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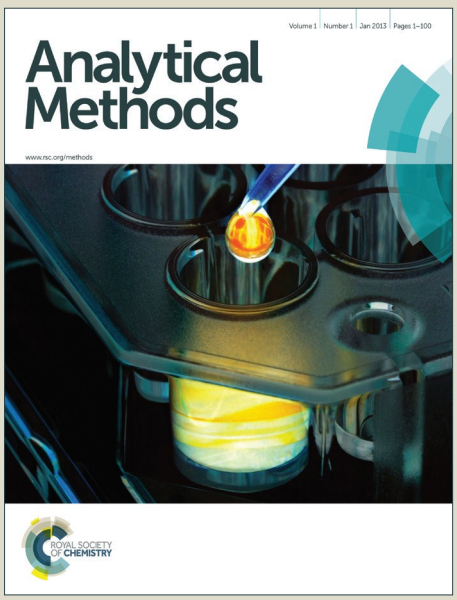

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Innovative application of Coomassie Brilliant Blue: a simple, economical, and effective determination of water-insoluble protein surface hydrophobicity

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Abstract

A simple, credible and economical method for water-insoluble protein surface hydrophobicity determination was developed and validated. The method is based on the non-covalent binding of Coomassie Brilliant Blue G-250 (CBBG) to aromatic and basic amino acid residues on the surface of proteins, generating insoluble protein-CBBG complexes that could be precipitated by centrifugation and cause a reduction in the absorbance at 585 nm in the supernatant. The amount of protein-bound CBBG is applied as an indicator of protein surface hydrophobicity. Thermally treated, water insoluble myofibrillar proteins (MP) were chosen to study the validity of the proposed method. The amount of protein-bound CBBG increased in thermally treated protein samples and the results were compared with the surface hydrophobicity (S_0) calculated from the commonly accepted fluorescence method using 1-anilino-8-naphthalenesulfonate (ANS) probe and intrinsic tryptophan fluorescence. Results from CBBG-binding method linearly correlated to the S_0 of ANS fluorescence method (R = 0.95), the maximum emission wavelength of protein intrinsic fluorescence (R = 0.96) and the intrinsic fluorescence intensity at 337 nm (R = –0.73), strongly suggesting the validity of the proposed CBBG-binding method.

Keywords: Coomassie Brilliant Blue; Surface hydrophobicity; Myofibrillar proteins; Water-insoluble protein.

1. Introduction

Hydrophobicity is the property that nonpolar solutes have the tendency to exclude water molecules and simultaneously adhere to one another in aqueous environments.[1,2] Protein hydrophobicity is closely related to the conformation and functionalities of proteins, protein-protein interactions, binding capacity to nonpolar substances, and their susceptibility to proteolysis.[3–7] Bigelow[8] suggested that surface hydrophobicity is one of the two major controlling factors of protein solubility which governs the functionalities of proteins. The lower the surface hydrophobicity is, the higher the solubility of the protein. Nakai[6] reported significant correlations between protein surface hydrophobicity and its emulsifying capacity, emulsion stability, fat binding capacity, and gelation. Therefore, quantitative determination of surface hydrophobicity has practical implications to precisely predict protein functionalities.

A number of physical and spectroscopic methods have been developed to determine protein hydrophobicity, including reverse-phase and hydrophobic interaction chromatography, intrinsic fluorescence, fluorescence probe methods, and binding of hydrophobic or amphiphilic compounds to proteins.[1,3,9–11] Chromatography method is time-consuming, column-dependent, and could induce protein denaturation.[1] Fluorescent probe methods are the most commonly used among the existing methods. However, contradictory results could be obtained due to different fluorescent probes employed.[9] In addition, most of these methods are only suitable for soluble protein samples. In view of the above, a simple, credible and economical method for water-insoluble protein surface hydrophobicity determination is needed. Coomassie Brilliant Blue G250 (CBBG) and R250 (CBBR) play a crucial role in protein analyses. CBBR is the more

sensitive form of the two and has been commonly used to stain proteins in polyacrylamide gels. However, CBBG can bind to proteins more rapidly. The balance can be established in two minutes with reasonable color stability for approximately one hour.[12] Binding of CBBG to proteins is attributed to hydrophobic interactions and Van der Waals forces between the anionic form of CBBG and primarily the aromatic (Phe, Tyr, Trp) and basic amino acid residues on proteins.[13–15] As a result, insoluble protein-CBBG complexes are formed.[16] Based on these facts, we hypothesized that the amount of protein-bound CBBG could be proportional to the surface hydrophobicity of the proteins. Insoluble complexes formed due to the binding of CBBG to proteins could be precipitated by centrifugation, causing a fade in the color of the supernatant (Fig. 1). The more hydrophobic the protein is, the more CBBG would be precipitated in the form of protein-CBBG complex, and the lighter the supernatant becomes.

In this paper, myofibrillar proteins (MP), which are critical to the meat functional properties and cannot be solubilized in low ionic strength aqueous buffers, were chosen an example of water-insoluble protein. Thermal treatment was applied to MP due to its known effect on protein unfolding and thus protein surface hydrophobicity.[17–21]

2. Material and methods

2.1. Materials

Porcine Longissimus muscle (24 h postmortem) was obtained from the Meat Laboratory of the University of Kentucky. After visible fat and connective tissues were carefully removed, individual samples (about 100 g, 1.5 cm thick slice) were vacuum packaged and stored in a

−30 °C freezer until use. Coomassie Brilliant Blue G250 (electrophoresis pure) was purchased from Bio-Rad Laboratories Ltd. (Hercules, CA, USA), 1-anilino-8-naphthalenesulfonate (ANS) was purchased from Thermo–Fisher Scientific (Altham, MA., USA). All the other chemicals were purchased from Sigma–Aldrich (St. Louis, MO., USA) or Thermo–Fisher Scientific and were at least of analytical grade. Distilled deionized ultrapure water (NANOpure Diamond, Barnstead, USA) was used throughout the experiments.

2.2 Absorption spectra of CBBG in solutions

The maximum absorption wavelength (λ_{max}) of CBBG and the effect of sodium on λ_{max} were determined by scanning CBBG solutions (20 μg/mL, final concentration, dissolved in ultrapure water, 20 mM phosphate buffer (pH 6.0) with or without 0.6 M NaCl, respectively) from 400 to 700 nm using a Shimadzu UV-2401PC spectrophotometer (Shimadzu Inc., Columbia, MD, USA). Correlation between CBBG content and the absorbance at its λ_{max} was investigated by analyzing a series of concentrations (0–50 μg/mL, final concentration) of CBBG dissolved in 20 mM phosphate buffer (pH 6.0). The effect of pH on the absorption spectra of CBBG was studied by dissolving CBBG (20 μg/mL, final concentration) in 20 mM phosphate buffer of varying pHs (0–14). The effect of protein binding on the λ_{max} of CBBG was studied in 20 mM phosphate buffer (pH 6.0) by mixing 1.0 mg/mL bovine serum albumin (BSA) and 20 μg/mL CBBG (final concentrations).

2.3 Extraction of myofibrillar proteins

One random bag of the frozen muscle samples was thawed in a 4 °C refrigerator for 4 h and then used for MP extraction according to the method described by Cao and Xiong.[22] The

entire preparation process was carried out at under 4 °C. The extracted protein was kept in a tightly capped bottle, stored on ice, and utilized on the same day. Protein concentration was measured by the Biuret method[23] using BSA as the standard.

2.4 Heat treatment of MP

Heat treatments were implemented in an orbital reciprocating water bath (Boekel Scientific, Feasterville, PA, USA). Ten milliliters of MP suspension (5 mg/mL, in 20 mM phosphate buffer, pH 6.0) were transferred into a 15 × 125 mm glass centrifuge tube with PTFE (Teflon) lined screw cap, and then heated in a constant temperature for 10 minutes, ranging from 30 to 70 °C. The centrifuge tubes were lightly closed to avoid evaporation during treatments. The tubes were immediately cooled in an ice-water bath for 10 min after each heat treatment to avoid further thermal denaturation. The treated samples were brought to room temperature before subsequent tests.

2.5 Determination of MP surface hydrophobicity by CBBG-binding method

The CBBG-binding method is outlined in Fig. 1. In brief, 300 μL of 0.1 mg/ml CBBG (in ultrapure water) was added to 1.2 ml of 5 mg/ml MP suspension (dispersed in 20 mM phosphate buffer, pH 6.0) in a 2 mL plastic centrifuge tube and well mixed. For control, the same amount of CBBG was added to 1.2 ml phosphate buffer. The samples and the control were vortexed for 3 min at room temperature, and then centrifuged at 2000 × g for 10 min. The supernatants were transferred to clean plastic centrifuge tubes and centrifuged again under the same condition. The absorbance of the supernatant was measured at 585 nm against a reagent blank. The amount of CBBG bound is given by the formula:

$$\text{CBBG bound } (\mu g) = 30\ \mu g \times \frac{(A_{Control} - A_{Sample})}{A_{Control}}$$

With A = absorbance at 585 nm.

2.6 Determination of MP surface hydrophobicity by ANS fluorescence method

Surface hydrophobicity change induced by heat treatment was also determined using ANS fluorescence method developed by Li-Chan et al.[24] MP was diluted to 0.01, 0.02, 0.03, 0.04, and 0.05 mg/ml in 20 mM phosphate buffer containing 0.6 M NaCl, pH 6.0. To 4.0 ml of diluted protein solution, 20 µL of 8.0 mM ANS (dissolved in the same phosphate buffer) was added and mixed thoroughly. The fluorescence intensity was measured exactly 45 min after ANS addition using a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA), with the excitation and emission wavelengths set at 390 nm and 470 nm, respectively. The fluorescence intensity values of sample blank (protein solution without ANS added) and reagent blank (phosphate buffer without protein, but ANS added) were measured and subtracted from those of ANS-protein conjugate samples (protein solutions with ANS added). Protein surface hydrophobicity was expressed as the initial slope (S_0) of the fluorescence intensity versus protein concentration plot calculated by linear regression analysis.

2.7 Intrinsic tryptophan fluorescence

Intrinsic tryptophan fluorescence was determined using a FluoroMax-3 spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA) according to the method described by Utrera and Estévez[25] with modifications. The protein concentration of all treated samples was diluted to 30 µg/ml in 20 mM phosphate buffer containing 0.6 M NaCl (pH 6.0). The excitation wavelength was set at 283 nm and the emission spectra were recorded from 300 to 400 nm. The data

collection rate was 500 nm/min and both the excitation and the emission slit widths were set at 5 nm. For the reagent blank, the same phosphate buffer was scanned under the same conditions.

2.8 Statistical analysis

Data with three independent trials (n = 3) were subjected to the analysis of variance using the general linear model's procedure of the Statistix software 9.0 (Analytical Software, Tallahassee, FL). Significant ($P < 0.05$) differences between means were identified by LSD all-pairwise multiple comparisons.

3. Results and discussion

3.1. Absorption spectra of CBBG in solutions

The absorption spectra of CBBG dissolved in ultrapure water, 20 mM phosphate buffer (pH 6.0) with or without 0.6 M NaCl are shown in Fig. 2 (A). Phosphate and sodium chloride had no significant influence on the λ_{max} of CBBG, which was at 585 nm. However, the magnitude of the peak at 585 nm decreased in the presence of phosphate and further decreased with sodium addition. The absorbance of CBBG in a pH 6.0 phosphate buffer at its λ_{max}, 585 nm, was analyzed in a concentration range of 0–50 μg/mL. As shown in Fig. 2 (B), there was a nearly perfect linear correlation (R > 0.99) between the amount of CBBG and its absorbance value.

Spectra of CBBG (20 μg/mL, final concentration) were affected by the pH of the solutions (Fig. 2 (C) and Table 1.). The λ_{max} was about 470 nm and the solution had a light brick red color at pH 0. The λ_{max} shifted to around 640 nm when the color of the solution changed to greenish-blue at pH 1. The λ_{max} shifted from 594 nm to 585 nm when the pH was increased from

2 to 4. In the pH range of 4 to 10, CBBG solutions have a blue color with a stable λ_{max} at 585 nm. However, there were slight but statistically significant differences in the maximum absorbance values (A_{max}) of CBBG spectra in the pH range of 4 to 10 (Table 1). Under extremely alkaline conditions (pH > 11), the A_{max} of CBBG solutions markedly decreased and the λ_{max} shifted (Fig. 2 (C)). These changes could be a result that the CBBG molecules were destroyed under these conditions. Similar results have been reported by other researchers.[13–14] The CBBG exists in different forms at different pH: cationic (pH < 0.39, λ_{max} = 470 nm, red), neutral (pH ~ 1.3, λ_{max} = 650 nm, green), and anionic (pH > 1.3, λ_{max} = 595 nm, blue).[14]

In addition to pH, binding of CBBG to proteins affected the absorption spectrum of CBBG. Our result showed that the λ_{max} converted to about 610 nm from 585 nm after the CBBG anionic species bound to BSA. Similar result has been reported that BSA could bind to the anionic species of CBBG, causing the peak to shift from 590 nm to 615 nm.[14] It is also known that BSA binding to CBBG neutral species resulted in a shift of the λ_{max} from 650 nm to 615 nm.[14] Sedmak and Grossberg[26] reported that the λ_{max} of CBBG cationic species shifted from 470 nm to 595 nm after it bound to proteins. The λ_{max} of anionic CBBG was reported to be 590 nm and 595 nm while in our study the λ_{max} was 585 nm. This difference may be attributed to the variations in CBBG chemical. Based on the spectral characteristics of CBBG, we proposed the CBBG-binding method for water-insoluble proteins surface hydrophobicity determination at neutral pH based on the absorbance change at 585 nm.

3.2. MP surface hydrophobicity measured by CBBG-binding method

The heat-induced change in surface hydrophobicity of MP was analyzed using the

innovative CBBG-binding method. As shown in Fig. 3, no significant difference was detected in the amount of MP-bound CBBG between samples heated at 30 °C and 40 °C for 10 min, indicating that MP was stable under these heating conditions. The amount of MP-bound CBBG continually increased with the rise of temperature from 40 °C to 70 °C, corresponding to the unfolding of MP and the exposure of hydrophobic amino acid residues to protein surface. This result was consistent with Boyeqa et al.[17] which detected increased surface hydrophobicity of rabbit myosin after heat treatment at 30–80 °C determined by fluorescence method using ANS and cis-parinaric acid (CPA) probes. Yongsawatdigul and Park[21] reported that the surface hydrophobicity (determined using ANS probe) of threadfin bream actomyosin began to increase at above 30 °C and continued to rise with elevated heating temperature until 70 °C. Xiong and Brekke[20] reported a marked increase in the surface hydrophobicity of salt-soluble proteins (SSP) prepared from chicken myofibrils at 35 °C and it rapidly increased upon further heating above 65 °C. The difference in the initial temperature at which protein surface hydrophobicity began to increase can be attributed mainly to muscle source and type, and partly to ionic strength, pH and heating rate. Chelh, et al.[3] developed a bromophenol blue (BPB)-binding method to determine the surface hydrophobicity change after thermal treatments. They found that binding of BPB to porcine myofibrils continuously increased as the temperature increased from 30 °C to 70 °C. According to their results, the amount of protein-bound BPB was approximately two times higher at 70 °C than that at 30 °C after 10 min's heating. In our study, the MP-bound CBBG increased by 5.6 fold under the same heating conditions, implying that CBBG is more sensitive for protein surface hydrophobicity determination.

3.3. MP surface hydrophobicity measured by ANS fluorescence method

The proposed CBBG-binding method was compared to the commonly accepted ANS fluorescence method. Because CBBG primarily binds to aromatic and basic amino acid residues,[13–15] ANS, which is composed of aromatic rings,[9] was chosen for this comparison. The ANS fluorescence intensity and the calculated surface hydrophobicity (S_0) of thermally treated MP are shown in Fig. 4. The ANS fluorescence intensity significantly increased with the elevation of heating temperature. The calculated MP surface hydrophobicity (S_0) was correlated to the amount of protein-bound CBBG in Fig. 3. The linear regression curve had a Pearson's correlation coefficient (R) of 0.95, strongly suggesting the suitability and validity of the CBBG-binding method for surface hydrophobicity measurement.

3.4. Effect of heat treatments on intrinsic tryptophan fluorescence

Intrinsic tryptophan fluorescence properties are particularly sensitive to the polarity of proteins' microenvironments. The more polar the microenvironments, the lower quantum yields and fluorescence intensities the tryptophan groups have. As summarized in Fig. 5, overall, the intrinsic tryptophan fluorescence intensity of MP decreased as the temperature increased, indicating changes in the microenvironments of MP tryptophan residues. In the folded state, tryptophan residues are mainly located in the hydrophobic core of the proteins. When they become exposed to solvents (a hydrophilic environment) in an unfolded state, they will have a reduced fluorescence intensity.[22] Heat treatment caused a red shift of the maximum fluorescence emission wavelength for MP, further suggesting protein unfolding (Fig. 5). There was no notable difference in either the maximum fluorescence emission wavelength or the fluorescence intensity

between 30 °C and 40 °C treatments, consistent with the CBBG-binding results (Fig. 3). Tryptophan residues totally buried in protein core have the maximum fluorescence emission wavelength at about 330 nm, however, it shifts to about 340–350 nm when exposed to water. The maximum fluorescence emission wavelength of MP was 337 nm at 30°C in our study, implying that the majority of tryptophan residues were buried in the protein core with part of them located on the surface of MP. The maximum fluorescence emission wavelength shifted to 342 nm after heating at 70 °C for 10 min, suggesting large amounts of tryptophan residues buried in protein core were exposed to the surface. The amount of MP-bound CBBG was inversely correlated to the maximum absorbance values (A_{max}) of CBBG at 337 nm (R = – 0.73, linear regression). In addition, a stronger correlation (R = 0.96) was found between the amount of MP-bound CBBG and the maximum fluorescence emission wavelength, once again suggesting the validity of the proposed CBBG method.

4. Conclusion

In conclusion, a simple, credible, and economical CBBG-binding method was developed and verified for surface hydrophobicity determination of water-insoluble proteins using myofibrillar proteins as an example. The proposed method well correlated to the widely accepted ANS fluorescence method (R = 0.95) and protein intrinsic fluoresce maximum emission wavelength (R = 0.96), indicating its suitability and validity for protein surface hydrophobicity determination. Further studies are needed to verify the suitability of this method for other protein categories and under different processing conditions.

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Conflict of Interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. This article does not contain any studies with animals or human participants performed by any of the authors.

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Captions of Figures

Fig. 1. Schematic of the proposed Coomassie Brilliant Blue G-250 (CBBG)-binding method for protein surface hydrophobicity measurement. MP: myofibrillar proteins.

Fig. 2. (A) Effect of phosphate buffer and sodium chloride on the absorption spectra of CBBG. (B) Absorption spectra of CBBG (0–50 μg/mL) dissolved in 20 mM phosphate buffer, pH 6. The corresponding regression curve is shown as the insertion. (C) Effect of pH on the absorption spectra of CBBG. CBBG: Coomassie Brilliant Blue G-250.

Fig. 3. Effect of thermal treatment on the surface hydrophobicity of MP expressed as the amount of protein-bound CBBG. All values are reported as the mean ± SD (standard deviation) of three measurements. $^{A–D}$ Means without a common letter differ significantly ($P < 0.05$).

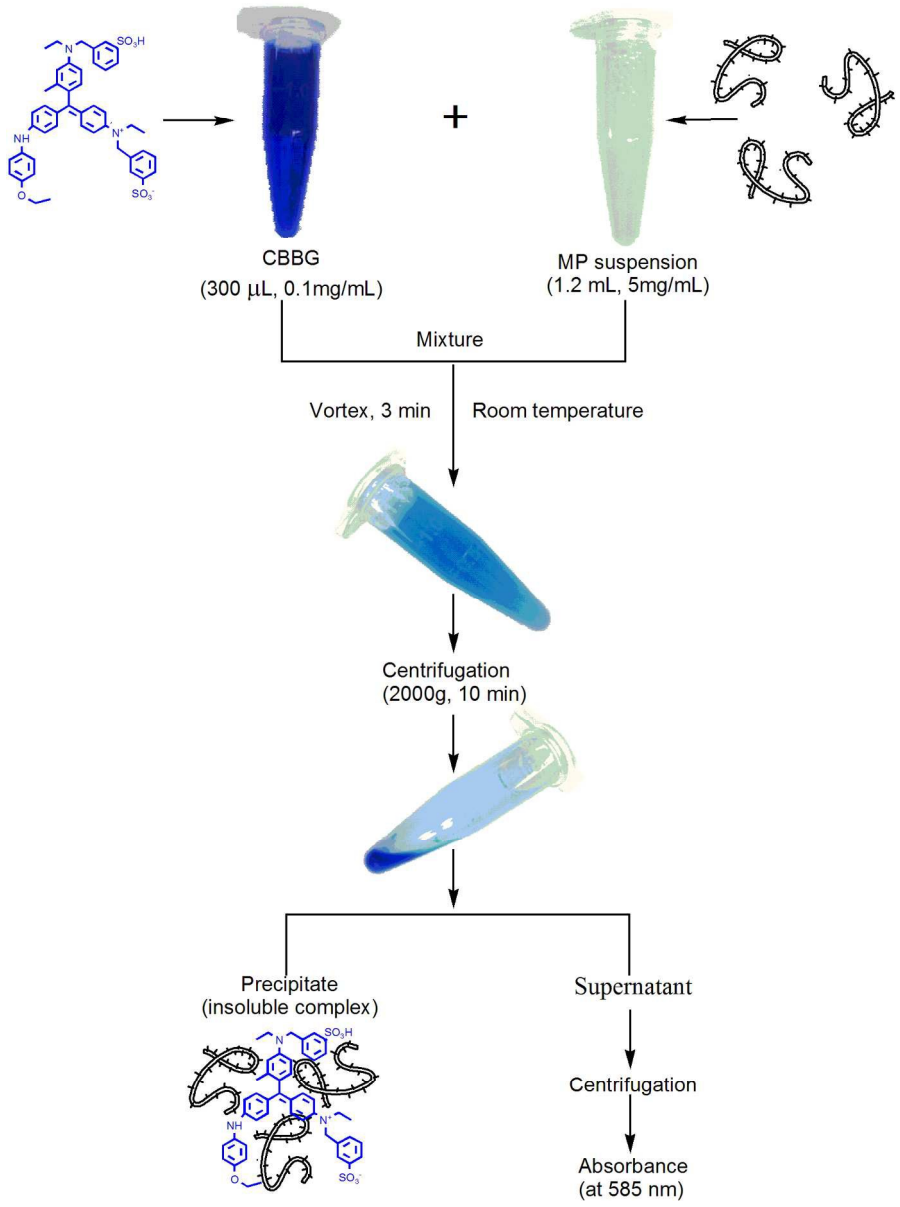
Fig. 4. Effect of thermal treatment on the ANS fluorescence intensity and surface hydrophobicity of MP measured using ANS fluorescence method. Excitation and emission wavelengths were set at 390 nm and 470 nm, respectively. MP: myofibrillar proteins. ANS: 1-anilino-8-naphthalenesulfonate. $^{a–d}$ Means without a common letter differ significantly ($P < 0.05$).

Fig. 5. Intrinsic tryptophan fluorescence of thermally treated MP. The excitation wavelength was set at 283 nm. Emission spectra were recorded from 300 to 400 nm. λ_{max}: the maximum intrinsic fluorescence emission wavelength. MP: myofibrillar proteins.

Table 1. The effect of pH on the maximum absorption wavelength (λ_{max}) and the maximum absorbance values (A_{max}) of CBBG (20 μg/mL, final concentration).

pH	λ_{max}* (nm)	A_{max}*	pH	λ_{max}* (nm)	A_{max}*	pH	λ_{max}* (nm)	A_{max}*
0	469.5±0.71	0.316±0.02	5	584.5±0.71	0.908±0.01	10	585.0±0.00	0.922±0.03
1	639.5±0.71	0.373±0.04	6	585.5±0.71	0.948±0.03	11	588.0±1.41	0.820±0.09
2	594.0±1.41	0.647±0.09	7	585.0±0.00	0.915±0.03	12	594.5±0.71	0.563±0.06
3	588.0±2.83	0.800±0.08	8	585.0±0.00	0.925±0.03	13	544.5±2.12	0.347±0.03
4	585.5±0.71	0.876±0.05	9	585.0±0.00	0.929±0.01	14	513.5±2.12	0.331±0.01

* All values are reported as the mean ± standard deviation of three measurements.



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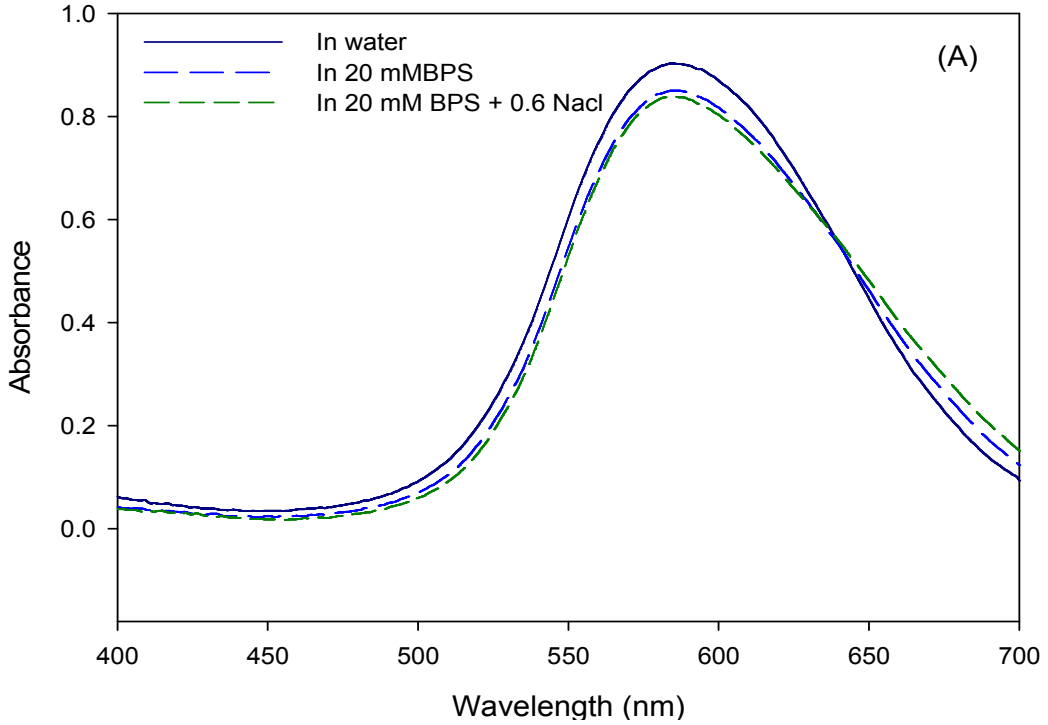

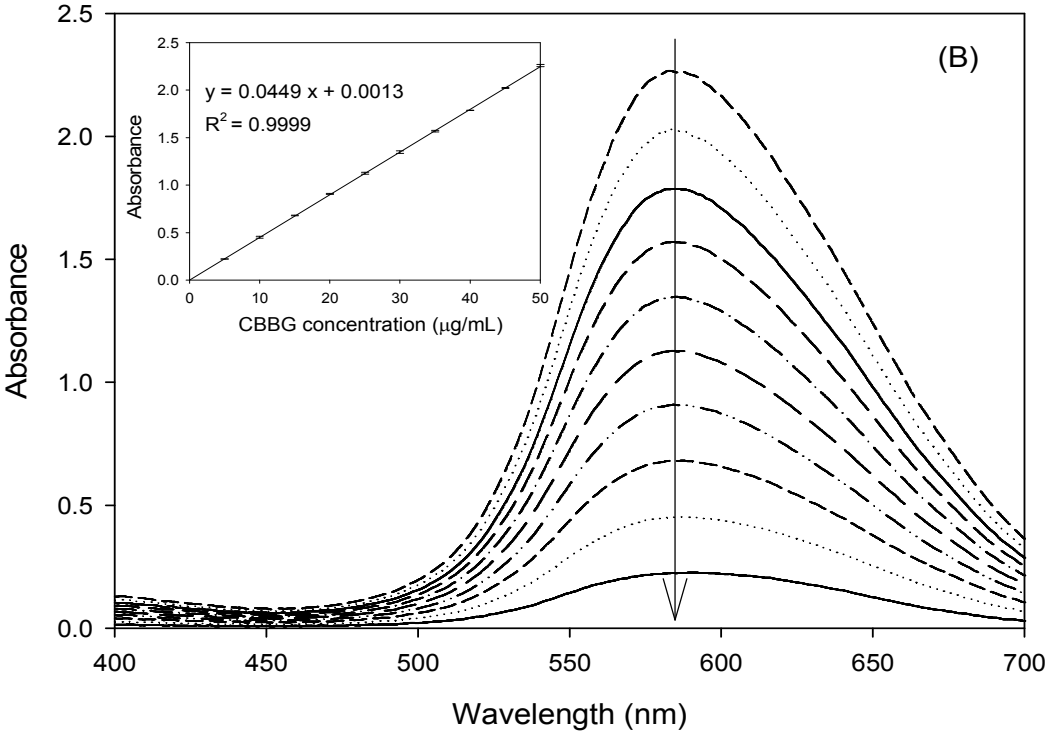

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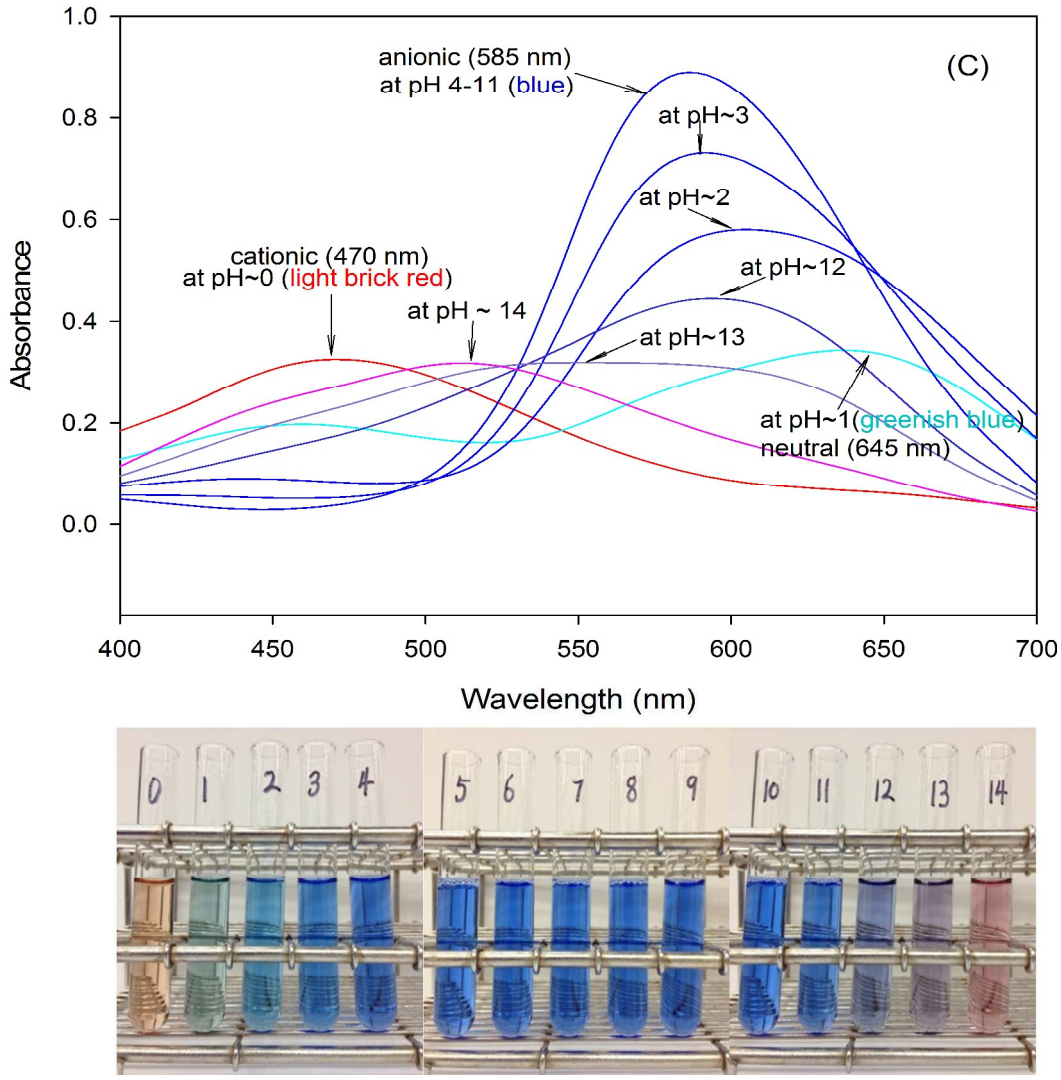

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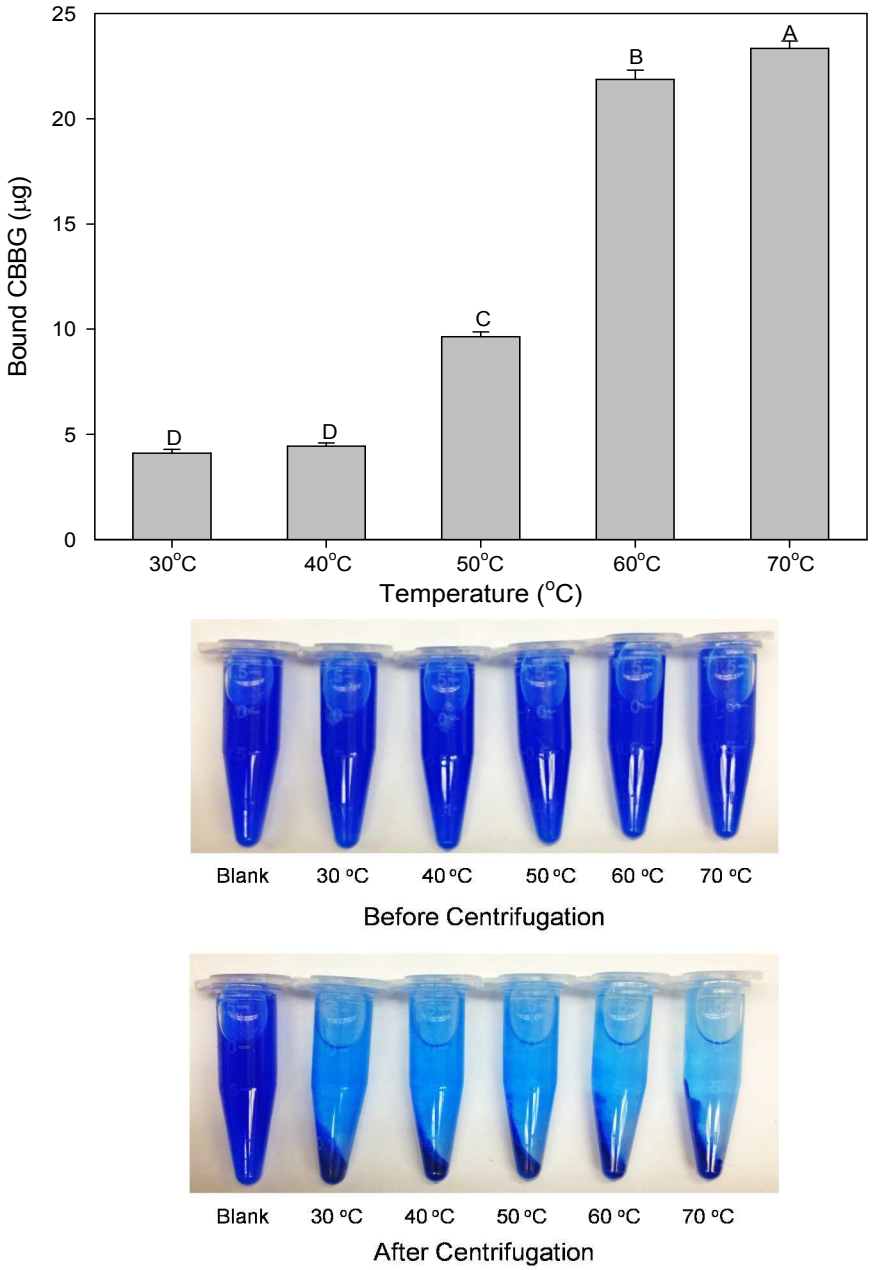

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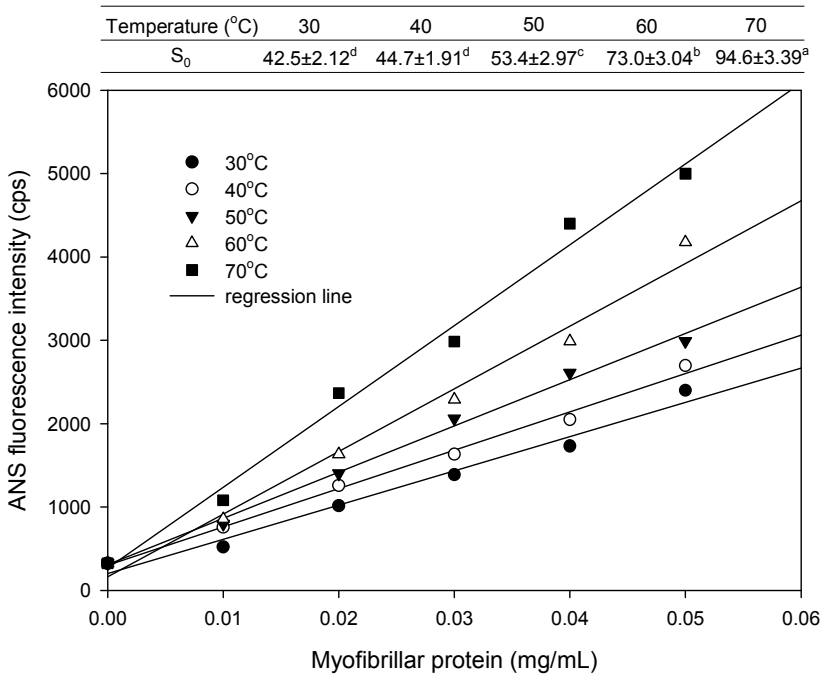

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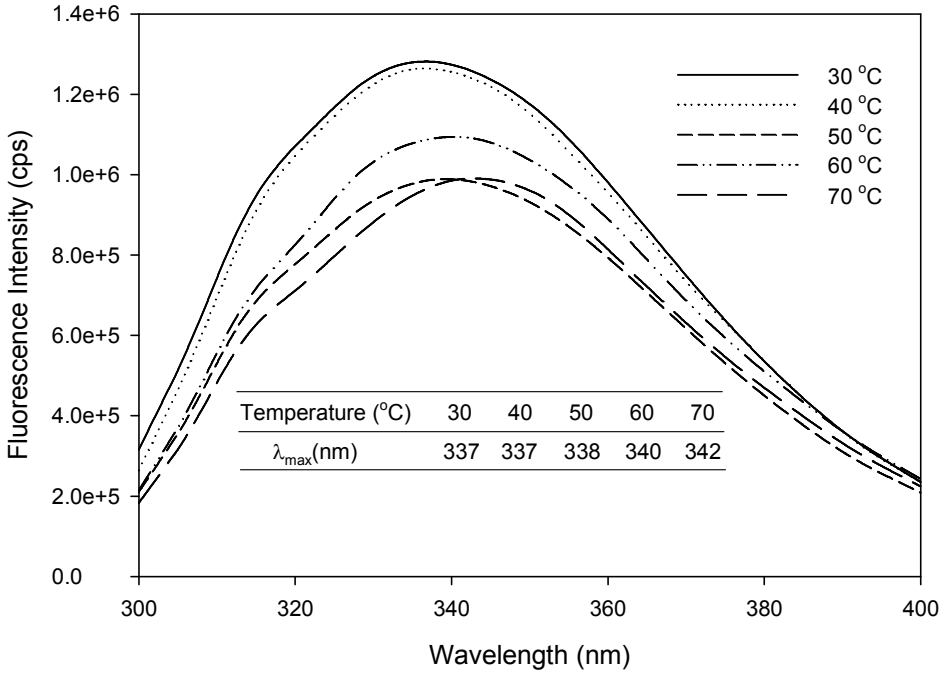

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Highlights

►A novel CBBG-binding method was developed to determine surface hydrophobicity for water-insoluble proteins.

►Results from the proposed CBBG-binding method well correlated to that of the commonly accepted ANS fluorescence method (R = 0.95) and the intrinsic fluorescence change in proteins.