heme iron was determined using the method of Hornsey (1956). Beef (2 g) was transferred into a 50-mL polypropylene tube, and 9 mL of acidic acetone (90% acetone + 8% DW + 2% HCl) was added. The beef was macerated with a glass rod and allowed to stand 1 h in a dark cabinet at room temperature. The extract was filtered with Whatman filter paper #42, and the absorbance was read at 640 nm against the acidic acetone blank. Total pigments, as acid hematin, were calculated using the formula:

\[
\text{Total pigments (ppm)} = A_{640} \times 680
\]

and heme iron was calculated as follows (Clark et al., 1997):

\[
\text{Heme iron (ppm)} = \text{total pigment (ppm)} \times 8.82 / 100
\]

**TBA values.** Thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). Samples (0.5 g) were mixed with 2.5 mL of 0.375% TBA-15% TCA-0.25 N HCl stock solution. The mixture was heated for 10 min in a boiling water bath (95-100 °C) to develop a pink color, cooled with tap water, centrifuged at 5,500 rpm for 25 min in a Beckman centrifuge (Model J-21C, Palo Alto, CA). Absorbance of the supernatant was measured spectrophotometrically at 532 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). TBARS
were calculated from a standard curve of malondialdehyde, a breakdown product of TEP. TBA number was calculated as mg MDA/kg sample.

**Metmyoglobin.** Minced meat (5 g) was placed into a 50-mL polypropylene centrifuge tube and 25 mL ice-cold phosphate buffer (pH 6.8, 40 mM) was added. The mixture was homogenized for 10 s at 13,500 rpm with an Ultra-Turrax T25 (Janke & Kunkel GmbH, Staufen, Germany). The homogenized sample was allowed to stand for 1 h at 4 °C and centrifuged at 5,000 rpm for 30 min at 4 °C. The supernatant was filtered through Whatman #1 filter paper, and the absorbance was read at 700, 572, and 525 nm with a UV-VIS recording spectrophotometer (Model UV 2100 U, Shimadzu Co., Kyoto, Japan). Percent metmyoglobin was determined using the formula by Krzywicki (1982):

\[
\% \text{ MetMb} = \left(1.395 - \frac{(A_{572} - A_{700})}{I (A_{525} - A_{700})}\right) \times 100
\]

**Meat color.** Instrumental measurement of meat color was determined with a Digital Color Difference Meter D25D2A (Hunter Associates Laboratories, Inc., Reston, VA). The instrument was standardized with a red plate: L=25.9, a=27.4, b=13.1. Readings were taken directly on samples in petri dishes.

**Glycogen.** Glycogen concentration in muscle was determined using the method described by Dalrymple and Hamm (1973). Finely chopped meat samples (3 g) were placed into a 50-mL polypropylene tube, and 15 mL cold 0.6 N perchloric acid was added. The sample was homogenized for 10 s at 13,500 rpm with an Ultra-Turrax T25 (Janke & Kunkel GmbH, Staufen, Germany) in an ice-bath. Then 0.5 mL homogenate was transferred with a 1-mL syringe into a test tube, and 1.0 mL α-amylglucosidase (E.C. 3.2.1) solution (1 mg protein/mL, 0.2 M acetate buffer, pH 4.8) and 0.1 mL of 5.4 N KOH were added. The mixture was incubated for 1.5 h at 40 °C. After centrifugation of the incubated mixture at 2,000 x g for 20 min at 4 °C, the supernatant was neutralized
with 5 N NaOH and analyzed for total glucose using a commercial enzyme kit (Sigma). The remaining homogenate was centrifuged 4,000 x g for 20 min at 4 °C; the resulting supernatant was filtered with Whatman #1 filter paper and neutralized with 5.4 N KOH. The resulting potassium perchlorate was allowed to settle for 20 min at 0 °C. This extract was used for free glucose determination using an enzyme kit (Sigma). Glycogen concentration was expressed as glucose concentration, calculated as “total glucose minus free glucose.”

Statistical analysis

Data were analyzed using SAS (1985) program on triplicate samples with two replications. The least significant difference procedure was used to determine significant differences at ≤ 0.05% between means of treatment groups. ANOVA tables (A.1-A.5) are shown in appendix A.

RESULTS & DISCUSSION

Meat pH and glycogen

The pH of beef muscle decreased with storage time at 4 °C with an ultimate pH of 5.75 after 24 h post-mortem (Fig. 5-1). When the pH was measured at 2, 5, or 8 h post-mortem, 1 mM carnosine and 1 mM phytic acid had accelerated the decrease of meat pHs and were found to be notably lower than the control (p<0.05). However, there was no pH difference among treatment groups after 24 h post-mortem.

Muscle glycogen content decreased with post-mortem time (Fig. 5-2). Phytic acid significantly lowered muscle glycogen concentration during post-mortem storage at 4°C, compared with the control and carnosine groups (p<0.05). After 20 h post-mortem
Fig. 5-1—Effect of carnosine (CAR) and phytic acid (PA) on pH of raw, pre-rigor beef during post-mortem storage at 4 °C. *significantly different from the control within the same time period (p<0.05).

storage, there was no significant difference in glycogen content of muscle among treatment groups. The rapid drop of muscle glycogen concentration by phytic acid may be related to the low meat pH.

Glycogen in muscle can be used for energy for short periods after slaughter (Hamm, 1977). Anaerobic metabolism of glycogen results in an accumulation of lactic acid, resulting in a drop in meat pH. The rapid drop of meat pH may be associated with the prevention of bacterial invasion and the maintenance of meat red color. A low glycogen content of beef muscle causes dark-cutting beef, perhaps resulting from high meat pH. In our study, processes such as blending and adding 10% water might increase
Fig. 5-2—Effect of carnosine (CAR) and phytic acid (PA) on depletion of muscle glycogen in pre-rigor beef during post-mortem storage at 4 °C. *significantly different from the control within the same time period (p<0.05).

air contact and accelerate loss of cellular reductants, and also cause a rapid drop of glycogen and pH. Newbold and Scopes (1971) reported that 50 mM and 100 mM potassium phosphate stimulated phosphorylase and phosphofructokinase activity in pre-rigor sternomandibularis minces, thereby resulting in higher ultimate lactate concentrations and lower ultimate pH, compared with undiluted minces. Hamm (1977) reported that 1.0% sodium diphosphate stimulated various glycolytic enzymes in unsalted
minces, resulting in low ultimate glycogen and high lactate concentration. Carnosine has been reported to have an accelerating effect on the number of enzymatic reactions involved in anaerobic and aerobic metabolism of carbohydrates in muscle. It was also reported to have a stimulating effect on phosphorylase $b$ for glycogen metabolism (Severin, 1964). However, our results showed no effect of carnosine on glycolysis in post-mortem beef muscle.

The glycogen concentration in muscle depends on the type of fiber, age, and stress. Slow, oxidative (SO) fibers typically contain less glycogen than fast, glycolytic (FG) fibers (Lacourt and Tarrant, 1985). Pre-stress (ACTH, adrenaline, or a long period in transportation) before slaughter depletes glycogen reserves from muscle, resulting in dark cutting condition after slaughter (Sanz et al., 1996). The beef muscle we used had a glycogen content similar to that reported by Dalrymple and Hamm (1973).

**Metmyoglobin and meat color**

Metmyoglobin formation occurred when meat samples were stored for 9 days at 4 °C (Table 5-1). Treatment with carnosine or phytic acid inhibited metmyoglobin formation ($p<0.05$). The effectiveness of carnosine and phytic acid in inhibiting metmyoglobin formation was more pronounced with increasing storage time.

The formation of metmyoglobin was negatively associated with red color (Hunter ‘a’ values) of meat ($r=-0.91$). The red color faded with increasing storage time at 4 °C (Table 5-2). The red color of the control meat faded very rapidly. Meat samples treated with carnosine or phytic acid exhibited a color difference from that of the control treated with deionized water ($p<0.05$). The meat colors were also visually distinctive: bright red (carnosine), purple red (phytic acid), and brown (the control). However, there were no significant differences in hunter meat red color between carnosine and phytic acid treatments.
Table 5-1—Effect of carnosine and phytic acid on metmyoglobin (%) formation in a raw beef model system during storage at 4 °C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Storage time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control (DW)</td>
<td>27.5 aw</td>
</tr>
<tr>
<td>Carnosine, 0.1 mM</td>
<td>27.4 aw</td>
</tr>
<tr>
<td></td>
<td>27.0 aw</td>
</tr>
<tr>
<td></td>
<td>26.8 aw</td>
</tr>
<tr>
<td>Phytic acid, 0.1 mM</td>
<td>27.8 aw</td>
</tr>
<tr>
<td></td>
<td>27.3 aw</td>
</tr>
<tr>
<td></td>
<td>26.5 aw</td>
</tr>
</tbody>
</table>

Mean values in the same column with different superscripts are significantly different at p<0.05.

The color stability of meat is directly related to shelf-life. The bright red color of oxymyoglobin indicates high-quality fresh meat that is attractive to consumers. The influence of biological factors on meat discoloration depends largely on the nature of fibers. Color stability is muscle-dependent (Reddy and Carpenter, 1991). After 8 days storage, the metmyoglobin percentage at the meat surface in beef varied from 25-50% of total myoglobin content (Renerre et al., 1996). In our results, the higher % metmyoglobin might be the result of the chopping and blending preparation of samples. Although many factors can influence meat color stability, myoglobin oxidation by free radicals or lipid peroxidation products is predominant (Gray et al., 1996; Renerre and Labas, 1987). The color-stabilizing effects of carnosine and phytic acid may be the result of their ability to chelate transition metals involved in free radical generation and/or free...
Table 5-2—Effect of carnosine and phytic acid on the Hunter red color values (‘a’) of a raw beef model system during storage at 4 °C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Storage time (day)</th>
<th>Hunter (red) color (a) value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Control (DW)</td>
<td>13.1&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>10.7&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carnosine, 0.1 mM</td>
<td>12.7&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>11.6&lt;sup&gt;awx&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>12.9&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>11.0&lt;sup&gt;ax&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>13.1&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>13.1&lt;sup&gt;bw&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phytic acid, 0.1 mM</td>
<td>12.5&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>11.5&lt;sup&gt;awx&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>12.8&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>12.0&lt;sup&gt;awx&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>12.8&lt;sup&gt;aw&lt;/sup&gt;</td>
<td>12.4&lt;sup&gt;bw&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>abc</sup>Means in same column with different superscripts different at p<0.05.

<sup>wxyz</sup>Means in same row with different superscripts different at p<0.05.

radical scavenging, thereby delaying the oxidation of oxy myoglobin to metmyoglobin. Carnosine can act as a reductant (Kohen et al., 1988). The function of carnosine to donate an electron to free radicals is generally related to its antioxidant activity (Kohen et al., 1988). Decker and Crum (1991) reported that carnosine had a color-protecting effect on salted ground pork, and its effectiveness was greater than that of butylated hydroxytoluene, sodium polyphosphate, or α-tocopherol. Decker et al. (1995) reported that carnosine accelerated the conversion of metmyoglobin to oxy myoglobin at pH > 7.0 with carnosine > 25 mM. However, these conditions are not found in meat. Moreover, carnosine (1-50 mM) also accelerated the metmyoglobin formation at low pH in a dose-dependent manner. In our results, low concentrations (0.1, 1, and 5 mM) of carnosine inhibited metmyoglobin formation in a dose-dependent manner during storage for 9 days.
Lipid peroxidation in raw beef

The total iron of beef muscle was 26.5 ± 1.0 μg/g wet sample. TBARS formation increased sharply with increasing storage time at 4 °C (Table 5-3). Treatment with carnosine or phytic acid inhibited lipid peroxidation in beef muscle (p<0.05). The inhibitory effects of carnosine and phytic acid were dose-dependent. The inhibition of lipid peroxidation by phytic acid was stronger than with carnosine at the same concentrations.

The development of off-flavor in meat results from lipid peroxidation during storage. Oxidation of muscle lipids involves the peroxidation of membrane polyunsaturated fatty acids (Keller and Kinsella, 1973). Transition metals such as iron

Table 5-3—Effect of carnosine and phytic acid on TBA number (mg MDA/kg meat) in raw beef model system during storage at 4 °C

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Storage time (day)</th>
<th>TBA number (mg MDA/kg meat)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Control (DW)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carnosine, 0.1 mM</td>
<td>0.16</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>aw</td>
<td>ax</td>
</tr>
<tr>
<td>1 mM</td>
<td>0.16</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>aw</td>
<td>bx</td>
</tr>
<tr>
<td>5 mM</td>
<td>0.11</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>bw</td>
<td>cx</td>
</tr>
<tr>
<td>Phytic acid, 0.1 mM</td>
<td>0.14</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>abw</td>
<td>cw</td>
</tr>
<tr>
<td>1 mM</td>
<td>0.13</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>abw</td>
<td>cw</td>
</tr>
<tr>
<td>5 mM</td>
<td>0.12</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>bw</td>
<td>dw</td>
</tr>
</tbody>
</table>

Means in same column with different superscripts different at p<0.05.

Means in same row with different superscripts different at p<0.05.
and copper and heme are important in the reaction (Kanner et al., 1988; Sato and Hegarty, 1971; Schricker and Miller, 1983). Phytic acid can chelate the transition metal ions inhibiting metal-catalyzed lipid peroxidation or free radical formation from the Fenton reaction. Carnosine can chelate the transition metals and also scavenge free radicals such as superoxide anions, hydroxyl radicals, and peroxy radicals (Brown, 1981; Chan et al., 1994; Kohen et al., 1988). Such actions of carnosine and phytic acid may be involved in the inhibition of lipid peroxidation. There are reports on antioxidant activity of carnosine and phytic acid in several systems. Carnosine (25 mM) inhibits the catalysis of lipid peroxidation by iron, hemoglobin, lipoxidase, and singlet oxygen from 35 - 96%, suggesting that its antioxidant mechanism is not solely due to metal chelation (Decker and Faraji, 1990). Decker and Crum (1991) reported that carnosine (0.5 and 1.5%) effectively inhibited TBARS formation in frozen pork stored up to 6 mo. Carnosine was more effective than sodium triphosphate and other lipid-soluble antioxidants such as alpha-tocopherol and BHT. Lee and Hendricks (1995) reported that phytic acid inhibited lipid peroxidation effectively and dose-dependently in beef homogenates. The inhibition by phytic acid may be largely due to its ability to chelate transition metals and/or facilitate oxidation of ferrous ion (Graf and Eaton, 1990).

**Heme iron content during storage of cooked beef**

Muscle tissue contains considerable iron bound to proteins. Myoglobin is the most abundant hemoprotein in muscle tissue. Our total iron and heme iron content of ground beef were 25.7 ± 2.0 and 16.8 ± 1.2 μg/g wet weight, respectively, corresponding to 65.1% heme iron of total iron. The values were similar to those reported by Schricker et al. (1982) but lower than those reported by Carpenter and Clarks (1995). The heme
samples, cooking increased nonheme iron release by 4.4 μg/g sample, corresponding to decrease in heme iron by 16.9%.

Cooking destroys the porphyrin rings of heme pigments, resulting in nonheme iron release from heme pigments. The release of nonheme iron depends on temperature, time, type (slow or fast), and method of cooking (boiling, baking, microwaving) (Schricker et al., 1982; Schricker and Miller, 1983; Chen et al., 1984). Ascorbic acid

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**Fig. 5-3**—Effect of L-carnosine and phytic acid on heme iron content in cooked beef during storage for 9 days at 4 °C. Data points represent mean values of treatment (n=6).
increases nonheme iron release, while nitrite inhibits nonheme iron release in meat (Schricker and Miller, 1983) and a meat-free model system (Graf and Panter, 1991).

Heme iron content in cooked beef decreased with increasing storage time (Fig. 5-3). Both carnosine and phytic acid inhibited nonheme iron release (p<0.05). The inhibitory effect of phytic acid on iron release from degradation of pigments was stronger than that of carnosine during storage for 9 days (p<0.05). Harel et al. (1988) reported that the amounts of free iron increased almost four-fold during storage of turkey dark muscle at 4 °C for 7 days. Although the mechanisms of inhibition of carnosine and phytic acid on nonheme iron release were not reported, their chelating or free radical scavenging activity might be involved (Brown, 1981; Chan et al., 1994; Graf et al., 1984; Graf and Eaton, 1990; Kohen et al., 1991).

The contents of heme and nonheme iron in meat affects its availability in the intestine (Cook and Monsen, 1976). Phytate is known to inhibit nonheme iron absorption but it does not affect heme iron absorption (Carpenter and Mahoney, 1992). Since heme iron is fixed in a complex, it cannot be converted into an unabsorbable form, indicating that it cannot form into complexes with phytates that would become unavailable (Rogov et al., 1989a). Kim et al. (1993) reported that meat (beef, pork, and chicken) enhanced nonheme iron absorption by iron-deficient rats only in the presence of meals containing added phytate. Rogov et al. (1989b) reported that the higher amounts of dietary fibers in the presence of cysteine (meat factor) caused lower binding of iron to the dietary fibers (increased iron solubility). Therefore, phytate as a meat additive may not result in a decrease in total iron absorption during digestion in the small intestine. Carnosine may also increase total iron absorption since it inhibits nonheme iron release from meat pigments during cooking.
Lipid peroxidation in cooked beef

Lipid peroxidation as expressed by TBA number was increased with cooking and with increasing storage after cooking (p<0.05) (Fig. 5-4). Both carnosine and phytic acid inhibited TBARS formation (p<0.05) and the effect was dose-dependent. The inhibitory effect by phytic acid was much greater than by carnosine (p<0.05).

This was probably due to strong chelation by phytic acid of iron released from degradation of heme pigments. When all six coordination sites in Fe (III)- phytate chelate are occupied, the chelate cannot participate in the Fenton reaction (Graf et al., 1984). This

Fig. 5-4—Effect of L-carnosine and phytic acid on TBA number in cooked beef during storage for 9 days at 4 °C. Data points represent mean values of treatment (n=6).
explains how phytic acid inhibits \textsuperscript{·}OH generation and subsequent lipid peroxidation (Graf et al., 1984). We have also shown that phytic acid effectively inhibited iron- and hemeprotein-catalyzed lipid peroxidation in beef homogenate and a linoleic acid model system (Lee and Hendricks, 1995; 1997b). There is no direct evidence for chelation of iron by carnosine. Decker et al. (1992) reported that carnosine did not form a complex with iron. Carnosine can be broken down in the presence of pro-oxidants such as transition metals (Kondrat'eva et al., 1993). In our results, the weaker inhibitory effect of carnosine than of phytic acid might be explained by those findings.

The phospholipids in muscle membrane provide an ideal substrate for lipid peroxidation. Iron bound to negatively charged phospholipids promotes lipid peroxidation, resulting in generation of warmed-over flavor (Empson et al., 1991).

Both nonheme and heme iron in beef muscle can catalyze lipid peroxidation (Kanner et al., 1988; Love, 1983; Monahan et al., 1993). However, Sato and Hegarty (1971) reported that nonheme iron was the active catalyst in cooked meats. Chen et al. (1984) and Igene et al. (1979) had also reported that iron was released from heme pigments during cooking and proposed that the resultant increase in nonheme iron was responsible for lipid peroxidation.

There was a negative relationship (Fig. 5-5) between heme iron content (Fig. 5-3) and TBA number (Fig. 5-4) of cooked beef ($r=-0.92$). This supports the view that iron release from heme in cooked meat is responsible for catalyzing lipid peroxidation resulting in warmed-over flavor.
Fig. 5-5—The correlation between heme iron and TBA number. All heme iron data were plotted against all TBA number data obtained by the same treatment and at the same storage time.

CONCLUSIONS

Carnosine and phytic acid as antioxidants inhibited metmyoglobin formation and stabilized red meat color when added to pre-rigor beef muscle. In addition, they inhibited lipid peroxidation and degradation of heme pigments caused by cooking and storage. These actions of carnosine and phytic acid indicate they may be useful as additives for meat processing to prevent off-flavor formation and increase shelf-life.
REFERENCES


CHAPTER 6
A COMPARISON OF CARNOSINE AND ASCORBIC ACID ON COLOR AND LIPID STABILITY IN A GROUND BEEF PATTIE MODEL SYSTEM

ABSTRACT

Ascorbic acid (0.1%) significantly inhibited metmyoglobin formation on the surface of ground beef but not in the bulk of the product where oxygen tension was lower. Carnosine (1.0%), however, significantly inhibited metmyoglobin formation and brown color development throughout the product. The combinations of carnosine and ascorbic acid were also very effective on inhibition of metmyoglobin formation and brown color development. Carnosine increased meat pH, cook yield, and salt-soluble protein, but ascorbic acid had no effect on cook yield, and decreased meat pH and salt-soluble protein. Carnosine was more effective for preventing lipid peroxidation than ascorbic acid. Carnosine inhibited copper (II)-catalyzed ascorbate oxidation in a dose-dependent manner.

INTRODUCTION

Meat color is a primary criterion by which consumers evaluate meat quality and acceptability (Cornforth, 1994). The relatively short shelf-life of fresh meats is the single greatest concern to retail meat markets. When brown metmyoglobin reaches 30-40% of total pigments on the surface of fresh retail beef, consumers make a no-purchase decision (Greene et al., 1971). Therefore, the meat industry and researchers have continuously looked for new ingredients or additives to extend fresh meat color stability. Ascorbic acid is now approved by the USDA for application to the surface of fresh beef or lamb cuts to
delay discoloration (USDA, 1994). However, hamburger per se may not be treated with
ascorbic acid or any added substance. Ground beef with added substance must be
labelled as "patties," and the substances added must be prominently indicated on the
label.

Lipid peroxidation products and free radicals can cause oxidation of
oxymyoglobin to metmyoglobin, indicating discoloration of meats (Renerre and Labas,
1987). Non-meat ingredients that stabilize color can improve shelf-life of meat and meat
products (Mitsumoto et al., 1991a, b; Trout and Dale, 1990). Ascorbic acid has been
widely used as a food ingredient for its reducing and antioxidative activity (Bendich et al.,
1986). Ascorbic acid prevents pigment and lipid peroxidation in ground beef and beef
steaks, sometimes synergistically in combination with phenolic antioxidants, vitamin E,
butylated hydroxyanisole, and propyl gallate (Greene et al., 1971; Mitsumoto et al.,
1991a, b; Okayama et al., 1987). Several mechanisms, including regeneration of primary
antioxidant, inactivation of prooxidant metals, and scavenging of reactive oxygen radicals,
may be involved in its antioxidant activity (Bauernfiend and Pinkert, 1970; Bendich et al.,

Muscle foods contain negligible amounts of ascorbic acid (Graby and Singh,
1991). The addition of ascorbic acid to meats may be beneficial as a human nutrient as
well as for improving color and shelf-life (Bauernfiend and Pinkert, 1970). However,
ascorbic acid is highly susceptible to oxidation, especially when catalyzed by metal ions
such as Cu (II) and Fe (III) (Khan and Martell, 1967). Muscle foods contain high
amounts of the metal ions, possibly resulting in rapid oxidation of ascorbic acid when
ascorbic acid is added to meats. The potency of metal ions in catalyzing ascorbic acid
degradation depends on the metal involved, its oxidation state, and the presence of
-chelators (Buettner, 1988). Catalytic potency is as follows: Cu (II) is about 80 times more potent than Fe (III), and the Fe (III)-EDTA complex is 4 times more catalytic than free Fe (III) (Buettner, 1988).

The phenolic antioxidants do not protect ascorbic acid from oxidation when they are used in meat processing in combination with ascorbic acid (Greene et al., 1971). In this respect, carnosine (β-alanyl L-histidine) may be useful for protecting ascorbic acid against metal-catalyzed oxidation, especially Cu (II)-catalyzed ascorbic acid oxidation, because carnosine can form catalytically inactive complexes with Cu (II) (Brown, 1981) and scavenge reactive free radicals (Chan et al., 1994; Lee and Hendricks, 1997a). In addition, carnosine showed a color stabilizing activity in pre-rigor beef (Lee et al., 1998) and salted ground pork (Decker and Crum, 1991). Carnosine also inhibits lipid peroxidation in several systems (Decker et al., 1992; Lee and Hendricks, 1997a; Lee et al., 1998). Furthermore, carnosine has several biological actions such as improving wound healing (Nagai et al., 1986) and buffering skeletal muscle (Harris et al., 1990), and it can be applied as a therapeutic agent for mammary cancer (Boissonneault et al., 1994), atherosclerosis (Borgadus et al., 1993), gastritis (Yoshikawa et al., 1991), and cataracts (Babizhayev, 1989).

These properties may render carnosine useful as health-improving agents as well as a natural food antioxidant. Furthermore, the conjunction of carnosine with ascorbic acid may increase their positive effects on foods. In this study, we evaluate the ability of carnosine and ascorbic acid to stabilize meat color and lipids in ground beef and the possible protective effect of carnosine against Cu (II)-catalyzed ascorbic acid oxidation.
MATERIALS & METHODS

Materials

L-carnosine, ascorbic acid, 2-thiobarbituric acid (TBA), ferrozine [3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine], Hepes (N-2-hydroxyethyl piperazine N'-2-2-ethanesulfonic acid), 2,6-dichlorophenol indophenol, and tetraethoxypropane (TEP), were purchased from Sigma Chem. Co. (St. Louis, MO). Trichloroacetic acid (TCA) and sodium hydroxide were obtained from EM Science (Cherry Hill, NJ). Hydrogen peroxide, potassium phosphate (monobasic anhydrous), nitric acid, and ammonium acetate were obtained from Mallinckrodt Inc. (Paris, KY).

Methods

Sample preparation. Ground beef (lean, ≤ 5% fat) was obtained from the Meat Laboratory at Utah State University. The meat sample (135 g) was blended with 15 mL test solutions using a food processor (Braun Mutipractic MC100, Braun Co., Lynnfield, CA). There were seven treatment groups, including 1) control (deionized water), 2) 1% carnosine, 3) 0.1 % ascorbic acid, 4) 1% carnosine + 0.1 % ascorbic acid, 5) 1% carnosine + 0.05% ascorbic acid, 6) 0.5% carnosine + 0.1% ascorbic acid, and 7) 0.5% carnosine + 0.05% ascorbic acid. The pH of test solutions was not adjusted. The solution pH values of carnosine (10%) and ascorbic acid (1%) before treatment were 8.3 and 2.5, respectively. Aliquots of the treated beef samples were stored in petri plates for 1-3 days in a cold room (4 °C) in the dark, with the lid on to minimize dehydration. The top surface was aerobic, similar to fresh meats wrapped with oxygen-permeable PVC overwrap. The bottom surface was anaerobic, or nearly so, as occurs in the center of bulk hamburger chubs, or in center patties, when patties are stacked (6-10/stack), as is
common prior to freezing of retail patties. Thus, placing our samples in a petri plate was a convenient way to observe color changes in both the surface and anaerobic interior of our pattie system during storage. Sampling and weighing were done in a cold room (4 °C).

**pH measurement.** The pH of meat sample was measured using a glass pH electrode (Orion Research Incorporated Co., Boston, MA) after homogenizing 1 g sample in 9 mL deionized water for 10 sec at 13,500 rpm with an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany).

**Iron and copper content.** The total iron and copper concentrations were determined in wet-ashed samples using an atomic absorption spectrophotometer (Perkin Elmer 3100, Perkin Elmer Co., Norwalk, CT). Beef muscle (0.2-0.3 g) in a test tube (16 x100 mm) was digested with concentrated nitric acid and 30% hydrogen peroxide on a hot plate until a white ash formed. The ash was dissolved in 0.2 mL of 1.0 N HCl and diluted with 2.8 mL 1.0 N nitric acid. The samples and standard solutions were used for determination of iron and copper.

**Heme iron.** Heme iron was determined using the method described by Homsey (1956). Total pigments were extracted with 90% acid acetone. Beef (2.0 g) was transferred into a 50-mL polypropylene tube, and 9 mL of acidic acetone (90% acetone + 8% deionized water + 2% HCl) was added. The beef was macerated with a glass rod and allowed to stand 1 h at room temperature. The extract was filtered with Whatman paper #42, and the absorbance was read at 640 nm against an acidic acetone blank. Total pigments as hematin were calculated using the formula:

\[ \text{Total pigments (ppm)} = A_{640} \times 680 \]

and the heme iron was calculated using the formula:
Heme iron (ppm) = total pigment (ppm) x 8.82 / 100

**TBA number.** Thiobarbituric acid-reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). Samples (0.5 g) were mixed with 2.5 mL of 0.375% TBA-15% TCA-0.25 N HCl stock solution. The mixture was heated for 10 min in a boiling water-bath (95-100 °C) to develop a pink color, cooled with tap water, and centrifuged at 5,500 rpm for 25 min in a Beckman centrifuge (Model J-21C, Palo Alto, CA). The supernatant was measured spectrophotometrically at 532 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). TBARS were calculated from a standard curve of malondialdehyde, a breakdown product of TEP. TBA number was calculated as mg MDA/kg sample.

**Metmyoglobin.** Minced meat (5 g) was placed into a 50-mL polypropylene centrifuge tube, and 25 mL ice-cold phosphate buffer (pH 6.8, 40 mM) was added. The mixture was homogenized for 10 s at 13,500 rpm with an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany). The homogenized sample was allowed to stand for 1 h at 4 °C and centrifuged at 5,000 rpm for 30 min at 4 °C. The supernatant was filtered with Whatman #1 filter paper, and the absorbance was read at 700, 572, and 525 nm by scanning the visible spectrum (Shimadzu Co., Kyoto, Japan). Metmyoglobin was determined using the following formula (Krzysicki, 1982).

\[
\% \text{MetMb} = \{1.395 - [(A_{572} - A_{700}) / (A_{525} - A_{700})]\} x 100
\]

**Hunter meat color.** The meat color was determined using a Hunter Lab Digital Color Difference Meter D25D2A (Hunter Associates Laboratories, Inc., Reston, VA). The instrument was standardized with a red plate: L=25.9, a=27.4, b=13.1. The meat color was read directly on raw beef in a petri dish.
Cook yield (WHC) in cook beef. Percent cook yield was calculated as [(weight after cooking/15 g weight) x 100]. A mixture containing 15 g ground beef in a 50-mL polypropylene test tube was placed in a boiling water-bath for 30 min (internal cooking temperature, 70 ± 2 °C). The tube was allowed to cool with tap water, and the cooked meat sample was filtered through a Whatman #4 filter paper for 1 h at 4 °C, blotted with a paper towel, and weighed for the determination of cook yield (based on 15 g ground beef).

Salt soluble protein. Beef (1 g) was homogenized in 5 mL 0.6 N NaCl at 13,500 rpm for 10 s with an Ultra-Turrax T25 (Janke & Kunkel, Staufen, Germany) and allowed to stand for 1 h at 4 °C (Knipe et al., 1985). The homogenates were centrifuged at 6,000 rpm for 30 min at 4 °C (Beckman, Model J-21C, Palo Alto, CA). The supernatants (0.1 mL) or standard solutions (bovine serum albumin) were diluted with 0.9 mL of 0.6 N NaCl. The diluted solutions were used in the determination of protein by the method of Lowry et al. (1951).

Oxygen uptake rate. The uptake of oxygen was measured with an oxygen electrode in an Orion EA 940 expandable ion analyzer (Orion Research Incorporated Co., Boston, MA). Oxygen consumption rate was calculated by measuring changes in dissolved oxygen concentration for 3 min at room temperature. The reaction mixtures contained 35 mM Hepes buffer (pH 7.0), 10 μM cupric chloride, and 100 μM ascorbic acid. Ascorbic acid was added to the reaction solution, and the dissolved oxygen concentration was measured using an oxygen electrode in an ion/pH meter.

Ascorbic acid. Ascorbic acid was determined spectrophotometrically using the dye 2,6-dichlorophenol indophenol, which is reduced from a blue to a colorless form by ascorbic acid (Brewster and Turley, 1987). Beef homogenates (10% in DW) were
centrifuged at 6,000 x g for 10 min at 2 °C, and the supernatant was filtered with Whatman #42 filter paper. The filtered solution or the diluted solution (1 mL) was used in the determination of ascorbic acid in beef. For a model system, reaction mixtures (1 mL) at various pHs, including 35 mM Hepes buffer, 100 μM ascorbic acid, 10 μM Cu (II), and 1 mM carnosine, were allowed to stand for 5 min at room temperature. To 1 mL of the filtered solution and the reaction mixture, 0.2 mL of 1 M metaphosphoric acid and 20 μL of 10 mM 2,6-dichloroindophenol were added, and then the absorbance was measured at 520 nm. When excess dye was added to the reaction mixtures containing ascorbic acid, the decrease in color was related to the amount of reduced ascorbic acid. As another method, ascorbic acid was also monitored by directly reading the absorbance at 265 nm following removal of aliquots of reaction mixtures at timed intervals. The reaction mixtures (1 mL, pH 7.0) included 0.1 M phosphate buffered saline, 100 μM ascorbic acid, 1 μM Cu (II), and test solutions (phosphate buffer for control, 0.1 and 1 mM carnosine, 1 mM L-histidine, and 1 mM β-alanine). The experiment was performed at room temperature.

**Statistical analysis**

Data were analyzed using the SAS (1985) program on triplicate samples with two replications. The least significant difference procedure was used to compare significant differences at level of p≤0.05 between the means of treatment groups. ANOVA tables (B.1-B.8) are shown in appendix B.
RESULTS & DISCUSSION

Meat pH, cook yield, TBA value, and salt soluble protein

Carnosine is known to have buffering capacity in skeletal muscle (Harris et al., 1990). Treatment with carnosine increased meat pH, but ascorbic acid decreased meat pH through day 3 (Table 6-1). The combination of carnosine and ascorbic acid counteracted the drop of meat pH seen with ascorbic acid alone. Carnosine actually increased meat pH regardless of ascorbic acid level, in part due to the use of the higher level of carnosine (1%) than of ascorbic acid (0.1%). Carnosine significantly increased cook yield, but ascorbic acid had no effect (Table 6-1). The increase in meat pH by carnosine or the combinations of carnosine and ascorbic acid probably accounts for the observed increase in cook yield. There is a clear tendency for the water-holding capacity to increase with increasing meat pH (Thomsen and Zeuthen, 1988).

Carnosine treatment significantly increased salt-soluble protein level, while ascorbic acid significantly decreased salt-soluble protein in meat (Table 6-1). The combination of carnosine and ascorbic acid counteracted the effect of ascorbic acid. Carnosine (1%) + ascorbic acid (0.05%) showed highest value for the salt-soluble protein. The higher salt-soluble protein values in ground beef can also be partly explained by the increased meat pH (Bernthal et al., 1991). Honikel et al. (1981) reported that as pH values are increased away from the isoelectric pH of proteins, there is an increased water-holding capacity and protein solubility.

TBARS formation in the control ground beef was storage time-dependent at 4 °C (Table 6-1). Treatment with carnosine and/or ascorbic acid significantly inhibited TBARS formation in ground beef, regardless of storage time (p<0.05). The inhibition of
Table 6-1—Effect of carnosine and ascorbic acid on pH, cook yield, salt soluble protein, and TBA number in ground beef

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Cook yield (%)</td>
</tr>
<tr>
<td>Control (deionized water)</td>
<td>5.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>63.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carnosine (CAR), 1%</td>
<td>6.31&lt;sup&gt;b&lt;/sup&gt;</td>
<td>68.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ascorbic acid (AA), 0.1%</td>
<td>5.51&lt;sup&gt;c&lt;/sup&gt;</td>
<td>63.8&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAR (1%) + AA (0.1%)</td>
<td>6.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAR (1%) + AA (0.05%)</td>
<td>6.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>67.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAR (0.5%) + AA (0.1%)</td>
<td>6.07&lt;sup&gt;d&lt;/sup&gt;</td>
<td>65.1&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAR (0.5%) + AA (0.05%)</td>
<td>6.08&lt;sup&gt;d&lt;/sup&gt;</td>
<td>64.8&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>abcdef</sup> Means in each column with different superscripts are significantly different (p<0.05).

SSP: salt soluble protein, TBA No; TBA number.
lipid peroxidation by carnosine was stronger than by ascorbic acid (p<0.05). The inhibitory effects of carnosine and ascorbic acid on lipid peroxidation might be due to scavenging free radicals, chelating transition metals, or changing redox potential of the transition metals (Brown, 1981; Chan et al., 1994; Lee and Hendricks, 1997a; 1998; Niki, 1991).

Lee et al. (1998) reported that carnosine (0.1-5 mM) treatment to pre-rigor beef significantly inhibited lipid peroxidation during 9 days storage. Decker and Crum (1991) also reported that carnosine (0.5 and 1.5%) more effectively inhibited lipid peroxidation in frozen pork stored up to 6 mo, compared with sodium triphosphate and other lipid-soluble antioxidants such as alpha-tocopherol and butylated hydroxytoluene. Meanwhile, antioxidant activity of ascorbic acid may depend on its concentration in a model system. Mitsumoto et al. (1991a) reported that treatment with 500 ppm ascorbic acid to ground beef decreased lipid peroxidation. However, Benedict et al. (1975) reported 50 ppm ascorbic acid addition to ground beef caused increased lipid peroxidation. We also showed that the antioxidant or prooxidant activity of ascorbic acid depended on the concentration of ascorbic acid and Fe (III) in a linoleic acid micelle system (Lee and Hendricks, 1997b). Ascorbic acid showed an antioxidant activity at high concentrations, while it showed a prooxidant activity at low concentrations. The ratio of Fe (II) to Fe (III) may be very important in the initiation or propagation of lipid peroxidation (Miller and Aust, 1989).

**Metmyoglobin and color change during storage**

Ground meat tends to become brown and rancid more rapidly than whole muscle retail cuts. In this study, ground beef was blended with the test solutions. These
processes not only increased air contact, but also may accelerate loss of intracellular reductants, thereby resulting in higher metmyoglobin formation. However, treatment with carnosine and/or ascorbic acid inhibited (p<0.05) metmyoglobin formation (Table 6-2). Ascorbic acid (0.1%) was more effective for inhibition of metmyoglobin formation than 1% carnosine (p<0.05). The inhibitory effect by a combination of carnosine and ascorbic acid was stronger than carnosine or ascorbic acid alone after storage for 1 day.

Treatments (control, carnosine, ascorbic acid) and exposure to air had a significant effect on sample color during refrigerated storage of beef samples. The ground beef control stored in a petri dish maintained reasonably good red color through 3 days storage at 4°C, on the top surface, exposed to air. However, when viewed through the bottom of the petri plate, where air contact was limited, the control samples were very brown after even 1 day storage, as would be expected, since it is well known that metmyoglobin formation occurs most rapidly at low oxygen tension (George and Stratman, 1952).

Table 6-2—Effect of carnosine and ascorbic acid on % metmyoglobin in ground beef

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (deionized water)</td>
<td>47.7 ± 1.5a</td>
<td>55.0 ± 1.2a</td>
</tr>
<tr>
<td>Carnosine (CAR), 1%</td>
<td>30.5 ± 1.3b</td>
<td>34.6 ± 1.0b</td>
</tr>
<tr>
<td>Ascorbic acid (AA), 0.1%</td>
<td>26.7 ± 0.5c</td>
<td>32.2 ± 1.3c</td>
</tr>
<tr>
<td>CAR (1%) + AA (0.1%)</td>
<td>22.8 ± 0.4d</td>
<td>32.9 ± 0.6c</td>
</tr>
<tr>
<td>CAR (1%) + AA (0.05%)</td>
<td>24.7 ± 0.4e</td>
<td>32.3 ± 0.9c</td>
</tr>
<tr>
<td>CAR (0.5%) + AA (0.1%)</td>
<td>24.5 ± 1.1e</td>
<td>35.6 ± 1.5b</td>
</tr>
<tr>
<td>CAR (0.5%) + AA (0.05%)</td>
<td>24.6 ± 0.5f</td>
<td>35.7 ± 0.7b</td>
</tr>
</tbody>
</table>

*abcde* Means in each column with different superscripts are significantly different (p<0.05).
The control samples had the highest % metmyoglobin of all samples at both 1 and 3 days storage (Table 6-2) and low values for Hunter color ‘a’ or redness (Table 6-3 & 6-4). After 1 day storage, samples treated with 0.1% ascorbic acid had the most bright red surface color, with highest ‘a’ values, whereas treatment with 1% carnosine resulted in a more purplish red color (Table 6-3). Samples treated with carnosine also maintained a reddish color and higher ‘a’ values on the bottom surface (Table 6-4), in contrast to controls or samples treated with ascorbate, which turned brown (Table 6-4). There was no increase in surface redness values of samples containing both ascorbate and carnosine compared to ascorbate alone (Table 6-3). Neither were there any synergistic effects of ascorbate + carnosine for the prevention of browning on the bottom surface of stored samples (Table 6-4).

Table 6-3—Effect of carnosine and ascorbic acid on Hunter color values of top (aerobic) side of ground beef stored in a petri dish

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L  a  b</td>
<td>L  a  b</td>
</tr>
<tr>
<td>Control (deionized water)</td>
<td>35.7\textsuperscript{a} 13.9\textsuperscript{a} 9.3\textsuperscript{a}</td>
<td>37.3\textsuperscript{a} 8.1\textsuperscript{a} 8.7\textsuperscript{a}</td>
</tr>
<tr>
<td>Carnosine (CAR), 1%</td>
<td>30.7\textsuperscript{b} 14.0\textsuperscript{a} 8.0\textsuperscript{b}</td>
<td>32.0\textsuperscript{b} 9.9\textsuperscript{b} 7.4\textsuperscript{b}</td>
</tr>
<tr>
<td>Ascorbic acid (AA), 0.1%</td>
<td>36.4\textsuperscript{a} 17.8\textsuperscript{b} 10.4\textsuperscript{c}</td>
<td>35.6\textsuperscript{c} 10.9\textsuperscript{c} 8.9\textsuperscript{a}</td>
</tr>
<tr>
<td>CAR (1%) + AA (0.1%)</td>
<td>31.6\textsuperscript{b} 15.5\textsuperscript{c} 9.0\textsuperscript{a}</td>
<td>30.1\textsuperscript{d} 11.2\textsuperscript{c} 7.5\textsuperscript{b}</td>
</tr>
<tr>
<td>CAR (1%) + AA (0.05%)</td>
<td>31.0\textsuperscript{b} 15.8\textsuperscript{c} 8.7\textsuperscript{a}</td>
<td>30.9\textsuperscript{d} 10.8\textsuperscript{c} 7.6\textsuperscript{b}</td>
</tr>
<tr>
<td>CAR (0.5%) + AA (0.1%)</td>
<td>33.0\textsuperscript{c} 17.0\textsuperscript{b} 9.4\textsuperscript{a}</td>
<td>32.8\textsuperscript{e} 10.6\textsuperscript{c} 8.4\textsuperscript{a}</td>
</tr>
<tr>
<td>CAR (0.5%) + AA (0.05%)</td>
<td>33.8\textsuperscript{c} 16.2\textsuperscript{c} 9.9\textsuperscript{c}</td>
<td>33.3\textsuperscript{e} 11.5\textsuperscript{d} 8.4\textsuperscript{a}</td>
</tr>
</tbody>
</table>

\(L = \) lightness, \(a = \) redness, \(b = \) yellowness.

\textsuperscript{a,b,c,d,e} Means in each column with different superscripts are significantly different (p<0.05).
However, after 1 day storage, all samples containing carnosine were more red on the bottom surface than was the case for ascorbate alone, probably in part due to the increased meat pH, which is associated with darker, reddish color (Egbert and Cornforth, 1986).

Both ascorbate and carnosine inhibited metmyoglobin formation compared to controls (Table 6-2). The brown bottom surface seen after day one for control or ascorbate-treated samples actually increased somewhat in redness by 3 days storage, perhaps indicative of some metmyoglobin reduction with increased storage time. Metmyoglobin reduction and brown to red color reversion has also been reported in vacuum-packed meats with increasing storage time, due to action of endogenous muscle reductants in the absence of oxygen (Pierson et al., 1970). The increase in redness seen

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Day 1</th>
<th>Day 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>a</td>
</tr>
<tr>
<td>Control (deionized water)</td>
<td>37.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carnosine (CAR), 1%</td>
<td>31.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ascorbic acid (AA), 0.1%</td>
<td>36.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAR (1%) + AA (0.1%)</td>
<td>31.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>10.1&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAR (1%) + AA (0.05%)</td>
<td>30.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.0&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAR (0.5%) + AA (0.1%)</td>
<td>33.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>11.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>CAR (0.5%) + AA (0.05%)</td>
<td>34.6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>10.8&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

L = lightness, a = redness, b = yellowness.

<sup>abcde</sup>Means in each column with different superscripts are significantly different (p<0.05).
on the bottom of sample from 1 to 3 days storage was not accompanied by a decrease in % metmyoglobin (Table 6-2). Metmyoglobin values reported in Table 6-2 reflect mean % metmyoglobin in a 5.0-g core from top to bottom of the sample, while the redness or ‘a’ values reported in Table 6-4 are indicative of just the very bottom surface.

Although many factors can influence meat color stability, the oxidation of myoglobin by free radicals is paramount (Renerre and Labas, 1987). Ascorbic acid may be able to function as a MetMb reductant until lipid peroxidation products or free radicals become numerous, thereby either destroying the ascorbic acid activity or overpowering ascorbic acid (Greene et al., 1971). Carnosine can act as a reductant. The ability of carnosine to donate an electron to free radicals is generally related to its antioxidant activity (Kohen et al., 1988). Our laboratory showed that carnosine can slowly but effectively reduce Cu (II) to bathocuproine-reactive Cu (I) in a time- and dose-dependent manner (Lee and Hendricks, 1998, unpublished data). The Cu (II)- reducing potential of carnosine was 1/10-1/20 that of ascorbic acid. The reducing activity of ascorbic acid or carnosine may be involved in the stabilization of meat color. Our results indicate that the effect of a combination of carnosine and ascorbic acid on meat color was more beneficial than carnosine or ascorbic acid alone.

Protection of carnosine against Cu (II)-catalyzed ascorbic acid oxidation

Ground beef used in this study contained 24.5 ± 2.1 μg/g of total iron, 16.7 ± 0.2 μg/g of heme iron, and 0.82 ± 0.05 μg/g copper. We quantified concentration of ascorbic acid in meat using the dye 2,6-dichlorophenol indophenol. The ascorbic acid content of the control meat was negligible (< 0.001 mg/g), but 0.1% ascorbic acid-treated meats contained approximately 0.11 - 0.15 mg ascorbic acid/g sample after storage for 3 days.
One percent carnosine + 0.1% ascorbic acid-treated meats contained approximately 0.20 - 0.25 mg ascorbic acid /g sample. This result indicates that carnosine may inhibit ascorbic acid oxidation catalyzed by transition metals such as iron and copper. Cu (II) is a well-known strong catalyst for ascorbic acid oxidation; therefore, we used in vitro model systems to investigate the possible protective effect of carnosine against Cu (II)-catalyzed ascorbic acid oxidation.

In the presence of 10 μM cupric ion, the concentration of dissolved oxygen rapidly decreased in the reaction mixtures, but carnosine strongly inhibited oxygen consumption associated with ascorbate oxidation (Table 6-5). The indophenol method was also used to determine the protective effect of carnosine against ascorbate oxidation

<table>
<thead>
<tr>
<th>Carnosine (mM)</th>
<th>Oxygen uptake rate (nmole/min)</th>
<th>Inhibition (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 (control)</td>
<td>21.0 ± 3.0</td>
<td>-</td>
</tr>
<tr>
<td>0.05</td>
<td>15.4 ± 0.4</td>
<td>26.7</td>
</tr>
<tr>
<td>0.1</td>
<td>9.6 ± 0.6</td>
<td>54.3</td>
</tr>
<tr>
<td>1.0</td>
<td>5.4 ± 1.0</td>
<td>74.3</td>
</tr>
<tr>
<td>2.5</td>
<td>5.8 ± 0.2</td>
<td>72.4</td>
</tr>
<tr>
<td>5.0</td>
<td>7.2 ± 0.3</td>
<td>65.7</td>
</tr>
<tr>
<td>10.0</td>
<td>8.8 ± 0.4</td>
<td>58.1</td>
</tr>
</tbody>
</table>

Data represent mean ± SE of three determinations.

$^a$Inhibition (%) = \frac{\text{Oxygen uptake (control) - Oxygen uptake (carnosine)}}{\text{Oxygen uptake (control)}} \times 100
in reaction mixtures including 10 μM Cu (II) and 100 μM ascorbic acid (Fig. 6-1). The higher the concentration of reduced ascorbic acid, the lighter the color in the reaction with indophenol. In this study, blank (buffer only) contained no ascorbic acid, resulting in pink (red) color (Curve 1). Ascorbic acid (100 μM) reduced indophenol, accompanied by loss of the color (slightly pink color, Curve 4). The addition of 10 μM Cu (II) in the presence of 100 μM ascorbic acid increased absorbance at 520 nm, which indicated oxidation of ascorbic acid (Curve 2). The oxidation of ascorbic acid increased with increasing pH (4.5 to 9.0) of reaction mixtures. Carnosine (1 mM) effectively inhibited the ascorbic acid oxidation regardless of the pH levels (Curve 3).

Fig. 6-1—Effect of 1 mM L-carnosine (CAR) on oxidation of ascorbic acid (AA) at various pHs. Data represents mean of three determinations. 1: buffer only (no AA), 2: 10 μM Cu (II) + 100 μM ascorbic acid, 3: 10 μM Cu (II) + 100 μM ascorbic acid + 1 mM carnosine, 4: 100 μM ascorbic acid only (no copper).
In addition, when ascorbic acid concentrations were monitored by UV absorbance at 265 nm, ascorbic acid was completely oxidized within 25 min in the presence of 1 μM Cu (II) (Fig. 6-2). Carnosine (1 mM) inhibited the Cu (II)-catalyzed ascorbate oxidation by 65%. L-histidine (1 mM) also strongly inhibited the Cu (II)-catalyzed ascorbic acid by 70%, but β-alanine did not inhibit the reaction (Fig. 6-2). This result suggests that the inhibitory effect by carnosine on the Cu (II)-catalyzed ascorbate oxidation may be due to the chelating action of the imidazole ring of the histidine moiety of carnosine.

Fig. 6-2.—Carnosine (CAR) inhibits the Cu (II)-catalyzed oxidation of ascorbic acid. The absorbances of reaction mixtures in 0.1 M sodium phosphate buffered saline (pH 7.0), including 100 μM ascorbic acid, 1 μM Cu (II), and test solutions (buffer only for control, 0.1 and 1 mM carnosine, 1 mM L-histidine, and 1 mM β-alanine) were monitored spectrophotometrically at 265 nm. Data points represent the mean of three determinations.
These actions of carnosine in simple solutions may be different from those of actual food systems because other constituents such as proteins, amino acids, or other chelates may associate with transition metals and influence ascorbate oxidation. Carnosine is known to form three structurally different complexes with Cu (II) at physiological pH and temperature, depending on the relative concentrations of Cu (II) and carnosine (Brown, 1981). These complexes may not be catalytically inactive for free radical reactions such as ascorbate oxidation and lipid peroxidation.

Although our system using ground beef with added water is actually not practical in the meat industry, our results can be applied for production of meat products such as fresh pork sausage. Furthermore, a dietary manipulation such as histidine supplementation to farm animals can be performed to increase carnosine content of tissues (meat).

CONCLUSIONS

Both carnosine and ascorbic acid are well-known antioxidants. They can inhibit metmyoglobin formation as well as lipid peroxidation of meats by several mechanisms including their reducing, free radical scavenging, and/or chelating activities. Carnosine is superior to ascorbic acid in preventing lipid peroxidation. Ascorbic acid is superior to carnosine for lower % metmyoglobin and higher ‘a’ surface value, but carnosine is also superior to ascorbic acid for bottom surface ‘a’ value. Carnosine can also protect metal-catalyzed ascorbic acid oxidation. The combination of carnosine and ascorbic acid as food or meat additives or ingredients may be useful due to their synergistic effects to prevent off-flavor formation and increase shelf-life of meat and meat products.
REFERENCES


USDA. 1994. Ascorbic acid, erythobic acid, citric acid, sodium ascorbate, and sodium citrate on beef, lamb, and pork cuts. Federal Register 59 (52) : 12536-12538.

CHAPTER 7
EFFECT OF CARNOSINE AND PHYTATE ON WATER-HOLDING CAPACITY, COLOR, AND TBA VALUES OF A DILUTE BEEF MODEL SYSTEM

ABSTRACT

The effects of carnosine and phytate on water-holding capacity (WHC) and color of meat were investigated in ground beef. Ground beef (15 g) was soaked in 15 mL of carnosine (5 or 25 mM), phytate (5 or 25 mM), or carnosine (12.5 mM) + phytate (12.5 mM) solutions for 1 and 5 days at 4 °C (for raw ground beef). A duplicate set of these preparations was cooked at 70 ± 2 °C for 30 min in a water-bath. After filtration of the mixture through Wattman #1 filter paper, the weight change, as an indicative of water-holding capacity (WHC), was calculated in both raw and cooked ground beef. Compared with the control, phytate greatly increased WHC of raw and cooked meat, while carnosine only slightly increased WHC. There was a strong correlation of WHC between raw and cooked meat on the same storage day (1 or 5 day). Phytate strongly decreased the Hunter ‘L’ value (whiteness) while carnosine strongly increased the Hunter ‘a’ value (redness), compared with the control. Carnosine and phytate significantly inhibited metmyoglobin formation in raw beef and lipid peroxidation in both raw and cooked beef. These results indicate that carnosine and phytate may be useful as meat additives for increasing shelf-life as well as WHC of meat and meat products if they are cheaply obtained.
Water-holding capacity (WHC) of muscle is important because it affects both qualitative and quantitative aspects of meat and meat products (Honikel and Hamm, 1994; Winger and Hagyard, 1994). Fresh meat at slaughter contains about 72-75% water, while most post-rigor muscle contains about 70% water, depending primarily on lipid content and the physiological maturity of muscle (Honikel and Hamm, 1994). The water is either very tightly bound to other molecules such as proteins, is loosely associated with other charged molecules, or is in the extracellular spaces free to move into the external environment (Trout, 1988). Weight gains or losses of meat occur during processing, cooking, storage, or evaporation. Such gains or losses are important because meat is sold by weight and because consumers prefer meat with less drip when fresh and less cook loss (Winger and Hagyard, 1994).

Meat pH is strongly associated with WHC of meat (Roseiro et al., 1994). A fast drop of muscle pH increases protein denaturation and tendency of actomyosin to contract, thereby affecting the amount of fluid free to enter extracellular spaces and the meat structure and color (paleness). Meat pH also affects a change in the charge of molecules (Gault, 1985). At pH above 6.0 or below 4.0 (isoelectric point of actomyosin = 5.0) the number of available charges is enhanced, thereby increasing WHC. Addition of salt or phosphates to meats also enhances WHC (Kenney and Hunt, 1990, Lee et al., 1998b). Sodium pyrophosphate and sodium tripolyphosphate improved binding strength and cook yield in cooked beef rolls when they were added at the concentration of 0.5% (Lee et al., 1998b). The possible mechanisms of phosphates include 1) raising the pH, 2) increasing the anionic electrolytes, 3) sequestering cations, 4) raising ionic strength, and 5) dissociating actomyosin (Trout and Schmidt, 1983).
Carnosine (β-alanyl L-histidine) and phytate (myoinositol hexaphosphate) are naturally occurring antioxidants found in several mammalian tissues and plants, respectively (Crush, 1970; Reddy et al., 1989). Carnosine and phytate inhibit lipid peroxidation in several systems (Decker et al., 1992; Lee and Hendricks, 1995; 1997a, b). The antioxidant properties of carnosine and phytate may result from their ability to chelate transition metals (Brown, 1981; Graf et al., 1984) and their enzyme-like activity (Babizhayev et al., 1994; Graf and Eaton, 1990). These properties may enhance the antioxidant potential of muscle and may render carnosine and phytate useful as natural food antioxidants (Decker and Crum, 1991; Lee and Hendricks, 1997a, b). Our previous work showed that treatment with carnosine and phytate to pre-rigor beef stabilized meat color by decreasing in metmyoglobin formation (Lee et al., 1998a). Also, sodium phytate (0.5%, pH 10.4) increased cook yield in beef rolls, partially due to increased meat pH (Lee et al., 1998b).

In this study, to minimize the high pH effect of carnosine and phytate on WHC, metmyoglobin formation, and lipid peroxidation, we adjusted the pHs of carnosine and phytate solutions to the same as initial meat pH (5.8). The dilute beef model system contained 50% ground beef and 50% water to investigate the effect of carnosine and phytate on WHC of meat in the presence of a great excess of water.

**MATERIALS & METHODS**

**Materials**

L-carnosine, phytate, 2-thiobarbituric acid (TBA), and tetraethoxypropane (TEP), were purchased from Sigma Chemical Company (St. Louis, MO). Trichloroacetic acid (TCA) and sodium hydroxide were obtained from EM Science (Cherry Hill, NJ).
Potassium phosphate (monobasic anhydrous) and acetone were obtained from Mallinckrodt Inc. (Paris, KY).

Methods

Sample preparation. Ground beef (*biceps femoris*, lean, 24 h post-mortem) was obtained from the Meat Laboratory at Utah State University. The meat sample (15 g) was soaked in 15 mL test solution [control (deionized water), carnosine (5 and 25 mM), phytate (5 and 25 mM), or a mixture (12.5 mM carnosine and 12.5 mM phytate)] in a 50-mL polypropylene test tube for 1 or 5 days at 4 °C. The pH of test solutions was adjusted to 5.8 with HCl and NaOH to reduce the pH effect on WHC of muscle (meat pH at starting time was 5.8). After filtration of the mixture of meat and test solution, the meat samples were used for determination of WHC, the Hunter color value, MetMb formation, and TBARS formation. A duplicate set of the preparation (mixture of ground beef and test solution) was stored for 1 or 5 days at 4 °C, then cooked to 70 ± 2 °C (internal temperature) in a water-bath, and cooled with tap water. The cooked meat samples after filtration were used for determination of cook yield (gain or loss) and TBARS formation. Sampling and weighing were performed in a cold room (4 °C).

Water holding capacity (WHC) in raw beef. WHC [(meat weight after storage / 15 g) x 100] in raw ground beef was determined after filtering the mixture (15 g meat + 15 mL test solution) through Whatman #4 filter paper for 1 h at 4°C.

Cook yield (WHC) in cook beef. Percent cook yield [(weight after cooking/15 g weight) x 100] was used as a measure of WHC in cooked beef. A mixture containing 15 g ground beef and 15 mL of test solution in a 50-mL polypropylene test tube was placed in a boiling water-bath for 30 min (internal cooking temperature, 70 ± 2 °C). The tube
was allowed to cool with tap water, and the cooked meat sample was filtered through a Whatman #4 filter paper for 1 h at 4 °C, blotted with a paper towel, and weighed for the determination of cook yield (based on 15 g ground beef).

**pH measurement.** The pH of ground meat samples or filtrates was directly measured using a glass pH electrode (Orion Research Inc., Boston, MA).

**Moisture content.** Moisture content was determined as weight loss after samples were dried in a convection oven at 100 °C for 16 h (AOAC, 1990).

**TBA number.** Thiobarbituric acid reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). Samples (0.5 g) were mixed with 2.5 mL of 0.375% TBA-15% TCA-0.25 N HCl stock solution. The mixture was heated for 10 min in a boiling water-bath (95-100 °C) to develop a pink color, cooled with tap water, and centrifuged at 5,500 rpm for 25 min in a Beckman centrifuge (Model J-21C, Palo Alto, CA). The supernatant was measured spectrophotometrically at 532 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). TBARS were calculated from a standard curve of malondialdehyde (MDA), a breakdown product of TEP used in preparation of the standard curve. TBA number was calculated as mg MDA/kg sample.

**Metmyoglobin.** Ground beef (5 g) was placed in a 50-mL polypropylene centrifuge tube, and 25 mL ice-cold phosphate buffer (pH 6.8, 40 mM) was added. The mixture was homogenized for 10 s at 13,500 rpm with an Ultra-Turrax T25 (Janke & Kunkel GmbH, Staufen, Germany). The homogenized sample was allowed to stand for 1 h at 4 °C, then centrifuged at 5,000 rpm for 30 min at 4 °C. The supernatant was filtered through Whatman #1 filter paper, and the absorbance was read at 700, 572, and 525 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). Percent metmyoglobin was determined using the formula by Krzywicki (1982):
% MetMb = \{1.395 - [(A572 - A700) / (A525 - A700)]\} \times 100

**Hunter color measurements.** Meat color was determined instrumentally using a Hunter Lab Digital Color Difference Meter D25D2A (Hunter Associates Laboratories, Inc., Reston, VA). The instrument was standardized with a red plate; L=25.9, a=27.4, b=13.1. After filtration of the mixture containing 15 g beef and 15 mL test solution, the meat was transferred into a petri dish. Hunter readings were taken directly read on the meat sample in the petri dish.

**Statistical analysis**

Data were analyzed using SAS (1985) program on triplicate samples with two replications. If overall F-test was significant, the least significant difference procedure was used to determine significant differences at the level of p<0.05 between the means of treatment groups. ANOVA tables (C.1-C.3) are shown in appendix C.

**RESULTS & DISCUSSION**

**Water-holding capacity**

**In raw ground beef.** Although the pH of test solutions was initially adjusted to 5.8, the pH of the mixture of 15 g beef and 15 mL test solution decreased to 5.58 - 5.65 after storage for 1 day at 4 °C, perhaps due to the further breakdown of ATP and glycogen (Table 7-1). The pH of the mixture was 5.66 - 5.79 after storage for 5 days at 4 °C. Hamm (1986) reported a decrease in pH of beef up to 1.5 days after slaughter, then a steady increase through 13 days. Lee et al. (1998b) reported that treatment of post-rigor beef with 0.5% sodium phytate (4.5 mM) without adjustment of pH increased meat pH by 0.6 unit. In this study, carnosine (25 mM) and phytate (25 mM) seemed to have a
buffering effect, thereby inhibiting the drop of the meat pH, even after pH adjustment of test solutions (Table 7-1).

The weight gain of meat after filtration of the mixture was evident among all treatments including the control (deionized water), compared with untreated ground beef. At day 1, the weight gain of the control beef was 3.45 g, corresponding to 23% increase of meat weight (Table 7-1). Kenney and Hunt (1990) reported that soluble protein and water retention after centrifugation increased as preblend water content increased from 0 to 80% of the formulation water. Compared with the untreated ground beef, carnosine (5 and 25 mM) treatments increased the meat weight by 25.4% and 26.1%, respectively, only slightly more than the control (23.0%). Phytate (5 and 25 mM) greatly increased the weight of meat by 29.8% and 50.8%, respectively, indicating a strong WHC of beef with phytate. Carnosine (12.5 mM) + phytate (12.5 mM) had an intermediate weight gain between 5 mM and 25 mM of phytate. The weight gain of meat after 5 days storage was increased by about 3% more in the control and carnosine groups, and 5.4-12.6% more in phytate groups than those after 1 day storage, perhaps partially resulting from an increase in meat pH (Table 7-1). Ockerman and Crespo (1982) reported that WHC of lean beef preblends containing 20% water increased as storage time increased. Our data confirm their findings. The weight gain of meat was dependent on the concentration of phytate. The slight increase of weight gain in the carnosine group, compared with the control, might reflect slightly higher meat pH than the control because there is a clear tendency for the WHC to increase with increasing pH of meat (Thomsen and Zeuthen, 1988). A low pH may cause denaturation of meat proteins, thereby resulting in a lower WHC. However, phytate evidently enhanced WHC of the meat in our study.
Table 7-1—Effect of carnosine (CAR) and phytate (PA) on pH, water-holding capacity (WHC), and moisture of raw ground beef

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>WHC (%)</td>
</tr>
<tr>
<td>Untreated ground beef (B)</td>
<td>5.60 b</td>
<td>100.0 b</td>
</tr>
<tr>
<td>+ Water (control)</td>
<td>5.58 b</td>
<td>123.0 c</td>
</tr>
<tr>
<td>+ CAR (5 mM)</td>
<td>5.60 b</td>
<td>125.4 d</td>
</tr>
<tr>
<td>+ CAR (25 mM)</td>
<td>5.65 c</td>
<td>126.1 d</td>
</tr>
<tr>
<td>+ PA (5 mM)</td>
<td>5.61 b</td>
<td>129.8 e</td>
</tr>
<tr>
<td>+ PA (25 mM)</td>
<td>5.63 c</td>
<td>150.8 f</td>
</tr>
<tr>
<td>+ CAR (12.5 mM) + PA (12.5 mM)</td>
<td>5.65 c</td>
<td>135.7 g</td>
</tr>
</tbody>
</table>

*a* Ground beef (15 g) was placed in 15 mL of test solution for 1 or 5 days at 4°C. After filtration for 1 h at 4°C, the weight of beef was measured. WHC (%) = \[ \text{weight (g) of beef after filtration} / 15 \text{ g} \] \times 100

bcdefg Means in the same column with different superscripts are significantly different (p<0.05).
The moisture content directly reflected the WHC of muscle. Untreated ground beef contained 73.4% (11.01 g) moisture and 26.7% (3.99 g) solids \[15 \text{ g} \times 0.734 = 11.01 \text{ g} \text{ (water)}, \ 15 \text{ g} - 11.01 \text{ g} = 3.99 \text{ g} \text{ (solids)}\]. Control ground beef treated with deionized water contained 81.0% moisture (Table 7-1). Theoretically, the control meat should have contained 14.46 g water (18.45 g total weight - 3.99 g solids weight), corresponding to a 78.3% moisture \[14.46 / 18.45 \times 100\). The actual moisture value of the control beef in this study was higher than the theoretical moisture value. This result indicates that the solids of the control might be extracted into filtrates. In contrast, the ground beef treated with 25 mM phytate theoretically contains 18.63 g \[22.62 - 3.99\) water, corresponding to 82.4% \[18.63 / 22.62 \times 100\) moisture, but the actual moisture value of the beef in this study was 82.8%, indicating that the loss of solids in the ground beef treated with 25 mM phytate was negligible.

**In cooked ground beef.** Cooking increased the meat pH (Table 7-2), compared with raw beef (Table 7-1). Treatment of ground beef with carnosine (12.5 mM) + phytate (12.5 mM) increased the meat pH after cooking, compared with the control (Table 7-2). However, 25 mM phytate slightly but significantly decreased the meat pH after cooking at day 5. Untreated ground beef showed 32-33% cook loss, but ground beef treated with water showed 0.6% cook gain at day 1. Carnosine showed a slightly higher cook yield than the control (Table 7-2), and 25 mM phytate significantly increased cook yield, regardless of the storage time (Table 7-2). The cook yield at day 1 was much higher than that at day 5, perhaps resulting from loss of solid matter after 5 days storage.

The moisture of the cooked beef was correlated to the cook yield of meat. At day 1, the weight of the control was 15.09 g, and the moisture value was 76.3% (Table 7-2).
Table 7-2—Effect of carnosine (CAR) and phytate (PA) on pH, cook yield, and moisture of cooked ground beef

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH</td>
<td>Cook yield (%)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Untreated ground beef (B)</td>
<td>5.83&lt;sup&gt;b&lt;/sup&gt;</td>
<td>66.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ Water (control)</td>
<td>5.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>100.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ CAR (5 mM)</td>
<td>5.85&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>102.2&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ CAR (25 mM)</td>
<td>5.86&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>101.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ PA (5 mM)</td>
<td>5.82&lt;sup&gt;b&lt;/sup&gt;</td>
<td>99.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ PA (25 mM)</td>
<td>5.81&lt;sup&gt;b&lt;/sup&gt;</td>
<td>119.4&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ CAR (12.5 mM) + PA (12.5 mM)</td>
<td>5.90&lt;sup&gt;c&lt;/sup&gt;</td>
<td>110.4&lt;sup&gt;f&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ground beef (15 g) was placed in 15 mL of test solution for 1 or 5 days at 4 °C, then cooked at 70 ± 2 °C for 30 min in a water bath. After filtration for 1 h at 4 °C, the weight of the cooked beef was measured.

<sup>b</sup>Cook yield (%) = [weight (g) of cooked beef after filtration / 15 g] x 100.

<sup>bcdef</sup> Means in each column with different superscripts are significantly different (p<0.05).
Theoretically, the control should have contained 11.1 g water (15.09 g - 3.99 g), corresponding to 73.6% moisture (11.1 / 15.09 x 100). In the 25 mM phytate group, the measured moisture level was 77.8%, and the theoretical value was 77.7% (13.93 / 17.92 x 100). During the cooking process, components other than moisture, such as protein and fat, are degraded, solubilized, and lost in the drip (Hamm, 1986). Our results indicate that phytate may protect the solids against solubilization.

Changes in the WHC are very sensitive indicators of changes in the charge and structure of myofibrillar proteins. A loosening of the microstructure and, consequently, an increase of immobilized water is caused by raising the protein net charge. Increasing the electrostatic charge on myofibrillar protein increases the repulsive force and causes swelling (Hamm, 1986). Phytate may affect the protein net charge or structure by binding to the proteins.

Phytate has six phosphate groups which can be negatively charged (Graf and Eaton, 1990). In acidic conditions such as in post-mortem meat, phytate can bind to basic amino groups of proteins, thereby changing net charge of proteins. Thus, the protein-phytate complex may increase the net negative charge of proteins, which is strongly associated with the WHC of meat.

Shrinkage of meat on cooking was significantly influenced by the extent of raw meat swelling (Gault, 1985). In this study, the highest cooking losses were associated with the lowest raw meat swelling. There was a strong correlation of WHC between raw and cooked meat (Fig. 7-1). The correlation coefficient ($R^2$) between WHC in raw versus cooked meat was 0.90 after 1 day storage, and 0.94 after 5 days storage. Similarly, Boakye and Mittal (1993) reported that there is a positive correlation between press juice and cooking loss.
Fig. 7-1—The correlation of WHC in raw meat with WHC (cook yield) in cooked meat. The correlation coefficient ($R^2$) was 0.902 at day 1 and 0.944 at day 5.

**Meat color and MetMb formation**

Raw meat after filtration was used for the Hunter color determination. Phytate (25 mM) significantly lowered ‘L’ (lightness) values compared with the control after storage for 1 or 5 days at 4 °C (Table 7-3). Carnosine (5 and 25 mM) significantly increased ‘a’ (redness) values after storage for 1 or 5 days. Carnosine and phytate also significantly lowered ‘b’ (yellowness) values at day 5. In this study, the ‘L’ values of meat were negatively related to the WHC of beef ($R^2 = 0.989$ at day 1, $R^2 = 0.943$ at day 5), while the ‘a’ values were negatively related to the percent metmyoglobin in meat ($R^2 = 0.949$).
Table 7-3—Effect of carnosine (CAR) and phytate (PA) on the Hunter color values and metmyoglobin formation in ground beef

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Day 1</th>
<th>Day 5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>a</td>
</tr>
<tr>
<td>Untreated ground beef (B)</td>
<td>28.3</td>
<td>15.5</td>
</tr>
<tr>
<td>+ Water (control)</td>
<td>41.0</td>
<td>16.5</td>
</tr>
<tr>
<td>+ CAR (5 mM)</td>
<td>40.2</td>
<td>18.6</td>
</tr>
<tr>
<td>+ CAR (25 mM)</td>
<td>39.3</td>
<td>19.8</td>
</tr>
<tr>
<td>+ PA (5 mM)</td>
<td>38.7</td>
<td>16.7</td>
</tr>
<tr>
<td>+ PA (25 mM)</td>
<td>33.0</td>
<td>17.6</td>
</tr>
<tr>
<td>+ CAR (12.5 mM) + PA (12.5 mM)</td>
<td>36.9</td>
<td>18.6</td>
</tr>
</tbody>
</table>

L = whiteness, a = redness, b = yellowness.

Means in each column with different superscripts are significantly different (p<0.05).
for day 1 and day 5). The discoloration of meat (high 'L', low 'a', and high 'b' values) might be due to the extraction of myoglobin into the filtrate solution. Carnosine effectively inhibited metmyoglobin formation in meat during storage for 5 days at 4 °C (Table 7-3). Phytate (25 mM) also significantly inhibited the metmyoglobin formation in meat, but not as well as carnosine (Table 7-3). The color stability of meat is important for shelf-life. Biological factors such as fiber type and metmyoglobin reductase activity may affect meat discoloration (Reddy and Carpenter, 1991). Also, the process of myoglobin oxidation by free radicals and/or lipid peroxidation products is important (Renerre and Labas, 1987). We reported that treatment with carnosine or phytate to pre-rigor beef stabilized meat color by inhibiting metmyoglobin formation and lipid peroxidation (Lee et al., 1998a). In this study, we used post-rigor beef and a dilute beef model in the presence of excess water. The color stabilizing effect of carnosine and phytate in both studies might be due to their antioxidant activities (Lee and Hendricks, 1995, 1997a, b).

In addition, carnosine can act as a reductant (Kohen et al., 1988). Boldyrev et al. (1993) proposed that carnosine forms a bi-cyclic ring chain tautomeric structure, resulting in release of hydrogen atoms. The tautomeric carnosine possesses strong biological activity as a quencher of free radicals and as a reducing agent. Our laboratory also showed that carnosine slowly but effectively reduced Cu (II) to bathocuproine-reactive Cu (I) in a time- and dose-dependent manner (unpublished data). The reducing potential of carnosine was 5-10% reducing potential of the ascorbic acid. Decker et al. (1995) reported that carnosine affected oxidation and reduction of myoglobin dependent on the pH of the reaction mixture. Carnosine (> 25 mM) accelerated the conversion of metmyoglobin to oxymyoglobin at pH>7.0. However, carnosine (1-50 mM) also accelerated the metmyoglobin formation at lower pHs (<7.0) in a dose-dependent
manner. In our model systems, carnosine inhibited metmyoglobin formation when added to both pre-rigor beef (Lee et al., 1998a) and post-rigor beef in this study.

**Lipid peroxidation**

All treatment groups including the control showed much lower TBARS formation than untreated ground beef (Table 7-4). This may be due in part to a simple dilution of the meat with 50% water or a decrease in oxygen dissolved in water solution. Carnosine and phytate significantly inhibited lipid peroxidation in both raw and cooked meat (Table 7-4). Cooking significantly increased TBARS formation in this model system (Table 7-4), probably due to an increase in iron release from heme proteins. Lee et al. (1998a) reported that phytate and carnosine inhibited degradation of heme pigments

<table>
<thead>
<tr>
<th>Table 7-4—Effect of carnosine (CAR) and phytate (PA) on TBA number in raw and cooked beef samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Untreated ground beef (B)</td>
</tr>
<tr>
<td>B (15 g) + Water (15 mL)</td>
</tr>
<tr>
<td>+ CAR (5 mM)</td>
</tr>
<tr>
<td>+ CAR (25 mM)</td>
</tr>
<tr>
<td>+ PA (5 mM)</td>
</tr>
<tr>
<td>+ PA (25 mM)</td>
</tr>
<tr>
<td>+ CAR (12.5 mM)</td>
</tr>
<tr>
<td>+ PA (12.5mM)</td>
</tr>
</tbody>
</table>

*abcdedf* Means in each column with different superscripts are significantly different (p<0.05).
during cooking. There was a negative relationship between heme iron content and lipid peroxidation in a beef model system. Phytate more strongly inhibited lipid peroxidation in cooked beef than did carnosine (Table 7-4).

In cooked meat, free or low molecular weight iron is the most important for the lipid peroxidation (Lee et al, 1998a). Phytate can chelate transition metal ions and stimulate oxidation of ferrous to ferric ion, thereby inhibiting metal-catalyzed lipid peroxidation or free radical formation from the Fenton reaction (Graf and Eaton, 1990; Lee and Hendricks, 1995; 1997b). Carnosine can chelate transition metals and it can also trap free radicals such as superoxide anions, hydroxyl radicals, and peroxyl radicals (Babizhayev et al., 1994; Brown, 1981; Chan et al., 1994; Kohen et al., 1988). These actions of carnosine and phytate may be involved in the inhibition of lipid peroxidation in this beef model system.

Lee and Hendricks (1995; 1997a, b; 1998a, b) investigated antioxidant properties of carnosine and phytate using several model systems such as liposomes, micelles, deoxyribose, and beef homogenate as well as beef. In all the systems, carnosine and phytate strongly inhibited lipid peroxidation. Decker and Faraji (1990) also reported that carnosine (25 mM) inhibited the catalysis of liposomal lipid peroxidation by iron, hemoglobin, lipoxidase, and singlet oxygen from 35 - 96%, suggesting that the antioxidant mechanism of carnosine was not solely due to metal chelation. Decker and Crum (1991) reported that carnosine (0.5 and 1.5%) effectively inhibits TBARS formation in frozen pork stored up to 6 mo. Moreover, carnosine was more effective than sodium triphosphates and other lipid-soluble antioxidants such as α-tocopherol and butylated hydroxytoluene.
CONCLUSIONS

Phytate greatly enhanced WHC of meat in our model system, but was not due to simply increased meat pH since systems with added phytate were adjusted to the same level as controls. Carnosine and phytate inhibited metmyoglobin formation and lipid peroxidation, thereby stabilizing meat color. Carnosine was more effective for preserving 'a' value and phytate was more effective for preserving 'L' value. These properties of carnosine and phytate can be applied as meat additives for the meat industry. If there is a cost problem for their use in the meat industry, several possible approaches can be made, such as increasing content of carnosine in tissues by dietary supplement of histidine to farm animals or using phytate-rich plants as meat additives.

REFERENCES


CHAPTER 8

EFFECT OF SODIUM PHYTATE, SODIUM PYROPHOSPHATE AND SODIUM TRIPOLYPHOSPHATE ON PHYSICO-CHEMICAL CHARACTERISTICS OF RESTRUCTURED BEEF

ABSTRACT

The effects of 0.5% sodium phytate (SPT), sodium pyrophosphate (SPP), and sodium tripolyphosphate (STPP), along with 1% NaCl, on physico-chemical properties of restructured raw and cooked beef were evaluated. In raw beef stored for 1 day at 4°C, the SPT, SPP, and STPP increased pH and salt-soluble protein level and decreased %MetMb and thiobarbituric acid reactive substances (TBARS), compared to the control with salt alone (p<0.05). In cooked beef, SPT, SPP, and STPP increased bind strength, cook yield, moisture level, and pH, and decreased TBARS (p<0.05). SPP and STPP increased orthophosphate in both raw and cooked beef (p<0.05), compared to the SPT and control. SPT, SPP, and STPP decreased the Hunter color L and b values and increased a value in raw beef (p<0.05) but had no effect on the Hunter color values in cooked beef. The binding value of SPP and STPP were similar over time, and the time to reach maximum binding strength was 10 s longer than SPT and 25 s longer than the control. These results indicate that SPT compares favorably with traditional phosphates for increasing bind strength and cooked yield, but SPT was slightly more effective than other phosphates for reducing TBARS 1 day after cooking.

INTRODUCTION

The use of inorganic, alkaline sodium phosphates in processed meat products of beef, pork, and chicken has been increasing due to their beneficial effects in improving the functionality, palatability, and storage stability of meat (Knipe et al., 1985; Anjaneyulu et al., 1989; Bernthal et al., 1991; Moiseev and Cornforth, 1997). Polyphosphates or their blends are incorporated in sausages and restructured meat products to enhance their quality in respect to water-holding capacity (Knipe et al., 1985; Anjaneyulu et al., 1989; Bernthal et al., 1991), meat particle-particle binding (Dobson et al., 1993; Moiseev and Cornforth, 1997), emulsion stability (Knipe et al., 1985), cook yield (Anjaneyulu et al., 1989; Moiseev and Cornforth, 1997), and color, flavor and texture (Smith et al., 1973; Young et al., 1987).

In addition, the utilization of condensed phosphates lowers the concentration of NaCl required to obtain beneficial functional characteristics. Offer and Trinick (1983) showed that 10 mM sodium pyrophosphate (SPP) halved the concentration of NaCl needed to elicit swelling and water uptake in isolated myofibrils. Neer and Mandigo (1977) reported a synergistic effect of NaCl and sodium tripolyphosphate (STPP) on smokehouse yields in flaked, cured pork products.

Phytate (myoinositol hexaphosphate) is a natural antioxidant constituting 1-5% of most cereals, nuts, legumes, oil seeds, pollen, and spores (Reddy et al., 1989). Harland and Peterson (1978) suggested that the average American consumes about 750 mg phytate per day. Cereal may be the major source of dietary phytate for American men consuming an omnivorous diet. Phytate can tightly bind to metal ions, and the metal phytate complexes are highly insoluble over a wide pH range (Graf and Eaton, 1990). Although phytate has antinutritional properties with respect to mineral absorption
(Harland and Morris, 1995), a few studies have suggested that phytate has no inhibitory effect (Hunter, 1981; Graf and Eaton, 1984) or may even enhance mineral absorption in certain circumstances (Kim et al., 1993). Furthermore, several animal and epidemiological studies indicate that phytate has an anticarcinogenic effect on mammary and colon carcinogenesis (Shamsuddin et al., 1988; Nelson et al., 1989; Pretlow et al., 1992; Graf and Eaton, 1993; Vucenik et al., 1992). Phytate also decreases kidney stones, lowers blood cholesterol, and improves glycemic index in humans (Kaufman, 1986; Yoon et al., 1983). Phytate inhibits iron-driven hydroxyl radical formation and lipid peroxidation (Graf et al., 1984; Lee and Hendricks, 1995). Graf and Eaton (1985) proposed that inhibition of intracolonic hydroxyl radical generation via the chelation of reactive iron by phytate may help explain the suppression of colonic carcinogenesis and other inflammatory bowel diseases by diets rich in phytate.

In addition, the lower phosphorylated forms of inositol such as inositol tri- or tetraphosphate may play an important role in the signal transduction mechanism by regulating a variety of cellular processes including proliferation (Michell, 1986). Therefore, Shamsuddin et al. (1989) proposed that inositol phosphates may prevent colon cancer and immunosuppressive disease by modulating cellular proliferation and differentiation. Phytase and phosphatase in foods and in the intestine dephosphorylate inositol hexaphosphate to the lower phosphorylated forms (Shamsuddin et al., 1988). Commercial phytate also contains lower phosphorylated inositol (Shamsuddin et al., 1988).

Although phytate, a natural phosphate, is known to be an antioxidant, no data are available on the possible use of phytate to increase bind strength or cooked yield of cooked meats. In this study we evaluated the effects of sodium phytate (SPT) on
physicochemical properties of meat and meat products, compared with traditional phosphates (SPP and STPP), which have been widely used in the meat industry.

MATERIALS AND METHODS

Materials

Sodium phytate, ascorbic acid, 2-thiobarbituric acid (TBA), Folin & Ciocalteu’s phenol reagent, and tetraethoxypropane (TEP) were obtained from Sigma Chemical Co. (St. Louis, MO). Sodium pyrophosphate was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). Sodium tripolyphosphate and cupric sulfate were purchased from Fisher Scientific Co. (Fair Lawn, NJ). Trichloroacetic acid (TCA), sodium hydroxide, potassium phosphate (monobasic anhydrous), sulfuric acid, and ammonium molybdate were obtained from Mallinckrodt Inc. (Paris, KY).

Methods

Experimental design

Beef rolls (2.3 kg, from EA Miller Co.) were prepared with 3 different added phosphates and a control as follows: (a) 1% salt (control), (b) 1% salt + 0.5% sodium phytate, (c) 1% salt + 0.5% sodium pyrophosphate, and (d) 1% salt + 0.5% sodium tripolyphosphate. The raw meat for all four treatments was obtained from a single beef inside round. The experiment was done with three replications. All measurements were done in duplicate.

Sample preparation

Select beef inside rounds (10 kg) were trimmed of fat and passed once through a 2.5 cm grinder plate of a Hobart grinder (Model 4152, Hobart Mfg Co., Troy, OH). Ten
percent of the meat was fine ground through a 0.31 cm plate. Salt and test compounds were separately dissolved with hot (65 °C) deionized water (DW). Hot water was needed to dissolve SPP and STPP (SPT is dissolved well in cool (20 °C) water). The solutions were allowed to cool to 7 °C (Meat Lab room temperature) before mixing with the meat at 1-2 °C. For each treatment, 115 ml of the salt solution and 115 ml of the test solution were separately added to 2.3 kg of coarse and fine grind meat in a ratio 90:10, to give 10% added water, 1% salt, and 0.5% phosphate as a percent of raw meat weight. On a millimolar basis, the concentrations of 0.5 % SPT, SPP, and STPP are equivalent to 4.5, 11.2, and 13.6 mM, respectively. The salt and phosphate solutions were manually mixed with the raw meat for 2 min. The meat was manually stuffed (F. Dick Corp., Farmingdale, NY) into 15 cm diameter water-impermeable casings (0.4 ml polyethylene, Vista International Packing Inc., Kenosha, WI). One roll (ca. 2.3 kg) was made per treatment. A portion (100 g) of the remaining beef was taken for determination of physico-chemical characteristics of raw beef. The restructured beef was stored for 1 day at 2 °C to allow equilibration and salt soluble protein extraction before cooking. Weighed rolls were cooked about 6 hr to 74 °C internal temperature in an electrically heated smokehouse (Model TR2-1700, Vorton, Inc., Beloit, WI) at 82 °C with manual cook controls (dampers partially open). Cooked beef rolls were stored at 2 °C until evaluation.

**pH measurement**

Meat samples (1 g) were homogenized in 9 ml deionized water for 10 s at 13,500 rpm with an Ultra-Turrax T25 (Janke & Kunkel GMBH, Staufen, Germany). The pHs of the homogenized samples were measured using a glass pH electrode (Orion Research Incorporated Co., Boston, MA).
Moisture content

Moisture content was determined as weight loss after samples were dried in a convection oven at 100°C for 16 h (AOAC, 1990).

Cook yield

After the meat rolls were cooked and cooled, one end of the casing was punctured just sufficiently to drain off the broth. The rolls were then reweighed, and the percentage cook yield was determined [(weight after cooking/weight before cooking) x 100].

TBA number

Thiobarbituric acid reactive substances (TBARS) assay was performed as described by Buege and Aust (1978). Samples (0.5 g) were mixed with 2.5 ml of 0.375% TBA-15% TCA-0.25 N HCl stock solution. The mixture was heated for 10 min in a boiling water-bath (95-100 °C) to develop a pink color, cooled with tap water, and centrifuged at 5,500 rpm for 25 min in a Beckman centrifuge (Model J-21C, Palo Alto, CA). The supernatant was measured spectrophotometrically at 532 nm using a UV 2100 U spectrophotometer (Shimadzu Co., Kyoto, Japan). TBARS were calculated from a standard curve of malondialdehyde, a breakdown product of tetraethoxypropane (TEP) used in preparation of the standard curve. TBA number was calculated as mg MDA/kg sample.

Metmyoglobin (%) and percent myoglobin denatured (PMD) in cooked meat

Minced meat (5 g) was placed in a 50-ml polypropylene centrifuge tube, and 25 ml ice-cold phosphate buffer (pH 6.8, 40 mM) was added. The mixture was homogenized using a polytron homogenizer (Omni 5000 International Co., Waterburg,
(CT) for 1 min at level 4. The homogenized sample was allowed to stand for 1 hr at 4 °C, then centrifuged at 5,000 rpm for 30 min at 4 °C. The supernatant was filtered with Whatman #1 filter paper, and the absorbance was read at 700, 572, and 525 nm with a spectrophotometer (Shimadzu Co., Kyoto, Japan). Metmyoglobin (% of undenatured meat pigments) was determined using the following formula (Krzywicki, 1982):

\[
\% \text{ MetMb} = \{1.395 - [(A_{572} - A_{700}) / (A_{525} - A_{700})]\} \times 100
\]

Percent myoglobin denatured (PMD) in cooked meat was also determined as described by Krzywicki (1982):

\[
\text{Mb (mg/mL)} = (A_{525} - A_{700}) \times 2.303 \times \text{dilution factor}
\]

\[
\text{PMD} = \{1 - (\text{Mb conc. after heating} / \text{Mb conc. before heating})\} \times 100
\]

Orthophosphate

Soluble orthophosphate was determined by modification of methods of Chen et al. (1956) and Molins et al. (1985). Beef (2 g) was homogenized in 18 ml of DW (10% homogenate) at 13,500 rpm for 10 s with an Ultra-Turrax T25 (Janke & Kunkel GMBH & Co. KG, Germany) and allowed to stand for 30 min at 4 °C. The homogenates were filtered with Whatman #1 filter paper, and 1 ml filtrate was transferred into a test tube. TCA (10%, 1 ml) was added to the test tube, then centrifuged at 2,000 x g for 10 min. The supernatant (0.1 ml) and phosphorus standard solutions (25, 50, and 100 ppm) were diluted with 0.5 ml DW and 1 ml of color reagent (6 N sulfuric acid + water + 2.5% ammonium molybdate + 10% ascorbic acid, Volume 1:2:1:1, respectively) was added and mixed thoroughly. The reaction mixture was incubated for 10 min at 37°C, and absorbance was read at 690 nm. The concentration of orthophosphate was calculated.
using the standard curve prepared from potassium phosphate (KH$_2$PO$_4$) and multiplied by the dilution factor (20).

**Salt soluble protein**

Beef (1 g) was homogenized in 5 ml 0.6 N NaCl at 13,500 rpm for 10 s with an Ultra-Turrax T25 (Janke & Kunkel GMBH & Co. KG, Germany) and allowed to stand for 1 hr at 4°C (Knipe *et al.*, 1985). The homogenates were centrifuged at 6,000 rpm for 30 min at 4°C with a refrigerated centrifuge (Beckman, Model J-21C, Palo Alto, CA). The supernatant (0.1 ml) or standard solutions (Bovine serum albumin) were diluted with 0.9 ml 0.6 N NaCl. The diluted solutions were used for determination of protein by the method of Lowry *et al.* (1951).

**Hunter color measurements**

Meat color was determined instrumentally using a Hunter Lab Digital Color Difference Meter D25D2A (Hunter Associates Laboratories, Inc., Reston, VA). The illuminant was a DZA low voltage halogen lamp. The instrument was standardized with a red plate; L=25.9, a=27.4, b=13.1. After mixing raw beef with added ingredients, a portion (100 g) was stored in a plastic bag at 2°C until color readings were taken (within 1 hr). The sample retained a normal red color during Hunter color measurement. To obtain Hunter color values, the chilled meat samples were placed in a petri plate (8.5 cm diameter and 1.4 cm depth). Four Hunter readings were taken per sample as follows; 1) bottom, 2) bottom, rotated 90°, 3) top, 4) top, rotated 90°. With three different samples (replications), there were 12 readings/treatment. Readings were completed within 10 min after samples were placed in petri plates.
For cooked meat samples, slices (1.5 cm thick x 10 cm diameter) were cut using a Berkel slicer (Berkel, Inc., New York, NY). Slices were wrapped individually in a plastic wrap to prevent oxidation or drying. Four Hunter color measurements were taken per slice, as described for raw samples. Readings were taken within 10 min after slicing, and the slices were then used for binding strength measurements.

**Bind strength measurements**

Bind strength measurements were made using the penetrometer described by Dobson et al. (1993). Following the Hunter color reading, the slices were mounted on a plexiglass cylinder and held in place by tapered needles, 0.4 cm apart and protruding 1.25 cm above the surface of cylinder. The circle formed by needles was 9 cm in diameter. The meat slice + cylinder was placed on a top loading balance with digital readout and 1 g readability (Sartorius PT6, 6000 g capacity, Baxter Scientific Products, Salt Lake City, UT), centered under the penetrometer rod, and tared to zero. The rod was advanced at maximum speed (2 cm/min), and applied force (g) was read at 5-s intervals until the polished steel ball (1.9 cm diameter) on the end of the rod penetrated the meat slice.

**Statistical analysis**

Data were analyzed using SAS (1985) for one-way ANOVA with three replications. The least significant difference (LSD) procedure was used to compare treatment means. Significance was accepted at the 5% probability level (p<0.05). ANOVA tables (D.1-D.18) were shown in appendix D.
RESULTS AND DISCUSSION

Treatment with all phosphate compounds, including 0.5% sodium phytate (SPT), sodium pyrophosphate (SPP), and sodium tripolyphosphate (STPP), significantly affected physicochemical characteristics of raw restructured beef after storage for 1 day at 2°C (Table 8-1). Sodium phytate, SPP, and STPP inhibited (p<0.05) MetMb formation, and the inhibitory effect of SPT and SPP was stronger than with STPP. Sodium phytate, SPP, and STPP increased salt soluble protein concentration in homogenates (p<0.05). Sodium phytate, SPP, and STPP had lower TBARS values than the control (p<0.05). Sodium pyrophosphate and STPP also increased soluble orthophosphate level compared with the control and SPT (p<0.05). Predictably, treatments with SPT, SPP, and STPP had higher pH (p<0.05) because the pH of 1% solutions of SPT, SPP, and STPP were 10.5, 10.2, and 9.8, respectively.

Cooked beef rolls treated with SPT, SPP, and STPP had significantly (p<0.05) higher bind strength, cook yield, and moisture level than did the control (Table 8-2). There were no significant differences in bind strength, moisture, and PMD among SPT, SPP, and STPP. The binding values in Table 8-2 were the means of peaks for six measurements per treatment group. Soluble orthophosphate was increased with treatment of SPP and STPP (p<0.05). Sodium phytate, SPP, and STPP decreased TBARS formation in cooked beef rolls, and the inhibitory effect of SPT was higher than with other phosphate compounds. Phytate, SPP, and STPP also increased meat pH (p<0.05). Compared with raw beef control treatment, cooking increased meat pH but decreased soluble orthophosphate in meat treated with SPP and STPP. A decrease in soluble orthophosphate in cooked meat was observed during the first day, possibly due to phosphate absorption by meat components (Molins et al., 1985).
Table 8-1
Physico-Chemical Characteristics of Raw Restructured Beef Rolls After Storage for 1 Day at 2°C.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MetMb (%)</th>
<th>Salt soluble protein (mg/ml)</th>
<th>TBA number (mg MDA/kg meat)</th>
<th>Orthophosphate (ppm)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>41.5 ± 3.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.81 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1135.3 ± 27.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.32 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SPT</td>
<td>28.3 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.56 ± 0.34&lt;sup&gt;bc&lt;/sup&gt;</td>
<td>0.31 ± 0.03&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1127.4 ± 103.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.91 ± 0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SPP</td>
<td>29.2 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.04 ± 0.34&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.24 ± 0.01&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1817.8 ± 172.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.80 ± 0.04&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>STPP</td>
<td>35.6 ± 2.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8.96 ± 0.15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.24 ± 0.02&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2206.5 ± 78.1&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.80 ± 0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup>Means within columns sharing the same superscript letter were not significantly different at p < 0.05.

SPT: sodium phytate, SPP: sodium pyrophosphate, STPP: sodium tripolyphosphate.
### Table 8-2

Treatment Means for Various Physicochemical Characteristics of Cooked Beef Rolls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Binding Strength (g)</th>
<th>Cook Yield (%)</th>
<th>Moisture (%)</th>
<th>PMD&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Orthophosphate (ppm)</th>
<th>TBA number (mg MDA/kg meat)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>761 ± 148&lt;sup&gt;a&lt;/sup&gt;</td>
<td>76.8 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>69.0 ± 1.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>83.8 ± 13.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1148.3 ± 44.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.49 ± 0.08&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.51 ± 0.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SPT</td>
<td>1878 ± 251&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.3 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>70.9 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>76.1 ± 8.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1167.2 ± 65.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.46 ± 0.02&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5.98 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SPP</td>
<td>1965 ± 224&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.6 ± 1.4&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>71.5 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>81.0 ± 4.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1657.4 ± 60.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.66 ± 0.09&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.85 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>STPP</td>
<td>1996 ± 416&lt;sup&gt;b&lt;/sup&gt;</td>
<td>85.8 ± 1.7&lt;sup&gt;c&lt;/sup&gt;</td>
<td>71.0 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.3 ± 5.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1837.8 ± 136.0&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.57 ± 0.10&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.82 ± 0.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a-c</sup> Means within columns sharing the same superscript letter were not significantly different at p< 0.05.

<sup>a</sup> PMD: Percent myoglobin denatured in cooked beef
SPT: sodium phytate, SPP: sodium pyrophosphate, STPP: sodium tripolyphosphate.
The soluble orthophosphate content increased linearly during 4 days storage, suggesting enzymatic hydrolysis of phosphates by meat phosphatases (Awad, 1968). One mole of STPP is known to hydrolyze to one mole of orthophosphate and one mole of pyrophosphate (Awad, 1968). However, phytic acid is very stable to heat and pH. The release of 50% of the phosphorus requires acid hydrolysis in 5 N HCl at 100°C for at least 6 hr (Cosgrove, 1980). Both acid hydrolysis and enzymatic treatment with phytase result in dephosphorylated inositol or low phosphorylated inositol.

There was a high correlation ($R^2 = 0.958$) between % metMb values (Table 8-1) and Hunter color redness (‘a’) values (Table 8-3) of raw beef rolls. SPT, SPP, and STPP significantly decreased the Hunter color L value (lightness) and b value (yellowness) but significantly increased ‘a’ value of raw beef rolls (p<0.05). In cooked beef, there were no significant differences among treatment groups, including the control. However, the control quickly turned to a greenish color within about 1 hr after slicing, indicating a rapid deterioration of quality. We noticed a pronounced off-flavor in the cooked beef control. However, there was not any flavor difference among groups treated with SPT, SPP, and STPP. Although sensory panel comparisons were not the objective of this study, a controlled sensory evaluation would be useful to evaluate 1) the effect of phytate in meats on metallic or other taste changes and 2) the effect on meat flavor determination over time.

Bind strength curves for 1.5 cm slices of cooked beef rolls are shown in Fig. 8-1. Data points represent mean values (n=6) with time. The control, SPT, SPP, and STPP showed peak bind strength at $35 \pm 5$, $50 \pm 6$, $65 \pm 7$, and $61 \pm 6$ s, respectively. Sodium phytate treatments showed an earlier peak than either SPP or STPP, indicating that the texture of beef rolls treated with SPT was slightly more brittle than those treated with SPP.
Table 8-3

Effect of Sodium Phytate and Other Phosphates on Hunter Color Values of Restructured Beef Rolls.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Raw</th>
<th>Cooked</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$L$</td>
<td>$a$</td>
</tr>
<tr>
<td>Control</td>
<td>30.9 ± 2.2$^a$</td>
<td>12.8 ± 1.5$^a$</td>
</tr>
<tr>
<td>SPT</td>
<td>25.9 ± 1.6$^b$</td>
<td>16.5 ± 0.4$^b$</td>
</tr>
<tr>
<td>SPP</td>
<td>27.8 ± 1.8$^b$</td>
<td>16.1 ± 0.8$^b$</td>
</tr>
<tr>
<td>STPP</td>
<td>26.3 ± 1.7$^b$</td>
<td>15.1 ± 1.3$^b$</td>
</tr>
</tbody>
</table>

$L =$ lightness, $a =$ redness, $b =$ yellowness.

$^a,b$ Means in columns with the same superscript letter were not significantly different ($p<0.05$).

SPT: sodium phytate, SPP: sodium pyrophosphate, STPP: sodium tripolyphosphate.
and STPP. Phytate, SPP, and STPP significantly increased maximum binding strength by about 2.5-fold and peak time by 15-30 s, compared with the control. The control slices were less cohesive, thereby resulting in faster penetration with less applied force by the advancing penetrometer rod.

The peak mean values in Fig. 8-1 were generally lower than listed in Table 8-2 because the peak occurred at slightly different times for each run. Moiseev and Cornforth (1997) reported that the binding strength depends on the added water content and pH of meat. Bind values were lower in beef rolls with 20% added water than with 5 or 10% added water. NaOH also increased binding strength but significantly less than STPP.

![Graph showing penetrometer binding strength values with time for beef rolls. DW (control): deionized water (no added phosphate), SPT: sodium phytate, SPP: sodium pyrophosphate, STPP: sodium tripolyphosphate. For each point, n=6 (3 replications, duplicate measurements/replication).]
even if the pH of beef treated with NaOH was higher, indicating that a simple increase in pH is not the only mechanism by which phosphates increase bind strength (Moiseev and Cornforth, 1997). The increase of cook yield in SPT, SPP, and STPP-treated rolls may partly reflect the higher meat pH than the controls because there is a clear tendency for the water-holding capacity to increase with increasing meat pH (Thomsen and Zeuthen, 1988). In addition, the higher salt-soluble protein values in raw beef treated with SPT, SPP, and STPP can be partly explained by the combined influence of phosphate acting as ATP analogues for actomyosin dissociation and high meat pH (Bernthal et al., 1991). Honikel et al. (1981) reported that as pH values are increased away from the isoelectric pH of proteins, there is an increased water-holding capacity and protein solubility. According to Hamm (1971), phosphates also increase binding strength through their ability to dissociate actomyosin into actin and myosin, thereby increasing protein extraction from post-rigor meats. The degree of extraction of myofibrillar protein in meat products is related to both cook yield and binding strength (Theno et al., 1978; Turner et al., 1979). Although the mechanisms by which SPT increased cook yield, binding strength, and salt soluble protein were uncertain in this study, an increase in meat pH by SPT can partially explain the effects. Phytate has six phosphate groups that may increase ionic strength of meat, thereby resulting in interaction with proteins and minerals. Phytate can form strong electrostatic linkages with basic amino acyl residues at low pH, thus precipitating most protein below pH 5.0. At neutral pH, both phytate and proteins have a net negative charge that leads to their dissociation from each other, thereby perhaps increasing binding strength and cook yield (Graf and Eaton, 1990).

Transition metals such as iron and copper and the heme moiety play an important role in peroxidation of membrane polyunsaturated fatty acids of muscle foods (Keller and
Phytate and phosphates can chelate the transition metals. These actions of phytate and phosphates might be involved in the inhibition of lipid peroxidation in raw and cooked beef. Especially in cooked beef, phytate may tightly bind the nonheme iron released from heme during cooking, and result in the lowest TBARS formation among treatments as found in this study because the phytate iron complex is not catalytically active for free radical formation (Graf and Eaton, 1990; Lee and Hendricks, 1995). Our unpublished data also confirm these findings: 5 mM phytate strongly inhibited lipid peroxidation in cooked beef through 9 days storage at 4 °C, and STPP had no effect on lipid peroxidation in cooked chicken breast. The chelating and buffering activity of phytate and phosphate might also contribute to color stability in raw restructured beef because myoglobin oxidation is known to be accelerated by the presence of free radicals or low meat pH (Gray et al., 1996).

Although phytate is known to inhibit mineral absorption, it may be beneficial as a meat additive. Phytate has no effect on heme iron absorption (Carpenter and Mahoney, 1992). Kim et al. (1993) reported that meat (beef, pork, and chicken) enhanced nonheme iron absorption by iron-deficient rats only in the presence of meals containing added phytate. An increase in net per capita red meat supply (195 g/day in the 1930s to 322 g/day in 1988) may be positively associated with a rising incidence of colorectal cancer (rates per 100,000, from about 47 in the 1940s to about 65 in the late 1980s) in the United States (Wynder et al., 1991). Body iron stores and dietary iron intake have both been shown to be positively associated with the risk of colon cancer (Siegers et al., 1988; Nelson et al., 1989, 1994). Iron is a strong catalyst for oxygen free radical formation and lipid peroxidation. Red meat, high in iron and fat, may provide copious substrates for lipid peroxidation (Souci et al., 1989). The peroxidation products may also be
mutagenic and carcinogenic (Janero, 1990; Halliwell and Chirico, 1993). Several animal and epidemiological studies have shown that phytate was protective against colon carcinogenesis (Graf and Eaton, 1985; Shamsuddin et al., 1988; Nelson et al., 1989). Phytate is a powerful inhibitor of iron-driven hydroxyl radical formation because it can form a unique iron chelate that becomes catalytically inactive (Graf et al., 1984). Therefore, inhibition of intracolonic hydroxyl radical generation may help explain the suppression of colonic carcinogenesis and other inflammatory bowel diseases by diets rich in phytate (Graf and Eaton, 1985, 1993).

CONCLUSIONS

Sodium phytate was nearly as effective as traditional meat phosphate additives such as STPP and SPP for increasing bind strength and cook yield of restructured beef rolls. Phytate was more effective as an inhibitor of lipid peroxidation than SPP or STPP, even though it was used at a much lower millimolar concentration (4.5 mM SPT, vs 11.2 mM SPP or 13.6 mM STPP). In view of the possible health benefits from phytate use, SPT may have potential as another phosphate additive for use in restructured meat products. However, SPT is substantially more expensive than STPP. The development of new techniques for mass production of phytic acid or the use of phytate-rich plants as meat additives may solve the cost problem.

REFERENCES


The possible antioxidant activities of carnosine and phytic acid were investigated using several in vitro and in situ model systems. Carnosine and phytic acid inhibited metal ion-catalyzed deoxyribose degradation, perhaps by chelating metal ions or by reacting with free radicals that otherwise degrade deoxyribose to MDA-like materials. Carnosine strongly inhibited metal ion-catalyzed lipid peroxidation in liposomes and ground beef homogenates. The beef homogenates are rich in lipid substrates and catalysts for lipid peroxidation. Phytic acid facilitated oxidation of Fe (II) to Fe (III), and it inhibited hemeprotein + H$_2$O$_2$-catalyzed lipid peroxidation in linoleic acid micelles.

The antioxidant properties of carnosine and phytic acid were also applied in meat-based systems. In pre-rigor beef muscle, carnosine and phytic acid increased the rate of drop in meat pH during post-mortem storage at 4 °C. Both carnosine and phytic acid stabilized the red color of meat by reducing metmyoglobin formation and lipid peroxidation over 9 days storage at 4 °C. Carnosine and phytic acid inhibited lipid peroxidation and iron release from the heme in cooked beef over 9 days storage at 4 °C. Heme iron content remaining after aging was negatively related to lipid peroxidation in cooked beef (r= -0.92, p<0.001). Phytate was also more effective than carnosine in inhibiting iron release from heme during cooking. Thus, phytate is recommended over carnosine as an antioxidant in cooked meats.

Ascorbic acid protects meat color and prevents lipid peroxidation. The color-stabilizing ability of carnosine was compared to that of ascorbic acid in a ground beef pattie model system. Ascorbic acid significantly inhibited metmyoglobin formation on
the surface of ground beef patties, but not in the bulk of the product. In contrast, carnosine significantly inhibited metmyoglobin formation and brown color development throughout the product. Carnosine increased cook yield and salt-soluble protein, but ascorbic acid had no effect on cook yield and decreased salt-soluble protein. Carnosine was more effective at inhibiting lipid peroxidation than was ascorbic acid. The combination of carnosine and ascorbic acid protected against ascorbate degradation in the ground beef patties. Carnosine strongly inhibited Cu (II)-catalyzed ascorbate oxidation in in vitro model systems.

The effects of carnosine and phytate on water-holding capacity (WHC) and color of raw and cooked meat were investigated in a model system of dilute ground beef. Ground beef (15 g) was soaked in 15 mL of carnosine (5 or 25 mM), phytate (5 or 25 mM), or carnosine (12.5 mM) + phytate (12.5 mM) solution for 1 and 5 days at 4°C. A duplicate set of these preparations was cooked to 70 ± 2°C for 30 min in a water-bath. After filtration of the mixture, the water-holding capacity (WHC) was calculated as the weight change in both raw and cooked ground beef. Compared with the control, phytate significantly increased WHC of raw and cooked meat, while carnosine only slightly increased WHC. Phytate decreased the Hunter ‘L’ value (whiteness), and carnosine increased the Hunter ‘a’ value (redness) compared with the control. Carnosine and phytate independently inhibited metmyoglobin formation in raw beef and lipid peroxidation in both raw and cooked beef.

Effects of 0.5% sodium phytate (SPT), sodium pyrophosphate (SPP), and sodium tripolyphosphate (STPP), along with 1% NaCl, on physicochemical properties of restructured raw and cooked beef were evaluated. In raw beef stored for 1 day at 4°C, the treatments with SPT, SPP, and STP increased pH and salt-soluble protein level and
inhibited metmyoglobin formation and lipid peroxidation, compared to the control (p<0.05). In cooked beef, SPT, SPP, and STPP increased bind strength, cook yield, moisture level, and pH, and inhibited lipid peroxidation (p<0.05). Sodium pyrophosphate and STP increased orthophosphate in both raw and cooked beef (p<0.05), compared to the SPT and control. Sodium phytate, SPP, and STPP decreased the Hunter color ‘L’ and ‘b’ values and increased ‘a’ value in raw beef (p<0.05), but had no effect on the Hunter color values in cooked beef. The binding strength values of SPP and STPP were similar over time, and the time to reach maximum binding strength was 10 s longer than SPT and 25 s longer than the control. These results indicate that SPT increased binding strength and cook yield, similar to traditional phosphates, but SPT inhibited lipid peroxidation after cooking more effectively than other phosphates were able to do.

In these studies, carnosine and phytate showed antioxidant properties in several model systems. The antioxidant activities of carnosine and phytate were associated with their color stabilizing activity in meat model systems by preventing metmyoglobin formation and lipid peroxidation. Phytate enhanced water-holding capacity of beef, thereby increasing cook yield in cooked beef. The effects of phytate to enhance WHC were comparable with traditional phosphates such as sodium pyrophosphate and tripolyphosphate. These results indicate that carnosine and phytate can be useful as meat additives for improving color and shelf-life of meat and meat products. The use of carnosine and phytate in meat processing may also benefit human health. However, carnosine and phytate are expensive. The development of new techniques for mass production of carnosine and phytate or the use of phytate-rich plants as meat additives may solve the cost problem.
Future studies that may be indicated from this research include: 1) the relationship between carnosine content and shelf-life of meats, 2) the effects of carnosine and phytate on color and lipid stability of fresh meat cuts using other application methods such as dipping or injection, 3) the antibacterial effects of carnosine and phytate in meat and meat products, and 4) the effects of phytate-treated meats on mineral absorption in humans. These future studies will give more information about the benefits of using carnosine and/or phytate as meat additives.
APPENDIX A

ANOVA TABLES FOR CHAPTER 5
Table A.1. Analysis of variance for metmyoglobin (%) of raw beef.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>Pr &gt; F</th>
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<tbody>
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<td>11704.29</td>
<td>377.56</td>
<td>389.61</td>
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</tr>
<tr>
<td>Error</td>
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<td>31.01</td>
<td>0.97</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
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<td>11735.29</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day (D)</td>
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<td>2859.47</td>
<td>2950.76</td>
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<td>Treatment (T)</td>
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<td>12.60</td>
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</tr>
<tr>
<td>D * T</td>
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<td>92.32</td>
<td>30.77</td>
<td>31.76</td>
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</tr>
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<td>Level (L)</td>
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<td>487.57</td>
<td>503.13</td>
<td>0.0001</td>
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<tr>
<td>D * L</td>
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</tr>
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<td>61.26</td>
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Table A.2. Analysis of variance for Hunter red color (a) value of raw beef.

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<td>0.69</td>
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<tr>
<td>Corrected total</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day (D)</td>
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<td>170.64</td>
<td>56.88</td>
<td>81.88</td>
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<td>0.10</td>
<td>0.14</td>
<td>0.7113</td>
</tr>
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<td>0.56</td>
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<td>19.89</td>
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<td>D * L</td>
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<td>4.16</td>
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<tr>
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<td>1.23</td>
<td>1.77</td>
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<td>D * T * L</td>
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<td>1.73</td>
<td>0.19</td>
<td>0.28</td>
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</table>
Table A.3. Analysis of variance for TBA number of raw beef.

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<tr>
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<td></td>
<td></td>
<td></td>
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<td>3.360</td>
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<td>0.142</td>
<td>19.71</td>
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<td>Level (L)</td>
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<td>1.375</td>
<td>190.47</td>
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<td>T * L</td>
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<td>0.020</td>
<td>2.72</td>
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</table>

Table A.4. Analysis of variance for heme iron of cooked beef.

<table>
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<td>Error</td>
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<tr>
<td>Corrected total</td>
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<td>332.38</td>
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<td>Day (D)</td>
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<td>0.81</td>
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Table A.5. Analysis of variance for TBA number of cooked beef.

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<th>Mean square</th>
<th>F value</th>
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APPENDIX B

ANOVA TABLES FOR CHAPTER 6
Table B.1. Analysis of variance for pH of raw ground beef pattie.

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Table B.2. Analysis of variance for cook yield (%) of ground beef pattie.

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<td>118.67</td>
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Table B.3. Analysis of variance for Hunter color ‘L’ values of top side of ground beef pattie stored in a petri dish.

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Table B.4. Analysis of variance for Hunter color ‘a’ values of top side of ground beef pattie stored in a petri dish.

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Table B.5. Analysis of variance for Hunter color ‘b’ values of top side of ground beef pattie stored in a petri dish.

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Table B.6. Analysis of variance for Hunter color ‘L’ values of bottom side of ground beef pattie stored in a petri dish.

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Table B.7. Analysis of variance for Hunter color ‘a’ values of bottom side of ground beef pattie stored in a petri dish.

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Table B.8. Analysis of variance for Hunter color ‘b’ values of bottom side of ground beef pattie stored in a petri dish.

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APPENDIX C
ANOVA TABLES FOR CHAPTER 7
Table C.1. Analysis of variance for Hunter color ‘L’ values of dilute ground beef model.

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Table C.2. Analysis of variance for Hunter color ‘a’ values of dilute ground beef model.

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Table C.3. Analysis of variance for Hunter color ‘b’ values of dilute ground beef model.

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APPENDIX D
ANOVA TABLES FOR CHAPTER 8
Table D.1. Analysis of variance for metmyoglobin (%) of raw restructured beef.

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Table D.2. Analysis of variance for salt-soluble protein level (mg/ml) of raw restructured beef.

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</table>

Table D.3. Analysis of variance for TBA number (mg MDA/kg meat) of raw restructured beef.

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</table>
Table D.4. Analysis of variance for orthophosphate level (ppm) of raw restructured beef.

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</table>

Table D.5. Analysis of variance for pH of raw restructured beef.

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<th>Mean square</th>
<th>F value</th>
<th>Pr &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>1.2521</td>
<td>0.4174</td>
<td>329.93</td>
<td>0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>0.0253</td>
<td>0.0013</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>23</td>
<td>1.2774</td>
<td></td>
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</tr>
</tbody>
</table>

Table D.6. Analysis of variance for binding strength (g) of cooked beef.

<table>
<thead>
<tr>
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<th>Mean square</th>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>6346551.0</td>
<td>2115517.0</td>
<td>26.60</td>
<td>0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>1590670.3</td>
<td>79533.5</td>
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<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>23</td>
<td>7937221.3</td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>
Table D.7. Analysis of variance for cook yield (%) of cooked beef.

<table>
<thead>
<tr>
<th>Source</th>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>131.4700</td>
<td>43.8233</td>
<td>33.75</td>
<td>0.0001</td>
</tr>
<tr>
<td>Error</td>
<td>8</td>
<td>10.3867</td>
<td>1.2983</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>11</td>
<td>141.8567</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table D.8. Analysis of variance for moisture (%) of cooked beef.

<table>
<thead>
<tr>
<th>Source</th>
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<th>Pr &gt; F</th>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>22.4500</td>
<td>7.4833</td>
<td>11.12</td>
<td>0.0002</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>13.4633</td>
<td>0.6732</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corrected total</td>
<td>23</td>
<td>35.9133</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table D.9. Analysis of variance for PMD (%) of cooked beef.

<table>
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<tr>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>219.0245</td>
<td>73.0082</td>
<td>1.14</td>
<td>0.3554</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>1276.0517</td>
<td>63.8026</td>
<td></td>
<td></td>
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<td>Corrected total</td>
<td>23</td>
<td>1495.0763</td>
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</tr>
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</table>
Table D.10. Analysis of variance for orthophosphate level (ppm) of cooked beef.

<table>
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<th>Source</th>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>2186509.3062</td>
<td>728836.4354</td>
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<tr>
<td>Error</td>
<td>20</td>
<td>142322.8892</td>
<td>7116.1445</td>
<td></td>
<td></td>
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<tr>
<td>Corrected total</td>
<td>23</td>
<td>2328832.1954</td>
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</tbody>
</table>

Table D.11. Analysis of variance for TBA number (mg MDA/kg meat) of cooked beef.

<table>
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<tr>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>3.9722</td>
<td>1.3241</td>
<td>222.26</td>
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<td>Error</td>
<td>20</td>
<td>0.1191</td>
<td>0.0060</td>
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</tr>
<tr>
<td>Corrected total</td>
<td>23</td>
<td>4.0913</td>
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</table>

Table D.12. Analysis of variance for pH of cooked beef.

<table>
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</thead>
<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>0.7172</td>
<td>0.2391</td>
<td>100.23</td>
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<tr>
<td>Error</td>
<td>20</td>
<td>0.0477</td>
<td>0.0023</td>
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<tr>
<td>Corrected total</td>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>92.6713</td>
<td>30.8904</td>
<td>8.37</td>
<td>0.0008</td>
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<tr>
<td>Error</td>
<td>20</td>
<td>73.8550</td>
<td>3.6928</td>
<td></td>
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</tr>
<tr>
<td>Corrected total</td>
<td>23</td>
<td>166.5263</td>
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</thead>
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<tr>
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<td>49.9512</td>
<td>16.6504</td>
<td>12.45</td>
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<td>Error</td>
<td>20</td>
<td>26.7450</td>
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<tr>
<td>Corrected total</td>
<td>23</td>
<td>76.6963</td>
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</table>

Table D.15. Analysis of variance for Hunter ‘b’ value of raw restructured beef.

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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>9.8433</td>
<td>3.2811</td>
<td>6.28</td>
<td>0.0035</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>10.4567</td>
<td>0.5228</td>
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</tr>
<tr>
<td>Corrected total</td>
<td>23</td>
<td>20.3000</td>
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<table>
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<tbody>
<tr>
<td>Model</td>
<td>3</td>
<td>20.9283</td>
<td>6.9761</td>
<td>1.16</td>
<td>0.3511</td>
</tr>
<tr>
<td>Error</td>
<td>20</td>
<td>120.6967</td>
<td>6.0348</td>
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<td>Corrected total</td>
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<td>141.6250</td>
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Table D.17. Analysis of variance for Hunter ‘a’ value of cooked beef.

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<tbody>
<tr>
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<td>1.1082</td>
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<td>0.3514</td>
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<td>22.5096</td>
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Table D.18. Analysis of variance for Hunter ‘b’ value of cooked beef.

<table>
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</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
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<td>11.8713</td>
<td>3.9571</td>
<td>1.04</td>
<td>0.3974</td>
</tr>
<tr>
<td>Error</td>
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<td>76.2883</td>
<td>3.8144</td>
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<tr>
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<td>88.1596</td>
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</tr>
</tbody>
</table>
APPENDIX E

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Department of Nutrition & Food Sciences
Utah State University, Logan, UT 84322-8700 USA
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Chicago, IL 60601 USA

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BEOM JUN LEE

EDUCATION:


ACADEMIC ACTIVITY:

Member of association of Korean Public Health
Member of Association of Korean Laboratory Animals
Member of Korean Toxicological Association
Seoul National University Scholarship (1978-1980)

EXPERIENCE:

Research Assistant (10/1990 present). Dept. of Nutrition and Food Sciences, Utah State University, Logan, Utah. Worked for an iron-fortified cheese
project and investigated antioxidant properties of carnosine and relative compounds. Investigated antioxidant activity of phytate: possible use as a meat preservative.

Research Assistant (3/1988-2/1989). Dept. of Veterinary Public Health, College of Veterinary Medicine, Seoul National University, Seoul, Korea. Administered laboratory fund and purchased equipment and reagents. Researched on the effect of foods (ginseng and fish oils) on human diseases (atherosclerosis, liver cancer, stomach cancer, etc.)


PUBLICATION:


INTEREST:

All kinds of sports including golf, tennis, soccer, football, etc.