

Processing, Nutrition, and Functionality of Hempseed Protein: A Review

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Abstract: The increasing demand for plant proteins, along with rising awareness of the nutritional and functional roles of dietary proteins, has prompted the nutrition and food industry to explore nontraditional protein sources. Hempseed protein with its excellent nutritional value and superior digestibility has drawn great interest in both scientific and industrial fields. In this review, the chemistry and nutritional quality of hempseed protein and the health benefits of bioactive peptides derived thereof are highlighted. Processing, functionality, and application of various hemp protein products, including hempseed meal, hemp protein concentrates, and hemp protein isolates, are discussed. Moreover, recent advances in technological innovation to improve hempseed protein functionality are introduced. The objective of this review is to provide an updated summary of the research on hempseed protein and to fill the knowledge gap in the exploitation of potential nutritional, health, and processing benefits of this emerging protein source.

Keywords: functionality, health, hempseed protein, nutrition, peptides

Introduction

Hemp (*Cannabis sativa* L.), an annual herbaceous plant that belongs to the Cannabinaceae family, has been an important source of food, fiber, medicine, and a psychoactive/religious drug (Cromack, 1998). Historically, the cultivation of hemp has been limited due to the presence of the psychoactive compound tetrahydrocannabinol (THC). Since 1990, dozens of countries have authorized the licensed growth and processing of the hemp cultivars with substantially reduced levels (<0.3%) of THC (Cherney & Small, 2016). Canada, Australia, Austria, China, Great Britain, France, and Spain are among the most important agricultural producers of hempseeds (Rodriguez-Leyva & Pierce, 2010). In recent years, some states in the United States, including North Dakota and Kentucky, have also passed legislation approving its production.

Hempseeds, a by-product obtained after the commercial utilization of fiber, are achieving growing popularity as an excellent source of nutrients. Whole hempseeds contain 25% to 35% oil, 20% to 25% protein, 20% to 30% carbohydrates, 10% to 15% insoluble fibers, and vitamins and minerals such as phosphorus, potassium, magnesium, sulfur, calcium, iron, and zinc (Callaway, 2004; House, Neufeld, & Leson, 2010). After removal of the hull, the edible portion of the seeds contains, on average, 46.7% oil and 35.9% protein. The concentration of antinutritional compounds, such as phytic acid, condensed tannins, and trypsin inhibitors, is very low in hempseeds (Russo & Reggiani, 2015). The oil extracted from hempseeds is rich in polyunsaturated fatty acids,

especially linoleic (ω -6) and α -linolenic (ω -3) acids with a desirable ratio between 2:1 and 3:1 for optimal health (Callaway, 2004; Porto, Decorti, & Natolino, 2015). The by-product (hemp cake or meal) after oil extraction is abundant in high-quality storage proteins. Hempseed protein has been well known for its excellent digestibility and desirable essential amino acid composition (Tang, Ten, Wang, & Yang, 2006; Wang, Tang, Yang, & Gao, 2008). The arginine content in hempseeds, at 12%, is also remarkably high. A recent proteomic characterization of hempseed concluded that hempseed is an underexploited nonlegume, protein-rich seed (Aiello et al., 2016).

Because of the high nutritional value, hempseed protein has drawn increasing attention in scientific research, and this is well reflected in the progressive increase in the number of scientific publications related to the term “hemp protein” in the title, abstract, and keywords of the publications (Figure 1). Of particular interest is the purported health benefit of bioactive peptides prepared from hemp protein as well as its technological functionality, such as foaming, emulsifying, gelling, and film-forming capabilities. On the other hand, in the food industry, a wide range of products have been developed from hempseed proteins, for example, beverages, functional ingredients, nutritional supplements, and various personal-care products. The value and application of hemp protein in food products are closely related to the protein structure and functional properties.

This article provides a comprehensive review of available research on the structure and chemical composition of hempseed proteins, processes for hemp protein extraction, functional properties, and their potential use in food matrices. Moreover, the quality and potential use of hempseed protein for bioactive peptide preparations are discussed. Recent advances in improving functional properties of hemp proteins are also highlighted.

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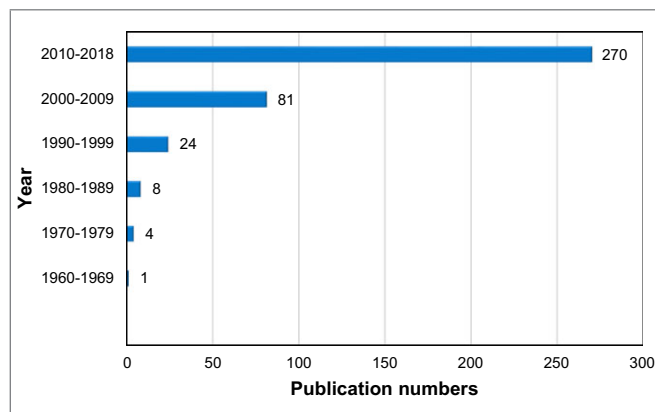


Figure 1—Number of publications reported in the Scopus system containing the term "hemp protein" in the title, abstract, and keywords within the years 1960 to 2018 (as of November 17, 2018).

Major Hempseed Proteins

Protein fractions

Hempseed protein consists mainly of globulin (edestin) and albumin. Edestin accounts for approximately 60% to 80% of the total protein content (Odani & Odani, 1998; Tang et al., 2006), while albumin constitutes the rest.

Edestin. The globular edestin is located inside the aleurone grains as large crystalline substructures (Angelo, Yatsu, & Altschul, 1968). Using crystallographic techniques, edestin is shown to have a structure similar to that of the hexamer of soy glycinin; it is composed of six identical subunits, each consisting of an acidic (AS) and a basic (BS) subunit linked by one disulfide bond (Patel, Cudney, & McPherson, 1994). The molecular weight (MW) of edestin is estimated to be approximately 300 kDa (Wang et al., 2008). The AS is approximately 34.0 kDa and relatively homogeneous, while BS consists mainly of two subunits of about 20.0 and 18.0 kDa (Figure 2).

A fine characterization of edestin (globulin) from a Korean variety has been reported by Kim and Lee (2011). The authors isolated the edestin protein and analyzed the N-terminal amino acid sequence of the first seven and six amino acid residues of the AS and BS, respectively, by the automated Edman degradation method. The seven amino acid residues in AS had a sequence of Ile-Ser-Arg-Ser-Ala-Val-Tyr in the N-terminus, while two constituents of BS showed an identical N-terminus of Gly-Leu-Glu-Glu-Thr-Phe. The 11S-rich and 7S-rich hemp protein isolates (HPIs) have been prepared by Wang et al. (2008) using a similar extraction method for 7S and 11S fractions of soy protein isolate (SPI). SDS-PAGE analysis indicates that the main component in HPI-11S is edestin, and the BS of edestin, along with a subunit of about 4.8 kDa, makes up the HPI-7S. Further analysis shows that the 7S polypeptide has no thermal transition, while the 11S protein exhibits a similar denaturation temperature as HPI at 91.9 °C, indicating the HPI thermal property was due mainly to the 11S component (Wang et al., 2008).

Docimo, Caruso, Ponzoni, Mattana, and Galasso (2014) isolated seven cDNAs encoding for edestin, suggesting that they were divergent forms of two edestin types (CsEde1 and CsEde2) based on the sequence similarity. Both edestin types exhibit high amounts of arginine (11% to 12%), but CsEde2 is particularly rich in methionine (2.36%), which is even higher than the methionine-rich 2S albumin (8 Met) isolated from hempseed (Odani & Odani, 1998), and also exceeds the methionine contents in soybean glycinins

(Docimo et al., 2014). Ponzoni, Brambilla, and Galasso (2018) identified a type3 edestin gene, CsEde3, which shows approximately 65% and 58% sequence homology when compared to the genomic forms of CsEde1 and CsEde2, respectively.

Albumin. The albumin fraction constitutes about 25% of hempseed storage protein. Malomo and Aluko (2015a) isolated the globulin and albumin fractions by dialysis of the salt extract of hempseed meal against water. The albumin fraction was found to contain fewer disulfide-bonded proteins and hence a less compact structure with greater flexibility than the globulin fraction. This was also confirmed by both intrinsic fluorescence and circular dichroism analysis which illustrated greater exposures of tyrosine residues when compared with globulin. Furthermore, albumin exhibited significantly higher solubility and foaming capacity (FC) than globulin, while no differences in emulsion-forming ability were observed between the two protein fractions (Malomo & Aluko, 2015a).

Additionally, a methionine- and cystine-rich seed protein (10 kDa protein, 2S albumin) has been isolated from hempseed (Odani & Odani, 1998). The protein contains 18% (w/w) sulfur amino acids and consists of two polypeptide chains (small and large) with 27 and 61 amino acid residues, respectively. The two polypeptide chains are held together by two disulfide bonds. This sulfur-rich protein has no inhibitory activity against trypsin and could serve as a rich thiol source to formulate highly nutritious foods, since various plant food proteins, especially legumin proteins from soybean, pea, and beans, are deficient in sulfur. The gene families encoding the precursor polypeptides of 2S albumin have recently been identified by Ponzoni et al. (2018), and two genomic isoforms for 2S albumin were obtained, namely, Cs2S-1 and Cs2S-2. The alignment of the deduced gene with the mature 2S protein sequence published in the literature (Odani & Odani, 1998) showed that Cs2S is 97% identical to the mature 2S protein.

Protein processing

Hemp protein meal. The oil extraction by-product of crushed hempseeds is commonly referred to as hemp protein meal (HPM). The protein content in HPM ranges from 30% to 50% in dry matter depending on the variety of hemp used and the oil extraction method (cold-pressing or solvent) and efficiency (Malomo, He, & Aluko, 2014). Pojić et al. (2014) separated HPM into four fractions by particle size (>350, >250, >180, and <180 μm). The two cotyledon-containing fractions (>180 and <180 μm) were found to be significantly richer in protein and higher in free radical-scavenging capacity compared with the hull-containing fractions (>350 and >250 μm). Antinutrients (trypsin inhibitors, phytic acid, glucosinolates, and condensed tannins) were mostly located in the cotyledon fractions. Moreover, Russo and Reggiani (2015) showed that the dioecious varieties have lower contents of antinutritional compounds than monoecious varieties.

Hemp protein concentrate. Hemp protein concentrate (HPC) is prepared from dehulled and defatted hempseed or HPM by removing most of the water-soluble, nonprotein constituents. HPC contains at least 65% protein (N × 6.25) on a dry weight basis. Malomo and Aluko (2015b) obtained HPC by enzymatic digestion (carbohydrase and phytase) of fiber coupled with membrane ultrafiltration that enriched protein content up to 70%. Protein digestibility of the HPC was significantly higher than that of HPM and traditional isoelectric protein isolate.

Hemp protein isolate. The most purified and enriched form of commercial protein product, HPI (>90% protein), is prepared to meet food processing needs that entail minimal influence of

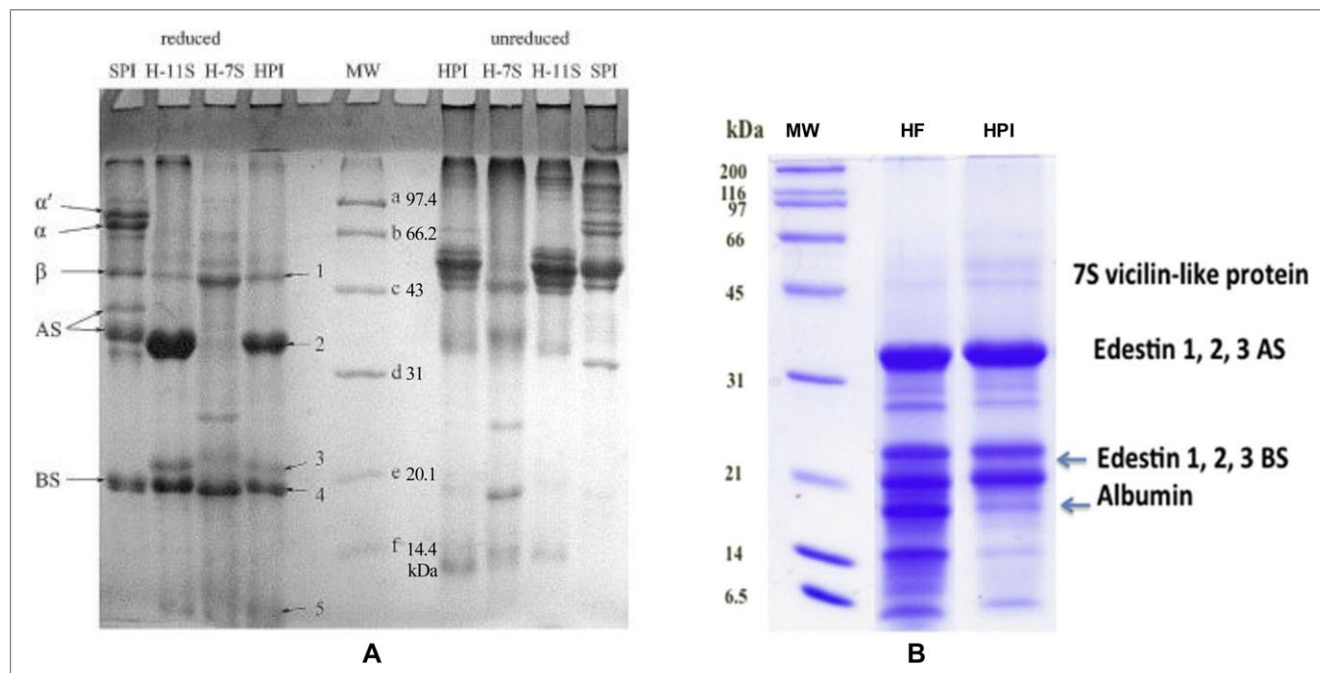


Figure 2—(A) SDS–PAGE profiles of HPI in comparison with SPI in the presence (reduced) or absence (unreduced) of 2-mercaptoethanol. AS and BS represent the acidic and basic subunits of soy glycinin, respectively, and α' , α , and β represent the corresponding major subunits of soy β -conglycinin (Wang et al., 2008). (B) SDS–PAGE in reducing conditions of hemp flour and HPI showing edestin dominating in both samples (Mamone et al., 2019).

unwanted nonprotein components. Depending on the method of extraction employed, the final HPI could vary in protein content, composition, solubility, and, when applied as a functional ingredient, the reactivity with food additives. Alkaline extraction followed by isoelectric precipitation is the most common method for the preparation of HPI (Lu et al., 2010; Malomo et al., 2014; Orio et al., 2017; Raikos, Duthie, & Ranawana, 2015; Ren et al., 2016; Tang et al., 2006; Wang et al., 2008). The method can produce an isolate with a purity up to 94% depending on the specific extracting conditions, for example, pH, temperature, extraction time, and centrifugal force. The alkaline extraction pH is generally 9 to 10, higher than that for legume protein extraction (pH 8), because native hempseed proteins are tightly compacted, and may be closely integrated with other components, for example, phenolic compounds.

To maximize the yield, some researchers use elevated extracting temperatures, for example, 35 to 40 °C, to improve the protein solubility. It should be noted that adverse chemical reactions, such as the conversion of cysteine and serine residues to nephrotoxic lysinoalanine compounds, can occur under highly alkaline and heating conditions. As reported by Wang, Jin, and Xiong (2018), HPI extracted at pH 10 and room temperature had a low level of lysinoalanine (0.8 mg/100 g protein). However, if the extracted HPI was held at pH 12 for as short as 5 min at 40 °C, the lysinoalanine content would increase to 4 mg/100 g protein. Corresponding to alkaline extraction, acid extraction was also adopted to prepare HPI (Teh, Bekhit, Carne, & Birch, 2014). The yield of protein extracted at acidic pH was lower than that extracted at alkaline pH. Compared to alkaline-extracted HPI, this protein isolate had a lower lightness (L^* value), higher redness (a^* value), and lower yellowness (b^* value). Another method, known as “salt extraction with micellization”, has been described for HPI preparation in a recent report (Hadnađev et al., 2018a). HPI obtained by this method has a very high purity (98.9% protein, on a dry basis)

and significantly greater colorimetric values (L^* , a^* , and b^*), but lower recovery yield, in comparison with the common alkaline extraction–isoelectric precipitation method. The color difference is because the alkaline condition employed to extract HPI favors the coextraction of phenolics from hempseed meal, resulting in the development of dark green to brown color of protein isolates from the exposure to molecular oxygen. Based on the SDS–PAGE profiles, it appears that the salt extraction minimally influences the subunit composition of hemp protein, differing from the high pH–isoelectric precipitation method that could cleave disulfide bonds between some subunits. For salt extraction, extreme alkaline or acidic pH and temperature elevation are not necessary, as compared with acid and alkaline extraction. Protein extraction occurs at a slightly acidic pH (5.5 to 6.5), although Aiello, Lammi, Boschini, Zaroni, and Arnoldi (2017) and Zaroni et al. (2017) also isolated hemp protein by salt extraction under a slightly alkaline pH to maximize protein extractability.

Nutrition and Health Benefits of Hempseed Protein

Nutritional quality

Amino acid composition. The dietary requirements of humans are not for protein *per se*, but for specific amounts of indispensable or essential amino acids (building blocks of protein). Hemp protein provides all the essential amino acids with a balanced amino acid profile (Table 1). The essential amino acids are comparable to other high-quality proteins, such as casein and soy protein (Tang et al., 2006), and are sufficient for the Food and Agriculture Organization (FAO)/World Health Organization (WHO) suggested requirements for 2- to 5-year-old children. Hemp protein contains an exceptionally high amount of arginine and glutamine (Lu et al., 2010). Arginine accounts for approximately 12% of hempseed protein when compared with less than 7% for most other food proteins, including the proteins from potato, wheat, maize, rice, soy, rapeseed, egg white, and whey (Callaway, 2004).

Table 1–Amino acid composition (% w/w) of hemp protein products and selective nonhemp proteins for comparison.

Amino acids	Hempseed		Meal		Concentrate		Protein isolate				Protein fraction		Other protein		FAO/WHO/UNU	
	Whole seed ^a	Dehulled seed ^a	HPM ₁ ^a	HPM ₂ ^b	HPC ^b	HPI ₁ ^b	HPI ₂ ^c	HPI ₃ ^d	HPI ₄ ^d	HPI ₅ ^c	Albumin ^e	Globulin ^e	SP ^f	Casein ^g	Infant	Child2–5 y
Asx	11.23	10.68	10.69	10.78	11.17	11.31	10.06	9.38	9.38	10.27	7.93	9.47	11.47	6.30		
Thr	4.75	3.70	3.94	3.75	3.77	3.42	3.24	4.76	4.81	3.27	4.63	2.60	3.98	3.70	4.3	3.4
Ser	5.59	4.96	5.05	5.65	5.55	5.58	5.00	5.48	5.59	5.16	5.12	5.73	5.32	4.60		
Glx	17.58	18.17	17.61	18.45	17.53	19.10	16.92	15.99	16.41	17.60	20.37	21.48	20.67	19.00		
Pro	4.23	4.73	4.64	4.37	4.98	4.30	3.64	4.00	3.81	3.79	3.82	3.87	5.13	N/A		
Gly	4.98	4.70	4.85	4.67	4.86	4.31	3.68	4.64	4.57	3.73	8.26	4.10	3.74	1.60		
Ala	4.51	4.43	4.70	4.19	4.25	3.81	3.76	4.14	3.91	4.09	3.91	2.84	3.72	2.70		
Cys	1.93	1.90	2.04	1.52	1.62	1.25	1.46	1.64	1.42	1.49	3.20	3.32	0.05	0.04		
Val	5.36	5.19	5.58	4.69	5.34	4.66	5.33	5.80	5.59	5.67	2.90	3.41	4.28	6.00	5.5	3.5
Met	2.63	2.74	2.57	2.28	1.94	1.92	2.02	4.23	2.33	2.12	1.74	4.07	0.92	2.60		
Ile	3.76	3.76	4.23	3.59	3.81	3.63	3.64	4.46	4.47	4.66	2.02	2.86	4.35	4.90	4.6	2.8
Leu	7.00	6.24	6.86	6.87	6.88	6.46	6.54	7.23	7.02	6.53	4.05	5.57	6.79	8.40	9.3	6.6
Tyr	3.20	3.73	3.36	3.04	2.79	3.61	3.64	3.49	3.88	3.67	2.02	3.41	3.61	5.50	1.7	1.1
Phe	4.84	4.17	4.73	4.68	4.74	4.72	4.23	5.41	5.53	4.20	1.32	3.27	5.14	4.50		
His	2.58	2.83	2.72	3.26	3.48	3.15	2.71	2.52	3.19	2.81	3.68	3.87	2.81	2.70	2.6	1.9
Lys	4.04	3.68	3.86	3.94	4.21	2.73	2.89	2.77	2.82	2.87	7.37	3.69	5.23	7.10	6.6	5.8
Arg	10.71	13.27	11.42	13.20	11.98	15.06	10.02	13.98	15.24	9.98	12.82	16.12	7.35	3.30		
Trp	1.08	1.11	1.14	1.06	1.05	1.00	N/A	0.09	0.03	N/A	0.16	0.34	N/A	N/A		
Arg/Lys	2.65	3.61	2.96	3.35	2.85	5.52	3.47	5.05	5.40	3.48	1.74	4.37	1.41	0.46		
Met+Cys	4.56	4.64	4.61	3.80	3.56	3.17	3.48	5.87	3.75	3.61	4.94	7.39	0.97	2.64	4.2	2.5
Phe+Tyr	8.04	7.90	8.09	7.72	7.53	8.33	7.87	8.90	9.41	7.87	3.34	6.68	8.75	10.00	7.2	6.3
E/T	0.36	0.33	0.36	0.34	0.35	0.32	0.34	0.37	0.36	0.38	0.29	0.29	0.35	0.48		

N/A, not available; E/T, the proportion of essential amino acids to total amino acids; Asx, Glx, sum of asparagine and aspartic acid, and glutamine and glutamic acid, respectively. Data are from a House et al., 2010. b Malomo and Aluko, 2015b. c Hadnadev et al., 2018a. d Teh et al., 2014. e Malomo and Aluko, 2015a. f Wang et al., 2008. g Tang et al., 2006.

Arginine is a precursor of nitric oxide, the vasodilating agent that enhances blood flow and contributes to the maintenance of normal blood pressure (Wu & Meininger, 2002). The Arg/Lys ratio is a determinant of the cholesterolemic and atherogenic effects of a protein. The Arg/Lys ratio of hemp protein, at 3.0 to 5.5, is remarkably higher than that of SPI (1.41) or casein (0.46), making hemp protein particularly valuable as a nutritional and bioactive ingredient for the formulation of foods that promote cardiovascular health. Furthermore, hemp protein products (HPM, HPC, and HPI) contain excellent amounts of the sulfur amino acids cysteine and methionine (Callaway, 2004; Russo & Reggiani, 2013). Total sulfur-containing amino acids are in the range of 3.5% to 5.9%, which is close to the reference protein profiles established by FAO/WHO/United Nations University (UNU) as requirements for infants and preschool children 2- to 5-year-old (Table 1). House et al. (2010) calculated the respective amino acid scores of hemp protein, identifying that lysine (score 0.5 to 0.62) was the first-limiting amino acid in hemp protein, followed by leucine and tryptophan.

When the amino acid composition of whole hempseed or meal is compared with protein products (Table 1), the influence of protein composition (types) of the final product can be observed. As addressed by Malomo and Aluko (2015b), the contents of essential amino acids and branched-chain amino acids of HPC were higher than HPM and isoelectric precipitated HPI, which could contribute to better muscle metabolism and maintenance of protein homeostasis (Herman, She, Peroni, Lynch, & Kahn, 2010). Isoelectric precipitated HPI has a higher Arg/Lys ratio than HPM and HPC. Hadnađev et al. (2018a) have demonstrated that HPI prepared by micellization (salt extraction followed by membrane filtration) has a similar amino acid pattern to HPI obtained by isoelectric precipitation (Table 1). However, acid-extracted hemp protein was slightly different in amino acid composition from alkali-extracted (isoelectric precipitated) HPI, especially for methionine, histidine, and arginine (Teh et al., 2014). It is not a surprise that different fractions of HPI (albumin and globulin) possess different amino acid compositions (Malomo & Aluko, 2015a). The globulin fraction has a higher content of sulfur amino acids, especially methionine, and is also higher in hydrophobic and aromatic amino acids when compared to albumin. A higher Arg/Lys ratio (4.37) in globulin, when compared with albumin (1.74), suggests strong potential for globulin utilization in the formulation of cardiovascular health-promoting food products.

Digestibility. The degree of digestion of dietary proteins depends on enzyme accessibility, which is affected by the molecular structure as well as other components associated with proteins. House et al. (2010) measured the protein digestibility-corrected amino acid score (PDCAAS) of whole hempseed, dehulled hempseed, and HPM using a rat bioassay for protein digestibility and the FAO/WHO amino acid requirement of children (2 to 5 years of age) as reference. The protein digestibility of dehulled hempseed, depending on the sources, was 90.8% to 97.5%, almost comparable to 97.6% for casein. The PDCAAS value for hemp protein, depending on the source, was 0.48 to 0.61. These values are within the range of major pulse proteins, for example, from beans, but are above those of cereal grain products, for example, whole wheat. Lysine and tryptophan are the main limiting amino acids in hempseeds, which presumably contribute to the relatively low PDCAAS score. Removal of the hull improved the protein digestibility and the corresponding PDCAAS. It is worth noting that, currently, there is no literature report comparing digestibility and PDCAAS for HPIs prepared with different methods.

However, a digestibility study for HPIs (HPI, 7S, and 11S) and SPI using an *in vitro* digestion model that measures nitrogen release has been published (Wang et al., 2008). During the pepsin digestion, edestin (including AS and BS) was rapidly degraded within 1 min, similar to the digestion of the AS and BS of soy glycinin, releasing oligo-peptides with MWs less than 10 kDa. Although HPIs displayed a similar digestibility to SPI when only digested by pepsin, the total digestibility (pepsin plus trypsin digestion) of HPIs (88% to 91%) was distinctly higher than that of SPI (71%), hinting that HPI is an efficient source of protein nutrition for human consumption. Mamone, Picariello, Ramondo, Nicolai, and Ferranti (2019) also showed evidence that hemp flour and HPI had a high degree of digestibility. A very limited number of peptides survived in the simulated intestinal digestion, whereas some free aromatic amino acids Tyr, Phe, and Trp were also detected. This finding supports, on a molecular basis, the claim of good digestibility with the ready release of bioaccessible amino acids from HPI (Wang et al., 2008).

Health benefits

Allergenicity. Hempseeds are considered to be a low-allergen food material. The allergenicity associated with hemp, including allergens from its seeds, roots, fibers, leaves, and flowers, has been comprehensively reviewed by Decuyper et al. (2017). Five allergens have been identified in the hemp plant, including *C. sativa* 3/ns-LTP (nonspecific lipid transfer proteins, 9 kDa), profilin (14 kDa), oxygen-evolving enhancer protein (23 kDa), thaumatin-like protein (38 kDa), and ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCo, 50 kDa). Of these allergenic proteins, only RuBisCo has been detected in hempseeds through proteomic analysis (Aiello et al., 2016; Park, Seo, & Lee; 2012). A systematic evaluation of HPI by Mamone et al. (2019) concluded that all known hemp allergens, including the major thaumatin-like protein and LTP, were entirely eliminated by the HPI production process. Neither fragments of the proteins were present after gastrointestinal digestion. Although there are limited reports on immunological responses to hempseed proteins, the presence of allergen-homolog proteins (for example, serpins) in HPI and their digestion-resistant peptide fragments warrants further research to establish the potential health impact.

Bioactivity. Aside from their nutritive values, proteins from both plant and animal sources have been hydrolytically converted to peptides that exhibit a wide range of bioactivity both *in vitro* and *in vivo*, for example, antioxidant, antihypertensive, antimicrobial, antithrombotic, hypocholesterolemic, mineral-binding, immunomodulatory, and cytomodulatory properties (Mine, Li-Chan, & Jiang, 2010; Sánchez & Vázquez, 2017; Sharma, Singh, & Rana, 2011). Enzymatic digestion of proteins can break down long polypeptide chains into short fragments that fit in, hence, disrupt the specific active site of the disease-related metabolic enzymes. The peptides may also bind to nonactive sites on the target disease-causing enzymes through hydrophobic or electrostatic interactions.

Hemp protein has been explored for its feasibility and potential in the production of bioactive peptides during the past decade. The various health benefits and pharmaceutical value of peptides generated by controlled hydrolysis of hemp proteins are summarized in Table 2. The specific health-promoting activities include angiotensin I-converting enzyme (ACE) inhibition, renin inhibition, acetylcholinesterase (AChE) inhibition, metal-binding capacity, antioxidant activity, hypocholesterolemic effect, and serum glucose regulation. The bioactivity is elicited through the

Table 2–Reported bioactivity of peptides derived from hempseed protein.

Bioactivity	Study details	Reference
Angiotensin I-converting enzyme (ACE) inhibition, <i>in vitro</i> and <i>in vivo</i>	Peptide fractions (<1 and 1 to 3 kDa) had less potency against ACE than the original hempseed protein hydrolysate (HPH) produced from pepsin–pancreatin hydrolysis. Small-size peptides (<1 kDa) were more effective than larger size peptides (1 to 3 kDa) as ACE inhibitors.	Girgih et al., 2011b
	Plasma ACE activity was remarkably suppressed in hempseed meal protein hydrolysate (HMH)-fed rats compared to control rats.	Girgih et al., 2014a
	HT (bacterial) produced the highest ACE-inhibitory activity of alkali- and acid-extracted HPI among all microbial and plant proteases (AFP, HT, protease G, actinidin, and zingibain).	Teh et al., 2016
	Four peptides (GVLY, IEE, LGV, and RVR) were identified from HPH by extensive chemical hydrolysis (6 M HCl). GVLY possessed the highest ACE-inhibitory activity, while IEE was almost inactive in inhibiting ACE.	Orio et al., 2017
Renin inhibition	Peptide fractions (<1 and 1 to 3 kDa) exhibited moderate renin inhibitory activity, which was lower than HPH.	Girgih et al., 2011b
	Plasma renin level was decreased for HMH-fed rats compared to rats fed only casein and HPI.	Girgih et al., 2014a
	Novel peptides WVYY, WYT, SVYT, and IPAGV were generated from an enzymatic digest of hempseed protein. WYT and SVYT had similar renin-inhibitory activity, which was significantly better than that of IPAGV.	Girgih et al., 2014c
Antihypertensive effect	Oral administration of HPH to spontaneously hypertensive rats led to significant reductions in systolic blood pressure, while the antihypertensive effect of peptide fractions (<1 and 1 to 3 kDa) was significantly lower than that of HPH.	Girgih et al., 2011b
	WVYY and PSLPA showed maximum systolic blood pressure (SBP) reduction in spontaneously hypertensive rats among 23 identified short-chain (≤ 5 amino acids) peptides.	Girgih et al., 2014d
	HMH had stronger hypotensive effects in spontaneously hypertensive rats when compared to HPI and casein.	Girgih et al., 2014a
	The 1% alcalase HPH was most effective in reducing systolic blood pressure among different HPHs produced with 2% pepsin, 4% pepsin, 1% alcalase, 2% alcalase, 2% papain, and 2% pepsin + pancreatin, while the pepsin HPHs produced longer lasting effects.	Malomo et al., 2015
Acetylcholinesterase (AChE) inhibition	The pepsin–HPH was the most active ACE inhibitor when compared to HPHs obtained by papain, thermoase, flavourzyme, alcalase, and pepsin + pancreatin.	Malomo and Aluko, 2016
Antioxidation, <i>in vitro</i> and <i>in vivo</i>	HPHs by six proteases (alcalase, flavourzyme, neutrase, protamex, pepsin, and trypsin) exhibited varying antioxidant properties indicated by DPPH radical scavenging and Fe^{2+} chelating abilities and reducing power, depending on the yield of trichloroacetic acid (TCA)-soluble peptides and surface hydrophobicity.	Tang et al., 2009
	HPH by Neutrase[®] exhibited antioxidant activities (DPPH radical scavenging ability, reducing power and Fe^{2+} chelating ability) to various extents, depending on TCA-soluble peptide yields.	Wang et al., 2009
	HPH separated with macroporous adsorption resin after alcalase hydrolysis of HPI displayed improved DPPH radical scavenging activity. The active fraction was further separated by gel filtration and RP-HPLC to obtain two purified peptides, namely NHAV and HVRETALV.	Lu et al., 2010
	HPH exhibited a significantly weaker ferric-reducing power and hydroxyl radical scavenging activity in comparison with the fractionated peptides. HPH and peptide fractions significantly inhibited linoleic acid oxidation compared to HPI.	Girgih et al., 2011a
	Eight peptide fractions were separated by reverse-phase (RP)-HPLC from HPH produced through simulated gastrointestinal tract. Partially purified HPH peptides demonstrated stronger antioxidant properties by scavenging free radicals, and the antioxidant activity was closely associated with the amino acid composition and hydrophobicity.	Girgih et al., 2013
	WVYY and PSLPA were the most active antioxidant peptides of 23 identified short-chain (≤ 5 amino acids) peptides, with metal chelation activity of 94% and 96%, respectively.	Girgih et al., 2014d
	HMH diets reduced the rate of lipid peroxidation in spontaneously hypertensive rats with evidently higher plasma superoxide dismutase and catalase levels and lower total peroxide levels.	Girgih et al., 2014b
	HPH produced by protease N exhibited comparatively superior radical scavenging activity in DPPH system compared to HPHs by protease A and papain.	Das, 2015
	Among all hydrolysates (prepared by AFP, HT, protease G, actinidin, and zingibain), HT hydrolysates exhibited the highest oxygen radical absorbance capacity (ORAC) and DPPH scavenging activity in the shortest hydrolysis time (2 hr).	Teh et al., 2016
	The hydrolysates obtained using pancreatin exhibited stronger DPPH antiradical activity as well as reducing power than that derived by alcalase treatment.	Hadnadev et al., 2018b
Metal binding	Zn^{2+}-binding capacity of HPHs was lower than that of HPI, but the water-soluble Zn^{2+}–HPH complex was more abundant in HPHs. Peptides by flavourzyme possessed the highest Zn^{2+}-binding activity, while those with pepsin exhibited the maximum solubility.	Wang et al., 2018
Cell growth	Purified peptides from alcalase HPH by gel filtration and RP-HPLC possessed protective effects for PC12 cells against oxidative stress-mediated (H_2O_2) injuries.	Lu et al., 2010

(Continued)

Table 2–Continued.

Bioactivity	Study details	Reference
Hypocholesterolemic effects	- Pepsin and trypsin HPHs showed higher inhibitory activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) than pancreatin HPH. - HPH can inhibit the catalytic activity of HMGCoAR and modulate the HMGCoAR regulation by the activation of the phospho-5'-adenosine monophosphate-activated protein kinase (AMPK) pathway. Applying HepG2 cells as model system, HPH induced upregulations of the protein levels of regulatory element binding proteins 2 (SREBP2), low-density lipoprotein receptor (LDLR), and HMGCoAR, and increased the LDL-uptake and the proprotein convertase subtilisin/kexin type 9 (PCSK9) levels.	Aiello et al., 2017 Zanoni et al., 2017
Serum glucose regulation	- HPH yielded higher dipeptidyl peptidase IV (DPP-IV) inhibitory potency than the intact protein when subjected to simulated gastrointestinal digestion. - High α-glucosidase inhibitory activity was obtained from HPH by alcalase at the degree of hydrolysis 27.2%. Two novel α-glucosidase inhibitory oligopeptides were isolated and identified having the sequences of Leu-Arg (287.2 Da) and Pro-Leu-Met-Leu-Pro (568.4 Da).	Nongonierma and FitzGerald, 2015 Ren et al., 2016

hydrolysis with gastrointestinal enzymes (pepsin, pancreatin, and trypsin) (Aiello et al., 2017; Girgih et al., 2014d,b; Girgih, Alashi, He, Malomo, & Aluko, 2014a; Girgih, He, & Aluko, 2014c; Girgih, Udenigwe, & Akuko, 2011a, 2013; Girgih, Udenigwe, Li, Adebisi, & Aluko, 2011b; Zanoni, Aiello, Arnoldi, & Lammi, 2017) or exogenous proteases such as alcalase, papain, flavourzyme, neutrase, and thermolysin (Hadnadev et al., 2018b; Lu et al., 2010; Malomo & Aluko, 2016; Malomo, Onuh, Girgih, & Aluko, 2015; Tang, Wang, & Yang, 2009; Wang, Tang, Chen, & Yang, 2009). Peptides with ACE inhibitory activity have also been produced from hemp protein with acid hydrolysis (Orio et al., 2017).

To increase the efficacy, peptides in the protein hydrolysates can be fractionated, purified, and enriched. Girgih, Udenigwe, and Aluko (2011a,b) subjected HPI to the sequential action of pepsin and pancreatin and applied membrane ultrafiltration to separate the protein hydrolysate into different MW fractions. Three peptide fractions with MWs of 1 to 3, 3 to 5, and 5 to 10 kDa exhibited significantly greater radical scavenging activity but lower metal-chelation activity, than the whole protein hydrolysate (HPH, that is, mixed peptides) (Girgih et al., 2011a). Moreover, HPH was faster acting and more effective than the peptide fractions in reducing blood pressure in spontaneously hypertensive rats (Girgih et al., 2011b), indicating concerted actions of peptides present in the HPH. Reverse-phase HPLC (RP-HPLC) was subsequently used by these authors to obtain eight less heterogeneous peptide fractions based on the elution time (Girgih, Udenigwe, & Aluko, 2013). Some of the peptide fractions demonstrated excellent antioxidant activity with higher oxygen radical-absorbance capacity as well as the ability to scavenge superoxide anion and hydroxyl radicals than HPH. Several amino acids, including Lys, Leu, and Pro, appeared to contribute to the observed radical-scavenging activity. Tang et al. (2009) and Wang et al. (2009) also witnessed significant Fe^{2+} -chelating capability of HPH, reporting a strong correlation between the antioxidant activity and the hydrophobicity (hydrophobic amino acids) and the trichloroacetic acid (TCA)-soluble peptide contents. It should be noted that some of the previous antioxidant studies also employed DPPH (2,2-diphenyl-1-picrylhydrazyl) in a single electron transfer test. However, because of the poor solubility of peptides in the DPPH ethanol assay solution, caution must be taken when interpreting the radical-scavenging test results.

Great efforts have been made to identify the specific bioactive peptides from HPH and their sequences using robust analytical tools, such as mass spectrometry. A number of short-chain peptides (≤ 5 amino acids) with remarkable bioactivity have been identified and purified. Girgih et al. (2014d) have isolated and

sequenced 23 short-chain peptides from the pepsin and pancreatin HPI digests through RP-HPLC separation followed by tandem mass spectrometry analysis. Trp-Val-Tyr-Tyr (WVYY) and Pro-Ser-Leu-Pro-Ala (PSLPA) were found to be the most potent antioxidant peptides, which were also capable of lowering the systolic blood pressure in spontaneously hypertensive rats. Furthermore, Trp-Tyr-Thr (WYT) and Ser-Val-Tyr-Thr (SVYT) isolated from the same HPH showed strong dual inhibition *in vitro* of ACE activity (89% and 79%, respectively) and renin (77% and 86%, respectively). Four ACE inhibitory peptides, namely, Gly-Val-Leu-Tyr (GVLY), Ile-Glu-Glu (IEE), Leu-Gly-Val (LGV), and Arg-Val-Arg (RVR), have also been identified from acid-hydrolyzed HPI by Orio et al. (2017), of which GVLY exhibited the lowest ACE-inhibition IC_{50} values. Lu et al. (2010) also purified two peptides, Asn-His-Ala-Val (NHAV) and His-Val-Arg-Glu-Thr-Ala-Leu-Val (HVRETAIV), from HPH that exhibited high antioxidant activity (*in vitro*) and protective effects against oxidative stress. More recently, two α -glucosidase inhibitory peptides (Leu-Arg and Pro-Leu-Met-Leu-Pro) were isolated from alcalase-hydrolyzed HPI (Ren et al., 2016). It seems that hydrophobic, acidic, and branched-chain amino acids, which are abundantly present in these bioactive oligopeptides, positively contribute to the peptides' function both *in vivo* and *in vitro*.

The production of bioactive peptides from hemp protein is obviously dependent upon the type and specificity of the proteases used as well as the degree of hydrolysis (DH). Malomo and Aluko (2016) applied six different proteases (pepsin, papain, thermoase, flavourzyme, alcalase, and pepsin + pancreatin) with concentrations ranging from 1% to 4% to hydrolyze HPI, finding that the resulting HPH prepared with 1% pepsin was the most active AChE inhibitor. The high AChE-inhibitory effects of pepsin-generated peptides are ascribed to increased synergistic actions from the broad size range when compared to other proteases used that produce narrower size range peptides.

Recently, our group (Wang & Xiong, 2018) investigated the metal-binding potential of soluble compared with insoluble peptides obtained from HPH. Our results demonstrated different Zn^{2+} -binding activities for the hydrolysates produced with pepsin, alcalase, flavourzyme, papain, protamex, or trypsin (Figure 3). Site-specific cleavages played a major role in producing the observed diverse peptide profiles that varied in available Zn^{2+} -binding groups, that is, N-H, C = O, C-N, and COO^- . Of the various protein hydrolysates, flavourzyme-generated HPH exhibited the highest Zn^{2+} -binding capacity (88.8%), while pepsin-HPH had the lowest (61.0%). Zinc was found to bind to both water-soluble (small) and insoluble (large) peptides to form complexes. However, the two

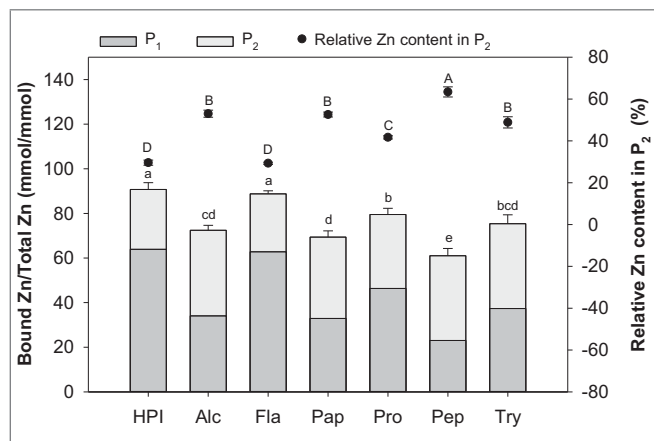


Figure 3—Zn²⁺-binding capacity of combined water-soluble fraction (P₁) and ethanol-extracted insoluble fraction (P₂), and the relative zinc content in P₂ [that is, P₂/(P₁+P₂)] of hemp protein isolate (HPI) and its hydrolysates (HPHs) prepared with different enzymes: alcalase (Ala), flavourzyme (Fla), papain (Pap), protamex (Pro), pepsin (Pep), or trypsin (Try). Means without a common lowercase letter (a to e) or a common uppercase letter (A to D) within the respective parameter groups differ significantly ($P < 0.05$) (Wang et al., 2018).

fractions of peptides possessed different Zn²⁺-binding sites, where N–H groups were the dominant site in insoluble peptides and C = O groups were the primary binding site in soluble peptides.

Thermal Properties of Hempseed Protein

The stability against thermal denaturation is an important quality characteristic of proteins because conformational changes induced by heat play a crucial role in the functionality of proteins. HPI exhibits a typical endothermic peak with a denaturation temperature (T_d) of about 92 to 95 °C when analyzed at pH 7 at a heating rate of 5 °C/min. (Hadnađev et al., 2018a; Tang et al., 2006; Wang et al., 2008). This peak corresponds to the edestin component, especially the hexamer form. As evinced by Wang et al. (2008), the 11S fraction of hemp protein displayed a similar melting profile to HPI when subjected to differential scanning calorimetry (DSC). However, no thermal transition signals were detected in the 7S fraction (Figure 4A). Tang et al. (2006) found that the thermal stability of HPI was mainly maintained by disulfide bonds rather than hydrophobic interactions. A much lower T_d (68.3 °C) was reported for HPI by Teh et al. (2014). This may be caused by the difference in variety and extraction conditions that influenced the structure of isolated proteins. Furthermore, micellized HPI has a slightly higher denaturation temperature (T_d = 96.2 °C) and enthalpy (ΔH = 21.7 kJ/mg) than regularly extracted HPI (alkaline extraction–isoelectric precipitation) (T_d = 93.5 °C and ΔH = 11.8 kJ/mg) (Figure 4B). This can be explained because the milder extraction condition of micellization has a minimal disrupting effect on protein intramolecular bonds, thereby allowing edestin to largely maintain its higher structural order and thermal stability (Hadnađev et al., 2018a). Teh et al. (2014) found that HPI prepared by alkaline (or acid) extraction–isoelectric precipitation had a lower T_d than defatted hempseed cake. The lower stability of protein due to the exposure to high or low pH during protein extraction indicates that HPI prepared in this way may not be considered to be truly “native.” Furthermore, the HPI extracted with alkaline pH was less heat-resistant than that extracted with the acid pH. In comparison to flaxseed and canola protein isolates prepared with the same alkaline or acid extraction,

HPI showed a higher T_d than flaxseed protein but lower T_d than canola protein (Teh et al., 2014).

Functionality of Hempseed Protein

The value and usefulness of a protein ingredient depend on its functionality, namely, its behavior and performance in food systems during preparation, processing, storage, and consumption. The functional properties of proteins depend on the nature and extent of interactions among protein molecules and with the presence of other components (for example, water and oil) in the food system. Since many formulated foods exist as beverages, emulsions, foams, and viscoelastic solids, the ability of proteins to bind water or fat and their solubility, foaming, gelation, emulsification, and film formation properties are essential to the quality of food products. Although not widely researched yet, some of the aforementioned functional properties for hemp proteins have been reported.

Water binding

The ability to interact and entrap water voluminously is extremely important for the application of proteins in food products. In the scientific literature, water binding, water absorption, and water holding are often used interchangeably to characterize the interactions between proteins and water, although, strictly speaking, the actual concepts differ. Water-holding capacity (WHC), a common term that describes the amount of water entrapped in the protein matrix and the ability of proteins to retain water against gravity, is widely used to refer to water binding by oilseed proteins. As reviewed by Moure, Sineiro, Domínguez, and Parajó (2006), WHC is related to many intrinsic and extrinsic factors, for example, the amino acid profile, protein concentration, conformation, hydrophobicity, pH, temperature, and ionic strength. The study reported by Raikos, Neacsu, Russell, and Duthie (2014) showed that hemp flour had a stronger WHC than flours prepared from fava bean, buckwheat, green pea, and wheat due to its high protein content. Malomo and Aluko (2015b) prepared HPC by enzymatic digestion of fiber coupled with membrane ultrafiltration, noting significantly higher WHC when compared to HPI and HPM. The removal of some nonprotein components during HPC preparation and more loose protein structure by the enzyme treatment appeared to promote the interactions between hydrophilic groups with water. A comparison between two kinds of protein isolates—extracted by alkaline or micellization—showed that alkaline extracted HPI had greater ability to prevent water and oil losses from the hydrated three-dimensional structure than micellized HPI (Hadnađev et al., 2018a). This difference can be attributed to more conformational changes in the former where a higher number of hydrophilic, as well as hydrophobic, groups were exposed to the alkali extraction condition.

The study conducted on alkaline- and acid-extracted HPI showed that alkaline-extracted HPI had a higher WHC (Teh et al., 2014). The high degree of protein subunit dissociation and the exposure of polar amino acid sidechain groups with the alkaline extraction were implicated in the observed WHC variation. As reported by Tang et al. (2006), the WHC of HPI was significantly lower than that of SPI, presumably because of the larger extent of protein aggregation (low solubility) in HPI at neutral pH, since the polar groups were buried in the interior of the aggregates. Nevertheless, compared with other protein isolates (flaxseeds and canola), HPI from both alkaline and acid extractions exhibited a superior WHC (Teh et al., 2014).

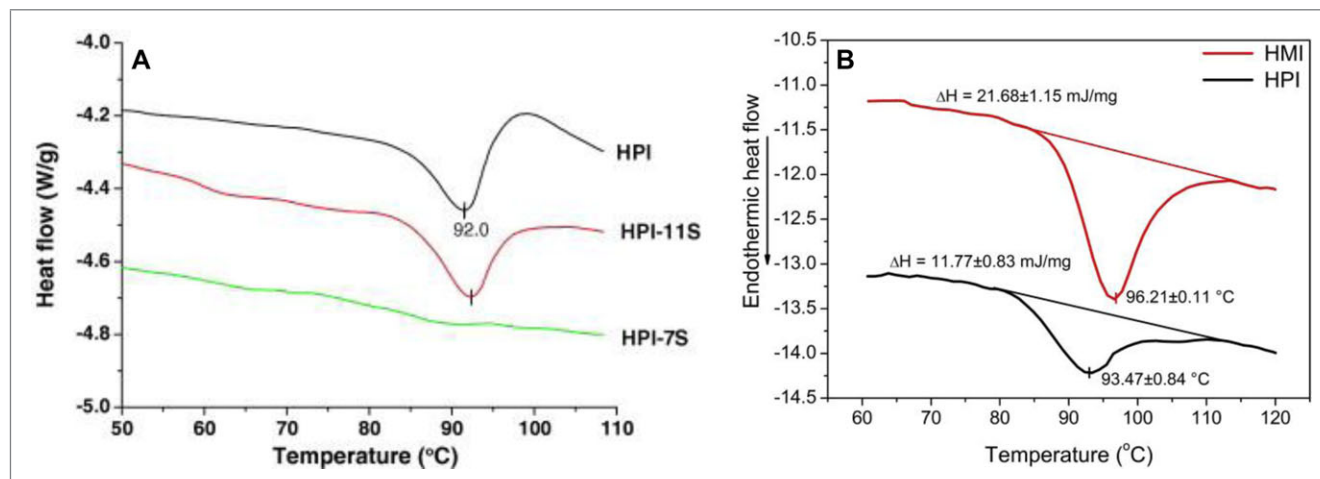


Figure 4—Typical DSC thermograms of: (A) HPI, HPI-7S, and HPI-11S in 0.05 M phosphate buffer (pH 7.0) (Wang et al., 2008) and (B) 15% aqueous dispersions of HPI (in 0.3 M NaCl, pH 7.0) prepared by the micellization (HMI) or isoelectric precipitation (HPI) procedures (Hadnađev et al., 2018a).

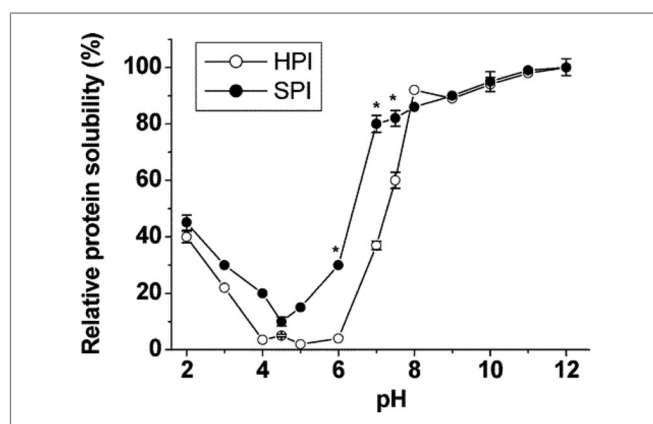


Figure 5—Protein solubility profiles of HPI and SPI at different pH values. *The asterisk above the HPI curve denotes significant ($P < 0.05$) difference between HMP and SPI (Tang et al., 2006).

Solubility

Protein solubility, a manifestation and result of strong interaction of polar groups with water molecules, is a prerequisite to many other functional properties of proteins. It has a great influence on the colloidal structure development, such as gelation, foaming, and emulsification, hence, plays a crucial role in the specific applications in fabricated foods. Hemp protein displays a typical U-shaped pH–solubility profile (Figure 5) with an isoelectric point at about pH 5.0 (Hadnađev et al., 2018a; Tang et al., 2006). At neutral pH, HPI generally has poor solubility, and the value ranges from 8% to 38%, depending on the centrifugation force applied and the solubility determination protocol (Hadnađev et al., 2018a; Malomo & Aluko, 2015a,b; Tang et al., 2006). The protein solubility of the albumin fraction, ranging from 57% at pH 3.0 to 84% at pH 8.0, was found to be significantly greater than that of the globulin fraction (from 20% at pH 3.0 to 50% at pH 8.0) (Malomo & Aluko, 2015a). The low solubility of HPI at pH less than 7.0 was ascribed to the propensity of edestin to aggregate (Tang et al., 2006). However, at pH greater than 8.0, the protein solubility can increase to 65% to 90% (Hadnađev et al., 2018a; Tang et al., 2006), suggesting that HPI in a strict sense is a type of alkali-soluble protein. The underlying mechanism of

solubilization at alkaline pH (especially at pH > 10.0) could be related to the dissociation of edestin (Goring & Johnson, 1955), much like the alkaline effect on soy glycinin or β -conglycinin (Jiang, Xiong, & Chen, 2010). In comparison with soy protein, HPI has a higher solubility at pH values less than 8.0 (Figure 5). At pH above 8.0, the solubility of the two types of proteins was similar. The difference in protein solubility in the acidic pH range may be attributed to differences of protein constituents and aggregation extent of hexamers (glycinin or edestin). The high content of cysteine residues in edestin may predispose it to intermolecular disulfide bond formation, and thereby increasing the extent of aggregation.

According to Malomo and Aluko (2015b), HPC had a higher solubility than HPI and HPM in the pH 3 to 9 range. HPM was very insoluble due to the cross-linking by phytate which reduced the opportunity for the protein to interact with water. The low solubility of HPI, on the other hand, may be caused by the strong protein–protein association during the isoelectric pH protein isolation leading to aggregation and ultimate precipitation. Similarly, Hadnađev et al. (2018a) found that micellized HPI had a higher protein solubility at a pH below the isoelectric point (3 to 5), but less solubility at pH 6 to 9 in comparison with regular alkaline extraction–isoelectric precipitation HPI. The high protein solubility at pH 3 to 5 for micellized HPI was due to the minimal disruption of edestin's native structure during the isolation process.

Gelation

Gel formation is a complex process that involves multiple kinetic steps, including denaturation, dissociation–association, and aggregation. Protein gelation can be induced by heat treatment, pH, salts, pressure or shearing, and the presence of various solvents. Heat-induced protein gels are important for the structure and properties of many food products. Malomo et al. (2014) studied the least gelling concentration of hemp protein, finding that a 22% protein concentration was necessary for HPI prepared by alkali extraction–isoelectric precipitation and only a 12% protein concentration for HPM. They suggested that a high level of protein aggregates of HPI generated during isoelectric precipitation reduced the molecular flexibility required for the gel network formation. Besides, nonprotein materials, especially sugars and polysaccharides, may also contribute to the superior gelling

properties of HPM. However, Isinguzo (2011) reported that HPI by isoelectric precipitation formed a self-supporting gel at 12% protein concentration, which was in agreement with the result obtained by our work (not yet published). The inconsistency in the least gelling concentration is probably associated with the different protein compositions as affected by hempseed variety and specific extraction conditions. It can also be attributed to the varying degrees of protein denaturation that occurs prior to gelation as a result of the preheating treatment of hempseed.

The influence of isolation techniques (alkaline extraction–isoelectric precipitation compared with micellization) and NaCl concentration on gelation properties of HPI has been investigated by Dapčević–Hadnađev, Hadnađev, Lazaridou, Moschakis, and Biliaderis (2018). The dispersions of isoelectric precipitated HPI had a paste-like consistency, while dispersions prepared from micellized HPI behaved as concentrated liquid-like dispersions. This was revealed in the microscopic images (Figure 6I), where the former HPI exhibited a more densely packed particle structure with substantially smaller sizes in comparison to micellized HPI. The addition of NaCl into the micellized HPI dispersions induced a charge-shielding effect, thus, suppressed electrostatic repulsion between protein molecules and promoted aggregation (Figure 6I). The increase of the protein particle size in micellized HPI dispersions resulted in substantial losses in the elastic modulus (G'). However, NaCl concentration did not have a pronounced impact on the rheological properties and microstructure of the isoelectric HPI dispersions.

Thermally induced gels from isoelectrically recovered HPI were characterized by fairly large globular entities, whereas the respective micellized HPI gels exhibited a finer network (Figure 6II). However, isoelectric precipitated HPI gels actually had a slightly higher G' value than the gels formed by micellized HPI (Dapčević–Hadnađev et al., 2018). An irregular aggregate structure with larger pore sizes in the micellized HPI gels was displayed as the salt concentration was increased to 300 mM, which most likely resulted in intensive protein–protein interactions. In regard to the gel color, isoelectrically precipitated HPI gels were darker than the gels prepared by micellized HPI, and coexistence of polyphenols in HPI during alkaline extraction was responsible for the color development. The addition of salt reduced the lightness of the gel due to changes in the network microstructure.

Overall, the isolation methods used in hemp protein preparation greatly affect the quality of HPI, therefore, its gelling potential and response to salt, and also the microstructural properties of the gel. The more gentle nature of the micellization isolation method, as opposed to the alkali solubilization–isoelectric precipitation procedure, clearly favors the formation of finely structured and textured HPI gels (Dapčević–Hadnađev et al., 2018).

Foaming

The foaming properties of proteins depend on their ability to readily adsorb at the air–water interface, undergo rapid conformational change and rearrange at the interface, and form a cohesive viscoelastic film via intermolecular interactions (Damodaran, 1994; Kinsella, 1981). Inherent factors, such as protein concentration, solubility, and surface hydrophobicity, affect foaming ability (Malomo et al., 2014). The foaming properties of different hemp protein products (HPM, HPC, and HPI) have been compared by Malomo et al. (2014, 2015b). HPC was shown to have a significantly higher FC than HPI and HPM at all tested pH values (3 to 9) and protein concentrations (20 to 60 mg/mL). The higher FC of HPC indicated a more flexible structure with a minimal level of

protein aggregation, corresponding to a high protein solubility. Although not as strong a foaming agent as HPC, HPI was able to produce a higher foam volume than HPM at pH 3 and 5, and the superiority at pH 3 was particularly notable because of its greater solubility. On the other hand, through forming thick and strong interfacial membranes favored by the aggregated nature, the HPI foams were more stable than foams formed with HPC or HPM (Malomo & Aluko, 2015b). The lower protein contents and presence of nonprotein substances in both HPC and HPM seem to weaken the interfacial membranes against the gravitational force, thus causing a higher foam drainage than HPI.

A comparison between hemp albumin and globulin fractions showed that albumin produced a significantly higher foam volume (FC) than the globulin over a broad pH range and protein concentrations (Malomo & Aluko, 2015a). The greater solubility for albumin was credited for the FC. Nevertheless, the globulin had higher foaming stability (FS) than albumin at most of the pH and protein concentrations studied. The higher percentage of hydrophobic amino acids in globulin, which is conducive to protein–protein hydrophobic association favoring a cohesive interfacial membrane at the air–water interface, appears to contribute to the greater FS.

Oil binding

Protein–lipid interactions occur through hydrophobic attractions of the aliphatic chains in fatty acids and nonpolar side chains in some amino acids. Oil absorption capacity (OAC), defined as the amount of oil absorbed per gram of protein, has been used to describe oil binding by plant proteins, such as quinoa protein isolate (Abugoch, Romero, Tapia, Silva, & Rivera, 2008). According to Malomo and Aluko (2015b), HPC and HPI, having a similar OAC, were more capable of binding oils than HPM. The higher OACs of HPC and HPI than HPM may result from their higher protein contents, since interactions with the lipid phase are enhanced more by protein molecules than by insoluble polysaccharides. Conversely, Teh et al. (2014) noted that HPI by acid or alkaline extraction had a lower OAC than HPM. Moreover, they found acid extraction allowed a lower OAC in HPI than alkaline extraction. The effect of different extraction procedures on the OAC of HPI was also reported by Hadnađev et al. (2018a), who noted a higher OAC for alkaline-extracted HPI than micellized HPI. This may be due to partial protein denaturation (unfolding) by alkaline extraction that causes an increase in surface hydrophobicity (Jiang, Chen, & Xiong, 2009). The OAC of HPI was essentially comparable to that of SPI (Tang et al., 2006), and significantly superior to that of flaxseed and canola isolates (Teh et al., 2014).

Emulsification

Emulsifying properties of proteins are related to many factors, for example, the rate of protein adsorption at the oil–water interface, the amount of protein adsorbed (loading), the conformational rearrangement at the interface, the extent of interfacial tension reduction, and the rheology of the cohesive film (Amagliani, O'Regan, Kelly, & O'Mahony, 2017). A number of quality indexes, such as emulsifying activity (EA), emulsifying capacity (EC), emulsion stability (ES), and droplet size, are commonly used to evaluate the emulsifying properties of proteins. According to Malomo and Aluko (2015b), HPC had a generally low EC, and the size of oil droplets was typically 6 to 15 $\mu\text{m}(d_{3,2})$. By contrast, HPM and HPI formed emulsions with oil droplet sizes less than 1 μm . As aforementioned, the partially unfolded HPI molecules would

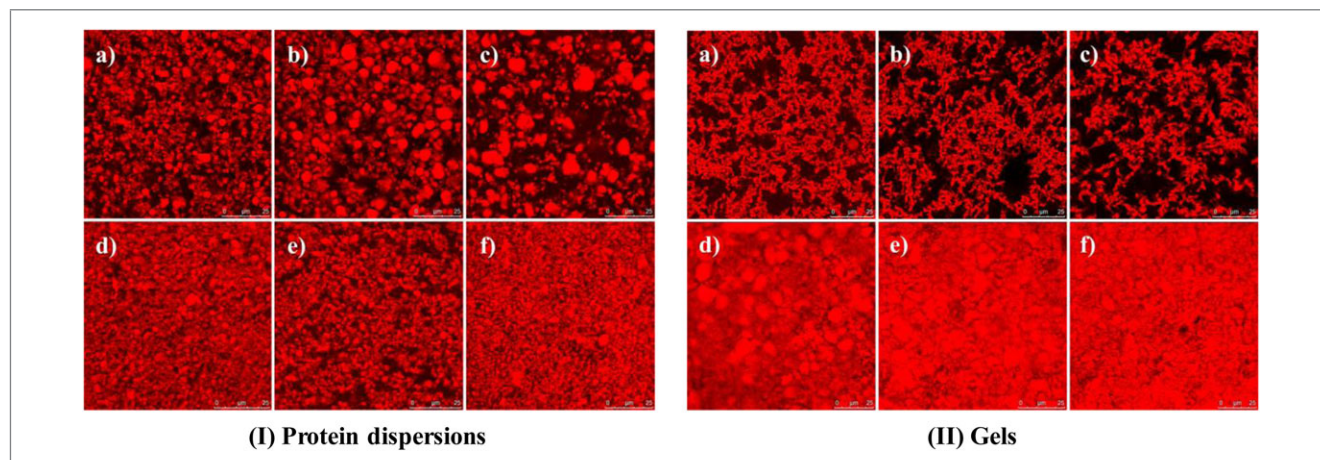


Figure 6—Confocal images of: (I) 30% (w/w) micellized HPI dispersions in 0 mM (a), 50 mM (b), and 300 mM NaCl (c), and isoelectrically precipitated HPI dispersions in 0 mM (d), 50 mM (e), and 300 mM NaCl (f), and (II) 30% (by weight) micellized HPI gels in 0 mM (a), 50 mM (b), and 300 mM NaCl (c), and isoelectrically precipitated HPI gels in 0 mM (d), 50 mM (e), and 300 mM NaCl (f). All samples were at pH = 7.0 (Dapčević-Hadnađev et al., 2018).

have more hydrophobic groups exposed, allowing stronger interactions between proteins with the oil core. On the other hand, the high level of insoluble polysaccharides, which are more interactive with oil rather than water, may be responsible for the good EC of HPM. In fact, due to strong effects of nonprotein materials, HPM showed an even better EC than HPI, especially at low protein concentrations (Malomo et al., 2014). In general, the emulsions formed by HPM and HPI were very stable. The stability of HPC emulsions, due to the molecular flexibility, was protein concentration dependent. The emulsions were stable at lower protein concentrations (10 and 25 mg/mL), but less stable when the protein concentration was increased to 50 mg/mL (Malomo & Aluko, 2015b). This phenomenon can be explained by protein crowding, which will disrupt ordered protein–protein interactions.

Differing from their mixture (whole hemp protein), fractionated hemp protein samples (albumin and globulin) were able to produce emulsions with small particle sizes (500 to 600 nm) over a broad pH range (3.0 to 9.0) (Malomo & Aluko, 2015a). There was no significant difference in emulsion droplet size between the two protein fractions, except at the 50 mg/mL protein concentration where albumin emulsions at pH 7.0 and 9.0 were composed of larger droplets than globulin emulsions. Although both protein fractions were expected to carry more negative charges, which would hinder the construction of an interfacial membrane due to charge repulsions, such electrostatic effects at neutral and alkaline pH were obviously greater on albumin than on globulin proteins.

Furthermore, at a 10 mg/mL protein concentration, the globulin fraction displayed a higher ES at pH 3.0 and 5.0 and a lower value at pH 7.0 than albumin. However, at higher protein concentrations (25 and 50 mg/mL), no remarkable difference in ES was observed between the two protein fractions over the entire pH range tested (3.0 to 9.0). It is worth noting that when compared with SPI, perhaps the generally agreeable plant protein standard, the EA of HPI, was generally much less, as demonstrated by Tang et al. (2006). On the other hand, HPI prepared by alkaline or acid extraction–isoelectric precipitation had a comparable EC to canola protein in spite of a lower ES (Teh et al., 2014).

Film formation

Proteins under appropriate denaturing conditions (heat, acid, base, oxidation, organic solvent, and so on) can develop an extended structure and many can form a cohesive protein film

through hydrogen, ionic, hydrophobic, and covalent bond interactions (Krochta, 2002; Wang, Xiong, Rentfrow, & Newman, 2013). Films with reported good mechanical properties have been made from whey and soy proteins, zein, wheat gluten, and gelatin. Film formation of HPI has been investigated and compared to those produced by SPI (Yin, Tang, Wen, & Yang, 2007). As shown in Figure 7, HPI films exhibited a higher tensile strength and smaller elongation at break. The total soluble mass in HPI films was markedly less than that in SPI films, giving the HPI films a more cohesive and filled microstructure. Disulfide bonds were the predominant interactive force in the network of HPI films, while in the case of SPI film formation was the result of collective contribution of disulfide and hydrogen bonds along with hydrophobic interactions. Having a contact angle values above 65°, HPI films can be considered to possess a hydrophobic surface, which means they are more resistant to water and adaptable to commercialization relative to SPI films.

Structural Modification for Functionality Improvement

As described above, due to its compact structure, hemp protein generally has limited functionality and industrial application when compared to many other food proteins. Therefore, structural modifications to improve the functionality have been conducted. Several physical, chemical, and enzymatic treatments have been applied to hemp protein, of which heat treatment, enzymatic hydrolysis, acylation, and pH shift have demonstrated promising efficacies.

Heat treatment

HPI was heated from 40 to 100 °C for 10 min to modify the protein structure (Raikos et al., 2015). Heating at 80 °C or higher temperatures was found to produce insoluble large molecular aggregates via covalent linkages as could be shown by SDS–PAGE. Approximately 60% to 80% of native protein was converted to an aggregated state. A similar observation was reported by Yin et al. (2008), who heated HPI at 95 °C for 10 min, noting that the thermally treated HPI had a significantly lower protein solubility than untreated HPI at pH 3.0 to 10.0. However, Wang et al. (2018) found that heating at 80 °C up to 60 min actually slightly improved the solubility of HPI (Figure 8). The discrepant findings

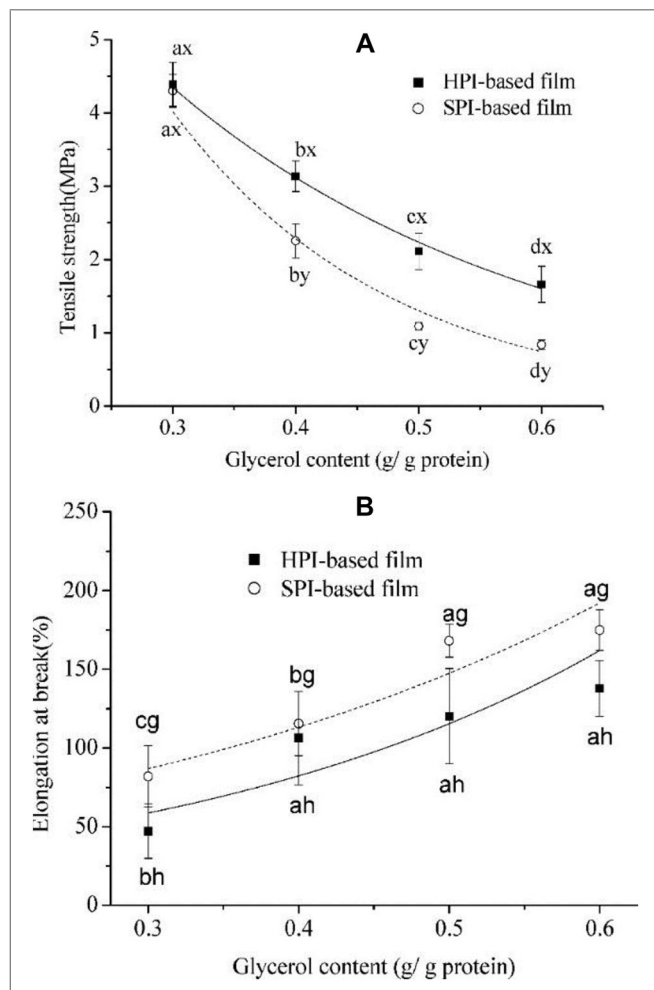


Figure 7—Tensile strength (A) and elongation at break (B) of HPI- and SPI-based films as a function of glycerol concentration (Yin et al., 2009).

may be due to different protein concentration testing methods, as well as the protein composition. Yin et al. (2008) also addressed that heat treatment increased EA of HPI at pH values away from the isoelectric point. The strong correlation between EA and the amount of exposed hydrophobic groups (loss of protein solubility) suggested enhanced protein interactions with oil for particle size reduction. Moreover, heat treatment significantly increased the ES at acidic and alkaline pH values. The heat treatment had no effect on FC of HPI, but it significantly increased foam stability. The lack of a significant impact on foaming activity by heat treatment was attributed to the decreased protein solubility. Additionally, the WHC of HPI was slightly improved by the heat treatment, while the OAC was significantly decreased (Yin et al., 2008). It should be noted that controlling the specific heating conditions, to allow deliberate conformational or structural changes, is crucial to achieving improved functionality for hemp protein. Because studies on the heating effect have been very limited, more research is required to expand the information in this area.

Enzymatic hydrolysis

Peptides produced by limited proteolysis have smaller molecular sizes and reduced spatial structures than the original proteins. Therefore, a low degree of enzyme cleavage can potentially improve hydrophobicity, solubility, and emulsifying properties.

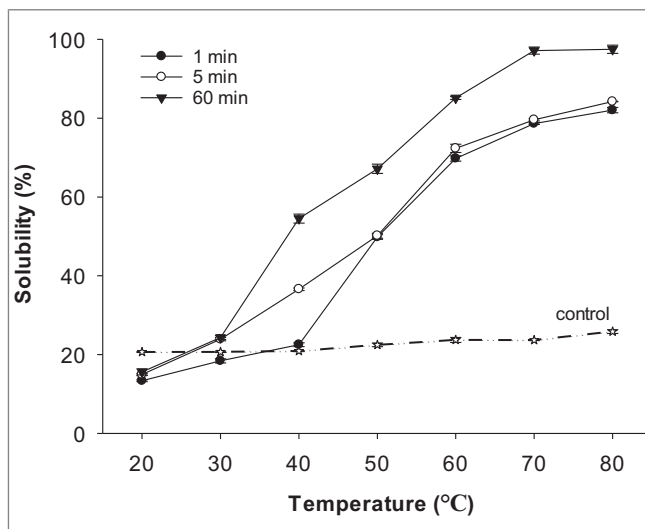


Figure 8—Solubility of HPI samples (pH 7.0) subjected to pH₁₂ shift treatment under different temperatures (20 to 80 °C) for 1, 5, and 60 min. pH₁₂ shift: exposing protein to pH₁₂ (unfolding) then to pH_{7.0} (partial refolding) (Wang et al., 2018).

Several researchers have applied this structure-modifying technique to improve the functionality of HPI (Isinguzo, 2011; Tang et al., 2009; Wang et al., 2009; Yin et al., 2008). Commercial proteases, such as alcalase, flavourzyme, neutrase, protamex, pepsin, and trypsin, are generally used.

Wang et al. (2009) and Teh, Bekhit, Carne, and Birch (2016) confirmed that hydrolysis improved surface hydrophobicity of HPI, but extensive hydrolysis decreased it. Soluble aggregates can form by the hydrophobic association of peptides. Yin et al. (2008) investigated the effect of trypsin proteolysis on the functionality of HPI, and they showed marked improvements of protein solubility at pH 3 to 10, especially for the alkaline pH where up to 85% solubility enhancements were produced. Protein solubility at pH < 6.0 was positively correlated to the DH, coinciding with the increased digestion of insoluble protein aggregates. The production of TCA-soluble peptides depended on the type of protease used as well as the time of hydrolysis (Tang et al., 2009).

The enzymatic hydrolysis led to significant reductions in EA at neutral and acidic pH values (Yin et al., 2008). The effect of the hydrolysis on the EAI at pH 7 was more remarkable than at pH 3 and 5, which is most likely due to the difference in protein solubility at different pH values. In contrast, enzymatic hydrolysis resulted in a significant enhancement of ES at acidic and alkaline pHs, but a decrease at pH 7. WHC and OAC of HPI were significantly reduced following trypsin hydrolysis (Yin et al., 2008). Furthermore, decreases in peptide chain length had a general deleterious effect on FC and FS of HPI. This was consistent with the results obtained by Isinguzo (2011) who used alcalase, pepsin, and thermolysin to hydrolyze HPI. Moreover, as expected, enzyme hydrolysis has an adverse effect on the gelling properties of HPI (Isinguzo, 2011).

Heat treatment under mild alkali conditions has been investigated as a means to aid in enzymatic degradation of HPI (Yin et al., 2008). As reported, pretreatment at 75 or 90 °C had little effect on the early digestion process (0 to 5 hr) by trypsin. However, preheating at 80 °C accelerated the HPI degradation within the first 5 hr when compared with unheated HPI. Yet, for all heat-treated HPI samples, the final protein digests had a

reduced DH when compared with the unheated control. It appears that preheat treatments had a dichotomous role: unfolding certain polypeptide chain segments within the protein structure to allow greater enzyme accessibility to the susceptible peptide bonds, or burying them through thermal aggregation. Hence, to obtain desirable functional properties, the hydrolysis process must be carried out under strictly controlled conditions.

Acylation

Chemical derivatization through acylation of amino acid residues with acetic and succinic anhydrides has been used to improve functional properties of many plant proteins, such as soybean, pea, chickpea, rapeseed, sunflower, oat, peanut, mung bean, wheat, and cotton seed (El-Adawy, 2000). Acylation eliminates the positive charges by substituting hydrophobic acetyl groups for ϵ -amino groups and enables an increased electronegativity, whereas succinylation introduces anionic succinate residues covalently linked to the ϵ -amino groups of lysine residues.

Succinylation, as well as acetylation, has been applied to the structural modification to improve the functional properties of HPI (Yin, Tang, Wen, & Yang, 2009). Acylation caused gradual unfolding of hemp protein at high levels of acetic anhydride (>0.05 g/g) and shifted the isoelectric pH of HPI toward more acidic values. Both acetylation and succinylation had an adverse effect on protein solubility at pH below 5.0, but, conversely, increased the protein solubility above pH 5.0. Succinylation was particularly efficacious: it improved the solubility of HPI from 30% to 85% with 0.1 g/g succinic anhydride. The solubility improvement was attributed to the introduction of negatively charged succinate groups. Similarly, for acetylation, the introduction of electrostatic charges contributed to the improvement of protein solubility. However, extensive acetylation could cause a marked solubility loss due to the formation of edestin aggregates via hydrophobic interactions.

The EA of both acetylated and succinylated HPI samples was found significantly elevated upon acylation, and the impact for succinylation was more pronounced (Yin et al., 2009). The greater exposure of hydrophobic groups in anhydride-treated HPI allows the protein molecules to effectively interact with lipids at the oil-water interface, leading to fine and well-dispersed oil droplets. However, succinylated HPI produced less stable emulsions than control protein, while acetylation had a minimal effect on ES. In addition, *in vitro* trypsin digestion showed that succinylation could improve the digestibility of HPI at succinic anhydride levels of 0.05 to 0.1 g/g, while acetylation exhibited no measurable effect. Overall, succinylation was preferable over acetylation for the modification of hemp protein. Studies also showed that succinylation and acetylation can improve the FC of proteins (El-Adawy, 2000; Lawal, Adebawale, & Adebawale, 2007). According to Lawal et al. (2007), at a broad pH range (4 to 10), the FC of bambarra groundnut protein was markedly enhanced by both succinylation and acetylation, which can be attributed to the increased protein-water interaction caused by the unfolding of proteins by acylation.

pH shift

Based on the principle that high alkaline or low acidic pH levels can disrupt protein intramolecular as well as intermolecular ionic bonds, a conformation-modifying technique known as "pH shift" has been introduced to food processing. In essence, the pH shift is a process that subjects proteins to an extreme pH condition to actuate protein structure unfolding and then to a

neutral environment to induce refolding. It has been successfully applied to improve the functionality of various proteins, including muscle protein (Kristinsson & Hultin, 2003), egg albumen (Liang & Kristinsson, 2007), soy protein (Jiang et al., 2009), and pea protein (Jiang, Zhu, Liu, & Xiong, 2014). Jiang et al. (2009) have reported that pH shift, especially under extreme alkaline conditions (pH 10 to 12), could induce a molten globule structure in both glycinin (11S) and β -conglycinin (7S). Such pH-modified proteins show remarkably improved solubility and EA. Liu, Geng, Zhao, Chen, and Kong (2015) described that moderate heating could complement the acid pH shift effect to promote the unfolding process for soy protein which will facilitate gelation.

The pH shift treatment, aided by controlled heating, was investigated by Wang et al. (2018) to modify the structure of HPI for functionality improvement. The pH₁₂ shift conducted at high temperature (80 °C) instantly increased the protein solubility from approximately 20% to 82% within the first minute, and to ultimately 97% within 60 min (Figure 8). The solubility improvement under such alkaline pH conditions was attributed to the strong charge repulsion (ionized carboxyl groups) and the weakening of hydrogen bonds (heating) between polypeptides, as well as cleavages of some peptide bonds as evidenced by electrophoresis. The enhancement of EA resulted from increased solubility and surface hydrophobicity. Additionally, the emulsion formed by pH₁₂-modified HPI exhibited superior stability with a more uniform oil droplet distribution. Hence, the heating-aided pH shift and mild heating could be a useful method to improve the functionality of hemp protein when carefully conducted. This reported study also addressed the production of lysinoalanine, a potentially harmful amino acid derivative. The content of lysinoalanine in the pH₁₂ shift HPI samples had a very slight increase (from 0.08 to 0.24 mg/g) at low heating temperatures (20 to 50 °C). At 60 °C, there was a rapid formation of lysinoalanine; however, the level did not exceed 1 mg/g if the heating time was kept within 5 min.

Other structure-modifying techniques

Several emerging technologies to modify proteins for functionality improvement are gradually becoming available. Among them, ultrasonication, high hydrostatic pressure, irradiation, filtration, supercritical carbon dioxide, plasma technology, and electric field are attracting considerable attention (Mirmoghataie, Aliabadi, & Hosseini, 2016). In our preliminary study, we applied ultrasound to treat HPI, finding that the protein solubility of HPI increased from 28% to 70% after the treatment by ultrasound at an intensity of 160 W for 30 min. The same ultrasound process produced a 3.2-fold increase in HPI gel strength. Dynamic rheological analysis confirmed that the high-intensity ultrasound can remarkably improve the G' value (results not presented). Although ultrasonication appears to be a promising technology to improve hemp protein functionality, more research is required to explore the most effective processing conditions and to understand how the cavitation works to modify the structure of hemp proteins to maximize the benefit.

Applications in Food Products

Due to hemp's well-recognized nutritional value, food manufacturers have developed a wide range of retail products from hemp, such as nuts, oil, protein flour, energy bars, granola, hemp nut butter, pasta, and ice cream (Leson, 2006). A recent emphasis has been on hemp protein, which is used not only as a nutritive additive but also as a functional ingredient in formulated foods to enhance the product quality attributes. The low allergenicity of

Table 3—Edible uses reported for hemp protein and products.

Application	Reference
Bakery products	
- Hemp flour up to 40% (w/w) can be incorporated into cracker formulation to replace rice flour. - Hemp flour used to replace wheat flour up to 10% (w/w) did not have any negative effect on dough stability or strength, and enhanced nutritional value with increased protein and macro- and microelement contents. - Incorporation of hemp flour resulted in significantly higher specific volume and spread ratio of baked cookies. - Baking loss was reduced when adding hemp protein to wheat flour. - Replacing a portion of starch with hemp flour resulted in the weakening of dough structure, while HPC reinforced the structure. Hemp protein products, especially HPC, resulted in a significant increase in bread volume and limited hardening of the crumb and recrystallization of amylopectin during storage. - The partial replacement of corn flour by hemp flour increased the hardness and nutritional value of biscuits but reduced sensory scores.	Radočaj et al., 2014 Pojić et al., 2015; Apostol et al., 2015 Hrušková and Švec, 2016 Ruban et al., 2016 Korus et al., 2017b
Bread production using hemp flour reduced proofing time for the dough, and also the baking time.	Korus et al., 2017a Lukin and Bitiutskikh, 2017
Extruded products	
- Using hemp powder to replace rice flour increased the bulk density and antioxidant activity of extruded energy bars. - Defatted hemp cake was added to corn grits to make corn snack products.	Norajit et al., 2011 Jozinović et al., 2017
Beverages	
- Bioactive compounds from muscadine grape and blackcurrant juice or muscadine pomace incorporated into hemp flour and HPI provided greater inhibition on L-dopamine oxidation by tyrosinase and microbial proliferation. - HPI can bind with a broad range of polyphenolic compounds in cranberry juice concentrate. - Hemp protein added to muscadine and kale juices significantly suppressed proinflammatory gene expression.	Plundrich et al., 2013 Grace et al., 2013 Grace et al., 2014
Dairy and infant formula	
- Hemp protein added to raw milk before pasteurization decreased syneresis and improved viscosity of yogurt more than pumpkin seed flour, soy and pea protein isolates, and wheat gluten. - Various hemp protein products (hulled or dehulled hempseed, HPC, hemp protein powder, and HPI) can be made into infant formula, including hemp milk and nutritional powder.	Dabija et al., 2018 Wright and Sprague, 2015
Processed meat products	
Adding hemp flour to the recipe of cutlets does not adversely affect the physicochemical quality parameters of semfinished meat products.	Naumova et al., 2017

hemp protein when compared with most of other plant proteins also permits it as a substitute for other proteins in some food products. Table 3 lists food applications of hemp protein products, which are separated into five major categories: bakery products, extruded products, beverages, dairy and infant formula, and processed meat products. The use of hempseed protein products as an alternative to the commonly used casein, whey, wheat, and soy protein is on a rise. For instance, some studies have shown that hemp protein products can be used as value-added ingredients in the production of bread with increased protein and macro- and microelement contents, and lower baking loss and baking time (Korus, Witczak, Ziobro, & Juszczak, 2017b; Lukin & Bitiutskikh, 2017; Pojić et al., 2014). The color of hempseed protein products can range from light tan to dark brown, depending on the pH condition used during processing and the temperature involved in the final product drying. The key to keeping hemp protein competitive in the plant protein market is to assure its nutritional value, functionality, safety, and acceptable sensory characteristics.

Conclusions

Hempseeds, an emerging protein-rich plant material, are becoming an important alternative protein source in the food and nutraceutical industry. Since the interest of consumers worldwide for ingredients derived from natural sources is ever-growing, the demand for hempseed protein is expected to grow rapidly. Although research has made progress in recent years in understanding the chemical composition, nutritional and health benefits, processing properties, and functional behavior of hempseed proteins in food processing, much remains unknown about it. For example, many of the minor protein components present have not been characterized. Therefore, it is clear that more systematic

research is required to explore the structure–functionality relationship of hemp protein, especially its subunits. In spite of its nutritional value, the poor solubility of native hempseed protein impedes its specific functions and applications in food systems. Therefore, structure-modifying technologies must be vigorously explored through scientific research to convert hemp protein into a more soluble and diversely functional protein. Additional research is also needed to investigate the health benefits using molecular, cellular, and animal models. Such basic and applied research is essential to the development of this valuable protein source and broadening its market potential in the food industry.

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Authors' Contributions

Qingling Wang compiled and analyzed the literature and drafted the manuscript. Youling Xiong conceived and designed the study and revised and edited the manuscript.

Abbreviations

THC	tetrahydrocannabinol
AS	acidic subunit
BS	basic subunit
HPI	hemp protein isolate
SPI	soy protein isolate
HPM	hemp protein meal

HPC	hemp protein concentrate
HPH	hemp protein hydrolysate
FAO	Food and Agriculture Organization
WHO	World Health Organization
UNU	United Nations University
PDCAAS	protein digestibility-corrected amino acid score
ACE	angiotensin I-converting enzyme
AChE	acetylcholinesterase
ORAC	oxygen radical-absorbance capacity
DPPH	2,2-diphenyl-1-picrylhydrazyl
DSC	differential scanning calorimetry
WHC	water-holding capacity
FC	foaming capacity
FS	foam stability
OAC	oil absorption capacity
EA	emulsifying activity
EC	emulsifying capacity
ES	emulsion stability

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