Chapter 8
Functional and Bioactive Properties of Hemp Proteins

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Abstract Hemp seeds primarily represent a source of edible oil, which comprises over 30% of the whole seed. However, the role of the hemp seed, as a valuable material for protein extraction, could not be neglected. While the hemp seed is characterized with the protein content of 25%, after oil extraction, the protein content in a hempseed cake, a by-product of the oil extraction process, can increase up to 50%. Hempseed protein mainly consists of a legumin type protein, edestin, which accounts for 60–80% of the total protein content, followed by albumin. Recently, the ability of hemp protein to act as a techno-functional agent in different food applications has been investigated. The role of hemp protein as emulsifiers, foaming agent, gel-forming and biodegradable film-forming material was studied pointing the possibility to replace synthetic agents with the natural ones. Moreover, a large number of studies have revealed a bio-functionality of hemp proteins, i.e. application of enzymatic hydrolysis for the production of bioactive peptides. Bioactivity was mostly investigated by determining antioxidant properties and antihypertensive effects of enzymatic hemp seed protein hydrolysates and their peptide fractions. The hydrolysis was achieved by employing a range of proteases as well as different degrees of hydrolysis, which resulted in significant differences in the antioxidant properties of obtained hemp protein hydrolysates. The present chapter is a review of recent information on hemp protein extraction techniques, with the special emphases to its techno- and bio-functionality.

Keywords Hemp protein · Isolation technique · Hydration · Gelling · Interfacial properties · Bioactive peptides

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Abbreviations

ACE	angiotensin-I-converting enzyme
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPH$^{\bullet}$	2,2-diphenyl-1-picrylhydrazyl radical
DSC	differential scanning calorimetry
HPH	hemp protein hydrolysate
HPI	hemp protein isolate
IEP	alkaline extraction-isoelectric precipitation
MW	molecular weight
OHC	oil holding properties
pI	isoelectric point
S	Svedberg unit
WHC	water holding properties

8.1 Introduction

Investigations on different alternative sources of proteins have experienced a boom in the last decade, due to the growing interest in the economical valorisation of agro-food by-products, along with concerns about food allergies, animal welfare, higher demands for dietary protein due to population increase, as well as the negative impact on the environment associated with the animal-derived proteins (Söderberg 2013; Hadnađev et al. 2017; Pihlanto et al. 2017). However, plant protein sources are still underutilized compared to animal proteins because of their lower nutritional values due to their amino acid profile and the presence of anti-nutrients, which can impair protein digestion and increase consumption toxicity (Pihlanto et al. 2017). Nevertheless, the processed forms of plant-based food (protein concentrates or isolates) are found to have lower levels of anti-nutritional factors such as trypsin inhibitors, alkaloids, lectins, tannins, phytic acid and hemagglutinins than their corresponding raw materials (Arntfield et al. 1985; Rodríguez-Ambriz et al. 2005; Mondor et al. 2009). Moreover, in comparison to animal-derived proteins, plant proteins exhibit poorer physico-chemical and sensory properties such as solubility, bitterness, off-flavour, dark colour which may hinder their maximal utilization (Hadnađev et al. 2017; Pihlanto et al. 2017). Therefore, there has been considerable effort to improve both nutritional and functional properties of plant proteins through the exploitation of innovative processing conditions as well as potential novel applications (Gómez-Guillén et al. 2011; Pihlanto et al. 2017).

Hemp seeds, which are primarily grown for oil production, are rich in proteins. After oil extraction, the protein content in a hempseed cake or meal, a by-product of the extraction process, can increase up to 44%, making this product a valuable material for protein isolation (Pojić et al. 2014). Hempseed meal and obtained

isolates can be incorporated into various food systems, such as bread (Pojić et al. 2015; Korus et al. 2017) to increase its nutritional value and/or to provide specific functional attributes, such as rheological, texture and sensory characteristics. However, most of the studies investigating hemp proteins focus on its functional characteristics and the antioxidant potential of its hydrolysates. In order to improve its functionality and bioactivity, hemp proteins are isolated from hempseed meal using various techniques (Malomo and Aluko 2015b; Hadnađev et al. 2018a) and/or modified by physical (Yin et al. 2008), enzymatic (Yin et al. 2008; Tang et al. 2009a) and chemical (Yin et al. 2009) processes.

This chapter gives an overview of the relationships between the hemp protein structure and physico-chemical properties and its functionality. Strategies to improve hemp protein techno-functional (e.g. gelling, foaming, emulsifying) and bio-functional (e.g. antioxidant activity, cholesterol-lowering) properties by innovative isolation and modification processes will be also discussed.

8.2 Techniques for Hemp Protein Extraction

Extraction of proteins is generally governed by many different parameters such as pH, temperature, duration of extraction, type of solvent/salt used, ionic strength, solid to solution ratio, particle size of starting raw material, etc. In order to optimize protein yield, different protein extraction procedures have been developed (Singhal et al. 2016). While dry processing (air classification) has been widely used to separate protein fraction from some legume crops, due to high fat content, hemp proteins are typically extracted using wet processing. The wet extraction processes which are being exploited in the preparation of protein-rich material (isolates or concentrates) include alkaline extraction-isoelectric precipitation, salt extraction (micellization) and ultrafiltration.

8.2.1 Alkaline Extraction-Isoelectric Precipitation

One of the most widely used protein extraction technique is alkaline extraction-isoelectric precipitation (IEP) procedure. This procedure involves several steps and has been commonly used for oilseed and legume sources. First step is alkaline extraction procedure under which proteins are dissolved. It is assumed that at pH higher than pI protein-water interactions are promoted and dispersibility and solubility of protein is improved (Singhal et al. 2016). In order to facilitate the hemp protein extraction procedure, Tang et al. (2006) proposed a previous defatting step of hemp seed meal. After alkaline extraction, insoluble materials such as insoluble fibres, carbohydrates and insoluble proteins are removed by centrifugation step. The pH value of the obtained supernatant is adjusted to pI value of extracted protein (cca 5.0 for hemp proteins). Around the pI value proteins tend to aggregate via van der

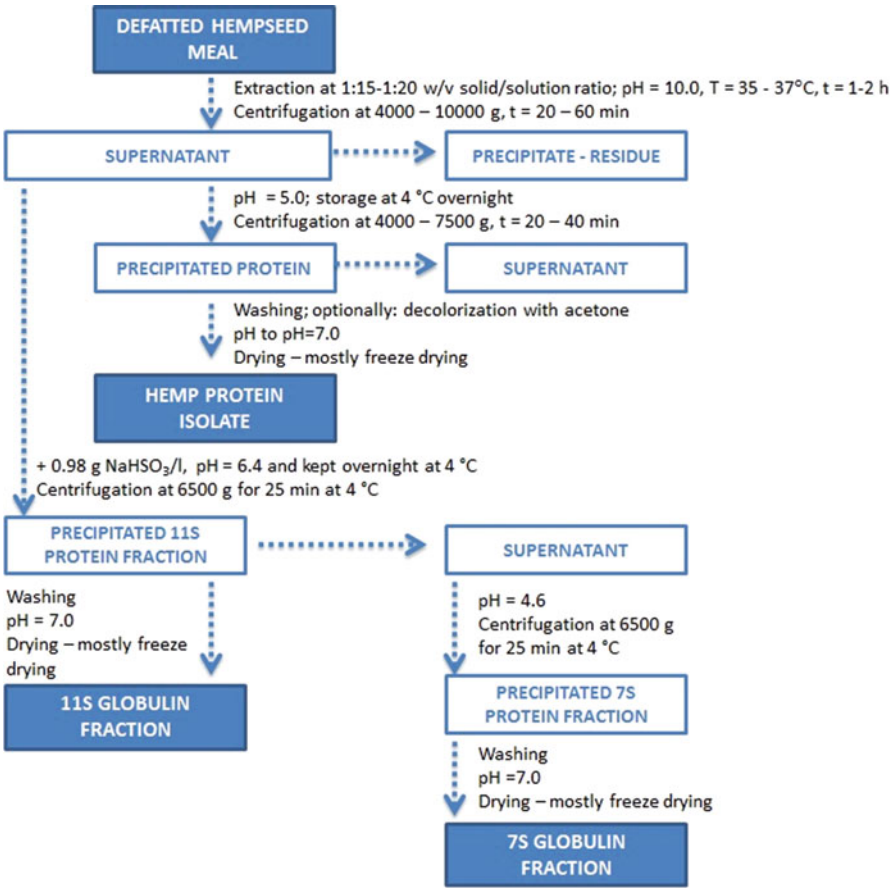

Fig. 8.1 Flow chart depicting the steps of hemp protein extraction by alkaline extraction-isoelectric precipitation technique and fractionation into 11S – legumin-type globulins and 7S – vicilin-type globulins. Starting material and obtained products are presented in text boxes, while the overview of the used procedures is listed along the arrows. *T* temperature, *t* time

Waals forces and hydrophobic interactions which are now more dominant than protein-water interaction resulting in protein precipitation from the solution (Singhal et al. 2016). After the precipitation, centrifugation procedure followed by several washing steps is commonly employed. Consequently, the obtained precipitate is optionally dispersed in distilled water and its pH value is adjusted to 7.0. The final step involves drying of obtained protein precipitate; most commonly by freeze drying technique (Fig. 8.1).

In order to fractionate hemp globulin protein, Wang et al. (2008) proposed the addition of $NaHSO_3$ in supernatant obtained after alkaline protein extraction and after centrifugation procedure (Fig. 8.1), followed by pH adjustment to 6.4 and precipitation. The obtained precipitate is referred to 11S protein fraction whereas the

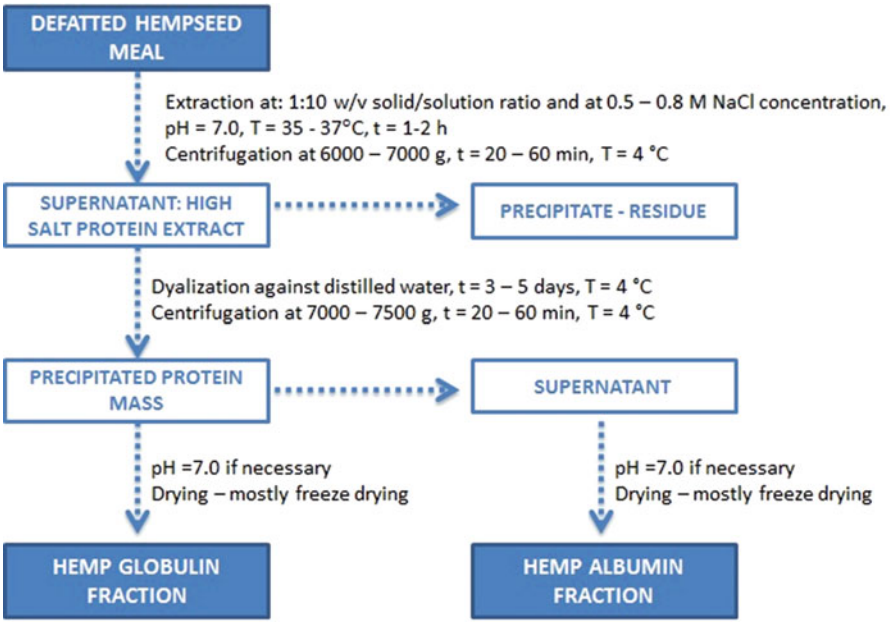

Fig. 8.2 Flow chart depicting the steps of hemp protein extraction by salt extraction (micellization) technique and fractionation into globulin and albumin fractions. Starting material and obtained products are presented in text boxes, while the overview of the used procedures is listed along the arrows. *T* temperature, *t* time

residual supernatant represents dissolved 7S protein fraction. The precipitation of 7S protein fraction is generally conducted by adjusting to pH = 4.6, followed by centrifugation step.

In order to optimize protein extraction procedure concerning the protein yield, purity, quality, etc., different approaches were discussed and different extraction conditions are summarised in Figs. 8.1 and 8.2. Zhang et al. (2008) found that optimum extraction conditions, obtained by response surface methodology, were T = 39.58 °C, extraction pH = 9.27, ratio of liquid to solid 24.22 and time 0.5 h, precipitation pH = 5.0. Under these conditions 96.4% of protein was precipitated from supernatant, the obtained protein yield was 52.3% and protein content was 83.8%. According to Hadnađev et al. (2018a) hemp proteins were extracted at pH = 10.0 at 1:20 w/v solid/solution ratio, the obtained supernatant was precipitated at pH = 5.0 and the obtained precipitate was washed several times in order to remove soluble molecules. The authors recovered 50.6% of proteins found in starting hemp meal and the obtained protein content was 91.4%. Karaca et al. (2011), who investigated the isolates prepared from legume sources, obtained about 85% of overall protein content using this extraction procedure. Moreover, Malomo et al. (2014) obtained 37.9% of hemp protein yield, whereas Tang et al. (2006) reported a recovery of 73% protein relative to total protein content in hemp meal which can be related to different shearing history of raw material during pressing/extracting of oil,

milling, etc. According to Malomo and Aluko (2015a) different mechanical stresses can influence protein/protein interaction resulting in decreased protein solubility and thus lower protein recovery in extraction process.

8.2.2 Salt Extraction (Micellization)

Salt extraction technique known as micellization or "salting in – salting out" procedure has been also employed for protein extraction from different seed sources. In comparison to IEP procedure, salt extraction results in protein isolates with more preserved native state (Murray, et al. 1979). This method involves a separation of globulin proteins from albumins fraction based on their different solubility. At low level of salts protein-water interactions is promoted resulting in solubility of globulin protein fraction. The most commonly used salts are $(NH_4)_2SO_4$ and NaCl. Sodium chloride solution at a concentration of 0.3–0.5 M is the most frequently used solution for this extraction procedure (Singhal et al. 2016). Salt extraction procedure generally involves: (i) protein extraction using a salt solution (salting-in step), followed by centrifugation step to remove insoluble material and (ii) precipitation of the extracted proteins in supernatant (Arntfield et al. 1985; López and Ordorica-Falomir 1986; Hadnađev et al. 2018a), which can be conducted using two different approaches (salting-out step). First method is to dilute supernatant with water in order to reduce ionic strength and second is to use dialysis to remove salts. The dilution method involves the addition of large quantity of water which has to be removed after protein precipitation. Therefore, the advantage is given to dialysis method which is easier to perform. According to Malomo and Aluko (2015a) and Hadnađev et al. (2018a) hemp proteins have been extracted at 1:10 w/v (solid to solution ratio) and at 0.5 M NaCl and 0.8 M NaCl solution concentration, respectively, followed by dialysis using ultrafiltration cellulose membranes during 3–5 days at 4 °C. The precipitated proteins referring to globulin fraction were centrifuged and the obtained supernatant represents albumin protein fraction (Malomo and Aluko 2015a). Summarized conditions for salt extraction technique is presented in Fig. 8.2.

According to Alsohaimy et al. (2007) ammonium sulphate precipitation of legume proteins resulted in higher protein content in comparison to IEP procedure. However, study performed by Karaca et al. (2011) showed opposite trend according to which alkaline extraction procedure resulted in higher protein levels in comparison to salt extraction technique of legume proteins. Hadnađev et al. (2018a) prepared protein isolates from hempseed meal using IEP and salt extraction techniques. The latter method resulted in lower yield (19.24%) and higher protein content (98.87%) in comparison to the former method (yield – 24.24%, protein content – 91.44%). The lower yield can be related to higher selectivity of salt extraction technique towards a particular protein fraction (globulin) in comparison to more generic procedure of IEP. The yield of protein isolate relative to total protein in hemp meal obtained for salt extraction was 40.17%, which was lower in comparison to IEP (50.6%).

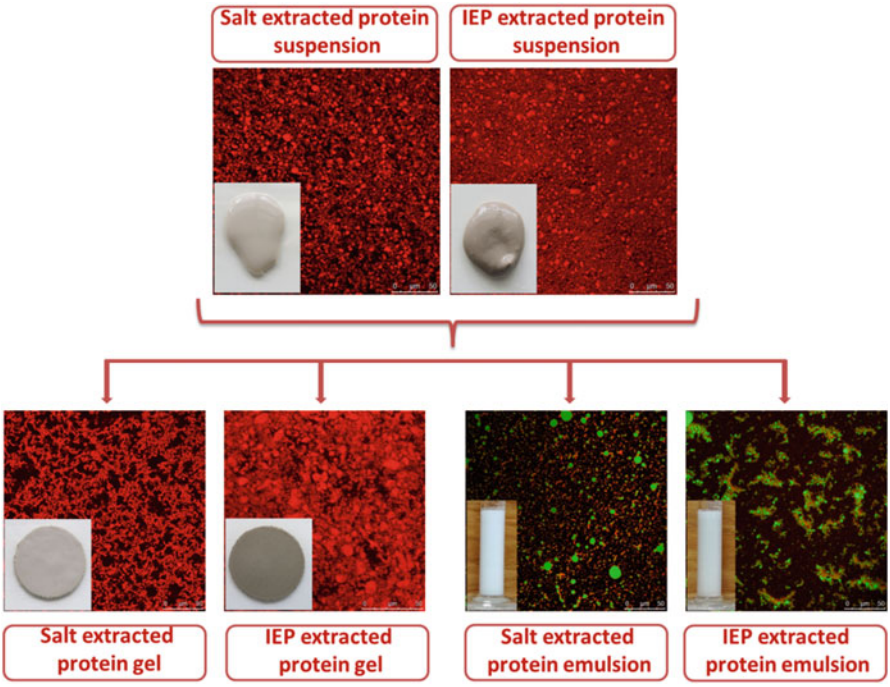

Fig. 8.3 Confocal laser scanning micrographs and photographs of 30% aqueous suspensions, 30% heat-induced gels and 10% sunflower oil-in-water emulsions (at pH = 3.0, emulsifier concentration = 1.5% calculated on continuous phase) prepared with alkali extracted-isoelectric precipitated (IEP) and salt extracted hemp proteins

Although both IEP and salt extraction procedure were characterized with high protein content, a higher purity for isolates obtained by micellization technique was observed by different authors (Mwasaru et al. 1999; Hadnađev et al. 2018a). Salt extraction procedure resulted in stronger protein/protein interactions resulting in exclusion of non-proteinaceous fractions. Moreover, the IEP procedure favoured co-extraction of phenolics from starting hemp meal material, resulting in dark green colour of obtained isolates and higher total phenolics in comparison to isolates prepared by salt extraction (Hadnađev et al. 2018a). Milder extraction conditions of micellization technique resulted in lower co-extraction of seed phenolics and obtained isolates were significantly lighter (Fig. 8.3).

8.2.3 Ultrafiltration

Ultrafiltration represents technique which involves implementation of different membranes and pressure as a driving force for protein separation (Singhal et al. 2016). Fredrikson et al. (2001) and Fuhrmeister and Meuser (2003) concluded that

protein isolates obtained by membrane separation procedure exhibited higher functionality, whereas Singh (1988), Waggle et al. (1989) and Mondor et al. (2009) showed that those protein isolates had decreased level of anti-nutritional properties such as protease and amylase inhibitors, lectins, etc. The main principle of this method is that supernatant obtained after alkaline or acidic extraction is subjected to ultrafiltration or diafiltration in order to isolate proteins. Generally, extraction procedure involving ultrafiltration/diafiltration gave better results concerning protein content for different legume seed material in comparison to IEP (Vose 1980; Boye et al. 2010). According to Papalamprou et al. (2009, 2010) and Kiosseoglou and Paraskevopoulou (2011), ultrafiltration resulted in different protein composition of obtained proteins rather than one prepared using IEP procedure in terms that these protein isolates comprised of both albumin and globulin fraction while isolates obtained by IEP method contained only globulin fraction. Malomo and Aluko (2015b) treated hemp seed meal characterized with 37% protein content with different enzymes and the obtained digest was subjected to membrane ultrafiltration. As a result a membrane ultrafiltration protein concentrate had 74% protein content characterized by increased digestibility and solubility in comparison to isoelectric protein isolate and commercial protein concentrate.

8.3 Hemp Protein Structure and Physico-chemical Properties

Physico-chemical properties of proteins influence its quality and potential application and they are highly related to its structure. Structural features of hemp proteins have been studied by different techniques such as electrophoretic analysis (Tang et al. 2006; Wang et al. 2008; Yin et al. 2008; Malomo et al. 2014; Raikos et al. 2015; Malomo and Aluko 2015a, b; Hadnađev et al. 2018a), differential scanning calorimetry (DSC) (Tang et al. 2006; Wang et al. 2008; Yin et al. 2008; Hadnađev et al. 2018a), Fourier transform infrared spectroscopy (Hadnađev et al. 2018a), spectrofluorimetry (Malomo et al. 2014, 2015; Malomo and Aluko 2015a), circular dichroism (Malomo et al. 2014; Malomo and Aluko 2015a), size exclusion chromatography (Yin et al. 2008; Malomo et al. 2015), real-time qPCR (Ponzoni et al. 2018), mass spectrometry (Aiello et al. 2016), viscoelasticity (Dapčević-Hadnađev et al. 2019a), confocal microscopy (Dapčević-Hadnađev et al. 2019a).

It was shown that the majority of hemp proteins are globulin and albumin fractions, where globulins comprise up to 85% and albumins constitute about 15% of the total hemp protein (Tang et al. 2006; Wang et al. 2008). The salt soluble globulins can be further divided into 11S (S = Svedberg Unit) legumin-type globulins and 7S vicilin-type globulins (Singhal et al. 2016; Ponzoni et al. 2018). The 11S fraction of hemp proteins is called edestin. Ponzoni et al. (2018) reported detailed information on hemp storage protein genes and their genomic organization.

Their results demonstrate that two 2S albumin, one 7S vicilin-like, and six edestin genes are present in the hemp genome.

The 11S edestin is the main storage protein representing approximately 80% of the total seed protein (Tang et al. 2006; Wang et al. 2008). It is a hexamer (MW of ~300 kDa) composed of six identical subunits joined by non-covalent interactions. Each subunit pair consists of an acidic and basic chain linked by a disulfide bond (Patel et al. 1994; Kim and Lee 2011). The SDS-PAGE profiles, under non-reducing conditions, have indicated a major band of about 52 kDa corresponding to acidic-basic subunits. Upon adding 2-mercaptoethanol (reducing conditions), the disulphide bond between the acidic and basic subunits is disrupted. While acidic subunit (~34.0 kDa) is relatively homogenous, basic subunit (~18.0 and 20.0 kDa) consists of two bands (Wang et al. 2008; Raikos et al. 2015). According to amino acid composition Ponzoni et al. (2018) identified three edestin types (type1, −2 and −3). All edestin types showed typical 11S globulin features and were very rich in arginine and glutamic acid, but edestin type3 was the richest in cysteine and methionine.

Separation of the hemp protein in the 11S and 7S fractions using pH shift (pH = 6.4 and 4.6, respectively) and comparison to hemp protein isolate (HPI) precipitated at pH = 5.0 has indicated that 11S fraction showed similar SDS-PAGE profile to that of HPI. On the contrary, in the case of 7S fraction, the corresponding acidic-basic units (band of about 52 kDa) were almost absent (non-reducing conditions), and the relative content of the subunit with MW of ~48.0 kDa (reducing conditions) increased by almost fivefold relative to that of HPI (Wang et al. 2008). Ponzoni et al. (2018) indicated that Cs7S might be the gene that encodes the 7S minor polypeptide of approximately 48.0 kDa, which corresponds to the SDS-PAGE band reported by Wang et al. (2008).

Tang et al. (2006) and Wang et al. (2008) have found that water-soluble protein 2S albumin constitutes about 13% of the total hemp protein. According to Malomo et al. (2014), a minor band at SDS-PAGE profile of HPI with less than 14 kDa in size is likely to correspond to the albumin fraction. Gel electrophoresis, as well as intrinsic fluorescence and circular dichroism data, have indicated that the albumin is characterized with less disulfide bonds and hence a more open (flexible) structure in comparison to globulins (Malomo and Aluko 2015a).

According to the amino acid profiles of hemp seed products, hemp proteins contain a high amount of arginine (94–128 mg/g protein) (House et al. 2010), relative to other oilseeds, such as soybean (73.5 mg/g protein) (Wang et al. 2008). Hemp protein also represents a good source of the sulfur-containing amino acids methionine and cystine (15.5 mg/g protein, in comparison to 9.6 mg/g protein detected in soybean protein) (Callaway 2004; Wang et al. 2008). Compared to FAO/WHO reference protein for children 2–5 years of age, lysine is the first limiting amino acid in hemp protein, followed by leucine and tryptophan. The limitation in the lysine content of hemp protein results in its low amino acid scores (0.50–0.62) which are comparable to those of cereals (0.44 for whole wheat), but significantly lower relative to oilseeds (1.05 for soybean) (House et al. 2010). However, in vivo hemp protein digestibility, determined using casein (digestibility = 97.6%) as a

reference standard, ranged between 85.2% and 86.7% (House et al. 2010), which was in a good agreement with in vitro digestibility (measured by nitrogen release) of hemp protein isolates (88–91%) (Wang et al. 2008). In general, hemp proteins are described as easily digested (Aiello et al. 2016).

It has been shown that separation of hemp protein into different fractions can significantly improve its amino acid profile. For example, the globulin fraction has a higher content of sulfur-containing amino acids, especially methionine, as well as aromatic amino acids and branched chain amino acids when compared to albumin fraction (Malomo and Aluko 2015a). Comparing two globulin fractions, edestin (11S globulin) is nutritionally superior protein in terms of essential and conditionally essential amino acids content than 7S globulin since it is characterized with higher content of methionine, arginine and tyrosine (Wang et al. 2008).

Differential scanning calorimetry (DSC) has shown that, unlike 7S protein fraction, edestin expressed ordered structure (Wang et al. 2008). DSC results were also highly impacted by isolation technique. Salt extracted isolates exhibited significantly higher structural order and thermal stability than IEP isolates which reflected the higher enthalpy and peak denaturation temperature of the former (Hadnađev et al. 2018a). While milder extraction conditions during micellization do not cause any irreversible changes in protein structure, hence allowing proteins to maintain their native conformation, highly alkaline conditions during IEP protein extraction led to extensive protein denaturation (Arntfield et al. 1985; Hadnađev et al. 2018a). In comparison to proteins isolated from other oilseeds, the enthalpy values of the hemp seed isolates (9.4–11.9 J/g protein) recovered by IEP technique (Tang et al. 2006; Wang et al. 2008; Yin et al. 2008; Hadnađev et al. 2018a) were close to that reported for canola (9.66 J/g protein) and soybean (14.23 J/g protein), but higher than that obtained for flaxseed (8.25 J/g), peanut (6.26 J/g) and pumpkin (5.1 J/g) protein preparations isolated with the same technique (Murray et al. 1985; Liu et al. 2011; Rezig et al. 2013; Kaushik et al. 2016). Hemp protein isolates obtained by the salt extraction also exhibited denaturation enthalpy values comparable to those found for canola (24.06 J/g protein) and soybean (24.56 J/g protein) micellar protein isolates, and almost two times higher than that reported for native peanut protein extracted by ammonium sulfate (12.4 J/g) and salt extracted pumpkin seed proteins (12.6 J/g) (Murray et al. 1985; Liu et al. 2011; Rezig et al. 2013).

Protein surface composition (presence of polar/non polar amino acids), together with different environmental conditions (pH, temperature, ionic strength and the type of ions present in the solution) greatly influence protein solubility. This physico-chemical property plays a key role for protein food applications as functional properties such as gelling, emulsification, foaming are closely related to protein solubility (Singhal et al. 2016). In general, proteins exhibit minimum solubility at their isoelectric point (pI) where they form aggregates and precipitate due to zero net surface charge. However, at pH values higher or lower that the pI, proteins possess negative or positive net charge which influence the formation of repulsive inter-molecule forces and increase in their solubility (Singhal et al. 2016). The solubility profiles of hemp protein isolates obtained by IEP and micellization were found to be the lowest between pH 5.0 and 6.0 (Tang et al. 2006; Yin et al. 2008;

Hadnađev et al. 2018a). The study by Hadnađev et al. (2018a) showed higher solubility at acidic conditions of hemp isolates prepared by the salt extraction method as compared to ones prepared by IEP method. This was ascribed to preparation conditions which in the case of IEP method favoured higher co-extraction of phytic acid and, consequently, formation of insoluble phytic acid-protein complexes at pH bellow pI (Hadnađev et al. 2018a). In general, hemp globulins exhibit low solubility in comparison to other vegetable proteins, such as soy protein (Tang et al. 2006), which is attributed to high free sulfhydryl content from sulfur-containing amino acids which favour the formation of covalent disulfide bonds between individual proteins and subsequent aggregation at neutral or acidic pH (Pihlanto et al. 2017). Due to reduced level of aromatic and hydrophobic amino acids and less rigid conformational structure, hemp albumins are characterized with higher solubility compared to the globulins (Malomo and Aluko 2015a). It was shown that hemp globulin solubility can be significantly increased by limited or extensive enzymatic hydrolysis (Yin et al. 2008) and succinylation or acetylation at low anhydride levels (Yin et al. 2009).

8.4 Techno-functionality of Hemp Protein

Apart from basic physicochemical properties, such as composition parameters, structural features, solubility, colour, taste, etc., the most important techno-functional properties of hemp proteins can be divided into: (i) properties associated with their gelling behaviour, i.e. water binding capacity, gel formation, thickening, (ii) properties related to their surface behaviour, which include emulsion and foam formation and stabilisation, and (iii) film-forming properties.

8.4.1 Gelling, Water and Oil Holding Properties

Water holding capacity represents the amount of water entrapped in the protein matrix, i.e. the ability of the protein to prevent water loss from its hydrated three-dimensional structure after applying centrifugal force (Ly et al. 1998). The water (WHC) and oil (OHC) holding properties of proteins are important in formulation of food products, its quality, shelf life and consumer acceptability since they influence product texture and mouth feel (Singhal et al. 2016). WHC and OHC values of hemp proteins have been determined by different authors (Tang et al. 2006; Teh et al. 2014; Malomo et al. 2014; Hadnađev et al. 2018a) and fall in the range of 0.80–12.01 g/g and 1.62–13.70 g/g, respectively, suggesting that both protein isolation procedure and method used to determine WHC influence values. Namely, as there are no standard methods for WHC and OHC determination, the results among studies are largely influenced by centrifugation force, time allowed for hydration, absorption, etc. Hadnađev et al. (2018a) reported that IEP protein isolates

had higher WHCs than the ones prepared by micellization procedure. However, no significant differences in OHC were observed between the isolates produced by different techniques (Hadnađev et al. 2018a). Higher WHCs values of proteins isolated by IEP were ascribed to partial denaturation of the protein during extraction which led to extensive conformational changes and exposure of polar amino acid side chains and, consequently, increased water uptake through capillary mechanisms and formation of hydrogen bonding between the exposed amino acid residues and water molecules (Hadnađev et al. 2018a).

According to Tang et al. (2006) hemp protein isolates showed significantly lower water absorption capacity in comparison soy protein isolates. This could be explained by the fact that the polar groups of the proteins are found in the interior of the aggregates resulting in lower WHC values of hemp protein isolates in comparison to soy proteins. However, oil holding capacity was almost the same as of the soy protein isolates suggesting that surface hydrophobic groups can interact with lipids causing higher OHC values. Concerning the flax seed and canola proteins, Teh et al. (2014) revealed that hemp protein isolates demonstrated the highest WHC as well as OHC values especially in comparison to flax seed proteins. Moreover, hemp and canola protein isolates expressed higher WHC and lower OHC values in comparison to hemp and canola seed meal suggesting that alkaline or acid extraction resulted in conformational changes of hemp and canola proteins leading to more pronounced hydrophilic surface and WHC values than the starting raw material.

Formation of more or less ordered macroscopic structures, such as gels, is governed by the tendency of proteins to aggregate or to form intermolecular cross-links on heating and change of pH (Caetano da Silva Lannes and Natali Miquelim 2013). In order to measure the least gelation concentration of hemp products, Malomo et al. (2014) placed samples of different concentrations in water bath at 95 °C and left them in refrigerator for 14 h. The concentration of protein samples at which the gel was formed, i.e. it did not slip by inverting the tube was referred to the least gelation concentration. The authors revealed that gelation capacity of hemps seed meal was 12%, whereas for hemp protein isolate was 22%. This was most likely attributed to poor gel-forming ability of hemp protein isolates probably caused by significant protein aggregation in IEP procedure resulting in reduced protein flexibility needed for network structuration. However, hemp meal which was not treated by alkaline/acid was not affected by protein aggregation and consequently had lower least gelation concentration values (Malomo et al. 2014).

Dapčević-Hadnađev et al. (2019a) compared gelling properties of hemp protein isolates obtained by alkaline extraction-isoelectric precipitation and salt extraction procedure at 30% protein concentration. At lower protein concentrations, isolates obtained by salt extraction gave low viscous dispersions which did not built homogenous gels upon heating. Alkaline extraction procedure resulted in protein gels that upon heating had initially higher storage modulus values and less pronounced drop in system stiffness in comparison to salt extracted proteins. This could be attributed to lower dissociation of edestin subunits during heating since the part of dissociation had already taken place in more aggressive alkaline extraction procedure.

Subsequent heating, however, resulted in gels of similar strengths, i.e. they had similar values of elastic modulus. The gels of differently isolated proteins were also prepared by pouring protein dispersions in hermetically sealed disc shape vessel and immersing them in oil bath at 120 °C which enabled fast gel formation. According to Dapčević-Hadnađev et al. (2019a) slight frequency dependence of both moduli, which is typical for gel-like behaviour, was observed. Moreover, according to strain sweep measurements earlier decrease in yield stress values was observed for gels prepared by salt extracted proteins in comparison to gels obtained by alkaline extraction. This could be a result of weaker transient network structure of those gels. Generally, both gels were characterized by low tan δ values (<0.4) indicated that they built true gel structure. Confocal micrographs (Fig. 8.3) have shown that IEP isolated protein gel networks were characterized by fairly large globular entities, whereas salt extracted protein gels exhibited a finer network microstructure.

8.4.2 Interfacial Properties

One of important techno-functionalities of proteins is surface activity, i.e. ability to reduce the surface tension at interface between a solid, liquid or gas phase. Surface phenomena play a key role in food processing and knowledge of colloidal interactions at interface represents important prerequisite for controlling food physical properties, such as stability (Caetano da Silva Lannes and Natali Miquelim 2013).

Emulsions and foams as dispersed systems are often present in food. Proteins are widely used as functional ingredients for the formation and stabilisation of emulsions and foams, due to their propensity to concentrate at most interfaces. Protein surface properties are based on the presence of both polar or hydrophilic, and non-polar or lipophilic groups. During the emulsion and foam formation, they migrate toward surface, adsorb and form a film at the surface of the oil droplets or gas bubbles, thus protecting the system against destabilisation (Rouimi et al. 2005; Caetano da Silva Lannes and Natali Miquelim 2013).

8.4.2.1 Emulsifying Properties

Unlike small molecular weight emulsifiers, proteins have the ability to act as both emulsifier and stabilizer since they have high tendency to adhere to the oil–water interfaces and form viscoelastic films at the surface of the oil droplets providing electrostatic and steric stabilization (Rouimi et al. 2005; Tcholakova et al. 2006; Lam and Nickerson 2013).

The emulsifying properties of food proteins are mostly evaluated by measuring: (i) emulsion activity, i.e. the maximum interfacial area per unit mass of protein in a stabilized solution; (ii) emulsion capacity, i.e. the maximum amount of oil per unit mass of protein that can be emulsified prior to a phase inversion taking place, (iii) emulsion stability, i.e., the capacity of a protein to form an emulsion that remains

unchanged for a certain time period at a specific conditions (Amarowicz 2010; Lam and Nickerson 2013).

According to the values of emulsion activity and stability indexes Tang et al. (2006) concluded that emulsifying activities of hemp protein isolate are poor, when compared to the once of soy protein. Both proteins exhibited the highest emulsifying activity indexes at pH = 3.0 which was attributed to their high solubility at that pH value. Studies concerning emulsifying properties of hemp protein prepared by IEP, as well as globulin and albumin fractions prepared by salt extraction revealed that all the fractions can form emulsions with small particle sizes ($d_{3,2}$ less than 0.6 μm at protein concentration 50 mg/ml) which are comparable to ones observed for whey protein isolate and lower in comparison to other plant proteins (Malomo et al. 2014; Malomo and Aluko 2015a). In the range of pH 3.0–9.0, IEP extracted isolate exhibited the lowest emulsification ability at pH 3.0 where higher solubility value was recorded (Malomo et al. 2014) which was in contrast to a report of Tang et al. (2006). On the contrary, hemp globulin fraction obtained by salt extraction did not express pH-dependant emulsion droplet size, while albumin stabilized emulsions had higher droplet sizes at pH 7.0 and 9.0 as measured at protein concentration of 50 mg/mL. All the studies reported that emulsion stabilities were nearly unchanged with pH. The differences between the studies (Tang et al. 2006; Malomo et al. 2014; Malomo and Aluko 2015a) could be ascribed to the differences in emulsion preparation. While Tang et al. (2006) prepared emulsions containing 1 part of oil in 3 parts of aqueous phase with 0.2% (w/v) proteins, emulsions investigated by Malomo et al. (2014) and Malomo and Aluko (2015a) had 1 part of oil in 5 parts of aqueous phase containing 1–5% (w/v) of proteins. Dapčević-Hadnađev et al. (2019b) studied the interfacial and emulsifying properties of hemp proteins extracted with IEP and micellization, in order to evaluate the differences in their ability to stabilize 10% oil-in-water emulsions at pH = 3.0. They have shown that emulsification mechanism of two hemp proteins was influenced by the differences in their denaturation degree which contributed to the alteration in their solubility, adsorption rate and conformational flexibility at the interface. While salt extracted protein formed emulsions with small droplets and enough droplet-droplet static repulsion, in the emulsions stabilized by IEP extracted protein, isolation technique favoured pH-induced structural unfolding of protein molecules, exposure of hydrophobic sites and sulfhydryl groups and subsequent droplets bridging flocculation (Fig. 8.3) (Dapčević-Hadnađev et al. 2019b).

Although enzymatic hydrolysis is considered a powerful tool in the modification of emulsifying characteristics of proteins (Amarowicz 2010), Yin et al. (2008) have shown that limited enzymatic hydrolysis with trypsin led to significant decline in emulsifying activity index at neutral and acidic pH values, relative to the hemp protein isolate. On the contrary, it was shown that hemp protein physical modification (thermal treatment) or chemical modification by acetylation or succinylation can led to increase in its emulsifying activity index (Yin et al. 2008, 2009). The improvement of emulsifying activity index by thermal treatment or acylation is attributed to unfolding of protein molecule and subsequent exposure of hydrophobic groups initially buried in the interior of protein molecules (Yin et al. 2008, 2009).

8.4.2.2 Foaming Properties

Proteins are considered to be good foaming agents since they can strongly adsorb at the gas-liquid interface, provide steric and electrostatic stability, form films with cohesive structure and a high module of rheological interface as a result of the interactions between the adsorbed molecules. Their adsorption at the gas-liquid interface is facilitated by the presence of hydrophobic regions due to non polar amino acids (Caetano da Silva Lannes and Natali Miquelim 2013). Foaming properties are mostly characterized by measuring: (i) foaming capacity, i.e. increase in volume of the protein dispersion upon mixing and (i) foam stability, i.e. the percentage of foam remaining after certain period of time. Higher foaming capacities of hemp proteins were recorded at lower pH values, which was attributed to their higher solubility at those pHs, as well as greater exposure of aromatic groups leading to enhanced protein–protein interactions and formation of cohesive viscoelastic interfacial membranes. However, the pH value did not influence foam stability (Malomo et al. 2014). It was also revealed that hemp albumins have higher foaming capacity than hemp globulin due to higher solubility of the former which enable their diffusion and unfolding at the air-water interface to encapsulate air bubble. However, globulins had higher foam stability than albumins which may be attributed to higher contents of hydrophobic amino acids in globulin fraction, which enhance protein-protein interactions and formation of a strong interfacial viscoelastic membrane (Malomo and Aluko 2015a). Hemp protein physical modification via heat treatment did not affect foaming capacity, while it influenced an increase in foam stability. On the contrary, enzymatic modification with trypsin led to decreases in both foaming capacity and foam stability indicating the role of the polypeptide chain length in foam stabilization (Yin et al. 2008).

8.4.3 Film-Forming Properties

Edible films either formed as food coatings or free-standing films are defined as a thin layer of material which can be consumed and provides a barrier to food from moisture, oxygen and solute migration (Bourtoom 2008). Different biological compounds such as polysaccharides, proteins, lipids and their derivatives can be used for the biopolymer films and coatings preparation (Yin et al. 2007). Proteins are characterized by the ability to form continuous network and wide range of different proteins (wheat gluten, maize zein, soy proteins, gelatine, collagen, egg proteins, pea proteins, milk proteins, etc.) have already been investigated for this purposes (Bourtoom 2008).

Yin et al. (2007) compared cast films obtained from hemp protein isolate and from soy protein isolate in terms of moisture content, total soluble mass, tensile strength and elongation at break as well as surface hydrophobicity. The amount of glycerol which served as plasticizer and its influence on abovementioned parameters

were also monitored. At certain glycerol amounts, films prepared with hemp protein isolates expressed similar moisture content, but much less total soluble mass and elongation at break, higher surface hydrophobicity as well as higher tensile strength in comparison to films prepared by soy protein isolates. Moreover, surface hydrophobicity increased with plasticizer amount increase. According to protein films solubility in different solvents as well as to free sulfhydryl group content it was estimated that disulfide bonds were the most dominant force in the formation of films prepared from hemp protein isolates, whereas in the formation of soy protein films hydrogen bonds and hydrophobic interactions were also employed. The obtained findings showed that hemp protein isolates might have good properties for film preparations characterized by low solubility and high surface hydrophobicity.

8.5 Bio-Functionality of Hemp Protein Hydrolysates

Biopeptides are natural chemical compounds found in plants and animals that have the ability to improve certain health functions when found in the human organism (Hernandez-Ledesma et al. 2007). They represent short amino acid sequences (2–20 amino acid residues) found in the primary protein structure. Biopeptides are derived from food proteins by the action of enzymes and when they enter the body they can act as modulators of certain physiological processes, similar to endogenous peptides with hormonal activity (Tang et al. 2009b). Potential physiological effects of biopeptides are related to: regulation of elevated blood pressure (inhibition of angiotensin and converting enzyme), antioxidant activity, prevention of platelet aggregation, modulation of the immune system, lowering of cholesterol and triglycerides levels in plasma, stimulation of the nervous system, antimicrobial effect, improvement of the transport and absorption of minerals (Korhonen and Pihlanto 2006; Hartmann and Meisel 2007).

Besides abovementioned bio-functionalities, it was shown that protein hydrolysates can exhibit a variety of techno-functionalities (Fig. 8.4) (Amarowicz 2010).

8.5.1 Antioxidant Properties

Antioxidants are important to the human body as they may provide a defence against Reactive Oxygen Species (Ryan et al. 2011). The supplementation with the antioxidants may provide additional support to endogenous antioxidants in the defence against oxidative stress (Kunwar and Priyadarsini 2011). The antioxidant activities of enzymatic hydrolysates from animal and plant food proteins, including bovine caseins and whey proteins (Pihlanto 2006), soy proteins (Moure et al. 2006), wheat protein (Zhu et al. 2006), porcine haemoglobin, collagen and myofibrillar protein (Chang et al. 2007), and fish proteins (Rajapakse et al. 2005; Kim et al. 2007), have been widely investigated by use of many *in vitro* antioxidant evaluation systems

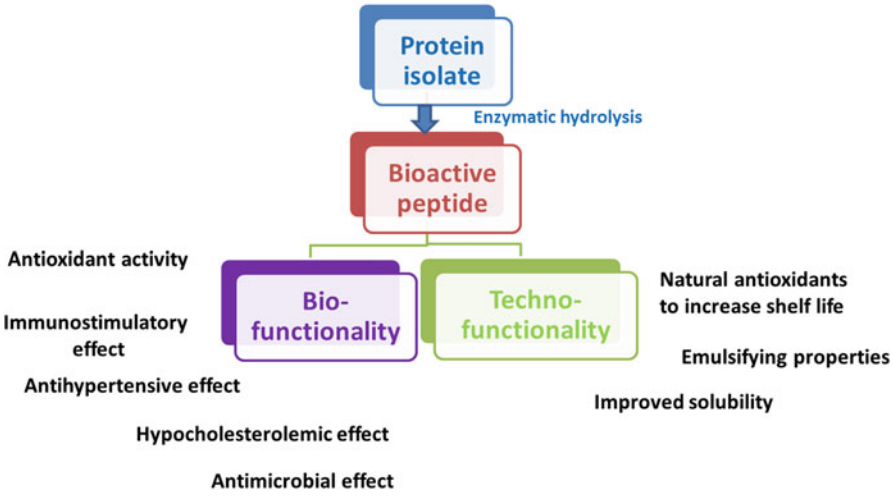

Fig. 8.4 Versatile functionalities of protein hydrolysates obtained by hydrolysis of protein isolate. List of investigated bio- and techno-functionalities

(water-soluble and oil-soluble). The antioxidant property of these hydrolysates was shown to be dependent on protease specificity, degree of hydrolysis and nature of released peptides (e.g. molecular weight and amino acid composition) and it has 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.

The initial HPI extracts have limited bioactive properties as measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, oxygen radical absorbance capacity and angiotensin-I-converting enzyme (ACE)-inhibitory activity. The bioactivities of the HPI extracts can be increased upon hydrolysis by the proteases. The degree of hemp protein hydrolysates (HPH) bioactivity depends on the hydrolysis time, as well as the type of protease and substrate (Teh et al. 2016).

To examine the antioxidant properties of hemp seed peptides, protein hydrolysates are prepared using different enzymes, and different hydrolysis times (Tang et al. 2009a). Tang et al. (2009a) conducted hemp protein isolate hydrolysis with alcalase, flavourzyme, neutrase, protamex, pepsin and trypsin for 2 and 4 h, separately, followed by testing for antioxidant activities such as $DPPH^{\bullet}$ scavenging, iron (III) reduction, and iron chelation. They have found that the DPPH radical scavenging and Fe^{2++} chelating abilities of the hydrolysates were positively correlated with the yield of trichloroacetic acid-soluble peptides or surface hydrophobicity values. Similar results were obtained when HPH were produced using Neutrase® (Wang et al. 2009).

Girgih et al. (2011a) performed simulated gastrointestinal hydrolysis of hemp seed proteins using pepsin and pancreatin followed by membrane ultrafiltration fractionation to obtain fractions with peptide sizes less than 1, 1–3, 3–5, and

5–10 kDa. While fractionation of the HPH led to improvements in ferric reducing power, DPPH radical, and hydroxyl radical scavenging activities, a decrease in metal chelation capacity occurred. In another paper, Girgih et al. (2013) confirmed that the peptide fractions exhibited higher oxygen radical absorbance capacity as well as scavenging of DPPH, superoxide and hydroxyl radicals when compared to HPH.

Numerous research papers compared the action of different enzymes on the antioxidant activity of the protein hydrolysates by keeping the time of hydrolysis constant. However, in the research published by Hadnađev et al. (2018b), where HPH were obtained by alcalase and pancreatin, the degree of hydrolysis for both enzymes was kept constant enabling comparison of enzymes specificity to certain protein sites rather than hydrolysis degrees on peptides antioxidant capacity. Utilizing the same amount of protein substrate, as well as the same amount of enzyme, the required degree of hydrolysis (3, 6 and 9%) was achieved for the shorter period of time using alcalase enzyme than pancreatin. In the case of alcalase time range was between 1.8 and 13.5 min, and in the samples with pancreatin it was between 10.9 and 90.9 min (Hadnađev et al. 2018b). This can be explained by the fact that alcalase has a greater affinity for peptide cleavage. Therefore, alcalase could be observed as a more effective choice for the hemp proteins hydrolysis than pancreatin. In general, alkaline proteases, such as alcalase, show better activity in peptide hydrolysis in comparison to acid and neutral proteases (Klompong et al. 2007).

Literature data shows that the ability of the sample (Tang et al. 2009a) to scavenge $DPPH^{\bullet}$ is closely related to protein hydrolysis degree. The strongest antioxidant activity was detected in samples characterized by the highest degree of hydrolysis (9%) regardless of the enzyme employed. Comparing the IC50 values (the concentration of a sample needed to quench 50% of the initial amount of $DPPH^{\bullet}$), it can be concluded that hydrolysates obtained by pancreatin showed a stronger antioxidant activity than hydrolysates derived by alcalase (Hadnađev et al. 2018b). Previous studies showed that the ability of protein isolates to scavenge $DPPH^{\bullet}$ was associated with the high hydrophobicity of the proteins themselves. It was also indicated that smaller protein fractions possessed a higher proportion of hydrophobic amino acids (Pownall et al. 2010). Moreover, it is well known that the antioxidant activity of the proteins is related to amino acid composition (Chen et al. 1995). Since pancreatin represents a mixture of enzymes with different specificities, results of scavenging activity on $DPPH^{\bullet}$ indicated that in the case of pancreatin hydrolysis, bioactive peptides with a higher proportion of hydrophobic amino acids have been released. The results confirmed that protein hydrolysates exhibited greater scavenging activity on $DPPH^{\bullet}$ than the isolate (HPI). Moreover, it can be assumed that certain DPPH antiradical activity of HPI was probably due to polyphenol compounds and pigments that have been co-extracted with the isolate, as indicated by the green colour of the isolate itself (Hadnađev et al. 2018b). Namely, phenolic compounds are the reason of the dark colour and undesirable taste of protein isolates. According to Xu and Diosady (2002), phenolic compounds bind to proteins in aqueous solution through the following mechanisms: hydrogen bonds, covalent bonds, hydrophobic interactions and ionic bonds, and their removal represents a major challenge.

IC50 values for reducing power of the protein hydrolysates were in the range of 1.98–4.70 mg/mL, depending on the enzyme used, as well as the hydrolysis time or degree (Hadnađev et al. 2018b). These results are in agreement with the results obtained by Tang et al. (2009a) who investigated the effect of various proteases on the antioxidant properties of the resulting hemp protein hydrolysates. Glutathione exhibited the greatest ability in reducing Fe^{3+} compared to hemp protein isolates and hydrolysates. It is a tripeptide containing cysteine – amino acid with sulfhydryl group. Sulfhydryl groups are considered to be responsible for the expression of the reducing activity (Battin and Brumaghim 2009). The hydrolysates obtained by using pancreatin showed a higher reducing power relative to those obtained by alcalase treatment. Battin and Brumaghim (2009) stated that the type of amino acids, as well as peptide type plays an important role in reducing power activity. Since hydrolysates obtained using pancreatin and alcalase treatment had a lower proportion of sulfhydryl groups in their composition than glutathione, they exhibited poorer ability to reduce Fe^{3+} (Isinguzo 2011).

By fractionation of HPH, Girgih et al. (2014) identified Trp-Val-Tyr-Tyr (WVYY) and Pro-Ser-Leu-Pro-Ala (PSLPA) as the most active antioxidant peptides with 67% and 58% $DPPH^{\bullet}$ scavenging and metal chelation activity of 94% and 96%, respectively.

8.5.2 Antihypertensive/ACE Inhibitory Activity

Elevated blood pressure is one of the major risk factors for cardiovascular disease, a leading cause of death in the developed world (Erdmann et al. 2008). Angiotensin I-converting enzyme (ACE) plays a crucial role in the regulation of blood pressure as it promotes the conversion of angiotensin I to the vasoconstrictor angiotensin II as well as inactivates the vasodilator bradykinin. ACE is an exopeptidase that cleaves dipeptides from the C-terminal of peptide substrate. Binding to ACE is mainly affected by the C-terminal tripeptides sequence of the substrate. Though the mechanism is not well known, previous studies showed that the presence of aromatic, hydrophobic and positively charged amino acid residues at the C-terminus has a major influence in the ACE inhibitory potency of the peptides. Hydrophobic residues tryptophan, tyrosine, phenylalanine and proline were shown to be the most potent ACE inhibitory peptides (Nagpal et al. 2011). Synthetic ACE inhibitors have been used for decades as the antihypertensive agents. In recent years, some food proteins have been identified as sources of ACE inhibitory peptides and are currently the best known class of bioactive peptides (FitzGerald et al. 2004). Although additional investigation is needed, the ACE inhibitory peptides have shown to have potential to be used in initial treatment in mildly hypertensive individuals or as supplemental treatment. They would also represent a less costly alternative in treatment of hypertension. Another advantage is that these peptides have not been associated with the some side effects reported for synthetic ACE inhibitors such as

dry cough, skin rashes and angioedema, probably due to the lower ACE inhibitory activity determined in vitro (FitzGerald and Meisel 2000).

Short-term (24 h) oral administration (200 mg/kg body weight) of hemp seed protein hydrolysate (HPH) to spontaneously hypertensive rats was shown to reduce blood pressure (−30 mmHg after 8 h) and was positively correlated with the in vitro ACE and renin inhibitions (Girgih et al. 2011b). Spontaneously hypertensive rats are considered one of the best experimental models for evaluating antihypertensive drugs or food-based inhibitors (Quiñones et al. 2010).

Long term effect of hemp protein products was also determined by Girgih et al. (2014) who investigated the ability of diet containing HPH and HPI to attenuate elevated blood pressure development in spontaneously hypertensive rats. They have showed that after 8 weeks HPH more significantly reduced blood pressure than HPI, demonstrated that a simulated gastrointestinal digested hemp seed protein hydrolysate inhibited renin (IC_{50} of 0.81 mg/mL) and ACE (IC_{50} of 0.67 mg/mL) activities in vitro. Weaker antihypertensive effect of the unhydrolysed protein (HPI) reflects the superior absorption properties of predigested peptides. The higher potency of the HPH diets may also be due to the higher contents of aromatic and hydrophobic amino acids when compared to the HPI. Those results are similar to the once reported for soybean protein hydrolysate (Yang et al. 2004).

The results confirm the potential of HPH as an ingredient that can be used to formulate functional foods for the prevention and for the support in therapy of hypertension.

8.6 Conclusion

Although having a range of techno-functionalities (gelling, emulsifying, foaming, film-forming agents), utilization of hemp proteins in different food matrices is limited since their behaviour highly depend on their structure and composition, environmental factors (pH, ionic strength, type of solvent/salt used, temperature) and isolation technique and conditions. Therefore, incorporation of hemp proteins into food products represents a technological challenge. At the same time, knowledge about hemp proteins limitation to adapt their functionality to environmental and isolation conditions represents a technological advantage which is reflected in target application of the one type of protein for different techno-functionalities in food systems. It was shown that poor solubility of hemp proteins (an important prerequisite for a protein to be an effective techno-functional agent) can be altered by changing the isolation procedure or by application of enzymatic or chemical modification. Enzymatic hydrolysis of hemp protein proved as a powerful tool in providing versatility in its techno-functionalities, as well as in enabling simultaneous expression of techno- and bio-functionality in the same food system since protein hydrolysates can also act as antioxidants in foams, emulsions, edible film, etc.

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