JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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Food and Beverage Chemistry/Biochemistry

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J. Agric. Food Chem., **Just Accepted Manuscript** • DOI: 10.1021/acs.jafc.8b01619 • Publication Date (Web): 22 May 2018

Nitration of tyrosine in the mucin glycoprotein of edible bird's nest changes its color from white to red

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ABSTRACT

The edible bird's nest (EBN) of the swiftlet *Aerodramus fuciphagus*, a mucin glycoprotein, is usually white in color, but there also exist the more desirable red or 'blood' EBN. The basis of the red color has been a puzzle for a long time. Here, we show that the nitration of the tyrosyl residue to the 3-nitrotyrosyl (3-NTyr) residue in the glycoprotein is the cause of the red color. Evidences for the 3-NTyr residue come from: (a) the quantitative analysis of 3-NTyr in EBN by ELISA, (b) the UV-Vis absorption spectra of red EBN as a function of pH being similar to 3-nitrotyrosine (3-NT), (c) the change in the color of red EBN from yellow at low pH to red at high pH just like 3-NT, and (d) strong Raman nitro bands at 1330 cm^{-1} (symmetric $–NO_2$ stretch) and 825 cm^{-1} ($–NO_2$ scissoring bend) for red EBN. The high concentrations of nitrite and nitrate in red EBN are also explained.

KEYWORDS

red edible bird's nest; mucin glycoprotein; nitration; 3-nitrotyrosine; ELISA; UV-Visible spectroscopy; Raman microspectroscopy

1. INTRODUCTION

Swiftlets of genus *Aerodramus fuchiphagus*, native to the Indo-Pacific region, build edible bird's nest (EBN), also known as cubilose or nest cement, which is an unusual amorphous, composite material of strands of mucin glycoprotein (*ca*. 90% by weight) strengthened largely with its own bird feathers (*ca*. 10% by weight). The mucin glycoprotein is secreted like saliva in copious amounts from a pair of sublingual glands under the tongue of the swiftlet during breeding. In living systems, mucin glycoprotein is only present in tiny amounts, but EBN is a material with the highest known concentration of mucin glycoprotein and more than a million kg of EBN is harvested annually. The raw EBN is moistened and cleaned of feathers, sand grains, etc., with tweezers, leaving behind strands of clean, white glycoprotein to be used as food or medicine. When cooked, EBN is believed to have exceptional nutritional and medicinal properties. It is one of the most expensive Asian food delicacies today, and has been prescribed in traditional Chinese medicine for more than a thousand years. Recent studies have shown that EBN can inhibit influenza virus infection, stimulate the immune system, and promote epidermal growth.[1]

The EBN mucin glycoprotein is a polymer with a protein backbone and many dendritic carbohydrate chains attached to it. The proximate analysis of EBN revealed that it contains *ca.* 62% by weight of protein, made up of 17 types of amino acids, with the most abundant being serine, valine, tyrosine, isoleucine, aspartic acid and asparagine, glutamic acid and glutamine, and phenylalanine, and the distinct absence of tryptophan, cysteine, and proline.[2-5] The dendritic carbohydrates make up *ca.* 27% by weight and the major saccharides are sialic acid, galactose, N-acetylgalactosamine, N-acetylglucosamine, fucose, and mannose.[5, 6] The rest are moisture (*ca.* 8%), ash/minerals (*ca.* 2%),[7] and fat (< 1%).[5]

There are several colors of EBN available in the market – white (Figure 1A shows a natural white EBN), various shades of light yellow or golden, and red EBN (Figure 1B shows

a natural red EBN from a birdhouse). The latter is often addressed as 'blood' nest due to its color. Commercially, the more common cleaned, white EBN costs about US$3,000/kg today, with the price fluctuating based on supply and demand, while the less common red EBN costs several times more, as the red EBN is perceived to have superior nutritional and medicinal properties over the white EBN. Today, the trade in EBN is a multi-billion dollar industry annually. Due to the lucrative return of red EBN, some EBN processors have used various methods, including dyeing and fumigation with swiftlet 'bird soil' (feces), to convert the white EBN into red 'blood' nest.[8]

Contrary to a long-held belief, red 'blood' EBN is a misnomer as it contains no hemoglobin.[9] In any case, we would not expect hemoglobin to be present in swiftlet saliva unless the bird was injured or sick to regurgitate blood. Marcone found that: (a) the substance giving the red color is neither water soluble nor lipid/solvent extractable, (b) white and red EBN both have ovotransferrin, and red EBN has a relatively higher amounts of iron compared to white EBN, and so it was suggested that an ovotransferrin-iron complex could be the cause of the red color in red EBN.[5] The red color is also not a structural coloration (light interference effect) as both the white and red nests are made of similar amorphous cement-like material from the dried, clear liquid gel of mucin glycoprotein initially secreted through the beak of the same species of swiftlet. The coloration appears after the initial white EBN has been formed.

Recently, But *et al.*[8] showed that red EBN could be produced by fumigating white EBN either with vapor from unwashed swiftlet bird soil (feces), simulating the environment in some bird houses that accumulate the bird soil to produce colored nests, or with nitrous acid (HNO_2) vapor from a solution of sodium nitrite in 2% hydrochloric acid (Figure 1C shows a red EBN prepared by HNO_2 fumigation for 30 days). The similarity in the outcome is because the vapor from the bird soil also contains HNO_2 produced by bacterial

decomposition of the protein/nitrogen-rich bird soil. Clearly, an ovotransferrin-iron complex cannot be the cause of the red color in these experiments. In analogy to sodium nitrite liberating nitric oxide (NO) and forming NO-myoglobin to give the deep red color of cured meat, it was conjectured that perhaps NO produced in the nitrous acid vapor equilibrium could similarly bind to the glycoprotein of EBN to give a red color. However, But *et al.*[8] noted that the chemistry of the color change from white to red EBN remains unknown.

The xanthoproteic reaction, using a mixture of concentrated nitric and sulfuric acids, is a standard biochemical test that gives a positive yellow to a deep red substance in proteins with aromatic amino acids, e.g. tyrosine, phenylalanine or tryptophan, due to nitration of the aromatic ring. Now, EBN is a glycoprotein containing substantial amounts of the aromatic amino acids tyrosine and phenylalanine, and it reacts positively at room temperature, giving a red color (Figure 1D shows a xanthoproteic reacted EBN). Clearly, the natural red EBN, the HNO_2 fumigated EBN, and the xanthoproteic reacted EBN in Figures 1B-D all have a similar red color. It is known that nitration of phenylalanine by the xanthoproteic reaction requires heat as the aromatic ring is not activated, but for tyrosine, it proceeds readily at room temperature due to ring activation by the phenolic –OH group. So, the red color is most likely due to the nitration of the glycoprotein tyrosine residues to give 3-nitrotyrosyl (3-NTyr) residues. It remains for us to show that the 3-NTyr residue is practically absent in the white nest, but present significantly in the natural red EBN, the HNO_2 fumigated EBN, and the xanthoproteic reacted EBN, and 3-NTyr is the cause of the red color.

An anti-3-NTyr antibody enzyme-linked immunosorbent assay (ELISA) kit can be used to detect and quantitatively measure the 3-NTyr in EBN. Further evidences for 3-NTyr come from: (a) the UV-Vis absorption spectroscopy of solubilized red EBN showing pH-dependent absorption spectra similar to that of free 3-nitrotyrosine (3-NT), (b) the change in the color of red EBN strands from yellow at low pH to red at high pH, consistent with the UV-Vis

absorption spectra of red EBN and 3-NT, and (c) Raman microspectroscopy showing the presence of the nitro group in red EBN.

2. MATERIALS AND METHODS

Edible bird's nest (EBN) and other materials

Raw white and natural red EBN samples produced by the species *Aerodramus fuciphagus* were obtained from birdhouses from different geographical locations in South-East Asia: Port Dickson (West Malaysia), Lahad Datu (East Malaysia), and Makassar (Indonesia). All standard chemicals used were analytical grade. Ultrapure water (18.2 MΩ cm) was prepared with Sartorius Arium Lab water purification system (Germany) and used for all experiments. Sodium nitrite, silver nitrate, and citric acid were purchased from Sigma-Aldrich Chemicals (St Louis, MO). Hydrochloric acid, sulfuric acid, and nitric acid were purchased from Fisher Scientific Chemicals (Hampton, NH). Urea, disodium hydrogen phosphate, sodium hydroxide, sodium carbonate, and sodium bicarbonate were purchased from VWR chemicals (Radnor, PA). L-tyrosine and L-phenylalanine were purchased from NowFoods (Bloomingdale, IL). 3-NT ELISA Kit was purchased from Abcam (UK). Citric acid and disodium hydrogen phosphate were used to make buffers from pH 4 to 8, and sodium carbonate and sodium bicarbonate were used to make buffers from pH 9 to 10.

Fumigation of white EBN by nitrous acid to produce red EBN

EBN was fumigated with nitrous acid vapor to convert to red EBN.[8] A solution of sodium nitrite (200 mg) in 2% HCl (20 mL) was poured into a 250 mL glass beaker. A small empty glass bottle was placed at the bottom of the beaker as support for a glass petri dish containing the EBN so that it is above the fumigating solution. The glass beaker was then sealed with a paraffin film and kept in the dark at room temperature, and the color change in the EBN was monitored at regular intervals for up to 30 days, with a change of the $NaNO_2$-

HCl solution every 7 days, during which time the EBN changed color from white to yellow to orange and finally to red.

Xanthoproteic reaction to produce red EBN

A whole piece of moistened raw white EBN (about 6 g) was dipped in a nitrating acid mixture (500 mL; 69% nitric acid: 98% sulfuric acid: water (5: 4: 95 v/v)) for *ca.* 5 seconds, removed and fan-dried for *ca.* 10 min at 25 ˚C and *ca.* 50% relative humidity, and the dipping and drying was repeated for about 30 times, until the EBN turned yellow. The dipping and drying helped to retain the shape of the EBN and to better control the rate of nitration. Next, the dipping and drying was repeated for 30 times with water to rinse off residual acid to stop the reaction. Finally, 1 mM sodium hydroxide solution was sprayed on the surface of the nitrated EBN to neutralize any remaining acid, followed by fan-drying for *ca.* 10 min. The process was repeated several times, and the EBN turned red as shown in Figure 1D.

Synthesis of 3-nitrotyrosine (3-NT) and 4-nitrophenylalanine (4-NP)

3-NT was synthesized by reacting L-tyrosine with an acid mixture of nitric acid and sulfuric acid to generate the nitronium ion for electrophilic aromatic substitution.[10] The product was characterized by nuclear magnetic resonance (NMR) spectroscopy and liquid chromatography-mass spectroscopy (MS). ^{1}H NMR spectra were recorded on a JOEL 400 (Japan) at 400 MHz and mass spectra were recorded on a Thermo Finnigan LCQ Fleet Mass Spectrometer (San Jose, CA) using water as a solvent. ^{1}H NMR (400 MHz, D_2O) δ/ppm: 8.03 (s, 1H, aromatic proton), 7.57 (dd, 1H, J_1 = 9 Hz, J_2 = 2 Hz, aromatic proton), 7.17 (dd, 1H, J_1 = 9 Hz, J_2 = 2 Hz, aromatic proton), 4.22 (t, J = 8 Hz, 1H, -CH), 3.26 (multiplet, 2H, -CH_2). LC-MS (ESI) m/z: $[M + H]^+$ Calcd for $C_9H_{10}N_2O_5 + H^+$, 227.19; found 226.88.

4-NP was synthesized from phenylalanine in the same way as 3-NT by electrophilic aromatic substitution of the nitronium ion,[11] and subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) for further purification. The mixture was injected onto the Agilent ZORBAX SB-C18 column (9.4 x 250 mm, 5 μm) (Santa Clara, CA) and eluted with eluent A (water: acetonitrile: trifluoroacetic acid = 95: 5: 0.1) and a linear gradient of eluent B (water: acetonitrile: trifluoroacetic acid = 50: 50: 0.1) for 20 min, followed by a washing and re-equilibration step. The injection volume was 400 μL at a flow rate of 3 mL min^{-1} and the UV absorbance of the eluents was monitored at 280 nm. The major product eluted at 11.5 min was characterized by NMR and LC-MS. ^{1}H NMR (D_2O) δ/ppm: 8.20 (d, 2H, J = 9 Hz, aromatic proton), 7.51 (d, 2H, J = 8 Hz, aromatic proton), 4.40 (t, 1H, J = 7 Hz, -CH), 3.39 (multiplet, 2H, $-CH_2$). LC-MS (ESI) m/z: $[M + H]^+$ Calcd for $C_9H_{10}N_2O_4 + H^+$, 211.19; found 210.99.

Microscopic images of red EBN strands with change in pH

Carl Zeiss Stemi 305 Trino Greenough system with Stand K EDU and spot illuminator K LED (Germany) was used to obtain the microscopic images of EBN strands. The images were captured and recorded with Microscopy Camera Axiocam 105 color and Carl Zeiss ZEN Lite software. EBN strands were soaked in the respective buffer solutions for 10 min and fan dried for 15 min at 25 ˚C and *ca.* 50% relative humidity, before the images were taken.

Solubilization and lyophilization of EBN

About 0.1g of white or red EBN was frozen in liquid nitrogen, pulverized, and sieved through 0.5 mm mesh. The EBN was rinsed with water (4 L) to remove any nitrate/nitrite in the EBN. Subsequently, the residue was further soaked and rinsed with 0.05 M urea (200 mL) to remove any residual nitrite, and finally rinsed with water (4 L) again. We will call this cleaned EBN residue. It was then added to 1 M sodium hydroxide (5 mL) and stirred until the

EBN dissolved (*ca*. 12 hours). The solubility of the EBN was about 20 g/L. The solution was centrifuged and the supernatant was ultra-filtered with Sartorius Vivaspin 20 ultrafiltration tube (MWCO 3000 Da) using water as eluent to remove any excess sodium hydroxide and salt present. The neutral EBN solution was lyophilized and stored at 4 ˚C. We will call the product lyophilized EBN.

Enzyme-linked immunosorbent analysis (ELISA)

The ELISA was conducted using the commercially available 3-NTyr sandwich ELISA kit. A calibration curve utilizing 3-NTyr labeled bovine serum albumin (BSA) in the working range from 8 to 1000 ng mL^{-1} was fitted using a 4-parameter logistic algorithm in Origin Pro 9.0 (Northampton, MA). Lyophilized white or red EBN samples, silver nitrate, and sodium nitrite were appropriately diluted for the analysis, and triplicate measurements were carried out for each sample. 3-NTyr labeled BSA and incubation buffer were used as the positive and negative control, respectively. The absorption at 600 nm was recorded at 15 min after the addition of the horseradish peroxidase enzyme (HRP) development solution with Tecan 96-wells plate spectrophotometer (Switzerland). The concentrations of 3-NTyr in the lyophilized EBN samples were determined.

UV-Visible spectroscopy

A series of solutions (~1 g L^{-1}) at different pH were made by adding the lyophilized EBN, 4-NP or 3-NT into pH buffers with pH from 4 to 10. The pH of the solutions was monitored with a Mettler Toledo benchtop pH meter (Switzerland). The absorption spectra of the solutions, in Fisher-Scientific 1 cm path-length quartz cuvette cell, were then recorded from 250 to 600 nm with a Shimadzu UV-1800 UV-Vis spectrophotometer (Japan).

Raman microspectroscopy

Cleaned white or red EBN residues were pre-soaked in 0.01 M sodium hydroxide to deprotonate the 3-NTyr residue so as to intensify the Raman bands of the nitro group, and fan dried for *ca.* 12 hours at 25 ˚C and *ca.* 50% relative humidity before measurement. Raman spectra of the EBN were collected with the Nanophoton Ramantouch microspectrometer (Nanophoton Corporation, Osaka, Japan) using the 532 nm laser excitation and instrument parameters (LU Plan Fluor 100 X, 600 gr mm^{-1}, 28 mW, 30 s, 24 ˚C).

Pure 3-NT (0.1 mg) was dissolved in 1 M sodium hydroxide (10 μL), deposited on a silicon substrate, and the solvent was allowed to evaporate slowly in a covered petri dish for *ca.*72 hours before the Raman measurement. The Raman spectrum of 3-NT was collected also using the 532 nm laser excitation, but with lower power (3 mW, 180 s, 24 ˚C) to minimize fluorescence.

Each sample was measured in triplicate over different spots and the average Raman spectrum was baseline corrected with the Origin Pro 8.0 software.

3. RESULTS AND DISCUSSION

Fumigation of white EBN with nitrous acid vapor and by xanthoproteic reaction to produce red EBN

But *et al.*[8] showed that red EBN can be formed by fumigating the white EBN with HNO_2 vapor formed by the reaction between sodium nitrite and hydrochloric acid or the vapor from 'bird soil' collected from birdhouses. Figure 1 shows the natural white nest (A), natural red nest (B), red nest produced by HNO_2 fumigation (C), and red nest produced by the xanthoproteic reaction (D).

Figure 2 shows the red color development of a piece of raw white EBN when fumigated with a mixture of sodium nitrite and hydrochloric acid over a period of 30 days (d). The white EBN had turned yellow/golden after 1 d, orange-red after 5 d and gradually a terracotta red

after 30 d. This can be explained by the increasing concentration of 3-NTyr residue in the EBN as the fumigation time increases.

In fumigation, sodium nitrite and hydrochloric acid react steadily to yield nitrous acid vapor,

$$NaNO_2(aq) + HCl(aq) \rightarrow HNO_2(aq, g) + NaCl(aq) \quad . \qquad (1)$$

The nitrous acid undergoes disproportionation to yield reactive nitrogen species (RNS), e.g. $\bullet NO_2$ and $\bullet NO$,[12, 13]

$$2HNO_2 \rightleftharpoons \bullet NO_2 + \bullet NO + H_2O \quad . \qquad (2)$$

Besides reacting with the glycoprotein tyrosine, the radicals can also undergo the following reactions,

$$2\bullet NO_2 \rightleftharpoons N_2O_4 \qquad (3)$$

$$N_2O_4 + 2H_2O \rightarrow NO_3^- + NO_2^- + 2H_3O^+ \quad . \qquad (4)$$

In the presence of atmospheric O_2, the oxidation of $\bullet NO$ can also occur,[14]

$$4\bullet NO + O_2 + 6H_2O \rightarrow 4NO_2^- + 4H_3O^+ \quad . \qquad (5)$$

The EBN in the birdhouse is thus exposed to an atmosphere containing $\bullet NO_2$, $\bullet NO$, NO_2^-, and NO_3^-.

In analogy to protein tyrosine nitration in living systems, the reaction between the RNS and protein tyrosine can occur by a radical mechanism as shown in Scheme **1**. The one electron oxidant $\bullet NO_2$ can undergo a slow reaction with the glycoprotein tyrosine to give the tyrosyl radical, and a further fast reaction with $\bullet NO_2$ gives 3-NTyr.[15, 16] The tyrosyl radical can also react (slowly) with $\bullet NO$ to form the intermediate 3-nitrosotyrosyl residue which is rapidly oxidized to 3-NTyr, e.g. by HNO_3.[13, 15, 17] The intermediate 3-nitrosotyrosyl residue

can be trapped by the addition of Hg(II) to give a red pigment as in Millon's test for tyrosine.[18]

The natural red EBN, Figure 1B, came from a birdhouse. In birdhouses, there's swiftlet bird soil on the floor. The bird soil is rich in protein and nitrogenous compounds because the swiftlets feed on a high protein diet of aerial insects. Aerobic bacteria can feed on the bird soil and its metabolism produces nitrous acid and nitric acid vapors. If the concentration of nitrous acid reaches a sufficient level in the largely enclosed birdhouse, it will be absorbed by the white EBN initially built by the swiftlet on the walls and ceiling of the birdhouse, and reacts by Scheme **1** first to a yellow/golden color, then to red, and finally to a terracotta red as the concentration of 3-NTyr increases in the EBN, just as in Figure 2. Thus, even the white nest can have a small amount of 3-NTyr. Equations (4) and (5), and also nitric acid vapor from the bird soil, explains why red EBN would have a significant amount of nitrite and nitrate. This makes red EBN an unsafe food or health supplement, if not properly cleaned. The toxicity of high concentrations of nitrite and nitrate (found in thousands of ppm in red EBN) was what led China to put a ban on the sale of both white and red EBNs at one time.[19] The nitrate and nitrite in EBN, being water soluble, can be rinsed away, and this is carried out before analyzing for 3-NTyr to avoid interference.

In the xanthoproteic reaction of EBN with a mixture of nitric and sulfuric acid to give red EBN, significant amounts of 3-NTyr is formed by Scheme **2**. By conjugating with sulfuric acid, nitric acid produces a nitronium ion (NO_2^+), which is the active species in electrophilic aromatic nitration,

$$HNO_3(aq) + 2H_2SO_4(aq) \rightleftharpoons NO_2^+(aq) + H_3O^+(aq) + 2HSO_4^-(aq) \quad . \qquad (6)$$

The nitronium ion reacts with the aromatic ring to form a nitrotyrosyl arenium ion, and subsequently the H^+ is eliminated by a nucleophile, H_2O, and a neutral 3-NTyr is formed. [10, 20]

Determination of 3-NTyr in white and red EBN with ELISA

An anti-3-NTyr ELISA kit was used to detect and quantify the 3-NTyr in natural white EBN, natural red EBN, HNO_2 fumigated EBN and xanthoproteic reacted EBN. The concentrations of 3-NTyr detected and the degree of protein tyrosine nitration are shown in Table 1.

The concentrations of 3-NTyr in red EBNs are hundreds to thousands of times higher than in white EBN. In the natural white EBN, the concentration of 3-NTyr is very low 2.5 ± 1.2 ppm, while natural red EBN and HNO_2 fumigated EBN contained $(9.84 \pm 1.24)x10^2$ ppm and $(2.53 \pm 0.45)x10^3$ ppm of 3-NTyr, respectively. It was observed that a higher degree of nitration could be achieved if the fumigation time was lengthened or the surface area of the EBN was increased, e.g. by pulverization. The xanthoproteic reaction gave a more effective nitration of the glycoprotein tyrosine, and the red EBN produced had an even higher concentration of 3-NTyr, $(8.28 \pm 1.64)x10^3$ ppm. The EBN tyrosyl nitration degree in Table 1, ranging from <0.0034% for natural white EBN to about 7.6% for the xanthoproteic reacted EBN, was calculated based on 87.2 mg tyrosine per g of EBN.[5]

Color change in the red EBN strands as a function of pH

A color change as a function of pH, like an indicator, was observed in strands of red EBNs when they were soaked in the respective buffers and air-dried. Figure 3 shows the microscopic images where a color change from light yellow/golden to a deep red can be observed when the buffer changed from acidic to alkaline pH. The natural red EBN and HNO_2 fumigated EBN were pale yellow from pH 2 to 5, then an orange hue developed from pH 6 to 9, and finally a deep red was seen from pH 10 to 13. The color intensity of natural red EBN is lower than HNO_2 fumigated EBN due to the lower concentration of the 3-NTyr chromophore. A similar color change (not shown) occurred with strands of the xanthoproteic reacted red EBN.

UV-Vis absorption spectra of 4-nitrophenylalanine and 3-nitrotyrosine as a function of pH

Figure 4A shows the absorption spectra of 4-nitrophenylalanine (4-NP) as a function of pH from 4 to 10. It absorbs in the UV region and would not show any visible color. The absorption bands are similar with a maximum at 280 nm for pH 4-8, and a maximum at 290 nm for pH 9-10. There would be a gradual change in the absorption band from one form to another at around pH 9, due to the change from protonated to deprotonated amine on the amino acid as phenylalanine has $pK_{a2} \approx 9.1$.

In the UV-Vis absorption spectra of 3-NT, the hydroxyl (-OH) auxochrome in 3-NT, conjugated with the nitro ($-NO_2$) group chromophore, when deprotonated (to $-O^-$) will give a bathochromic shift of the absorption. The absorption spectra of 3-NT (Figure 4B) in acidic solutions, with –OH intact, show an absorption maximum at 358 nm. As the pH is increased, the absorption at 358 nm gradually decreases, and the absorption with a 427 nm maximum for the deprotonated phenolate $-O^-$ form of 3-NT increases. The color change due to the phenol-phenolate reversible equilibrium and the presence of an isosbestic point at 380 nm makes 3-NT, with a pK_a of 7.1, an indicator.[21, 22]

The color change of 3-NT solutions from light yellow to red as the pH is changed from 2 to 13, is shown in Figure 4C. Clearly, the color change with pH for red EBN strands in Figure 3 is similar. It suggests that the chromophore present in red EBN is likely to be 3-NT. The phenylalanine in EBN is much less readily nitrated compared to tyrosine, and even if it is nitrated, its absorption is in the UV region and will not show any visible color change with pH.

UV-Vis absorption spectra of white and red EBN as a function of pH

To ascertain that the absorbing chromophore in red EBN is 3-NTyr, the absorption spectra of solubilized white and red EBNs were investigated. Figure 5 shows the absorption

spectra of white EBN as a function of pH. The absorption falls in the UV region and is nearly pH independent. The absorption band at 280 nm is due to the π-π* transition of the aromatic amino acids – tyrosine and phenylalanine – in EBN.[23]

Figures 6A-C show the absorption spectra as a function of pH for the three types of red EBNs – natural, HNO_2 fumigated, and xanthoproteic reacted. The corresponding difference spectra between the red and white EBNs, Figures 6D-F, would reveal the absorbing chromophore. They each show a bathochromic shift with a clear resemblance to the pH-dependent absorption spectra for 3-NT (Figure 4B), with absorption maxima at *ca.* 360 nm in acidic medium, shifting to *ca.* 430 nm in basic medium, and an isosbestic point at *ca.* 380 nm. The absorptivity ε in Figures 6A-C is given in terms of the concentration (g L^{-1}) of EBN, and then was scaled using the concentration of 3-NTyr determined by ELISA in Table 1 for the respective red EBNs to give the molar absorptivity of 3-NTyr in EBN in Figures 6D-F. It corresponds well to the molar absorptivity of pure 3-NT at the 427 nm absorption maximum, within the margin of error of the determination of 3-NTyr concentration by ELISA. The nitration could also produce a small amount of 4-NP residue on the glycoprotein and this has not been subtracted out in Figures 6D-F; it would raise the difference absorption spectra only in the 290-350 nm region slightly, but would not affect the major band at around 427 nm.

Raman spectra showing the nitro group in red EBN

Raman microspectroscopy with 532 nm laser excitation was used to confirm the presence of the nitro group in red EBN. The Raman spectra of white EBN, natural red EBN, HNO_2 fumigated EBN, xanthoproteic reacted EBN, and pure 3-NT are shown in Figures 7A-E, respectively. The two rectangular boxes in Figures 7B-D frame the spectral regions where the red EBNs differ significantly from the white EBN. The red EBNs show a strong Raman band at 1330 cm^{-1} which is due to the symmetric $–NO_2$ stretch, and a strong Raman band at 825

cm^{-1} which is due to the $–NO_2$ scissoring bend.[24, 25] These two $–NO_2$ Raman bands are also present in the Raman spectrum of 3-NT, Figure 7E.

EBN as a scavenger for reactive nitrogen species

In summary, our findings, based on the color change with pH, UV-Vis spectra as a function of pH, and Raman microspectroscopy on red EBN, provide conclusive evidence for the nitration of tyrosine residues in the mucin glycoprotein of EBN to yield 3-NTyr which gives rise to the red color. The presence of 3-NTyr in red nest is a post-translational modification. The 3-NTyr pigment, with a $pK_a \approx 7.1$, behaves like an indicator and exhibits a color change from yellow in acid to red in alkali, with the color change occurring around pH 7.

Generally, protein nitration by RNS is a complex process. It has been suggested that protein nitration is dependent on the proximity of the protein tyrosine to the nitration source, but not all tyrosine residues in a protein are equally susceptible to nitration. There is no specific amino acid consensus sequence or specific protein primary structure that determines the site of tyrosine nitration.[26, 27] From our results, it was found that EBN is quite readily nitrated. The high concentration of asparagine/aspartic acid (60.1 mg g^{-1}), glutamine/glutamic acid (48.9 mg g^{-1}), and glycine (21.1 mg g^{-1}) residues[5] in EBN might be responsible for this, as it has been noted that the acidic amino acids (glutamic acid or aspartic acid) or turn-inducing amino acids (proline and glycine) in the vicinity of the targeted tyrosine are the characteristic features observed for nitrated proteins.[28]

In vivo, a high concentration of RNS can induce pathological effects on our body, and the protein-bound tyrosine in our organs and tissues react with RNS to form 3-NTyr.[29, 30] The levels of protein nitration is clinically viewed as a marker of oxidative or nitrative stress.[31, 32] For this reason, a food material like EBN that is able to mop up RNS may help to alleviate disease-related nitrative stress.[33-36] This could be one of the new benefits in the prescription

of EBN in traditional Chinese medicine. White EBN, where >99% of the tyrosine residues are not nitrated would therefore be more beneficial than the colored yellow, golden, orange or red EBNs where a portion of the tyrosine residues would have already been spent, i.e. converted to 3-NTyr.

Preliminary results show that EBN is also a very strong antioxidant, with higher antioxidant capacity than black garlic, ginseng or goji berry, and white EBN is a stronger antioxidant compared to red EBN. Part of the antioxidant capacity of EBN arises from the protein bound tyrosine, so tyrosine plays multiple roles of mopping up RNS as well as free radicals and oxidants. This work is ongoing.

ABBREVIATIONS USED

EBN, Edible bird's nest; RNS, reactive nitrogen species; 3-NT, pure 3-nitrotyrosine; 4-NP, 4-nitrophenylalanine; 3-NTyr, 3-nitrotyrosine residue in the glycoprotein; ELISA, Enzyme-linked immunosorbent analysis; NMR, nuclear magnetic resonance; LC-MS, liquid chromatography-mass spectroscopy; ESI, electrospray ionization; RP-HPLC, reversed-phase high-performance liquid chromatography; HRP, horseradish peroxidase

FUNDING SOURCES

We gratefully acknowledge the financial support of the School of Physical and Mathematical Sciences, Nanyang Technological University, for consumables and instrument time.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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473

474 **FIGURE CAPTIONS**

475 **Figure 1.** A. White EBN; B. Natural red EBN; C. HNO_2 fumigated EBN; and D.
476 Xanthoproteic (HNO_3+H_2SO_4) reacted EBN.

477

478 **Figure 2**. Development of the red color in EBN by HNO_2 fumigation from 0 – 30 days (d).

479

480 **Figure 3.** Color change in strands of natural red EBN (top panel) and HNO_2 fumigated EBN
481 (bottom panel) as pH is varied from 2 to 13, observed under the microscope.

482

483 **Figure 4.** UV-Vis absorption spectra of A. 4-nitrophenylalanine, and B. 3-nitrotyrosine – pH
484 10 (black), pH 9 (red), pH 8 (orange), pH 7 (green), pH 6 (blue), pH 5 (indigo) and pH 4
485 (purple); and C. Color change of 3-nitrotyrosine solution (5 g L^{-1}) from yellow to red with pH
486 from 2 to 13.

487

488 **Figure 5.** pH independent UV-Vis absorption spectra of solubilized white EBN – pH 10
489 (black), pH 9 (red), pH 8 (orange), pH 7 (green), pH 6 (blue), pH 5 (indigo) and pH 4
490 (purple).

491

492 **Figure 6.** pH dependent UV-Vis absorption spectra (A - C) of A. natural red EBN (top panel),
493 B. HNO_2 fumigated EBN (middle panel), and C. xanthoproteic reacted EBN (bottom panel)
494 and the corresponding difference spectra (D - F) between red and white EBN – pH 10 (black),
495 pH 9 (red), pH 8 (orange), pH 7 (green), pH 6 (blue), pH 5 (indigo) and pH 4 (purple). In (A-

C), the absorption spectra in the region between 320-600 nm have been scaled up by an appropriate factor, as indicated, for visualization.

Figure 7. Comparison of Raman spectra of A. raw white EBN (black), B. natural red EBN (red), C. HNO_2 fumigated EBN (orange), D. xanthoproteic reacted EBN (green), and E. 3-nitrotyrosine (blue). The boxes over the red EBNs show: (a) the 1330 cm^{-1} Raman band which is due to the symmetric $–NO_2$ stretch, and (b) the 825 cm^{-1} Raman band which is due to the $–NO_2$ scissoring bend. These bands are also present in 3-nitrotyrosine.

Scheme 1. The reactions between the protein tyrosine residues and reactive nitrogen species - $•NO_2$ and •NO – to produce 3-NTyr during HNO_2 fumigation. The illustration is not to scale as the mucin glycoprotein is a 3D giant macromolecule in comparison to the tyrosine residue. For the sake of clarity, only one tyrosine residue is shown.

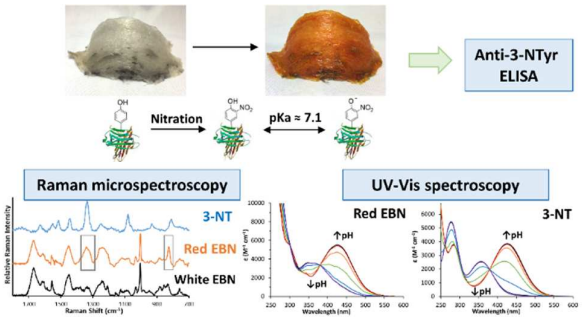
Scheme 2. Electrophilic aromatic substitution reaction by the nitronium ion, NO_2^+, on the protein tyrosine residues in the xanthoproteic reaction to produce 3-NTyr.

513 **TABLES**

514 **Table 1.** Concentrations of 3-nitrotyrosine residue found in white and red EBNs by ELISA
515 test.

sample	**[3-nitrotyrosine] (ppm)**	**EBN tyrosyl nitration degree (%)**
natural white EBN	2.5 ± 1.2	< 0.0034
natural red EBN	$(9.84 \pm 1.24)\times10^{2}$	0.90 ± 0.11
EBN fumigated with $NaNO_2$/HCl for 30 days	$(2.53 \pm 0.45)\times10^{3}$	2.32 ± 0.41
xanthoproteic reacted EBN	$(8.28 \pm 1.64)\times10^{3}$	7.60 ± 1.50

GRAPHIC FOR TABLE OF CONTENTS



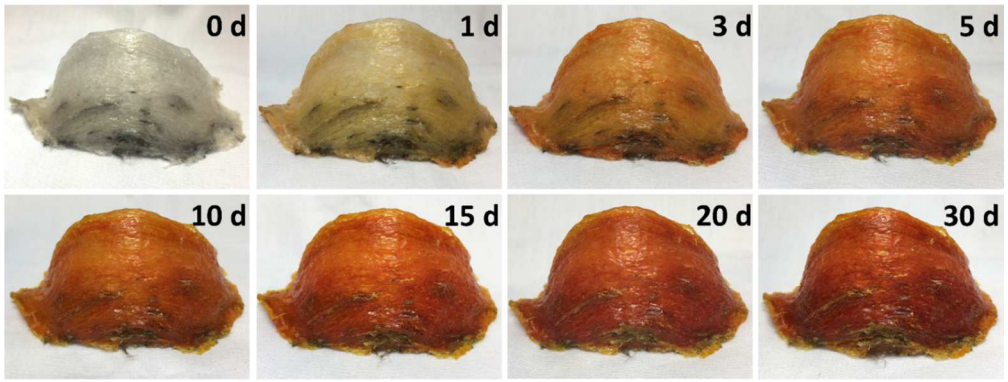


Figure 2. Development of the red color in EBN by HNO_2 fumigation from 0 – 30 days (d).

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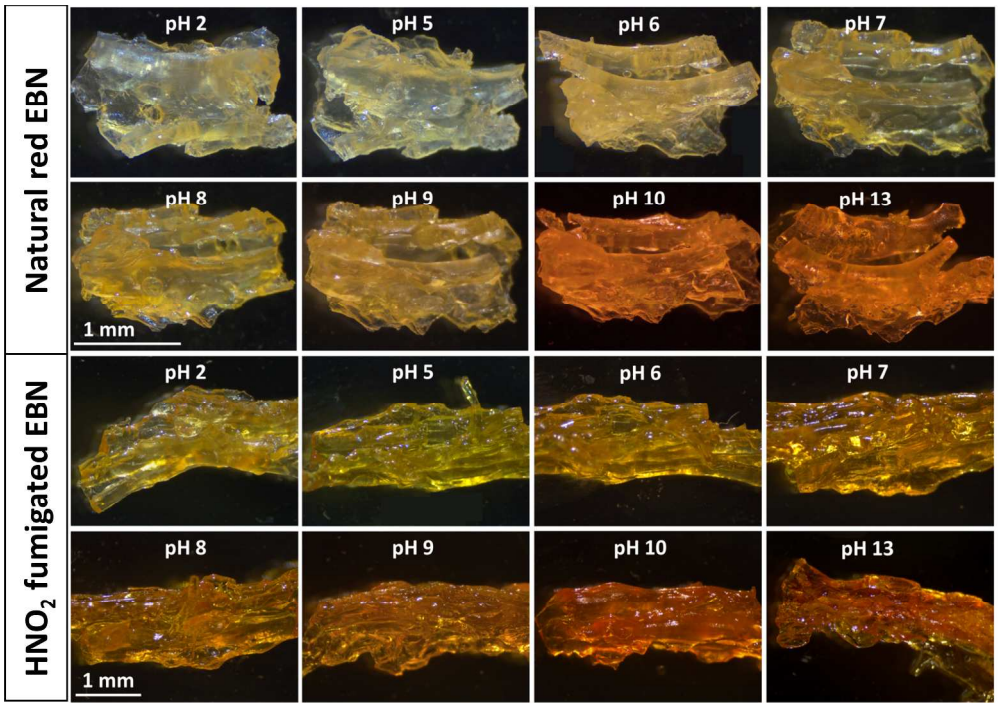

Figure 3. Color change in strands of natural red EBN (top panel) and HNO_2 fumigated EBN (bottom panel) as pH is varied from 2 to 13, observed under the microscope.

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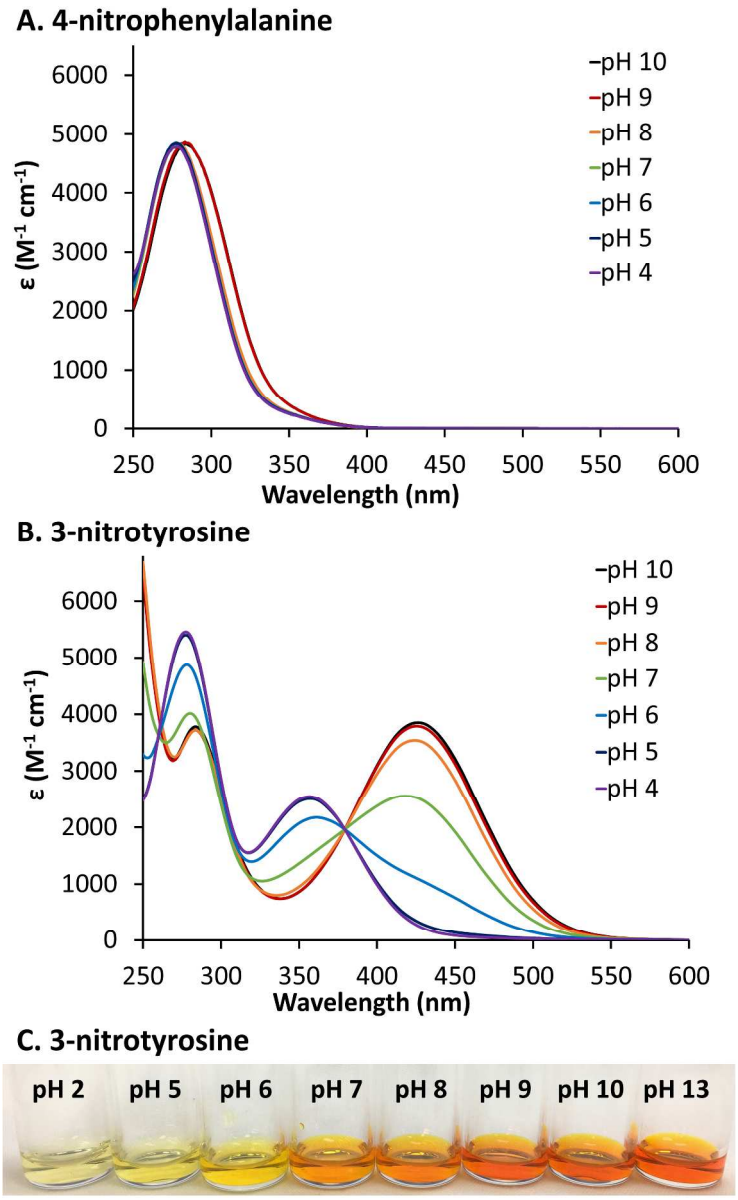

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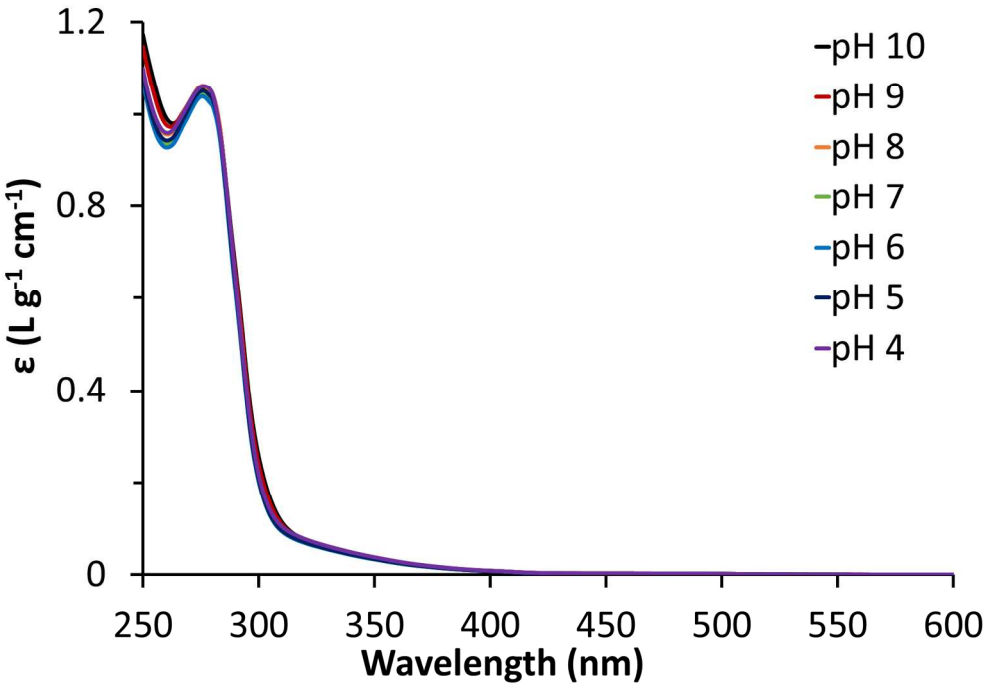

Figure 5. pH independent UV-Vis absorption spectra of solubilized white EBN – pH 10 (black), pH 9 (red), pH 8 (orange), pH 7 (green), pH 6 (blue), pH 5 (indigo) and pH 4 (purple).

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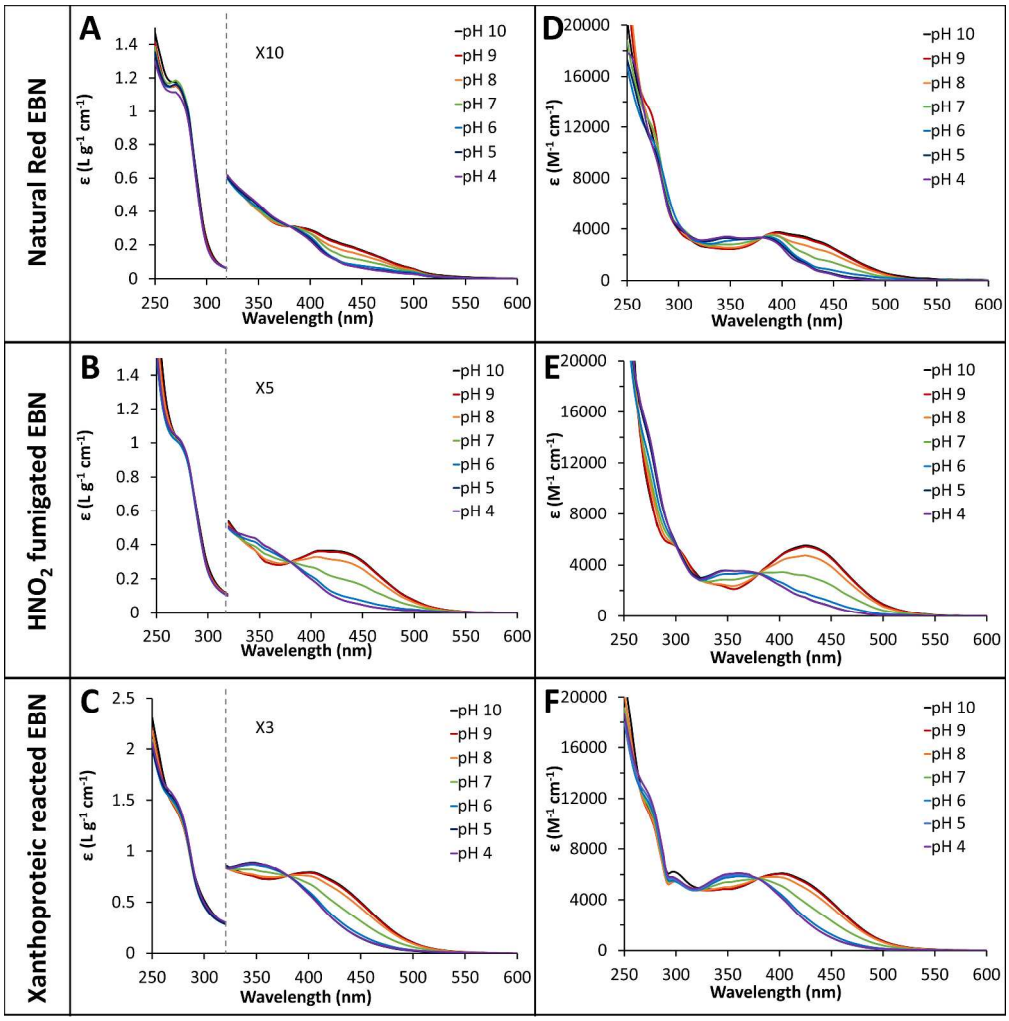

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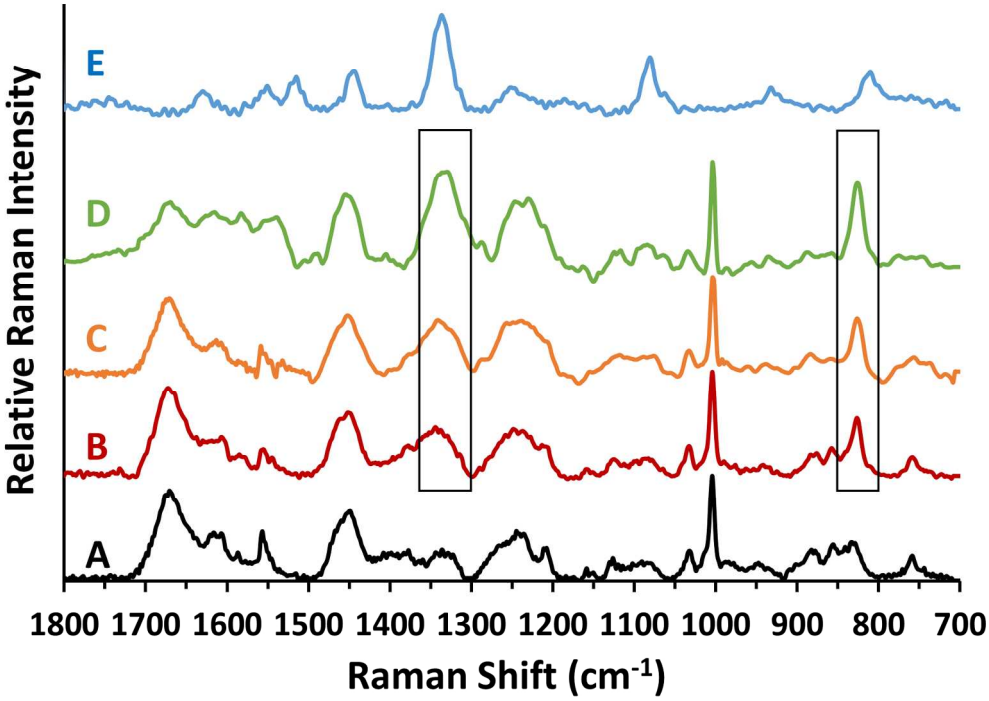

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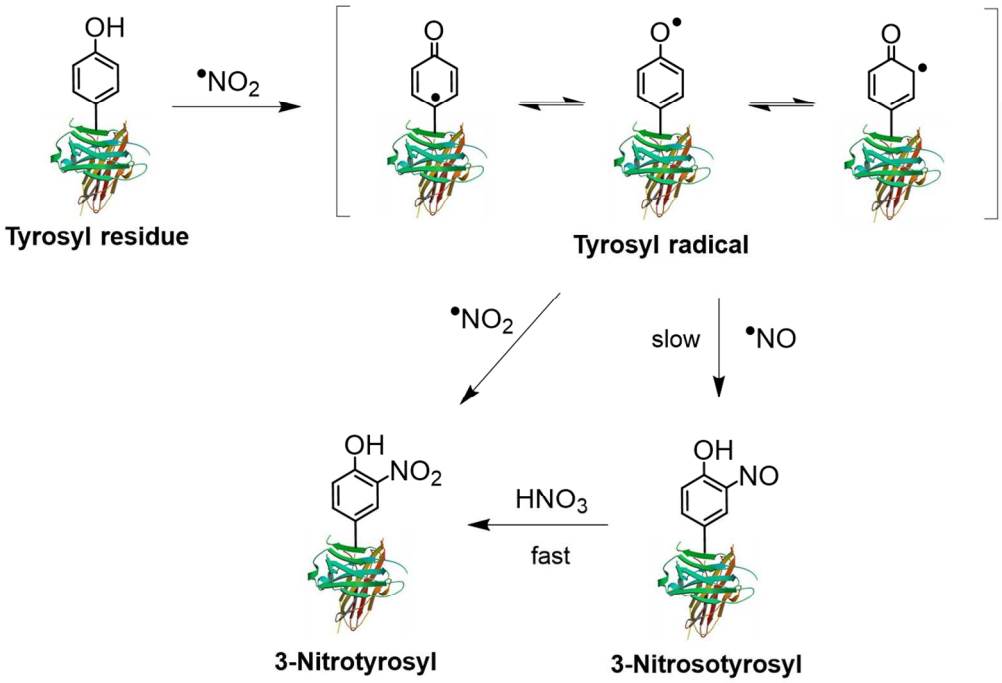

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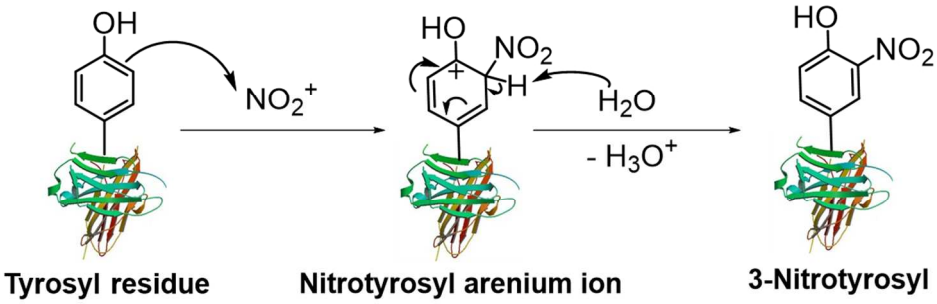

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