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Evaluation of Several Natural and Synthetic Antioxidants By the Induction Period Method

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Abstract

Motivation.. Quantitative in vitro studies of the antioxidants and the inhibition of autooxidation by various antioxidants have been performed under aerobic conditions and hence, appeared to be critically reviewed, because the effectiveness of antioxidants was not related to that of biological system. Since the biological system is in lower oxygen tension, the effectiveness of antioxidants may be considerably different between nonbiological and biological conditions. To clarify this points, we evaluated here the efficiency of natural and synthetic antioxidants and free-radical scavengers by the induction period method, under nearly unaerobic conditions

Method. Polymerization of methyl methacrylate in the presence or absence of antioxidants was carried out by BPO or AIBN at 70 □ using a DSC. Based on the time-conversion curves the induction period and initial rate of polymerization were determined. Stoichiometric factors (*n*) and k_{inh} for antioxidants and free-radical scavengers were calculated**.**

Results. The *n* for the AIBN system (BPO system) was as follows. *a*-T:1.7 (0.3), BHA: 2.2 (2.0), BHT:1.5 (2.4), DPA:2.95 (0.1), DPPH:1.9 (1.0), galvinoxyl:0.3 (0.4), trolox: 2.2 (0.04). The *n* of *a*-T, trolox, DPPD and DPPH for the BPO was markedly less than that for the AIBN system. The *n* of BHA and BHT for the both system was about 2. The k_{inh} of trolox, DPPD and galvinoxyl was markedly grater than that for the AIBN.

Conclusions BHA and BHT can be an efficient scavenger that scavenged about two free-radicals, whereas *a*-T, trolox and DPPD cannot be an efficient scavenger of BPO radicals. The effectiveness of antioxidants that is involved in biological system should be measured under nearly unaerobic conditions.

Keywords. Antioxidants; stoichiometric factors (n); inhibition rate constants; radical polymerization of methylmethacrylate ; BPO; AIBN.

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Abbreviations and notations

AIBN: 2,2'-azobisisobutyronitrile; BPO: benzoyl peroxide; α-T: α-tocopherol; DPPD: *N.N*'-diphenyl-1,4-phenylenediamine; DPPH: 2, 2-diphenyl-1-picrylhydrazyl; BHA: 2-*tert*-butyl-4-methoxyphenol; BHT: 2, 6-di-*tert*-butyl-4-methylphenol; *n*: stoichiometric factor)(*i.e.* numbers of free radicals trapped by 1 mole phenol or amine); k_{inh}: inhibition rate constant; DH(Ar(O-H)): the O-H bond dissociation enthalpy; SAR: structure-activity relationship

1 INTRODUCTION

Natural and synthetic antioxidants are widely used in the food industry. Antioxidants have been implicated in preventing various diseases. It is therefore important to understand the behavior of antioxidants and to have measures of the rates at which they react with free radicals such as peroxy radicals, alkyl radicals, reactive oxygen species and NO radicals. There have been numerous quantitative studies, using induction period methods, of the reactivity of antioxidants (*e.g.* α-T, BHA and BHT) with peroxy radicals and of their inhibition of auto-oxidation [1-5]. However, these studies have used *in vitro* systems, because more biologically relevant systems are complex and not stoichiometric, making quantitative study difficult. These *in vitro* studies have been critically reviewed, possibly due to the differences between cell-free and biological conditions. We thought that these differences may be associated with the oxygen levels in the experimental systems. The

oxygen tension under a 15 torr oxygen atmosphere is similar to that in many tissues [6], suggesting that oxygen is scarce in living cells. Since previous quantitative *in vitro* studies were performed under aerobic conditions, the efficiency of antioxidants such as α-T, BHA and BHT may differ considerably between aerobic and anaerobic conditions. We previously reported evaluation of the radical-scavenging ability of polyphenols and β-carotene related-compounds by induction methods [7-9]. In the present study, we investigated the radical-scavenging activity of 1 natural and 4 synthetic antioxidants and 2 free-radical scavengers in the polymerization of methyl methacrylate (MMA) initiated by thermal decomposition of AIBN or BPO, using the induction period method. For each antioxidant, the stoichiometric factor (*n*) (i.e. the number of free radicals trapped by 1 mole of phenol) and the inhibition of rate of polymerization (R_{inh}) were determined. The kinetic effects of antioxidants are discussed from an SAR perspective.

2 MATERIALS AND METHODS

Antioxidants were purchased from commercial sources. MMA was purified by distillation. AIBN and BPO were recrystallized from methanol and chloroform methanol system (1:2.5 v/v), respectively.

2.1 Chemical Data

2.1.1 Stoichiometric factors (*n*)

The *n* values were determined by the induction period method previously reported (9). In brief, the experimental resin consisted of MMA and BPO or AIBN with or without antioxidants or radical scavengers, *i.e.* inhibitors. The concentration of BPO or AIBN was 1 mole% and that of the inhibitors was 0-0.5 mole%. About 10 microL of the experimental resin (MMA) was loaded into an aluminum sample container and sealed by applying pressure. The container was placed in a differential scanning calorimeter (model DSC 3100; MAC Science Co., Tokyo, Japan) maintained at 70 °C. Thermal changes induced by polymerization of MMA were 13.0 kcal/mole in this experiment. Polymerization curves were derived from DSC thermograms using the integrated heat evoked by polymerization of MMA. Curves of inhibitors in the AIBN or BPO system are shown in Figure 1. The time-conversion curves in the absence (control) and the presence of inhibitors showed a break when the additives had been completely consumed. These breaks were sharp and provided a reliable measure of the induction (inhibition) period and initial rate of polymerization. Such a break was detected in the absence of inhibitor (control) because oxygen acts as an inhibitor. In the present study, the induction period (τ) for test compounds was presented as the difference from controls. Tangents were drawn to polymerization curves at an early stage in the run. The τ of test compounds was determined from the length of time between the zero point on the abscissa and the point of the intersection of tangents drawn to the early stage of polymerization. The τ values were calculated from the difference in the induction periods between the test compounds and controls. The *n* values were calculated per one unit of phenolic moiety by using Eq. (1):

$$n = \tau R_i / [IH] \quad \text{(1)}$$

in which R_i (the rate of initiation) for AIBN and BPO was 2.28×10^{-6} and 5.66×10^{-6} $Ml^{-1}s^{-1}$ at 70 °C, respectively. These R_i values were determined by the induction period method using 2,6-di-*tert*-butyl-4-methoxyphenol (DTBMP) with a stoichiometric factor of 2.00 [2]. IH represents inhibitor.

2.1. 2 Inhibition rate constants (k_{inh})

The k_{inh} values were calculated from the equations described below. The initial rate of polymerization (R_p) and the inhibited rate of polymerization (R_{inh}) were calculated from the slope of the first linear portion of the conversion rate of polymerization of MMA (Figure 1). R_p and R_{inh} were calculated from Eq. (2) and Eq. (3), respectively [1-5,7,8]:

$$R_p = \{k_p / (2\,k_t)^{1/2}\} / \{[MMA]\,R_i^{1/2}\} \quad \ldots\ldots\ldots\ldots \quad (2)$$

$$R_{inh} = \{k_p\,[MMA]\,R_i\} / \{n\,k_{inh}\,[IH]\} \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

where k_p and k_t represent the rate constants of propagation and of termination, respectively, and MMA is methyl methacrylate.
The rate of decrease in polymerization curves can be expressed by Eq. (4):

$$R_{inh}/R_p = \{(2\,k_t\,R_i)^{1/2}\} / \{n\,k_{inh}\,[IH]\} \quad \ldots\ldots\ldots\ldots\ldots\ldots (4)$$

3 RESULTS AND DISCUSSION

We investigated the relative activities of α-T, BHA, BHT, DPPD, DPPH, trolox and galvinoxyl at 0-0.05 mole% in the AIBN and BPO systems. The τ values for these compounds increased linearly in a dose-dependent manner (data not shown). We present here data on the reactivity of the natural and synthetic antioxidants and free radical scavengers at concentrations of 0.05 mole% (Table 1 and Figure 1).

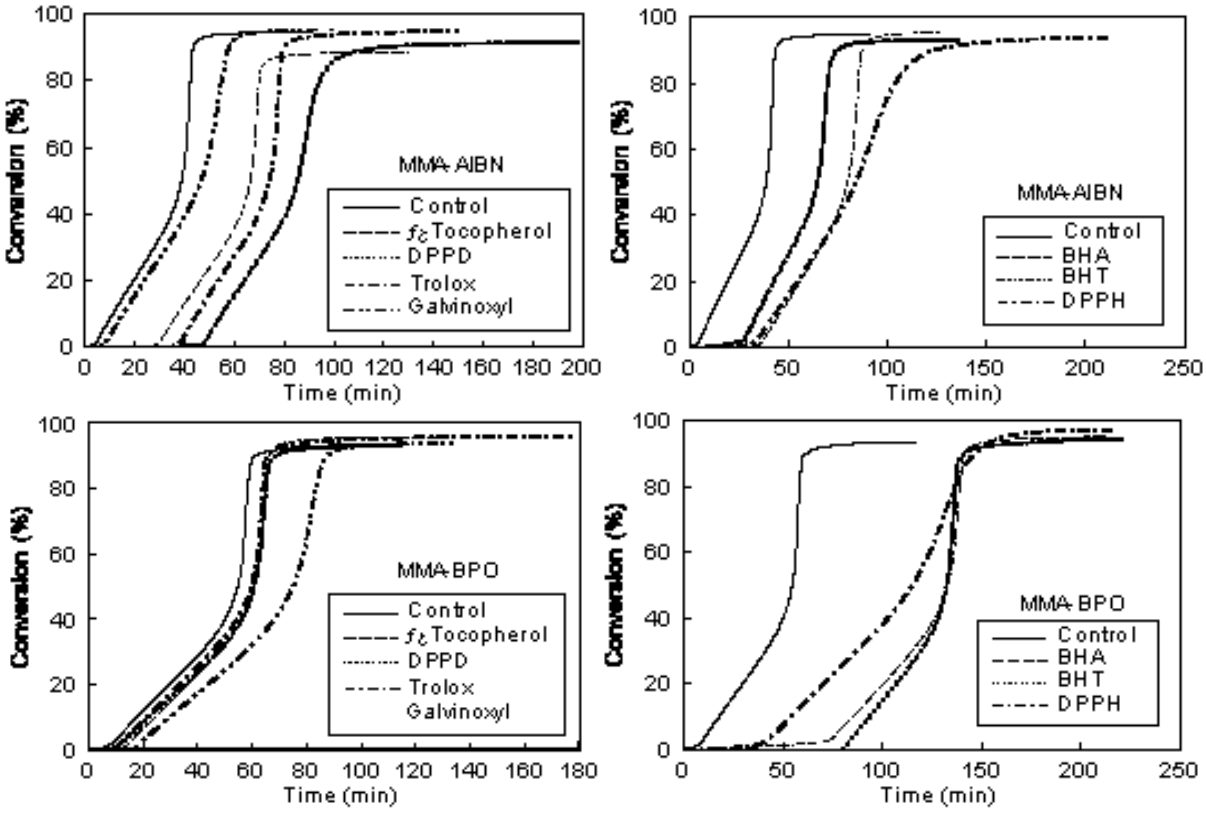

Figure 1 Time-conversion curves for the polymerization of MMA in the presence of additives

Table 1. Induction period, stoichiometric factors (*n*), R_{inh}/R_p and K_{inh} for antioxidants and free radical scavengers

A) AIBN

Antioxidants	Induction period s	*n*	R_{inh}/R_p	$K_{inh}x10^{-3}$ $M^{-1}s^{-1}$
ƒ¿-Tocopherol	1,532	1.73	0.84	2.94
BHA	1,912	2.16	0.71	2.70
BHT	1,328	1.50	1.01	0.71
DPPD	2,614	2.95	0.91	1.60
DPPH	1,652	1.87	0.62	3.70
Trolox	1,984	2.24	0.93	2.00
Galvinoxyl	232	0.26	0.85	1.94

B) BPO

Antioxidants	Induction period s	*n*	R_{inh}/R_p	$K_{inh}x10^{-3}$ $M^{-1}s^{-1}$
ƒ¿-Tocopherol	592	0.31	0.95	9.40
BHA	3,790	2.01	0.83	2.30
BHT	4,463	2.37	0.90	1.30
DPPH	1,824	2.37	0.64	4.50
Trolox	53	0.04	0.93	107.50
Galvinoxyl	660	0.35	0.73	10.90

The *n* and K_{inh} were calculated from Eq (1) and Eq (4), respectively (See text) R_{inh} and R_p reprent the initial rate of polymerization and inhibited rate of polymerization of MMA, respectively. MMA: 9.4mol/l, AIBN or BPO: 1 mol%

The *n* and k_{inh} were calculated by Eq. (1) and Eq. (4), respectively. The *n* of DPPH was approximately 2,

suggesting the regeneration of DPPH during the induction period because DPPH scavenges one radical. Since the peroxy radicals (ROO□) produced by the reaction of AIBN with oxygen do not cause the regeneration of DPPH [10], the radical species in the present AIBN system must have been alkyl radicals (R□), *i.e.* cyanoisopropyl radicals. In contrast, the n of DPPH for the BPO system was approximately 1.0, indicating that DPPH scavenged one benzoate radical (ROO□). Based on this finding, R_i was determined from Eq. (1), producing 2.74×10^{-6} $Ml^{-1}s^{-1}$. This value agreed well with that for DTBMP (n = 2.00) measured by us in this study. In contrast, the n value of galvinoxyl for both the AIBN and the BPO systems was 0.2-0.35, with a relatively greater decrease in R_{inh}/R_p values. The long-lived free radical galvinoxyl can produce coupling products with AIBN or BPO [11], and therefore showed less reactivity by the present induction method.

One of the most striking results from our study became apparent when the n values for α-T, trolox and DPPD in the AIBN and BPO systems were compared. Their values (n = 0.04-0.31) for BPO were markedly less than those (n = about 2) for AIBN, suggesting the lower reactivity of these compounds toward peroxy radicals. Fully oxidized α-T scavenges two radicals [2,12] but the n of α-T for AIBN was 1.73, suggesting dimerization of this compound [12]. The n for DPPD (about 3.0) suggested that oxidized DPPD-producing substances could scavenge radicals because fully oxidized DPPD scavenges two radicals [10]. Next, we examined n and k_{inh} for the synthetic antioxidants BHA and BHT. Interestingly, the n or k_{inh} for BHA and BHT did not depend on the radical species, *i.e.* they did not differ between BPO or AIBN. This was greatly different from the results for α-T, trolox and DPPD. BHT, a hindered phenol, is known to have a noticeable steric hindrance to the approach of radical species. The n = 1.5 of BHT in the AIBN system suggested dimerization due to the *para-para* coupling reaction of this compound, producing a stilbenequinone. Okatsu *et al*. previously reported n = 1.71 for BHT *via* the formation of a stilbenequinone [1]. In contrast, BHA, a less-hindered phenol with a single *ortho tert*-butyl group, showed n = about 2. In general, less hindered phenols cannot intercept two peroxy radicals, and consequently have low n values [1] . However, in the present study the n for BHA was 2, indicating that dimerization did not occur. It is of interest why the peroxy radical (ROO□) from BPO showed lower n values and rate retardation with α-T or trolox, but not with BHA or BHT. There is a known relationship between k_{inh} and the O-H bond dissociation enthalpy, DH(Ar(O-H)) (kcal M^{-1}) [2]. DH(Ar(O-H)) for α-T, BHA, BHT, DTBMP and galvinoxyl is 78.93, 82.19, 80.07, 77.61 and 78.80, respectively [13, 14]. However, no relationship was apparent in this study, because k_{inh} ($1\text{-}3 \times 10^3$ $M^{-1}s^{-1}$) for BHA, BHT and DTBMP was similar, despite greatly different values of DH(Ar(O-H)). On the other hand, DH(Ar(O-H)) for α-T was previously reported to be affected by addition of benzene or water due to the effect of the chroman-ring ether-oxygen atom [5]. Boozer *et al*. previously reported that some hydrocarbon inhibitors function by reactivity with alkyl radical (R□) rather than alkyl peroxy radical (ROO□) at low oxygen pressure [11]. This suggests that the reactivity of the chroman-ring ether-oxygen atom in α-T and trolox might be modulated by ROO□ derived from BPO at low oxygen pressure, because the n of α-T and trolox for the BPO system was close to zero. The lower n values of α-T or trolox and, consequently, the retardation by inhibitor radicals derived from BPO-induced α-T or trolox, may be associated with the effect of the modulated chroman-ring ether-oxygen atom. Assuming the usual reaction of polymerization, where one inhibitor radical stops one growing radical, a relationship between the n and the k_{inh} values could be found. The k_{inh} values were markedly enhanced when n was close to zero.

The k_{inh} values ($\times 10^4$ $M^{-1}s^{-1}$) for some antioxidants in the present study were compared with values previously reported. Burton and Ingold reported k_{inh} values for α-T (235) and BHT (1.2) in benzene-styrene solution with AIBN [2]. Niki *et al* reported a k_{inh} for α-T (51) in methyl linoleate [3] . Ohkatsu *et al*. reported a k_{inh} for BHT (2.2) in AIBN-initiated auto-oxidation of styrene in chlorobenzene at 70 °C [1]. Also, Pryor *et al*. reported that the k_{inh} values for α-T and BHT in aqueous sodium dodecylsulfate micelle solution with an azo-initiator were 3.7 and 1.1, respectively [5]. Furthermore, Mukai *et al*. reported a k_{inh} for α-T (0.5) with a stopped flow technique for phenoxy radicals [4]. The k_{inh} for α-T in the present study was in satisfactory agreement with that obtained by Mukai *et al*. These results indicate large variations of the

k_{inh} for α-T, by one or two orders of magnitude. It is of interest why the k_{inh} for α-T exhibits such great variation. The experiments of Burton and Ingold, and also of Niki *et al*., were performed under aerobic conditions, whereas our experiment was performed under nearly anaerobic conditions. When the addition of oxygen is efficient, the k_{inh} for antioxidants appears to become markedly greater. This change of inhibition rate may be ascribed to effects of oxygen on substrates such as styrene, methyl linoleate or linoleic acid, and additionally on the antioxidants themselves. To evaluate the effects of antioxidants on biological activity, their efficiency should be measured at low oxygen tension. Our findings suggest that the k_{inh} values for antioxidants in biological systems may be considerably less than those previously reported. A detailed examination of this problem requires investigation of other radical species.

4 CONCLUSIONS

The *n* and k_{inh} for antioxidants were determined under nearly anaerobic conditions. The *n* of α-T, trolox and DPPD for the BPO system was close to zero, whereas that for the AIBN system was about 2. The *tert*-butyl-substituted phenols BHA and BHT showed an *n* of 2 for both systems. The k_{inh} was markedly enhanced when *n* was close to zero. The k_{inh} of trolox for the BPO system was the largest, followed by that of DPPD. The present results suggest that previously reported k_{inh} values for antioxidants evaluated by the induction period method may be of doubtful biological relevance because the *in vitro* quantitative evaluations were performed under aerobic conditions. Measurements of antioxidants should be performed under nearly anaerobic conditions, since biological systems are at a low oxygen pressure.

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