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Enzymatic hydrolysis of hemp (*Cannabis sativa* L.) protein isolate by various proteases and antioxidant properties of the resulting hydrolysates

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ABSTRACT

Enzymatic hydrolysis of hemp protein isolate (HPI) by six proteases (alcalase, flavourzyme, neutrase, protamex, pepsin and trypsin) and antioxidant activities of the resulting hydrolysates, obtained for 2 and 4 h were investigated. The yield of trichloroacetic acid (TCA)-soluble peptides (Y_{sp}), protein composition and surface hydrophobicity (H_o) of the hydrolysates were evaluated. The results showed that the hydrolysates exhibited varying DPPH radical scavenging (with lowest IC_{50}, ~2.3 mg/mL) and Fe^{2++} chelating (with lowest IC_{50} of 1.6–1.7 mg/mL) abilities and reducing power (with highest absorbance at 700 nm of 0.31–0.35), depending on their Y_{sp} and H_o values. The DPPH radical scavenging and Fe^{2++} chelating abilities of the hydrolysates were positively correlated with their Y_{sp} or H_o values. The results suggest that enzymatic hydrolysis can be used as an effective technique to produce high value-added products of hemp proteins.

1. Introduction

The seed of *Cannabis sativa* L., commonly referred to 'hempseed', is an excellent source of nutrition (Callaway, 2004). Hempseed has been documented as a source of food throughout recorded history – raw, cooked or roasted, and its oil has been used as a food/medicine for at least 3000 years (Callaway, 2004). The hemp milk obtained from the crashed seed meal is a popular traditional drink in South China. The seed usually contains over 30% oil and about 25% protein of high quality. The hempseed oil is rich in polyunsaturated fatty acid, especially linoleic (ω-6) and α-linolenic (ω-3) acids, while the storage protein is superior in essential amino acid composition. 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; Wang, Tang, Yang, & Guo, 2008). Hemp storage proteins mainly consist of edestin (legumin) and albumin. Like the hexamer of soy glycinin, the edestin molecule is also composed of six identical subunits, and each consists of an acidic subunit and a basic subunit linked by one disulphide bond (Patel, Cudney, & McPherson, 1994). In our previous paper, it was confirmed that hemp protein (especially the edestin component) is a kind of easily digested proteins, which is highly suitable for human consumption (Wang et al., 2008).

However, hemp protein isolate (HPI), isolated from defatted hempseed flour by alkali solubilisation/acid-precipitation, showed much poorer functional properties (especially protein solubility) relative to soy protein isolate (Tang et al., 2006). The protein solubility of HPI could be effectively improved by limited enzymatic hydrolysis with trypsin, but other surface-related functional properties on the contrary decreased (Yin et al., 2008). In this work, the decreases were largely attributed to formation of aggregates in the hydrolysates, obtained by limited hydrolysis. Apart from the functional properties, potential health effects (e.g., antioxidant activities) of hemp protein hydrolysates obtained by enzymatic hydrolysis have not investigated. The investigation for these health effects will expand the use of this valuable protein or its hydrolysates as a food material.

To date, the antioxidant activities of enzymatic hydrolysates from animal and plant food proteins, including bovine caseins and whey proteins (Pihlanto, 2006), soy proteins (Chen, Muramoto, & Yamauchi, 1995; Chen, Muramoto, Yamauchi, & Kenshiro, 1998; Moure, Domínguez, & Parajó, 2006), wheat protein (Zhu, Zhou, & Qian, 2006), chickpea protein (Li, Jiang, Zhang, Mu, & Liu, 2008), porcine haemoglobin, collagen and myofibrillar protein (Chang, Wu, & Chiang, 2007; Li, Chen, Wang, Ji, & Wu, 2007; Saiga, Tanabe, & Nishimura, 2003), and fish proteins (Kim, Je, & Kim, 2007; Rajapakse, Mendis, Byun, & Kim, 2005), have been widely investigated using many *in vitro* antioxidant evaluation systems (water-soluble and oil-soluble). The antioxidant properties of these hydrolysates, largely depending on protease specificity, degree of hydrolysis (DH) and nature of released peptides (e.g., molecular weight and

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amino acid composition), have been attributed to cooperative or combined effects of a number of properties, including their ability to scavenge free radicals, to act as chelating agents of metal ions, or act as hydrogen donator.

Thus, the main aim of this study was to evaluate antioxidant potential of hemp protein hydrolysates, obtained by six proteases after various periods of hydrolysis time, using selected *in vitro* antioxidant evaluation systems. The process of enzymatic hydrolysis and some physicochemical properties of the resulting hydrolysates were analysed. Furthermore, possible relationships between antioxidant activities of the hydrolysates and the yield of trichloroacetic acid (TCA)-soluble peptide or H_o were also evaluated.

2. Materials and methods

2.1. Materials

Defatted hemp meal was kindly supplied by YUNNAN Industrial Hemp Co., Ltd. (China). Hemp protein isolate (HPI) was prepared from this meal, according to the same process as described by Tang et al. (2006). The protein content of this protein was about 87% (wet basis). Alcalase 2.4 L FG (2.4 Au/g; alkaline protease), flavourzyme (1.5 Au/g; exopeptidase), neutrase 1.5MG (1.5 Au/g; neutral protease) and protamex (1.5 Au/g; combined proteases) were kindly supplied by Guangzhou Office (China) of Novo Co. (Novo Nordisk, Bagsvaerd, Denmark). Pepsin (catalogue number: P7000, 1:10,000, 600–1000 units/mg; acidic protease) and trypsin (an alkaline protease from porcine pancreas; catalogue number T4799, 1000–5000 BAEE units/mg) were purchased from Sigma Chemical Co. (St. Louis, USA). Low molecular weight protein markers were purchased from Shanghai DINGUO Biotech. Co., Ltd. (China). All the chemicals were of analytical or better grade.

2.2. Enzymatic hydrolysis of HPI and/or preparation of the hydrolysates

Ten grams of freeze-dried HPI were dispersed in 200 mL of deionised water at room temperature. The dispersions were pre-incubated at optimal catalytic temperatures of individual proteases, prior to adjusting the pH of the dispersions to required values. The hydrolysis by various proteases was carried out according to the following reaction conditions: (1) *alcalase:* at pH 8.5 and 55 °C; (2) *flavourzyme:* at pH 7.0 and 50 °C; (3) *neutrase:* at pH 7.0 and 55 °C; (4) *protamex:* at pH 7.0 and 50 °C; (5) pepsin: at pH 1.5 and 37 °C; and (6) trypsin: at pH 7.0 and 37 °C. The enzyme-to-substrate (E/S) ratio for all cases was 5% on the weight basis. The mixtures of protein and enzyme were incubated in a temperature-controlled water bath. The pH of the mixtures was kept constant during hydrolysis, by addition of 1.0 mol/L NaOH or HCl. At the end of the reaction, the mixtures were stopped by heat treatment in boiling water for 10 min, and cooled immediately in ice water to room temperature. The pH of the cooled enzyme digests were adjusted with 1.0 mol/L NaOH, or 1.0 mol/L HCl to 7.0. The digests were centrifuged at 4000*g* for 20 min to remove insoluble residues. The supernatants were then lyophilised to produce the hydrolysates, which were stored at −20 °C before further analysis.

2.3. Determination of yield of TCA-soluble peptide (Y_{sp})

The Y_{sp} was determined from peptide solubility of enzyme-digested hydrolysates in 10% (w/v) TCA solution. Aliquots (10 mL) of enzyme digests of hemp protein, obtained after hydrolysis for 0, 30, 60, 90, 120 and 240 min, respectively, were fully mixed with same volumes of 20% (w/v) TCA solution. The mixtures were placed at room temperature for 10 min, and centrifuged at 3000*g* for 20 min. The nitrogen contents of the supernatants were determined by conventional Kjeldahl method ($N \times 6.25$) (AOAC, 1990). The Y_{sp} was defined as: Y_{sp} (%) = $(N_2 - N_1) \times 100/(N_0 - N_1)$, where N_0 is total nitrogen content in undigested HPI (g), N_1 and N_2 TCA-soluble nitrogen contents (g) of undigested HPI and enzyme-treated digests, respectively.

2.4. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE experiments were performed on a discontinuous buffered system according to the method of Laemmli (1970) using 12% separating gel and 4% stacking gel. The hydrolysates obtained at various periods of hydrolysis time were directly mixed with 4-fold volume of 0.0125 mol/L tris–HCl buffer containing 1% (w/v) SDS, 2% (v/v) 2-mercaptoethanol, 5% (v/v) glycerol and 0.025% (w/v) bromophenol blue. The samples were then heated for 5 min in boiling water before electrophoresis. Every sample (10 μL) was applied to each lane. The gel was stained with 0.25% Coomassie brilliant blue (R-250) in 50% methanol, and destained in 7% acetic acid in methanolic solution (50%, v/v).

2.5. Size exclusion chromatography (SEC)

The SEC experiment was performed using a Sephadex G-50 column (2.0 × 100 cm) at room temperature. The hydrolysate samples (2 mL) at a concentration of 50 mg/mL were applied to the column, eluted with 50 mmol/L phosphate buffer (pH 7.0), at a rate of 1 mL/min. The effluent was collected at 3 mL per tube. The UV absorbance at 280 nm of the collected effluent was recorded.

2.6. Surface aromatic hydrophobicity (H_o)

H_o was determined with the hydrophobicity fluorescence probe 1-anilino-8-naphtalene-sulphonate (ANS) according to Alizadeh-Pasdar and Li-Chan (2000). Serial dilutions in 0.01 mol/L phosphate buffer (pH 7.0) were prepared with the hydrolysates to a final concentration of 0.005–0.2% (w/w). ANS solution (8.0 mmol/L) was also prepared in the same phosphate buffer. Twenty microlitres of ANS solution was added to 4 mL of each dilution and fluorescence intensity (FI) of the mixture was measured at 365 nm (excitation) and 484 nm (emission) using F4500 fluorescence-spectrophotometre (Hitachi Co., Japan). The initial slope of the FI versus protein concentration (mg/mL) plot (calculated by linear regression analysis) was used as an index of surface aromatic hydrophobicity (H_o).

2.7. 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH radical scavenging activity was determined by the method described by Shimada, Fujikawa, Yahara, and Nakamura (1992). Two millilitres of the sample solution with various solid concentrations were fully mixed with 2 mL of 2.0×10^{-4} mol/L DPPH methanolic solution (freshly prepared). The resulting solution was then left to stand for 30 min, prior to being spectrophotometrically measured at 517 nm. A low absorbance at 517 nm indicates a high DPPH scavenging activity. The methanol was used as the blank. The DPPH scavenging activity as a percentage is calculated by [1 − (test sample absorbance/blank sample absorbance)] × 100.

2.8. Reducing power

The reducing power of the hydrolysates was evaluated by the method developed by Oyaizu (1986), with slight modifications. The sample solution (10 mL) was mixed with 2.5 mL of phosphate