Postprint of Food and Function, 10 (10): 6732-6739 (2019)
DOI: https://doi.org/10.1039/C9FO01904A

Neuroprotective protein hydrolysates from hemp (*Cannabis sativa* L.) seeds

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DOI: 10.1039/C9FO01904A

ABSTRACT

Hemp (*Cannabis sativa* L.) seeds are well known for their potential use as source of nutrients, fiber, and bioactive compounds. Hemp protein isolate, prepared from defatted hemp flour, was hydrolyzed by Alcalase and Flavourzyme under specific conditions. The resulting hydrolysates were evaluated to the selection of potentially bioactive hemp protein hydrolysates (HPHs) by its DPPH scavenging and ferric reducing antioxidant power activity. *In vitro* cell-free experiments led to the identification of two bioactive HPHs, HPH20A and HPH60A+15AF, which were used at 50 and 100 μg/mL on BV-2 microglial cells in order to evaluate the anti-neuroinflammatory activities. Our results showed that HPH20A and HPH60A+15AF down-regulated *TNF-α*, *IL-1β*, and *IL-6* mRNA transcriptional levels in LPS-stimulated BV-2 microglial cells. In addition, HPH20A and HPH60A+15AF up-regulated gene expression of anti-inflammatory cytokine IL-10. This study suggests for the first time that HPHs may improve neuroinflammatory and inflammatory states, supporting the nutraceutical value of hemp seeds.

Keywords: Hemp; Cannabis sativa; protein hydrolysate; microglia; neuroinflammation.

Introduction

Non-drug varieties of *Cannabis sativa* L. (Cannabaceae), collectively known as "hemp", are a well-known plant of industrial value as an important source of nutrients, fiber, and bioactive compounds.[1] In the last years, industrial hemp global market is rapidly increasing, since low-Δ9-tetrahydrocannabinol (THC) cultivars are legal and available.[2] Hemp seeds are raising great interest as valuable crop, not requiring fertilizers, herbicides, and pesticides.[3] Currently, the great attention for hemp seed is related to its nutritional content: 25-35% oil, 20−30% carbohydrates, 20-25% proteins, 27.6% total fiber, vitamins, and minerals.[4,5]

Up-to-now, there is increasing attention for hemp seed proteins owing to their digestibility, satisfactory essential amino acid composition, and techno-functional properties.[6,7] Nevertheless, the poor water solubility of hemp protein isolate (HPI) hinders its application as a functional ingredient in food industry. Recent research has shown that hydrolysates obtained by treating HPI with different enzymes, such as pepsin, alcalase, papain, pancreatin, or other proteases, containing from 60 % to 80 % of protein content.[8] Induced proteolysis not only modifies HPI hydrophobicity, but also changes protein molecular weight and conformation, obtaining high availability of bioactive peptides.[1]

In this context, hemp protein hydrolysates (HPHs) contain a mixture of polypeptides, oligopeptides, and amino acids that can be used as a key factor in the development of applications in nutraceuticals and functional foods.[9] To date, the characterization of the bioactive HPHs remains largely unstudied. In

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particular, few studies have showed that HPHs obtained from enzymatic hydrolysis demonstrated antihipertensive, hypocholesterolemic, antioxidant, anthrombotic, and immunomodulatory effects.[8–14] However, to our knowledge, neuroprotective HPHs have not been described in the literature.

Microglia are phagocytic macrophages present in all regions of the adulthood brain and spinal cord, and represent between 10-15% of the total cells in the central nervous system (CNS).[15] The principal function of microglia is to destroy and to clear foreign materials through phagocytic and cytotoxic mechanisms.[16] Microglia cells also contribute to inflammation and homeostatic mechanisms by secreting cytokines and other signaling molecules, and they act as antigen presenting cells.[17] When local inflammation in the CNS and brain is chronically active (neuroinflammation), it can produce a wide variety of neurodegenerative disorders including Alzheimer's disease, Parkinson's disease, Huntington's disease, tauropathies, age-related macular degeneration, and diseases of autoimmune origin such as multiple sclerosis.[18]

On the basis of these considerations, the overall objective of the present study was to investigate the effect of different HPHs from hemp seed on neuroinflammation in LPS-activated BV-2 microglial cells. In particular, to achieve this goal, the specific targets were (i) the chemical characterization of hemp products, (ii) the evaluation of the biological activity of HPHs on *in vitro* cell-free experiments, and (iii) the identification of neuroprotective effect of the two HPHs selected on BV-2 microglial cells.

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Materials and methods

Chemicals and sampling

Seeds of *C. sativa* L. were provided by Sensi Seeds Bank. Alcalase 2.4 L (2.4 AU/g) and Flavourzyme (1000 L) were a gift from Novozymes (Bagsvaerd, Denmark). The murine BV-2 cell line was gently provided by Cell Biology Unit (Institute of Fats, CSIC, Spain). Primers were purchased from Eurofins Biolab S.L.U (Barcelona, Spain). Total RNA was isolated using Trisure (Bioline, Meridian Life Science, Inc. Memphis, USA) and reverse transcribed to cDNA using a synthesis kit of Bio-rad (California, EE. UU). Quantitative PCR was performed by iTaq™ Universal SYBR® Green Supermix of Bio-rad. All chemicals (reagents and solvents) were of analytical grade and provided by Sigma Chemical Co. (St. Louis, MO, USA), Bachem AG and Gibco.

Preparation of hemp protein isolate (HPI)

The HPI was obtained using the method of Lqari et al.[19] Briefly, hemp defatted flour (HDF) was extracted with 0.25% Na_2SO_3 (w/v) at pH 10.5 for 1 h. After centrifuging the extract at 7500 rpm for 15 min, the supernatant was recovered and the pellet was extracted again. Both supernatants were adjusted to the isoelectric point of hemp proteins (pH 4.3) and the precipitate was washed with distilled water adjusted to pH 4.3 and centrifuged to remove residual salts and other non-protein compounds. Finally, the precipitated proteins were lyophilized and stored at room temperature.

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Hydrolysis of hemp protein isolate

Hydrolysis of HPI was performed in a bioreactor under continuous stirring at a controlled pH and temperature. HPI was suspended in distilled water (10% w/v) and temperature was adjusted to 50ºC. Alcalase 2.4 L (2.4 AU/g, a serine endo-peptidase which was kindly donated by Novozymes, Bagsvaerd, Denmark) was added at enzyme/substrate = 0.3 AU/g protein at pH 8 for 3 h. After 60 min of hydrolysis with Alcalase, Flavourzyme (1000 L, a leucine amino-peptidase which was kindly donated by Novozymes) was added at enzyme/substrate = 60 LAPU/g protein at pH 7 for 2 h. During peptic hydrolysis, the pH was constantly kept by titration with NaOH solution in appropriate concentration. Each hydrolytic time-point sample was stopped by holding at 85 °C for 15 min to ensure a complete inactivation of residual enzyme activity. The supernatant obtained by centrifugation at 7500 rpm for 15 min constituted the HPH. The HPHs obtained using only Alcalase were designated as 10A, 20A, 30A, 45A, and 60A. The HPHs obtained using Alcalase followed by Flavourzyme were designated as 60A+15AF, 60A+30AF, 60A+60AF, 60A+90AF, and 60A+120AF. The number indicates the time of hydrolysis in minutes.

Hemp product compositional analysis

The protein concentration was determined by elemental microanalysis as % nitrogen content x 6.25 using a Leco CHNS932 analyser (St. Joseph, MI, USA). Total dietary fiber was determined by the gravimetric method.[20] Ash content was determined according to the direct ignition method (550 ºC for 36 h). Polyphenols and soluble sugars were measured using standard curves of chlorogenic acid and glucose, respectively.[21,22] Amino acid quantification by

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HPLC was done according to Alaiz et *al*.[23] Finally, tryptophan content was analyzed according to the method of Yust et *al*.[24]

Evaluation of hydrolysis degree (HD)

The HD, defined as the percentage of peptide bonds cleaved, was calculated by the TNBS method which determine free amino acid groups.[25] The total number of amino groups was determined in a sample that had been 100% hydrolyzed in 6 N HCl at 110 ºC for 24 h.

Molecular profile by Fast Protein Liquid Chromatography (FPLC)

The molecular profile of the samples was determined using FPLC Akta purifier 10 (GE Healthcare Bio-sciences AB, Uppsala, Switzerland). Superose 12 HR 10/300 GL, a prepacked column for high-performance size exclusion chromatography with a broad fractionation range for molecules with molecular weights between 1 and 300 kDa, was used. The column was calibrated with proteins of known molecular weights. From the logarithms of the molecular weights of these proteins and their elution volumes, the calibration line was made. The elution was carried out with 50 mL of 0.05 M sodium phosphate buffer, 0.5 M sodium chloride and 0.02% sodium azide (w/v) at pH 7.5, with a flow of 1 mL/min. The injected volume of the samples and its concentrations were of 500 µL and 1 mg/mL of protein, respectively. The elution of the proteins was recorded by measuring their absorbance at 280 nm.

DPPH radical scavenging activity

The scavenging effect of the samples on DPPH free radical was measured according to Wu et al.[26] Briefly, 1.5 mL of each sample (at concentration of 10

mg of protein/mL) was added to 1.5 mL of 0.1 mmol/L DPPH in ethanol. The mixture was shaken and left for 30 min at room temperature, and absorbance was measured at 517 nm. The control was conducted in the same manner but distilled water was used instead of sample. Butylated hydroxytoluene (BHT) at 0.08 mg/mL was used as positive standard.

Ferric reducing antioxidant power (FRAP)

The ability of the samples to reduce iron (III) was measured according to Oyaizu.[27] Briefly, 0.1 mL of sample (at concentration of 10 mg of protein/mL) was added to 0.25mL of 0.2 mol/L sodium phosphate buffer pH 6.6 and 0.25 mL of 0.03 mol/L potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min. Then 0.25 mL of 0.6 mol/L trichloroacetic acid was added and the mixture was centrifuged at 1300 x g for 10 min. 500 µL of the upper layer were mixed with 0.5 mL of distilled water and 0.1 mL of 3.7 mmol/L ferric chloride. After 10 min, absorbance of the resulting solutions was measured at 700 nm. Increased absorbance of the reaction mixture indicated increased reducing power. BHT at 0.08 mg/mL was used as standard.

BV-2 Cell Culture and Treatments

BV-2 is a murine cell line used extensively in research related to neurodegenerative disorders.[28,29] BV-2 microglial cells were obtained from the Cell Biology Unit at the Instituto de la Grasa (Seville, Spain). The cells were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin/streptomycin. They were maintained in 5% CO2 at 37 °C in a CO2 incubator (Thermo Con Electron Corporation, Waltham, MA, USA). Experiments

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were done in twelve-well plates. BV-2 microglial cells were seeded at a density of 5 x 10^5 cells/well. Different conditions were used: Untreated cells (negative control), cells exposed to LPS at 100 ng/mL (positive control), and cells exposed to LPS at 100 ng/mL + different treatments (HPI, HPH20A, and HPH60A+15AF) at 50 or 100 µg/mL. LPS (E. coli 055: B5) (Sigma-Aldrich) was added for 1 h and then the hemp product. After incubation for an additional period of 24 h, cells were collected for RNA extraction.

Cell Viability Assay

BV-2 microglial cells were incubated with different concentrations of hemp products in 96-well plates (1 x 10^5 cells/well) during 24 h. Concentrations used were the following: 10, 25, 50, 100, and 200 µg/mL. Afterwards, the MTT solution (Sigma-Aldrich) was incubated in the well until a purple precipitate was visible. MTT-formazan crystals were solubilized with DMSO (Sigma-Aldrich), and then measured with a microplate reader at 570 nm corrected to 650 nm.[30] Cell survival was expressed as the percentage of absorbance compared with that obtained in control, non-treated cells.

RNA Isolation and Real-Time quantitative PCR Analysis

RNA from BV-2 microglial cells was isolated to quantify gene expression by RT-qPCR. Total RNA was extracted by using TRIsure Reagent (Bioline). RNA quality was assessed by A260/A280 ratio in a NanoDrop ND-1000 Spectrophotometer (ThermoFisher Scientific, Madrid, Spain). Briefly, RNA (250 ng) was subjected to reverse transcription (iScript, Bio-Rad). An amount of 40 ng of the resulting cDNA was used as template for qRT-PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-

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Rad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT) as housekeeping genes. All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (HPRT) gene content and expressed as relative fold-change of control. The sequences of the designed oligonucleotides are shown in **Supplementary Table 1**.

Statistical analysis

All values are expressed as arithmetic means ± standard deviations (SD). Data were evaluated with Graph Pad Prism Version 5.01 software (San Diego, CA, USA). The statistical significance of any difference in each parameter among the groups was evaluated by one-way analysis of variance (ANOVA), following Tukey multiple comparisons test as post hoc test. P values less than 0,05 were considered statistically significant.

Results and discussion

Chemical characterization of hemp products

Hemp has been cultivated since ancient times to produce fiber and seeds. Nowadays, seeds are essentially used for extracting high ω-3 fatty acids-containing oil.[2] The HDF, a by-product of hemp industry from oil extraction,

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contains high amount of fibers and proteins. Hemp seeds were defatted obtaining HDF with a protein content of 33.3%, as reported in **Table 1**. HPI was separated in 13.8% yield from defatted hemp seed flour by basic extraction followed by acidic precipitation and the obtained HPI had 96.5% protein content. The protein content in our study was higher than previously described for HDF and HPI.[1]

Amino acidic composition of hemp products

Amino acid profile might influence to peptide activity in hemp products. Hence, this study analyzed the amino acid profile in HDF, HPI, and two HPHs: HPH20A and HPH60A+15AF (**Table 2**). All these hemp products had similar amino acid profile and have been identified as a great source of essential amino acids. Briefly, hemp products were rich in negatively charged amino acid like Asp + Asn (11-12%) and Glu + Gln (18%), and also rich in Arg (12-13%). These amino acid profiles have some similarities with the amino acid profile of edestin, the main globulin protein in hemp seed, reported in the literature.[31] The importance of these amino acids, Asp and Glu, are that they are involved in electron donation mechanism between reactive oxygen species and Arg for nitric oxide (NO) production,[32] which may have contributed to regulate the hemp protein products bioactivity.

Among the differences between hemp products and edestin amino acid profiles it turns out to appear a higher percentage of apolar amino acids in edestin than in HPI and HPHs, as a result, it could indicate that HPI and HPHs arises better solubility properties.[1,7,10,]

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Analysis of hydrolysis degree and molecular profile of hemp products

Extent of hydrolysis can be estimated from the DH values, which can be used an indication of peptide chain length, higher and lower values indicate mean shorter and longer lengths, respectively. Previous results indicated that the use of a single enzyme, Alcalase or Flavourzyme, led to the production of protein hydrolysates with HD < 30%, where peptides of 3–20 kDa are predominant.[33] However, most bioactive peptides described up to date have a molecular weight under 1 kDa. In this context, the extensive hydrolysis of HPI was performed using both enzymes in a sequential way as depicted in **Figure 1**. Herein, HPI was hydrolyzed for 1 h with Alcalase, which facilitated the subsequent action of Flavourzyme. The rate of hydrolysis was fast during the first 30 min of hydrolysis with Alcalase and remained almost stable in the following 30 min (approximately 30% of HD). The addition of Flavourzyme led to a new increase of HD up to 60% after 75 min with Alcalase plus 15 min with Flavourzyme (HPH60A+15AF).

As shown in **Figure 2**, the peptide sizes in hemp products were estimated by FPLC and the results showed distribution into five major peaks with approx. MW ranging from 0.3 (peak e) to 300 kDa (peak a). These bigger peptide peaks (peaks a-c) were most remarkable for HPI. The hydrolysis with Alcalase and Flavourzyme contained a minor peak with size 1.45 kDa (peak d), which indicates a polypeptide that is resistant to digestion by these enzymes. In addition, sequential hydrolysis with Alcalase and Flavourzyme released an oligopeptide with approx. MW ranging from 300-400 Da. The interrelationship between the MWs of hydrolysates and their bioactivities in human health application is very crucial in functional foods and nutraceutical formulations.

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Thus, the presence of low molecular weight peptides enhances the potential amino acid absorption and their ability to have bioactive effects during *in vivo* tests.[10]

Cell-free *in vitro* evaluation of hemp product biological activity

Biological activity of peptides is related to chain length, HD, amino acid composition, sequence, and conformation.[34] Some literature evidence indicates that the most effective hemp products are ACE-inhibitory peptides identified up-to-now contain 2–20 amino acids and must have a good hydrophobicity/hydrophilicity balance and some particular structural characteristic related to its sequence.[8-11] Herein, we evaluated the potential antioxidant activity of HPI, five HPHs with Alcalase, and five HPHs with Alcalase and Flavourzyme (**Figure 3**) in cell-free *in vitro* experiments. The DPPH radicals have been widely used to investigate the scavenging activity of protein hydrolysates. As shown in **Figure 3A**, all HPHs tested showed DPPH radical scavenging activity. Furthermore, all hydrolysates had higher activity than HPI, so it maybe concluded that hydrolysis generated peptides that could react with free radicals to convert them to more stable products and complete the radical chain reaction. Hydrolysis with Alcalase raised up DPPH scavenging effect to 21-36%. The subsequent hydrolysis with Alcalase and Flavourzyme slightly increased the DPPH scavenging activity even more, obtaining values of approximately 40%. Similar results were founded in HPHs obtained by different proteases (AFP, HT, ProG, actinidin and zingibain) by Teh et *al*.[35] A previous study documented that the smaller size peptide fraction (<1 kDa) of the pepsin hydrolysate – followed by hydrolysis with pancreatin, had a higher DPPḢ

scavenging activity (25%) than a larger sized peptide fraction (5–10 kDa) (20%), while the unfractionated HPI had less than 5% DPPḢ scavenging activity.[14] As compared to our study here, HPHs hydrolyzed with Alcalase and Flavourzyme had higher DPPḢ scavenging activity (40%). The FRAP was used in order to found a direct correlation between antioxidant activity and the donate-electron potential of bioactive compounds into HPI and HPHs as other studies have reported in similar seeds hydrolysates.[36] For FRAP value (**Figure 3B**), significant differences were observed for the HPHs with Alcalase compared to those hydrolyzed with Alcalase and Flavourzyme. HPHs hydrolyzed with Alcalase and Flavourzyme had higher FRAP value compared to those hydrolyzed with alcalase. All peptide fractions had significantly lower ($p < 0.05$) absorption values when compared to BHT action. Similar results were founded in HPHs obtained by reverse-phase HPLC by Girgih et *al.*[13]

Neuroprotective properties of hemp protein hydrolysates

Oxidative stress has been associated with chronic inflammation in neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis, which increasing numbers of elderly individuals suffer.[18] The brain is particularly vulnerable to oxidative stress and damage, because of its high oxygen consumption, low antioxidant defenses, and high content of polyunsaturated fats very prone to be oxidized.[37] Thus, it is not surprising the importance of protecting systems, including antioxidant molecules, to maintain brain homeostasis. After analyzing the previous results, we decided to select two hydrolysates considering the HD, hydrolysis time, and antioxidant activity. The HPHs were HPH20A and HPH60A+15AF. At first, to evaluate the cytotoxic effects of hemp products on BV-2 microglia cell viability,

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the cells were treated with HPI, HPH20A, and HPH60A+15AF up to 200 μg/mL for 24 h. The cell viability evaluated by the MTT method was not affected by any hemp product concentration (data not shown). In order to evaluate the neuroprotective properties of HPHs, gene expression of cytokines TNF-α, IL-1β, IL-6, and IL-10 was measured by RT-qPCR in BV-2 microglial cells (**Figure 4**). As expected, LPS increased mRNA levels of TNF-α, IL-1β, and IL-6. Meanwhile, these LPS-induced changes were blocked in the presence of HPH20A and HPH60A+15AF at 50 or 100 μg/mL. HPI were also effective to down-regulate the expression levels of TNF-α and IL-6. Hemp products also showed a statistically significant dose-dependent effect on IL-6 gene expression. Furthermore, HPH20A and HPH60A+15AF induced a increase in mRNA levels of anti-inflammatory IL-10 gene at 100 μg/mL. In the process of developing new strategies to modulate the inflammatory response in neurodegenerative diseases, a number of phytochemicals, mainly phenolic compounds, have been shown to target neuroinflammation in Alzheimer's disease due to their anti-oxidant and anti-inflammatory activities,[38] however, some of the benefits of these natural products were further dependent on their poor bioavailability or activity when metabolically transformed into conjugates. One promising attempt for an improved and safety therapy of neurodegenerative diseases by naturally occurring compounds has been recently reported with the soy-derived peptide lunasin.[39] Inspired in part by these studies, we envisioned that HPHs might be an ideal candidate to test its efficacy in mitigating a neuroinflammatory environment. To date there is little if any studies on the impact of HPHs on microglial activation. The present study contributes to establishing novel data for a hemp seed global database of

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bioactive constituents. Based on previous studies, hemp seeds contain a highly amount of bioactive chemicals as canabisin-F or grossamide (lignanamides), which improve neuroinflammatory states in LPS-stimulated BV-2 microglial cells through down-regulation of TLR-4 mediated NF-κB pathway.[40,41] In particular, this study showed for the first time that the treatment with hemp protein products obtained by enzymatic digestion decreased inflammatory state in LPS-stimulated BV-2 microglial cells. However, the study had limitations. First, the responsible bioactive peptides were not identified in this work and second, the study on activity-structure relation is missing. These two limitations of this study can be future research directions.

To conclude, the initial HPI has limited bioactive properties as measured by DPPḢ scavenging and FRAP activities. The bioactivity of the HPI was increased upon hydrolysis by Alcalase and Flavourzyme, obtaining HPHs with small peptides that exert bioactivities. Our findings advocate to considerate that HPHs may inhibit oxidative stress and inflammatory response in activated microglia. HPHs should be the focus of future analysis of the bioactive peptides generated in the prevention or treatment of neurodegenerative diseases, confirming that hemp seeds are a valuable source of bioactive compounds.

Acknowledgements

This work was supported by grant CYTED-2019 119RT0567 from the Spanish Ministry of Science, Innovation, and Universities. Authors thank Cell Biology Unit of Instituto de la Grasa for its assistance during the fulfilment of this work. SMP acknowledges financial support from “V Own Research Plan” (University of Seville).

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DOI: 10.1039/C9FO01904A

Conflicts of interest

All the authors declare that they do not have conflicts of interest.

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Figure legend

Figure 1. Time-course of hydrolysis degree of HPI during enzymatic hydrolysis with Alcalase at 10 (HPH10A), 20 (HPH20A), 30 (HPH30A), 45 (HPH45A), and 60 (HPH60A) min, and with Flavourzyme at 15 (HPH60A+15AF), 30 (HPH60A+30AF), 60 (HPH60A+60AF), 90 (HPH60A+90AF), and 120 (HPH60A+120AF) min. Data, expressed as the percentage of peptide bonds cleaved, are mean ± standard deviation of three determinations.

Figure 2. Molecular weight (MW) profiles by size-exclusion FPLC of HPI, HPH20A, and HPH60A+15AF. MW ranging from 0.3 (peak e) to 300 kDa (peak a). The bigger peptide peaks (peaks a-c) were most remarkable for HPI.

Figure 3. DPPH radical scavenging activity (**A**) and ferric reducing antioxidant power (FRAP) (**B**) by HPI and HPHs obtained using Alcalase and Flavourzyme in cell-free *in vitro* experiments. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($p < 0.05$).

Figure 4. Gene expression of TNFα (**A**), IL-1β (**B**), IL-6 (**C**), and IL-10 (**D**) in BV-2 microglial cells after 24 h incubation with or without LPS (100 ng/mL) and HPI, HPH20A, and HPH60A+15AF at 50 and 100 μg/mL. Values are presented as means ± SD (n = 3) and those marked with different letters are significantly different ($p < 0.05$).

Table 1. Chemical composition of HDF and HPI. Data, expressed as percentage in dry basis, are mean ± standard deviation of three determinations.

Fat (seed) (%)	32.35 ± 0.57	
	HDF	**HPI**
Proteins (%)	33.28 ± 1.39	96.46 ± 0.93
Moisture (%)	6.18 ± 0.02	2.19 ± 0.01
Ash (%)	7.01 ± 0.11	1.06 ± 0.10
Fiber (%)	40.90 ± 1.98	0.00 ± 0.00
Sugar (%)	5.24 ± 1.05	0.00 ± 0.00
Polyphenols (%)	0.48 ± 0.03	0.04 ± 0.00
Fat (%)	---	0.25 ± 0.00

Table 2. Amino acid composition of hemp products. Data, expressed as percentage of amino acids on total amino acid content, are mean ± standard deviation of three determinations.

Aa (%)	HDF	HPI	HPH20A	HPH 60A+15AF	FAO/ WHO
Asp + Asn	11.34±0.37	12.35±0.34	12.40±0.29	12.08±0.51	
Glu + Gln	18.41±0.13	18.86±0.35	18.83±0.25	18.58±0.12	
Ser	5.57±0.11	6.31±0.21	6.36±0.19	6.31±0.22	
His	3.07±0.01	2.85±0.05	2.80±0.02	2.79±0.03	1.9
Gly	4.74±0.07	4.79±0.23	4.64±0.11	4.64±0.12	
Thr	3.79±0.08	3.79±0.10	3.89±0.06	4.02±0.07	3.4
Arg	12.68±0.21	13.44±0.03	13.34±0.24	13.78±0.23	
Ala	4.89±0.10	5.01±0.14	4.99±0.10	5.00±0.11	
Pro	2.66±0.69	3.02±0.00	2.83±0.05	1.29±0.19	
Tyr	3.57±0.06	4.08±0.06	3.98±0.03	4.06±0.12	
Val	5.27±0.11	3.77±0.40	4.05±0.42	4.71±0.19	3.5
Met	2.14±0.02	0.89±0.34	0.60±0.03	0.64±0.04	2.5[a]
Cys	1.22±0.02	1.98±0.08	1.81±0.18	1.65±0.21	
Ile	4.23±0.08	3.02±0.40	3.57±0.15	3.85±0.20	2.8
Trp	0.76±0.05	0.55±0.02	0.66±0.01	0.82±0.03	1.1
Leu	7.12±0.05	7.13±0.11	7.12±0.13	7.34±0.07	6.6
Phe	4.76±0.09	5.12±0.02	5.04±0.05	5.19±0.08	6.3[b]
Lys	3.78±0.09	3.04±0.08	3.10±0.06	3.23±0.04	5.8

[a]Met + Cys, [b]Phe + Tyr.

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Figure 1

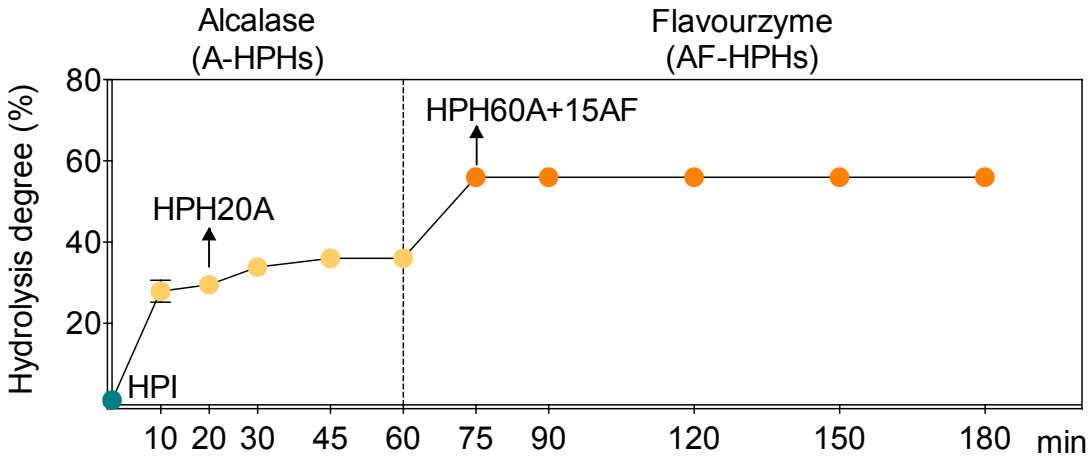

Figure 2

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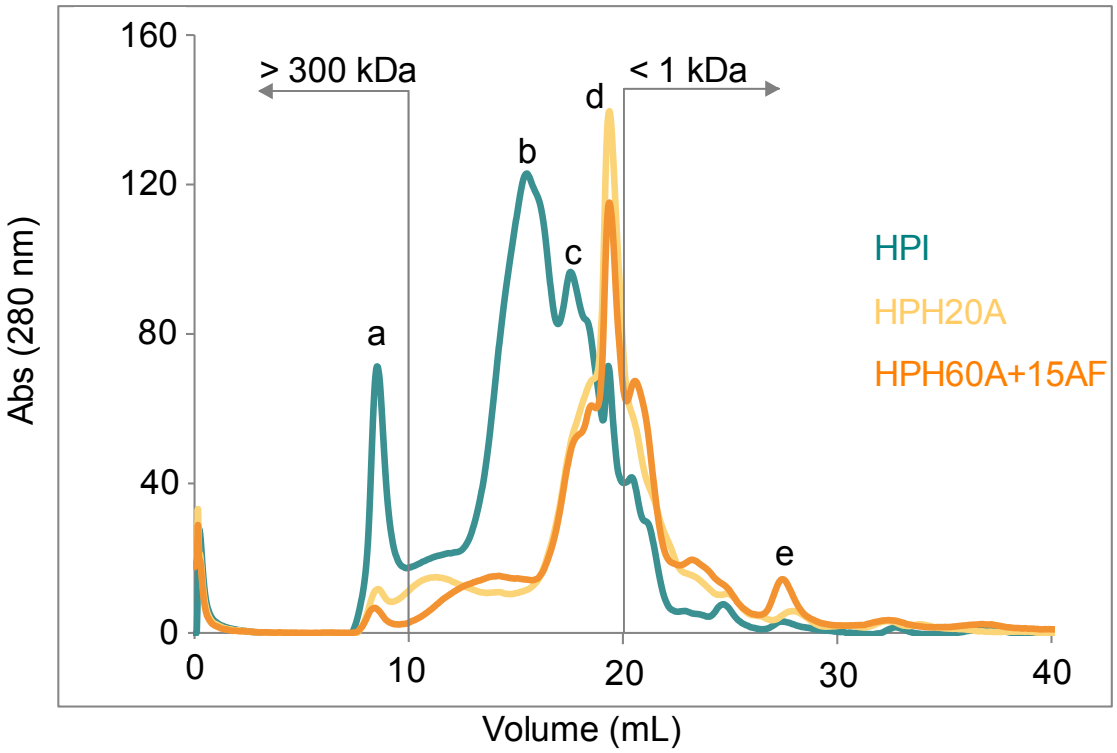

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Figure 3

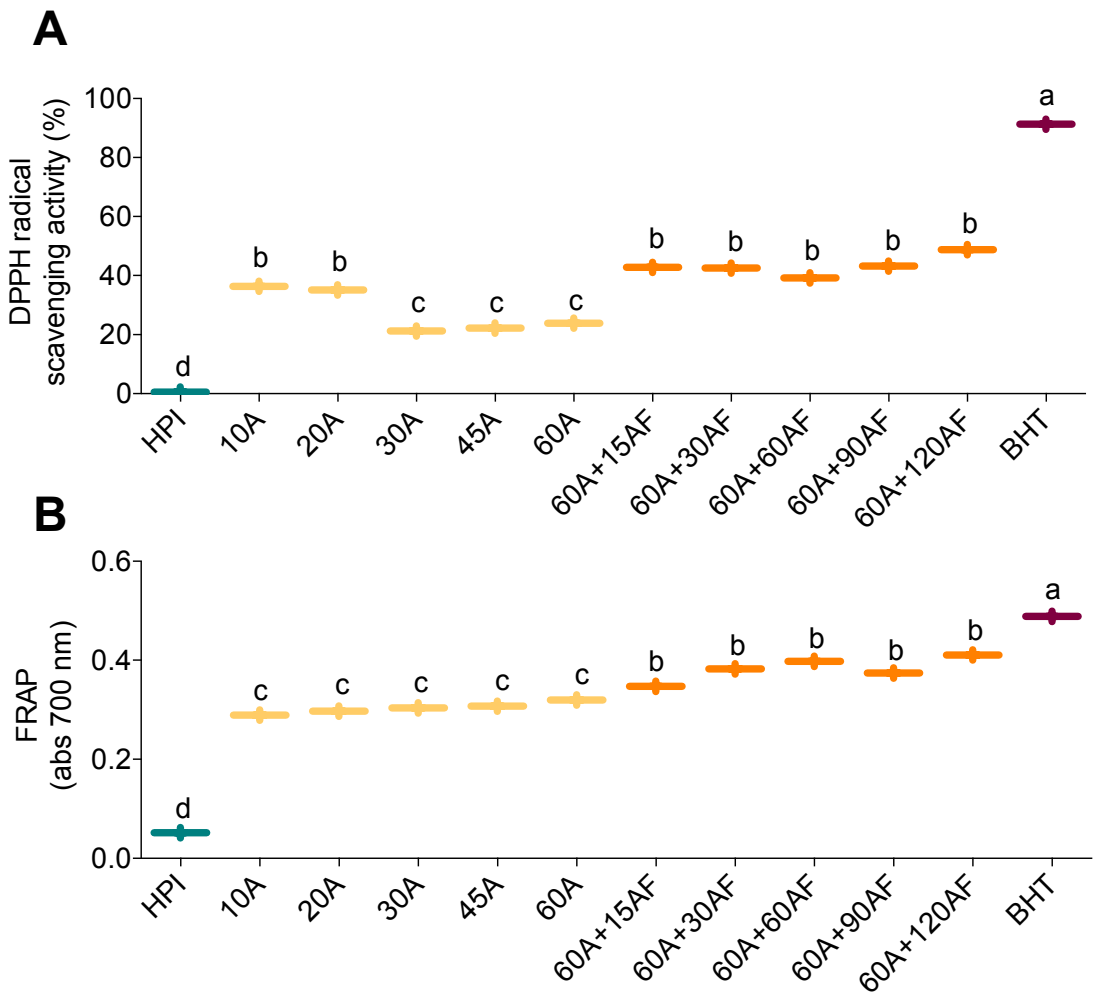

Figure 4

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