Food Chemistry 115 (2009) 859–866

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Heat-induced modifications in the functional and structural properties of vicilin-rich protein isolate from kidney (*Phaseolus vulgaris* L.) bean

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ARTICLE INFO

Article history:
Received 23 September 2008
Received in revised form 6 December 2008
Accepted 29 December 2008

Keywords:
Kidney protein isolate
Phaseolus vulgaris L.
Heat treatment
Functional property
Structural conformation
Modification

ABSTRACT

Heat-induced changes in the physico-chemical (and/or functional) and structural properties of protein isolate from kidney beans (KPI) were characterised. The extent of protein denaturation, free sulphydryl contents, surface hydrophobicity, as well as structural characteristics of the proteins were evaluated. Analyses of size-exclusion chromatography combined with laser scattering showed that the heating at 95 °C led to transformation of 7S-form vicilin to its 11S-form, and even higher molar mass (MW) oligomers or polymers. Moderate heating (for 15–30 min) significantly improved protein solubility, emulsifying and foaming activities (at neutral pH), whilst extensive heating (for 60–120 min) on the contrary decreased these properties. Spectral analyses of fluorescence and/or Raman spectroscopy showed that tertiary and secondary conformations of protein in KPI were remarkably affected to a varying extent by the heating. The results suggested a close relationship between functional properties of the vicilin from kidney bean and its conformational characteristics.

1. Introduction

There is a growing interest in the utilisation of proteins from different legumes, due to increased needs for protein nutrition and food formulation. Kidney (*Phaseolus vulgaris* L.) bean is the most widely produced and consumed food legume in Africa, India, Latin America and Mexico. The protein (20–30%) from this legume has been shown to have a good amino acid composition but be lack of sulphur-containing amino acids (notably methionine) and tryptophan (Sathe, 2002). Vicilin, sometimes also named as phaseolin (or G1 globulin), is the major storage protein. It is an oligomeric protein consisting of three polypeptide subunits α-, β- and γ-phaseolin with molecular weight distribution from 43 to 53 kDa (Romero, Sun, McLeester, Bliss, & Hall, 1975).

The flour from kidney bean has been proved to have higher functional properties, e.g., gelation capacities, emulsifying activity and emulsion stability, relative to soybean flour (Chau & Cheung, 1998). Clearly, these good properties of the flours are largely contributed by the proteins contained in this flour. However, the thermal and surface active properties (e.g., foaming) of proteins from kidney beans were different depending on the method of preparation. The alkali-extracted protein isolates (amorphous) had lower denaturation enthalpy change (ΔH), and higher surface hydrophobicity and foam expansion than acid-extracted protein isolates (crystalline) (DiLollo, Alli, Biliarderis, & Barthakur, 1993). In our previous study, it was found that the alkali-extracted protein isolate of kidney bean (KPI) showed much higher heat-induced gelation properties than those from other *Phaseolus* legumes, and their thermal denaturation and gelation seemed to be largely related to their free sulphydryl and/or disulfide bonds (Tang, 2008).

The heat treatment has been widely applied to improve the nutritional properties of legume proteins, e.g., especially *in vitro* protease digestibility, by means of inactivating protease inhibitors or denaturing the proteins. For example, it was observed that heating treatment (at 100 or 121 °C for 15 min) could remarkably improve the *in vitro* protease digestibility of the major globulin (vicilin or phaseolin) of kidney bean (Romero & Ryan, 1978). In this case, the improvement of *in vitro* digestibility was attributed to the disruption of conformational constraints (on protease hydrolysis) of the native molecule. Although the influence of the thermal treatment on the properties of soy protein or its purified glycinin (11S) and β-conglycinin (7S) was well recognised, little information is available concerning about the effects on other legume proteins.

In our previous study (Yin, Tang, Wen, Yang, & Li, 2008), we investigated the influence of high pressure treatment on the functional and nutritional properties of KPI, and found that the treatment at 200–600 MPa led to gradual unfolding of protein structure and subsequent aggregation, in a pressure dependent manner, and the emulsifying activities were improved only at pressures of 200 or 400 MPa. However, the *in vitro* trypsin digestibility and thermal stability of proteins was unaffected by high pressure treatment. Thus, the high pressure treatment is not a good choice to modify these vicilin-rich protein isolates from legumes. In the

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doi:10.1016/j.foodchem.2008.12.104

present study, we applied a thermal treatment (at 95 °C for 15–120 min) to modify the physico-chemical and functional properties of KPI. The heating temperature (95 °C) applied was slightly higher than the denaturation temperature (about 94 °C) of the vicilin component in KPI (Yin et al., 2008). Additionally, the heat-induced structural conformational changes were also characterised.

2. Materials and methods

2.1. Preparation of KPI

Red kidney bean (*Phaseolus vularis* L.) seeds, cultivated in Gangshou province of China were purchased from a local supermarket (Guangzhou, China). The preparation of KPI was according to the same process as described in our previous paper (Tang, 2008). After soaked, de-hulled and dried in the oven, the de-hulled beans were smashed and passed an 80-mesh to produce the flour. The flour was defatted by solvent extraction with *n*-hexane (the ratio of the flour to *n*-hexane = 1:5 g/ml) for three times. The defatted flour was then dispersed with 10-fold distilled water. The dispersions were adjusted to about pH 8.0 with 2 mol/l NaOH, and magnetic-stirred at room temperature for more than 2 h, and then centrifuged at 9000g for 30 min to obtain the protein supernatant. The supernatant were adjusted to pH 4.6 using 2 mol/l HCl, and placed at 4 °C for 2 h, and then centrifuged at the same condition. The obtained precipitate was washed with pre-cooled water for several times, and re-dispersed in distilled water, and adjusted to neutral pH. Last, the protein dispersion was dialysed three times at 4 °C against desalted water (1:100 v/v, 3 times), and then lyophilised to yield KPI. The protein content of this protein was about 92.5% (wet basis), as determined by micro-Kjeldahl method, using a nitrogen conversion factor of 6.25.

2.2. Heat treatment

The KPI solutions (1 or 2% w/v) were prepared with 50 mM phosphate buffer (pH 7.4) containing 50 mM NaCl. Aliquots of KPI solutions (50 or 100 ml) were sealed in plastic containers with thin wall, and incubated in a water bath at 95 °C. At accurate periods of heating time (15, 30, 60 and 120 min), the samples were immediately taken out and cooled in an ice bath. Then, the cooled samples (with plastic containers) were frozen in liquid nitrogen. The unheated KPI sample (control) was also frozen in the same mode. Last, the frozen KPI samples (unheated and heated) were lyophilised. The untreated and heat-treated (15, 30, 60 and 120 min) KPI samples were denoted as control, K-15, K-30, K-60 and K-120, respectively.

2.3. High-performance size-exclusion chromatography (HPSEC) combined with multi-angle laser light scattering (MALLS)

The HPSEC and the MALLS systems were the same as those described by Zhao, Mine, and Ma (2004). Two TSK columns (G4000 PW_{XL} + TSK G6000 PW_{XL}) were connected in series (TOSOH Corp., Montgomeryville, PA). The fractionation range of these two columns was 2000–8000 000 for proteins. The mobile phase (50 mM phosphate buffer, pH 7.4, containing 50 mM NaCl) was filtered through 0.2 μm (Whatman International Ltd., Maidstone, England) and then 0.02 μm filters (Millipore Corp., Bedford, MA). The flow rate was 0.8 ml/min.

A Dawn EOS photometer (Wyatt Technology Corp., Santa Barbara, CA) was used. Two auxiliary analogue inputs enabled interfacing to external detectors such as refractive index (RI) and UV detectors. The instrument was placed directly before the RI detector and after the SEC columns and UV detector to avoid backpressure on the RI cell. Dynamic light scattering measurement was performed on-line in the flow cell using a QELS metre (Wyatt Technology Corp., Santa Barbara, CA). An optical fibre receiver was mounted in the read head of one of the MALLS detectors (detector 13 in our works). Chromatographic data were collected and processed by the ASTRA software (Wyatt Technology Corp.). The M_w of protein eluting in small and individual slices of the SEC chromatogram was determined, based on the Debye plot (Zhao, Ma, Yuen, & Phillips, 2004b). The d*n*/d*c*, where *n* and *c* present the refractive index and sample concentration for each data slice, is reckoned to be constant (±0.185 ml/g) across the sample peak, and nearly independent of its amino acid composition. Bovine serum albumin monomer (Sigma, St. Louis, MO) was used for normalising various detectors' signals relative to the 90° laser light detector signal.

2.4. Differential scanning calorimetry (DSC)

DSC experiments were performed on a TA Q100-DSC thermal analyser (TA Instruments, New Castle, DE), according to the procedure of Meng and Ma (2001), with some modifications. Approximately 2.0 mg of untreated or heated KPI samples were accurately weighed into aluminum liquid pans, and 10 μl of 50 mM phosphate buffer (pH 7.4) was added. The pans were hermetically sealed and heated in the calorimeter from 20 to 120 °C at a rate of 10 °C/min. A sealed empty pan was used as a reference. Peak or denaturation temperature (T_d), enthalpy change of denaturation (ΔH) and width at half peak height of endothermic peak ($\Delta T_{1/2}$) were computed from the thermograms by the universal analyser 2000, version 4.1 D (TA Instrument-Waters LLC, USA). All experiments were conducted in triplicate.

2.5. Free sulphydryl (SH) content

Free sulfhydryl groups (SH) contents of protein isolates, including total and exposed SH contents, were determined according to the method of Ellman (1959) and Beveridge, Toma, and Nakai (1974), as described by Yin et al. (2008). The SH contents were expressed as μmol/g of protein. The determinations were conducted in duplicate.

2.6. Surface hydrophobicity (H_o)

H_o was determined using ANS, according to the method of Haskard and Li-Chan (1998). In brief, stock solutions of 8×10^{-3} M ANS^-, and 1.5% (w/v) protein were prepared in 10 mM phosphate buffer (pH 7.0). To successive samples containing 4 mL of buffer and 20 μl of ANS^- stock solution were added 10, 20, 30, 40, and 50 μl of 1.5% protein solution. The mixtures were shaken in a vortex mixer for 5 seconds. Fluorescence intensity (FI) was measured at wavelengths of 390 nm (excitation) and 470 nm (emission) using a RF-5301 PC spectrofluorometer (Shimadzu Corp., Kyoto, Japan) at 20 ± 0.5 °C, with a constant excitation and emission slit of 5 nm. The FI for each sample with probe was then computed by subtracting the FI attributed to protein in buffer. The initial slope of the FI versus protein concentration plot was calculated by linear regression analysis and used as an index of H_o. Additionally, the extrinsic fluorescence spectroscopy of ANS in the presence of proteins was obtained at a protein concentration of 7.5×10^{-5} g/ml (or 20 μl of 1.5% protein solution mixed with 4 ml of the buffer). The determinations were conducted in duplicate.

2.7. Protein solubility (PS)

An aqueous solution (1% w/v) of samples in distilled water was stirred magnetically for 30 min, and then with either 0.1 N HCl or

0.1 N NaOH, the pH was adjusted to pH 7.0. Then it was centrifuged at 8000*g* for 20 min at 20 °C in a CR22G centrifuge (Hitachi Co., Japan). After appropriate dilution, the protein content of the supernatant was determined by the Lowry method (Lowry, Rosembroug, Lewis, & Randall, 1951) using bovine serum albumin (BSA) as a standard. PS was expressed as g of soluble protein/100 g of protein. All determinations were conducted in duplicate.

2.8. Emulsifying activity index (EAI) and emulsion stability index (ESI)

EAI and ESI were determined according to the method of Pearce and Kinsella (1978), with minor modifications. For emulsion formation, 15 ml of 0.1% (w/v) untreated or heated KPI solutions in 50 mM phosphate buffer (pH 7.0) and 5 ml of corn oil were homogenised in ULTRA-TURRAX® T25 digital homogenizer (IKA Co., Germany) at 24,000 turn/min for 1 min. Fifty micro-litres of emulsion were taken from the bottom of the homogenised emulsion, immediately (0 min) or 10 min after homogenisation, and diluted (1:100 v/v) in 0.1% (w/v) SDS solution. After shaking in a vortex mixer for 5 s, the absorbance of dilute emulsions was read at 500 nm using a Spectrumlab 22PC spectrophotometer (Shanghai Lengguang Technology Co. Ltd., Shanghai, China). EAI and ESI values were calculated using the following equations:

$$EAI(m^2/g) = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \phi \times (1-\theta) \times 10000}, \; ESI(min) = \frac{A_0}{A_0 - A_{10}} \times 10,$$

where DF is the dilution factor (100), c the initial concentration of protein (g/ml), Φ the optical path (0.01 m), θ the fraction of oil used to form the emulsion (0.25), and A_0 and A_{10} the absorbance of diluted emulsions at 0 and 10 min respectively. Measurements were performed in triplicate.

2.9. Foaming properties

Foaming properties including foaming capacity (FC) and foam stability (FS) were determined by the method of Fernandez and Macarulla (1997) with minor modifications. Aliquots (10 ml) of sample solutions (1%, w/v) at pH 7.0 in measuring cylinder (25 ml) were homogenised with an ULTRA-TURRAX® T25 digital homogenizer (IKA Co., Germany) at 24,000 turn/min for 2 min. FC was calculated as the percent increase in volume of the protein dispersion upon mixing, whilst FS was estimated as the percentage of foam remaining after 30 min. Measurements were performed in triplicate.

2.10. Raman spectroscopy

Raman spectra with an excitation at 632.8 nm (laser He/Ne, less than 10 mW on the sample) were obtained at room temperature, using a LABRAM-Aramis spectrometer (Horiba Jobin-Yvon, France). The laser was focused on the solid samples which were placed on microscope slides. Each spectrum was obtained under the following conditions: 32 scans, 2 s exposure time, 2 cm^{-1} resolution. The averaged spectral data from the scans of samples in the Raman spectrophotometer were baseline corrected and normalised against the phenylalanine band at 1003 cm^{-1}.

2.11. Statistics

An analysis of variance (ANOVA) of the data was performed, and a least significant difference (LSD) or Tamhane's with a confidence interval of 95% was used to compare the means (n = 2 or 3).

3. Results and discussion

3.1. Thermal denaturation and/or aggregation

3.1.1. SEC-MALLS analysis

The untreated and heated KPI samples were analysed by SEC-MALLS-RI technique, and typical SEC-RI elution profiles of control, K-30 and K-60 are shown in Fig. 1. In this figure, molar mass distribution profiles of these proteins are also included, which were cal-

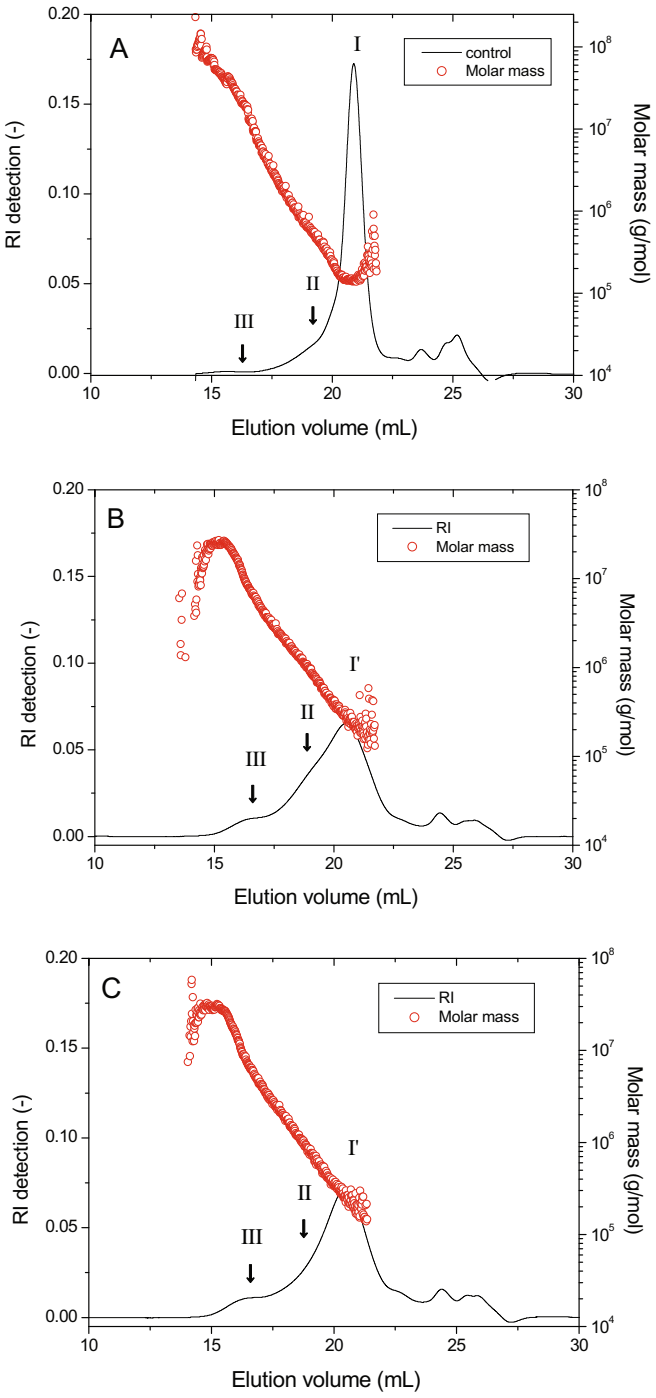

Fig. 1. Typical SEC-RI elution and molar mass distribution profiles of control (A), K-30 (B) and K-60 (C), respectively. Control, K-30 and K-60 represent the KPI samples, obtained by heating at 95 °C for 0, 30 and 60 min, respectively. The numbers (I or I′, II and III) indicate the major peaks of vicilin components.

culated from combined LS (at 90°) and RI signals. In the RI profile of untreated KPI, one major peak (peak I) eluting at 20.6 ml and several minor peaks eluting before or after the major peak were observed (Fig. 1A). The molecular weight (M_w) of this major peak was calculated to be about 161 kDa (within elution volume range of 20–21 ml), clearly attributed to trimeric form (7S) of vicilin. There was a shoulder peak (peak II) eluting at about 17–19 ml, with calculated M_w 9-fold higher than that of peak I. The aggregate peak (peak III) eluting at near void volume (14–15 ml) was minor for the untreated KPI. In this study, the relative content of the vicilin component (corresponding to the RI elution peaks in the range of 0–22 ml) in untreated KPI was calculated to account for about 84% total integrated RI peaks.

The heat treatment changed SEC elution profile of KPI to a varying extent, indicating changes in M_w distribution and relative content of protein constituents (e.g., peaks I, II and III). In the heated KPI samples, the major peak (peak I′) almost eluted at the same volume as peak I in untreated KPI (Fig. 1 B and C). Table 1 summaries percentages of integrated area (from the RI profile) and mean M_w and/or polydispersity of peaks I or I′, II and III for untreated and heated KPI samples. The major peak (peak I) of untreated KPI decreased upon heat treatment, and concomitantly, the aggregate and shoulder peaks (peaks II and III) increased. The percentages of the peaks were similar, except that in the case of K-15, the percentage of peak II was distinctly higher than that of KPI-30 (Table 1). The decrease in percentage of peak II upon further heat treatment is clearly related to formation of higher M_w aggregate (peak III).

The influence of heat treatment on SEC profiles of KPI was clearly different from that observed by Carbonaro, Cappelloni, Nicoli, Lucarini, and Carnovale (1997), in which the major elution peak (7S vicilin) in SEC profile of kidney bean was mainly shifted to higher retention times (with M_w < 83 kDa), after cooking treatment. The difference of the form of protein products subject to heat treatment may account for this difference. The heating at temperatures close to or higher than denaturation temperature (e.g., 95 °C) of vicilin can disrupt the oligomeric structure and denature the monomeric proteins (Carbonaro et al., 1997; Meng & Ma, 2002). In the present study, the unfolded monomeric proteins associated together to form the corresponding aggregates (mainly the vicilin dimers), whilst in the case of Carbonaro et al., the aggregation of unfolded monomeric proteins in the legume beans was largely inhibited, since the proteins (only 20–25%) in beans are evenly dispersed and not easily accessible each other. On the other hand, the SEC profiles of different heated KPI samples were almost the same (Fig. 1 and Table 1), indicating similar extent of heat-induced aggregation. The high charged residues (e.g., aspartic acid and glutaminc acid) of kidney bean protein (Carbonaro et al., 1997) may account for this phenomenon, since the charged residues are mostly located on the surface of the protein molecule, and the increase in electrostatic repulsive force between initially formed aggregates may inhibit further aggregation (Visschers & de Jongh, 2005).

The mean M_w of peak I increased from 2.0×10^5 to $3.5–4.0 \times 10^5$ g/mol by the heat treatment (Table 1). This data suggests that the heat treatment resulted in dimerization of 7S-form vicilin, and the formed 11S-form vicilin had more compact structure (relative to the 7S-form). Whereas, the mean M_w of peaks II and III on the contrary decreased, upon heating (Table 1). For the peak II, the polydispersity (M_w/M_n) of protein constituents was remarkably decreased by the treatment, reflecting increased homogenous M_w distribution of the proteins. The data confirmed that the heat treatment at 95 °C changed the relative content, M_w and polydispersity of protein constituents of KPI.

3.1.2. DSC analysis

The thermal denaturation of vicilin component in untreated and heated KPI samples was analysed by DSC, and extent of protein denaturation obtained by changes in enthalpy (ΔH) of the major endotherm before and after heat treatment. Table 2 shows DSC characteristics of vicilin component in untreated and heated KPI samples. The heat treatment remarkably decreased the ΔH of the endotherm with increasing heating time. Almost 86% protein was denatured in K-15, and the extent of protein denaturation reached 91.4% in the K-30 (Table 2). The decreases in ΔH suggest thermal denaturation of the vicilin component, since partially unfolded protein would require less heat energy (lower enthalpy) to denature (Meng & Ma, 2002).

Unexpectedly, the denaturation temperature (T_d) of the vicilin component gradually decreased with heating time increasing from 0 to 30 min (Table 2), indicating gradual structural unfolding upon

Table 2
DSC characteristics of untreated (control) and heated KPI samples, in 50 mM phosphate buffer (pH 7.0). Each data was the means and standard deviations of three measurements (Tang et al.).

Protein samples	T_d (°C)	ΔH (J/g)	$\Delta T_{1/2}$ (°C)	Extent of denaturation (%)[a]
Control	98.8 ± 0.6^{A}	9.3 ± 0.3^{A}	7.0 ± 0.2	0.0
K-15	98.1 ± 0.1^{AB}	1.3 ± 0.3^{B}	8.7 ± 1.1	86.0
K-30	97.6 ± 0.3^{B}	0.8 ± 0.2^{C}	5.2 ± 0.1	91.4
K-60	–[b]	–[D]	–	100.0
K-120	–	–[D]	–	100.0

Different superscripts (*A–D*) indicate the significant difference at $p < 0.05$ level amongst a same column. For control, K-15, K-30, K-60 and K-120, refer to Table 1.
[a] The extent of denaturation for control was defined as 0%.
[b] The character '-' indicates no thermal transition in the DSC thermogram.

Table 1
Percentages of integrated area (relative to total integrated area of peaks I or I′, II and III), mean M_w and polydispersity (M_w/M_n) of protein constituents of the peaks (I or I′, II and III) for untreated and heated KPI samples (Tang et al.).

KPI samples	Peak I or I′ [a]			Peak II			Peak III		
	Percentage (%) [b]	Mean M_w (g/mol)	Polydispersity (M_w/M_n) [c]	Percentage (%)	Mean M_w (g/mol)	Polydispersity (M_w/M_n)	Percentage (%)	Mean M_w (g/mol)	Polydispersity (M_w/M_n)
Control	91.1	0.20×10^6 (6%)	1.157 (8%)	8.4	1.61×106 (3%)	1.453 (6%)	0.5	42.3×10^6 (8%)	1.518 (10%)
K-15	65.4	0.39×10^6 (8%)	1.224 (16%)	25.7	1.80×10^6 (3%)	1.208 (4%)	8.9	13.3×10^6 (13%)	1.414 (15%)
K-30	65.0	0.36×10^6 (3%)	1.108 (5%)	23.5	1.54×10^6 (1%)	1.189 (1.4%)	11.5	12.5×10^6 (15%)	1.640 (15%)
K-60	65.1	0.35×10^6 (9%)	1.211 (19%)	23.0	1.60×10^6 (4%)	1.209 (6%)	11.9	13.7×10^6 (20%)	1.617 (22%)
K-120	65.4	0.40×10^6 (9%)	1.152 (13%)	23.4	1.73×10^6 (4%)	1.222 (6%)	11.2	15.7×10^6 (27%)	1.558 (30%)

[a] Peaks (I or I′, II and III) are defined as the peaks eluting at 19–22, 17–19 and 14–17 ml, respectively.
[b] Total integrate areas of Peaks I or I′, II and III for one sample was defined as 100%.
[c] Polydispersity of proteins included in the Peaks was defined by relative ratio of calculated weight-average (M_w) molecular weight (g/mol) to number-average (M_n) molecular weight. Control, K-15, K-30, K-60 and K-120 represent the KPI samples, heat-treated at 95 °C for 0, 15, 30, 60 and 120 min, respectively. The values within parentheses indicate standard deviations for calculated mean M_w or other values.

heating. The T_d (97.6 °C) for K-30 was significantly ($p < 0.05$) lower than that of control (98.8 °C). This phenomenon is deviating from the general view that the thermal stability of globulins is improved by thermal treatment, since the preheated globulins may aggregate to form a more compact structure with higher thermal stability and cooperativity (Gorinstein, Zemser, & Paredes-López, 1996; Meng & Ma, 2002). This phenomenon is consistent with the SEC analysis (Fig. 1), further confirming the inhibition of protein aggregation following denaturation, due to electrostatic repulsive interactions.

3.2. Total and exposed free SH contents

Table 3 shows total and exposed free SH group contents of untreated and heated KPI samples. In the untreated KPI (control), there were about 3.8 and 1.9 μmol/g protein for total and exposed free SH contents, respectively. These free SH groups were clearly attributed by non-vicilin components (about 16%) in the KPI, since the vicilin (or phaseolin) is devoid of cysteine residues (Refer to Biological Macromolecule Crystallization Database of NIST, the U.S. Commerce Department's). The heat treatment resulted in gradual and significant decreases in total and exposed free SH contents, with heating time increasing from 0 to 30 min, and further treatment didn't change it any more, except that severest heat treatment (120 min) led to further decrease in exposed free SH content (Table 3). The heat-induced decreases in free SH groups and concomitant increases in disulfide bonds have been confirmed in albumin fractions of kidney bean (Genovese & Lajolo, 1998) and egg white proteins (mainly ovalbumin) (Van der Planken, Van Loey, & Hendrickx, 2006). In the case of egg white proteins, the decrease of total free SH content by heating was attributed to oxidation of the sulfhydryl groups. On the other hand, in the purified globulins from red bean, no marked changes in free SH contents were observed (Meng & Ma, 2002). Thus, the decreases in free SH contents by heating may be largely attributed to formation of disulfide bonds (Visschers & de Jongh, 2005).

3.3. Surface hydrophobicity (H_o)

The H_0 of untreated and heated KPI samples was determined with ANS as the fluorescence probe at neutral pH, as also shown in Table 3. With increasing heating time from 0 to 30 min, the H_0 gradually increased from 1186 to 1518. However, further heating on the contrary gradually decreased the H_0, and the H_0 of K-120 was even significantly lower than that of control. Similar changes in H_0 have been observed in red bean globulin, before and after heating at 90 °C for 0–60 min (Meng & Ma, 2002). Clearly, the exposure of buried hydrophobic residues of globular proteins during thermal denaturation accounts for the increases in H_0. The decreases in H_0 upon further heating might be attributed to hydrophobic interactions of exposed hydrophobic groups to form aggregate with low H_0. The results confirmed heat-induced denaturation and/or protein unfolding, and subsequent rearrangement or re-association of the vicilin component, as suggested by similar extent of protein aggregation in different heated KPI samples (Fig. 1).

3.4. Functional properties

3.4.1. Protein solubility (PS)

Table 3 also shows the PS of untreated (control) and heated KPI samples at neutral pH. Heating led to gradual and significant increases in PS with heating time increasing from 0 to 30 min, whilst the PS gradually and significantly decreased upon further heating from 30 to 120 min ($p < 0.05$). The solubility of a protein is usually affected by its hydrophilicity/hydrophobicity balance, depending on the amino acid composition, particularly at the protein surface (Moure, Sineiro, Domínguez, & Parajó, 2006). Thus, the increases in PS by moderate heating may be attributed to increases in charged residues, initially buried within the molecules, as a result of protein denaturation and/or unfolding. The decreases in PS upon extensive heating are clearly due to formation of aggregates with altered hydrophobicity/hydrophilicity ratio of the surface. The data seems to contradict the result of SEC analysis (Fig. 1), where the integrated areas under the peaks in the RI profiles for different KPI samples, reflecting protein solubility in the eluting buffer were similar (Data not shown). The difference could be largely attributed to the difference in ionic strength (higher ionic strength was applied in the SEC analysis), since the vicilin component in KPI is a kind of salt-soluble proteins.

3.4.2. Emulsifying activities (EAI and ESI)

The EAI and ESI values of untreated and heated KPI samples were determined at neutral pH, as also included in Table 3. The heating treatment led to gradual and significant increases in EAI and ESI of KPI, reaching a maximum at 30 min, and then, the EAI and ESI decreased gradually upon further heating (60–120 min). Generally, the emulsifying activities of proteins are affected by their molar mass, hydrophobicity, conformation stability and charge and physico-chemical factors such as pH, ionic strength and temperature. Solubility also plays an important role, since highly insoluble proteins are not good emulsifiers and can generate coalescence (Moure et al., 2006).

In this study, the pattern of changes in EAI and ESI of KPI upon heating was similar to that of its H_0 and PS, suggesting a close relationship between the emulsifying activities of KPI and its H_0 and/or PS. The improvement of emulsifying properties by heating may be mainly attributed to increased hydrophobic surface and flexibility, as a consequence of thermal denaturation (Raymundo, Franco, Gallegos, Empis, & Sousa, 1998). However, the aggregation or the

Table 3
Free SH contents, surface hydrophobicity (H_0), protein solubility and surface-related functional properties (including emulsifying and foaming activities) of untreated (control) and heated KPI samples. The data were the means and standard deviations of duplicate or triplicate measurements (Tang et al.).

KPI samples	Free SH contents (μmol/g of protein)		H_o	Protein solubility (%)[a]	Emulsifying activities[b]		Foaming activities[c]	
	Total	Exposed			EAI (m^2/g)	ESI (min)	FC (%)	FS (%)
Control	3.85 ± 0.08^{A}	1.94 ± 0.19^{A}	1186 ± 30^{D}	80.0 ± 1.7^{C}	23.7 ± 0.8^{D}	30.9 ± 1.6^{C}	244.9 ± 1.6^{A}	87.8 ± 1.0^{A}
K-15	3.12 ± 0.02^{B}	0.91 ± 0.01^{B}	1358 ± 21^{B}	82.8 ± 1.1^{B}	30.2 ± 1.0^{C}	42.4 ± 2.7^{B}	221.2 ± 2.6^{B}	77.4 ± 0.8^{C}
K-30	1.27 ± 0.02^{C}	0.75 ± 0.01^{C}	1518 ± 43^{A}	87.3 ± 1.1^{A}	41.7 ± 1.5^{A}	55.1 ± 2.9^{A}	139.0 ± 3.4^{C}	80.6 ± 1.2^{B}
K-60	1.21 ± 0.01^{D}	0.70 ± 0.05^{C}	1258 ± 25^{C}	78.5 ± 1.3^{C}	33.6 ± 0.6^{B}	33.0 ± 2.3^{C}	136.7 ± 3.4^{C}	80.5 ± 0.5^{B}
K-120	1.19 ± 0.06^{D}	0.33 ± 1.05^{D}	1092 ± 19^{E}	71.4 ± 2.6^{D}	30.5 ± 0.9^{C}	31.7 ± 1.4^{C}	125.2 ± 4.2^{D}	74.2 ± 5.4^{D}

Different superscripts (*A-E*) on the top of columns indicate significant difference at $p < 0.05$ level amongst a same column. Control, K-15, K-30, K-60 and K-120, refer to Table 1.

[a] PS was determined at pH 7.0.

[b] EAI and ESI: Emulsifying activity index and emulsion stability index, respectively.

[c] FC and FS: Foaming capacity and foam stability, respectively.

decrease in PS of proteins would impair their emulsifying properties. So, the decreases in emulsifying activities upon extensive heating (60–120 min) were largely attributed to the decreases in PS, by the heat-induced aggregation. Of course, the decrease in flexibility as a result of structural rearrangement of unfolded proteins may also to a varying extent account for the decreases in the emulsifying activities.

3.4.3. Foaming activities

The foaming activities (FC and FS) of untreated and heated KPI samples were also included in Table 3. Good foaming proteins must (i) rapidly adsorb during whipping and bubbling, (ii) have a rapid conformational change, rearranging at the air–water interface with reduction of surface tension and (iii) form a viscoelastic cohesive film through intermolecular interactions (Hettiarachchy & Ziegler, 1994). As shown in Table 3, the FC of KPI gradually (and significantly) decreased with heating time. Especially when the heating time was increased from 15 to 30 min, the FC was remarkably decreased by about 37%. In contrast, the FS was slightly affected by the heating. The decreases in FC are clearly due to the heat-induced denaturation and subsequent aggregation, since the aggregation of proteins would impair their ability to adsorb to air–water interface and form a viscoelastic cohesive film. In a previous study of protein isolates from *Phseolus* beans, a good and positive relationship between foam expansion (and surface tension) and hydrophobicity was observed (DiLollo et al., 1993). However, in the present study, the relationship was not observed. This may be because the negative influence of thermal aggregation on the foaming properties of KPI pre-dominates the beneficial effect of the increases in surface hydrophobicity.

3.5. Structural characterisation

3.5.1. Fluorescence spectroscopic analysis

The emission fluorescence spectroscopic technique was used to characterise the conformational changes of KPI, induced by heat treatment. Typical fluorescence emission spectra of ANS (a polarity-sensitive fluorescent probe) upon binding to untreated and heated KPI samples are shown in Fig. 2. At a constant pH (e.g., pH 7.0), ANS binds to exposed hydrophobic surfaces in partially unfolded proteins with much higher affinity than to native or completely unfolded proteins, resulting in an increase in fluorescence emission compared with the emission of free ANS in aqueous solution (Pallarès, Vendrell, Avilès, & Ventura, 2004). As expected, the ANS fluorescence emission spectra upon binding to untreated and heated KPI samples were distinctly affected by the heating. However, the changes in fluorescence intensity and wavelength at maximum fluorescence intensity varied with the time of heating. The heating led to gradual increases in fluorescence intensity, reaching a maximum at 30 min, and it then decreased gradually upon further heating (60–120 min) (Fig. 2). This phenomenon is consistent with

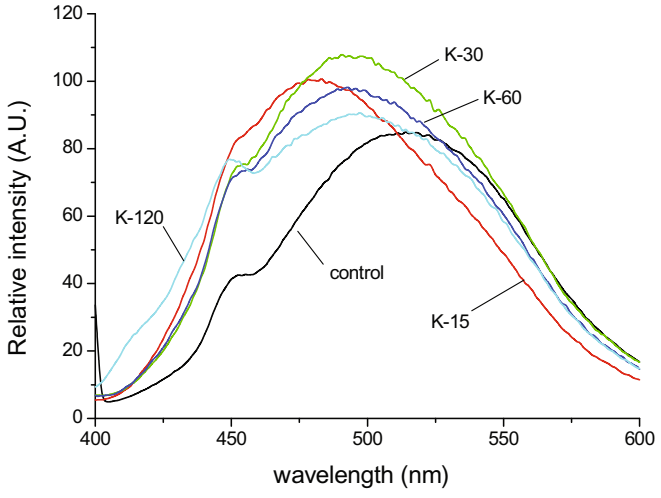

Fig. 2. Typical extrinsic fluorescence spectra of ANS, in the presence of untreated (control) and heated KPI samples. For labels within the figure, refer to Table 1.

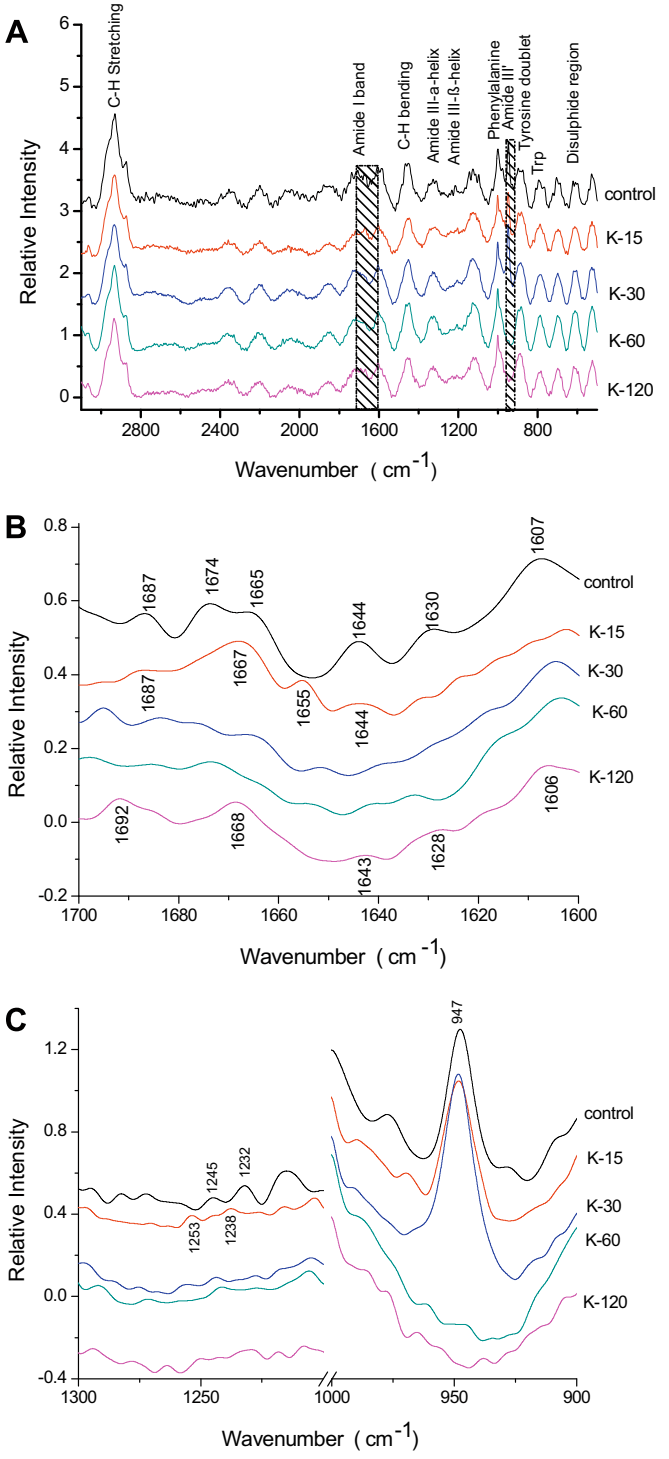

Fig. 3. Stacked FT-Raman spectra of untreated and heated KPI samples (freeze-dried). Panel A: all region (3100–500 cm^{-1}); Panel B: amide I region (1700–1600 cm^{-1}); Panel C: amide III (1300–1200 cm^{-1}) and III′ (1000–900 cm^{-1}) regions. The spectra were baselined and normalised to the phenylalanine peak at 1004 cm^{-1}. For labels within the figure, refer to Table 1.

the H_0 data (Table 3), indicating that a moderate heating resulted in gradual exposure of hydrophobic clusters initially buried in the native proteins, whilst extensive heating subsequently led to structural rearrangement of initially unfolded proteins.

The wavelength at maximum fluorescence intensity remarkably blue-shifted from 516 to 479 nm when KPI was heated for 15 min, whilst further heating (30–120 min) reduced this shift (Fig. 2). The fluorescence spectrum is determined chiefly by the polarity of the environment of the chromophores (e.g., ANS), and the fluorescence maximum suffers a blue shift when chromophores become less exposed to solvent (Pallarès et al., 2004). The data indicated that the moderate heating increased ANS binding to hydrophobic clusters of proteins in KPI, whilst the further heating on the contrary decreased this binding. This is also an indirect evidence for heat-induced unfolding of protein molecules and/or subsequent structural rearrangement of unfolded proteins.

3.5.2. Raman spectroscopic analysis

To further reveal the heat-induced conformational changes of KPI, we analysed Raman spectra of untreated and heated KPI samples, in the freeze-dried powder state, as shown in Fig. 3. Tentative assignment of the major bands in the spectra of untreated KPI is denoted within Fig. 3 A, based on comparison with Raman spectral data reported by previous workers (Li-Chan, 1996; Li-Chan & Nakai, 1991; Ngarize, Herman, Adams, & Howell, 2004). Fig. 3B and C shows Raman spectra of amide I (1700–1600 cm^{-1}), III (1300–1200 cm^{-1}) and III′ (1000–900 cm^{-1}) regions for untreated and heated KPI samples. In the untreated KPI, the amide I and III bands were centred at 1665–1674 cm^{-1} and 1232–1245 cm^{-1}, respectively, indicating that β-sheets and random coils were the major secondary structures (Li-Chan, 1996; Li-Chan & Nakai, 1991; Zhao et al., 2004a). This is in agreement with the data reported by Deshpande and Damodaran (1989), where the secondary structure composition of native phaseolin (the major protein in KPI), measured by circular dichroism spectroscopy, was 50% β-sheets, 31% random coils, 10.5% α-helix, and 8.5% β-turns.

The Raman spectra of amide I and III regions were distinctly affected by the heating, but to varying extents (Fig. 3 B), indicating secondary structural changes. The heating for 15 min led to transformation of amide I bands at 1665 and 1674 cm^{-1} into the predominant band at 1667 cm^{-1}, and the latter is a characteristic Raman band of random coil (Zhao et al., 2004 a). In the amide III profiles, the intensity of bands at 1230–1245 cm^{-1} decreased (Fig. 3 C), further indicating that the heating for 15 min led to transformation of β-sheets to random coils. The amide I bands (e.g., at 1667 and 1655 cm^{-1}) in K-15 were considerably decreased by further heating for more than 30 min (Fig. 3 B). The decreases in band intensity by heating indicated the disruption of secondary structure. In the K-120 case, the random coil band (at 1668 cm^{-1}) became very prominent when compared to K-30 or K-60. This consolidates our previous presumption that the heating led to structural unfolding of the proteins in KPI, whilst the denatured proteins might rearrange and/or re-associate their structure to form a more stable intermediate (e.g., aggregate or oligomers of vicilin) after extensive heating.

Interestingly, a prominent amide III′ band at 947 cm^{-1}, attributed to α-helix (Ngarize et al., 2004) was observed in the untreated KPI (Fig. 3C). The heating up to 30 min distinctly increased the intensity of this band, indicating gradual unfolding of protein, however, this band completely disappeared after heating for above 30 min (Fig. 3C). The disruption of α-helix was conformable to the complete denaturation of the protein (Table 2). On the other hand, taking the emulsifying and foaming activity data (Table 3) together, it is possible to suggest that the maintenance (and even enhancement) of α-helix structure in KPI may be beneficial for its surface-related properties. The decreases in PS by extensive heating (Table 3) seem to be also closely related to the disruption of the α-helix secondary structure. Thus, the band at 947 cm^{-1} in the amide III′ region of Raman spectra could be used as an indication to modulate the some physiochemical and surface-related functional properties of KPI.

4. Conclusions

The heat treatment clearly led to aggregation of proteins in KPI, however, the extent of the aggregation was relatively limited, possibly due to high charged residues on the surface of the proteins. The heat-induced modifications in physiochemical and/or functional and structural properties of vicilin-rich KPI have been confirmed, but the extent of these modifications was closely related to the extent of heating. Moderate heating (e.g., at 95 °C for 15–30 min) greatly improved the protein solubility, emulsifying and foaming abilities, whilst these properties were contrarily decreased by extensive heating (e.g., for 60–120 min). The modifications in functional properties were associated with heat-induced changes in structural conformations of KPI, especially those in secondary structures. The results confirmed close relationships between functional properties of the proteins from kidney beans and their conformational structures.

Acknowledgment

This work is part of the research projects of National Natural Science Foundation of China (serial number: 20876057).

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