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Conformational Study of Red Kidney Bean (*Phaseolus vulgaris* L.) Protein Isolate (KPI) by Tryptophan Fluorescence and Differential Scanning Calorimetry

Shou-Wei Yin,* Chuan-He Tang, Xiao-Quan Yang, and Qi-Biao Wen

Department of Food Science and Technology, South China University of Technology, Guangzhou 510640, People's Republic of China

Fluorescence and differential scanning calorimetry (DSC) were used to study changes in the conformation of red kidney bean (*Phaseolus vulgaris* L.) protein isolate (KPI) under various environmental conditions. The possible relationship between fluorescence data and DSC characteristics was also discussed. Tryptophan fluorescence and fluorescence quenching analyses indicated that the tryptophan residues in KPI, exhibiting multiple fluorophores with different accessibilities to acrylamide, are largely buried in the hydrophobic core of the protein matrix, with positively charged side chains close to at least some of the tryptophan residues. GdnHCl was more effective than urea and SDS in denaturing KPI. SDS and urea caused variable red shifts, 2−5 nm, in the emission λ_{max}, suggesting the conformational compactness of KPI. The result was further supported by DSC characteristics that a discernible endothermic peak was still detected up to 8 M urea or 30 mM SDS, also evidenced by the absence of any shift in emission maximum (λ_{max}) at different pH conditions. Marked decreases in T_d and enthalpy (ΔH) were observed at extreme alkaline and/or acidic pH, whereas the presence of NaCl resulted in higher T_d and ΔH, along with greater cooperativity of the transition. Decreases in T_d and ΔH were observed in the presence of protein perturbants, for example, SDS and urea, indicating partial denaturation and decrease in thermal stability. Dithiothreitol and *N*-ethylmaleimide have a slight effect on the thermal properties of KPI. Interestingly, a close linear relationship between the T_d (or ΔH) and the λ_{max} was observed for KPI in the presence of 0−6 M urea.

KEYWORDS: Red kidney bean protein isolate (KPI); conformational properties; DSC; tryptophan fluorescence; fluorescence quenching

INTRODUCTION

Legumes have been used as a major source of protein nutrition in many developing countries, especially where animal proteins are scarce or expensive. In 2001, the world production of legumes was estimated to be 52.3 million metric tonnes, and dry beans accounted for 32% (16.8 million metric tonnes) of this total production (*1*). Kidney bean (*Phaseolus vulgaris* L.), one of the main dry beans, is the most widely produced and consumed food legume in Asia, South America, and Africa (*1*). This bean usually contains 20−30% protein on a dry basis, and the protein has a balanced amino acid composition but is low in sulfur-containing amino acids (notably methionine) and tryptophan (*2*, *3*). Vicilin (also named phaseolin), the major storage protein of this bean, has unique structural peculiarity, for example, lower susceptibility to trypsin digestion and high-pressure treatment, as well as higher subunit homogeneity, as compared to other vicilin components (*4*−*6*). It is an oligomeric protein consisting of two or three polypeptide subunits, depending on its generic type, namely, α-, β-, and γ-phaseolin with a molecular weight distribution from 43 to 53 kDa (*7*). Unlike soy protein, the protein composition of kidney bean protein isolate (KPI) is much more homogeneous with the major vicilin content of 83−86%, relative to total protein content (*8*), and thus this protein isolate may be much more easily processed to act as a functional ingredient. KPI had the best gelling ability, as compared with those of protein isolates from red bean or mung bean (*9*). In another previous work, it was also indicated that the vicilin (or phaseolin) from kidney bean also exhibited the best emulsifying ability, as compared to those of pea, fava bean, cowpea, and soybean (*10*). Thus, KPI exhibits good potential to be applied as an excellent food functional ingredient.

A better understanding of the functional and physicochemical characteristics of kidney bean proteins, including their conformational properties and thermal properties, can enhance their potential utilization as a kind of food ingredient. In our laboratory, we had characterized some selected functional and physicochemical properties of KPI (*8*), and a series of physicochemical means had been used to improve the functional properties of KPI (*6*, *11*, *12*). As a potential food ingredient, KPI will be subjected to various processing conditions during food manufacturing, leading to

*Author to whom correspondence should be addressed (phone +86-20-87114262; fax +86-20-87114263; e-mail feysw@scut.edu.cn).

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conformational and structural changes. Monitoring of these changes is important as they may affect the nutritional and functional properties of the processed foods (*13*).

In this study, intrinsic fluorescence spectroscopy and differential scanning calorimetry (DSC) will be employed to monitor conformational changes in the protein in KPI under various buffer conditions. Intrinsic fluorescence has been widely used to investigate protein conformation, because the fluorescence characteristics, such as spectrum, quantum yield, and quenching, are highly sensitive to the properties of the microenvironment of tryptophan and tyrosine residues in the protein macromolecule (*14*, *15*) and thus can provide a sensitive means of monitoring conformational changes in proteins and protein−protein as well as ligand−protein interactions (*16*). On the other hand, protein denaturation involves conformational changes from the native structure due to the disruption of chemical forces that maintain the structural integrity of the protein molecules, for example, hydrogen bonds, hydrophobic bonds, ionic interactions, and covalent disulfide bonds (*17*, *18*). DSC is a particularly pertinent technique for studying thermal denaturation of food proteins and the role of chemical forces in stabilizing the globulin conformation. Using the DSC analysis technique, the thermal denaturation of some food proteins, such as soybean proteins (*19*), α-lactalbumin and β-lactoglobulin (*20*), oat globulins (*17*, *18*), red bean globulins (*21*), flaxseed proteins (*22*), rice globulin (*23*), and buckwheat globulin (*8*, *24*), has been widely studied.

Despite the importance that thermal and conformational properties have in food processing, studies in this field related to KPI are limited. The objective of this investigation was to study the effect of medium composition, such as pH, salt, and protein structure perturbants, on the conformational and thermal characteristics of the protein in KPI by DSC and fluorescence emission spectroscopy. The microenvironment and accessibility of the tryptophan residues in KPI were probed by fluorescence quenching studies using a polar neutral quencher (acrylamide), an anionic quencher (nitrate), and a cationic quencher cesium ion (Cs^+). The possible relationship between DSC characteristics and fluorescence spectra was also discussed. Those studies would help to better utilize KPI as a food ingredient.

MATERIALS AND METHODS

Material. Red kidney bean (*P. vulgaris* L.) seeds, cultivated in Shandong Province of China, were purchased from a local supermarket (Guangzhou, China). The seeds were soaked in deionized water for 12 h at 4 °C and dehulled manually. The dehulled seeds were freeze-dried, ground, and defatted by Soxhlet extraction with hexane. All chemical reagents used were of analytical or better grade.

Preparation of KPI. KPI was extracted using the procedure of Yin et al. (*11*), with slight modifications. The defatted flour was dispersed in deionized water (1:20, w/v), and the pH of the dispersion was adjusted to 8.0 with 2 M NaOH. The resultant dispersion was gently stirred at room temperature for 2 h and then centrifuged at 12000*g* for 30 min at 20 °C in a CR22G centrifuge. The pellet was discarded, and the supernatant was adjusted to pH 4.5 with 2 M HCl and then centrifuged at 6000*g* at 20 °C for 20 min. The obtained precipitate was washed twice by deionized water (pH 4.5) and then redispersed in deionized water. The dispersion was homogenized and adjusted to pH 7.0 with 2 M NaOH and centrifuged at 10000*g* for 20 min at 20 °C to remove the insolubles. The supernatant was dialyzed against deionized water for 48 h and then freeze-dried to produce KPI. The protein content was determined according to the micro-Kjeldahl method, using a nitrogen conversion factor of 6.25.

Intrinsic Fluorescence Emission Spectroscopy. Intrinsic emission fluorescence spectra of the protein samples were obtained in an F-7000 fluorophotometer (Hitachi Co., Hitachinake, Japan). Protein solutions (0.10 mg mL^{-1}) were prepared in 10 mmol L^{-1} phosphate buffer (pH 7.0) in the absence or presence of protein perturbants, including 0−40 mM sodium dodecyl sulfate (SDS), 0−8 M urea, and 10 mM dithiothreitol (DTT). For experiments involving pH adjustment, aqueous dispersions (0.1 mg/mL) of KPI were stirred magnetically for 30 min and then with either 0.05−0.2 mol L^{-1} HCl or NaOH; the pH values of the solutions were adjusted to the desired values and equilibrated for 1 h prior to the measurements The protein solutions were excited at 295 nm, and emission spectra were recorded from 300 to 400 nm at a constant slit of 5 nm for both excitation and emission.

The solvent accessibility of the tryptophan residues of the protein was studied by tryptophan fluorescence quenching experiments carried out for protein samples under native or denaturing conditions. All of the protein concentrations for all solutions used were kept at 0.1 mg/mL. Quenching stock solutions used were 1 M acrylamide, 0.45 M CsCl, and 0.45 M $NaNO_3$. The ionic strengths of solutions quenched by CsCl and $NaNO_3$ were diluted to 0.045 M by the addition of 0.2 M NaCl. The quenching process of the tryptophan fluorescence was described by the Stern−Volmer equation

$$\frac{F_0}{F} = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher Q, respectively, [Q] is the concentration of the quencher, and K_{SV} is the Stern−Volmer quenching constant. This equation describes the well-known concept that fluorophores with longer lifetimes are quenched more than those with shorter lifetimes (*25*). If the relative intensity F_0/F increases linearly with [Q], the Stern−Volmer constant K_{SV} can be obtained from the (initial) slope of the linear Stern−Volmer plot (*25*). A nonlinear Stern−Volmer plot can also be observed in a multi-tryptophan protein with different tryptophan residues (buried and exposed). The fraction of total fluorophore accessible to the quencher can be calculated from the modified Stern−Volmer plot

$$\frac{F_0}{\Delta F} = \frac{1}{K_Q f_a[Q]} + \frac{1}{f_a} \quad (2)$$

where ΔF is the change in the fluorescence intensity due to quenching, F_0 and [Q] have the same meaning as in eq 1, K_Q is the Stern−Volmer quenching constant of the exposed tryptophan residues, and f_a is the fraction of the initial fluorescence that is accessible to the quencher.

DSC. DSC experiments were performed on a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE). Indium standards were used for temperature and energy calibration. Approximately 1.0 mg protein samples were accurately weighed into aluminum liquid pans, and 10 μL of 10 mM phosphate buffer (pH 7.0) was added. For experiments involving pH adjustment, aqueous dispersions (10%, w/v) of KPI were stirred magnetically for 30 min, and then with either 0.5−5.0 M HCl or NaOH, the pH of the solutions was adjusted to the desired values and equilibrated for 1 h prior to the measurements. Protein perturbants, including SDS, *N*-ethylmaleimiden (NEM), DTT, and urea were prepared in the required concentrations in phosphate buffer, and 10 μL of the solvent was added to the weighed protein samples. The pans were hermetically sealed and heated from 25 to 120 °C at a rate of 10 °C min^{-1}. A sealed empty pan was used as a reference. Peak or denaturation temperature (T_d) of different protein components, the enthalpy of denaturation (ΔH), and the width at half-peak height of the endothermic peak ($\Delta T_{1/2}$) were computed from the thermograms by the universal analyzer 2000, version 4.1D (TA Instrument-Waters LLC, New Castle, DE). All experiments were conducted in triplicate.

Statistics. An analysis of variance (ANOVA) of the data was performed using the SPSS 13.0 statistical analysis system, and a least significant difference (LSD) or Tamhane's T_2 with a confidence interval of 95 or 99% was used to compare the means.

RESULTS AND DISCUSSION

Proximate Analysis. The protein content in KPI used in this study is about 89% (on the basis). *Phaseolus* globulin phaseolin, a 7S globulin, accouts for about 83−86% of the proteins in KPI; the balance is albumin or G2 globulin, as evidenced by SDS-PAGE and SEC analysis (*11*).

Conformational Properties of KPI: A Steady-Stage Fluorescence Study. The emission fluorescence spectrum is determined chiefly

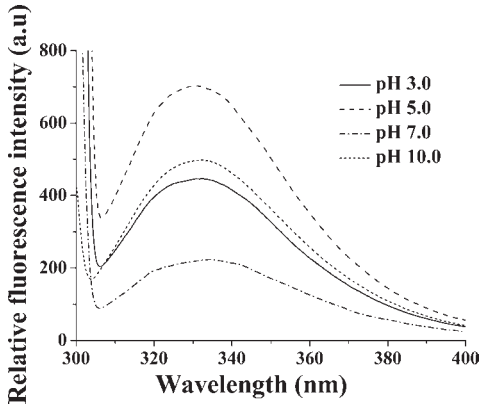

Figure 1. Fluorescence spectra of KPI at various pH values. Spectra were recorded using an excitation wavelength of 295 nm. Excitation and emission bandwidths were set at 5 nm.

by the polarity of the environment of the tryptophan and tyrosine residues and by their specific interactions and can provide a sensitive means of characterizing proteins and their conformation, because the fluorescence emission maximum suffers a red shift when chromophores become more exposed to solvent (*16*). At pH 7.0, KPI showed a typical intrinsic fluorescence spectrum, with an emission maximum (λ_{max}) at around 331 nm (**Figure 1**). This is a characteristic fluorescence profile of tryptophan residues in a relatively hydrophobic environment, such as the interior of a globulin (*26*).

Effect of pH. Due to the presence of ionizable amino acids (such as arginine, histidine, and aspartic acid), a change in pH can alter the overall charge on the protein and electrostatic interactions may change protein conformation. To find out the effect of pH on the microenvironment of tryptophan residues in KPI, the fluorescence spectra of KPI were also recorded at different pH values and plotted as a function of pH (**Figure 1**). This shows that the λ_{max} of the protein in KPI had no significant shift as the pH decreases from 10 to 3. This result indicates that the microenvironment of tryptophan residues does not change significantly as the pH decreases from 10 to acidic pH. The fact that the positions of the peak maxima of the protein in KPI at various pH values coincide suggests that the tertiary structure of the protein in KPI is insignificantly affected due to the change in pH from 10 to 3.

Effect of Protein Perturbants. **Figure 2** shows changes in fractional fluorescence intensity (F_0/F) and λ_{max} as a result of protein structure perturbants. Tryptophan fluorescence intensity increased in 0.5 mM SDS, 0.5 M urea, or 0.5 M GdnHCl and was thereafter quenched with increasing protein perturbants concentration (**Figure 2**). Similar results were observed for phaseolin (the main protein in KPI) by Deshpande and Damodaran (*27*). The observed increase in tryptophan fluorescence intensity at low denaturant concentration may be attributed to protein perturbant-induced dissociation of protein aggregates in KPI.

GdnHCl, owing to its poor hydration, is able to interact preferentially with poorly hydrated hydrophobic side chains on a protein's surface, leading to protein denaturation (*28*). No shift in λ_{max} was observed up to 3.5 M GdnHCl, and then a gradual red shift in emission λ_{max} and simultaneous decrease in FI_{max} was observed at $\geq$3.5 mol L^{-1} GdnHCl (**Figure 2A**), indicative of cooperative protein unfolding. The λ_{max} of KPI in 8 M GdnHCl was red-shifted to 351 nm, which is similar to the λ_{max} of free tryptophan (354.1 nm), indicating that 8 M GdnHCl resulted in extensive protein denaturation (or unfolding).

A red shift in λ_{max} of KPI (**Figure 2B**) at $\geq$1.5 M urea was recorded, and the λ_{max} of KPI red-shifted from 331 nm (in 0−1.5 M urea) to 335 nm (in 8 M urea), indicating a urea-induced slight increase in polarity of the microenvironment of tryptophan residues, also suggesting a slight comformational change due to the presence of 2−8 M urea.

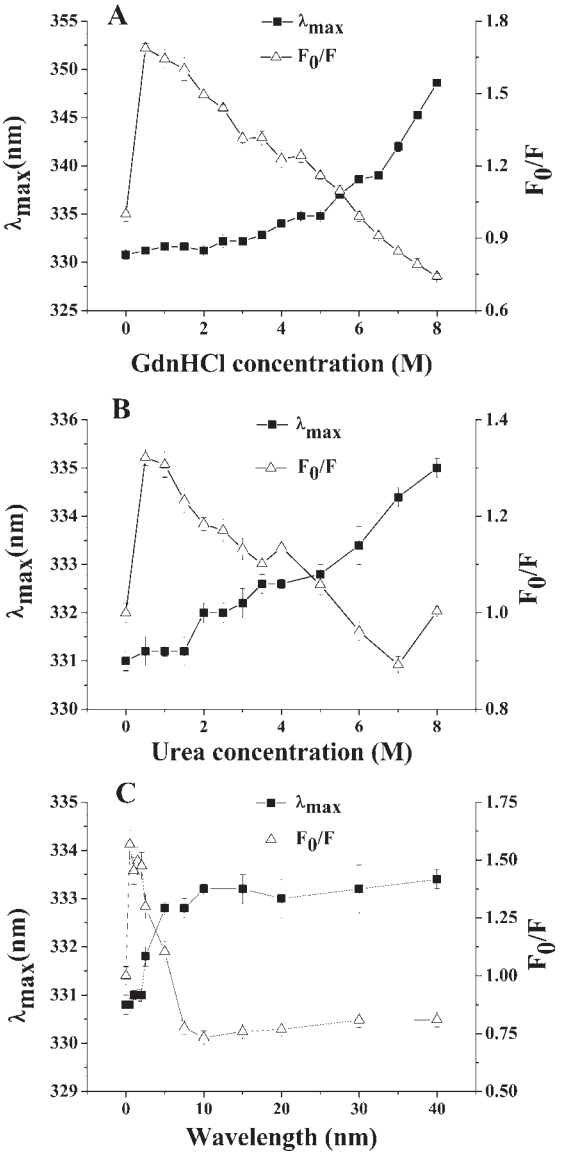

Figure 2. Changes in fractional fluorescence intensity (F_0/F) and emission maximum (λ_{max}) as a result of GdnHCl (**A**), urea (**B**), and SDS (**C**).

SDS exposure caused a small but significant red shift in λ_{max} of KPI at SDS > 5 mM (**Figure 2C**). However, only about 2−3 nm red shifts in λ_{max} in 10−40 mM SDS were observed as compared with that of the control, indicating slight exposure of tryptophan residues to a polar environment.

Phaseolin (also named G1 globulin) is the major storage protein in kidney bean seeds and exhibits structural peculiarity and compactness, as evidenced by the absence of any shift in emission maximum (λ_{max}) in the presence of structural perturbants (SDS, GdnHCl, urea, and DTT) (data not shown). Here, we found that tryptophan emission maxima of KPI in the presence of the denaturants shifted toward longer wavelengths. The extent of this red shift was in the order GdnHCl > urea > SDS, indicating that GdnHCl was the more effective in denaturing the KPI. This result was further confirmed by fluorescence quenching data (**Figure 3**). The different responses to protein structure perturbants of KPI and phaseolin may be associated with the loss of tertiary conformation of the proteins in KPI

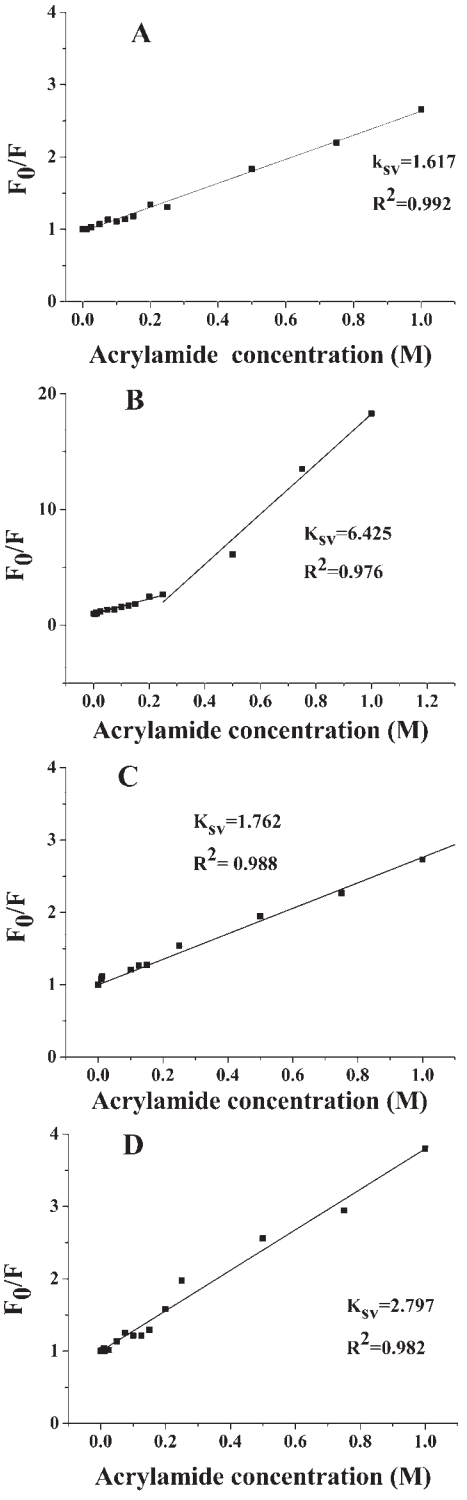

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Figure 3. Stern−Volmer plots for acrylamide quenching of KPI (0.1 mg/mL) in deionized water (**A**), 6 M GdnHCl (**B**), 20 mM SDS (**C**), and 8 M urea (**D**).

(relative to phaseolin) due to acid and alkali treatment during its processing.

Conformational Properties of KPI: Fluorescence Quenching. *Effect of Protein Perturbent*. Fluorescence quenching of KPI by acrylamide, a neutral quencher, in the absence or presence of protein perturbants was used to further study perturbant-induced conformational change of the proteins in KPI. **Figure 3** shows the Stern−Volmer plots for KPI exposure to deionized water, 20 mM SDS, 8 M urea, and 6 M GdnHCl. When the KPI was dissolved in deionized water, 20 mM SDS and/or 8 M urea acrylamide yielded linear quenching pattern, whereas in 6 M GdnHCl biphasic quenching patterns were observed, with a distinct upward curvature above 0.25 M acrylamide (**Figure 3**). The Stern−Volmer quenching constant, K_{SV}, denotes the accessibility of tryptophan residues in protein. K_{SV} increased gradually as the solvent was changed from deionized water to 20 mM SDS, 8 M urea, and 6 M GdnHCl (**Table 1**). The quenching data were in agreement with the steady-stage fluorescence of KPI under denaturing conditions.

Table 1. Summary of Parameters Obtained from the Intrinsic Fluorescence Quenching of KPI

quencher and condition	K_{SV}	f_a	K_a
acrylamide			
in water	1.617	0.749	1.972
in GdnHCl	6.425	1.053	5.588
in SDS	1.762	0.890	2.397
in urea	2.797	0.951	3.527

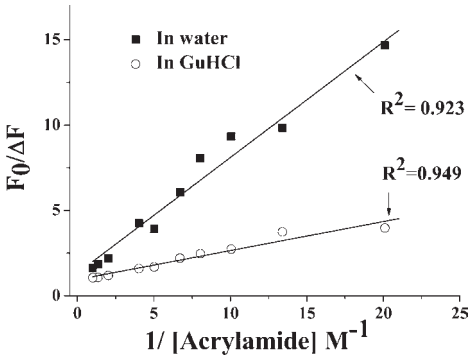

Figure 4. Modified Stern−Volmer plot for iodide quenching of KPI (0.1 mg/mL) in water or GdnHCl solution. Spectra were recorded using an excitation wavelength of 295 nm. Excitation and emission bandwidths were set at 5 nm.

The typical modified Stern−Volmer plots obtained with different solvent systems are shown in **Figure 4**, from which f_a and K_a were calculated according to eq 2 and are listed in **Table 1**. It can be seen from this table that 74.9, 89.0, 95.1, and ~100% of the total fluorescence of the KPI are accessible to acrylamide in deionized water, 20 mM SDS, 8 M urea, and 6 M GdnHCl, respectively. This further suggests that the presence of protein perturbants, especially GdnHCl, led to protein denaturation (or unfolding), with subsequent increase in tryptophan accessibility to the quencher. The observed results indicate that the more flexible the protein structure is and/or the higher the concentration of unfolded species is, the more pronounced is the Stern−Volmer plot (*29*).

According to Eftink and Ghiron (*30*), the upward curvature suggests either all fluorophores being equally accessible or a single fluorophore being predominant. To choose between these two possibilities, increments in K_{SV} upon GdnHCl treatments were compared. If only a single fluorophore existed, increments in K_{SV} would not be significant. In fact, a 3.97-fold increase in K_{SV} due to the presence of 6 M GdnHCl suggested multiple tryptophan fluorophores in KPI with different acrylamide accessibilities.

Effect of Quencher Type. Only 2−5 nm red shifts in the λ_{max} of KPI in the presence of 6−8 M urea or 10−40 mM SDS suggested the conformational compact or structural peculiarity of the protein in KPI. This result was further supported by quenching data of KPI in the presence of 8 M urea or 20 mM SDS. To elucidate the structural peculiarity of KPI, fluorescence

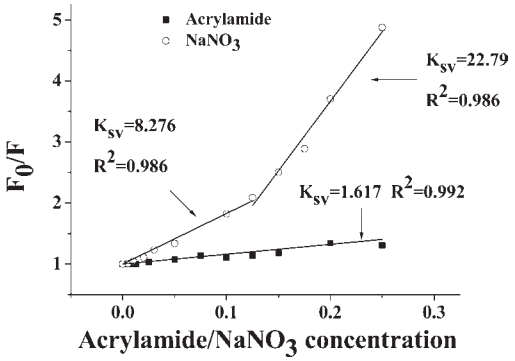

Figure 5. Stern−Volmer plots for acrylamide and $NaNO_3$ quenching of the protein in KPI (0.1 mg/mL) in deionized water.

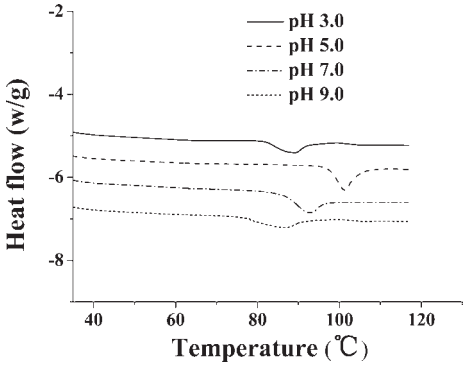

Figure 6. Typical DSC thermograms of KPI at various pH values. The pans were hermetically sealed and heated from 25 to 120 °C at a rate of 10 °C min^{-1}. A sealed empty pan was used as reference.

quenching experiments, using a polar neutral quencher (acrylamide), an anionic quencher (nitrate), and a cationic quencher [cesium ion (Cs^+)], were performed, as shown in **Figure 5**. The fluorescence intensity and spectral peak maximum of tryptophan residues in a protein are sensitive to the local environment of tryptophan. The quenching of intrinsic fluorescence emission of tryptophan residues in a protein therefore can be used to determine the surface accessibility of the tryptophan using different quenchers as well as to obtain information regarding the nature of the environment. The experiments were carried out by monitoring the change in intensity at the emission maximum. The fluorescence intensity of the protein in KPI decreased depending on the concentration of a quencher added for the anionic and neutral quenchers, whereas no quenching was observed with Cs^+ even up to 0.25 M under experimental conditions used. A striking difference between the two charged quenchers used is the lack of fluorescence quenching action by the cesium ion (Cs^+). This seems to suggest that fluorescence for the protein in KPI should be associated with the tryptophan located in close proximity to some positively charged amino acid residues (*31*). The extents of quenching observed with a polar neutral quencher (acrylamide) and an anionic quencher (nitrate) up to a final quencher concentration of 0.25 mol L^{-1} were compared, and the data indicate that nitrate quenched about 79.5% of the tryptophan fluorescence of the protein in KPI, whereas acrylamide quenched only 23.5% at the same concentration.

The fluorescence change was plotted according to Stern−Volmer eq 1. Only regions of low quencher concentrations were considered in the calculation of the K_{SV} constants (*32*, *33*). Typical quenching data are exhibited in **Figure 5** and summarized in **Table 1**. Protein quenching by acrylamide, a polar uncharged water-soluble molecule that can penetrate the matrix of the protein (*34*), gave a linear Stern−Volmer plot (**Figure 5**). The value of the Stern−Volmer constant K_{SV} was found to be 1.617 M^{-1}. The K_{SV} was higher than that of phaseolin obtained at the same condition (data not shown) and lower than the corresponding K_{SV} of 7S globulin (β-conglycinin). This observation suggests that tryptophan fluorophore in KPI was in a more hydrophilic, surface-accessible environment than that in phaseolin (the main globulin in kidney bean) obtained by acid extraction techniques. Acrylamide is known to exhibit static quenching (ground-state formation of a nonfluorescent complex) at higher concentrations. NO_3^-, like acrylamide, is a contact quencher (*32*). The high values of K_{SV} for nitrate (8.276 M^{-1}), relative to that of acrylamide, might be an indication of favorable charge interactions between the anionic quencher and positive charge on the protein in the vicinity of the tryptophan resulting in static quenching of the fluorescence (*33*, *35*). This result was consistent with quenching data of tryptophan fluorescence at a quencher concentration of 0.25 M. Furthermore, NO_3^- yielded biphasic quenching patterns, with distinct upward curvature above 0.1 M $NaNO_3$, indicating all fluorophores being equally accessible to NO_3^-.

In fact, the tryptophan residues in KPI are located in the hydrophobic core of the protein. Interestingly, quenching data from acrylamide and NO_3^-, as well as the lack of fluorescence quenching by Cs^+ on the KPI, also suggested the presence of charged basic amino acid residues in the vicinity of the tryptophan residue.

DSC Characteristics of KPI. The thermal property for a globulin protein is also important, because it may be related to its heat-induced aggregation and gelation behaviors. All tested samples exhibit a prominent endothermic peak. The major endothermic peak was clearly attributed to the thermal denaturation of vicilin-like globulin (the phaseolin).

Effect of pH. The effect of pH on thermal characteristics of KPI is shown in **Figure 6**. Close to the isoelectric point (iep) of the protein (pH 4.5), the endothermic peak is narrow and deep; the peak temperature at pH 5 was found to be 101.2 °C, and the enthalpy was 14.6 ± 0.3 J/g. The peak width increased and the depth of the peaks decreased at pH values deviating from the iep of KPI (**Figure 5**). Both T_d and ΔH values at extreme acidic and alkaline pH were decreased when compared to that at pH 5−7, whereas the $\Delta T_{1/2}$ value was increased (data no shown). Similar changes in thermal characteristics with pH were also reported for globulins from red bean (*21*) and fababean (*36*), buckwheat globulin, and β-lactoglobulin (*37*). Most proteins are stable near the isoelectric pH when the repulsive forces are low and the proteins remain in a native state. The narrow shape of the peak at pH 5 suggests that KPI dissociates and denatures in a cooperative way. At pH 7 and 9 the protein surfaces have a surplus of negative charges, whereas at pH 3.0 the protein surfaces have a surplus of positive charges as can be seen from the negative zeta-potential values (data no shown). The acidic and alkaline conditions did not affect the conformational stability of the protein in KPI at room temperature (25 °C), as shown in fluorescence data (**Figure 1**). Thus, the decrease in T_d and ΔH at acidic or alkaline conditions may be attributed to the synergistic effect between temperature and the repulsive forces when the pans were heated in the oven of DSC.

Effect NaCl Concentration. **Table 2** shows the thermal transition properties of KPI as a function of NaCl concentration (0, 0.5, 1.0, or 2.0 M). The T_d increased by approximately 10, 14, and 20 °C, respectively, when the ionic strength was increased from 0 to 0.5, 1, and 2 M NaCl. DSC data confirmed the stabilizing effect of NaCl on KPI. An identical increase in thermal stability has also been reported for β-lactoglobulin (*37*), red bean globulins (*21*), and flaxseed proteins (*22*) as well as globulin from

Table 2. Effect of NaCl Concentration on Thermal Characteristics of KPI[a]

	T_d[b] (°C)	ΔH[c] (J/g of protein)	$\Delta T_{1/2}$[d] (°C)
control	92.5 ± 0.2 d	11.2 ± 0.2 b	5.90 ± 0.10 a
0.5 M	102.6 ± 0.3 c	12.5 ± 0.3 a	5.60 ± 0.22 b
1 M	106.5 ± 0.2 b	11.8 ± 0.2 a	5.44 ± 0.31 b
2 M	112.3 ± 0.4 a	12.1 ± 0.4 a	5.39 ± 0.12 b

[a] Values are expressed as mean and standard deviations of triplicate measurements. Letters a−d indicate significant ($P < 0.05$) difference within the same column. [b] T_d, thermal denaturation temperature. [c] ΔH, enthalpy changes of the endotherm. [d] $\Delta T_{1/2}$, width at half-peak height of endothermic peak.

rice seed (*23*) and buckwheat (*24*). The heat stability of proteins is controlled by the balance of polar and nonpolar residues. This increased temperature stability can be explained by a reduction of intermolecular electrostatic repulsion leading to a growth in the association of native molecules. Therefore, the presence of a high concentration of NaCl favors the formation of more compact conformation of the proteins in KPI, with subsequent increase in thermal stability (higher T_d). The ΔH of the endothermic peak of KPI, to some extent, increased, whereas the width at half-peak height (ΔT) decreased with increasing NaCl concentration (**Table 2**), suggesting that the presence of a high concentration of NaCl enhanced the proportion of more compact proteins and the cooperativity of thermal transition process. Li-Chan and Ma (*22*) also indicated that higher salt condition (1.0 M) resulted in higher enthalpy values and better cooperativity of the transition of flaxseed proteins at pH 3−11 (as compared to 0.01 M salt condition).

Effect of Protein Perturbants. To gain the knowledge referred to the main interactions in stabilizing the protein conformation, the effects of protein structure perturbants, including SDS, urea, DTT, and the sulfhydryl blocking agent NEM, on the thermal transition properties of KPI were evaluated, as shown in **Table 3**.

With increasing concentration of urea, both T_d and ΔH were progressively decreased (**Table 3**). Generally, urea effectively disrupts the hydrogen bond structure of water and facilitates protein unfolding by weakening hydrophobic interaction (*38*). Moreover, urea also increases the "permittivity" of water for the nonpolar residues (*39*), causing loss of protein structure stability. Recently, Stumpe and Grubmüller (*40*) showed that, aside from interference on the hydrogen bond structure of water, urea molecules are located at the protein surface; in particular, they accumulate close to less polar residues and the backbone, inducing the displacement of water molecules and facilitating protein unfolding due to exposure of apolar groups. As a consequence, urea can be considered to denature proteins by interfacing between water and the natively buried parts of the protein. Apart from decreases in T_d and ΔH, urea caused a progressive broadening in the peak width ($\Delta T_{1/2}$), indicating a lack of cooperativity in the thermal denaturation process (*41*). Interestingly, a discernible endothermic peak, with T_d and ΔH of about 84.1 °C and 3.6 J/g of protein, respectively, was still detected up to 8 M urea. In general, urea at higher concentration resulted in extensive protein denaturation. No endothermic response was observed for globulin from rice (*Oryza sativa*) seeds (*23*), red bean (*21*), and buckwheat globulin (*8*) in the buffer with 8 mol L^{-1} urea. The data further confirmed the structural peculiarity and compactness of the proteins (mainly phaseolin) in KPI.

To further examine hydrophobic interactions, the influence of SDS on the denaturation parameters of KPI was studied (**Table 3**). The addition of 10−30 mmol L^{-1} SDS resulted in lower T_d and ΔH, as well as broader transitions of the protein in KPI. The thermal stability of this fraction was significantly affected starting from 10 mM SDS, in accordance with studies in peanut proteins (*42*). Generally, SDS as an anionic detergent is known to disrupt both hydrophobic and hydrogen bonding (*43*). SDS joins to proteins by means of interactions between sulfate groups and positively charged lateral chains and between alkyl chains and hydrophobic lateral chains and, thus, can cause ionic repulsion and unfolding of polypeptides (*43*, *44*), with decreases in thermal stability. Similar effects of SDS on thermal properties have been observed in the globulins from oat (*17*) and red bean (*21*).

Table 3. Effect of Some Perturbants on Thermal Transition Characteristics of KPI[a]

perturbant	concn	T_d[b] (°C)	ΔH[c] (J/g of protein)	$\Delta T_{1/2}$[d] (°C)
control		92.5 ± 0.1 a	11.2 ± 0.1 a	5.9 ± 0.1 f
urea	1 M	89.4 ± 0.2 c	8.9 ± 0.2 bc	5.8 ± 0.2 f
	2 M	87.8 ± 0.1 d	6.4 ± 0.2 cd	5.9 ± 0.2 f
	4 M	86.8 ± 0.2 e	5.7 ± 0.1 d	7.6 ± 0.3 c
	6 M	85.0 ± 0.3 f	3.9 ± 0.1 e	8.3 ± 0.4 b
	8 M	84.1 ± 0.2 f	3.6 ± 0.1 e	9.5 ± 0.3 a
SDS	10 mM	91.5 ± 0.2 b	11.3 ± 0.2 a	6.6 ± 0.1 e
	20 mM	89.3 ± 0.1 c	7.1 ± 0.2 bc	7.1 ± 0.2 d
	30 mM	89.2 ± 0.1 c	7.7 ± 0.3 b	6.8 ± 0.2 e
DTT	25 mM	91.0 ± 0.3 bc	7.8 ± 0.2 b	5.6 ± 0.1 f
	100 mM	89.1 ± 0.2 c	7.4 ± 0.3 b	5.8 ± 0.3 f
NEM	20 mM	91.2 ± 0.1 bc	10.2 ± 0.2 b	5.6 ± 0.3 f

[a] Values are expressed as mean and standard deviations of triplicate measurements. Letters a−f indicate significant ($P < 0.05$) difference within the same column. [b] T_d, thermal denaturation temperature. [c] ΔH, enthalpy changes of the endotherm. [d] $\Delta T_{1/2}$, width at half-peak height of endothermic peak.

To ascertain the contribution of disulfide bonds to the thermal stability, we also investigated the influence of DTT on the DSC characteristics of KPI. The addition of DTT caused slight decreases in both T_d from 92.5 °C (control) to 91 °C (25 mM DTT) and 89 °C (100 mM DTT) and ΔH from 11.2 (control) to 7.4−7.8 J/g of protein (25 and 100 mM DTT). Heating in the presence of reducing agents such as DTT could initiate the breakage of disulfide bonds, promoting denaturation of the monomers (*18*). The major globulin phaseolin in KPI is devoid of disulfide bonds between individual subunits (*10*). However, the PIL (protease inhibitor, lectin) fraction and/or albumin, with rich SH and disulfide content, may account for the high SH and SS contents in KPI (*45*), as well as DTT-induced destabilization of KPI. NEM, a sulfhydryl-blocking reagent, also led to the slight decrease in T_d and ΔH of KPI (**Table 3**), indicating that SS−SH interchange was also involved in stabilizing the conformation of the protein in KPI.

Possible Relationship between DSC Characteristics and Fluorescence. DSC characteristics can reflect the conformational change of a protein in tertiary and quaternary levels, particularly the tertiary conformation, whereas the emission fluorescence spectrum is also a sensitive means of characterizing proteins and their conformation, because the red or blue shift in the emission λ_{max} represents the tertiary conformational change of a protein (*46*). Here, we analyzed the data of fluorescence emission maxima (λ_{max}) and DSC characteristics (including denaturation peak temperature, enthalpy change) of KPI in the absence or presence of urea and found there were close linear relationships between the data of the T_d (or ΔH) and the λ_{max} for KPI in the presence of 0−6 M urea (**Figure 7**). This is the first evidence about a close relationship between the DSC characteristics and fluorescence spectra of KPI, indicating urea resulted in protein denaturation (unfolding), with decreasing thermal stability and

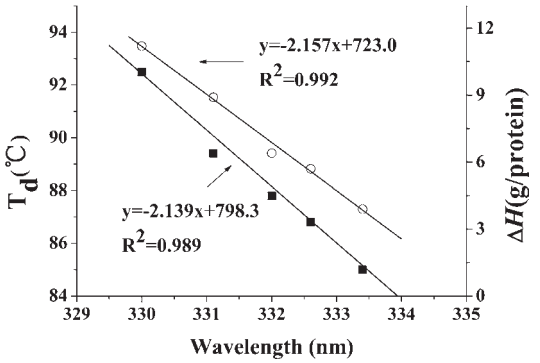

Figure 7. Relationship between T_d and ΔH of the endothermic peak and λ_{max} of KPI samples under different urea concentrations (0−6 M).

the proportion of undenatured protein and/or the extent of ordered structure in KPI. Close relationships between DSC characteristics and fluorescence data were further evidenced by DSC and fluorescence results of KPI in the presence of 0−40 mM SDS. The red shifts in λ_{max} of fluorescence spectra were accompanied with the decrease in denaturation temperature (thermal stability) at 0−10 mM SDS, whereas both the fluorescence parameters (λ_{max} and F_0/F) and thermal transition characteristics (T_d and ΔH) of KPI were nearly unaffected with further increase in SDS concentration (**Table 3** and **Figure 2**).

In conclusion, the presence of protein structure perturbants, especially GdnHCl, resulted in protein denaturation (unfolding), with subsequent increase in accessibility of tryptophan fluorophores to quencher. Acrylamide gave a linear Stern−Volmer, whereas NO_3^- yielded biphasic quenching patterns. The lack of fluorescence quenching by Cs^+ on the KPI was observed up to 0.25 M. These results suggested the presence of charged basic amino acid residues in the vicinity of the tryptophan residue. DSC analyses confirmed that different chemical forces, mainly hydrophobic and electrostatic interactions and hydrogen bonds, are involved in maintaining the native conformation of phaseolin. Disulfide linkages and SS−SH interactions make a slight contribution to protein stability. Interestingly, close relationships between DSC characteristics and tryptophan fluorescence data were observed for KPI in the presence of 0−6 M urea.

ABBREVIATIONS USED

DSC, differential scanning calorimetry; λ_{max}, emission maximum; SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; T_d, denaturation temperature; ΔH, enthalpy changes of the endotherm; $\Delta T_{1/2}$, width at half-peak height; SH−SS, sulfhydryl−disulfide.

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