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Effects of limited enzymatic hydrolysis with trypsin on the functional properties of hemp (*Cannabis sativa* L.) protein isolate

Shou-Wei Yin [a], Chuan-He Tang [a,*], Jin-Song Cao [a], Er-Kun Hu [b], Qi-Biao Wen [a], Xiao-Quan Yang [a]

[a] *Department of Food Science and Technology, South China University of Technology, Guangzhou 510640, PR China*
[b] *Henan Commercial Science Research Institute Co. Ltd., Zhengzhou 450003, PR China*

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Abstract

Effects of limited enzymatic hydrolysis induced by trypsin on the physicochemical and functional properties of hemp (*Cannabis sativa* L.) protein isolate (HPI) were investigated. The enzymatic hydrolysis was confirmed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and size exclusion chromatography (SEC). SEC and differential scanning calorimetry (DSC) analyses confirmed the presence of aggregates in the corresponding hydrolysates (with the degree of hydrolysis of 2.3–6.7%). Functional properties, including protein solubility (PS), thermal properties, emulsifying and foaming properties, and water holding and fat adsorption capacities (WHC and FAC) were evaluated. The PS was remarkably improved by the limited enzymatic hydrolysis at all tested pH values. However, the enzymatic hydrolysis led to the marked decreases in emulsifying activity index, foaming capacity and foam stability, WHC and FAC. These decreases were to a great extent related to the presence of aggregates in the hydrolysates.

Keywords: Hemp protein isolate (HPI); *Cannabis sativa* L.; Enzymatic hydrolysis; Functional property; Trypsin

1. Introduction

Cannabis sativa L., commonly referred to as hemp, is a widely cultivated plant of industrial importance, as an important source of food, fiber, and medicine. As the by-product during commercial utilization of the valuable fiber, hempseed contains over 30% oil and about 25% protein of high quality. The hempseed oil is rich in polyunsaturated fatty acids (PUFAs), especially linoleic (ω-6) and α-linolenic (ω-3) acids, while the high-quality storage proteins, with superior essential amino acid composition, are easily digested and rich in all essential amino acids (Callaway, 2004). Most of essential amino acids contained in hemp protein are sufficient for the FAO/WHO suggested requirements of infants or children (Tang, Ten, Wang, & Yang, 2006). Hemp proteins mainly consist of edestin (globulin) and albumin. Like the hexamer of soy glycinin, the edestin molecule is also composed of six identical subunits, and each subunit consists of an acidic subunit (AS) and a basic subunit (BS) linked by one disulfide bond (Patel, Cudney, & McPherson, 1994). In the hemp protein isolate (HPI), isolated from defatted hempseed, the edestin approximately accounts for 80% total hemp protein content (Tang et al., 2006). Although HPI has good potential to be applied as a source of protein nutrition, it shows much poorer functional properties, especially protein solubility, as compared to soy protein isolate (SPI) (Tang et al., 2006). The poor functional properties might greatly limit the application of this protein in many food formulations.

Many physical, chemical and enzymatic treatments have been widely applied to modify the functional properties of plant proteins, through changing the protein structure. Usually, the enzymatic modification is more preferable

* Corresponding author. Tel.: +86 20 87114262; fax: +86 20 87114263.
E-mail address: chtang@scut.edu.cn (C.-H. Tang).

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due to milder process conditions required, easier control of the reaction and minimal formation of by-products (Mannheim & Cheryan, 1992). In most enzymatic modification cases, enzymatic hydrolysis has been most widely used to improve the functional properties of proteins, such as solubility, emulsification, gelation, water- and fat- holding capacities, and foaming ability, or to tailor the functionality of certain proteins to meet specific needs (Arzu, Mayorga, Gonzalez, & Rolz, 1972; Kim, Park, & Rhee, 1990; Kuehler & Stine, 1974). However, in some cases, extensive enzymatic modification would on the contrary impair some functional properties of food proteins, or even cause off-flavors in the corresponding hydrolysates. Hence, the degree of hydrolysis (DH) should be controlled to obtain reproducible and optimum peptide size distribution, when the enzymatic hydrolysis is applied to modify the functional properties. To date, limited proteolysis has been used to modify the functional and physicochemical properties of soy protein (Jung, Murphy, & Johnson, 2005; Surówka, Żmudzinski, Fik, Macura, & Lasocha, 2004; Surówka, Żmudziński, & Surówka, 2004), sunflower protein (Martineza, Baeza, Millán, & Pilosof, 2005), rapeseed protein isolates (Vioque, Sanchez-Vioque, Clemente, Pedroche, & Millán, 2002), whey protein (Chobert, Bertrand-Harb, & Nicolas, 1988), oat bran protein concentrate (Guan, Yao, Chen, Shan, & Zhang, 2007), and legumin from faba bean (*Vicia faba*) (Dudek, Horstmann, & Schwenke, 1996). No the literature has been reported about this technique to modify hemp proteins, possibly due to the scarcity of commercial hempseed products. This situation may be changing, since hemp seeds have been obtained at a large scale during the commercial utilization of the valuable hemp fiber.

Trypsin (EC 3.4.21.4) is a member of a large family of serine proteases and cleaves the peptide bond on the carboxyl side of arginine and lysine (Kishimura et al., 2007). Studies on limited hydrolysis using trypsin to improve the properties of proteins have been carried out extensively (Barac, Jovanovic, Stanojevic, & Pesic, 2006; Giardina, Pelizzola, Avalli, Iametti, & Cattaneo, 2004; Guan et al., 2007; Henning, Mothes, Dudek, Krause, & Schwenke, 1997; Mutilangi, Panyam, & Kilara, 1996). On the whole, the protein solubility is one of the most outstanding functional properties that are improved by the limited hydrolysis. In some plant protein cases, the emulsifying and foaming properties are also improved, while in other cases, these properties are impaired. In fact, the modification by limited enzymatic hydrolysis is largely dependent upon the nature of protein and the DH. For example, Guan et al. (2007) showed that the enzymatic hydrolysis in a DH-dependent mode increased the solubility, water-holding capacity, emulsifying activity and foaming ability of oat bran protein concentrate. However, Henning et al. (1997) pointed out that the hydrolysis with a rather low DH led to the best emulsifying activity of legume while extensive hydrolysis on the contrary impaired the emulsifying properties.

It is well known that hemp proteins are a kind of easily digested proteins. Therefore, it will be a good choice to apply trypsin to modify the properties of HPI. Thus, one main objective of the present work was to investigate the effects of limited enzymatic hydrolysis by trypsin on some physicochemical and functional properties of HPI, including protein solubility, emulsifying and foaming properties, water holding and fat adsorption capacities, and thermal properties. The effects of thermal treatment on the enzymatic hydrolysis and properties of HPI were also evaluated.

2. Materials and methods

2.1. Materials

Defatted hempseed protein meal, a byproduct during the commercial utilization of the valuable hempseed oil and fiber, was kindly supplied by YUNNAN Industry Hemp Co. Ltd. (China). This meal had been obtained on a large scale from hemp (*Cannabis sativa* L.) seeds, through de-hulling, disintegrating and de-fatting with supercritical liquid (CO_2) at low temperatures (less than 40 °C). The denaturation extent of the protein components in this meal can be considered to be low, since all the steps were carried out at a temperature of less than 35 °C (except the disintegrating process). Trypsin powder (from porcine pancreas; catalog no. T4799, 1000–5000 BAEE units/mg solid) and Folin and Ciocalteu's Phenol Reagent (F-9252) were from sigma (St. Louis. MO, USA). All other chemicals used in the present study were of analytical or better grade.

2.2. Preparation of hemp protein isolate (HPI)

HPI was produced from the defatted hempseed meal according to Tang et al. (2006), with a slight modification. Defatted hempseed meal was dispersed in deionized water (1:20, w/v), and the pH of the dispersion was adjusted to 10.0 with 2 N NaOH. The resultant dispersion was gently stirred at 37 °C for 2 h, then centrifuged at 8000*g* at 20 °C for 30 min in a CR22G high-speed centrifuge (Hitachi Co., Japan). The pellet was discarded, and the supernatant was adjusted to pH 5.0 with 2 N HCl and then centrifuged at 5000*g* (Hitachi Co., Japan) at 20 °C for 20 min. The obtained precipitate was re-dispersed in deionized water. The dispersion was homogenized and adjusted to pH 7.0 with 2 N NaOH, then followed by freeze-drying to produce HPI product. The protein content of obtained HPI was 91.2% (determined by Kjeldahl method, $N \times 6.25$, wet basis).

2.3. Thermal pretreatment

To investigate the heat treatment on the enzymatic hydrolysis of HPI by trypsin, the HPI dispersions were prepared and thermally pretreated as follows. The 2% (w/v) HPI dispersions in deionized water were adjusted to pH

8.0 (25 °C) using 1 N NaOH. These dispersions in thin containers were incubated in a bath preset to the temperatures (75, 80 or 90 °C) for 10 min. After this treatment, these dispersions were immediately cooled in an ice bath to room temperature, to obtain the preheated HPI dispersions.

2.4. Enzymatic hydrolysis of HPI and preparation of its hydrolysates

The untreated and heat-pretreated HPI dispersions (2%, w/v) were adjusted to pH 8.0 with 1 N NaOH and incubated at 37 °C for about 10 min. Then, the enzyme trypsin was added at an enzyme to substrate ratio of 1:200 (w/w). The mixtures of protein and enzyme were incubated at 37 °C to start the enzymatic hydrolysis reaction. The classical pH-stat technique was used, and the pH was maintained at 8.0 by the addition of 0.5 N NaOH. DH was used as an indication of enzymatic hydrolysis (DH was determined as the following section).

To prepare the hydrolysates of heat-untreated HPI, the enzymatic hydrolysis was stopped by heating the mixtures (of enzyme and protein) in a 95 °C water bath for 10 min, at selected periods of incubation time. The DH was used to characterize the hydrolysates, obtained by pH-stat analysis technique for the enzymatic hydrolysis. After the thermal treatment, the obtained hydrolysates were freeze-dried and then preserved at −18 °C before use.

The properties of the hydrolysates may be affected by the additional thermal treatment (at 95 °C) used to inactivate the activity of trypsin. Furthermore, the endogenous protease enzymes may also induce the hydrolysis (autolysis). Thus, the HPI dispersion was treated as the same procedure to prepare the hydrolysates, with an exception that no enzyme was added. In the present study, this HPI treated by the thermal treatment at 95 °C was marked as the control.

2.5. Determination of DH

DH is defined as the ratio of the number of peptide bonds cleaved (number of free amino groups formed during proteolysis) expressed as hydrolysis equivalents (h), in relation to the total number of peptide bonds before hydrolysis (h_{total}). The h_{total} is equivalent to the amino acid composition of the protein, calculated from amino acid analysis by summing the mmoles of each individual amino acid per gram of protein (Adler-Nissen, 1986). The DH for enzymatic hydrolysis was measured by the pH-stat method (Adler-Nissen, 1986). The percent DH was calculated according to the following equation:

$$\mathrm{DH}\,(\%) = \frac{B \cdot N_{\mathrm{b}}}{\alpha \cdot M_{\mathrm{p}} \cdot h_{\mathrm{tot}}} \times 100,$$

where B is the base consumption in mL, N_{b} the normality of the base, M_{p} the mass of protein being hydrolyzed (g), and h_{tot} the total number of peptide bonds in the protein substrate (meqv/g protein). From previous amino acid composition analysis (Tang et al., 2006), h_{tot} was calculated to be 7.39 meqv/g for HPI.

The $1/\alpha$ is the calibration factor for pH-stat, and also the reciprocal of the degree of dissociation of the α-NH_2 groups. The α was calculated as follows:

$$\alpha = \frac{10^{(\mathrm{pH}-\mathrm{p}K)}}{1 + 10^{(\mathrm{pH}-\mathrm{p}K)}},$$

where pK is the average dissociation value for the α-amino groups, calculated according to the Gibbs–Helmholz equation (Adler-Nissen, 1986). At 37 °C as in the present study, the average dissociation value pK can be calculated to be 7.4.

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a discontinuous buffered system according to the method of Laemmli (1970), using 12% separating gel (pH 8.6) and 4% stacking gel (pH 6.7). The protein samples were solubilized in 0.125 M Tris–HCl buffer, containing 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol (2-ME), 5% (v/v) glycerol and 0.025% (w/v) bromophenol blue, and heated for 5 min in boiling water before electrophoresis. After the electrophoresis, the gel was dyed in 0.25% Coomassie blue (R-250) in the 50% trichloroacetic acid and then destained in methanol solution containing acetic acid [methanol:acetic acid:water, 227:37:236 (v/v/v)].

2.7. Size exclusion chromatography (SEC)

SEC experiments were carried out on a 2.5 × 90 cm column of Sepharose CL-6B (Pharmacia Co., Uppsala, Sweden). The column was equilibrated and eluted with 50 mM phosphate buffer (pH 7.6) containing 0.2 M NaCl. A 5-mL portion of 2% (w/v) protein dispersions was applied to the column and eluted at an elution rate of 1 mL per minute. Fractions (3 mL per tube) were collected. The UV absorbance of the collected fractions was measured at 280 nm.

2.8. Protein solubility (PS)

An aqueous solution (1%, w/v) of protein samples was stirred magnetically for 30 min, and then with either 0.5 N HCl or 0.5 N NaOH, the pH of the solutions was adjusted to the desired values. After 30 min of stirring, the pH was readjusted if necessary. Then it was centrifuged at 8000g 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 the standard. The PS was expressed as grams of soluble protein/100 g of sample. All determinations were conducted in duplicate.

2.9. Differential scanning calorimetry (DSC)

The thermal transition of HPI and its hydrolysates was determined using a TA Q100-DSC thermal analyzer (TA Instruments, New Castle, DE), according to the procedure of Meng and Ma (2001), with some modifications. Approximately 2.0–3.0 mg protein samples were accurately weighed into aluminum liquid pans, and 10 μL 50 mM phosphate buffer (pH 7.0) was added. The pans were hermetically sealed and heated from 20 to 120 °C at a rate of 5 °C/min. 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 endothermic peak ($\Delta T_{1/2}$) were computed from the thermograms by the universal analyzer 2000, version 4.1D (TA Instrument-Waters LLC, USA). All experiments were conducted in triplicate.

2.10. Emulsifying properties

Emulsifying activity index (EAI) and emulsion stability index (ESI) of the samples were determined according to the method of Pearce and Kinsella (1978), with minor modifications made by Tang, Yang, Chen, Wu, and Peng (2005). For the emulsion formation, 6 mL of 0.2% HPI dispersion (in deionized water adjusted to pH 3.0, 5.0, 7.0 and 9.0 with 1 N NaOH or HCl) and 2 mL of corn oil were homogenized in FJ-200 High-Speed Homogenizer (Shanghai Specimen Model Co., China) for one minute at the maximum velocity. Fifty micro-liters of emulsion was taken from the bottom of the homogenized-emulsion, immediately (0 min) or 10 min after homogenization, and diluted (1:100, v/v) in 0.1% (w/v) SDS solution. After shaking in vortex mixer for a moment (about 5 s), the absorbance of diluted emulsions was read at 500 nm in the spectrophotometer. EAI and ESI values were calculated by the following equations:

$$\text{EAI } (\text{m}^2/\text{g}) = \frac{2 \times 2.303 \times A_0 \times DF}{c \times \phi \times (1-\theta) \times 10,000}$$

$$\text{ESI } (\text{min}) = \frac{A_0}{A_0 - A_{10}} \times 10,$$

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 the diluted emulsions at 0 and 10 min. Measurements were performed in triplicate.

2.11. 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 homogenized with an FJ-200 high-speed homogenizer (Shanghai Co., China) at 10,000 rpm for 2 min. FC was calculated as the percent increase in volume of the protein dispersion upon mixing, while FS was estimated as the percentage of foam remaining after 30 min.

2.12. Water holding and fat absorption capacities (WHC and FAC)

WHC was determined according to the centrifugation method described by Cobb and Hyder (1972) with a slight modification. Duplicate samples (500 mg) were rehydrated with 10 mL of deionized water in centrifuge tubes and dispersed with a vortex mixer for 1 min. The dispersion was allowed to stand at room temperature for 3 h, and then centrifuged at 3000g for 30 min. The difference between the initial volume of deionized water added to the protein sample and the volume of the supernatant was determined, and the results were reported as mL of water absorbed per gram of protein sample. Each sample was determined in triplicate.

FAC was measured as the volume of edible oil held by 500 mg of materials, as described by Haque and Mozaffar (1992) with a slight modification. Each powder sample was added to 10 mL of commercial corn oil (JIALI group Co. Ltd., China) in a 15 mL plastic centrifuge tube, and mixed for 1 min in a vortex mixer. The dispersion was allowed to stand at room temperature for 30 min, then centrifuged at 3000g for 30 min at 25 °C. The volume of oil separated from the hydrolysates was measured and FAC was calculated as mL of oil absorbed per gram of protein sample. Each sample was determined in triplicate.

2.13. 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% or 99% was used to compare means.

3. Results and discussion

3.1. DH change during enzymatic hydrolysis

The trypsin-induced hydrolysis of HPI, with or without heat pretreatment (at 75, 80 and 90 °C for 10 min), was monitored using the pH-stat technique for up to 8 h by DH, as shown in Fig. 1. Interestingly, the hydrolysis of non-treated (raw) HPI by trypsin approximately consisted of two distinct digestion processes, that is, initial digestion process and further digestion process (Fig. 1). In the initial digestion process (0–300 min), the DH profile with time is similar to those of classic protease-induced hydrolysis of protein (Gbogouri, Linder, Fanni, & Parmentier, 2004; Kim et al., 1990). Upon further incubation for more than 300 min, the enzymatic hydrolysis reaction rate increased evidently, deviating from the original curve (at the same rate) (Fig. 1). The DH increase during last incubation may be related to the conversion of insoluble components to soluble ones, by the action of trypsin.

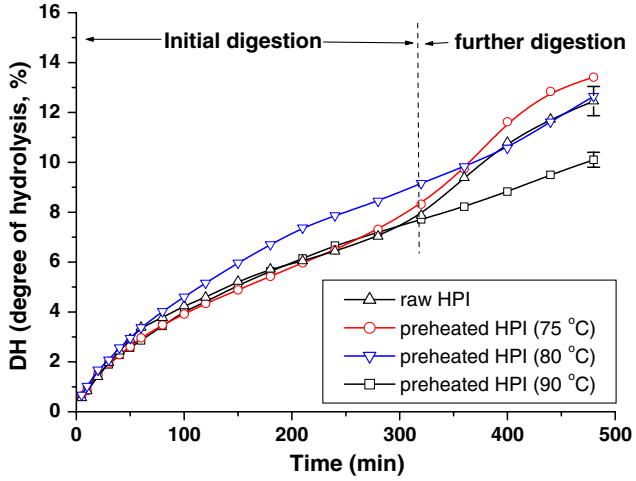

Fig. 1. DH changes during the trypsin-induced hydrolysis of raw and preheated HPIs as a function of incubation time. The protein concentration of HPI was 2% (w/v), and the ratio of enzyme to protein substrate was 1:200 (w/w).

These two processes of enzymatic hydrolysis were to a various extent affected by the thermal pretreatment. The initial digestion process was nearly unaffected by the pretreatment at 75 °C, while in the further process, the DH increased slightly higher than that of raw HPI (Fig. 1). The heat pretreatment at 80 °C increased the hydrolysis reaction rate during initial digestion, but the increase in hydrolysis rate during late digestion as untreated case was not observed. The further pretreatment at 90 °C resulted in almost complete inhibition of the increase in hydrolysis rate. Furthermore, DH at the end of hydrolysis (8 h) of raw HPI was significantly ($P < 0.05$) decreased by this pretreatment (Fig. 1). In usual, protein denaturation may be beneficial for the hydrolysis of protein by protease, while the protein aggregation may inhibit the hydrolysis reaction (due to the masking of catalytic sites by aggregation). Hence, the data reflect the relative contribution of aggregation and thermal denaturation by heating, since the heat pretreatment might lead to thermal denaturation and subsequent aggregation.

The DH profiles with hydrolysis time for various HPIs are well fitted with the Boltzmann model as follows:

$$\mathrm{DH} = \frac{A_1 - A_2}{1 + \mathrm{e}^{(t-t_0)/\mathrm{d}t}} + A_2,$$

where DH and t the degree of hydrolysis and incubation time (min), and A_1, A_2, t_0 and dt are various parameters for the Boltzmann model (as listed in Table 1). The regression coefficient (R^2) for the fitting in raw HPI case was 0.978, while that of the heat pretreated HPIs was in the range of 0.988–0.996. Thus, the Boltzmann model can be well applied to describe the hydrolysis process of hemp proteins.

3.2. SDS-PAGE and SEC analyses

Various HPI hydrolysates of raw HPI were prepared at some specific DH values. To prepare these hydrolysates, heat treatment at 95 °C for 10 min was used to inactivate the enzyme. Fig. 2 shows SDS-PAGE profiles of raw HPI and its hydrolysates in the presence of 2-ME. The heat treatment led to the formation of some aggregates, which appeared on the top of separating gel (Fig. 2, lane 2). After enzymatic hydrolysis by trypsin, both AS and BS of edestin were gradually decreased with DH increasing from 2.3 to 6.7% (Fig. 2, lanes 3–5). By comparison, BS was less susceptible to trypsin than AS. According to the structure

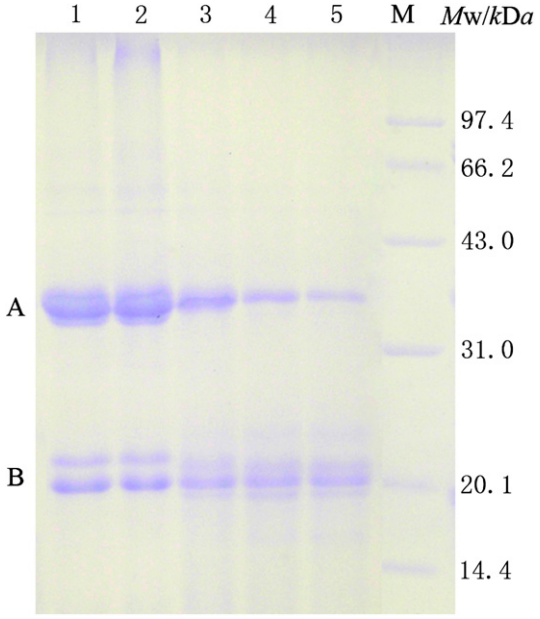

Fig. 2. SDS-PAGE profiles of raw HPI and its hydrolysates. Lane 1 represents raw HPI (untreated); lane 2 represents HPI heat-treated without the addition of trypsin (control); lane 3–5 indicate the hydrolysates with 2.3% DH, 4.5% DH and 6.7% DH, respectively; lane M represents the protein molecular weight marker. A and B indicate acidic and basic subunits of edestin.

Table 1
Parameters of Boltzmann model fitted for trypsin-induced hydrolysis of hemp proteins

Protein samples	Regression coefficient (R^2)	Parameters for Boltzmann model			
		A_1	A_2	t_0	dt
Raw HPI (untreated)	0.978	−530.5	32.0	−3030.6	1065.5
Pretreated HPI (75 °C)	0.988	−17.4	93.0	1178.6	740.8
Pretreated HPI (80 °C)	0.993	−439.1	16.9	−1330.9	400.9
Pretreated HPI (90 °C)	0.996	−491.2	12.6	−1204.4	338.7

model proposed by Plietz, Zirwer, Schlesier, Gast, and Damaschun (1984), AS is mainly located at the exterior of the protein molecules, while BS is usually buried in the interior. Thus, the susceptibility difference of BS and AS may be attributed to the availability difference of both subunits to the catalytic sites of trypsin. Similar results have been obtained in other plant 11 S globulins (Dudek et al., 1996; Kamata & Shibasaki, 1978; Plumb & Lambert, 1990; Schwenke, 2001).

We also investigated the SEC elution profiles of raw HPI and its hydrolysates (Fig. 3), using 50 mM phosphate buffer (pH 7.6) as the eluting solvent. In this buffer, only soluble protein components were solubilized. Thus, besides the molecular size distribution, the elution profile may also reflect the solubility of proteins. In the SEC profile of raw HPI, there were two major elution peaks (peak I and peak II) eluting at about 150 and 240 mL, indicating the presence of two kinds of protein components with different molecular weight distribution, respectively. As expected, the heat treatment led to remarkable decrease of peak I as observed in raw HPI (Fig. 3). The decrease was clearly attributed to the formation of insoluble aggregates or precipitates, induced by heat. The enzymatic hydrolysis with DH 2.3% not only remarkably increased the content of peak II, but also led to the appearance of soluble aggregate peak which eluting at the void volume (about 70–75 mL) (Fig. 3). This observation suggested that the protein solubility of HPI was remarkably improved by the hydrolysis, possibly due to the transformation of insoluble protein components to soluble ones (e.g., soluble protein aggregate). Compared to the SEC profile of the hydrolysate (DH 2.3%), further hydrolysis with DH 4.5–6.7% resulted in obvious decreases of the contents of soluble aggregates and peak I. These decreases can be attributed to the formation of more soluble peptides by more extensive hydrolysis, as evidenced by the shift of peak II to higher elution volume and the increase in the area of peak II (Fig. 3).

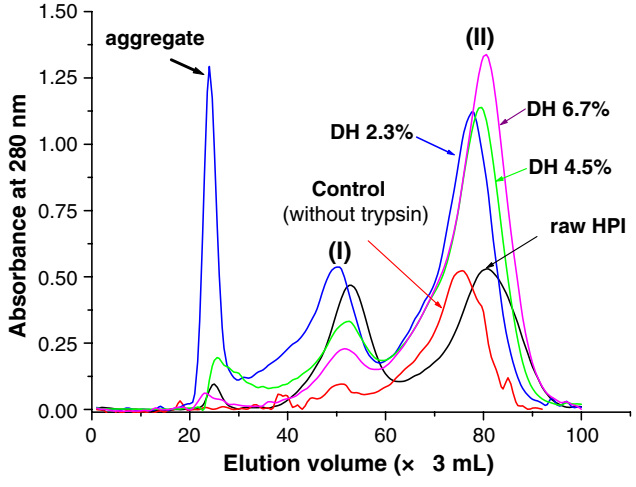

Fig. 3. SEC elution profiles of raw HPI and its hydrolysates (DH 2.3%, 4.5% and 6.7%, respectively). The protein samples were carried out on Sepharose CL-6B column (2.5 × 90 cm), eluted with 50 mM phosphate buffer (pH 7.6) containing 0.2 NaCl.

3.3. Functional properties

3.3.1. Protein solubility (PS)

Solubility is one of the most important functional properties of protein, since it can affect other functional properties, e.g., surface-active properties and rheological or hydrodynamic properties. In many protein-based formulations, for instance, emulsions, foams and gels, good solubility for the protein is usually required. Fig. 4 shows PS profiles of HPI and its hydrolysates obtained at DHs 2.3%, 4.5% and 6.7%, respectively, as a function of pH. The PS of raw HPI (untreated) was minimum at pH 5.0–6.0 and increased gradually below pH 5.0 and above 6.0 (Fig. 4). The PS profile of HPI at below pH 7.0 is consistent with our previous report, however, the PS at above pH 8.0 is much less (Tang et al., 2006). The PS difference at above pH 8.0 may be attributed to the difference of the pH-adjustment method. In this paper, we directly adjusted the pH of the protein solutions to some specific values, using 0.5 N NaOH or HCl, while in our previous paper, we used the buffers with specific pH values, to determine the protein solubility. The heat-treated HPI (control) had much significantly lower PS at below pH 4.0 and above pH 7.0, as compared to raw HPI ($P < 0.05$; Fig. 4). This data is consistent with the SEC data (Fig. 3), indicating the formation of insoluble aggregates, after the heat treatment.

Enzymatic hydrolysis (resulting in DH 2.3–6.7%) remarkably improved the PS of HPI at all tested pH values (pH 3–10) (Fig. 4). At above pH 7.0, the PS of various hydrolysates was about 85–86%, significantly higher than that of raw HPI ($P < 0.05$). The hydrolysates with DH of 2.3–6.7% showed different pattern of PS profile with pH, relative to raw HPI. The PS of the hydrolysates was minimal at pH 4.0–6.0, but increased suddenly as the pH changed to 3.0 or 7.0. At above pH 7.0, there was insignificant difference at $P < 0.05$ level among different pH values for various hydrolysates (with 2.3–6.7% DH). On the whole,

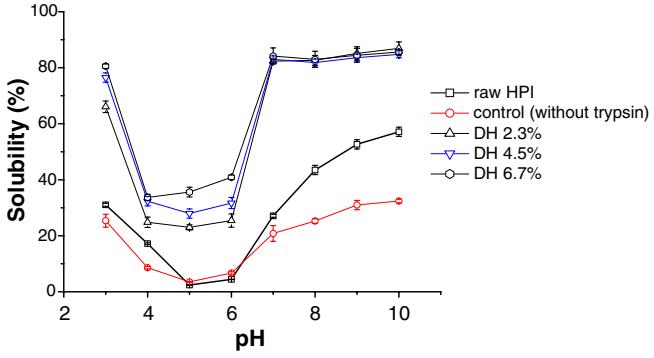

Fig. 4. Protein solubility profiles of HPI and its hydrolysates as a function of pH. Each value is the mean and standard deviation of duplicate measurements.

the PS at below pH 6.0 (especially at pH in the range of 4.0–6.0) increased with DH increasing from 2.3 to 6.7% (Fig. 4). The improvement in PS by the enzymatic hydrolysis, consistent with the SEC analyses, have been attributed to the release of soluble peptides from insoluble aggregates or precipitates and increased number of exposed ionizable amino and carboxyl groups (Panyam & Kilara, 1996). Thus, limited enzymatic hydrolysis could be used as an effective technique to improve the PS of hemp proteins.

3.3.2. Thermal properties

The thermal transition of raw HPI and its hydrolysates was investigated by DSC (Fig. 5), and the related DSC characteristics are listed in Table 2. Raw HPI and its hydrolysates exhibited different DSC patterns. Raw HPI presented a typical endothermic peak with thermal denaturation temperature (T_d) of about 95 °C (Fig. 5). This peak clearly corresponded to the edestin component, especially its hexamer form (Tang et al., 2006). The heat-treated HPI (control) had no detectable peaks in the DSC profile (data not shown), indicating complete denaturation of edestin after heat treatment at 95 °C for 10 min. In the hydrolysates (with 2.3–6.7% DH), two endothermic peaks with higher T_d than that of raw HPI were observed. Similar results have been obtained for the 11 S globulins of soy proteins treated by enzymatic hydrolysis (Feng, 2002). The T_d of major endothermic peak significantly ($P < 0.05$) increased from 95.5 to 97.3–97.8 °C when DH increased from 2.3% to 4.5 or 6.7%, while the T_d of minor endotherm was unrelated with the DH change (Table 2 and Fig. 5). Interestingly, the signal of the minor endotherm gradually increased with increasing DH from 2.3 to 6.7%. The presence of soluble and insoluble aggregates in hydrolysates, as shown in Fig. 3, may account for these two endothermic events.

The enthalpy change (ΔH) significantly decreased with DH increasing from 0 to 6.7% ($P < 0.05$; Table 2). The ΔH represents the extent of ordered structure of a protein

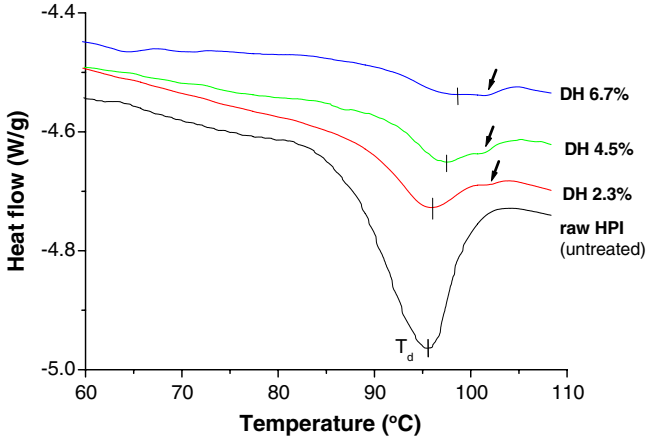

Fig. 5. Typical DSC profiles of raw HPI and its hydrolysates. The samples were dispersed in 0.05 M phosphate buffer (pH 7.0), and heated at a rate of 5 °C/min from 20 to 120 °C. The arrows within the figure indicate the appearance of minor endothermic peak.

Table 2
DSC characteristics of raw HPI and its hydrolysates in 0.05 M phosphate buffer (pH 7.0)

	T_d (°C)	ΔH (J g^{-1})	$\Delta T_{1/2}$ (°C)
Raw HPI (untreated)	95.1 ± 0.31^b	11.9 ± 0.85^a	8.1 ± 0.59^a
Hydrolysate (DH 2.3%)	95.5 ± 0.20^b	4.5 ± 0.30^b	6.7 ± 0.25^b
Hydrolysate (DH 4.5%)	97.3 ± 0.30^a	3.2 ± 0.15^c	7.9 ± 0.36^a
Hydrolysate (DH 6.7%)	97.8 ± 0.60^a	1.6 ± 0.30^d	8.7 ± 0.46^a

Means ± standard deviations of triplicate analyses are given.
T_d, thermal denaturation temperature of the endotherm (or the major endotherm); ΔH, total enthalpy changes of two endothermic peaks; $\Delta T_{1/2}$, width at half peak height of the major endothermic peak. Superscript letters (a and b) indicate significant ($P < 0.05$) difference within the same column.

(Arntfield & Murray, 1981). Thus, the data suggest that the extent of the ordered structure of HPI be gradually decreased by increasing DH from 0 to 6.7%. Furthermore, the width at half peak height of endothermic peak ($\Delta T_{1/2}$), indicative of the cooperativity of the thermal transition, was nearly unaffected by the enzymatic hydrolysis (Table 2).

3.3.3. Emulsifying properties

The EAI and ESI of raw HPI and its hydrolysates as a function of pH are shown in Fig. 6. All tested samples showed similar EAI profiles with pH. All the samples showed minimal EAI values at pH 5.0, and highest EAI values at pH 7.0 or 9.0. The thermal treatment significantly

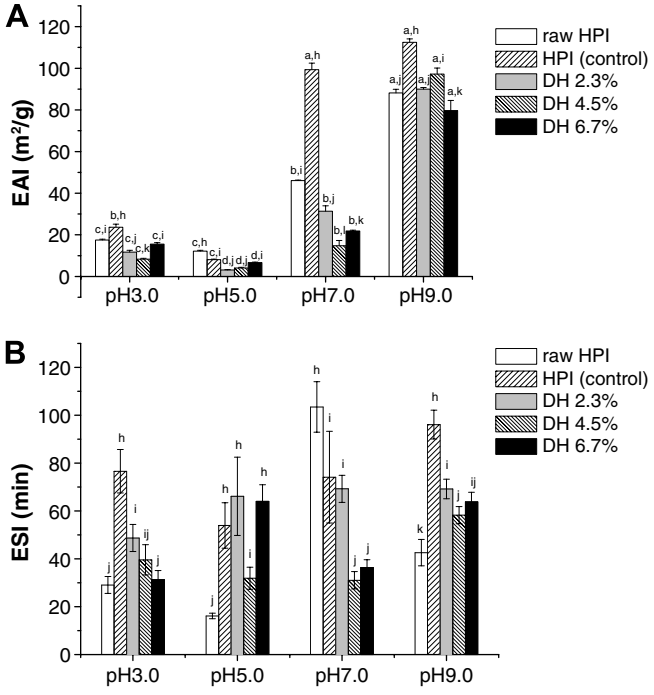

Fig. 6. Emulsifying activity index (A) and emulsion activity index (B) of HPI and its hydrolysates at pHs 3, 5, 7 and 9. Results are means and standard deviations of triplicate determinations. Different characters (*a–d*) on the top of columns within the figures represent the significance at $P < 0.05$ among various pH values, while different characters (*h–l*) indicate the significance at $P < 0.05$ among various samples at a specific pH value.

($P < 0.05$) increased the EAI values of HPI at pH values deviating from the isoelectric point (around pH 5.0) (Fig. 6A). This is consistent with the fact that heat treatment might lead to the exposure of hydrophobic groups initially buried in the interior of protein molecules, since proteins with high hydrophobicity would have higher EAI values (as compared to those with low hydrophobicity).

On the whole, the enzymatic hydrolysis led to significant declines in EAI at neutral and acidic pH values, relative to the control ($P < 0.05$). The influence of the hydrolysis on the EAI at pH 7.0 was more remarkable than at pHs 3 and 5. This might be mainly attributed to the difference in PS at these pH values. However, the influence of DH on the EAI was dependent upon the pH at which the EAI was measured. For example, at pHs 3 and 7, the hydrolysates with DH 4.5% had lowest EAI as compared to other DHs, while at pH 5.0, the lowest EAI was at DH 2.3–4.5%. At pH 9.0, only high DH values resulted in significant decline in EAI ($P < 0.05$). Generally, the hydrolysates are surface-active materials and can stabilize the oil-in-water emulsions because of their exposed hydrophilic and hydrophobic groups. Of course, the formation of the aggregates in the hydrolysates might inhibit the formation of a viscoelastic membrane on the oil-in-water surface, thus increasing the oil droplets coalescence (Gbogouri et al., 2004). Thus, the data is consistent with the SEC and DSC data (Figs. 3 and 5), further indicating the presence of aggregates.

The ESI data of the pH-dependence for various samples are inconsistent (Fig. 6B). The thermal treatment significantly ($P < 0.05$) increased the ESI values at tested acidic and alkaline pHs, while decreased the ESI at pH 7.0. Similarly, the enzymatic hydrolysis significantly ($P < 0.05$) increased the ESI values at the acidic and alkaline pHs, while at pH 7.0, the ESI was on the contrary significantly ($P < 0.05$) decreased by the hydrolysis. The reason for these phenomena is yet unknown.

3.3.4. Foaming properties

The FC and FS were determined at pH 7.0. Table 3 shows the effects of thermal treatment and enzymatic hydrolysis (with different DHs) on the FC and FS of HPI. The heat treatment did not affect FC of HPI ($P > 0.05$), while significantly increased FS ($P < 0.05$; Table 3). Townsend and Nakai (1983) had indicated that many factors mainly contributing to foaming capacity are viscosity, surface hydrophobicity, and solubility in descending order. Thus, no significant change in FC by thermal treatment may result from both increased hydrophobicity and decreased protein solubility.

Enzymatic hydrolysis with DH 2.3–6.7% led to significant decreases in FC and FS ($P < 0.05$; Table 3). The basic requirement for a protein to be a good foaming agent is the ability to rapidly adsorb at the air–water interface during bubbling, undergo rapid conformation change and rearrangement at the interface, and form a cohesive viscoelastic film via intermolecular interaction. Thus, the decreases in chain length of peptides as a result of enzymatic hydrolysis may mainly account for the decreases in FC and FS. The result is consistent with the general view of point that the larger the molecular size for a protein, the higher foaming stability (Damodaran, 1997).

Table 3
Foaming capability (FC) and foam stability (FS) of HPI and its hydrolysates

Samples	FC (%)	FS (%)
Raw HPI	150.0 ± 3.2^{a}	58.8 ± 0.57^{b}
Heat-treated HPI (control)	148.3 ± 15.3^{a}	63.6 ± 2.07^{a}
Hydrolysate (DH 2.3%)	133.3 ± 15.3^{b}	44.6 ± 0.76^{c}
Hydrolysate (DH 4.5%)	112.5 ± 17.7^{b}	47.0 ± 3.57^{c}
Hydrolysate (DH 6.7%)	122.5 ± 3.5^{b}	30.3 ± 0.47^{d}

All data were expressed as the means and standard deviations of triplicate. Different superscript characters (a–d) indicate the significant difference at $P < 0.05$ within the same column.

3.3.5. Water holding and fat adsorption capacities (WHC and FAC)

Fig. 7 shows WHC and FAC of raw HPI and its hydrolysates with DH 2.3–6.7%. The WHC of HPI was slightly

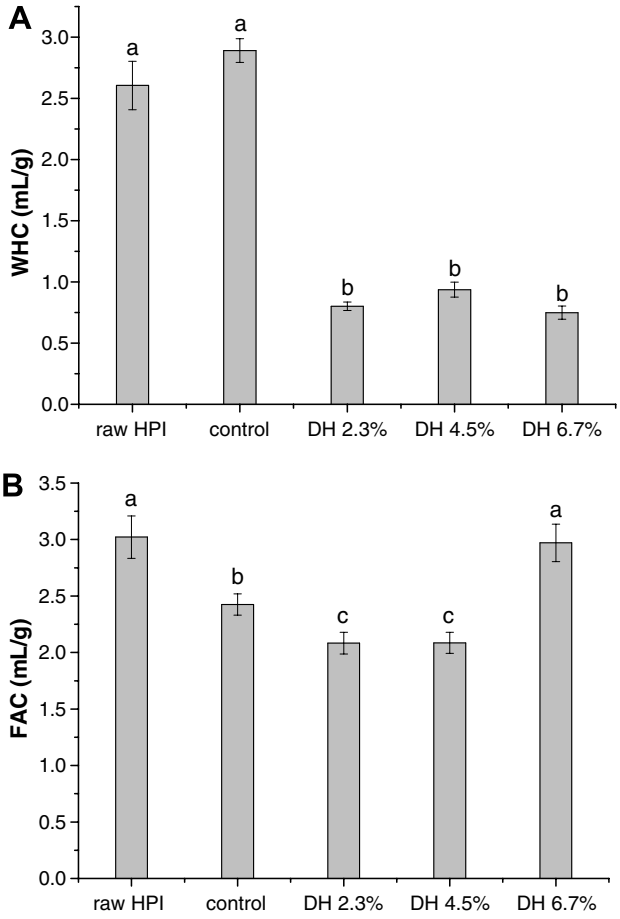

Fig. 7. WHC and FAC of HPI and its hydrolysates. Each value is the mean and standard deviation of triplicate. Different characters on the top of columns represent significant difference at $P < 0.05$.

but insignificantly increased by the thermal treatment. This may be attributed to the increase of functional groups (especially hydrophilic groups) as a result of heat treatment. The enzymatic hydrolysis with DH 2.3–6.7% led to significant decreases in both WHC ($P < 0.05$), however, in the tested DH range, the WHC of the hydrolysates was independent of DH (Fig. 7A). A contrary result of the influence of trypsin-induced hydrolysis on the WHC of oat bran protein concentrate was observed (Guan et al., 2007). In this case, the improvement of WHC was attributed to the increase in accessibility of non-protein components in the samples to the surrounding water (e.g. fibres, starches, etc.) by removal of protein. In the present study, the decrease of hydrophilic groups present in the exterior of hemp protein components (especially their insoluble aggregates) may account for the decrease in WHC, since the PS of HPI was remarkably improved by the enzymatic hydrolysis (Fig. 4).

The thermal treatment resulted in significant decrease in FAC ($P < 0.05$; Fig. 7B). This may be attributed to the formation of aggregates with higher molecular mass, in which more hydrophobic groups were buried in the interior as compared to those protein components of raw HPI. The enzymatic hydrolysis with DH 2.3–4.5% significantly decreased the FAC, while further hydrolysis with 6.7% on the contrary resulted in remarkable increase (Fig. 7B). A gradual decrease in FAC with increasing DH was observed in trypsin-modified oat or oat bran proteins (Guan et al., 2007; Ma, 1985). The mechanism of fat absorption has been attributed mostly to physical entrapment of oil (Kinsella, 1976; Wang & Kinsella, 1976). This is consistent with the view of Bernardi Don, Pilosof, and Bartholomai (1991) that protein seems to be in the form of network, and the disruption of network would decrease the protein absorption capability. Thus, the decrease in FAC by the enzymatic hydrolysis could be attributed to the disruption of protein network. The abnormal increase in FAC in the case of DH 6.7% is not a conflicting phenomenon, since the appearance of aggregates in the hydrolysates (as evidenced by Figs. 3 and 5) could re-strengthen the protein network.

4. Conclusion

The functional properties of HPI were remarkably affected by the trypsin-induced enzymatic hydrolysis with low levels of DH (2.3–6.7%). The thermal pretreatment at different temperatures resulted in different DH change patterns of enzymatic hydrolysis. The poor PS was remarkably improved by the enzymatic hydrolysis, especially at above pH 7.0. However, the enzymatic hydrolysis decreased the surface-related activities (including emulsifying and foaming properties), WHC and FAC. Further investigations, e.g., enzymatic hydrolysis combined with transglutaminase treatment or Maillard reaction with polysaccharide, should be carried out to achieve the improvement in protein solubility and other functional properties.

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