

In vitro studies on calf thymus DNA interaction and 2-*tert*-butyl-4-methylphenol food additive

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Abstract The interaction of native calf thymus DNA (CT-DNA) with 2-*tert*-butyl-4-methylphenol (TBMP) at physiological pH has been investigated by spectrofluorometric and viscosimetric techniques. TBMP molecules were found to intercalate between base pairs of DNA, demonstrated by an increase in the specific viscosity of DNA and decrease in the fluorescence of TBMP solutions in the presence of increasing amounts of DNA and the calculated binding constants (K_f) at different temperatures. Furthermore, the enthalpy and entropy of the reaction between TBMP and CT-DNA showed that the reaction is exothermic and enthalpy favored ($\Delta H = -19.18 \text{ kJ mol}^{-1}$; $\Delta S = -26.98 \text{ J mol}^{-1} \text{ K}^{-1}$) which are other evidences to indicate that TBMP is able to be intercalated in the DNA base pairs.

Keywords CT-DNA · *tert*-Butyl-4-methylphenol · Intercalation

Introduction

The synthetic phenolic antioxidants are frequently used to prevent food, pharmaceutical, and other commercial

products from oxidative rancidity. Various studies have shown that they could enter human body through the intake of foods, pharmaceuticals, etc. Therefore, the use of these additives is subject to regulations which define the permitted compounds and their concentration limits in animal and human food [1].

2-*tert*-Butyl-4-methylphenol (TBMP) is one of the synthetic phenols which are used as intermediates for the preparation of antioxidants and UV stabilizers added in rubbers, plastics, foods, and oils to inhibit or slow oxidative process. It has been considered that the antioxidant action of phenols depends on the hydrogen-donating capacity of a hydroxyl group in each molecule [2].

The synthetic antioxidants have been very thoroughly tested for their toxicological capacity, but some of them are coming after a long period of use or under heavy pressure as new toxicological data accumulate, which impose some cautions in their use [3]. When radicals oxidize phenolic antioxidants during the induction time, reactive intermediates such as quinone methides, quinones, and dimer can be formed which suggested that toxicological capacity of phenols may be dependent on radical reactions [4]. *p*-*tert*-butylphenol (PTBP) and 2-*tert*-butyl-4-methylphenol induced pronounced hyperplasia and papillomas in the hamster forestomach [5]. These findings indicate that PTBP and TBMP may cause carcinogenic effects for hamster forestomach after long-term administration. Moreover, both 1-hydroxy and *tert*-butyl substituents may have an active role at inducing hamster forestomach tumors [4].

In the present investigation in vitro interaction of TBMP with native calf thymus DNA in 10 mM Tris-HCl aqueous solutions at neutral pH 7.4 was examined in more details, utilizing spectrofluorometric and viscosimetric procedures.

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Materials and methods

Chemical and materials

The highly polymerized CT-DNA and Tris–HCl were purchased from Sigma Co. All solutions were prepared using double-distilled water. Tris–HCl buffer solution was prepared from (tris-(hydroxymethyl)-amino-methane–hydrogen chloride) and pH was adjusted to 7.4. The stock solution of DNA was prepared by dissolving CT-DNA in 10 mM of Tris–HCl buffer at pH 7.4 and dialyzing exhaustively against the same buffer for 24 h and used within 5 days. A solution of CT-DNA gave a ratio of UV absorbance at 260 and 280 nm more than 1.8, indicating that DNA was sufficiently free from protein [6]. The concentration of the nucleotide was determined by UV absorption spectroscopy using the molar absorption coefficient ($\epsilon = 6,600 \text{ M}^{-1} \text{ cm}^{-1}$) at 260 nm. The stock solution was stored at 4 °C. A TBMP stock solution ($1 \times 10^{-3} \text{ M}$) was prepared by dissolving an appropriate amount of compound in Tris–HCl buffer/DMSO (9:1). It has been verified that the low DMSO percentage added to DNA solution would not interfere with the nucleic acid DO measurement [7].

Instrumentation

Fluorescence quenching

All fluorescence measurements were carried out with a JASCO spectrofluorometer (FP6200). A 250 nm excitation wavelength was used for the fluorescence measurements and the emission spectra were recorded between 300 and 400 nm. The excitation and emission slits were both 5 nm, and the scan speed was 125 nm min^{-1} . Two milliliters of the TBMP (10^{-5} M) were placed in a 1-cm thermostated quartz fluorescence cuvette and titrated with 20 μL aliquots of 0.325 mM DNA with continuous stirring. After each titration, the solution was mixed thoroughly and was allowed to be equilibrated thermally for 5 min prior to the fluorescence measurements. The control fluorescence spectrum, TBMP solution titrated with 20 μL aliquots of the buffer, was also measured under the same conditions and was used to correct the observed fluorescence. The dilution effects of fluorescence titration experiments were performed by keeping the TBMP concentration constant and stoichiometrically varying the DNA concentration [8, 9].

Viscosity measurements

A viscosimeter (SCHOT AVS 450) thermostated at 25 °C in a constant temperature bath was used for viscosity

measurements. Flow time was measured with a digital stopwatch; the mean values of three replicated measurements were used to evaluate the viscosity (η') of the samples. The data were reported as η'/η'° versus the [TBMP]/[DNA] ratio, where η'° is the viscosity of the DNA solution alone [10].

Results and discussion

Fluorescence spectroscopic studies

Since luminescence was observed for the TBMP solution, it is possible to monitor the interaction of TBMP with DNA by employing direct fluorescence emission methods, so in order to investigate the interaction mode between the TBMP and CT-DNA; the fluorescence titration experiments were performed. TBMP can emit luminescence in Tris–HCl buffer in two areas from 285 to 400 nm and from 580 to 700 nm with maximum wavelengths of about 310 and 615 nm, but we preferred to work from 285 to 400 nm because this peak was sharper and better than the second peak. Figure 1 shows the emission spectra of TBMP in the absence and presence of varying amounts of CT-DNA. The emission intensity of TBMP decreases by increasing the amount of added DNA, which indicates that there is an interaction between DNA and TBMP.

Temperature effect on quenching efficiency

The titration data which were obtained from the interaction study of TBMP with DNA at different temperatures were fitted into the Stern–Volmer (Eq. 1). [9]:

$$F_0/F = 1 + K_{sv}[\text{DNA}] \quad (1)$$

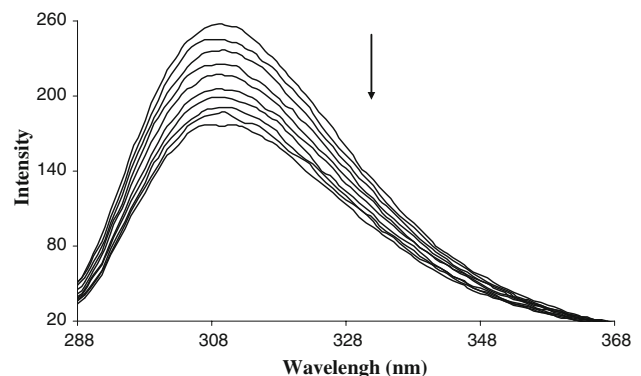


Fig. 1 Emission spectra of the TBMP in the presence of the increasing DNA concentrations in 0.01 M Tris–HCl buffer (pH 7.4) at room temperature ($r_i = [\text{DNA}]/[\text{TBMP}] = 0.0, 0.3, 0.6, 0.9, 1.2, 1.5, 1.8, 2.1, 2.4$ and 2.7), the emission intensity of TBMP decreases by increasing the amount of added DNA

where F_0 and F are the fluorescence intensities of the probe in the absence and presence of DNA, respectively, and K_{sv} is Stern–Volmer quenching constant which can be considered as a measure for efficiency of fluorescence quenching by DNA.

A variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangement, energy transfer, ground-state complex formation, and collisional quenching. Quenching normally refers to non-radiative energy transfer from excited species to other molecules. In fact, two quenching processes are known: static and dynamic. Dynamic quenching or collisional quenching requires contact between the excited lumophore and the quenching specie, the quencher. The rate of quenching is diffusion controlled and depends on temperature and viscosity of the solution. The quencher concentration must be high enough that the probability of collision between the analyte and quencher is significant during the lifetime of the excited species. As mentioned above, the other form of quenching is static quenching in which the quencher and the fluorophore in ground state form a stable complex. Fluorescence is only observed from the unbound fluorophore. The lifetime is not affected in this case; measurement of the lifetime provides a mean to distinguish between dynamic and static quenching [11, 12].

Dynamic and static quenching can also be discerned by their differing dependent on temperature [13]. Dynamic quenching depends upon diffusion. Since higher temperatures lead to larger diffusion coefficients, the K_{sv} can be increased by rising the temperature. In contrast, increased temperature is likely the result of decrease in complex stability, and thus lower values of the static quenching constants were resulted. The K_{sv} of TBMP fluorescence by DNA at different temperatures (283, 293, 303, and 310 K) was obtained and the results are shown in Table 1 (Fig. 2). These results show that DNA can quench TBMP fluorescence in a dynamic quenching procedure, because the K_{sv} has been increased by temperature rising [11, 14].

Equilibrium binding titration

Fluorescence titration data were used to determine the binding constant (K_f) and the binding stoichiometry (n) for

Table 1 The K_{sv} values of TBMP–DNA at different temperatures

Temperature (K)	$K_{sv} (\times 10^4)$	R^2
283	1.30	0.9923
293	1.43	0.9927
303	1.65	0.9945
310	1.75	0.9919

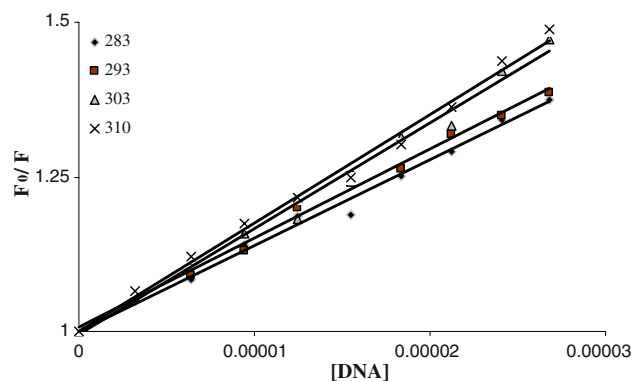


Fig. 2 Stern–Volmer plots of the quenching of fluorescence of TBMP with DNA at different temperatures (283, 293, 303 and 310 K)

the complex formation of TBMP with CT-DNA. It can be seen that the fluorescence intensity at 310 nm decreases in the presence of CT-DNA. This change in fluorescence intensity at 310 nm was used to estimate K_f and n for the binding of TBMP to CT-DNA from the following equation (Eq. 2) [15]:

$$\log(F_0 - F)/F = \log K_f + n \log[\text{DNA}] \tag{2}$$

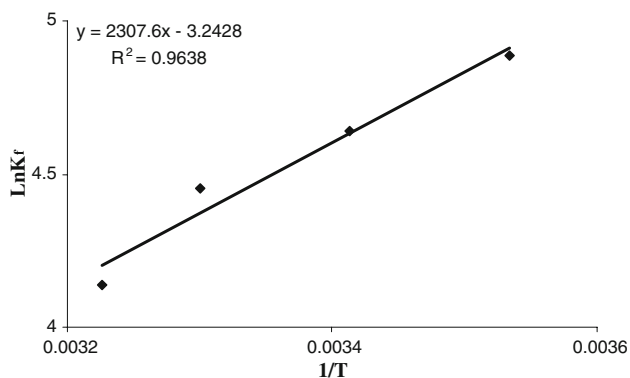
Here, F_0 and F are the fluorescence intensities of the fluorophore in the absence and presence of different concentrations of CT-DNA, respectively. The linear equations of $\log(F_0 - F)/F$ versus $\log[\text{DNA}]$ at different temperatures are shown in Table 2. The values of K_f clearly indicate the remarkably high affinity of TBMP for DNA. By comparing the calculated K_f value for TBMP and CT-DNA interaction with distinctive intercalators, it would be clear that the binding mode is intercalation of TBMP into DNA base pairs [16–18].

Thermodynamic studies

In order to have a better understanding of thermodynamic of the complexation reaction between TBMP and DNA, contributions of enthalpy and entropy should be determined in the reaction. Thermodynamic parameters describing the binding reactions can be divided into three contributions. The first contributions are due to hydrogen bonding and hydrophobic interactions between the TBMP and DNA binding sites. The next contribution is from the conformational change in either the nucleic acid or the TBMP upon binding. Finally, there are contributions from coupled processes such as ion release, proton transfer, or changes in the hydration water [19]. Evaluation of the formation constant for the TBMP–DNA complex at four different temperatures (283, 293, 303, and 310 K) allows thermodynamic parameters of TBMP–DNA formation via Van’t Hoff equation to be determined (Eq. 3) [20]:

Table 2 Linear equations of $\log(F - F_0)/F$ versus $\log[\text{DNA}]$, and K_f of TBMP with DNA at different temperatures

Temperature (K)	Linear equation	K_f
283	$Y = 1.1453X + 4.88$	7.69×10^4
293	$Y = 1.0918X + 4.64$	4.39×10^4
303	$Y = 1.0430X + 4.45$	2.82×10^4
310	$Y = 0.9778X + 4.13$	1.37×10^4

**Fig. 3** Van't Hoff plot for TBMP–DNA complex

$$\ln K_f = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

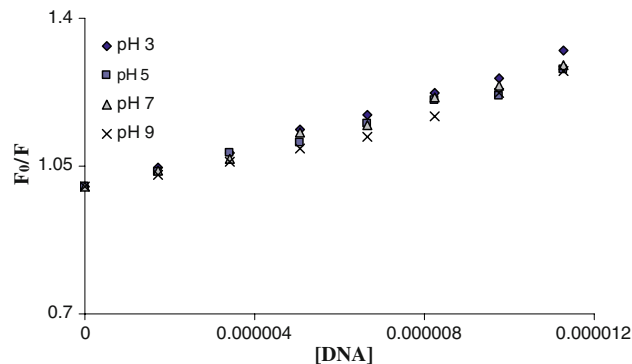
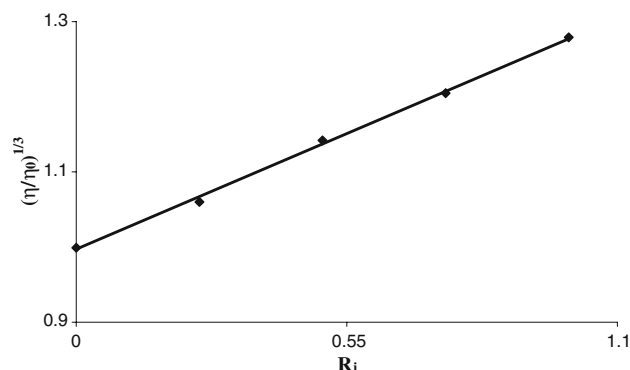
By plotting $\ln K_f$ versus $1/T$ (Fig. 3), ΔH and ΔS were determined. Knowing these two values, ΔG was calculated from the following standard equation (Eq. 4) [21]:

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

The results are shown in Table 3. The ΔH and ΔS values of the TBMP–DNA complex were $\Delta H = -19.18 \text{ kJ mol}^{-1}$ and $\Delta S = -26.98 \text{ J mol}^{-1} \text{ K}^{-1}$, respectively. It seems patent that the reaction is exothermic and enthalpy favored [22]. From the thermodynamic data, it is quite clear that while complex formation is enthalpy favored, it is also entropy disfavored. Therefore, formation of the complex results in a more ordered state, possibly due to the fixing of the motional freedom of both the TBMP and DNA molecules. In addition, negative entropy confirms the intercalative binding mode of TBMP to DNA [22].

Table 3 Thermodynamic parameters and binding constants for binding of TBMP to CT-DNA

T (K)	$\log K_f$ (M^{-1})	ΔG° (kJ mol^{-1})	ΔH° (kJ mol^{-1})	ΔS° ($\text{J mol}^{-1} \text{K}^{-1}$)
283	4.88	-11.557	-19.18	-26.96
293	4.64	-11.287	-19.18	-26.96
303	4.45	-11.018	-19.18	-26.96
310	4.13	-10.829	-19.18	-26.96

**Fig. 4** Stern–Volmer plots of the quenching of fluorescence of TBMP with DNA at different pH (3, 5, 7 and 9)**Fig. 5** Effect of increasing amounts of TBMP on the viscosity of CT-DNA ($5 \times 10^{-5} \text{ M}$) in 0.01 M Tris–HCl buffer (pH 7.4; $r_i = [\text{TBMP}]/[\text{DNA}] = 0.0, 0.25, 0.5, 0.75$ and 1)

The effect of pH

To clarify whether the only mode of TBMP interaction with DNA is via intercalation, its interaction at different pHs from 3 to 9 was conducted using fluorimetry. K_{sv} values for each of the pHs were calculated by means of Stern–Volmer equation (Eq. 1) [9].

No changes in K_{sv} have been observed at different pHs (Fig. 4). This suggests that there is no an electrostatic interaction or other outside binding such as hydrogen bonding between TBMP and DNA rather than intercalation which also matches other researchers' findings as well [8, 23].

Viscosity study

To further clarify the nature of the interaction between the TBMP and DNA, viscosity measurements were carried out by varying the concentration of TBMP added to DNA solution. Optical photophysical probes provide necessary, but not sufficient, evidence for supporting binding type of TBMP with DNA. Hydrodynamic measurements that are sensitive to length change (i.e., viscosity and sedimentation) are regarded as the least ambiguous and the most critical tests of a binding model in solution in the absence of crystallographic structural data [24, 25]. Intercalation is expected to lengthen the DNA helix as the base pairs are pushed apart to accommodate the bound ligand, leading to an increase in the DNA viscosity [26, 27]. In Fig. 5, the specific viscosity of the DNA sample clearly increases with the addition of the TBMP, which is indicative of TBMP insertion between base pairs. The viscosity studies provide a strong argument for intercalation [11].

The viscosity increase of DNA is ascribed to the intercalative binding mode of the TBMP molecule, due to effective DNA length increase [28]. So, it is deduced from viscosity measurements that the main mode of interaction between TBMP and DNA is an intercalation mode.

Conclusions

According to the results arise from fluorescence spectroscopy and viscosity, we conclude that TBMP binds to CT-DNA with a high affinity through intercalation mode, which could cause conformational changes in CT-DNA and therefore putative changes in the genic expression/regulation activity. Combining our result and that of the other researchers, we claim that it is about time to make a thorough analysis on TBMP widespread usage in food industry as an antioxidant candidate.

Moreover, it should be noted that the TBMP concentration which was used in this study (5×10^{-6} M) is much less than that of currently used as an antioxidant in food industry.

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