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ORIGINAL PAPER

Linking collective in vitro to individual in silico peptide bioactivity through mass spectrometry (LC-Q-TOF/MS) based sequence identification: the case of black cumin protein hydrolysates

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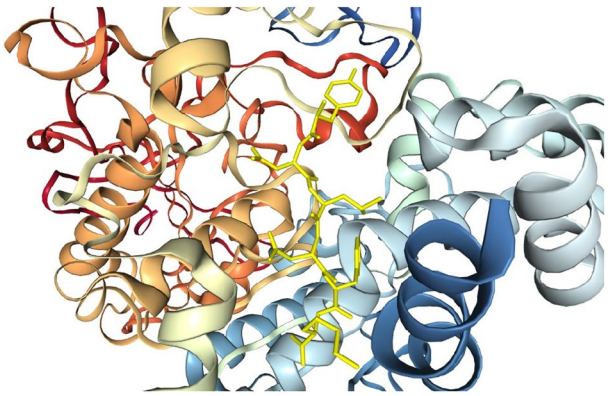
Abstract

Black cumin (*Nigella sativa* L.) is a seed that has been utilized in traditional medicine due to the bioactive characteristics of seed oil and therein solubilized components. In the present study, using enzymatic proteolysis (0–2 h) and fast protein liquid chromatography (FPLC)-based fractionation techniques, trypsin and papain hydrolysates of black cumin protein concentrates were investigated for their dual antioxidative and acetylcholinesterase (AChE) inhibitory activities. Peptides in the active fractions were identified using a liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF/MS) based analytical method, which further facilitated the in silico prediction of bioactivity for each and every characterized peptide sequence. While the extent of AChE inhibitory activity mostly decreased with proteolysis, various antioxidative activities increased during proteolytic treatments. Based on their relatively higher activities, 30 min papain treated hydrolysates were fractionated into four major fractions and their antioxidative capacities were verified in vitro. Peptide profiles of the fractions were investigated by LC-Q-TOF/MS analysis. Twenty different peptide structures were identified and their potential bioactivities were verified in silico. The interactions of the sample peptides with AChE were simulated via molecular docking. While anionic peptides were generated in this study, hydrophobic interactions possibly played a pivotal role in their dual bioactivities (i.e., AChE inhibitory and antioxidative) and peptide length could also be influential.

Graphic Abstract



Keywords Black cumin · Bioactive peptides · LC-Q-TOF/MS · In silico analysis · AChE-inhibitory activity · Molecular docking

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Extended author information available on the last page of the article

Introduction

Bioactive peptides are protein fragments that have positive effects on body functions. Based on their potential influence, they can be classified as anti-microbial, anti-thrombotic, anti-hypertensive, opioid, immunomodulatory, mineral binding and antioxidative peptides etc. They are normally hidden in the parent protein and often need to be extracted out of their sequence to demonstrate activity [1]. The manufacture process for these peptides may require fermentation, various food processing operations or enzymatic proteolysis [2]. Once an appropriate food protein source has been identified for the manufacture of bioactive peptides, utilization of a single protease or multiple proteases to release the peptides of interest is a commonly practiced method [3]. Food proteins can be hydrolyzed using proteases such as trypsin, pepsin, chymotrypsin, bromelain, ficin, or papain to obtain bioactive peptides [4]. Valorization of industrial by-products is a relatively economic route to generate bioactive peptides. For example, antioxidative and antihypertensive peptides were obtained from by-products of olive oil production [5].

Acetylcholinesterase (AChE) is an enzyme that degrades acetylcholine to acetic acid and choline. During the progression of Alzheimer's disease (AD), the activity of AChE increases in the brain which in turn leads to reduction in nerve conduction [6]. Therefore, inhibition of AChE has become one of the most widely used treatment options for AD [7].

Antioxidants from various sources play an important role in the prevention of cancer and cardiovascular diseases and in the reduction of the incidence of many other diseases including diabetes, cancer, schizophrenia, and AD [8]. It would be advantageous when an agent used against AD could also perform antioxidative effects [6]. In commercial uses, furthermore, natural antioxidants may be preferred due to their consumer appeal. Natural bioactive peptides with dual functionalities (i.e., AChE inhibitory and antioxidative) might be utilized in value-added formulations including functional foods and food supplements.

Black cumin (*Nigella sativa* L.) has been accepted as a medicinal plant due to some of its valuable components [9]. It is cultivated in many countries including Turkey and Eastern Mediterranean regions and has traditionally been used as an herbal medicine [10]. Previous studies have also shown that black cumin extracts demonstrated antimicrobial activity [11]. Its bioactive properties mostly originated from the presence of thymoquinone and essential oils [12, 13]. Recently, black cumin oil has become one of the most frequently used substances in food and health applications. Many biological activities have been attributed to black cumin seeds possibly indicating that black cumin components other than seed oil could also demonstrate bioactivities [9].

The consumption of black cumin seeds at reasonably high quantities is relatively difficult. Hence, the generation of functional or therapeutic components from the seeds could enhance their technical and biological potential. For example, protein concentrates and their derivatives could extend the functionality of seeds in industrial products [14, 15].

In this study, identification of the bioactive potential of black cumin protein hydrolysates and their corresponding active sequences were targeted using in vitro assays, in silico analysis techniques and mass spectrometry analyses which linked the two sets of findings. The aim of these studies included both the valorization of an industrial by-product and exploration of bioactive potential for future commercial uses.

Material and methods

Materials

Cold press deoiled black cumin cakes were donated by a local company that produces cold press oils (Oneva Foods, İstanbul, Turkey). The maximum temperature applied in cold press processing was < 40 °C in all of the cases, which limited the extent of lipid oxidation in the samples (data not shown). All the chemicals used in this study were obtained from Sigma-Aldrich Corp (Schnelldorf, Germany).

Methods

Preparation of protein concentrates

Protein concentrates were obtained from deoiled cakes using an alkali extraction-isoelectric precipitation technique [14]. Firstly, the size of the cake samples was reduced using a blender and the powdered samples were mixed with water (1:15 by mass; sample: water). The medium pH was set to pH 9.5 (1 M NaOH) to ensure solubilization of protein molecules and the mixture was kept stirred using a magnetic stirrer for 1 h. The resulting dispersion was centrifuged (HITACHI, CR22N) at 15000×g for 1 h (4 °C) to separate the undissolved matter. Medium was brought down to pH 4.5 using 1 N HCl and isoelectric precipitation was promoted. To ensure the separation of undissolved materials, centrifugation was administered under identical conditions as the previous centrifugation step. The precipitated proteins were collected and stored at −20 °C until lyophilization. Frozen samples were lyophilized using a Teknosem TRS 2/2 V instrument (Teknosem Corp., İstanbul, Turkey). The lyophilized samples were also stored at −20 °C until hydrolysis.

Protein contents of the lyophilized samples were determined based on the Kjeldahl method [15].

Proteolytic hydrolysis of protein concentrates

Proteolytic hydrolysis was performed using trypsin and papain enzymes [16]. Trypsin is a serine protease that cleaves proteins primarily at Arg-X and Lys-X regions [17]. Papain is an endolytic cysteine protease that cleaves peptide bonds containing basic amino acids (arginine, lysine and phenylalanine) [18]. An aqueous dispersion (1%) of black cumin protein concentrate was prepared in 50 mM potassium phosphate buffer (pH 7) using a magnetic stirrer (1 h, 25 °C). Then the dispersion (20000xg for 10 min) was centrifuged to separate the insolubles. The supernatant was transferred to Eppendorf tubes at an enzyme:protein ratio of 1:1000 for both enzymes and enzymatic hydrolysis was performed for 2 h using a thermomixer (MIULAB MTC-100 Thermo Shaker Incubator**,** 37 °C, 1000 rpm). In order to stop the enzymatic activity, the tubes were transferred to a 95 °C water bath. The hydrolysates were then quickly cooled using ice until the sample temperature reached to the ambient temperature. Once again, insolubles were separated using a microcentrifuge (Neuation, i Fuge M08). Finally, all samples were passed through cellulose acetate syringe filters (0.45 °m) and stored at −80 °C until further analyses.

Acetylcholinesterase (AChE) inhibitory activity test

AChE inhibitory activity test was used to examine potential anti-Alzheimer effects of the obtained protein concentrates and hydrolysates. An aliquot of each sample (10 µl) was mixed with 150 µl of sodium phosphate buffer (0.1 M, pH 8), and 1 ml of AChE enzyme solution (0.1 units.ml^{-1}). The mixture was kept incubated at 25 °C for 15 min on the thermomixer (1000 rpm). Subsequently, 10 µl 5,5-dithiobis-(2-Nitrobenzoic acid) (DTNB) solution (10 mM) and 10 µl of acetylthiocholine iodide (ATCI) solution (14 mM) were added and the color product formation was observed. After 10 min, absorbance measurements were performed at 412 nm [6].

Antioxidative activity tests

Various antioxidative activity tests were carried out to elucidate the potential mechanisms of oxidative reactions.

DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay

DPPH radical is a synthetic radical used to measure the free radical removal activity of compounds with antioxidative potential [19]. Ethanolic DPPH solution yields purple color, which is discolored by the antioxidants. Hydrolysate samples (0.1 ml) were mixed with 0.1 ml of DPPH solutions (53 mg DPPH. L^{-1} methanol). Samples containing only peptide preparation buffer, DPPH with buffer, DPPH with enzyme and DPPH with protein were also prepared for measurements as standards and references. All samples containing DPPH were incubated in the dark for 15 min before measurements. Then, absorbance measurements were performed at 515 nm [20, 21]. Inhibitory capabilities of the samples were evaluated in comparison to the control samples.

Iron chelation activity assay

In the iron chelation activity test, ferrozine, which is a strong iron binding reagent, and the sample to be analyzed are mixed in the presence of Fe^{2+} ions to compete for iron binding [21]. The Fe^{2+}/ferrrozine complex yields a strong red color and its intensity decreases as the complex formation is prevented by inhibitors [22]. In accordance with Ebrahimzadeh et al. [23], the capacity of black cumin proteins and peptides to chelate Fe^{2+} ions was determined. 0.5 ml of hydrolyzed peptide dispersions (02 h) were mixed with 1.6 ml of water and 0.05 ml of 2 mM $FeCl_2$. After 30 s of incubation at ambient temperature, 0.1 ml of 5 mM ferrozine was added to the mixture and incubated for 10 min in the dark. The absorbance of Fe^{2+}-ferrozine complex was measured at 563 nm. Ethylenediaminetetraacetic acid (EDTA) (0.2 mg.mL^{-1}), the most powerful iron chelator known, was a positive control in the evaluation of the chelation activity. Buffer, enzyme and protein concentrate samples were also measured as references or blanks.

Hydroxyl radical scavenging activity assay

Hydroxyl radical (OH^-) is the free radical that forms through the reduction of hydrogen peroxide and has the highest toxicity due to its high reactivity [24]. Since it is characterized with a short half-life, hydroxyl radical can cause great damage and lead to the formation of other radicals [25]. In this assay, an aliquot from 10 mM $FeSO_4$ solution (0.1 ml), 0.1 ml of 10 mM EDTA, 0.5 ml of 10 mM α-deoxyribose, 0.9 ml of potassium phosphate buffer solution (pH 7) and 0.2 ml of protein or peptide dispersions were mixed. 0.2 ml of 10 mM H_2O_2 was added to this mixture and the samples were incubated for 1 h at 37 °C (1000 rpm). Afterwards, 1 ml 2.8% trichloroacetic acid (TCA), and 1 ml 1.0% thiobarbituric acid (TBA) were added to the sample tubes and the samples were kept in a boiling water bath for 15 min. Absorbance values were measured at 532 nm when samples were cooled to ambient temperature. Similarly, absorbance values for the buffer solution and enzymes were measured in the absence of protein or peptide samples [26].

Fractionation of protein hydrolysates

Since the highest signal intensity and antioxidative activity were mostly observed in 30 min papain treated hydrolysates, these samples were used in the fractionation experiments. Black cumin protein hydrolysates were separated using 1 ml Capto DEAE (i.e., weak anionic) column at a rate of 1 ml per minute based on various pre-trials and fractionated using ÄKTA Pure 25 L (GE Life Sciences, Sweden) FPLC system supplied with UV and conductivity detectors. A 0–100% salt gradient (i.e., 0–1 M) was administered over 35 CV (column volumes), where 20 mM Tris–HCl (pH 8.0) sample buffer and 1 M NaCl containing identical Tris–HCl elution buffer were utilized. Fractions were collected by an automatic sampler and cationic peptides were mostly discarded in the waste stream [27].

LC-Q-TOF/MS analysis of hydrolysate fractions

Hydrolysate samples were incubated with 10 mM dithiothreitol (DTT) at 55 °C for 10 min to ensure the reduction of the peptides. The reduced peptide mixtures were then alkylated with 20 mM iodoacetamide (IAA) in the dark at ambient temperature conditions. Samples were filtered through 30 kDa filters. Peptide concentration of the samples was determined and the samples were taken into vials for LC-Q-TOF/MS analysis at 1 mg per injection. Prior to the analyses, the detector and calibration settings were made via MassLynx program specific to the Xevo G2-XS QTof device (Waters). The peptide fractions were further fractionated with an acetonitrile gradient (535%) in an HSS T3 column based on their hydrophobicity and the separated peptides were analyzed by mass spectrometry upon electrospray ionization. MS analysis was performed for 0.7 s and information was collected about the entire peptide. Afterwards, MS/MS analysis was performed for 0.7 s and the peptide fragmentation and sequence information was obtained. Peptides and proteins were identified using ProteinLynx Global Server (PLGS 3.0) software. Analysis was performed using the appropriate databanks for each sample type. The selected parameters for PLGS were based on the default settings of Labmed, Acıbadem University, İstanbul, Turkey where all the LC-Q-TOF/MS analyses were carried out.

In silico analyses and molecular docking

Upon the acquisition of MS-data, the determined peptide sequences were analyzed in silico for the prediction of their physicochemical and bioactive characteristics. Firstly, the isoelectric point, charge and toxicity assessment of the peptides were analyzed based on the work of Gupta et al. [28]. The probability of peptide sequences being bioactive was determined by PeptideRanker [29]. Finally, in cases where bioactivity was observed and these observations were supported by in silico calculations, the interactions between the respective peptides and the enzyme they inhibit (i.e., AChE from human) were analyzed based on the method of Trabuco et al. [30]. Using a molecular docking approach, peptide-protein interactions were also studied via HPEPDOCK, which generated docking scores and 3D protein-peptide interaction images [31]. In order to analyze the influence of the current hydrolysates in the human body, ADMET (absorption, distribution, metabolism, excretion and toxicity) properties of the hydrolysates were studied in silico [32].

Statistical analysis

The data collected in all of the experiments were reported as sample means ± standard deviation based on at least triplicate experiments. Whether differences existed between various treatments were studied based on statistical significance ($p \leq 0.05$).

Results and discussion

Acetylcholinesterase (AChE) inhibitory activity tests

Black cumin protein concentrate dispersions (1%) were subjected to proteolytic hydrolysis with papain and trypsin. The potential anti-Alzheimer activity of the hydrolysates were analyzed based on their acetylcholinesterase (AChE) inhibitory activities, as previously reported by Şenol et al. [6]. The results for these tests were summarized in Fig. 1. Firstly, black cumin protein concentrates demonstrated mild AChE inhibitory activities (28.72 ± 1.8%) prior to hydrolysis. For papain hydrolyzed samples, inhibitory activity gradually decreased in comparison to non-hydrolyzed concentrates.

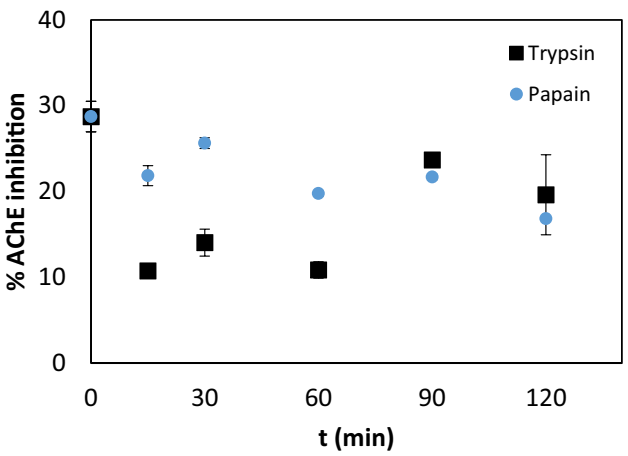

Fig. 1 AChE inhibitory activities of trypsin or papain treated (0–2 h) black cumin protein hydrolysates

Over a 2 h hydrolysis procedure, % inhibition values dropped down to approx. 16.8%. For the trypsin hydrolyzed samples, the relation between hydrolysis duration and % inhibition was not clear. An average value of approx. 17.5% was attained for all trypsin treatments. Consequently, in both cases, it was not possible to enhance the inhibitory activity based on enzymatic treatments and the concentrates performed as better AChE inhibitors than the hydrolysates, while the unprocessed concentrates were reasonably effective in AChE inhibition. Previously, the molecular sizes of peptides were found to be influential on the AChE inhibitory characteristics of hemp seed protein hydrolysates [33], which in turn might partly explain the reduction in AChE inhibitory characteristics of the black cumin protein hydrolysates. In most cases, the concentrates and hydrolysates generated a moderate AChE inhibitory effect, while the average AChE inhibitory activity of papain hydrolysates were slightly higher than that of trypsin hydrolysates.

Antioxidative activity assays

In this study, multiple antioxidative tests were carried out to determine the antioxidative performance of black cumin protein concentrates and their hydrolysates against various oxidative radicals. Black cumin protein concentrates treated with papain or trypsin enzymes were studied using DPPH inhibition, iron chelation and hydroxyl radical scavenging activity tests.

DPPH inhibitory activity assay

The antioxidative potential of the protein hydrolysates and fractions were analyzed based on the DPPH inhibition for the samples hydrolyzed with papain or trypsin. The results were summarized on Fig. 2a. DPPH inhibitory activity of the non-hydrolyzed protein concentrate was found to be approx. 30.4%. In papain hydrolysates, the activity generally increased with proteolysis duration. The highest activity was obtained from the concentrate which was hydrolyzed for 120 min (approx. 59.8%), while the results for 30 min were comparable at 52.8% inhibition. In trypsin hydrolysates, after 15 min, % inhibition was 41.7%. Beyond 15 min, no further increases in activity were recorded. In summary while increases in proteolysis duration enhanced activity for papain hydrolysates, that was not necessarily the case for trypsin hydrolysates treated beyond 15 min.

Iron chelation activity assay

Iron chelation activity of black cumin protein concentrate dispersions and their hydrolysates were analyzed after treatment with papain and trypsin (0–2 h) (Fig. 2b). Once again, in papain hydrolysates, iron chelation activity generally

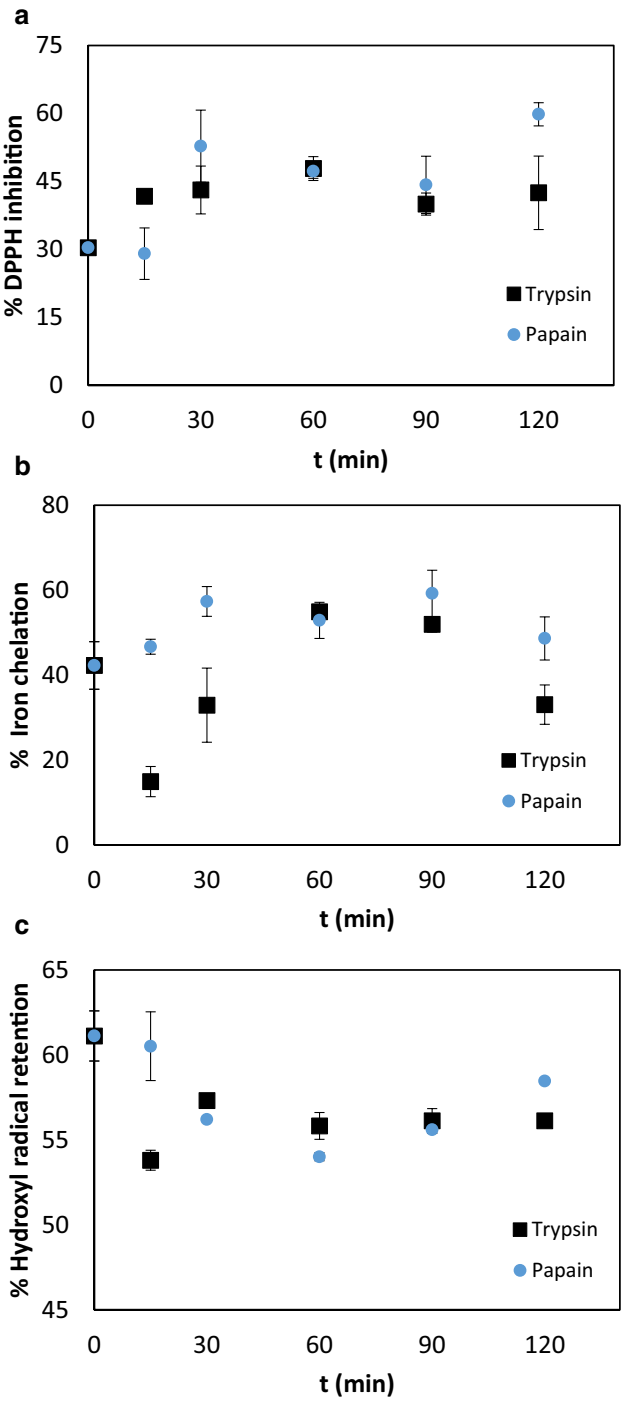

Fig. 2 **a** % DPPH inhibition, **b** % iron chelation activity, and **c** % hydroxyl radical scavenging activity of black cumin protein concentrates and hydrolysates (0–2 h) treated with papain or trypsin

increased with proteolysis duration. In most cases, the activity accounted for approx. 50% between 15 min and 2 h. In terms of shorter processing durations, the optimal activity could be attained after 30 min of papain hydrolysis (57.33% ± 3.5). The hydrolysis duration was not highly

correlated with the iron chelation activity in trypsin hydrolysates (Fig. 2b). While non-hydrolyzed protein concentrates demonstrated an activity level of 42.27% ± 5.6, and the highest activity was observed after 60 min (54.91% ± 1.5), further extension of hydrolysis treatment reduced the activity of the hydrolysates down to about 33%. Iron chelation activity test performed with EDTA (0.2 $mg.ml^{-1}$) demonstrated 89.03 ± 4.1% chelation activity. These results showed that although they are not as effective as EDTA, black cumin protein concentrates and their hydrolysates acted as natural antioxidants with iron chelating potential.

Hydroxyl radical retention activity test

The hydroxyl radical retention activities of the black cumin protein concentrate dispersions and their hydrolysates were measured (Fig. 2c). Non-hydrolyzed black cumin protein concentrate was observed to demonstrate a hydroxyl radical retention activity of 61.12 ± 1.5%. Papain hydrolysis generally leads to moderate reduction in the activity. Consequently, non-hydrolyzed concentrates were mostly higher in activity compared to the papain hydrolysates. Similar results were obtained for trypsin hydrolysates, although the extent of reduction in activity was mostly slight. In this test, hydroxyl radical is produced in vitro by reaction of Fe^{2+} and H_2O_2 and the hydroxyl radical retention activity of the tested sample is measured. The samples exhibiting antioxidant activity may also indirectly inhibit the formation of hydroxyl radicals by chelating the Fe^{2+} ion. Therefore, a good chelation or hydroxyl radical retention effect of the tested sample cannot be fully distinguished [25].

In order to enhance the antioxidative attributes of the protein hydrolysates, samples that demonstrated antioxidative activity were subjected to FPLC-based fractionation. In all antioxidative activity tests, maximum antioxidative activity of the hydrolysates was mostly obtained after 30 min of papain treatment. Consequently, fractionation was primarily carried out for 30 min papain hydrolysates.

DPPH inhibitory activity of the hydrolysate fractions

After a number of fractionation pre-trials with weak anionic, strong anionic or cationic columns (data not shown), the most intense peptide signals were obtained upon utilization of HiTrap Capto DEAE anion exchange column, which is a weak anionic column. Papain hydrolysates (P) were fractionated over a linear salt gradient and the major peaks in the chromatogram were divided equally into 4 major fractions named as PA, PB, PC, and PD (Fig. 3). In order to determine the corresponding antioxidative activities of the fractions, DPPH inhibitory activity tests were administered (Fig. 4). As the overall volume in the fractions was higher than the injected hydrolysate samples, peptide concentrations in the

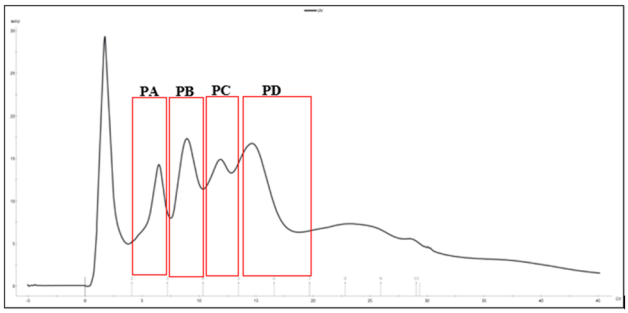

Fig. 3 Fractionation of papain treated (30 min) black cumin protein concentrates using a 1 ml HiTrap Capto DEAE weak anionic column, where a 0–100% salt (1 M NaCl) linear gradient was administered over 35 column volumes

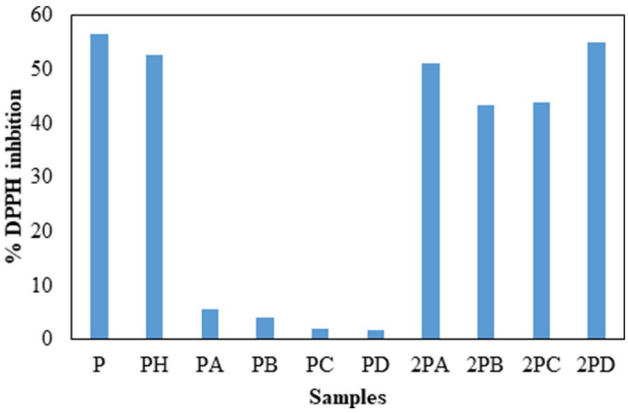

Fig. 4 DPPH inhibitory activities (%) of black cumin protein concentrates (P), their papain treated (30 min) hydrolysates and their corresponding fractions (PA, PB, PC and PD). In addition, hydrolysate volumes in the DPPH assay mixtures were doubled (2PA, 2 PB, 2PC and 2PD) to investigate concentration dependence. Standard deviation was < 5% of the sample mean in all cases

fractions can be anticipated to decrease. Consequently, the DPPH inhibitory activity of the fractions were lower compared to that of non-hydrolyzed concentrates. The highest activity was attained by the PA fraction which was the weakest anionic fraction. To ensure the presence of antioxidative capacity in the samples, a second set of DPPH analyses was carried out. In this case, twice the volume of hydrolysates was added to the assay medium to test the validity of findings. In all cases, fractions demonstrated significantly higher DPPH-inhibitory activity compared to the previous tests. These findings might be attributed to the relatively low concentrations of peptides in the fractionated hydrolysates. The double volume samples (2PA, 2 PB, 2PC and 2PD) demonstrated DPPH inhibitory activities (%) of 51.2, 43.4, 43.9, and 55.1%, respectively. While the concentration, anionic behavior and composition of the samples might be quite different, the inhibitory activity of each and every

fraction was comparable to that of the non-hydrolyzed protein concentrates.

Mass spectrometry and in silico analyses

Active fractions from papain treated hydrolysates were analyzed using an LC-Q-TOF/MS methodology and a sample spectrum is shown on Figure S1 of Supplementary Data. Among these fractions, 20 different spectra were generated and the spectral data were matched to the peptide structures listed in Uniprot and PLGS databanks in order to elucidate the sequences of active peptides. The determined sequences were listed in Table S1 of Supplementary Data along with their calculated physicochemical characteristics and bioactive potential.

As detailed on Table S1, none of the black cumin peptides identified here were found to be toxic agents in silico and pI values of these peptides were in the range of approx. pH 44.7 [28]. Since the majority of these peptides were not located in current databases, some of our findings possibly pointed out to novel proteins or peptides. This is not surprising since the current findings on black cumin proteins are mostly scarce [15]. Among the 20 different peptides listed on Table S1, 3 of them (ASADTSNTGSVSEANAQYYQQEAGKLK, YDLDFK, PICESLNILEYIDEIWPHNR) were found to demonstrate a PeptideRanker score > 0.5 indicating potential bioactivity which supported the in vitro findings. Thus, while the current peptides demonstrated antioxidative and/or AChE inhibitory activity in vitro, PeptideRanker [29] showed that some of them could demonstrate bioactivity.

Based on these findings, the potential interaction of a sample peptide (YDLDFK, PeptideRanker score > 0.5) with AChE was investigated in silico [30]. The findings are listed on Figure S2 of Supplementary Data and Table 1. Figure S2 demonstrated the interaction mechanism of the peptide and AChE visually, while Table 1 summarized which residues on AChE were likely to bind to the potentially inhibitor peptide. Consequently, in silico techniques predicted the probability of binding between the potential inhibitor and AChE enzyme was significant ($p \leq 0.05$) (Figure S2). According to the most probable model, 5 amino acids in YDLDFK peptide were predicted to actively bind AChE, while 12 potential binding sites existed on AChE for YDLDFK binding which in turn could cause inhibition. It should also be noted that human AChE consists of 614 amino acids, which demonstrates the relative effectiveness (i.e., 12 potential site among 614 amino acids) of the model.

Furthermore, protein-peptide interactions were studied through a molecular docking approach [31]. While information about the binding site can be predicted by global peptide interaction algorithms such as PepSite [30], HPEPDOCK can be utilized for local peptide interactions, where binding modes can be obtained by high resolution within the binding site. Table 2 and Fig. 5 demonstrate the docking scores and high resolution images of the most probable interaction models, respectively. In all cases, a significant interaction was predicted between the peptides and AChE.

ADMET properties

ADMET properties of the potentially bioactive black cumin peptides were investigated [32]. In terms of absorption characteristics (Table 3), these peptides could not penetrate the blood–brain barrier (BBB) or demonstrate Caco-2 permeability. However, they could potentially be absorbed in the human intestinal absorption (HIA) system enhancing their chances of demonstrating bioactivity.

In terms of metabolism characteristics (Table 3), these peptides were mostly not substrates or inhibitors for Cytochrome P450 (CYP450) enzymes. The only exception was that they could potentially serve as a substrate for CYP450 3A4 enzyme.

Table 2 Docking scores of the predicted protein-peptide interactions (A: YDLDFK, B: PICESLNILEYIDEIWPHNR and C: ASADTSNTGSVSEANAQYYQQEAGKLK)

Rank	Docking score-A	Docking score-B	Docking score-C
1	−173.050	−196.728	−188.242
2	−152.999	−189.498	−188.138
3	−152.466	−188.632	−185.240
4	−150.510	−186.037	−178.919
5	−148.780	−185.870	−177.819
6	−146.714	−182.937	−174.402
7	−146.642	−182.553	−170.679
8	−145.991	−182.372	−164.531
9	−145.886	−181.755	−163.081
10	−144.130	−175.471	−161.933

Table 1 List of the major amino acids in which YDLDFK peptide interacts with AChE

Sequence	Active amino acids	P-value	Potential binding sites on AChE
YDLDFK	Tyr-1 Leu-3 Asp-4 Phe-5 Lys-6	0.0005206	Tyr72, Asp74, Gly82, Trp86, Tyr124, Trp286, Phe297, Tyr337, Phe338, Tyr341, Tyr439, Met443

The most probable model (Model 1) was used

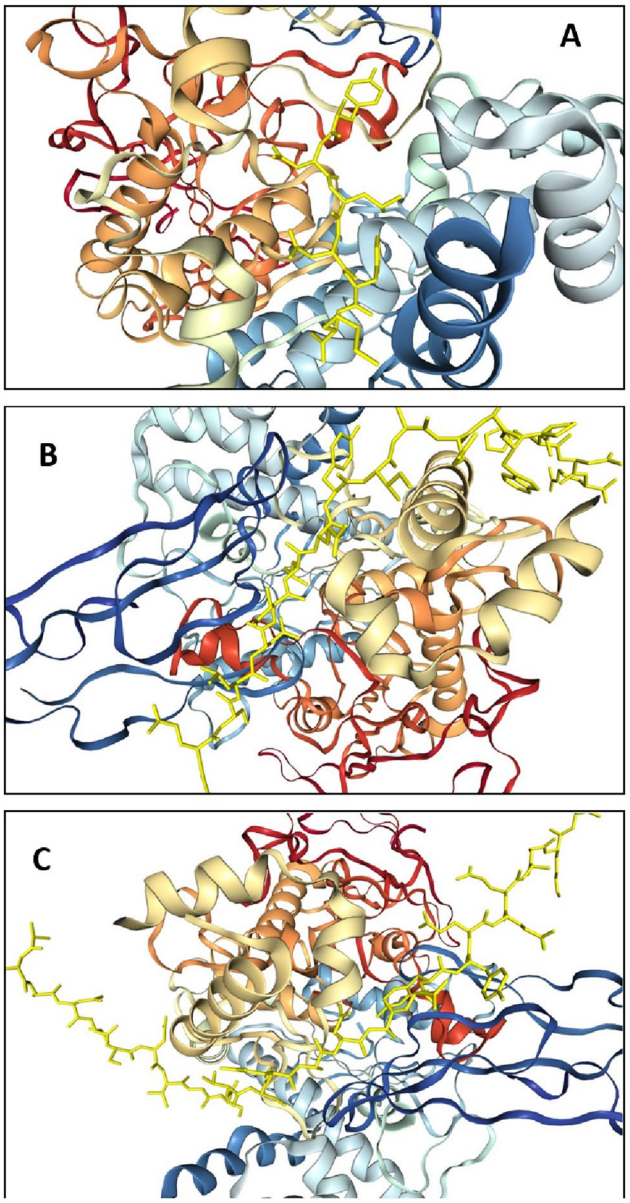

Fig. 5 Visual schematization of the interaction of the highest Peptide Ranker value peptides in each and every enzymatic digest with AChE. Molecular docking via HPEPDOCK was carried out for **a** YDLDFK, **b** PICESLNILEYIDEIWPHNR and **c** ASADTSNTGSVSEANAQYYQQEAGKLK peptides. The most probable model (Model 1) was used in all cases

Finally, none of the peptides were classified as carcinogens by the admetSAR system [32]. Based on these findings, papain hydrolysates generated from black cumin protein concentrates are well-tolerable, potentially non-toxic, non-carcinogenic components which do not or minimally affect the usual metabolism or pharmacokinetics of medicinal components. Meanwhile intestinal uptake capabilities seem to be present in the human body.

Discussion

In the previous studies, a novel peptide from *Ziziphus jujuba* fruits was shown to demonstrate both AChE inhibitory and antioxidative characteristics [34]. Furthermore, a pentapeptide (EQRPR) obtained from rice bran potentially possessed protective capabilities against Alzheimer's disease since the reduction in cell cytotoxicity was achieved on amyloid-induced neuronal cells [35]. Consequently, plant protein hydrolysates seem to be a rational source of AChE inhibitory and/or antioxidative active agents. Zare-Zardini et al. [34] indicated that dual bioactivity (i.e., cholinesterase inhibitory and DPPH scavenging activities) could be attributed to the hydrophobic characteristics of their active peptide (i.e., Smakin-Z), which consisted of approx. 80% of neutral and hydrophobic residues [36].

The three peptides investigated in detail here have anionic characteristics as anticipated by the separation method utilized (i.e., anion exchange) and demonstrated by their calculated pI values. Under most physiological pH conditions, negative charge potentially carried by these peptides could affect their interactions with AChE and their binding capabilities to positively charged metal ions, which otherwise could promote oxidative reactions. It is noteworthy that potential binding sites of YDLDFK peptide summarized on Table 1 consisted of 50% neutral, 41.67% hydrophobic and 8.33% acidic (i.e., 1 in 12) residues implying that electrostatics were possibly secondary in this potential interaction. Similar to Snakin-Z, other peptides listed on Table S1 (ASADTSNTGSVSEANAQYYQQEAGKLK and PICESLNILEYIDEIWPHNR) demonstrated roughly 70% and 80% of combined neutral and hydrophobic residues, respectively. It must be noted that in various cases, bioactive peptides with acetylcholinesterase inhibitory activities were characterized with relatively high molecular weights similar to the current peptides (for example, Ahn et al. [37]). Both ideas were coherent with the findings of Malomo and Aluko [38], which also pointed out to the presence of hydrophobic residues in their AChE inhibitory fractions prepared through reverse phase HPLC based separation and located up to 11 amino acid active peptides in these fractions.

Our group previously found out that black cumin protein concentrates and their corresponding hydrolysates demonstrated moderate ACE-inhibitory activities [14]. Here we have effectively demonstrated in silico and in vitro that these hydrolysates also possessed antioxidative and anti-AChE characteristics. Collectively the data point out to the fact that black cumin proteins and their hydrolysates have a good potential in the generation of bioactive components.

Table 3 Critical ADMET parameters for the most probable bioactive peptides among the identified peptides

		ASADTSNTGSVSE-ANAQYYQQEAGKLK	**YDLDFK**	**PICESLNI-LEYIDEI-WPHNR**
	Blood–Brain barrier	–	-	–
Absorption	Human intestinal absorption	+	+	+
	Caco-2 permeability	–	-	–
	CYP450 2C9 substrate	Non-substrate	Non-substrate	Non-substrate
	CYP450 2D6 substrate	Non-substrate	Non-substrate	Non-substrate
	CYP450 3A4 substrate	Substrate	Substrate	Substrate
Metabolism	CYP450 1A2 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
	CYP450 2C9 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
	CYP450 2D6 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
	CYP450 2C19 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
	CYP450 3A4 inhibitor	Non-inhibitor	Non-inhibitor	Non-inhibitor
Toxicity	Carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens

Conclusion

In terms of sustainability, valorization of industrial and/or agricultural byproducts is of paramount importance and their utilization exploits the potential to generate value added products and benefit human health. Here, we made an effort to generate peptides with dual (antioxidative and acetylcholinesterase inhibitory) functionality from industrially cold pressed black cumin cakes using enzymatic hydrolysis and FPLC-based fractionation. The protein concentrates, their hydrolysates and corresponding peptide fractions were observed to demonstrate antioxidative and/or AChE inhibitory activity to varying extents. Mass spectrometry was instrumental in linking the in vitro findings to the determination of specific and effective peptide sequences, for which the individual potential activities were also predicted in silico. Bioactive peptides obtained from deoiled black cumin cakes could facilitate the generation of value-added products through further studies. MS based identification of active black cumin peptides could lead the path to the design of functional agents that can be utilized in functional foods, food supplements and pharmaceutical formulations.

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Compliance with ethical standards

Conflicts of interest None.

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