α-Tocopherol Oxidation in Beef and in Bovine Muscle Microsomes

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The oxidation of α-tocopherol (TH) in beef was analyzed using a stable isotope dilution capillary gas chromatography−mass spectrometry assay. TH decreased while α-tocopherolquinone (TQ) and 2,3-epoxy-α-tocopherolquinone (TQE_2) increased in ground longissimus lumborum (LL) and psoas major (PM) muscles during storage ($P < 0.10$). In LL steaks, the relative concentrations of TH decreased and TQ and TQE_2 increased in surface samples; changes were less dramatic in deep samples. Deuterated α-tocopherolhydroquinone (THQ) standard was not recovered and endogenous THQ was not detected in meat; THQ was measurable in microsomes isolated from PM and incubated in the presence of 2,2′-azobis(2-amidopropane)HCl (ABAP) or myoglobin. ABAP-challenged microsomes yielded a tocopherol product profile which favored 5,6-epoxy-α-tocopherolquinone (TQE_1) and TQE_2, while the use of myoglobin as prooxidant resulted in a higher proportion of TQ and THQ. Results demonstrated that concentrations of TH decreased and TQ and TQE_2 increased in meat during storage and are consistent with the peroxy-radical scavenging function of tocopherol.

Keywords: *α-Tocopherol; oxidation; beef; meat*

INTRODUCTION

α-Tocopherol (TH) is a lipid-soluble antioxidant vitamin which acts principally by scavenging peroxy radicals (Burton and Ingold, 1986). Muscle-based foods which contain high concentrations of TH demonstrate greater stability of lipid and oxymyoglobin (Faustman et al., 1998). The basis for improved oxidative stability appears logical given the normal physiological antioxidant function of TH in vivo. A concentration of ca. 3.5 μg of α-tocopherol per gram of beef appears to be the threshold for realizing the maximal antioxidant effect in meat. Below this threshold, a significant positive correlation exists between the oxidative stability of a given piece of beef and its TH concentration; concentrations above the threshold do not appear to provide any additional antioxidant protection (Faustman et al., 1989; Arnold et al., 1993). To date, no studies have attempted to follow the oxidation of TH in beef during refrigerated storage.

Liebler et al. (1996) recently developed a stable isotope dilution capillary gas chromatography−mass spectrometry assay for TH and its principal oxidation products. The four major products of TH oxidation which can be readily measured are α-tocopherolquinone (TQ), α-tocopherolhydroquinone (THQ), 5,6-epoxy-α-tocopherolquinone (TQE_1), and 2,3-epoxy-α-tocopherolquinone (TQE_2) (Figure 1). The accumulation of these oxidation products in tissue or in vitro provide evidence of TH-mediated antioxidant reactions. Additionally, monitoring of antioxidant reaction products would represent a potential approach for following oxidative stress in muscle-based foods. The objective of this study was to determine if oxidation products of TH could be measured in postmortem beef muscle (i.e., meat).

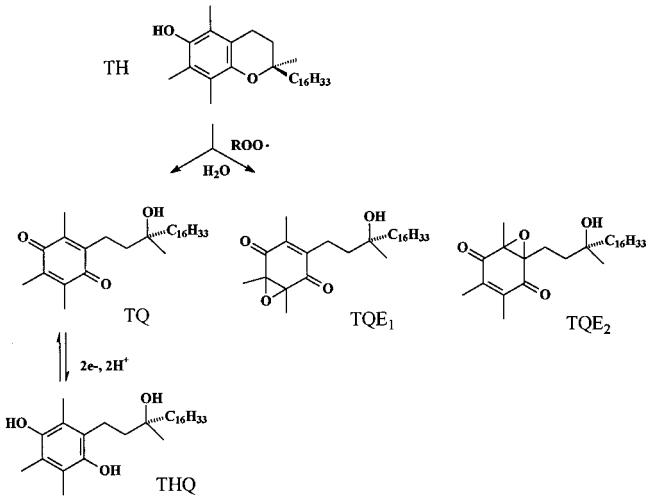

Figure 1. Oxidation pathway for α-tocopherol in peroxyl radical scavenging reactions: TH, α-tocopherol; TQ, α-tocopherolquinone; THQ, α-tocopherolhydroquinone; TQE_1, 5,6-epoxy-α-tocopherolquinone; TQE_2, 2,3-epoxy-α-tocopherolquinone.

MATERIALS AND METHODS

Reagents. TH (*R,R,R*-α-tocopherol), 5,7-dimethyl-[C^2H_3]-(*R,R,R*)-α-tocopheryl acetate, and 5-[$^2H_3$-methyl]-α-tocopheryl acetate were gifts of the Natural Source Vitamin E Association (Excelsior, MN). Deuterated TH, TQ, TQE_1, TQE_2, and THQ were prepared as previously described (Liebler et al., 1996). Trypsin-EDTA and phosphate-buffered saline (PBS; pH 7.4) were obtained from Gibco-BRL (Life Technologies, Gaithersburg, MD). Deferoxamine mesylate and horse heart myoglobin (95%+, met-form) were from Sigma Chemical Co. (St. Louis, MO). Dimethylformamide (DMF), trimethylchlorosilane (TMCS), and *N,O*-(bistrimethylsilyl) trifluoroacetamide (BSTFA) were purchased from Pierce Chemical Co. (Rockford, IL). Prior to

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use, DMF was passed over alumina to remove any potential peroxides. 2,2′-Azobis(2-amidopropane)HCl (ABAP) was from Polysciences Inc. (Warrington, PA). All glassware was silanized with dichlorodimethylsilane in toluene, rinsed with toluene and then methanol, and dried.

Meat. Whole muscle cuts ($n = 3$) of beef longissimus lumborum (LL) and psoas major (PM) were purchased locally. Where appropriate, beef was minced through a tabletop grinder (3.5 mm) and formed into miniature patties in the bottom half of a tissue culture dish (15 × 60 mm). Beef LL steaks (2.5 cm × 36 cm^2) were also prepared. Both patties and steaks were wrapped in oxygen-permeable PVC film (15500−16275 cm^3/m^2/24 h at 23 °C) and stored in the dark at 4 °C. Immediately prior to tocopherol oxidation product analysis, steaks were sampled by cutting perpendicularly through the central portion of the slice (with fiber direction) and dissecting 1−2 mm from the surface (∼100 mg) and a similar quantity of beef from the geometric center of the deep interior portion.

Extraction and Derivatization of TH and Its Oxidation Products from Beef. A representative sample of meat was freeze-clamped with liquid nitrogen and powdered using a stainless steel mortar and pestle. Samples were analyzed in triplicate. Approximately 50 mg of powdered tissue was placed in a silanized test tube (16 × 100 mm); a volume of 1 mL of 0.25% trypsin-EDTA (prewarmed for 15 min at 37 °C) was added to each tube, and tubes were incubated at 37 °C for 30 min. Following incubation, 0.35 mL of 1 mM BHT-ETOH and 1.65 mL of PBS-1 mM deferoxamine (pH 7.2) were added to each tube. Tubes were sonicated using a probe sonicator (Virsonic 50, Virtis Co. Inc., Gardiner, NY), and the sample was filtered through two layers of cheesecloth. One milliliter of filtered sample was transferred to a new tube, combined with 2.3 mL of ETOH, 0.83 mL of 10 mM sodium dodecyl sulfate, and deuterated standards of TH, TQ, THQ, TQE_1, and TQE_2, vortexed, and allowed to sit at room temperature for 15 min. α-Tocopherol and its oxidation products were extracted three times with 2 mL of hexane, and the solvent layers were combined into a separate tube. Hexane was evaporated under a constant stream of nitrogen and the residue resuspended in 1 mL of ETOH and 1 mL of 1 N HCl. This acid treatment converts acid-labile tocopherone precursors to TQ, TQE_1, and TQE_2 (Liebler et al., 1996). Following a 30 min incubation at room temperature, samples were again extracted with hexane (3 × 2 mL). Hexane was evaporated under nitrogen and the residue resuspended with a minimum volume of hexane. Samples were transferred to autosampler vials, and the hexane was evaporated under nitrogen. Each vial received 100 μL of DMF and was capped, and 100 μL of BSTFA (10% TMCS) was added. The vials were vortexed and contents allowed to derivatize for 2 h at room temperature. Recoveries of internal standards for TH, TQ, TQE_1, and TQE_2 in meat ranged from 95% to 111%.

Beef Muscle Microsomes. Microsomes were prepared from beef psoas major following the general procedure of Guengerich (1977). Oxymyoglobin was prepared from metmyoglobin by hydrosulfite-mediated reduction (Brown and Mebine, 1969); excess hydrosulfite was removed by passing oxymyoglobin over a PD-10 column (Pharmacia, Piscataway, NJ). Microsomes (1 mg of microsomal protein/mL) in 50 mM sodium citrate (pH 5.6) were incubated with 20 mM ABAP or 0.15 mM myoglobin at 37 °C for 30 min. Following incubation, a 1 mL sample was removed and combined with deuterated standards and extracted as outlined above.

GC−MS Analysis. GC−MS analyses were performed as previously reported (Liebler et al., 1996). Ions (m/z) used for selected ion monitoring analysis of the TMS derivatives for the endogenous and deuterated fragments were 502 and 508 for TH, 309 and 315 for TQE_1 and TQE_2, 293 and 299 for TQ, and 309 and 312 for THQ. Peak areas in selected ion chromatograms were obtained by integration with Fisons-VG LabBase data system software.

Thiobarbituric Acid Reactive Substances (TBARS). Lipid oxidation was measured by the thiobarbituric acid assay of Schmedes and Holmer (1989) according to Yin et al. (1993), and A_{532} was reported as TBA-reactive substances (TBARS).

Table 1. Concentrations of α-Tocopherol and Its Oxidation Products (μg/g) in Ground Beef Muscle during Storage at 4 °C[a]

	longissimus lumborum		psoas major	
	day 1	day 5	day 1	day 5
TH	1.39 (0.26)	0.11 (0.08)**	2.63 (0.20)	1.66 (0.28)*
TQ	0.33 (0.03)	0.42 (0.01)*	0.39 (0.02)	0.52 (0.03)*
THQ	nd[c]	nd	nd	nd
TQE_1	0.06 (0.02)	0.01 (0.01)	0.04 (0.01)	0.04 (0.01)
TQE_2	0.11 (0.03)	0.28 (0.03)*	0.10 (0.03)	0.27 (0.02)**
total[b]	1.89	0.82	3.16	2.49

[a] Abbreviations: TH, α-tocopherol; TQ, α-tocopherolquinone; THQ, α-tocopherolhydroquinone; TQE_1, 5,6-epoxy-α-tocopherolquinone; TQE_2, 2,3-epoxy-α-tocopherolquinone. Within a muscle type, means (SE) differ between days 1 and 5 at $p < 0.10$* or $p < 0.05$**; $n = 3$. [b] TH plus oxidation products. [c] Not detected.

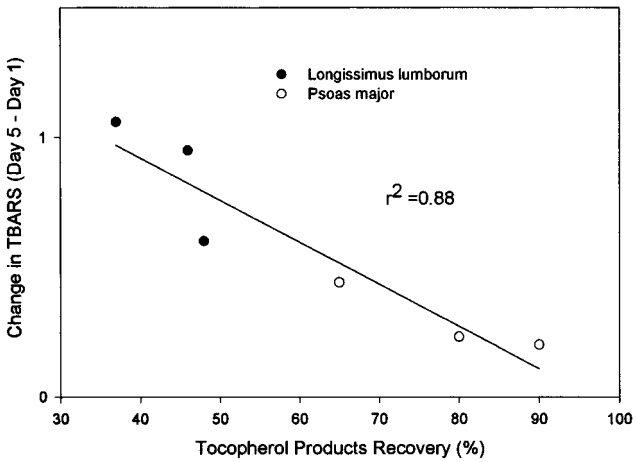

Figure 2. Relationship between lipid oxidation (TBARS) and recovery (%) of α-tocopherol and its oxidation products in ground beef stored at 4 °C for 5 days.

Statistical Analysis. Differences among means for day 1 and day 5 values were determined by Student's t test; correlation coefficients were calculated (Gilbert, 1981).

RESULTS AND DISCUSSION

The concentrations of α-tocopherol and its oxidation products in LL and PM muscles during storage are presented in Table 1. During 4 days of storage at 4 °C, the concentration of TH in LL decreased ($P < 0.05$), while that of TQ and TQE_2 increased ($P < 0.10$). TQE_1 concentration did not change significantly ($P > 0.05$). A reliable measurement of spiked deuterated THQ or endogenous THQ was not attained. PM muscle showed trends similar to those of LL with a decrease in TH, increases in TQ and TQE_2, and no change in TQE_1 concentrations. Recovery of total tocopherol plus products after 4 days was approximately 45% and 79% for LL and PM, respectively. We noted that recovery of tocopherol products was greatest in samples which experienced the smallest oxidative challenge (expressed as change in TBARS from day 1 to day 5). The relationship between recovery of tocopherol plus products and oxidative challenge is presented in Figure 2. Poor recovery could be due, in part, to further degradation of tocopherol to other oxidation products which would have escaped detection. Additionally, the inability to quantitate THQ would also provide a partial explanation for the apparent loss of recoverable tocopherol products following the 4 day storage period. The average (±SE) increase in the TBARS value from day 1 to day

Table 2. Relative Amounts of α-Tocopherol and Its Oxidation Products in Ground Beef Muscle during Storage at 4 °C[a]

	longissimus lumborum		psoas major	
	day 1	day 5	day 1	day 5
TH/T	0.72 (0.07)	0.12 (0.08)**	0.83 (0.01)	0.66 (0.04)*
TQ/T	0.18 (0.03)	0.51 (0.04)**	0.12 (0.00)	0.22 (0.03)
TQE_1/T	0.04 (0.02)	0.02 (0.01)	0.01 (0.00)	0.01 (0.01)
TQE_2/T	0.06 (0.02)	0.35 (0.05)**	0.03 (0.01)	0.10 (0.01)**

[a] Abbreviations: TH, α-tocopherol; TQ, α-tocopherolquinone; TQE_1, 5,6-epoxy-α-tocopherolquinone, TQE_2, 2,3-epoxy-α-tocopherolquinone; T = TH + TQ + TQE_1 + TQE_2. Within a muscle type, means (SE) differ between days 1 and 5 at $p < 0.10$* or $p < 0.05$**; $n = 3$.

5 of storage was 0.87 (± 0.13) for LL and 0.29 (± 0.08) for PM.

The decrease in the concentration of TH and the increase in concentrations of the oxidation products TQ and TQE_2 are consistent with the apparent antioxidant function of TH in the presence of oxidizing lipids in meat. The relative amounts of α-tocopherol and its oxidation products expressed as a percentage of total tocopherol detected are presented in Table 2. In LL, TH accounted for approximately 72% of tocopherol equivalents at day 1 and decreased to 12% at day 5. The decrease in the relative concentration of TH was not as extensive in PM with a change from 83% to 66%. After 4 days, TQ and TQE_2 each accounted for a greater relative proportion of tocopherol plus products than TH in LL. In PM, TH constituted the greatest relative portion of tocopherol equivalents at both days 1 and 5.

Relative changes in TH, TQ, and TQE_2 were also analyzed in the surface and deep portions of LL steaks. Results presented in Figure 3 demonstrate that relative loss of TH and accumulation of TQ and TQE_2 were greatest in surface samples; changes within the deep samples were less dramatic. These results are consistent with the oxidative challenge expected at deep and surface locations. Deep portions of beef steaks are relatively anaerobic and would not be expected to provide any substantial oxidative challenge. Oxygen penetration at beef surfaces would provide an opportunity for peroxy radical formation, and quenching by α-tocopherol would readily occur.

Liebler et al. (1996) utilized the stable isotope dilution capillary GC−MS assay to follow changes in the concentration of α-tocopherol and its oxidation products in rat liver microsomes subjected to radical generation by the azo initiator ABAP. Their recoveries of tocopherol plus products following an oxidant challenge were high, and THQ concentrations were readily assayed. Thus, we decided to determine if our inability to measure THQ and to achieve an acceptable recovery of tocopherol plus products was affected by the complex nature of meat. Microsomes were prepared from bovine psoas major and subjected to a prooxidative challenge from ABAP or oxymyoglobin. Results from this experiment are presented in Table 3 and demonstrate that THQ was readily detected in a repeatable manner. Additionally, recovery of tocopherol plus products in muscle microsomes was approximately 95% and 98% for ABAP and oxymyoglobin systems, respectively. Thus, it appears that some unknown factor or condition inherent to the meat matrix interferes with the ability to detect THQ and to obtain high recoveries of tocopherol products. The improved recovery of tocopherol plus products

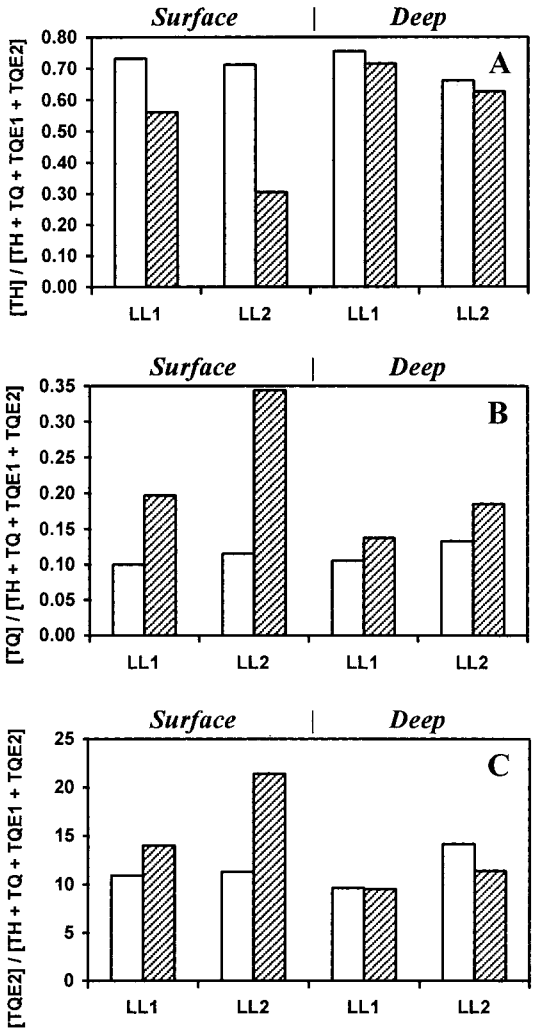

Figure 3. Change in relative concentrations of (A) α-tocopherol (TH), (B) tocopherolquinone (TQ), and (C) 2,3-epoxy-α-tocopherolquinone (TQE_2) in deep and surface sections of beef longissimus lumborum (LL) slices during 4 days of storage at 4 °C. Two different muscle samples were analyzed (LL1 and LL2); open bars indicate day 1 values and shaded bars indicate day 5 values.

Table 3. Oxidation of TH to TQ, THQ, TQE_1, and TQE_2 by ABAP or Oxymyoglobin in Bovine Muscle Microsomes Incubated at 37 °C, pH 5.6[a]

	concentration (pmol/mg microsomal protein)					
	TH	TQ	THQ	TQE_1	TQE_2	total
			$t = 0$ min			
ABAP	362 (19)	21 (4)	30 (3)	9 (1)	17 (2)	465
OxyMb	412 (26)	22 (6)	46 (15)	0 (1)	20 (4)	500
			$t = 30$ min			
ABAP	225 (10)	54 (15)	60 (12)	31 (1)	71 (4)	441
			$t = 60$ min			
OxyMb	205 (15)	63 (7)	155 (31)	6 (3)	61 (6)	490

[a] Means (SD), $n = 9$ for ABAP and OxyMb treatments.

in microsomes and not in meat under the conditions described is not readily explained.

Interestingly, we noted that the relative distribution of tocopherol and its oxidation products differed as a function of the oxidant used (Table 4). For both oxidant systems, approximately 44% TH remained following incubation. However, TQ and THQ constituted a greater product percentage and TQE_1 and TQE_2 a lower product percentage in myoglobin-challenged microsomes than

Table 4. Effect of ABAP and Myoglobin (Mb) on the Relative Distribution of α-Tocopherol and Its Oxidation Products in Bovine Muscle Microsomes Incubated at 37 °C, pH 5.6[a]

	relative distribution (%)[b]				
	TH	TQ	THQ	TQE_1	TQE_2
ABAP	44.4 (12.4)	11.9 (2.6)[a]	12.6 (1.5)[a]	9.1 (3.4)[b]	22.0 (9.1)[b]
Mb	42.2 (9.9)	20.6 (8.3)[b]	24.1 (4.9)[b]	1.9 (0.7)[a]	11.2 (1.5)[a]

[a] Means (SD) in columns with different superscripts (a, b) are different ($P < 0.05$); $n = 9$. [b] Relative distribution of each species is expressed as a percentage of total tocopherol and its oxidation products.

in microsomes treated with ABAP ($P < 0.05$). This is consistent with the nature of α-tocopherol and of these two different oxidants. ABAP is a peroxy radical generator, and in the presence of a high flux of peroxy radicals, the tocopherol oxidation pathway which generates TQE_1 and TQE_2 is favored (Liebler, 1993). The myoglobin system likely exerted its prooxidant effect via interaction between the heme protein and preformed peroxide (Kanner and Harel, 1985) to generate either high valent heme species or related oxidants that oxidize TH to the tocopherone cation by successive electron transfers. This would favor the production of TQ and THQ (Liebler, 1993).

The improved lipid and color stability of beef with elevated concentrations of α-tocopherol has been attributed to the peroxy radical scavenging function of this antioxidant vitamin without evidence that tocopherol was changed during retail display (Schaefer et al., 1995). The detection of decreased concentrations of TH and increased concentrations of tocopherol oxidation products, TQ and TQE_2, in ground beef stored for 4 days is consistent with an antioxidant role for α-tocopherol in postmortem muscle.

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