Multicharacteristic Behavior of Tyrosine Present in the Microdomains of the Macromolecule Gum Arabic at Various pH Conditions

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Supporting Information

ABSTRACT: Gum arabic (GA), the dried exudates of Acacia seyal and Acacia senegal trees, being a biopolymer, has found many applications in the food, pharmaceutical, cosmetic, and lithography industries. GA, a water-soluble food hydrocolloid, is a complex and variable mixture of arabinogalactan oligosaccharides, polysaccharides, and glycoproteins. It has been a subject of great interest and a wide range of research has been done on the polysaccharide structural aspects and the emulsifying properties only. In the present study, fluorescence spectral technique is employed as an analytical tool to understand the photophysics of GA. The tyrosine microenvironment of GA was explored by studying the steady-state absorption, emission, fluorescence lifetime, and three-dimensional (3D) emission contour spectra of GA at various pH conditions in aqueous solution. The multiple emissive states are attributed to the presence of intrinsic fluorophore tyrosine in a heterogeneous microenvironment. The study portrays the multicharacteristic behavior of tyrosine in various pH conditions and in different microdomains. The exposure of the buried tyrosine to the heterogeneous aqueous phase was authenticated by 3D emission contour spectral studies. An interesting visualization of tyrosine involving in hydrogen-bonding network with another tyrosine moiety at neutral pH was ascertained. The coexistence of hydrophilic carbohydrate and hydrophobic protein in GA enables its emulsification and stabilization properties. Hence, any advancement toward understanding the protein microenvironment of GA is of great significance for chemists, as the molecular modeling and biosynthesis of the gum with desired end product are underway in many research institutes.

1. INTRODUCTION

Gum arabic (GA), the dried exudates of Acacia seyal and Acacia senegal trees, being a biopolymer, has found many applications in the food, pharmaceutical, cosmetic, and lithography industries. GA is desirable for its emulsifying, stabilizing, binding, and shelf-life enhancing properties. Current literature suggests its cardio-, reno-, gut-, and dental-protective; satiety-inducing; antimicrobial; anti-inflammatory; and anticoagulant implications, which makes GA one of the best food hydrocolloids. Its foray into drug delivery, sensor, tumor imaging, and nanotechnology has met with appreciable success, fueling further investigation into its unexplored functionality. GA is a highly branched, neutral, or slightly acidic, polysaccharide complex, containing about 2% of polypeptide. Extensive research on the composition, structure, and conformation of GA has been reported in the literature. In general, three main fractions have been isolated by hydrophobic interaction chromatography. The three fractions are arabinogalactan—peptide or AG, arabinogalactan—protein or AGP, and glycoprotein or GP. The fraction (F) 1 (AG) is a thin oblate ellipsoid, branched disklike structure. The branches are mainly composed of 1,3-linked β-D-galactopyranosyl units with 1,6-linked β-D-galactopyranosyl side chains to which many arabinosyl, uronic acid, and rhamnose residues are linked, where the 43 amino acid residue peptide sequence was supposed to be totally buried. Fraction 2 (AGP) is a linear chain with branched building blocks closely resembling the wattle blossom model. The AGP consists of short arabinose side chains and much larger blocks of highly branched carbohydrates, linked to a polypeptide chain possibly containing approximately 250 amino acids, and the molecule adopts a very compact conformation with a radius of gyration ($R_g$) of 36 nm. Fraction 3 (GP) consists of spheroidal ringlike monomers of hydroxyproline—arabinogalactan (AG) subunits and more anisotropic oligomers resulting from monomer self-association. The compositions of the various fractions of GA are well established in the literature and are provided in Table 1.

GA has been a subject of great interest, and a wide range of research has been done on the structural aspects and the emulsifying properties only. The photophysical studies of GA were found to be very less in the literature. A valuable feature of intrinsic protein fluorescence is the high sensitivity to its local environment. Changes in the emission spectra often occur in response to conformational transitions, subunit association, substrate binding, or denaturation. Hence,
Table 1. Composition of the Three Fractions of GA

| fraction composition | F1 (AG) | F2 (AGP) | F3 (GP) |
|----------------------|---------|----------|---------|
| molecular weight (g mol⁻¹) | 2.86 × 10⁵ | 1.86 × 10⁵ | 2.95 × 10⁵ |
| total weight % | 88.3 | 10.3 | 1.3 |
| protein content % | 1.1 | 9 | 24.6 |
| no. of amino acids | 43–45 | 250–400 | No data |
| fluorescing amino acids | no | yes | yes |

!Figure 1. Absorption spectrum of GA (5.71 × 10⁻⁶ M) at various pH conditions.

valuable information about the protein microenvironment can be obtained through photophysical studies. Since the biosynthesis of the gum using biotechnological methods is of great interest, fluorescence spectroscopy can be applied as a tool to probe the microenvironment within the macromolecule.¹⁶

In our present study, we have recorded absorption, emission, fluorescence lifetime, and three-dimensional (3D) emission contour studies of GA in various pH conditions by buffering the medium using the universal Britton and Robinson buffer solution. The variation in the microdomains of the intrinsic fluorophore was ascertained by fluorescence spectral techniques.

2. RESULTS AND DISCUSSION

The three main fractions of GA have been isolated, analyzed, and reported in the literature.¹¹⁻¹⁴ Each fraction contained similar proportions of the various sugars and differed essentially in their molecular masses and protein contents,⁸ as provided in Table 1. The AGP and GP fractions of GA constitute to about 10.3 and 1.3% by total weight, and the protein contents were 9 and 24.6%, respectively. Earlier reports convey that these two fractions do exhibit fluorescence, but the origin of fluorescence was not emphasized. The AG fraction makes up to 88% of the total gum, made mostly of sugars and has the least protein content (1.1%) but does not exhibit any fluorescence property. All of the three fractions have similar sugar units and differ only in the protein content, which clearly reveals that the observed fluorescence for GA is not due to the sugar moieties but due to the protein content of AGP and GP fraction.

GA amino acid composition has been reported in the literature,¹⁵ and the compositions of fluorescing amino acids like phenylalanine, tyrosine, and tryptophan were found to be 6.33, 2.38, and 0 mol mg⁻¹ respectively. It is well known that tyrosine and tryptophan amino acids dominate the emission of most proteins compared to phenylalanine, which generally shows weak absorption and emission spectra at 260 and 282 nm, respectively.¹⁵ Tryptophan being absent in GA, the observed absorption and fluorescence spectra at 278 and 315 nm were ascribed predominantly to the tyrosine moieties only. The observations we made through all of the spectral studies are discussed in detail.

2.1. Absorption Spectral Studies. The absorption spectrum was measured for GA as such and for buffered solution of GA at various pH conditions. The absorption spectrum of GA exhibits a maximum at 278 ± 2 nm, which is correlated to tyrosine amino acid.¹⁵ As the pH of the medium was increased, the absorption maximum at 278 nm showed a slight increase in the absorbance along with a significant red shift, which is apparent in the normalized absorption spectrum of GA at different pH conditions (Figure 1). The observation is in accordance with the behavior of L-tyrosine amino acid in basic pH conditions, reported in the literature.¹⁷,¹⁸ Tyrosine can form ground-state complex with weak base near neutral pH, leading to the formation of tyrosinate anion.¹⁵ The Britton and Robinson buffer furnishes acetate and phosphate ions. Hence, the reason for red shift in the absorption maximum is the formation of a ground-state complex between tyrosine and the weak bases in the medium. The pKₐ of the aromatic hydroxyl group in tyrosine is 10 in the ground state. At pH 11.1 (pH > pKₐ), there is a clear red shift (278–293 nm) in the absorption maximum, indicating the formation of tyrosinate anion. To achieve pH 11.1, 0.2 M sodium hydroxide was used, while for all of the other pH conditions, Britton and Robinson buffer solution was used.

2.2. Fluorescence Spectral Studies. The fluorescence spectrum of GA exhibits a maximum at 315 ± 3 nm, a slight hump at 320 nm, and a broad shoulder from 390 to 450 (Figure 2). The observed fluorescence in GA is attributed to tyrosine moieties.¹⁵ At pH 2.18, the emission peak at 315 nm is blue-shifted to 311 nm, the hump at 320 nm decreases in intensity, while there is an increase in the intensity of the emission peak at 450 nm. It is well known that in acidic medium, the polysaccharides undergo cleavage at the glycosidic linkages forming mono-, di-, and oligosaccharides, and a similar phenomenon is reported for GA¹⁹ in dilute acids. Hence, a polysaccharide breakdown into minor units at pH 2.18 is presumed; in the process, the buried polypeptide is exposed to the aqueous phase. There are several reports signifying that protein-containing fluorescing amino acids on denaturation usually exhibit more than one emission apart from the native emission. An emission above 400 nm along with a high fluorescence lifetime results for many protein molecules on denaturation, which confirm the existence of fluorescing amino acids in a heterogeneous environment.¹⁵,²⁰⁻²² This, in general, is correlated to the presence of tryptophan moieties, and in our studies, tryptophan moieties being absent, the peak at 450 nm is attributed entirely to the exposure of tyrosine amino acids to the aqueous phase. A similar behavior is observed at various pH conditions, and exposure of tyrosine to the aqueous phase has occurred to varying extents, which is visualized by the change in the intensity of the emission peak at 450 nm. Maximum exposure of the peptide to the aqueous phase is observed at pH 9.20. In strong basic conditions, polysaccharides undergo a haphazard fragmentation into glycolic aldehyde, trioses, tetroses, formaldehyde, and glycolic acid. Such a happening in GA exposes the peptide to a greater extent to the aqueous phase at pH 9.20.
At pH 7.04, an interesting fluorescence pattern is observed (Figure 2B). Three distinct peaks are observed at 314, 333, and 450 nm. A unique and a prominent dip is seen at 317 nm. Several slight humps are observed at 320, 350, and 362 nm. Such occurrence of doublets (314 and 333 nm) are more probable for dimerization of fluorophore and inter- or intramolecular hydrogen bonding. 26 To get a better knowledge about the ground-state and excited-state properties and the microenvironment of the intrinsic fluorophore at pH 7.06, 3D emission contour spectral studies were performed. The results are discussed in Section 2.4.

2.3. Fluorescence Lifetime Studies. The fluorescence decay of aqueous solution of GA at various pH conditions such as 3.24, 4.63, 5.51, 6.22, 7.81, and 9.44 were measured. The excitation wavelength was fixed at 280 nm, and the decay at wavelengths 315, 335, and 450 nm were followed. The fluorescence decay of GA is triexponential at all pH conditions. The decay for GA in various pH conditions at 315 nm is shown in Figure 3 and its corresponding decay analysis is provided in Table 2. At pH 3.24, the lifetimes of the three components are 0.31, 0.94, and 2.13 ns and the relative amplitude percentages are 6, 86, and 9%, respectively. The second component has the highest relative amplitude percentage (86%) with a lifetime of 0.94 ns. As the pH of the medium was increased, τ2 gradually increases while A2 gradually decreases to about 1.45 ns and 35%, respectively. The observation clearly reveals that the component which was once emitting at 315 nm, as the pH of the medium is becoming more and more basic, is increasingly emitting at 450 nm. At 450 nm, all of the components at different pH values show an increased lifetime values compared to those at 315 nm. The fluorescence decay and decay analysis at 335 nm are given in the Supporting Information (Figure S1 and Table S1), where one can see the
the shift of the lifetimes for population. Hence, as observed in our studies, the increased which is a carbohydrate protein complex, leads to the exposure of tyrosine in the excited state is between 4 and 5; hence, the observed lifetimes for GA authenticate the predominant fluorophore to be tyrosine only.

2.4. Three-Dimensional (3D) Emission Contour Spectral Studies. The 3D emission contour spectra of GA at different pH conditions manifest the presence of multi-emissive environment. In the contour spectra of unbuffered GA (Figure 4A), two major regions are observed. The first region (R1) corresponds to the excitation centered at 280 nm and emission centered at 315 nm. The second region (R2) corresponds to the excitation centered at 280 and around 340 nm and a broader emission at 450 nm. Both the regions R1 and R2 are populated to varying extents depending on the pH of the medium. For unbuffered GA, R1 shows a strong fluorescence intensity and R2 shows a weak fluorescence intensity. On closer examination of R1 (Figure 4A), one could see that there is a separation of two minor segments within R1, one at 310 nm and the other at 330 nm (emission wavelengths). We observe that the two segments within R1 are being populated to varying extents at various pH conditions.

At pH 2.18 (Figure 4B), maximum intensity is at 310 nm supporting the emission spectrum observed for pH 2.18 (Figure 2). For pH values 3.20, 4.46, and 5.51, the segment at 310 nm gradually decreases in intensity, and at pH 6.22 (Figure 4C), the segment eventually vanishes and only a single segment is observed centered at 332 nm. On closer examination of R2 (Figure 4A), the aromatic hydroxyl group has the least ability to dissociate or to involve in hydrogen bonding with other amino acids in the vicinity or with the aqueous phase. Hence, the segment at 310 nm is predominant in R1 at pH 2.18. As the pH of the medium increases, the ability of the tyrosine to dissociate or to involve

| pH  | $r_1$ ± | $r_2$ ± | $r_3$ ± | $A_1$ | $A_2$ | $A_3$ | $\chi^2$ |
|-----|---------|---------|---------|-------|-------|-------|---------|
| 3.24 | 0.98 ± 0.07 | 2.74 ± 0.03 | 9.54 ± 0.22 | 51 | 35 | 15 | 1.15 |
| 4.63 | 0.92 ± 0.05 | 3.16 ± 0.1 | 9.5 | 0.2 | 44 | 38 | 18 | 1.09 |
| 5.51 | 0.74 ± 0.03 | 2.36 ± 0.08 | 7.54 ± 0.11 | 33 | 40 | 27 | 0.94 |
| 6.22 | 0.81 ± 0.03 | 2.84 ± 0.07 | 9.04 ± 0.18 | 37 | 47 | 17 | 1.15 |
| 7.06 | 0.69 ± 0.05 | 2.66 ± 0.06 | 8.39 ± 0.17 | 29 | 52 | 19 | 1.1 |
| 9.44 | 0.8 ± 0.06 | 2.86 ± 0.08 | 7.63 ± 0.16 | 24 | 55 | 21 | 1.07 |

“Excitation wavelength: 280 nm; emission wavelength: 450 nm.”
in hydrogen bonding also increases, which results in the decrease of intensity of the segment at 310 nm, and a new segment gradually appears and dominates R1 at pH above the $pK_a$ of tyrosine in the excited state, which is 6.22. A plot of pH versus normalized fluorescence intensity (Figure 2A) supports our discussion. At 311 nm (Figure 2A), the maximum fluorescence intensity is observed for pH 2.18, and the intensity decreases as the pH of the medium increases to 6.22. The opposite is observed at 334 nm, where the fluorescence intensity increases with the increase in pH. As we study the contour spectra at the high pH conditions, such as pH 8.43 (Figure 4D) and pH 9.20 (Figure 4E), one should expect to observe only one segment at 330 nm in R1; on the contrary, the segment at 310 nm continues to be present in minor intensities from pH 7.04 to 9.20. This contradiction can be explained considering the after effects of fragmentation of GA. As discussed earlier, on fragmentation, GA shows an unusual fluorescence at 450 nm due to the exposure of the fluorescing amino acids to a heterogeneous environment. The region R2 corresponds to the fraction of the amino acids completely

Figure 4. (A) Three-dimensional (3D) emission contour spectrum of GA as such (unbuffered). (B) Three-dimensional (3D) emission contour spectrum of GA at pH 2.18. (C) Three-dimensional (3D) emission contour spectrum of GA at pH 6.22. (D) Three-dimensional (3D) emission contour spectrum of GA at pH 8.43. (E) Three-dimensional (3D) emission contour spectrum of GA at pH 9.20. (F) Three-dimensional (3D) emission contour spectrum of GA at pH 7.04.
exposed to the aqueous phase. Hence, observing the R2 of all of the contour spectra, we conclude that the extent of fragmentation of GA is directly proportional to the increasing strengths of base and also with increasing strengths of acid. The trend in fragmentation is clearly evident in Figure 2A at 450 nm. The plot clearly reveals the ratio of fluorescence intensities at 311 and 450 nm for various pH conditions, which again ascertains the maximum exposure of tyrosine to the aqueous phase at pH values 2.18 and 9.44. So, on fragmentation, it is now evident that a portion of the polypeptide in GA is exposed while there still exists a portion of polypeptide that is not completely exposed to the aqueous phase. The fragmented sugar units surround the still buried polypeptide, further decreasing its ability to access the aqueous phase and hence the reason for the feeble appearance of the segment at 310 nm in R1 (pH 7.04–9.20). It is also supported by the increase in fluorescence intensity at 311 nm for pH 9.20 (Figure 2A).

At pH 7.04 (Figure 4F), a unique contour spectrum was observed. The emission spectrum (Figure 2) also recorded a doublet-like spectrum, which is observed as a single tyrosine moiety; hence, research can be performed to geometrically favorable position for HBN to form between two tyrosine HBN in GA. Tyrosine moieties must be present in a sequential emission contour spectral technique was used as an excellent tool to follow the chemistry of tyrosine. The study visually manifested the multicharacteristic behavior of tyrosine in various pH conditions and in different microdomains. The 3D emission contour spectral studies confirm the formation of tyrosine HBN in GA. Tyrosine moieties must be present in a geometically favorable position for HBN to form between two tyrosine moieties; hence, research can be performed to find out the possibility for the formation of tyrosine dimers (Tyr-O-O-Tyr) in GA. The dityrosine bridges are present in many proteins like resilin,28 wheat gluten, bread gluten, etc. Tyrosine dimers present in resilin are known for their remarkable elastic properties. GA is used as an adhesive and binder. Tyrosine dimers may have a role to play in the adhesive properties of GA. Another interesting fact is that many proteins containing tyrosine dimers are said to have similar protein sequence; hence, research on this area could lead us to the protein sequence of GA.

3. CONCLUSIONS

The photophysics of GA was explored by studying its steady-state absorption, emission, fluorescence lifetime, and 3D emission contour spectra at various pH conditions in aqueous solution. The red shift in the absorption (278–293 nm) and emission studies (310–335 nm) and the formation of HBN at neutral pH are only due to the presence of a hydroxyl group in tyrosine (absent in phenylalanine). Hence, these observations together with the average lifetime values for GA at various pH conditions authenticate the predominant fluorophore to be tyrosine only. The emission and 3D emission contour spectra clearly reveal the exposure of the intrinsic fluorophore to the aqueous phase, which was further ascertained by the high lifetime values in fluorescence decay analysis. Three-dimensional emission contour spectral technique was used as an excellent tool to follow the chemistry of tyrosine. The study visually manifested the multicharacteristic behavior of tyrosine in various pH conditions and in different microdomains.

4. MATERIALS AND EXPERIMENTAL METHODS

4.1. Sample Preparation. Acacia gum (gum arabic laboratory grade sample, CAS no 9000-01-05) was purchased from Merck. A 2% (w/v) solution of GA was prepared. Britton and Robinson buffer, a universal buffer, was used to prepare buffer solution with pH ranging from 2.0 to 10.0. The buffer was prepared by mixing appropriate volumes of acidic and basic components. The acidic buffer component comprises 0.04 M acetic acid, 0.04 M phosphoric acid, and 0.04 M boric acid. The basic buffer component comprises 0.2 M sodium hydroxide.
4.2. Steady-State Absorption and Fluorescence Measurements. The absorption spectra were recorded using an Agilent 8453 UV-visible diode array spectrophotometer. The lamp source used for the UV-visible range was deuterium and tungsten lamps. The fluorescence emission and 3D spectral measurements were performed in Fluoromax 4P spectrophotometer (Horiba Jobin Yvon) using fluorescence software provided by the manufacturer. Three-dimensional contour plots were obtained by simultaneously scanning the excitation and emission monochromator. The excitation and emission slits were set at 5 nm for all of the 3D measurements. The excitation wavelength range was 250–350 nm, and the emission wavelength range was 300–550 nm. The emission maximum corresponding to each excitation wavelength was recorded, and a plot was generated using Origin 8.0 Software.

4.3. Time-Correlated Single-Photon Counting (TCSPC) Technique. The fluorescence decay measurements of GA solutions were recorded using IBH time-correlated single-photon counting spectrometer with microchannel plate photomultiplier tube (Hamamatsu R3809U) as detector and 280 nm light-emitting diode (Spectra Physics) as an excitation source. TCSPC is a digital technique for counting photons, which are time correlated with the excitation pulse. The heart of the method is a time-to-amplitude converter. The fluorescence photons are collected at the magic angle (54.7°) to avoid the distortions due to the rotational polarization. The measured decay is the convolution of the true fluorescence decay, excitation function, and the instrument response function, which results in the fluorescence kinetics parameters like lifetime (τ) and relative amplitude (A). The data analysis was carried out by the software provided by IBH (DAS-6), which is based on reconvolution technique using iterative nonlinear least-squares methods. The reconvolution is preceded by the series of iterations until a χ² is reduced. The quality of fit is normally identified by the reduced χ², weighed residual, and autocorrelation function of the residuals.

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