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Thermal Degradation of Thaumatin at Low pH and Its Prevention Using Alkyl Gallates

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Abstract

Thaumatin, a potent sweet tasting protein extracted from the Katemfe Plant, is emerging as a natural alternative to synthetic non-nutritive sweeteners and flavor enhancer. As a food additive, its stability within the food matrix during thermal processing is of great interest to the food industry. When heated under neutral or basic conditions, thaumatin was found to lose its sweetness due to protein aggregation caused by sulfhydryl catalyzed disulfide bond interchange. At lower pH, while thaumatin was also found to lose sweetness after heating, it does so at a slower rate and shows more resistance to sweetness loss. SDS-PAGE indicated that thaumatin fragmented into multiple smaller pieces under heating in acidic pH. Using BEMPO-3, a lipophilic spin trap, we were able to detect the presence of a free-radical within the hydrophobic region of the protein during heating. Protein carbonyl content, a byproduct of protein oxidation, also increased upon heating, providing additional evidence for protein cleavage by a radical pathway. Hexyl gallate successfully inhibited the radical generation as well as protein carbonyl formation of thaumatin during heating.

Graphical Abstract

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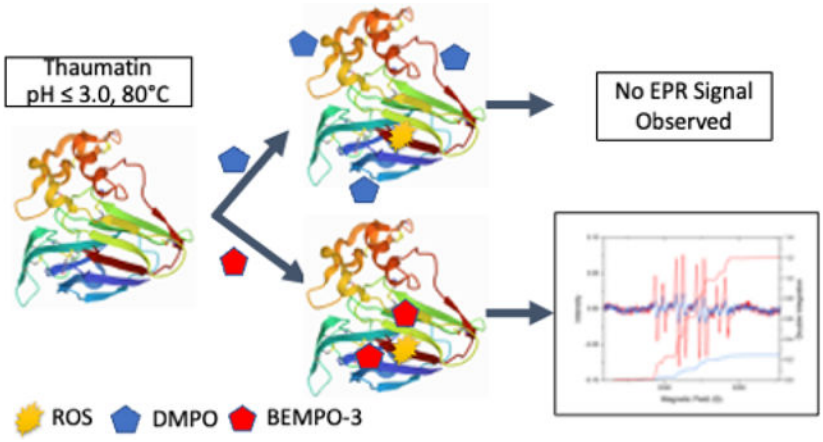
Declaration of competing interest

The authors declare no financial interests/personal relationships which may be considered as potential competing interests.

Conflict of Interest

The authors have no conflicts of interest to declare.



Keywords

Radical Organic Species; Thaumatin; Spin Probes; BEMPO-3; Antioxidants; EPR

1. Introduction

Thaumatin is a group of proteins extracted from the aril of the Katemfe Plant, *Thaumatococcus danielli* Benth. It is one of the few proteins recognized as sweet by the human palate, the others that have been identified are monellin, curculin, mabinlin, brazzein, pentadin, and egg white lysozyme (Joseph et al., 2019; Masuda et al., 2018). By mass, thaumatin is perceived to be 1600 times sweeter than sucrose and on a molar basis, 100,000 times sweeter (van der Wel & Loeve, 1972). The high sweetening power of thaumatin lends itself toward a non-nutritive sweetener as the amount needed for the same level of sweetness would be low enough for the energy contribution to be considered negligible (Fry, 2012; Glória, 2003). In this role, thaumatin has the advantage of being naturally derived, making it attractive to the increasingly health-conscious consumers (Joseph et al., 2019). In addition, it has also been approved as a flavor enhancer for peppermint and sugar alcohols (Gibbs et al., 1996; Glória, 2003). In commercial preparation, thaumatin extracted from the fruit seed aril exists in five isoforms (thaumatin I, II, III, a and b); among them thaumatin I and II are the major forms. These two forms differ only by five residues within the single 207 amino acid chain (Fry, 2012). The principal structural features of these proteins include eleven stacked antiparallel β-strands with small loops stabilized by eight disulfide bonds and a low percentage of α-helices (Gibbs et al., 1996; Ogata et al., 1992).

As with other sweet proteins, a clear consensus on the precise mechanism behind their tastes has not been reached (Kaneko & Kitabatake, 2001a; Masuda et al., 2018). Thaumatin shares no common structure with the others, though it is similar to monnellin in terms of its basicity. Chemical modification studies suggested that although this basicity plays a role in its sweetness, there are other factors (Shamil & Beynon, 1990; Van Der Wel & Bel, 1976). Selective modification, blocking, and mutagenesis of specific residues revealed that multiple lysine and arginine residues contribute to the sweetness of thaumatin (Kaneko & Kitabatake, 2001a; Ohta et al., 2008). Retention of the spatial arrangement as well as the surface charge

on thaumatin is vital to flavor recognition and its retention; a single charge inversion on Arg82 is enough to disable sweetness (Ohta et al., 2011a, 2011b).

Although the investigation into the molecular interaction underpinning the sweetness of thaumatin is important, equally crucial are the considerations for its application in the food industry, such as thermal stability. Although it was shown to be stable at room temperature up to a pH of 8.0 (Glória, 2003), heating above 70 °C at near neutral to basic pH levels causes the protein to aggregate and lose its sweetness. Although thaumatin possessed no free cysteine to catalyze the reaction, the disulfide bonds decomposed into lysinoalanine and cysteine during heating. These bond cleavages allowed the free cysteine to undergo interchange with other disulfide bridges and form different intra- and intermolecular bonds, the result was protein aggregation (Kaneko & Kitabatake, 1999). At pH levels below 6, however, thaumatin was reported to be remarkably stable, capable of surviving pasteurization temperatures (Fry, 2012; Glória, 2003; Kaneko & Kitabatake, 2001b). The protein becomes more resistant to heat with increasing acidity; it was found to suffer only a gradual decrease in sweetness at 80 °C for multiple hours (Kaneko & Kitabatake, 2001b). The observed stability was believed to be conferred by the disulfide bonds that stabilized the three-dimensional structure of the thaumatin (Fry, 2012; Witty, 1990).

While the disulfide bonds are thought to be the key to higher stability at low pH levels, the mechanism for the gradual loss of sweetness without any evident aggregation was not well understood. A study reported that at very low pH (2.0–3.0), thaumatin underwent fragmentation with extended heating but the cause of this was not identified (Kaneko & Kitabatake, 2001b). However, given that this phenomenon happens only at a narrow pH range where the protein was reported to be most stable with respect to sweetness, this suggested that there is more than one mechanism involved in the thermal denaturation of thaumatin at pH levels lower than 6.0 where aggregation was not a factor.

The goal of our work was to elucidate the mechanism by which thaumatin undergoes backbone cleavage at pH levels below 3.0. Electron paramagnetic resonance (EPR), protein carbonyl content, lipophilized antioxidant and other techniques were used to achieve a better understanding of the corresponding mechanism.

2. Materials & Methods

2.1 Materials

Thaumatin was purchased from TCI America (Portland, OR). Reagent grade 8-Anilino-1-naphthalenesulfonic acid (ANS), 2-bromohexanoic acid, guanidine hydrochloride (GuHCl), phosphorus tribromide, sodium nitrite, and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St. Louis, MO). Zinc dust (quality level 200) was purchased from Sigma-Aldrich (St. Louis, MO). 2,4-Dinitrophenylhydrazine (DNPH) was purchased from Spectrum Chemical (New Brunswick, NJ). The gel electrophoresis reagents were purchased from Bio-Rad Laboratories (Hercules, CA). DMPO was purchased from the Cayman Chemical Company (Ann Arbor, MI). All solvents used were at least reagent grade. Deionized water (18.2 MΩ/cm) from the Millipore water purification system was exclusively used for the experiment.

2.2 Synthesis of lipophilic spin trap

BEMPO-3 ((2R)-2-butyl-2-(ethoxycarbonyl)-4-methyl-3,4-dihydro-2H-pyrrole) was synthesized following the procedure reported by the literature (Stolze et al., 2005) with slight modification, synthetic methods and characterization can be found in the supporting information.

2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Thaumatin stock solution (2.00 mg/mL) was diluted to 1.00 mg/mL and half of the solution was adjusted to pH 5 and the other half was adjusted to pH 3 using 0.1 M HCl. The diluted and pH adjusted protein solutions (1 mL) were then heated at 80 °C between 1 to 4 hours in an aluminum heat block. For analysis, the samples were diluted in equal parts with 2X Laemmli sample buffer or 2X Laemmli sample buffer with 5 v/v % 2-mercaptoethanol for SDS-PAGE and SDS-PAGE-Reduced, respectively. To prevent thermal denaturation, the samples were incubated at room temperature for one hour. The samples (20 μL) were then injected into each well of the polyacrylamide gel, which was run with standard running buffer (192 mM glycine, 25 mM Tris base) at 200V for ~45 min. The gel was developed in Coomassie blue staining buffer for 30 minutes and destained overnight in a 1:2:7 mixture of acetic acid, methanol, and water.

2.4 Electron Paramagnetic Resonance (EPR) Spectroscopy

Thaumatin stock solution (2.00 mg/mL) was diluted to 1.00 mg/mL and half of the solution was adjusted to pH 3 and the other half was adjusted to pH 2 using 0.1 M HCl. A stock solution of 0.1 M BEMPO-3 was prepared in DMSO and 10 μL was added to each thaumatin sample (990 μL) to the final concentration of 1 mM. After vortex spinning thoroughly, the sample was transferred to a closed-end EPR tube, which was placed in the measurement chamber preheated to 80 °C.

The measurement was taken after 5 minutes of equilibration time with 20 scans from 3250 G to 3400 G. The signal was measured at 0 and 15 min to monitor the peak generation and the subsequent decay. The spectra were collected in a Bruker Elexsys X-band ESR spectroscopy (Bruker Instruments, Billerica, MA) at 80°C using an Intelligent Temperature Controller (Oxford Instruments, UK). The spectra were recorded using the following settings: sweep width, 120 G; center field, 3319.0 G; modulation amplitude, 2 G; microwave power, 2 mW; time constant, 40.96 ms, receiver gain 60 dB, scan time 43.13 s. The spectra were analyzed using Origin (OriginLab, Northampton, MA). The spectra with noisier signals were denoised using the Wavelet Denoising Package before the integration(M. Srivastava et al., 2016).

To determine the efficacy of lipophilic polyphenol to quench the free-radical, hexyl gallate was added to the sample (final concentration: 50 μM) prior to analysis and the results were compared with the control.

2.5 C11 BIODIPY 581/591 Fluorescence Quenching

Two thaumatin solutions (1 mg/mL) with 1 μM of C11 BIODIPY 581/591 fluorescence probe were prepared at pH 2 and 3. For each replicate, 20 mL of the sample was transferred

into a capped tube and heated in an 80 °C water bath. Aliquots (1 mL) were taken every 5 minutes for 60 min (12 separate samples). Each treatment was analyzed in six replicates.

For each sample collected a 250 μL aliquot was transferred to a 96-well opaque plate for fluorescence measurement in SpectraMax iD3–3020 plate reader (Molecular Devices, LLC, San Jose, CA), excitation wavelength of 488 nm and monitored emission wavelength of 591 nm. The fluorescence was expressed as a percentage of the maximum fluorescence intensity at time 0 of the sample.

2.6 Protein Carbonylation Assay

The protein carbonyl content quantification protocol was performed according to the literature with modification (Colombo et al., 2016).

Thaumatin stock solution (2 mg/mL) was diluted, and the pH adjusted to prepare 1 mg/mL samples at pH 3 and pH 5. Two controls were used, the first control treatment (native) contained the protein solution with no additive and no thermal treatment. The second control (heated) contained the protein solution and no additives but was subjected to heating. The final four treatments involved addition of polyphenols (gallic acid, ethyl gallate (C2-gallate), butyl (C4-gallate), hexyl gallate (C6-gallate)) to a concentration of 50 μM before thermal treatment. Using microcentrifuge tubes, 1.6 mL of each of the four sample solutions and the second control were heated at 80 °C in a water bath for 1 hour. All six treatments were prepared in triplicate.

For the carbonyl content quantification of each replicate, 200 μL of DNPH (10 mM in 2M HCl) was added to each tube. All treatments were incubated at room temperature in the dark for one hour with vortex spinning every 15 minutes. The protein samples were precipitated by adding 200 μL of 100 w/v % TCA, then incubated in ice for 15 minutes, followed by centrifugation at 10,000*g* for 5 minutes at 4 °C. The supernatant was then removed, and the resultant pellets washed again with 1 mL of 20 w/v % TCA and centrifuged again. The pellets were washed three additional times with 1:1 ethanol to ethyl acetate to remove unreacted DNPH. Finally, the supernatant was carefully discarded, and the pellets were dried under vacuum for 2 hours.

For analysis, the pellets for all treatments were resuspended in 250 μL of 6 M GuHCl, warmed gently at 37 °C on a heat block, and vortexed. An aliquot of 220 μL from each tube was transferred to a 96-well plate for absorbance measurement at 366 nm (SpectraMax iD3–3020, Molecular Devices, LLC, San Jose, CA).

To account for the possible intrinsic absorbance at 366 nm of thaumatin, an additional set of treatment samples were prepared and underwent the same carbonyl quantification protocol above, but with the addition of 200 μL of 2 M HCl instead of DNPH. The absorbance of these additional blanks was subtracted from the sample absorbance to yield the adjusted absorbance. The concentration of the protein carbonyl content was calculated from the adjusted absorbance using the molar absorptivity of 22,000 $M^{-1}cm^{-1}$.

2.7 Statistical Analysis

Where appropriate, the triplicate results were reported with average and standard deviation. ANOVA was used to determine the statistical difference between treatment groups ($p \leq 0.05$)

The EPR result reported was the average of 25 runs.

3. Results and Discussion

3.1 SDS-PAGE and SDS-PAGE-reduced

The speed at which proteins migrate through polyacrylamide gels is influenced by the structure and charge of the proteins. Anionic detergent (SDS) is used to eliminate these effects by binding to the backbone at a constant molar ratio, thus imparting a large negative charge and negating non-covalent bonding. This results in proteins adopting linear structures causing the migration rate to depends predominately on molecular weight (Nowakowski et al., 2014). SDS-PAGE analysis of our native thaumatin protein sample indicated that the sample was reasonably pure with the protein of interest appearing as an intense band around 20 kDa with some less intense bands indicating small impurities at higher and lower molecular weights.

With extended heating (4 h at 80 °C) at pH 5.0, no change was observed apart from the broadening of the impurity band at ~37 kDa (Figure 1A). However, the same thermal treatment at lower pH showed the appearance of faint new bands with a lower molecular weight (around 5–10 kDa) the intensity of which increased with heating time.. These changes suggested possible protein fragmentation during thermal treatment.

The inclusion of a reducing agent (2-mercaptoethanol) into the SDS-PAGE resulted in the cleavage of the covalent disulfide bonds. Thaumatin consists of a single peptide chain stabilized by eight such bonds. While the reducing treatment was expected to cause further disruption to the three-dimensional structure of the protein, the molecular weight should remain constant as the backbone remains intact (Nowakowski et al., 2014). The unheated thaumatin showed some smearing but no lower molecular weight fragments appeared as expected from its nature as single peptide chain.

Similar to the SDS-PAGE, at pH 5 the SDS-PAGE reduced showed no changes under heat treatment. However, at pH 3, the appearance of low molecular weight bands in heated sample became even more prominent in SDS-PAGE-reduced (Figure 1B). Thermal treatment for 4 h resulted in an intense low molecular weight band with fading of the parent thaumatin band. This suggested that heating at pH 3 resulted in the cleavage of the amino acid chain. The subsequent fragments were held together by the protein abundant disulfide bonds; hence the prominence of the fragments was only observed under reducing conditions when disulfide bonds were cleaved. This result matched the findings from Kaneko and Kitabatake (Kaneko & Kitabatake, 2001b). This fragmentation was initially thought to be attributed to the simple peptide hydrolysis process. However, when an *o*-phthalaldehyde assay was performed (data not shown), there was no appreciable increase in free amine groups within the sample under heat treatment.

3.2 Free radical generation during heating thaumatin at low pH

In the absence of hydrolysis, the fragmentation of thaumatin under low pH conditions could be mediated by a free-radical pathway. Reactive oxygen species (ROS) are known to cause backbone cleavage, resulting in the formation of carbonyl species (Colombo et al., 2016). Some proteins like C-phycocyanin were shown to generate free-radical species at very specific pH levels under photolysis (Li et al., 2021). Aromatic amino acids (tryptophan, tyrosine, phenylalanine) within immunoglobulin and albumins were also shown to absorb UV light to sensitize the generation singlet oxygen from dissolved oxygen (Bruskov et al., 2014; Wentworth et al., 2000). This was originally not considered because thaumatin lacks a chromophore that would normally generate free-radicals and the heating process was conducted in the absence of light. To test our hypothesis, the protein solution at pH 2 and pH 3 was heated with spin traps and monitored by EPR.

Using DMPO as a spin trap, no free radicals were detected across all pH levels. We postulated that either no free radical was generated or that the generation site was within the hydrophobic region of thaumatin. To investigate the latter possibility, we needed to explore this region.

To expose all regions of the protein, 6 M guanidine hydrochloride was chosen as a denaturant. Its highly favorable interactions with the hydrophilic regions of a protein allows the molecule to bind and unfold proteins (Monera et al., 1994; Qasim & Taha, 2013). Under this condition, DMPO was able to detect free-radical generation (Fig. S2) in thaumatin solution. However, the result did not coincide with the SDS-PAGE data. That is, the free-radical yield was shown to increase with pH, even though the fragmentation pattern in the SDS-PAGE only occurred at low pH levels. There was a possibility that guanidine hydrochloride at such high concentration permitted interactions that would not normally occur in native thaumatin. Hence, the ability for thaumatin to generate a free-radical in its native form under heating could not yet be confirmed by this method.

To investigate the hydrophobic region of thaumatin, a lipophilic spin trap, BEMPO-3, was used. This hydrophobic molecule can possibly penetrate into the hydrophobic region of protein due to the presence of the butyl group in the structure. Further, radical-BEMPO-3 adducts exhibited extremely long half-life from previous report by Stolze (Stolze et al., 2005), which makes BEMPO-3 a perfect candidate in the current study. Stolze successfully detected lipid derived radicals with this spin trap. Therefore, it is hypothesized that the free radical in the hydrophobic region can be detected if it is generated by heating thaumatin under acidic pH. At pH levels higher than 3.0, no free-radical generation was observed. However, at pH 3.0 or lower (Figure 2), a free radical was detected. The anisotropic shape of the EPR peaks suggested BEMPO-3 successfully trapped a small radical. It was further found that the free radical generation was pH dependent. The EPR spectrum intensity fell down approximately 80 % when the pH changed from 2 to 3. The obtained result matched with SDS-PAGE, which suggested that this fragmentation was confined to lower pH levels.

The observed EPR spectrum showed 8 peaks. We attribute this to 2 isomers of BEMPO-3 where each isomer-radical adduct exhibits 6 peaks in the EPR, and the four center peaks overlap, thus accounting for the 8 peaks (Figure 3). The hyperfine splitting constant were

calculated on EPR spectrum acquired at pH 2 for BEMPO-3 isomer 1 (a^N = 14.1, a^H = 18.7) and isomer 2 (a^N = 13.8, a^H = 9.0). This 6 peak pattern can be interpreted in themes of inequivalent hyperfine splitting from both the nitroxide nitrogen and the beta hydrogen atom as described for hydroxyl radical spin adducts (Janzen et al., 1978). The spectra are also similar to the reported spectrum of hydroxyl radical adducts of BEMPO-3 within a Fenton reaction mixture (Stolze et al., 2005). Therefore, we concluded that the hydroxyl radicals were the principal species generated and responsible for the fragmentation of thaumatin.

One probable source of hydroxyl radical generation in thaumatin is the Fenton reaction: the formation of hydroxyl radicals from trace iron and hydrogen peroxide. Not only are the BEMPO-3 adduct spectra from thaumatin and model Fenton reaction similar, a number of proteins were also reported to be capable of generating hydrogen peroxide in the absence of light, such as immunoglobulin with a chemical singlet oxygen source (Wentworth et al., 2000) and serum proteins with mild heating (Bruskov et al., 2014). Though happening at a neutral pH range, the latter suggested the possibility that thermal treatment alone could be adequate to induce hydrogen peroxide production in thaumatin, which then led to the generation of hydroxyl radicals.

For DMPO to be unable to interact and BEMPO-3 to interact with the free-radical, the site of the free-radical generation would need to be well isolated from the surface environment. Molecular oxygen access to the inner residues of the protein was shown to be possible (Chin et al., 2008), so the result did not agree with the above suggestion. As for the reason that the radicals were tightly confined within the hydrophobic region, it was known that the rigidity of the 3-dimensional structure of the protein could influence the oxygen diffusivity (Strambini & Cioni, 1999). The 8 disulfide bonds that offered structural stability to thaumatin under heating could also be the reason behind this phenomenon.

To investigate whether this free-radical generation would pose any problem in terms of application in food production, such as promoting lipid oxidation, oxidizable emulsions with and without thaumatin were heated at 80 °C for 24 hours (Figure S3). The samples with thaumatin showed markedly lower levels of oxidation, therefore it was unlikely for the protein to promote lipid oxidation.

3.3 C11 BODIPY 581/591 Fluorescence Quenching

To further probe the location of free radical generation during heating, thaumatin was incubated at the temperature of 80°C with the C11 BODIPY 581/591 fluorescence probe. C11 BODIPY 581/591 was chosen for its lipophilicity that allowed the molecule to interact with the hydrophobic region of the protein. When the large conjugation of C11 BODIPY is destroyed by radicals, a decrease of the fluorescence intensity is observed. Drummen applied this probe to monitor the lipid oxidation of egg-PC vesicles and observed a continuous decrease of emission intensity at 595 nm (Drummen et al., 2002). Therefore, in this study, the loss of emission intensity of C11 BODIPY at 595 nm due to radical generation was monitored as a function of heating time.

At pH 2 or 3, the fluorescence decreased rapidly within the first 5 min, then reached a plateau and remained consistent for the rest of the incubation period. Although the effect of pH was statistically significant, the practical difference was small.

It was shown that though the free-radical generation decayed after 15 min of heating at pH 2, there was still a significant proportion of free radical remaining, which would suggest a continual decrease beyond 5 min. The charge separation in the BODIPY moiety may also push the dye slightly out of the hydrophobic region of thaumatin where the free radical was generated, thus leading to an almost unchanged fluorescence intensity after 5 min. One possible explanation for the sudden emission intensity drop after 5 min heating was the destruction of C11 BODIPY by a burst of radicals generated in the thaumatin hydrophobic region when heated under an acidic environment. Quenching efficiency was significantly higher at pH 3 than at pH 2 when the samples were heated 10 to 60 min.

3.4 Protein Carbonylation Assay

EPR spectroscopy and fluorescence probe quenching supported the first part of the hypothesis that the free radical was being generated at low pH under heating. However, due to unidentifiable radical species, it was unconfirmed whether they would react in the same pathway as reactive oxygen species and result in peptide backbone cleavage. So, the protein carbonylation assay was used to detect and quantify the end product of radical mediated oxidation of protein.

Protein oxidation via free-radicals create a peptide-bound carbonyl group either through direct oxidation of amino acid residues, particularly arginine and lysine, or backbone cleavage. The latter started with hydrogen abstraction from the backbone, forming carbon radicals, which then further reacted with oxygen to become reactive protein peroxyl and alkoxyl radicals. These intermediates can then cleave the peptide backbone either through diamide or α-amidation pathway, resulting in carbonyl containing products (Colombo et al., 2016; Hawkins & Davies, 2001; Kehm et al., 2021; Sajapin & Hellwig, 2020; Stringfellow et al., 2014; Trnkova et al., 2015). Other sources of carbonylation include interaction between protein and reactive products in oxidation of lipid and reducing sugars (Colombo et al., 2016), but this was ruled out as the protein was purchased purified and contamination was assumed to be minimal. This still means that a part of the detectable increase in carbonyl content could be due to oxidized amino acid residues, but detection of carbonyl species can still be the link between free-radical generation and fragmentation.

The result of the carbonylation assay confirmed the observations from the SDS-PAGE and EPR spectroscopy (Figure 5). In an unheated sample, thaumatin at both pH levels exhibited a similar level of carbonyl content. After heating, the sample in pH 5.0 showed no increase, which agreed with the previous observation that indicated no fragmentation nor free-radical generation at higher pH levels (Figure 1B). However, the same thermal treatment at pH 3.0 resulted in drastic increase in carbonyl content (~23%).

To further ascertain the region where the radical was generated, we added lipophilized gallic acid derivatives to thaumatin at acidic pH and the samples were then heated. Gallic acid is an antioxidant that exists widely in natural fruits and plants and possesses strong

radical scavenging activity (Badhani et al., 2015). By esterifying gallic acid with various fatty alcohol, lipophilized gallic acid derivatives (alkyl gallates) with different carbon chain lengths were synthesized. From previous results (Zhao, 2018), longer carbon chains can effectively drag triphenol group into the hydrophobic region. Therefore, in this study, hydrophobic hexyl gallate was used to quench the heat-generated carbonyl species in thaumatin.

At pH 5.0, gallic acid and its derivatives caused no difference in the quantity of detected carbonyl groups within the sample. This was expected as no free-radical generation was observed at higher pH levels. However, at pH 3.0, inclusion of butyl and hexyl gallate into the thaumatin solution during heating effectively decreased the quantity of detected carbonyl groups to similar level as the unheated protein. This further reinforces that the cause of protein oxidation is the free radicals, which can be counteracted by antioxidants. Interestingly, gallic acid and ethyl gallate showed no effect compared to the control. This suggests that only the gallates of sufficiently high hydrophobicity are capable of reaching and quenching the free radicals, lending credence to the hypothesis that the radical generation is localized in the hydrophobic region.

3.5 Quenching free radical by alkyl gallates

To support the action of the hydrophobic antioxidant in protein carbonyl assay, thaumatin solutions with and without hexyl gallate were incubated at 80 °C within the EPR spectrophotometer. The spectra were obtained at the temperature equilibrium for peak radical yield and then monitored later to observe the decay. The addition of hexyl gallate decreased the peak area by 38.9 %, suggesting the peak production of the free radicals at pH 2 upon heating (0 min) (Figure 6B). Although effective, the incomplete quenching of the radicals possibly indicated that either the triphenol group in hexyl gallate still cannot penetrate deeply enough into the most hydrophobic region, or insufficient antioxidant was used. Further, the control sample took almost three times as long to achieve the same degree of decay as the sample with antioxidant; the control decayed ~17 % in 15 minutes and the gallate treated sample by ~29 % in 5 minutes Thus the use of a hydrophobic antioxidant was found to be an effective way to inhibit the free-radical generation within the protein. The test was also performed at pH 3.0 (Figure 7) where the already low radical generation was further reduced to baseline with the addition of hexyl gallate. This result correlated well with protein carbonyl species inhibition study, further indicating that the observed free radical was generated in the protein hydrophobic region.

4. Conclusion

The fragmentation of amino acid chains of thaumatin at lower pH was demonstrated to be possibly the result of free-radical generation within the hydrophobic region of the protein. The generated radical did not propagate beyond the hydrophobic region of the protein; hence it cannot be captured by DMPO. On the other hand, BEMPO-3, a hydrophobic synthetic spin trap, can effectively trap the generated radical during heating. The adduct has the hyperfine splitting constants of 14.1 G (a^N) and 18.7 G (a^H) for BEMPO-3 isomer 1 and 13.8 G (a^N) and 9.0 G (a^H) for isomer 2, which is very similar to the reported value of

hydroxyl radical generated via the Fenton reaction. Certain degree of hydrophobicity was also needed for the antioxidant to interact and quench the free radical generated. Only the more hydrophobic gallates were shown to be effective in inhibiting the formation of carbonyl species, oxidation products of protein, and hexyl gallate was directly shown to EPR to reduce the peak free radical production.

The oxidation resulting from this action may prove minor in the overall degradation of thaumatin's biological activity, loss of sweetness, due to its effect being most prominent at low pH where the sweetness was reported to be most stable. However, this study provides a new insight into the mechanism for thaumatin degradation under heating in an acidic environment in the absence of light, which serves a foundation for future related research.

For future avenues of research, the organoleptic impact of the fortification with hydrophobic antioxidants is of interest. Although promising in its inhibition of free radical generation, the sensory evaluation of thaumatin and protectant in food matrix context is needed. Further, the successful application of BEMPO-3 to investigate the presence of free radicals buried deep in the hydrophobic region of the protein raised the possibility of application within other food proteins.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- A lipophilic spin trap detected a free-radical upon heating of thaumatin at pH 2.0
- Thaumatin solution can oxidize C11-BODIPY dye.
- Alkyl gallates inhibits the protein the production of carbonyl species
- Free radicals generated heating were inhibited by alkyl gallates

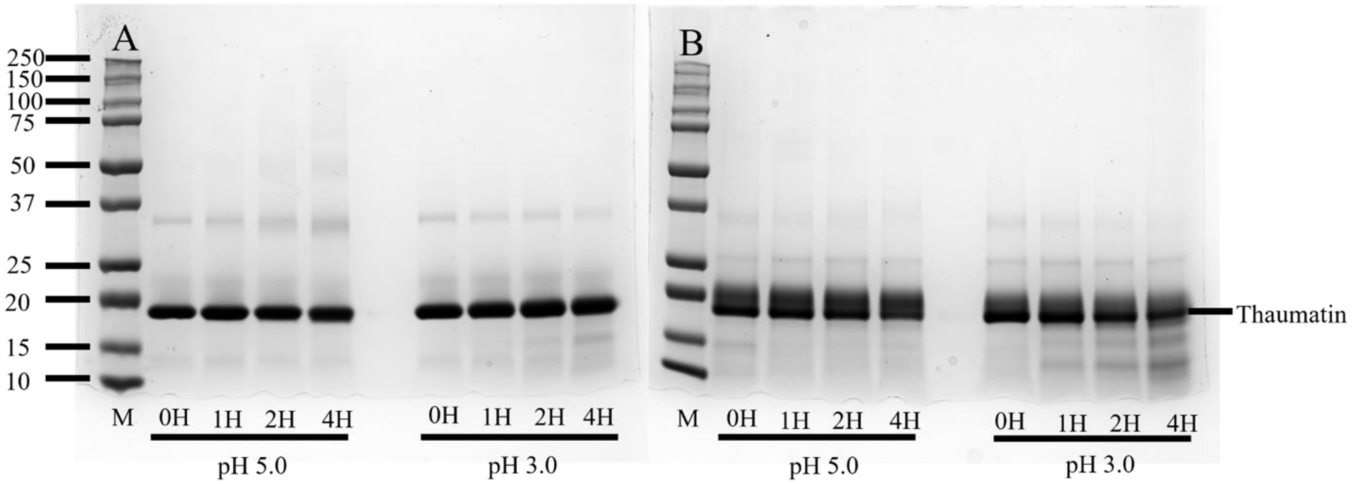

Figure 1:
(A) SDS-PAGE and (B)reduced SDS-PAGE of thaumatin heated at 80 °C, 0–4 hours.

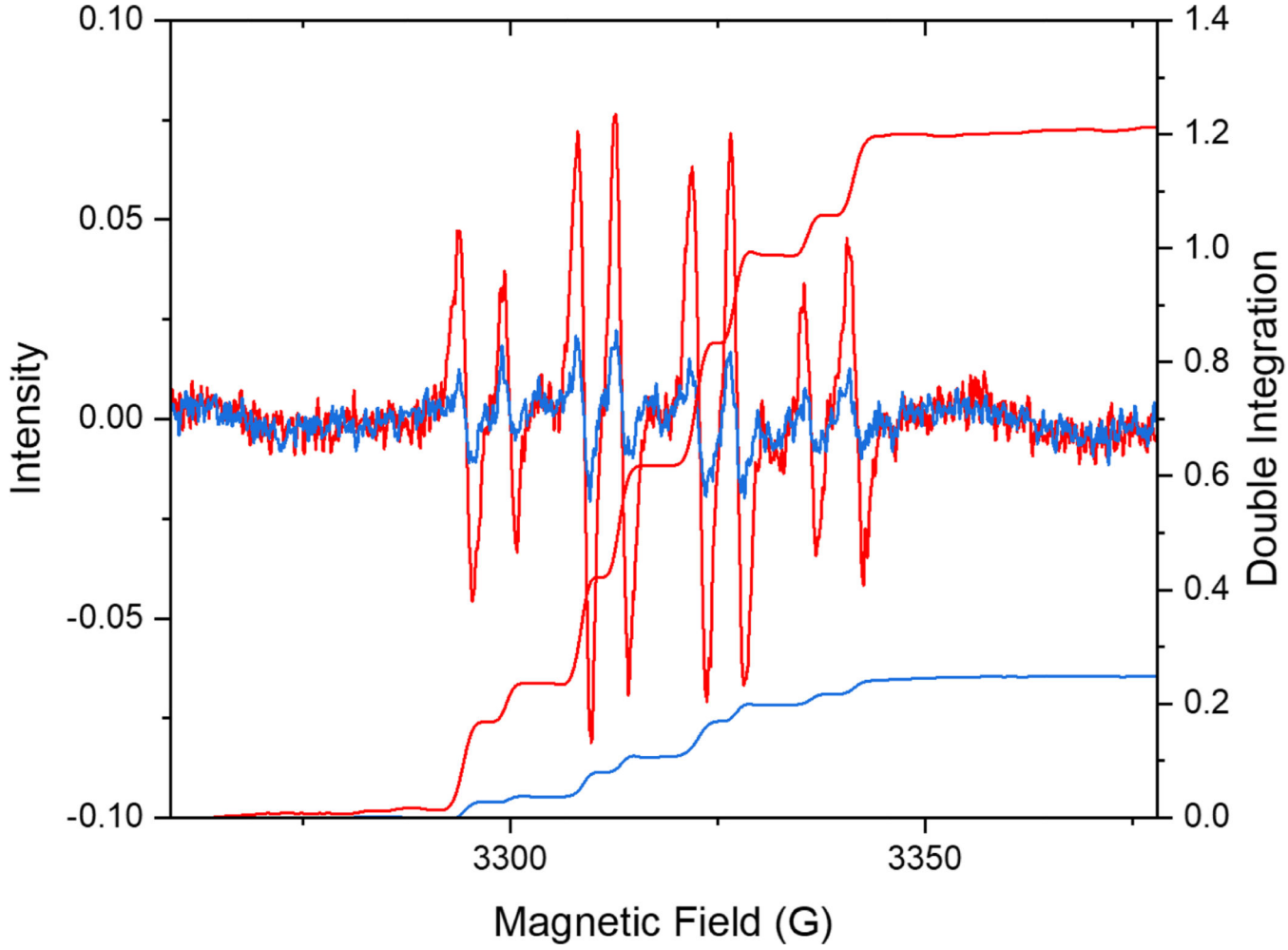

Figure 2:
EPR spectrum of the thaumatin sample with BEMPO-3 heated at 80 °C, 25 replications at pH 2 (red) and pH 3 (blue). Double integration value of the EPR spectrum is depicted on the right axis.

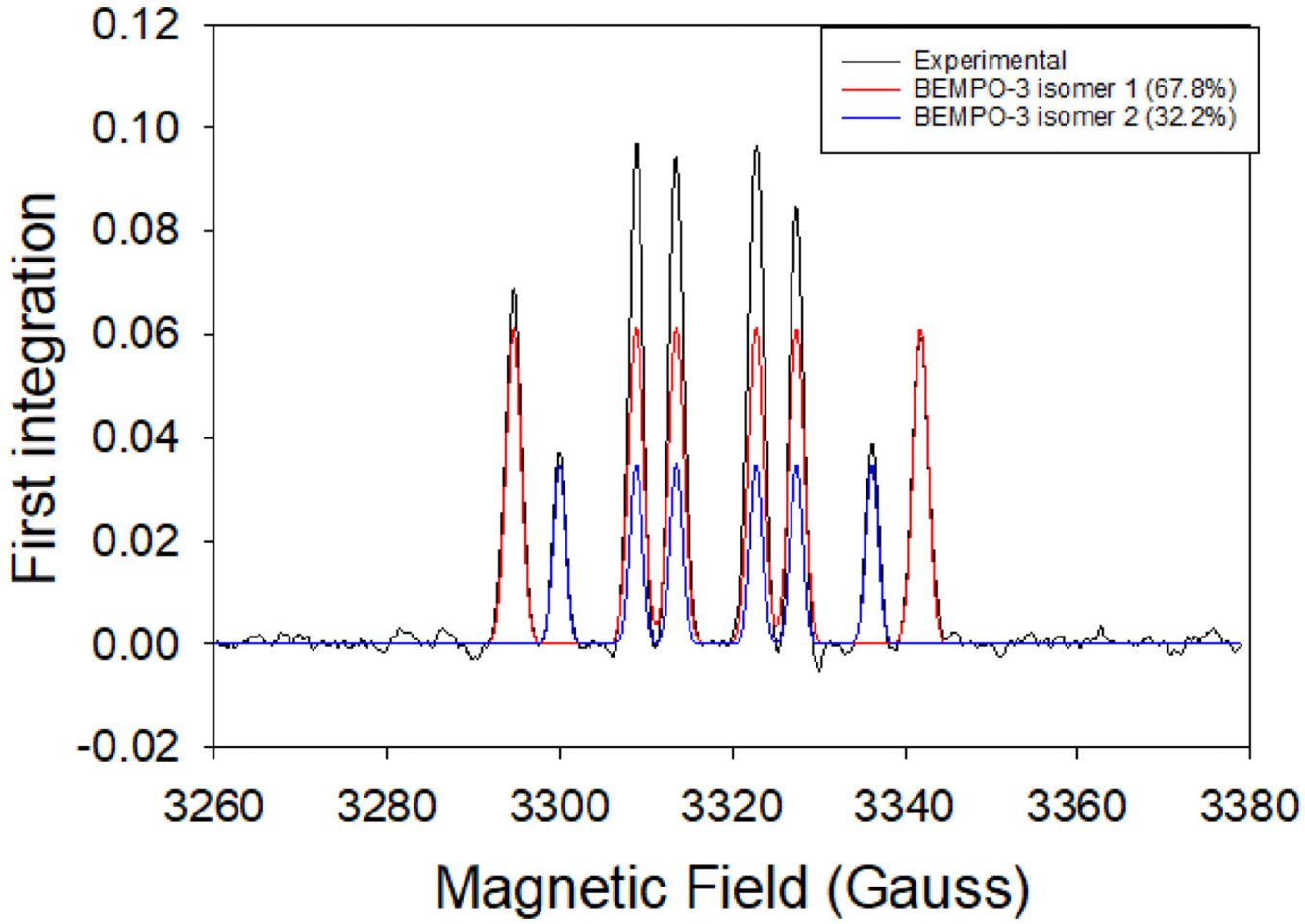

Figure 3:
Spectra deconvolution from EPR spectrum of the thaumatin sample with BEMPO-3 heated at 80 °C, 25 replications at pH 2

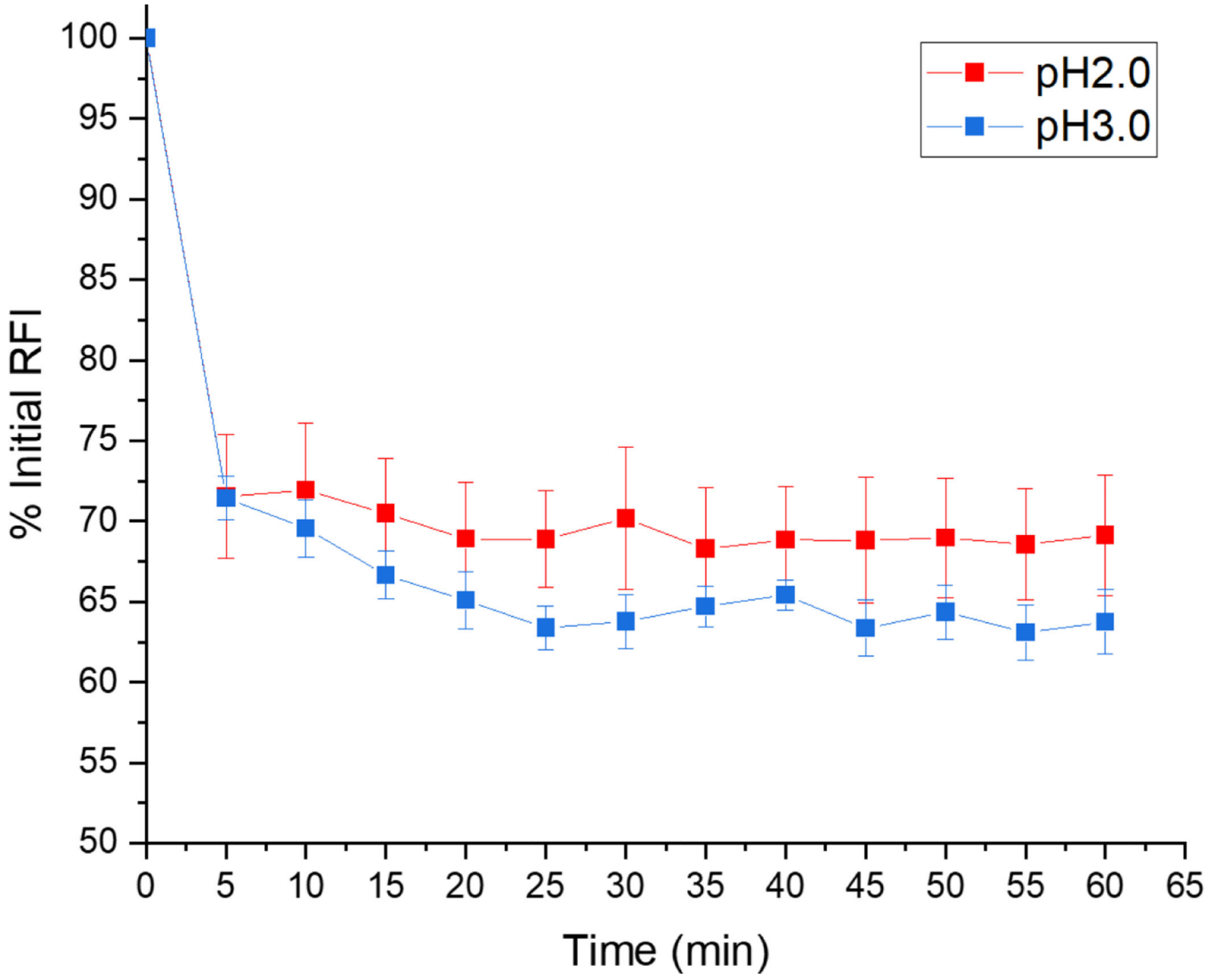

Figure 4:

C11 BIODIPY 581/591 red fluorescence (591 nm) decay over time in thaumatin solution heated at 80 °C. The interaction between pH and time was not statistically significant ($F_{4,155} = 1.621$, $p = 0.093$). However, the main effects of pH ($F_{4,155} = 76.61$, $p < 0.0001$) and time ($F_{4,155} = 134.93$, $p < 0.0001$) were statistically significant.

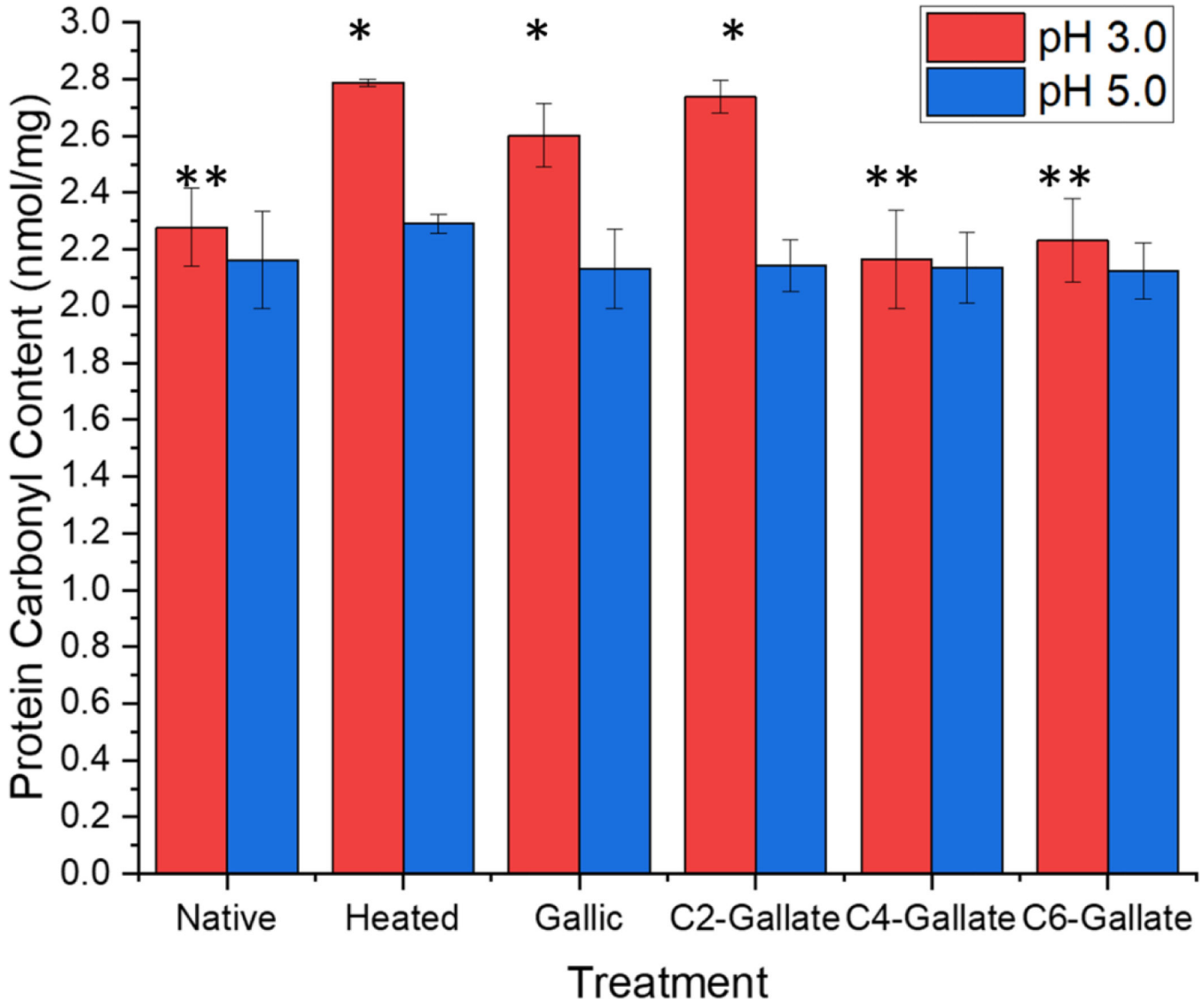

Figure 5:

Protein carbonyl content of thaumatin, thaumatin heated at 80 °C for 1 hour and thaumatin heated in the presence of 50 μM of alkyl gallates at pH 3.0 and 5.0. Within pH 3.0, there was statistical significance between treatments ($F_{5,36} = 22.244$, $p < 0.0001$), but not within pH 5.0 ($F_{5,39} = 0.759$, $p = 0.586$). Similar asterisks (* and **) indicate data with no statistically significant differences, between * and ** there is a significant difference.

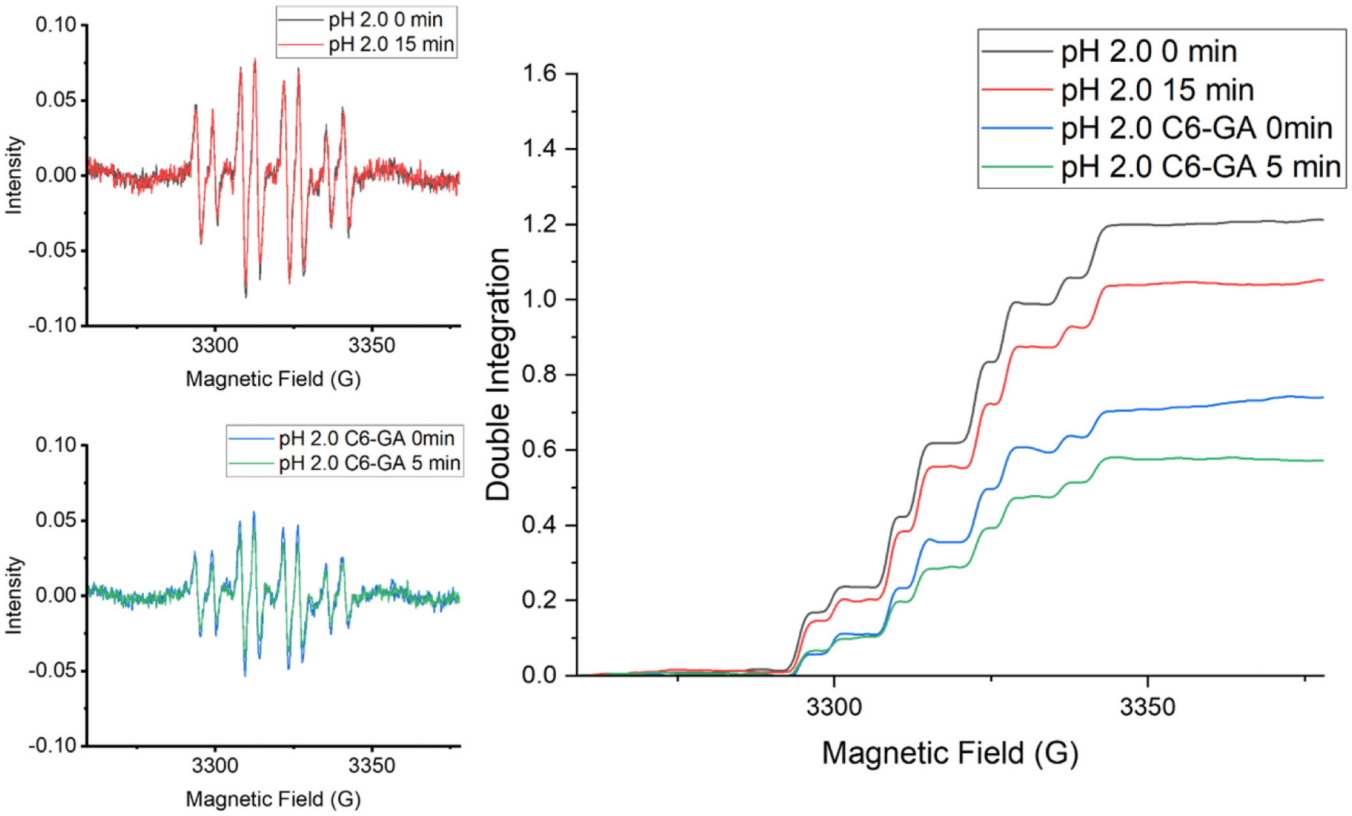

Figure 6:

A) EPR spectrum of thaumatin sample with BEMPO-3, heated at 80 °C at pH 2.0. Control sample of thaumatin solution at temperature equilibrium (time 0, black) and 15 minutes after (red). Sample treated with hexyl gallate at temperature equilibrium (time 0, blue) and 5 minutes after (green). **B)** Double integration of the EPR spectrum, indicating the free radical yield of each sample.

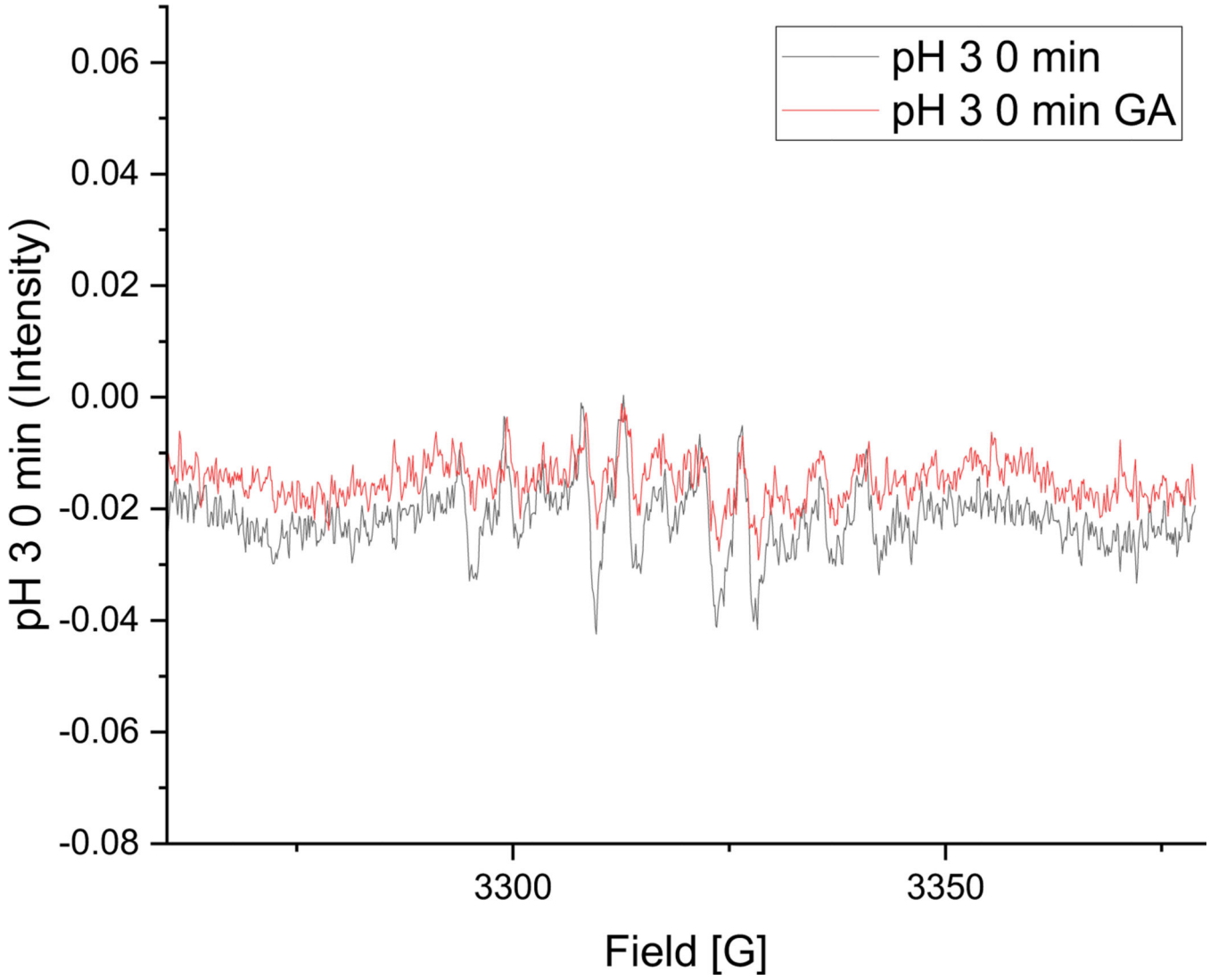

Figure 7:
EPR spectrum of thaumatin sample with BEMPO-3, heated at 80 °C at pH 3.0. Control sample of thaumatin solution at temperature equilibrium (time 0, black). Sample treated with hexyl gallate at temperature equilibrium (time 0, red)