Possible Arabinogalactan Stabilization of Unstable Phenoxy Radicals Produced by Horseradish Peroxidase

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Radical interactions between arabinogalactan (ABGT) and unstable phenoxy radicals are examined through EPR spectroscopy. Horseradish Peroxidase production of 3,5-di-t-butyl-4-hydroxyanisole (DTBHA) and 2-t-butyl-4-methoxyphenol (BHT) radicals were successful. ABGT was expected to increase radical stability, but data for both radical species did not prove this expectation. DTBHA radicals without ABGT remained more stable than DTBHA radicals with ABGT. This finding supports that ABGT does not increase radical stability. However, BHT radicals without ABGT were very short-lived when compared to the BHT radicals with ABGT. The two are not comparable however, because an unknown signal was discovered with the BHT radicals with ABGT. This unknown may have occurred from the dimerization of BHT molecules. This finding supports that ABGT increases radical stability in an unforeseen manner.

INTRODUCTION

The polymer Arabinogalactan (ABGT) is a food additive that is used in many gums and is produced in plants to seal wounds and in organisms to serve as a cell signaling molecule. It is a polysaccharide that contains galactose and arabinose in a six to one ratio, respectively. In previous experiments, regarding carotenoid radicals produced by photolysis, ABGT was found to increase the half-life decay by 100%.[1] This research was carried out with the carotenoid-ABGT complex immersed in a homogenous aqueous solution where water played a key factor in the increase of photostability.[1] ABGT was used in a similar manner to stabilize phenoxy radicals by surrounding them and sequestering them in a cage-like ABGT polymer.

A free radical is a positive, negative, or neutrally charged atom or molecule that contains unpaired electrons. In most cases, radicals are extremely chemically active and are typically very unstable, which results in a short lifespan. The free radicals used in combination with the ABGT polymer were produced through a catalytic process using the enzyme horseradish peroxidase (HRP). HRP is a well known peroxidase enzyme that contains heme as the active catalyst. Heme enzymes can catalyze the addition of oxygen to organic substrates through activation by H_2O_2 as well as other peroxides (Fig. 1).[2] The following, general equation portrays the HRP catalyzed oxidative reaction where H_2O_2 is used as the final electron acceptor:[2]

In order to observe the relative stabilities as well as the chemical interactions of the radicals, Electron Paramagnetic Resonance (EPR) spectroscopy was used in combination with a flat cell aspiration technique.

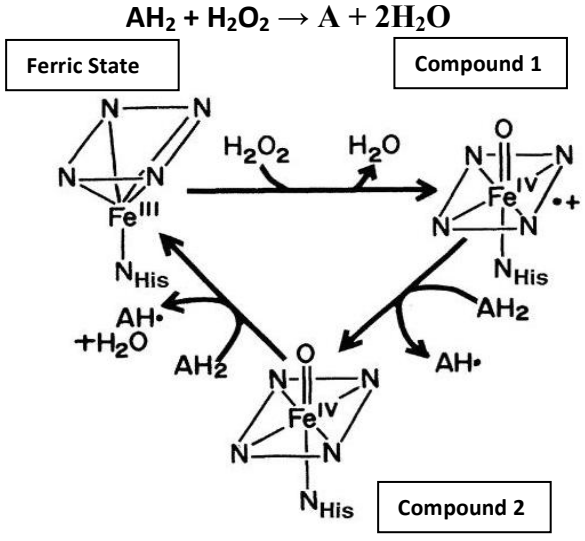

Figure 1: Here H_2O_2 activates the HRP resting ferric state through a double oxidation, thus producing compound 1 and H_2O. The first substrate reduces compound 1 into compound 2 and produces a radical intermediate. Then the second substrate reduces compound 2 which returns to the resting ferric state after the production of a second radical intermediate and H_2O.

The EPR spectroscopy instrument detects radicals by identifying unpaired electrons. After the unpaired electrons have been found, the spectrum can be assayed to discover the exact identity of the radical. The EPR instrument interacts with the experimental sample by applying a magnetic field. This field interacts with the unpaired electrons of the radical by causing them to occupy one of two energy

states: the high energy spin state or the low energy spin state (fig. 4).

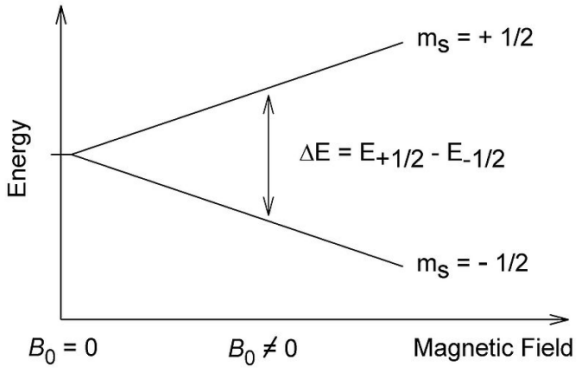

Figure 4: Here the two energy states, high (+1/2) and low (-1/2), are shown. Microwave energy absorption causes lower state electrons to excite to the higher state, but this transition only occurs when the microwave frequency and the energy difference of the two states are equal to each other.

The sample is subjected to microwave radiation which causes lower energy electrons to become excited. Radiation absorption takes place when the frequency of the radiation equals the energy difference between the magnetic moments.[1] This energy difference is known as the Zeeman Effect and is proportional to the strength of the magnetic field, $\boldsymbol{B_0}$. The energy of interaction can be formulated as:

$$\Delta \boldsymbol{E} = -\boldsymbol{\mu_E} * \boldsymbol{B_0} = (\boldsymbol{M_s \beta_E g_E}) * \boldsymbol{B_0}$$

Here $\mathbf{M_s}$ is the spin quantum number, $\boldsymbol{\beta_E}$ is the Bohr magneton, and $\mathbf{g_E}$ represents the g-factor.[4] The energy absorption can be converted into a graphical representation once the frequency of the radiation and the magnetic moment energy differences equal each other. The radical's unpaired electron also causes nearby nuclear spins to achieve different energy states, thus adding multiple peaks across the spectrum. This phenomenon is termed hyperfine interaction and results in the splitting of the EPR signal (Fig. 5).[3]

This splitting pattern can be predicted by using the formula:

$$\boldsymbol{2N_i I + 1}$$

where $\mathbf{N_i}$ is the number of equivalent nuclei of the associated spin **I** in set i.[4] Splitting interactions between two nuclei can be further represented by:

$$(\boldsymbol{2N_i I + 1})(\boldsymbol{2N_i I + 1})$$

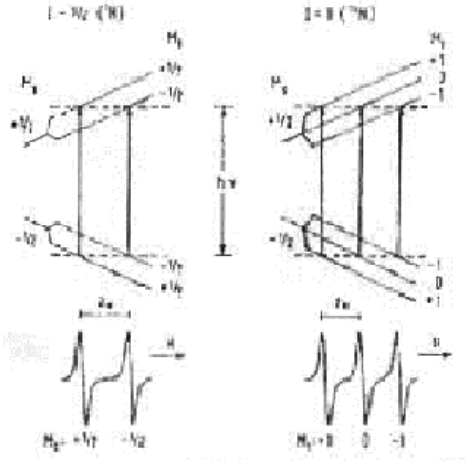

Figure 5: Hyperfine interaction. This diagram outlines the splitting of an EPR signal. This split is due to hyperfine interactions between unpaired electrons and a nucleus. The spectra that appear are derivatives of the absorption intensity

METHODS

The ABGT was purchased from the Food Science of Vermont company and was used to possibly stabilize the phenoxy radicals by surrounding them and sequestering them in a cage-like ABGT polymer. DTBHA and BHT were used from previous commercial samples recrystallized by former Hampden-Sydney College students. The HRP was purchased from Sigma-Aldrich. All instruments used, including the JEOL RE-1X EPR instrument, were provided by the Hampden-Sydney chemistry department. Ethanol and tris-HCl-buffer were used as obtained. All solutions, save ethanol, were kept refrigerated until use.

The radical samples were loaded into the EPR instrument through the use of the flat cell aspiration technique. This technique requires a steel tube to pull the solutions into the septum of the flat cell through the use of a vacuum. Any air bubbles or solution can be cleared through the use of de-ionized water, thus allowing the flat cell to be easily flushed. The flat cell is placed in the magnetic cavity so as to create an optimal space in which the microwave radiation as well as the magnetic field can interact with the unpaired electrons of the radical.

The Winsim program was used in order to interpret the data concerning radical signal strength and stability. It is an analytical program that correlates radical peaks and splitting patterns that are graphically represented in the form of EPR spectra. Winsim provides coupling values that are based on

the radical's nuclear interactions. This function aids in the identification of the desired radical spectra, and, in this experiment's interests, it allows the measurement of radiation absorption intensity.

For EPR measurement, a 5.02ml DTBHA sample was prepared; the concentrations are as follows: 1.01mM DTBHA, about 48% tris-HCl-buffer, about 48% EtOH, 10µg/ml HRP, 0.99mM H_2O_2. The solution was aspirated into the flat cell of the EPR instrument where a four-minute scan was conducted every five minutes for 75 minutes yielding 15 data points that were used in Winsim to measure the DTBHA radical decay rate. For the next step of the experiment, a 5.02ml DTBHA/ABGT solution sample was prepared with the following concentrations: about 48% ABGT (104.2µg ABGT/ml tris-HCL-buffer), 1.01mM DTBHA, about 48% EtOH, 10µg/ml HRP stock, and 0.99mM H_2O_2. After aspiration, a four-minute scan was conducted every five minutes for 85 minutes which yielded 17 data points that were simulated in Winsim in order to measure the DTBHA/ABGT radical decay rate.

For the next phase of the experiment, a 5.01ml BHT sample was prepared with the following concentrations: about 48% tris-HCl-buffer, 1.00mM BHT, about 48% EtOH, 10µg/ml HRP stock, and 50µl lf 1.00mMH_2O_2. After aspiration, EPR spectra were produced and simulated in Winsim. The last EPR measurement required scans of a 5.01ml BHT/ABGT solution. This solution contained the following concentrations: about 48% ABGT, 1.00mM BHT, about 48% EtOH, 10µg/ml HRP stock, and1.00mM H_2O_2. Following aspiration of the sample, the EPR instrument produced spectra which were simulated in Winsim. EPR conditions are explained in Figure 6, page 5.

RESULTS

The desired substrate used with HRP was 3,5-di-*t*-butyl-4-hydroxyanisole (DTBHA)(Fig. 2); in later experiments, 2-*t*-butyl-4-methoxyphenol (BHT)(Fig. 3) was substituted for DTBHA. DTBHA was chosen because it can be oxidized to a relatively more stable phenoxy radical than monosubstituted phenols. These radicals have been found to be more stable since both of the *ortho* positions are blocked by protecting groups in order to deter dimerization reactions.[3]

The initial experiments were geared towards the identification of the radicals produced from the DTBHA/ABGT solutions. The spectra yielded data that confirmed the radical identity as that of DTBHA when compared to data gained by Valoti *et al* (Fig. 6)[3]. EPR scans for DTBHA radicals without ABGT produced spectra that were confirmed in Winsim as DTBHA radicals.

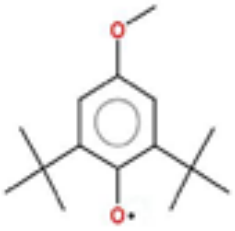

Figure 2: DTBHA radical. The two *t*-butyl blocking groups located at the ortho positions of the molecule allow greater radical stability.

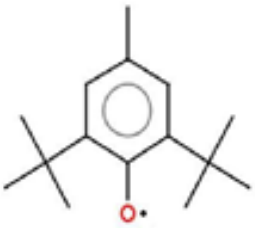

Figure 3: BHT radical. The two *t*-butyl blocking groups located at the ortho positions of the molecule allow greater radical stability by deterring radical dimerization.

After confirming the identities of the radicals, time sensitive scans were performed in order to produce decay rates for the DTBHA radicals (Fig. 7) and the DTBHA radicals in ABGT (Fig. 8).

For the DTBHA radical decay rate (Fig. 6), the arbitrary unit of signal intensity, which was measured in Winsim by measuring the global radical maximums and minimums and subtracting the two, was almost halved when compared to the DTBHA/ABGT radical decay rate (Fig. 7).

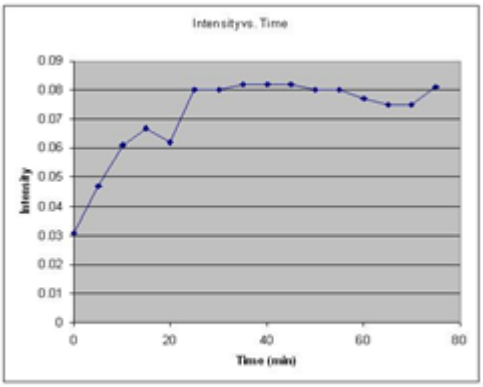

Figure 7: DTBHA radical decay rate: Signal intensity (Y-axis) vs. time in minutes (X-axis)

However, the DTBHA radicals remained more relatively stable and even increased in signal intensity at the seventy-fifth minute. The DTBHA/ABGT radical decay rate graph shows the H_2O_2 initiation of the reaction at the twenty-fifth minute and quickly drops to a signal intensity that can be considered noise around the sixtieth minute. A possible explanation for the ABGT-free DTBHA radicals' increased stability is

that the lack of ABGT molecules allows greater space between DTBHA radicals. In other words, the DTBHA radicals are not crowded by the ABGT molecules. In the DTBHA/ABGT radical solution, the DTBHA radicals are crowded and thus forced closer to each other which can result in radical neutralization through polymerization.

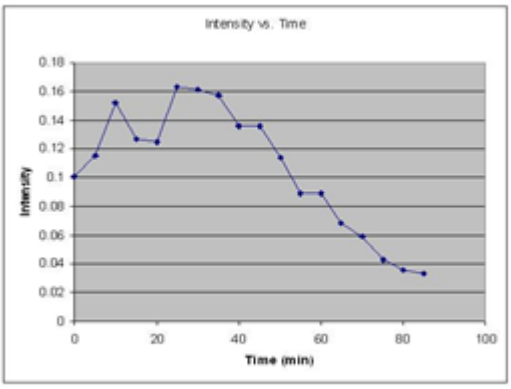

Figure 8: DTBHA/ABGT radical decay rate: Signal intensity (Y-axis) vs. time in minutes (X-axis)

EPR measurements of BHT without ABGT and BHT with ABGT produced mixed results. The BHT with ABGT sample produced a signal that showed the BHT signal as well as an unknown species signal between the methyl signals (Fig. 9). This signal may possibly arise from a dimer or polymer. It remained very stable when compared to the BHT without ABGT radical. Time sensitive scans were performed in hopes of producing a radical decay rate based on signal intensity.

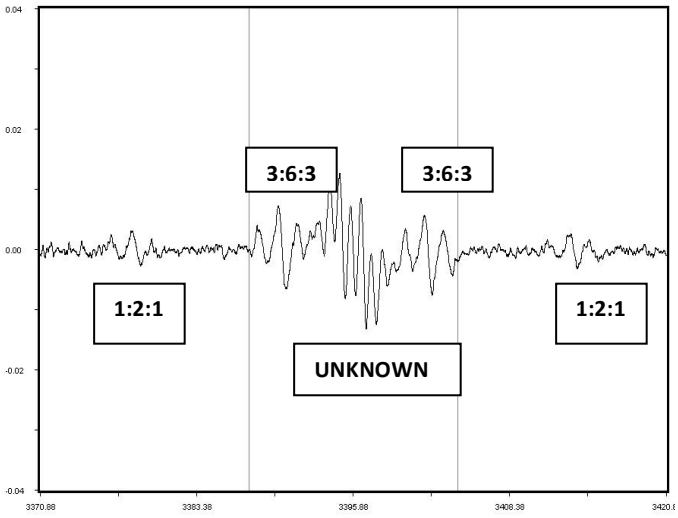

Figure 9: BHT/ABGT sample spectrum. The spectrum shows a second unknown species apart from the BHT radical. This species may have resulted from possible dimerization of two BHT radicals.

Between t=0min and t=80min, the BHT/ABGT radical's signal intensity changed very negligibly which confirms that the "dimer" was relatively very stable.

BHT without ABGT proved to be very unstable. The spectra showed that the signal intensity was comparably weak, and the radical decayed very quickly between t=0min and t=5min at which point the signal could be considered noise (Fig. 10).

The BHT without ABGT and BHT with ABGT data were not comparable in the end, so charts graphing signal intensity vs. time could not be produced. The possible dimerization of BHT radicals could have been induced through molecular packing by ABGT that could have pushed BHT molecules closer together thus resulting in radical interactions. This unknown species formation created a radical that was different from the BHT without ABGT radical. Due to this occurrence, the different sets of data incomparable.

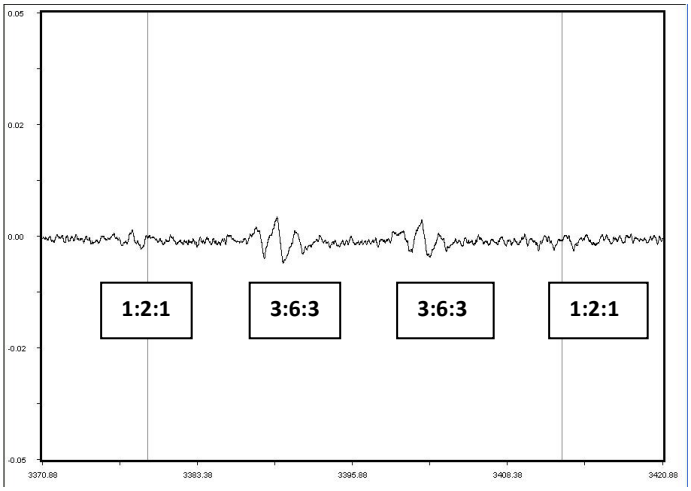

Figure 10: BHT without ABGT sample spectrum. The methyl protons create a quadruplet that is split by a triplet from the ring hydrogens. In figure 9, this BHT signal can be seen, but there is also an unknown species signal that could have formed through possible BHT dimerization.

DISCUSSION

The hypothesis that ABGT would increase radical stability could not be proved or disproved in the above set of experiments because of complicating results. HRP production of DTBHA and BHT radicals proved to be successful. The EPR spectra of DTBHA without ABGT showed that the radical decayed more slowly than the DTBHA with ABGT radical. This data

was the exact opposite of the hypothesis. The data collected on BHT without ABGT radicals and BHT with ABGT radicals could be interpreted as proving the hypothesis however. The BHT without ABGT radical was very unstable, and the radical's signal was initially very weak and lasted for less than five minutes. The BHT with ABGT radical proved to be very stable due to possible dimerization of BHT molecules which came as an unexpected discovery because it resulted in the spectrum of an unknown species. If ABGT played a role in this increased radical stability, then this outcome would directly uphold the hypothesis that ABGT increases radical stability. Due to the experiment resulting in two opposite outcomes, the conclusion that ABGT increases radical stability can neither be proven nor disproved. Future experiments will focus on EPR analysis of this unknown species to better understand the radical interactions that occurred in its production. Other experiments will focus on the exact mode of polymer sequestration of and interaction with known radicals.

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