

Antioxidant and Protein Protection Potentials of Fennel Seed-derived Protein Hydrolysates and Peptides

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Abstract: Fennel seed is an edible spice frequently used to enhance the aroma and taste of assorted cooking, and it is treasured across different cultures for its medicinal values. Previous studies have reported on the various bioactivities linked to fennel seeds. However, no much is known of hydrolyzed proteins derived from fennel seeds. In this paper, total proteins were isolated from fennel seeds, followed by enzymatic hydrolysis using four different proteases. The degree of hydrolysis, pH and temperature profiles were reported. Guided by ABTS radical scavenging assay, the alcalase-digested protein hydrolysates were further fractionated using centrifugal filtration, C18 reverse-phase and strong-cation exchange (SCX) fractionations. Compared to the crude hydrolysates, the fractionated hydrolysate demonstrated a 4.5-fold enhancement in its radical scavenging potential. The purified SCX fraction were further analyzed by de novo peptide sequencing, and two peptides sequences were identified and ranged from six to eight amino acids. The identified peptides were tested for their protein protection potential using albumin protein denaturation inhibitory assay, with one of the identified peptides (EDVDFR) showing comparable EC₅₀ value to that of commercial glutathione (GSH). In summary, fennel seed-derived protein hydrolysates and the identified peptide could potentially be exploited for utilization as health-promoting supplement or as food additive to preserve protein-rich foods.

Key words: antioxidant peptide; enzymatic hydrolysis, fennel seeds; protein protection; pH and thermal stability

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茴香种子蛋白质水解物和肽的抗氧化和蛋白质保护潜力

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摘要: 茴香种子是一种可食用的香料, 经常用于增进各种烹饪的香气和味道, 并且因其药用价值而在不同文化中被珍藏。之前的研究报告提及了与茴香种子相关的各种生物活性。然而, 对于来自茴香种子的水解蛋白质知之甚少。在本文中, 作者从茴香种子中分离出总蛋白质, 然后使用四种不同的蛋白酶进行酶水解。本文中也报道了水解度, pH和温度曲线。在ABTS自由基清除测定的指引下, 作者使用离心过滤, C18反相和强阳离子交换(SCX)来进一步分离碱性蛋白酶消化的蛋白质水解物。与粗水解物相比, 纯化水解物的自由基清除潜力增强了4.5倍。通过肽测序进一步分析纯化的SCX级分, 本文也鉴定出两个肽序列, 其范围为6~8个氨基酸。紧接着, 文中也报道了使用白蛋白变性抑制试验来测试茴香肽的蛋白质保护潜力, 其中一种茴香肽(EDVDFR)更被测出与纯谷胱甘肽(GSH)相当的EC₅₀值。总言之, 茴香种子衍生的蛋白质水解物和茴香肽有潜力被进一步开发成促进健康的补充剂或作为食品添加剂用于保存富含蛋白质的食物。

关键词: 抗氧化肽; 酶水解; 茴香籽; 蛋白质保护; pH和热稳定性肠稳定性

Foeniculum vulgare is a perennial, pleasant-smelling herb with a long cultivation history as a medicinal plant^[1]. *F. vulgare* seed or more commonly known as fennel seed is an important spice treasured worldwide for both its culinary and medicinal values^[2]. The fennel seeds have

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significant economic importance as they are widely used in the pharmaceutical, food, cosmetic, and healthcare industry^[3,4]. Additionally, in Middle East, Europe and Chinese traditional medicines, fennel seeds are used to treat bloat, diarrhea, menstrual problems, and piles. Likewise, fennel seeds are reportedly used as diuretics and relief for hypertension^[5,6]. Currently, many studies have reported on the medicinal properties of fennel

seed-derived phytochemicals including flavonoids, phenolic compounds, terpenoids and glycosides, along with their antioxidation, antitumor, and chemopreventive potentials^[6-9]. However, no much is known of protein hydrolysates and bioactive peptides derived from fennel seeds.

In general, protein hydrolysates and bioactive peptides are produced from their parental proteins *via* enzymatic hydrolysis, microbial proteolytic actions and fermentation^[10,11]. Among them, protein hydrolysates and bioactive peptides from food sources are especially noteworthy, as lower toxicity are implied. Previous studies have reported on their functional roles in scavenging free radicals, reducing oxidative stress, and possible alleviation for cellular inflammation and degenerative diseases^[12]. As a result of research and development efforts in this field, peptide-based therapeutic drugs have been developed and introduced, including but not limited to LupronTM for treating prostate cancer and VictozaTM for treating type 2 diabetes, with many other peptide-based therapeutic agents in various phases of clinical trials^[13].

In this study, protein hydrolysates were prepared from fennel seed proteins using four different proteolytic enzymes, namely alcalase, papain, bromelain, and trypsin. The protein hydrolysates were then tested for their radical scavenge potentials. Their stabilities under different thermal and pH treatments were also compared. Guided by ABTS radical scavenging assay, the alcalase-digested protein hydrolysates were further fractionated using centrifugal filtration, C18 reverse-phase and strong-cation exchange fractionations, followed by analysis using *de novo* peptide sequencing. The identified bioactive peptides were also tested for their protein protection potential using albumin protein denaturation inhibitory assay.

1 Experimental

1.1 Materials and reagents

Fennel seeds were purchased from a supermarket in Gopeng, Perak, Malaysia. Ammonium sulfate, o-phthaldialdehyde and ultrafiltration centrifugal units (MWCO 3 ku) were purchased from Merck. Trypsin was from Nacalai Tesque, while Alcalase and papain were

purchased from Calbiochem. Dialysis tubing was purchased from Fisher Scientific, phosphate-buffered saline (PBS) from Takara. Solid-phase extraction cartridges STRATA C18-E (sorbent mass: 500 mg; volume: 6 mL) and STRATA SCX (sorbent mass: 1000 mg; volume: 6 mL) were purchased from Phenomenex Inc. All reagents used were of analytical grade.

1.2 Preparation of fennel seed protein isolate and hydrolysates

Dried fennel seeds were firstly ground using a blender. Then, the fennel powder was suspended in chilled deionized water at the mass (g): volume (mL) ratio of 1:5 and stirred for 30 min at 4 °C. After heating at 90 °C for 20 min, the mixture was centrifuged at 9000 r/min for 20 min to remove water-insoluble substances. Next, the collected supernatant was adjusted to 80% ammonium sulfate saturation, followed by stirring at 4 °C for an hour. The mixture was then centrifuged at 9000 r/min at 4 °C for an hour to pellet the protein precipitates. Then, the isolated proteins were dialyzed overnight at 4 °C using dialysis tubing (molecular weight cut-off: 6000~8000 u). The dialyzed protein solution was freeze-dried and stored at -20 °C until use. Protein content was quantified by Bradford assay based on a bovine serum albumin (BSA) standard curve^[14].

For the preparation of protein hydrolysates, the freeze-dried protein isolate (0.5 g) was dissolved in 100 mL of 50 mM phosphate buffered saline (PBS) solution. Next, the proteases (alcalase, papain, bromelain, and trypsin) were added separately to the protein solution at a weight ratio of 1:10 (protease: protein), followed by incubation in water bath (50 °C for alcalase and bromelain; 37 °C for papain and trypsin) for one to eight hours. At the end of the incubation period, proteases were heat inactivated at 100 °C for 10 min, cooled on ice, and then freeze-dried. Degree of hydrolysis (DH) was determined as previously described^[15,16] for each protease treatment to identify the optimum proteolysis durations.

1.3 Determination of 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS^{·+})

radical cation scavenging potential

ABTS radical cation ($\text{ABTS}^{\cdot+}$) scavenging activities of the protein hydrolysates were determined as previously described previously, with some minor modifications [17,18]. ABTS^+ stock solution was prepared by mixing an equal volume of ABTS solution (8 mg/mL) with potassium persulfate (1.32 mg/mL). The mixture was kept for 12 hours in the dark at room temperature before use. Then, for ABTS^+ working solution preparation, the ABTS^+ stock solution was diluted with potassium phosphate buffer (100 mM, pH 7.4) to obtain an absorbance in the range of 1.000~1.099 at 734 nm. For measurements, 20 μL of sample was added to 200 μL of ABTS^+ working solution. The mixture was left in the dark for 10 min before its absorbance was read at 734 nm using a microplate reader (FLUO star Omega, BMG Labtech). ABTS^+ radical scavenging activity (%) was calculated as shown below:

$$\text{ABTS}^{\cdot+} \text{ radical scavenging ability (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100\%$$

Where A_{control} is the absorbance of control reaction (without protein hydrolysate) and A_{sample} is the absorbance in the presence of protein hydrolysate. EC50 is defined as the concentration required to scavenge 50% of the radicals. GSH and carnosine were used as the positive controls.

1.4 Determination of thermal and pH stability profiles

Thermal stability of the 2 h alcalase protein hydrolysate was determined according to published conditions, with some minor modifications [19,20]. Briefly, protein hydrolysate solutions were incubated for 30 min in a temperature-controlled water bath at 25, 65, 75, 85 or 100 °C. After thermal treatment, the ABTS scavenging activities were measured as described above. Likewise, for the pH stability, published method from was used with some modifications [21]. Here, sample solutions were adjusted to pH 3, 5, 7, 9, or 11, followed by incubation at 25 °C for 60 min. Then, all the sample solutions with different pH were adjusted back to pH 7 before measuring their ABTS scavenging activities.

1.5 Fractionation of alcalase hydrolysate by centrifugal filtration

After the identification of protein hydrolysate with promising activity, alcalase hydrolysate was further fractionated by membrane centrifugal filtration as reported previously with modifications [14]. Briefly, ten mL of the alcalase hydrolysate (2 mg/mL in deionized water) was added into a 3 ku MWCO ultrafiltration centrifugal unit. It was then centrifuged at 4000 r/min for 20 min. The retentate was labelled as >3 ku UF, whereas the permeate fraction was designated the as <3 ku UF. Then, peptide contents of the fractions were measured with the o-phthalaldehyde (OPA) method [22]. OPA reagent was prepared firstly by mixing disodium tetraborate decahydrate (0.953 g) and SDS (0.030 g) into 20 mL deionized water. The mixture was stirred at 25 °C for an hour. Then, OPA solution (0.020 g dissolved in 95% ethanol) and 0.022 g DTT were added to the mixture, before bringing the total volume up to 25 mL with deionized water. For measurements, 20 μL sample was added to 200 μL OPA reagent and left in the dark for 2 min, before its absorbance was read at 340 nm using a microplate reader. Casein peptone was used as the reference standard to plot the calibration curve. $\text{ABTS}^{\cdot+}$ scavenging activity was measured as described earlier.

1.6 Fractionation of alcalase hydrolysate by C18-E solid phase extraction (C18-SPE)

The <3 ku UF fraction was further fractionated by using Strata® C18-E SPE cartridges following the manufacturer instruction with modifications. Briefly, the <3 ku UF fraction (1.47 mg/mL, 1 mL) was applied to the C18-E cartridges which were preconditioned with methanol (3 mL) and equilibrated with deionized water (3 mL). After loading of sample, the cartridges were washed with 5% methanol (3 mL). The <3 ku UF was fractionated using a stepwise elution (3 mL) of pure methanol, isopropanol, acetonitrile, and hexane. This produced a series of fractions designated as C18-F1, C18-F2, C18-F3, and C18-F4 respectively as well. The peptide content and $\text{ABTS}^{\cdot+}$ radical scavenging activity

of each fraction were measured as described earlier.

1.7 Fractionation of alcalase hydrolysate by strong cation exchange solid phase extraction (SCX-SPE)

Further purification of the C18-F1 identified was done by using Strata® SCX-SPE cartridges following the manufacturer instruction with modifications. Briefly, SCX buffer A was prepared by adding KH_2PO_4 (10 mM) in 25% acetonitrile, and SCX buffer B was prepared by mixing KH_2PO_4 (10 mM) and KCl (350 mM) in 25% acetonitrile. The buffers were adjusted to pH 3 using 1 M phosphoric acid. Next, C18-F1 sample (7.6 mg/mL; 4 mL) was loaded to the pre-conditioned SCX-SPE cartridge, followed by washing with 4 mL of SCX buffer A. Then, for the elution step, SCX buffer B (12 mL) was applied and collected as twenty four 0.5 mL fractions labelled as SCX-F1 to SCX-F24. The peptide content and $\text{ABTS}^{\cdot+}$ scavenging activity of each fraction were measured as described earlier.

1.8 Determination of fennel seed peptide sequences

Purified peptides in the SCX-F4 were subjected to analysis using de novo peptide sequencing, as described previously [14]. Briefly, the analysis was performed by Proteomics Core Facility, Malaysia Genome Institute, National Institutes of Biotechnology Malaysia. The sample was analyzed on a Waters SYNAPT G2 high definition quadrupole-time-of-flight mass spectrometer, coupled to Waters nanoACQUITY UPLC. Peptide sequencing based on MS/MS spectral data was carried out by using the Protein Lynx Global Server Software (Version 2.4). Peptide sequences identified were synthesized and used for further characterization of their bioactivity. The synthetic peptides were purchased from Bio Basic Inc. (Canada).

1.9 Albumin protein denaturation inhibitory assay

The assay was carried out according to methods described by with some modifications [23,24]. Briefly, a reaction tube was prepared for each mixture containing 100 μL of 1 mg/mL bovine serum albumin (BSA), 140 μL of phosphate buffered saline (PBS), and 100 μL of the peptide sample tested. Deionized water instead of sample was used as a negative control. Then, the mixtures were incubated at 80 °C for 15 min, followed by cooling in ice water. After cooling, the absorbance was measured at 660 nm using a microplate reader. The inhibition percentage of albumin protein inhibition was determined as followed:

$$\text{Albumin protein denaturation inhibition (\%)} = (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100\%$$

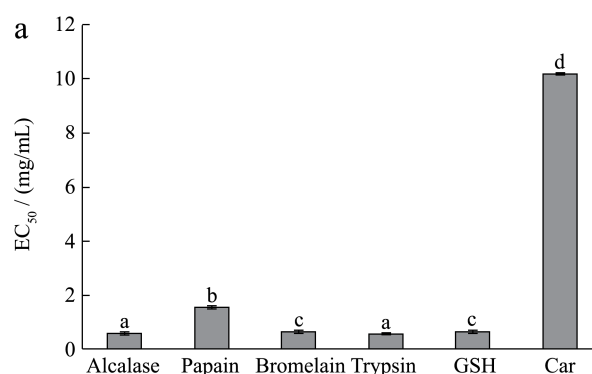
Where A_{control} is the absorbance of the control reaction (without peptide) and A_{sample} is the absorbance in the presence of peptides. The inhibition of albumin protein denaturation was reported as EC_{50} values (concentration required to achieve 50% albumin protein denaturation inhibition). GSH and carnosine were used as positive controls.

1.10 Data Analysis

Data are presented as mean \pm standard errors ($n = 3$). The statistical analysis was performed using SAS (Version 9.4). Data were analyzed by the ANOVA test and means of significant differences were separated using Fisher's Least Significant Difference (LSD) test at the 0.05 level of probability.

2 Results and discussion

2.1 Preparation of fennel seed protein hydrolysates



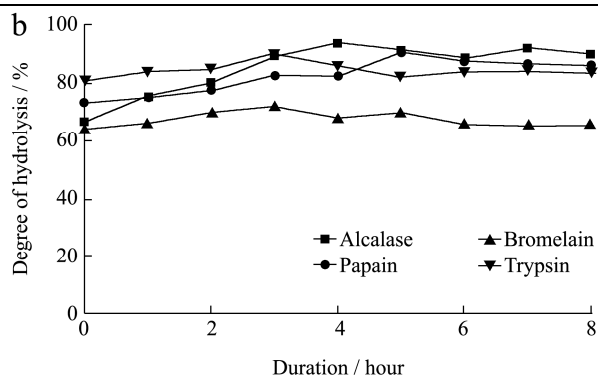


Fig.1 (a) EC₅₀ values of four protease hydrolysates and two antioxidant peptides (GSH, carnosine) tested in ABTS^{·+} radical scavenging assay; (b) Degree of hydrolysis of fennel seed proteins by four proteases over eight hours duration

Note: EC₅₀ values are defined as concentrations required to scavenging 50% of the radical in ABTS^{·+} radical scavenging assay. Data are reported as mean ± SE values (n=3). Different superscripts (a-d) indicate statistically significant differences (*p* < 0.05).

In this study, fennel seed proteins were isolated using ammonium sulfate precipitation method. The yield of the protein isolate was 1.25% of the dry weight of freeze-dried fennel seeds. Based on Bradford assay and bovine serum albumin standard curve, the soluble protein content of the protein isolate was determined at 0.15 g soluble protein/g protein isolate.

After protease treatments, changes in the degree of hydrolysis (DH) of fennel protein hydrolysates were observed over an eight hours duration. The results showed a rise in the DH of fennel proteins treated with alcalase and trypsin during the initial 4 h and 5 h respectively, with a slight declining trend thereafter (Figure 1b). On the other hand, DH of fennel proteins treated with papain and bromelain exhibited a rising trend up to 3 h, declining thereafter. Concurrently, the ABTS^{·+} radical scavenging activities of each protein hydrolysate were also determined. Based on analysis of the ABTS^{·+} scavenging activities of protein hydrolysates from 0~8 hours and DH values, we took optimum hydrolysis duration for all four protease to be 2 hours. Subsequently, the EC₅₀ values (the concentration need to achieve 50% radical scavenging activity) for alcalase, papain, bromelain, and trypsin hydrolysates at 2 h were determined as 0.63±0.00, 1.58±0.01, 0.68±0.01, and 0.59±0.00 mg/mL, respectively (Figure 1a). Among them, alcalase hydrolysate (0.63 mg/mL) and trypsin hydrolysate (0.59 mg/mL) had the lowest EC₅₀ values.

Interestingly, their EC₅₀ values were even lower compared to known antioxidant peptides such as glutathione (GSH, 0.69±0.00 mg/mL) and carnosine (Car, 10.18±0.03 mg/mL) (Figure 1a). On the other hand, the DH values (%) at the optimal duration (2 h) were 79.80±0.56, 85.37±0.17, and 70.21±0.10 and 77.66±0.44 for alcalase, papain, bromelain, and trypsin hydrolysates, respectively (Fig 1b). In summary, guided by the DH results and ABTS^{·+} radical scavenging potentials, we chose to focus on the 2 h alcalase hydrolysate for all further analysis in this paper.

2.2 Effects of thermal and pH treatments

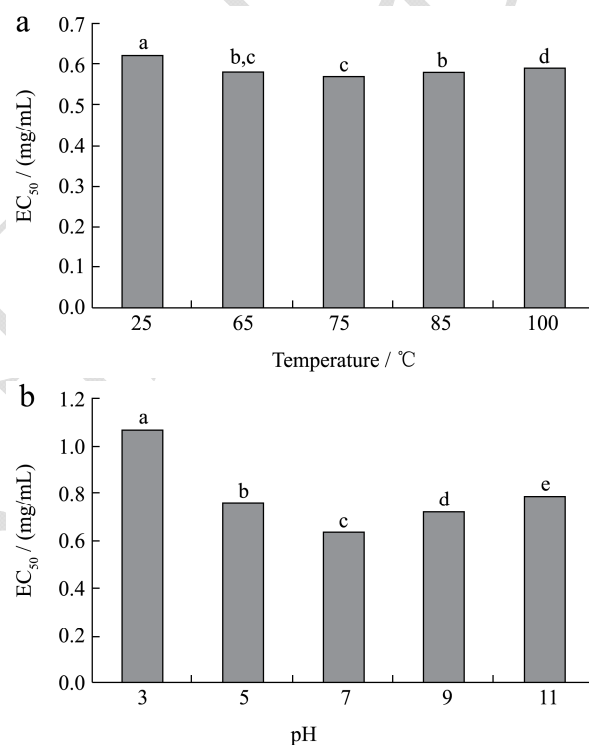


Fig.2 (a) Temperature activity profile and (b) pH activity profile of 2 h alcalase hydrolysate.

Note: EC₅₀ values are defined as concentrations required to scavenging 50% of the radical in ABTS^{·+} radical scavenging assay. Data are reported as mean ± SE values (n=3). Different superscripts (a-e) indicate statistically significant differences (*p* < 0.05).

In order to assess the thermal and pH stability, temperature activity profile (Figure 2a) and pH activity profile (Figure 2b) activity profiles were prepared for the 2 h alcalase hydrolysate. From our results, thermal treatments in the ranges of 25~100 °C did not drastically alter the ABTS^{·+} radical scavenging activity of 2 h alcalase hydrolysate, with EC₅₀ value ranged 0.57~0.62 mg/mL (Fig 2a), which corresponding to a 0.08 fold

difference compared to EC_{50} value at room temperature (25 °C). On the contrary, previous studies from other groups had reported of compromised antioxidant activity following thermal processing [25].

On the other hand, we also tested the radical scavenging potentials of 2 h alcalase hydrolysate following exposure to acidic and alkaline conditions. Here, exposure to pH treatment in the range of 5 to 11 did not drastically alter the $ABTS^{\cdot+}$ radical scavenging potential of the 2 h alcalase hydrolysate, with EC_{50} values ranged between 0.76 to 0.79 mg/mL, which corresponding to a 0.21 to 0.25 fold difference compared to EC_{50} value at pH 7 (Figure 2b). However, following exposure to pH 3, a more significant decrease in radical scavenging potential was observed (0.70 fold). Similar observation had previously been reported with pumpkin oil cake protein hydrolysate with reduced antioxidant potential at acidic and basic pH conditions [26]. This phenomenon may due to the possibility that at highly acidic or basic conditions, the net charges of the protein hydrolysates and bioactive peptides may be altered, which lead to compromised antioxidant potentials [20,27].

2.3 Purification of 2 h alcalase protein hydrolysate

In this study, the 2 h alcalase hydrolysate was subjected to fractionation and purification using centrifugal filtration, C18 solid phase extraction (SPE) and strong cation exchange (SCX) SPE, guided by the $ABTS^{\cdot+}$ radical scavenging assay. Following centrifugation filtration with a molecular weight cut-off of 3 ku, two fractions were produced, namely >3 ku UF and <3 ku UF. $ABTS^{\cdot+}$ scavenging assay revealed that > 3 ku UF had EC_{50} value of 0.45 ± 0.01 mg/mL, while the EC_{50} value of <3 ku UF was 0.58 ± 0.01 mg/mL (Table 1). Next, the <3 ku UF was further purified by using C18-SPE. This produced four SPE fractions, with the C18-F1 fraction demonstrated the strongest $ABTS^{\cdot+}$ radical scavenging activity (EC_{50} of 0.25 ± 0.01 mg/mL). Lastly, C18-F1 was further purified using SCX-SPE, with 24 fractions collected. Among them, the SCX-F4 fraction demonstrated the strongest $ABTS^{\cdot+}$ scavenging activity (EC_{50} of 0.14 ± 0.01 mg/mL). These results were tabulated in Table 1, and their values compared to that of two

commercially available antioxidant peptides (GSH and carnosine). Overall, from the crude 2 h alcalase hydrolysates to the last SCX fractionation step, we observed a 4.5 fold increase in the radical scavenging potentials.

Table 1 EC_{50} values of 2 h alcalase protein hydrolysate and its purification fractions.

Sample	EC_{50} value/(mg/mL)
2 h alcalase hydrolysate	0.63 ± 0.01^a
>3 ku UF	0.45 ± 0.01^b
<3 ku UF	0.58 ± 0.01^c
C18-F1	0.25 ± 0.01^d
SCX-F4	0.14 ± 0.01^e
GSH	0.69 ± 0.00^f
Carnosine	10.18 ± 0.03^g

Note: EC_{50} values are defined as concentrations required to scavenging 50% of the radical in $ABTS^{\cdot+}$ radical scavenging assay. Data are reported as mean \pm SE values (n=3). Different superscripts (a-g) indicate statistically significant differences ($p < 0.05$)

2.4 Identification of fennel seed peptides

In order to identified the bioactive peptide content, the purified SCX-F4 fraction was subjected to de novo peptide sequencing. Here, two peptides with novo score larger than 80 were identified from the SCX-F4 fraction, and their details were summarized in Table 2. These identified peptides ranged from six to eight amino acids, with molecular weight ranges from 779 to 901 u. Additionally, the peptide sequences were searched against the BIOPEP database (<http://www.uwm.edu.pl/biochemia/index.php/en/biopep>) to determine their novelty. We found no record of them in the BIOPEP database, thus confirming their novelty. Subsequently, these identified peptide sequences were synthesized and tested for their bioactivities.

The antioxidant potentials of the bioactive peptides are widely believed linked to their amino acid compositions. For instance, numerous research papers have reported on the important contributions of both hydrophobic and aromatic amino acids towards the antioxidant potentials [27-29]. These literature findings are agreeable with our identified peptides, whereby the sequences of Peptide 1 (EDVDFR) and Peptide 2 (ALEDVVEK) contain two (V, F) and three (A, L, V) hydrophobic or aromatic amino residues, respectively.

However, when tested in the ranges of 80 to 256 μM (result not shown), no $\text{ABTS}\cdot^+$ radical scavenging activity was detected with both of our identified peptides. One possible explanation could be due to the necessity of

these two peptides to work synergistically with other peptides in the fennel seed protein hydrolysates, in order to exert the full radical scavenging potentials.

Table 2 Characteristics and details of identified fennel seed peptides

Peptides	Experimental mass / (g/mol) ^a	Theoretical mass/ (g/mol) ^b	Net charge ^b	pI ^b
EDVDFR	779.36	779.79	-2	3.54
ALEDVVEK	901.49	902.00	-2	3.69

Note: ^aDetermined by using MS analysis. ^bPredicted by using the PepCalc online peptide property calculator.

2.5 Albumin protein denaturation inhibitory assay

Numerous studies have reported on the possible links between protein oxidation, protein denaturation, and aging-related diseases such as diabetes, Alzheimer's, and Parkinson's [12,24]. As cellular proteins are oxidized and denatured, the resultant protein aggregates may compromise many of the delicate cellular functions. Previously, albumin protein denaturation inhibitory assay had been applied to assess for the protein protection potentials of phytochemicals and peptides [23,24,30]. Here, the samples were tested for their abilities to protect albumin proteins from denaturation, by measuring the formation of protein aggregates using spectrophotometric method.

Table 3 EC₅₀ values of synthetic peptides in albumin protein denaturation inhibition assay

Sample	EC ₅₀ value /mM
EDVDFR	0.54±0.04 ^a
ALEDVVEK	>2
GSH	0.48±0.01 ^a
Carnosine	1.49±0.14 ^b

Note: Data are reported as mean \pm SE values (n=3). Different superscripts (a-b) indicate statistically significant differences ($p < 0.05$).

In our study, the identified peptides were also tested for their ability to protect albumin protein from denaturation. peptide 1 (EDVDFR) showed comparable ability to inhibit albumin protein denaturation as the commercial GSH peptide, whereby their EC₅₀ values are 0.54 mM and 0.48 mM respectively (Table 3). Whereas, for peptide 2 (ALEDVVEK) and commercial carnosine peptide, weaker protein protection potentials were observed, with EC₅₀ values determined at >2 mM and

1.49 mM, respectively (Table 3). These results suggested the possible applications of our peptide 1 (EDVDFR) as health-promoting supplement to alleviate aging-related diseases or as food additive to preserve and delay protein denaturation in protein-rich foods.

3 Conclusion

In summary, we presented the antioxidant potential of fennel seed-derived protein hydrolysates. Based on our temperature and pH profiles, the antioxidant activities of the protein hydrolysates are stable in the range of 25 to 100 °C, and pH 5~11. Following stepwise purification guided by radical scavenging assay, two peptide sequences were also identified. Additionally, we demonstrated the identified Peptide 1 (EDVDFR) with its ability to inhibit albumin protein denaturation, comparable to that of commercial GSH. In view of their broad range temperature and pH resistances, as well as the comparable protein protection and antioxidant potentials to commercial GSH, the fennel seed-derived protein hydrolysates and the identified Peptide 1 could potentially be exploited for utilization as health-promoting supplement or as food additive to preserve protein-rich foods.

References

- [1] Rather A, Dar B A, Sofi S N, Qurishi M A. *Foeniculum vulgare*: A comprehensive review of its traditional use, phytochemistry, pharmacology, and safety [J]. Arabian Journal of Chemistry, 2016, 9(2): 1574-1583
- [2] Abdellaoui M, Bouhlali E T. The effect of domestication on seed yield, essential oil yield and antioxidant activities of fennel seed (*Foeniculum vulgare Mill*) grown in Moroccan oasis [J]. Journal of the Association of Arab Universities for Basic and Applied Sciences, 2018, 1: 107-114
- [3] Picon P D, Picon R V, Costa A F. Randomized clinical trial of

- a phytotherapeutic compound containing *Pimpinella anisum*, *Foeniculum vulgare*, *Sambucus nigra*, and *Cassia augustifolia* for chronic constipation [J]. BMC Complement Altern Med., 2010, 1-10
- [4] Kooti W, Moradi M, Akbari S A, et al. Therapeutic and pharmacological potential of *Foeniculum vulgare* Mill: A review [J]. Journal of HerbMed Pharmacology, 2015, 4(1): 1-9
- [5] Preedy V R, Patel V B, Watson R R. Nuts and Seeds in Health and Disease [M]. London, Elsevier, 2011
- [6] Patel A, Bandivekar A H, Badgujar V V. *Foeniculum vulgare* Mill: a review of its botany, phytochemistry, pharmacology, contemporary application, and toxicology [J]. BioMed Research International, 2014, 1-32
- [7] Bano S, Ahmad N, Sharma A K. Phytochemical investigation and evaluation of anti-microbial and anti-oxidant activity of *Foeniculum vulgare* (fennel) [J]. International Journal of Pharma Sciences and Research, 2016, 7(7): 310-314
- [8] Beyazen A, Dessalegn E, Mamo W. Phytochemical screening, antioxidant and antimicrobial activities of seeds of *Foeniculum vulgare* [J]. World Journal of Pharmaceutical Sciences, 2017, 5(3):198-208
- [9] Shahmokhtar M K, Armand S. Phytochemical and biological studies of fennel (*Foeniculum vulgare*) [J]. Natural Products Chemistry & Research, 2017, 5(4): 1-4
- [10] Sarmadi B, Ismail A. Antioxidative peptides from food proteins: A review [J]. Peptides, 2010, 31(10): 1949-1956
- [11] Zambrowicz A, Timmer M, Polanowski A, et al. Manufacturing of peptides exhibiting biological activity [J]. Amino Acids, 2013, 44: 315-320
- [12] Chakrabarti S, Jahandideh F, WU Jian-ping. Food-derived bioactive peptides on inflammation and oxidative stress [J]. BioMed Research International, 2014, 1-11
- [13] Fosgerau K and Hoffmann T. Peptide therapeutics: current status and future directions [J]. Drug Discovery Today, 2015, 20(1): 122-128
- [14] Bradford M M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding [J]. Analytical Biochemistry, 1976, 72:248-254
- [15] CHEN Yi-Chao, CHANG Hsi-Shan, WANG Cheng-Taung, et al. Antioxidative activities of hydrolysates from duck egg white using enzymatic hydrolysis [J]. Asian Australasian Journal of Animal Sciences, 2009, 22:1587-1593
- [16] Quah Yixian, Nor Ismaliza M I, OOI Jillian-Lean-Sim, et al. Identification of novel cytotoxic peptide KENPVLSLVNG-MF from marine sponge *Xestospongia testudinaria*, with characterization of stability in human serum [J]. International Journal of Peptide Research and Therapeutics, 2018, 24(1): 189-199
- [17] Re R, Pellegrini N, Proteggente A, Pannala A, et al. Antioxidant activity applying an improved ABTS radical cation decolorization assay [J]. Free Radical Biology and Medicine, 1999, 26:1231-1237
- [18] WONG Fai-Chu, CHAI Tsun-Thai, HOO Yee-Wei. Antioxidation and cytotoxic activities of selected medicinal herbs used in Malaysia [J]. Journal of Medicinal Plants Research, 2012, 6(16): 3169-3175
- [19] WONG Fai-Chu, XIAO Jianbo, et al. Identification and characterization of antioxidant peptides from hydrolysate of blue-spotted stingray and their stability against thermal, pH and simulated gastrointestinal digestion treatments [J]. Food Chemistry, 2019, 271:614-622
- [20] Yarnpakdee S, Benjakul S, Kristinsson H G, et al. Antioxidant and sensory properties of protein hydrolysate derived from Nile tilapia (*Oreochromis niloticus*) by one- and two-step hydrolysis [J]. Journal of Food Science and Technology, 2015, 52(6): 3336-3349
- [21] ZHU Chao-Zhi, ZHANG Wan-Gang, KANG Zhuang-Li, et al. Stability of an antioxidant peptide extracted from Jinhua ham [J]. Meat Science, 2014, 96(2): 783-789
- [22] Nielsen P M, Petersen D, Dambmann C. Improved method for determining food protein degree of hydrolysis [J]. Journal of Food Science, 2001, 66:642-646
- [23] Kumari S, Yasmin N, Hussain M R, Babuselvam M. *In vitro* anti-inflammatory and anti-arthritis property of *Rhizopora mucronate* leaves [J]. International Journal of Pharmaceutical Sciences and Research, 2015, 6:482-535
- [24] Osman N I, Sidik N J, Awal A, et al. *In vitro* xanthine oxidase and albumin denaturation inhibition assay of *Barringtonia racemosa* L. and total phenolic content analysis for potential anti-inflammatory use in gouty arthritis [J]. Journal of Intercultural Ethnopharmacology, 2016, 5(4):343-349
- [25] WANG Xueqin, YU Huahua, XING Rongge, LI Pengcheng. Characterization, preparation, and purification of marine bioactive Peptides [J]. BioMed Research International. 2017, 1-16