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FT-Raman Spectroscopy, Fluorescent Probe, and Solvent Accessibility Study of Egg and Milk Proteins

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Due to possible contribution of both electrostatic and hydrophobic interactions, use of anionic fluorescent probes such as 1-anilinonaphthalene-8-sulfonic acid (ANS) and cis-parinaric acid (CPA) for the measurement of protein surface hydrophobicity (S_0) has been controversial. A neutral probe, 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN), may circumvent this problem. To select the best indicator of S_0, in this study, the data for nine model proteins in phosphate buffer, pH 7.5, measured using the above-mentioned probes, was compared to their FT-Raman spectra and calculated solvent accessibility values. Log S_0 measured using CPA had the highest correlation ($r =$ 0.874) with the intensities of Raman spectral signals at 760 cm^{-1} and 2800−3100 cm^{-1}, which were combined using a mixture design based on the random-centroid optimization. The order of correlation of Raman spectral parameters with S_0 values were CPA > PRODAN > ANS. FT-Raman spectroscopy, therefore, identified CPA, followed by PRODAN, as the fluorescent probe of choice for describing surface hydrophobicity. However, the amino acid surface accessibility calculated using the Predict-Protein software was not useful in identifying the best fluorescent probe for the measurement of S_0.

KEYWORDS: Protein surface hydrophobicity; fluorescent probe; ANS; CPA; PRODAN; Raman spectroscopy; surface accessibility

INTRODUCTION

Functionality or activity of a protein is influenced not only by hydrophobic, steric, and electronic parameters (*1*) but also by various intrinsic factors such as pH, temperature, salts, and presence of other molecules (*2*). However, for the investigation of protein structure−function relationships, great attention has been given to hydrophobicity due to its close correlation with protein stability. Importance of hydrophobicity in function has been reviewed by Nakai and Li-Chan (*3*).

Many scientists have tried to find the best method for the quantification of protein surface hydrophobicity (S_0); however, they still have not reached any agreement to identify a method as a "perfect standard". One common approach for quantifying protein hydrophobicity is through fluorescent probe methods. Many anionic probes such as 1-anilino-naphthalene-8-sulfonic acid (ANS) and cis-parinaric acid (CPA) have been widely used for the measurement of S_0, mainly because of their simplicity (*4*). However, due to possible contribution of both electrostatic and hydrophobic interaction to the binding of these anionic probes to proteins, interpretation of the relationship between functionality and hydrophobicity measured using S_0 values based on these probes is questionable. Use of a neutral probe such as 6-propionyl-2-(*N*,*N*-dimethylamino)naphthalene (PRODAN) may solve this problem.

Raman spectroscopy is a valuable technique for the study of the structure of molecules such as proteins in a solid or liquid form (*5*). Besides obtaining information regarding secondary structure of proteins, Raman spectroscopy can monitor environment around the amino acid side chains. Elucidation of the interaction of lysozyme and whey proteins (*6*), investigating hydrophobic interactions in the CH stretching region of proteins and amino acids (*7*), elucidation of protein−lipid interactions in a lysozyme-corn oil system (*8*), structural study of acid-induced myoglobin (*9*), and studying the interaction of hypericin with serum albumins (*10*) are some examples of the use of Raman spectroscopy to investigate hydrophobic interactions.

S_0 of three model proteins at various pH, with or without heating, was measured using ANS, CPA, and PRODAN in our earlier publication (*11*). However, although differences in S_0 measurement by the three probes were reported, the question still remains about which one of these fluorescent probes is the best indicator of S_0.

The objective of the present study was to obtain structural information from FT-Raman spectroscopy and solvent accessibility of amino acid residues in 3-D structure, to assist in better understanding of S_0 and in selecting the best fluorescent probe for the measurement. Nine model proteins (α-lactalbumin, β-lactoglobulin, α-casein, β-casein, κ-casein, lysozyme, ovalbumin, ovomucoid, and ovotransferrin) were used for this measurement.

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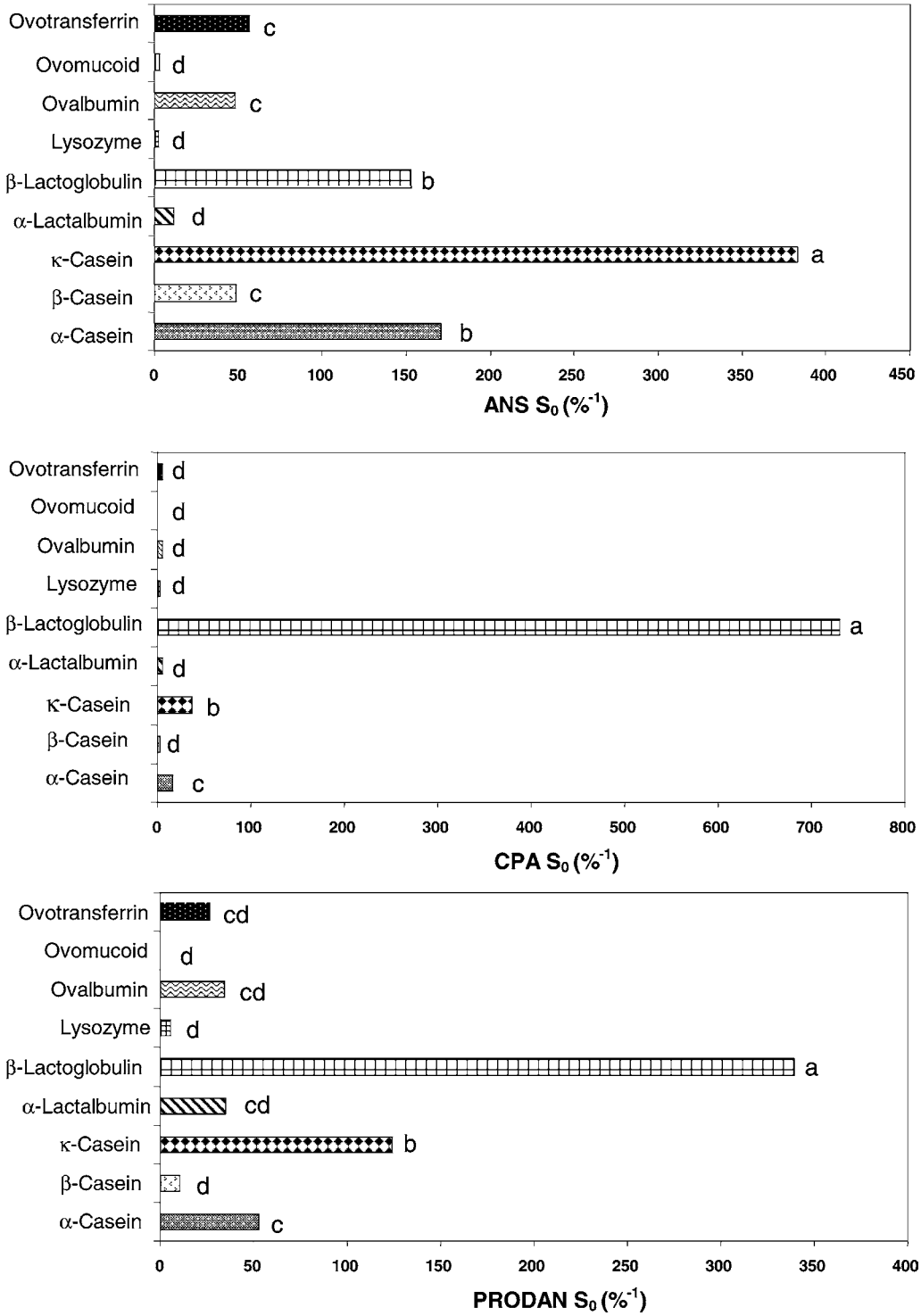

Figure 1. Surface hydrophobicity (S_0) of model proteins measured using ANS, CPA, and PRODAN (top, middle, and bottom graphs, respectively). (a–d) bars with different letters represent significant ($p \leq 0.05$) differences in S_0 values between proteins.

MATERIALS AND METHODS

Materials. α-Lactalbumin (L-5385), *β*-lactoglobulin (L-0130), α-casein (C-6780), *β*-casein (C-6905), *κ*-casein (C-0406), lysozyme (L-6876), ovalbumin (A-5503), ovomucoid (T-2011), and ovotransferrin (C-0755) were purchased from Sigma (St. Louis, MO). Three fluorescent probes, 1-anilinonaphthalene-8-sulfonic acid (ANS), cis-parinaric acid (CPA) and 6-propionyl-2-(dimethylamino)-naphthalene (PRODAN) were obtained from Sigma (A-5144), Molecular Probes (Eugene, OR, P-1901), and Molecular Probes (Eugene, OR, P-248), respectively. Sodium phosphate (0.1 M, pH 7.5 ± 0.1) was used as buffer, according to the method of Dennison (*12*). Sodium azide (0.02%, Sigma) was included to prevent bacterial growth.

Protein Solution Preparation. Stock protein solutions containing 0.05% (w/w) protein in double distilled water, with 0.02% sodium azide, were prepared in duplicate. Protein concentration was determined by measuring the absorbance at 280 nm using $E^{1\%}_{1\ cm}$ (absorptivity of a 1% solution measured in a 1 cm light path at 280 nm). Extinction coefficients of α-casein, *β*-casein, and *κ*-casein are 10.0, 4.0, and 10.5, respectively (*13*). Extinction coefficients of α-lactalbumin, *β*-lactoglobulin, lysozyme, ovalbumin, ovomucoid, and ovotransferrin are 20.1, 10.0, 26.0, 7.5, 4.55, and 11.1, respectively (*14*).

Fluorescent Probe Method. For the fluorometric probe assay, the stock protein solutions were diluted with phosphate buffer (pH 7.5 ± 0.1) to typical concentration ranges of 0.002−0.01% w/v (5 concentra-

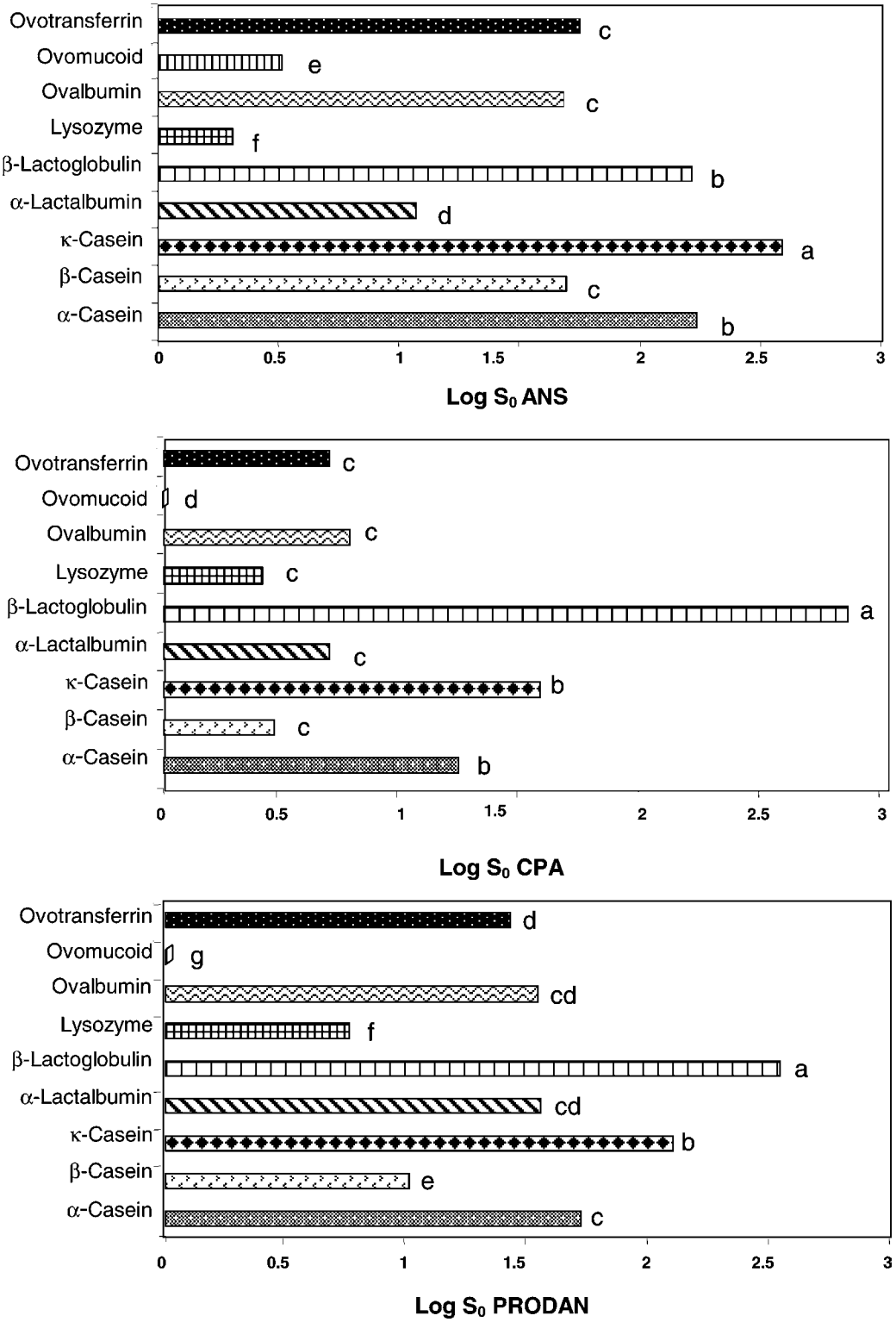

Figure 2. Logarithm of surface hydrophobicity (S_0) of model proteins measured using ANS, CPA, and PRODAN (top, middle, and bottom graphs, respectively). (a–g) bars with different letters represent significant ($p \leq 0.05$) differences in S_0 values between proteins.

tions). Protein surface hydrophobicity was measured as mentioned in our earlier publication (*11*). Surface hydrophobicity (S_0) values were determined using at least duplicate analysis. In all cases, R^2 values of 0.990 were noted for the linear regression analyses used to calculate surface hydrophobicity values.

FT-Raman Spectroscopy. Protein samples were prepared as a 10% solution in phosphate buffer (pH 7.5 ± 0.1) and were placed into glass NMR tubes (Kimble Glass Inc., Batavia IL, Art. No. 60830-542, size/cap 5 × 42 mm, 0.35 mL). The Raman scattering was measured at room temperature, using a Fourier transform (FT) Raman spectroscope from Thermo Nicolet Corp., Madison, WI; laser power, 500 mW, running 1000 scans for each sample at resolution of 4 cm^{-1}. The laser alignment was checked daily using polystyrene. KBr was used as a reference spectrum.

The Trp (760 cm^{-1}) and CH stretching (2880, 2930, 3060 cm^{-1}) bands were investigated, after normalization of spectral data to the intensity of the phenylalanine band at 1004 ± 1 cm^{-1}. The Raman spectra were plotted as intensity (arbitrary units) against Raman shift in wavenumber (cm^{-1}), using OMNIC Custom Software of Thermo Nicolet Corp.

Solvent Accessibility of Amino Acid Residues. Amino acid sequence of the model proteins was identified using genomic database, dbget program, available at http://www.genome.ad.jp.

These data were exported to PredictProtein software (available at http://cubic.bioc.columbia.edu/predictprotein/Dtab/phd_acc.html) for the prediction of solvent accessibility (*15*). This program is based on the solvent accessibility concept originally introduced by Lee and Richards (*16*). They defined solvent accessible surface area as "area on the surface of a sphere radius, on each point of which the center of a solvent molecule can be placed in contact with the atom without penetrating any other atoms of the molecule". Solvent accessibility was defined by the accessible surface area divided by $4\pi R^2$ and multiplied by 100 (*16*). Rost and Sander (*15*) then used amino acid profiles of over 100 known protein structures as input for an artificial neural network prediction system. The system can predict relative solvent accessibility. The correlation coefficient between observed and predicted solvent accessibility is about 0.54 (*15*). The correlation coefficient between % solvent accessibility and S_0 values measured using three fluorescent probes were then calculated using MINITAB (Version 12, Minitab Inc., State College, PA).

Random-Centroid Optimization. The method of Nakai et al. (*17*) was used.

Statistical Analysis. Analysis of variance (ANOVA) of the S_0 data was performed by using the General Linear Model (GLM) procedure of Minitab. Significant differences ($p \leq 0.05$) between treatment means were analyzed using Tukey's pairwise comparison test. Also, regression analysis was used to find out which of the fluorescent probe methods correlated most significantly with the Raman spectrum of the proteins. Significance was defined as $p \leq 0.05$. Logarithmic transformation of S_0 values was performed, due to the range in magnitude of data obtained (*18, 19*).

RESULTS AND DISCUSSION

Protein Surface Hydrophobicity. The surface hydrophobicity (S_0) values of the nine model proteins measured using the two anionic fluorescent probes, ANS and CPA, and the neutral probe, PRODAN, and their log values are depicted in **Figures 1** and **2**, respectively. Some similarity was observed between the trends of S_0 measured using CPA and PRODAN, which were different from the trends observed using ANS. For example, both CPA and PRODAN identified β-lactoglobulin as the most hydrophobic and the ovomucoid as the least hydrophobic proteins, while κ-casein and lysozyme were found to be the most and the least hydrophobic, using ANS probe, respectively. The 2nd least hydrophobic protein was lysozyme, using PRODAN and CPA. High affinity of CPA (C_{18} tetraenoic fatty acid) to β-lactoglobulin has been attributed to ionic as well as hydrophobic interactions (*4*). A C_{16} fatty acid, palmitate, better fits the central cavity (calyx) of β-lactoglobulin than other saturated fatty acids with different carbon chain lengths (*20*). The structural specificity of lipocalin protein superfamily (*21*) may not be ignored. A wide range of S_0 was observed using various probes. Rigidity of ovomucoid can explain its low S_0. On the other hand, higher S_0 of ovotransferrin, ovalbumin, κ-casein, β-casein, and β-lactoglobulin can be explained by their flexibility (*22*).

ANS is composed of aromatic rings, while CPA possesses an aliphatic hydrocarbon chain, and PRODAN contains both aromatic and aliphatic groups. ANS and PRODAN are known for sensing the polarity of environment in biological materials (*23*). It has been shown that the binding sites for CPA on protein molecules differ from the sites for ANS (*1*). The observed differences between S_0 measured by CPA or PRODAN with that of ANS suggests differences in characteristics of the probes to interact with protein hydrophobic groups that originate from aromatic amino acid side chains (e.g., Phe, Trp, and Tyr) and those of aliphatic amino acid side chains (e.g., Val, Leu, and Ile) (*24*) and the combination of the two in PRODAN.

Table 1. Normalized Intensity of Selected Bands of the Raman Spectrum of the Protein Samples

	wavenumbers (cm^{-1})			
protein samples	760 cm^{-1}	2880 cm^{-1}	2930 cm^{-1}	3060 cm^{-1}
α-casein	0.50	0.19	0.33	0.29
β-casein	0.70	0.33	0.60	0.67
κ-casein	0.82	0.16	0.67	0.62
α-lactalbumin	0.97	0.28	0.69	0.80
β-lactoglobulin	0.27	0.45	0.93	0.56
lysozyme	1.11	0.36	0.78	0.82
ovalbumin	0.69	0.28	0.54	0.68
ovomucoid	0.82	0.18	0.66	0.81
ovotransferrin	0.72	0.35	0.71	0.69

Table 2. Correlation between Surface Hydrophobicity of Proteins and Intensity of Several Raman Bands

Raman bands	Log S_0 ANS	Log S_0 CPA	Log S_0 PRODAN
760 cm^{-1}	–0.686[a]	–0.732[a]	–0.587[a]
2880 cm^{-1}	NS[b]	NS	NS
2930 cm^{-1}	NS	NS	NS
3060 cm^{-1}	–0.748[a]	–0.539	–0.560

[a] $p < 0.05$. No superscript, $0.05 < p < 0.10$. [b] NS, not significant.

Table 3. Mixture Design of Combination of Raman Signals Using RCO

combination	[760]	[2880]	[2930]	[3060]
1	0.25	0.33	0.39	0.03
2	0.17	0.52	0.08	0.23
3	0.16	0.29	0.17	0.38
4	0.45	0.41	0.08	0.06
5	0.11	0.30	0.28	0.31
6	0.09	0.73	0.07	0.11
7	0.22	0.33	0.17	0.28
8	0.09	0.58	0.23	0.10
9	0.27	0.15	0.26	0.32
10	0.19	0.27	0.22	0.32
11	0.18	0.31	0.20	0.31
12	0.20	0.32	0.17	0.31
13	0.16	0.36	0.17	0.31

FT-Raman Spectroscopy. Previous reports of Raman spectroscopy of milk and egg proteins have mostly involved the study of their secondary structure (*25*−*29*), while in this experiment, we are concerned about the Raman spectral bands that arise from amino acid side chains, namely, tryptophanyl ring vibrations (760 cm^{-1}) and CH stretching vibrations (2800−3100 cm^{-1}). Normalized intensity of selected bands of the Raman spectrum of protein samples and the correlation between surface hydrophobicity of proteins and the intensity of Raman bands are shown in **Tables 1** and **2**, respectively. Generally, indole rings in hydrophobic environments cause sharp bands in protein Raman spectra in the vicinity of the 760 cm^{-1} wavenumber (*30*). The band intensity at 760 cm^{-1} of the Raman spectrum of a protein has been reported to be related to its hydrophobicity (*31*, *32*). Hydrophobic groups of amino acids, peptides, and proteins exhibit CH stretching vibrational bands in the 2800−3100 cm^{-1} region. Bands near 2874−2897 cm^{-1} have been assigned to CH_3 symmetrical stretching and R_3C-H stretching bands of aliphatic amino acids, while $=C-H$ stretching bands of aromatic acids can be found around 3061−3068 cm^{-1}. Aromatic and aliphatic amino acids as well as charged amino acids, proline, threonine, and histidine, have C−H stretching bands near 2935−2955 cm^{-1} (*7*). Both the changes

Table 4. Correlation Coefficients of log S_0 vs Combined Raman Signals for 11 of the 13 Combinations Shown in **Table 3**

Log S_0	1	2	3	4	5	6	7	8	9	10	13
ANS	0.270	0.533	0.711	0.550	0.524	0.274	0.618	0.150	0.664	0.627	0.547
CPA	0.740	0.777	0.874	0.764	0.862	0.646	0.836	0.634	0.866	0.858	0.854
PRODAN	0.514	0.647	0.711	0.601	0.691	0.518	0.690	0.437	0.706	0.706	0.668

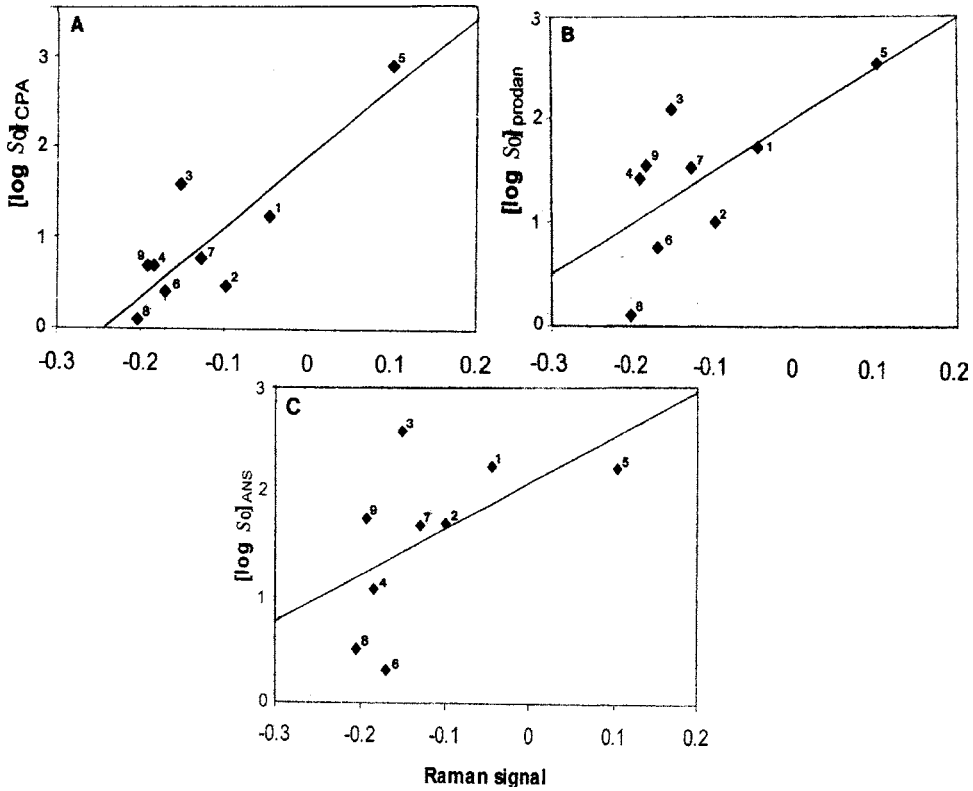

Figure 3. Relationships of log S_0 CPA (**A**), log S_0 PRODAN (**B**), and log S_0 ANS (**C**) of model proteins against combined Raman signal strength computed using combination 3 in **Table 3**. 1, α-casein; 2, β-casein; 3, κ-casein; 4, α-lactalbumin; 5, β-lactoglobulin; 6, lysozyme; 7, ovalbumin; 8, ovomucoid; 9, ovotransferrin.

in location and intensity of the vibrations of the C−H region represent changes in the environment of those groups that may be related to hydrophobic interactions (*18*, *33*−*35*). **Table 2** shows negative correlation of log S_0 against Raman signal intensities of the 760 and 3060 cm^{-1} bands (eq 1).

To obtain a combined response representing the intensities of all four Raman bands, the following formula was used:

$$\text{combined Raman signal} = -a[760] + b[2880] + c[2930] - d[3060]$$

where

$$a + b + c + d = 1.0 \quad (1)$$

By use of the RCO program (*17*), *a*, *b*, and *c* were randomly selected, then *d* was calculated using $d = 1 - (a + b + c)$ (combinations 1−9 in **Table 3**). For each protein, eq 1 was used to compute combined Raman values that were then correlated with Log S_0 of the nine proteins shown in **Table 1** (**Table 4**). Combinations 10−13 in **Table 3** were combinations in the centroid design, which were computed as the average of the best four combinations within five better/best combinations (2, 3, 5, 7, and 9) of the nine combinations in the random design (**Table 3**) and then the next four best combinations and so on, computed in the RCO optimization. The purpose of the centroid design is to search around the best response (*17*). Since there was not much variation in the four combinations 10−13, only combinations 10 and 13 were used for computing the correlation coefficients as shown in **Table 4**, which are the response values of the RCO search (**Table 3**). The best correlation coefficient of 0.874 was obtained for Log S_0 CPA in combination 3 with proportions of 0.16, 0.29, 0.17, and 0.38 for the four Raman signals (total is 1.0). Further cycle of RCO optimization was not attempted despite the existence of a slight possibility of better correlation coefficient if a proportion of [3060] in eq 1 slightly greater than 0.38 was tried, according to the mapping of approximated response surface. The regression lines illustrated in **Figure 3** as well as the correlation coefficients shown in **Table 4** show better correlation of the combined Raman signals with log S_0 CPA (correlation coefficient of 0.874 for combination 3) than with log S_0 PRODAN or ANS (correlation coefficients of 0.711 and 0.547, respectively).

It can be concluded that the order of correlation of Raman spectral parameters with S_0 values was CPA > PRODAN > ANS (**Table 4**). This may suggest CPA as the most suitable fluorescent probe for the measurement of fluorescent hydrophobicity, despite some electrostatic effects, which may be much less than that of ANS in terms of dissociation property. However, Drummen et al. (*36*), who used CPA as a peroxidative indicator, reported overestimation by CPA, which could also be the case in our study. Hayakawa and Nakai (*37*) and Alizadeh-Pasdar and Li-Chan (*11*) reported disagreement be-

Table 5. Predicted Solvent Accessibility of Protein Samples

protein samples	exposed[a]	buried[b]
ovotransferrin	54.24	45.76
ovalbumin	55.70	44.30
lysozyme	66.67	33.33
β-lactoglobulin	67.76	32.24
α-lactalbumin	62.60	37.40
κ-casein	86.84	13.16
β-casein	77.51	22.49
α-casein	77.89	22.11

[a] Exposed residues (with more than 16% of their surface exposed to solvent). [b] Buried residues (less than 16% solvent accessibility).

tween results obtained by ANS and CPA, which may be partly attributed to difference in the probe chemistry arising from the aromatic versus aliphatic nature of the probes, but also may have been due to interference of electrostatic interactions in the S_0 measurement (*38*).

The concentration of the proteins used in Raman spectroscopy (10%) is more relevant to concentrations in real food systems and is much higher than what is used in fluorescence spectroscopy (0.002−0.01%). Besides, intermolecular interactions have more important roles in protein solutions in higher concentrations (*39*), which may also explain why the intensity of Raman spectrum bands is not always in agreement with S_0 measured using various probes.

It should be noted that this study was conducted using proteins at pH 7.5. We may not obtain the same correlation between the S_0 and the Raman spectroscopic parameters for S_0 of proteins measured under basic and acidic pH using these probes, since the effect of ionic interactions may be more significant, due to the effect of charge. In addition, Howell et al. (*7*) recommended that the area under a peak is a better indicator of spectral intensity than the peak height, although the latter is more common (e.g., refs *33* and *40*). In contrast, an earlier study by Bouraoui et al. (*33*) did not find any disagreement between area of the peak and its height. In our study, we also measured the Raman peak areas and found similar correlations as described above using peak intensity.

Solvent Accessibility of Proteins. The predicted solvent accessibilities of the proteins are shown in **Table 5**. Chothia (*41*) reported a high correlation coefficient ($R^2 = 0.998$) between hydrophobicity of various amino acids and the accessible surface area. Stevens and Arkin (*42*) found a strong correlation between calculated solvent accessibility and hydrophobicity of membrane proteins. However, no significant correlation was found between the calculated surface accessibility of amino acids of each protein and S_0 measured using various methods or their log values in this study. This can be due to the limitation of the program used in terms of calculating only certain percentage of surface accessible residues at a time. The choice of 16% exposed surface threshold is rather arbitrary and is not well defined, and a variety of such cutoffs has been reported (*43*−*46*). Besides, the calculations are based on predictions and not measurements, and effects of environmental or processing conditions cannot be included. The inconsistency between calculated and experimental surface accessibility/hydrophobicity can also be attributed to the fact that the proteins used for experiments are not 100% pure, and some of the protein preparations might even be contaminated with other proteins or contain inhomogeneous fractions (native + denatured proteins), which could have an impact on the experimental surface hydrophobicity.

Conclusions. The results of FT-Raman spectroscopy have indicated that CPA and PRODAN in this order is appropriate to use as the fluorescent probe for describing surface hydrophobicity. Calculation of amino acid surface accessibility was not useful in identifying the best fluorescent probe for the measurement of S_0. Therefore, measurement of protein surface accessibility is recommended using experimental techniques rather than calculation.

Caution should be taken when choosing a fluorescent probe for measurement of protein surface hydrophobicity. Although ANS and CPA have been widely used for the measurement of S_0, their anionic nature may cause overestimation due to the contribution of both hydrophobic and electrostatic interactions. This effect might be less significant in some proteins than others due to their isoelectric point as well as under pH conditions being investigated.

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