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Zinc-binding behavior of hemp protein hydrolysates: Soluble versus insoluble zinc-peptide complexes

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ABSTRACT

Proteins and peptides when forming complexes with zinc can increase zinc bioavailability. Such complexation was investigated on hemp protein hydrolysates (HPHs) in the present study using Pepsin, Alcalase, Flavourzyme, Papain, Protamex, and Trypsin. Two solubility fractions of Zn^{2+}–HPH complexes, i.e., P_1 (water-insoluble large peptides) and P_2 (water-soluble small peptides, precipitable by ethanol), were collected. The FTIR analysis on Pepsin-HPH suggested that P_1 and P_2 peptides had different Zn^{2+}-binding sites where N—H and C═O were the primary sites in P_1 and P_2, respectively. Although the Zn^{2+}-binding capacity (P_1 and P_2 combined) of HPHs was lower than that of nonhydrolyzed hemp protein, the P_2-bound Zn^{2+} was more abundant in HPHs (up to 63.4%) than in nonhydrlyzed protein (29.6%). Isothermal titration calorimetry corroborated with Zn^{2+}-binding capacity for different HPH samples. Peptides produced with Flavourzyme had the highest Zn^{2+}-binding activity (88.8%) while those with Pepsin exhibited the maximum solubility.

1. Introduction

Zinc, the second most abundant inorganic micronutrient found in the human body (Cummings & Kovacic, 2009), is a crucial component within numerous metalloenzymes (Udechukwu, Downey, & Udenigwe, 2018). Moreover, it provides structural integrity to proteins such as transcription factors and hormones (McCall, Huang, & Fierke, 2000). Zinc plays important roles in cell growth and differentiation, protein and DNA synthesis, lipid metabolism, and immune functions (Hambidge, 2000; Ranasinghe et al., 2015). Zinc deficiency can lead to serious health problems, including growth retardation, cognitive impairment, testicular hypofunction, immune dysfunctions, neurological dysfunctions, increased oxidative stress, and increased generation of inflammatory cytokines (Bonaventura, Benedetti, Albarède, & Miossec, 2015; Chasapis, Loutsidou, Spiliopoulou, & Stefanidou, 2012).

Zinc is quite mobile from soils and consequently available to plants (Bielicka-Gieldon, Rylko, & Zamojc, 2013). As such, it can be easily incorporated into biogeochemical circulation. Regardless of the source (nuts, seeds, leafy vegetables, animal products, etc.) or form, the bioavailability of zinc depends on its intestinal absorption, which in turn is affected by its release from heterogeneous food matrices (Udechukwu et al., 2018). Some dietary components such as dietary fibers, tannins, and phytate can form insoluble complexes with Zn^{2+}, which render the metal unavailable for intestinal absorption (Baye, Guyot, & Mouquet-Rivier, 2017; Kumar, Sinha, Makkar, & Becker, 2010). Zinc supplements such as inorganic zinc salts have been incorporated into food products to prevent zinc deficiency. However, zinc salts can be unstable and cause gastrointestinal tract irritation, making them unsuitable for long-term intake (Akbar, et al., 2013).

On the other hand, food-derived peptides can serve as potential Zn^{2+} carriers in the diet due to their Zn^{2+}-binding capacity (Udechukwu, Collins, & Udenigwe, 2016). Zinc is better absorbed from Zn^{2+}–peptide complexes than from inorganic zinc salts (Udechukwu et al., 2016) or Zn^{2+}–protein complexes (Wang, Zhou, Tong, & Mao, 2011). Zn^{2+}-binding protein hydrolysates and peptides have been obtained from different food protein sources, including milk (Miquel & Farré, 2007; Udechukwu et al., 2018; Wang et al., 2011), chickpea (Torres-Fuentes, Alaiz, & Vioque, 2011), sesame (Wang, Li, & Ao, 2012), oyster (Chen, et al., 2013), silver carp (Jiang, Wang, Li, Wang, & Luo, 2014), rapeseed (Xie et al., 2015), wheat germ (Zhu, Wang, & Guo, 2015), and walnut (Liao et al., 2016).

Hemp seed protein has drawn increased commercial attention in recent years due to its purported nutritional value. Hemp protein consists mainly of globulin (edestin) and albumin and has a high digestibility and a well-balanced amino acid composition (Girgih, Udenigwe,

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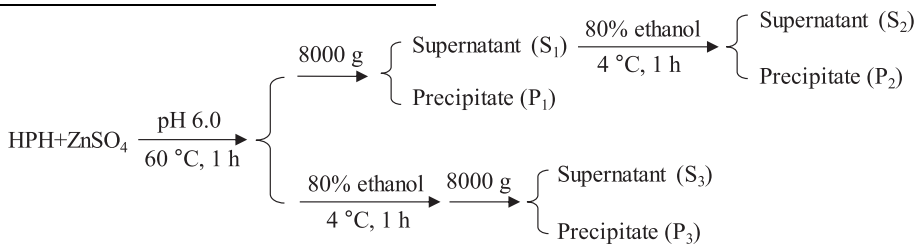
& Aluko, 2011). The nutritional value of hemp protein has been reported to be comparable to egg white and soybean proteins (Wang, Tang, Yang, & Gao, 2008). Thus, it has great potential to be applied as a valuable food ingredient and nutritional supplementary agent. Numerous studies have indicated that hemp protein is a good source for protein hydrolysates and peptides with substantial ability to scavenge free radicals, chelate metal ions, reduce linoleic acid oxidation, and inhibit ACE activity (Girgih et al., 2011; Orio et al., 2017). However, no study has been conducted on the binding of Zn^{2+} by hemp protein hydrolysate (HPH) as a possible means to improve the zinc bioavailability as well as expand hemp protein utilization.

In most Zn^{2+}–peptide complex studies, peptides in an aqueous solution are incubated with Zn^{2+} at certain pH, temperature and time conditions; the complexes are subsequently separated through different methods. Udechukwu et al. (2018) and Wang et al. (2011) obtained Zn^{2+}–YCH (yak casein hydrolysate) and Zn^{2+}–WPH (whey protein hydrolysate) complexes through dialyzing the resulting solutions with a semipermeable membrane to remove free Zn^{2+}. Wang, Li, Wang, and Xie (2015) and Xie et al. (2015) recovered the Zn^{2+}–peptides complex by collecting the precipitate after centrifugation at f g. Liao et al. (2016) precipitated Zn^{2+}–walnut peptide complexes in 75% ethanol (1:3, v/v) at 4 °C. Zn^{2+}-binding capacity, calculated based on the zinc content in the complex, may vary depending on the specific separation methods. On the other hand, zinc solubility has a strong correlation with zinc bioavailability. It has been reported that metal-chelating peptides increase mineral bioavailability through maintaining the mineral in a soluble form or increasing its absorption in carrier-mediated processes (Miquel & Farré, 2007).



The objective of the present study was to establish an analytical procedure for the determination of Zn^{2+}-binding capacity of HPHs and to characterize the chemical nature of insoluble versus soluble Zn^{2+}–peptide complexes. Our ultimate goal is to develop technologies for enhancing zinc bioavailability using HPH as the carrier agent.

2. Materials and methods

2.1. Materials

Hemp protein isolate (HPI) was prepared from raw hemp seeds which were obtained from Yunnan Industrial Hemp Co., Ltd. (Yunnan, China). Hemp seeds were milled and defatted with n-hexane (1:3, w/v) three times. Protein was extracted from the defatted flour dispersed in 15-fold volume (v/w) of water at pH 10.0 and then precipitated by adjusting the pH to 4.5 as described by Tang, Ten, Wang, and Yang (2006). The isoelectric precipitate was suspended in deionized water, adjusted to pH 7.0, then lyophilized. Alcalase, Flavourzyme, and Protamex were purchased from Novozymes North America, Inc. (Franklinton, NC, USA), while Papain, and Trypsin were obtained from Sigma Chemicals (St. Louis, MO, USA). All chemicals of at least a reagent grade were purchased from Sigma Chemicals or Fisher Scientific (Pittsburgh, PA, USA).

2.2. Preparation of HPHs

HPI (20 mg/mL) was hydrolyzed for 1 h with the following enzymes at their respective optimum conditions: Alcalase (pH 8.5, 55 °C), Flavourzyme (pH 7.0, 55 °C), Papain (pH 7.0, 55 °C), Protamex (pH 7.0, 55 °C), Pepsin (pH 2.0, 37 °C), or Trypsin (pH 8.0, 37 °C). The enzyme to HPI substrate ratio was 1:100. After incubation, the enzyme was immediately inactivated by heating in an 85 °C water bath for 10 min. The chilled hydrolysates were brought back to pH 7.0 with 1 N NaOH or 1 N HCl followed by centrifugation at 8000*g* for 10 min. The supernatants were lyophilized and stored at 4 °C for subsequent analysis.

2.3. Zn^{2+}–HPH binding

Zn^{2+}–HPH complexes were prepared and separated according to the method described by Jiang et al. (2014) with some modifications as shown in the following flow chart. HPHs (7.5 mg/mL) were incubated with 9 mM zinc sulfate ($ZnSO_4$) at pH 6.0 and 60 °C for 1 h. After rapidly chilling in an ice slurry, the incubates were centrifuged at 8000*g* for 15 min to obtain the supernatant (S_1) and precipitate (P_1). S_1 was mixed with ethanol (1:4, v/v), allowed to stand at 4 °C for 1 h, then centrifuged at 8000*g* for 15 min to yield the supernatant (S_2) and precipitate (P_2). For comparison, a 4-fold volume of ethanol was added directly to the incubates after reaction followed by centrifugation at 8000*g* for 15 min to separate the supernatant phase (S_3) and precipitate phase (P_3). Hence, in terms of protein recovery, these procedures yielded the following results: $S_1 = S_2 + P_2$; and $P_3 = P_1 + P_2$.

2.4. Determination of Zn^{2+}-binding capacity

To measure the zinc content, samples were first ashed in a Isotemp® Programmable Muffle Furnace (Fisher Scientific, Hampton, NH, USA) at 525 °C for 8 h. The zinc content in P_1, P_2, P_3 and the total zinc content added were measured by an AAnalyst 200 Atomic absorption spectrometer (PerkinElmer Inc., Waltham, MA, USA). The Zn^{2+}-binding capacity was defined as the bound zinc content (in P_1 or P_2) divided by the total zinc added.

2.5. Fourier transform infrared spectroscopy (FTIR)

The stretching or bending of chemical bonds in HPHs due to Zn^{2+} binding was characterized by a Nicolet Nexus 670 Fourier transform infrared spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The spectra were recorded in the wavenumber region from 600 cm^{-1} to 4000 cm^{-1} at a resolution of 4 cm^{-1} as an average of 64 scans. Significant shifts in absorption bands were used to deduce the possible Zn^{2+}-binding sites.

2.6. Electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed with a 4% acrylamide stacking gel and a 5–20% acrylamide gradient resolving gel. Protein samples (4 mg/mL) were dissolved in an equal volume of SDS–PAGE sample buffer (4% SDS, 20% glycerol, 0.125 M Tris buffer, pH 6.8). Aliquots of 60 μg of protein per lane were loaded onto the acrylamide gel. After electrophoresis, protein bands were detected through staining either with 1% Coomassie Blue R-250 (in 50% methanol and 6.8% acetic acid) or with silver ($AgNO_3$) as described by Swain and Ross (1995).