Technical

Evaluation of Antioxidant Activity: I. Application of an Enzyme-catalyzed System

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ABSTRACT

A study was made of the inhibitory effect of the antioxidants propyl gallate, tertiary butylated hydroxyanisol and ethoxyquin on the enzyme-catalyzed oxygenation of linolenic acid. Gas chromatography was used to measure unchanged fatty acid after varying incubation periods and at various concentrations of antioxidant. Propyl gallate was also tested for its influence on the oxygenation of an emulsion of linoleic acid. The effect of the antioxidants is compared with results obtained previously with a linoleic acid emulsion. It is concluded that it is advantageous to apply linoleic acid as substrate in the model system.

INTRODUCTION

A gas chromatographic method for evaluating the effect of antioxidants has been described in a previous paper (1). A well-defined system based on the lipoxygenase-catalyzed oxidation of linoleic acid was applied.

This communication reports a modification of the test system. Linolenic acid was applied as substrate and the antioxidants butylated hydroxyanisol (BHA), ethoxyquin (EMQ) and propyl gallate (PG) were tested. PG was also tested in the linoleic acid emulsion system. All data were tested statistically and compared with those reported previously (1).

EXPERIMENTAL PROCEDURES

Apparatus and Reagents

The gas chromatographic equipment and operating conditions have been described previously (1). Linolenic acid (99%) was obtained from Sigma Chemical Co. (St. Louis, MO). Propyl gallate (Tenox PG) was supplied by Eastman Chemical Products Inc. (Kingsport, TN). The other reagents were as described previously (1).

Analytical Procedure

Stock solutions of lipoxygenase, internal standard, antioxidant and fatty acid preparations were done as previously described (1), as were procedures for the enzyme reaction, analysis of the reaction mixture and data processing.

RESULTS

The percentage of unchanged fatty acid in the oxidative reaction systems was calculated at various incubation times and at various concentrations of antioxidant. Mean values are presented in Table I. Based on the parallels of experimental data, the calculated standard deviation of the modified method was $s_d = \pm 1.55\%$, as compared with $s_d = \pm 0.86\%$ for the linoleic acid-lipoxygenase system. The data in Table I show that linolenic acid was oxidized to a degree corresponding to the lipoxygenase-catalyzed oxidation of linoleic acid (1).

PG was tested in both the linolenic acid and linoleic acid emulsion systems and results are listed in Table I. The pronounced concentration effect of PG in both systems is notable. A Student's t-test (2) at 99% confidence level showed significant differences between the results with and without antioxidant and between the various antioxidant levels. The antioxidants BHA and EMQ were tested in the linolenic acid emulsion system. They showed almost no inhibitive effect under these experimental conditions.

A protective index (PI) was defined in our previous paper (1) to compare the activity of the various antioxidants. The PI for BHA, EMQ and PG tested in both oxidative systems are listed in Table II.

DISCUSSION

The properties of the enzyme lipoxygenase have been

TABLE I

Mean Values of Unchanged Fatty Acid Relative to Initial Amount of Fatty Acid (Percentage) at Varying Incubation Times and Concentrations of Propylgallate (PG)[a]

	Linolenic acid				Linoleic acid			
	Concentration of PG[b]				Concentration of PG[b]			
Incubation time (min)	32% (3.12 M)	8% (0.78 M)	0.8% (0.08 M)	Without antioxidant	32% (3.12 M)	8% (0.78 M)	0.8% (0.08 M)	Without antioxidant
1	99.2	97.8	93.7	96.3	99.0	99.2	97.2	96.0
3	96.7	93.1	87.9	86.8	98.1	95.4	89.9	86.4
5	93.1	86.9	81.0	80.2	97.4	94.9	83.9	77.7
7	91.4	82.6	72.9	70.9	96.0	91.4	78.1	71.0
10	89.4	75.5	66.8	61.5	94.0	85.8	70.9	60.8
12	86.8	73.3	58.9	54.8	93.6	83.4	64.3	53.0
15	83.3	69.6	55.7	48.8	89.4	82.2	56.2	45.1

[a]Lipoxygenase activity = 46, 000 units/mg.
[b]w/w of initial amount of linoleic acid.

TABLE II

The Effect of the Concentrations of the Antioxidants on the Protective Indices (PI) in an Enzyme-catalyzed System[a]

Substrate	Antioxidant	Concentration of antioxidant		
		32%	8%	0.8%
Linolenic acid	PG	5.8	2.0	1.1
	BHA	1.0	1.0	1.0
	EMQ	1.0	1.0	1.0
Linoleic acid	PG	7.2	4.0	1.42
	BHA	1.75	1.42	1.28
	EMQ	1.76	1.72	1.58

[a]See legend to Table I.

extensively studied (3-5). It catalyzes the oxygenation of unsaturated fatty acid by molecular oxygen and is highly specific for fatty acids containing a *cis,cis*-1,4-pentadiene unit. Recently, it has been demonstrated that iron is an essential component for the activity of lipoxygenase (6-8). Three isoenzymes, lipoxygenase 1, 2 and 3 have been isolated from soybeans. Their stereospecificity, however, is different (8-11). Lipoxygenase-1 favors the formation of 13-hydroperoxide, whereas lipoxygenase-2 favors oxidation in the 9-position. Based on these results, de Groot et al. (8) have proposed a reaction scheme for the activation of soybean lipoxygenase-1 in the presence of iron, and for catalytic activities at pH 9.0 under aerobic and anaerobic conditions. The enzyme is activated by the oxidation product hydroperoxy fatty acid, and a change between the ferric and ferrous species of the enzyme occurs during the reaction. A fatty acid radical-enzyme complex is formed which combines stereospecifically with oxygen. The enzyme complex dissociates to a small extent and free radicals leak out into the solution (8,12). Lipoxygenase-2 tends to be more leaky with respect to linoleic acid radicals than lipoxygenase-1, according to Boldingh (12). These free radicals may be subject to autoxidation. Kinetic analysis of the lipoxygenase-catalyzed oxygenation of linoleic acid has been described in recent papers (13,14).

Lipoxygenase-2 was applied in our test system. It is reasonable to assume that the reaction scheme proposed by de Groot et al. (8) will also be valid for this isoenzyme. That linoleic acid and linolenic had identical oxidation profiles is consistent with early observations by Holman and Elmer (15). Because of the low standard deviation of the linoleic acid-lipoxygenase system compared to that of linoleic acid, it is advantageous to apply linoleic acid when testing the antioxidants. The rate of autoxidation of the free radicals in the solutions is slower for linoleic acid than for linolenic acid. It is also possible that linolenic acid decomposes more readily than linoleic acid during the preparation of the sample for GC-injection. These factors affect analytical reliability. The antioxidant effects obtained in the enzyme-catalyzed system appeared to be completely different from those found in similar studies on a heme-catalyzed system (16). BHA and EMQ had only a weak effect on the linoleic acid oxidative system and no effect at all on the linolenic acid system, whereas PG was quite effective in both systems and showed a pronounced concentration effect. These features are expressed by the PI quoted in Table II.

Early works by Siddiqi et al. (17) and Tappel et al. (18,19) reported that PG is a potent lipoxygenase inhibitor. Recent studies on the effect of antioxidants on the lipoxygenase-catalyzed oxidation of arachidonic acid and on prostaglandin biosynthesis (20) showed that PG appeared to be an effective nonspecific inhibitor. Singleton et al. (21,22) studied the production of volatiles from lipoxygenase-catalyzed oxidation in food homogenates. Known lipoxygenase inhibitors were added and the results showed that PG effectively inhibited pentane production.

In the enzyme-catalyzed oxidation, the *cis,cis*-1,4-pentadiene group of the fatty acid is protected by the enzyme. Presumably the antioxidant will attack the complex and inhibit the formation of the free radicals (1), as well as the autoxidation of the free radicals that leak into the solution. Structurally, BHA and EMQ have large substituents in the *ortho* position to the active hydrogen, and it is reasonable to assume that the reactivity of BHA and EMQ is more sterically hindered than that of PG.

Gas chromatographical analysis measures the inhibitory effect of the antioxidants on the reaction of the fatty acid radicals. The analysis gives, however, no information concerning the inhibitory effect on the reaction of the oxygenated fatty acid radicals, the peroxy radicals. In order to explain the pronounced difference between the effect of the antioxidants on the linoleic acid system and on the linolenic acid system, we suggest that the oxygenation of the linolenic acid radicals is a faster reaction than the antioxidant reaction. Thus the antioxidants will mainly inhibit the reaction of the peroxy radicals in the linolenic acid system.

Several authors have reported a pro-oxidative effect of the antioxidants at high concentrations (23-25). The effect appeared, however, to be dependent on the solvent system. Fritsch et al. (23) tested phenolic antioxidants in palm oil, whereas various tocopherols have been tested in both aqueous and oil medium (24,25). No pro-oxidative effect was observed in our system and the antioxidative effect increased with increasing concentration.

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Evaluation of Antioxidant Activity: II. Application of a Heme-catalyzed System

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ABSTRACT

A study was made of the inhibitory effect of the antioxidants propyl gallate, *tert*-butylated hydroxyanisol, α-tocopherol and ethoxyquin on the hemoglobin-catalyzed oxygenation of linoleic acid. The concentration of unchanged fatty acid after varying incubation periods and at varying concentrations of antioxidant was measured by gas chromatography. The effect of the antioxidants is compared with results obtained previously from the lipoxygenase-catalyzed oxidation of a linoleic acid emulsion. It is concluded that all 4 antioxidants are good inhibitors of fatty acid oxidation catalyzed by hemoglobin.

INTRODUCTION

In a previous communication (1), we described a gas chromatographic method for evaluating the effect of antioxidants. The inhibitory effect of *tert*-butylated hydroxyanisol (BHA) and ethoxyquin (EMQ) on a lipoxygenase-catalyzed oxidation of linoleic acid was determined. In the first paper of this series (2), the system was modified and linolenic acid was applied as substrate. Linoleic acid appeared to be the more suitable substrate and propyl gallate (PG) a much more efficient antioxidant than BHA, EMQ and α-tocopherol (TO) when tested in the lipoxygenase-catalyzed system (1-3).

Lipoxygenase is a biological catalyst present mainly in plant tissues. It was therefore decided to extend measurements on antioxidative effect by applying a catalyst of animal origin and the results are described in this paper. Acceleration of the oxidation of animal fat depends on the presence of hemoglobin, myoglobin and their derivatives, which are distributed throughout the animal organism.

Tappel (4,5) has reviewed the catalytic activity of hematin compounds in biological systems. He and his coworkers studied the reaction rate and the mechanism of the hematin-catalyzed lipid oxidation (6,7) and the decomposition of lipid hydroperoxides (8,9).

More recently, Gardner (10) reviewed the decomposition of lipid hydroperoxides and the influence of heme compounds on the degradation process. Several workers have shown great interest in the reaction mechanism of the breakdown reaction, as well as the identification of the secondary products (11-17). It has been concluded that heme and metal ion catalysis plays an important role in the initiation and propagation steps of the reaction.

A variety of methods has been devised for testing the activity of food antioxidants on the heme-catalyzed oxidation of unsaturated lipids (6,18-20). Hamilton and Tappel (19) oxidized a methyl linoleate emulsion with hemoglobin and measured the oxygen consumption by a rapid polarographic method. This procedure was modified by Cort (20), who determined the activity of several food antioxidants by measuring oxygen removal with an oxygen analyzer. Tappel (4) has proposed a reaction mechanism for the antioxidant effect on the heme-catalyzed oxidation.

The measurement of oxygen uptake in a dynamic system has, however, proven difficult to standardize and reproduce (21). We therefore carried out a study on the effects of BHA, PG, TO and EMQ on the hemoglobin-catalyzed peroxidation of linoleic acid using the method developed in our laboratory (1).

EXPERIMENTAL PROCEDURES

Instrumental techniques have already been described (1).

Reagents

Hemoglobin from beef blood (type I) and DL-α-tocopherol (Vitamin E) were obtained from Sigma Chemical Co. (St. Louis, MO). The other reagents were as described previously (1,2).

Analytical Procedure

Hemoglobin stock solutions (5 mg/ml, 3 mg/ml and 0.5 mg/ml) were prepared in ice-cold 0.2 M phosphate buffer (pH = 7.0), stored at 5 C and kept in an ice-water bath while in use. About 0.2 ml was added to the reaction mixture. The amount of hemoglobin was calculated from constant weight ratio of substrate and catalyst. Stock solutions of fatty acid, antioxidants and internal standard