CARPATHIAN JOURNAL OF FOOD SCIENCE AND TECHNOLOGY

journal homepage: **http://chimie-biologie.ubm.ro/carpathian_journal/index.html**

IDENTIFICATION OF THE POTENTIAL BIOACTIVE PEPTIDES IN EDIBLE BIRD'S NEST

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https://doi.org/10.34302/crpjfst/2023.15.3.11

Article history:
Received: 14 October 2022
Accepted: 29 July 2023

Keywords:
Edible bird's nest;
Proteomic;
Bioactive peptides;
Protein;
In silico.

ABSTRACT

The major component in edible bird's nest (EBN) is protein. Thus, it is a potential source of bioactive peptides. Thus, this study aimed to determine the potential bioactive peptides from proteomic profiles of EBN using BIOPEP database. In this study, a proteomic profiling of soluble EBN proteins was carried out using high sensitivity liquid chromatography tandem mass spectrometry. Five proteins were selected as potential precursors for bioactive peptides which were deleted in malignant brain tumors 1, lysyl oxidase 3, acidic mammalian chitinase, NK-lysin and mucin-5AC for further analysis. It was found that the chosen proteins gave six dominant bioactivities which were angiotensin-converting enzyme (ACE) inhibitor, dipeptidyl peptidase-IV (DPP IV) inhibitor, dipeptidyl peptidase-III (DPP III) inhibitor, antioxidative, stimulating and renin inhibitor. Furthermore, the most potential bioactive peptides from soluble EBN proteins were angiotensin-converting enzyme (ACE) inhibitor and dipeptidyl peptidase-IV (DPP IV) inhibitor. Meanwhile for *in silico* proteolysis of EBN proteins using 33 type of enzymes, stem bromelain and pepsin were found to give the highest degree hydrolysis and to produce the highest number of bioactive peptides. Five tripeptides were generated after gastrointestinal digestion simulation for each ACE inhibitory activity, which were IRA, YPG, MKY, IVR and AVL and DPP IV inhibitory peptides that were WRD, WRT, WRS, VPL and APG, respectively. However, all these tripeptides have been reported in previous studies. This study showed that EBN has a promising source of bioactive peptide and *in silico* approach provide better understanding of theoretical and prediction of functional peptides.

1.Introduction

Edible bird nest (EBN) mainly comprises of the salivary gland secretion of several swiftlet species in the genus Aerodramus and Collocalia. These birds are found predominantly in Southeast Asia, e.g., Thailand, Vietnam, Indonesia, Malaysia and Philippines (Marcone, 2005). According to Wu et. al, (2010), swiftlet species that produced EBN belongs to the family Apodidae and genus Aerodamus. *Aerodamus fuciphagus* and *Aerodamus maximus*, commonly known as white-nest and black nest swiftlets respectively, are the two dominant EBN-producing species in Malaysia. EBN

produced by *A. fuciphagus* is more expensive compared to that of *A. maximus* in commercial market (Quek et al., 2015). EBN has been consumed as a Chinese delicacy and traditional Chinese medicine for centuries (Ma & Liu, 2012).

Protein is the major components in EBN which is around 62% protein, followed by carbohydrate, ash and fat (Marcone, 2005). Ma and Liu (2012) reported that medicinal and bioactive activities were only exhibited by hydrolyzed EBN by hydrolytic enzymes. While Syarmila et al. (2018) reported that glycopeptides in EBN in both crude EBN and EBN hydrolysate have functional bioactivities such as antimicrobial, antioxidant and ACE-inhibitory activities. Thus, EBN protein has potential to be used as a health enhancing ingredient in the nutraceutical formulation.

Poor solubility and low in extraction yield have become the major problems for EBN proteomic study (Wong et al., 2018) and several methods were used to enhance the solubility such as centrifugation, sonication, soaking and stewing in hot water. Several proteins have been identified in EBN, including mucin. A previous study showed that EBN was able to promote healthy stomach by preventing gastric caused by pathogens by releasing the fragments from EBN (Kong et al., 2016). Mucin layer has other compounds such as bicarbonate ions, epidermal growth factor, trefoil peptides, bactericidal factors, surface-active lipids and protease inhibitors that can make a layer to protect from degradation by gastric acid and pancreatic enzymes (Miner-Williams et al., 2009). As reported by Kong et al. (2016), in addition to mucin, carbonic anhydrase 9, acidic mammalian chitinase-like protein, immunoglobulin, NADH dehydrogenase, proline-rich protein and von Willebrand factors (VWF) were also detected in EBN. Other than mucin, Saengkrajang et. al (2013) reported that there were major essential amino acids found in EBN such as methionine and cysteine. These two amino acids contribute substantially to the maintenance and integrity of cellular systems by influencing cellular redox state and cellular capacity to detoxify toxic compounds, free radicals and reactive oxygen species. Glutamine also was found in EBN that may have benefits in inflammatory conditions such as infections and injuries. Besides these three amino acids, Zukefli et al. (2017) stated that EBN contain serine, valine and glutamic acid which have important role in immune system. However, there are differences in amino acids and nutrient composition content due to seasonal variation and breeding sites (Nur'Aliah et al., 2016).

Besides high in protein, EBN consists of glyconutrient was sialic acid that showed properties of neurological and intellectual advantages in infants as reported by Marcone et al., (2005). The other major glyconutrients include N-acetylgalactosamine (galNAc), N-acetylglucosamine (glcNAc), galactose and fructose. GalNAc is an amino sugar derived from galactose and a prominent precursor for glycosaminoglycans, a major component of joint cartilage that involved in the function of the synapses, the junction between nerve cells and deficiency can cause severe memory problem (Aswir & Nazaimoon, 2011). The EBN has unique structure and the findings on the identification of α 2–3 linked and α 2–6 linked sialoglycoproteins. Sialoglycoproteins that one of glycoproteins are rich in sialic acids and more than 10% were reported to have high biological and medicinal values. From the findings, α 2–3 linked sialoglycoprotein most likely an acidic mammalian chitinase-like protein, and α 2–6 linked sialoglycoprotein was an acidic mammalian chitinase (Zukefli et al., 2017).

Peptides released from dietary proteins by enzymatic hydrolysis have demonstrated bioactivities including, antioxidant, antidiabetic, anti-hypertensive,antithrombotic, immuno-modulating, osteoprotective, antimicrobial, anticarcinogenic and growth-promoting properties (Hall et al., 2018). Enzymatic hydrolysis is one of the most common method to hydrolyse protein other than microbial ferementation (Marciniak et al., 2018) due to cheap, more specific, release biological active,

produce antioxidative peptides and reduce allergenic potential of intact proteins (Singh et al., 2019). Other than that, the enzymatic reactions did not produce residual organic solvents or toxic chemicals. However, the process depends on several factors such as pH, temperature, enzyme, reaction time, enzyme concentration (Marciniak et al., 2018). In order to hydrolyse the protein, there are several enzymes can be chose in order to obtain the optimal results. Plant or microbial enzymes such as ficin, bromelain, papain, alcalase or flavourzyme are commonly employed in the production of protein hydrolysates. Ficin, papain and bromelain are cysteine endopeptidase, whereas alcalase is serine endopeptidase and flavourzyme is a mixture of endopeptidase and exopeptidase (Singh et al., 2019).

Since complete genome or protein database of swiftlet is not currently available, and due to the limitation of homology searching, further research is necessary to confirm the type and profile of proteins present in EBN. However, there are limited numbers of relevant protein sequences deposited in the database. More research is needed at the molecular level to explore the mechanisms behind the biological functions as well as the potential of bioactive peptide content.

In this study, *in silico* approach will be used to determine the potential bioactive peptides from EBN. Thus, proteomic profiles of soluble protein from EBN will be determined using LC-MS/MS method, and the profiles will be further analysed using BIOPEP database to determine the potential bioactive peptides from EBN.

2. Materials and methods

2.1. Materials

One kilogram of raw, cleaned EBN sample was purchased from a swiftlet farmer at Kampung Wa, Dungun, Terengganu, Malaysia. The protocols and method for protein extraction used in this study were performed as described by Kwan & Ismail (2018). Next the EBN solution was hydrolysed using trypsin and analysed using LC-MS/MS to determine its proteomic profiles. PEAKS studio 7.5 software was used to identify the protein.

2.2.Sample collection and protein extraction

First, the raw cleaned EBN was ground into fine powder. Then, 15 mg of the ground sample was dissolved in 1 ml extraction buffer (40 mM Tris-HCl, pH 8.8) and was kept for 20 minutes with occasional vortexing. Then, the sample undergone sonication for 15 minutes using a sonication probe at room temperature. The sample solution was then centrifuged at 12,000 g for 30 minutes, the supernatant was collected and then kept at -35^{o}C until further analysis.

2.3. Determination of protein content

Bradford assay was carried out to determine the soluble protein content in EBN sample as described by Bradford (1976). Five µL of EBN sample was mixed with 250 uL of Bradford reagent in a 96 well plate. Then the mixture was incubated at room temperature for 15 minutes. A standard curve ranging from 0.01-2 mg/mL was establish using the bovine serum albumin (BSA) by reading the absorbance at 595 nm. The total protein concentration was determined and calculated by comparing the absorbance value obtained for the sample against the standard curve.

2.4. Protein digestion

Protein digestion was carried out according to Kinter & Sherman (2005). The EBN sample was resuspended in 100 µL of 6 M urea, 100 mM Tris buffer. After that, 200 mM dithiothreitol (DTT) was added to the sample and was left at ambient temperature for 1 hour. Then, 200 mM of iodoacetamide was mixed into the sample solution and kept at room temperature for 1 hour, before another 20 µL of 200 mM DTT was added. Next, the sample was diluted with 775 µL of water. The EBN sample was digested with 20 µg of bovine trypsin where the mixture was incubated at 37°C, overnight. The digestion was stopped the next day by adjusting the pH of the mixture to pH less than 6 using concentrated acetic acid. The digested sample was then

concentrated to less than 20 µL using vacuum concentrator.

2.5. LC-MS/MS analysis

The digested EBN sample was reconstituted in 100 µL of 0.1% formic acid in deionized water and filtered using the 0.45 µm regenerated cellulose (RC) membrane syringe filter. Mass spectrometer was performed using the LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, CA, USA) coupled with Easy-nLC II nano-liquid chromatography system. The eluent was sprayed into the mass spectrometer at 2.1 kV (source voltage) at 220°C. Full scan mass analysis was done from m/z 300-2,000 at resolving power of 60,000 (at m/z 400, FWHM; 1-s acquisition) with data-dependent MS/MS analyses (ITMS) triggered by the 8 most abundant ions from the parent mass list of predicted peptides with rejection of singly or unassigned charge state. Collision-induced dissociation (CID) was applied as the fragmentation technique. Collision energy was set at 35. The sample was analyzed in duplicate readings.

2.6. Protein identification

PEAKS Studio Version 7.5 (Bioinformatics Solution, Waterloo, Canada) was used to perform de novo sequencing and database matching. UniProt *Avian* (bird) database from October 2019, was used for the database matching. Parameters of analyzing the sample were set according to Kwan & Ismail (2018). Parent mass and precursor mass tolerance was set at 0.1 Da. False detection rate (FDR) <0.1% and significant score (-10logP) for protein >20 was used for protein acceptance. Minimum unique peptide was set at 1.

2.7. Protein sequence evaluation of EBN as precursor for bioactive peptides

The identified EBN proteins in Section 2.4 were selected based on the Uniprot database (https://www.uniprot.org/). The proteins were analysed for potential bioactive peptides using BIOPEP (http://www.uwm.edu.pl/biochemia/index.php/en/biopep) database (accessed on August 8th , 2021) (Dziuba et al., 2004; Iwaniak et al., 2005). The PeptideRanker (Bioware.ucd.ie) was used in order to rank the predicted sequence according to bioactivity. The score presented by PeptideRanker which closer to 1 represent high chances to be bioactive. The toxicity of peptide was predicted by using ToxinPred (https://webs.iiitd.edu.in/raghava/toxinpred/design.php) while the solubility of the peptide was assessed by the online Innovagen server, available at http://www.innovagen.com/proteomics-tools.

2.8. *In silico* proteolysis to release ACE inhibitory peptides

The most effective proteases to prepare the bioactive peptides with the most dominant bioactivities from EBN, namely ACE inhibitory peptides and dipeptidyl peptidase IV inhibitory peptides, were evaluated using *in silico* proteolysis application in BIOPEP database. All 33 proteases available in BIOPEP were chosen in order to obtain the ACE inhibitory peptides and dipeptidyl peptidase IV inhibitory peptides. The 33 enzymes in BIOPEP are chymotrypsin A (EC 3.4.21.1), trypsin (EC 3.4.21.4), pepsin pH 1.3 (EC 3.4.23.1), proteinase K (EC 3.4.21.67), pancreatic elastase (EC 3.4.21.36), propyl oligopeptidase (EC 3.4.21.26), V8 protease pH 4 (EC 3.4.21.19), thermolysin (EC 3.4.24.27), chymotrypsin C (EC 3.4.21.2), plasmin (EC 3.4.21.7), catepsin G (EC 3.4.21.20), clostripain (EC 3.4.22.8), chymase (EC 3.4.21.39), papain (EC 3.4.22.2), ficin (EC 3.4.22.3), leucocyte elastase II (EC 3.4.21.37), metridin (EC 3.4.21.3), thrombin (EC 3.4.21.5), pancreatic elastase II (EC 3.4.21.71), stem bromelain (EC 3.4.22.32), glutamyl endopeptidase (EC 3.4.21.82), oligopeptidase B (EC 3.4.21.83), calpain 2 (EC 3.4.22.53), glycyl endopeptidase (EC 3.4.22.25), oligopeptidase F, proteinase P1 (EC 3.4.21.9), xaa-pro dipeptidase (EC 3.4.13.9), pepsin pH>2 (EC 3.4.23.1), coccolysin (EC 3.4.24.30), subtilisin (EC 3.4.21.62), chymosin (EC 3.4.23.4), ginger

protease (EC 3.4.22.67) and V-8 protease (EC 3.4.21.9).

3.Results and discussions

3.1. Protein content

It was found that the crude protein content of raw cleaned EBN using Kjeldahl method was 54.30%, which is in similar range as reported by Wong et al. (2018) (52%) and Nur 'Aliah et al. (2016) (56.47-60.63%). This shows that EBN has high protein content (Zukefli et al., 2017). However, determination of soluble protein extracted from EBN using Bradford assay only gave a protein content of 1.45 mg/ml. This may indicate the insolubility of EBN protein in aqueous as well as the inefficiency in extraction method (Zukefli et al., 2017).

3.2. Protein identification

There were five dominant proteins found in edible bird's nest (EBN) consisting of 115 to 1654 amino acid sequences while mass of proteins ranging from 13.232 to 58.700 kDa as stated in Table 1.

Table 1. Protein identification by de novo sequencing using ESI-ion-trap MS/MS

Protein name	UniProt Accession number	Species	Amino acid length	Average mass (kDa)	Protein sequence
Deleted in malignant brain tumors 1	A0A093BE17	*Chaetura pelagica (Chimney swift)*	115	13.232	PYHVDVNQDLFLEAYLHSSDPDLVLFLD TCVASPTPHNVSTVTYDIIRNGCAQDPTY TTYDSPSSYIVRFKFNAFRFVRKSPSVYL KCEFVVCRTYDYHSRCYQGCITTSRSQSS
Lysyl oxidase 3	A0A093BIT2	*Chaetura pelagica (Chimney swift)*	527	58.700	GRIWLDNVNCAGGEKSIGDCKHRGWGN SDCSHEEDAGVICKDERIPGFKDSNVIET EQSQGEEVRLRPVVSGARRLLPVTEGIVE LRYKDGWAQICDQGWDSRNSRVVCGM MGFPAEKKVNRNFYKLASKSQPKQKRR EDVGSKKSWQPLSCTVACQAVLSHAILS HVPCHVSHVPHHVSHIPRHALTRSLQARI RLKGGAKVGEGRVEVLRSSEWGTICDD RWNLQSASVVCRELGFGSAKEALTGAR MGQGTGPIHLNEVQCLGTEKSLWSCPYR NITREDCKHTEDAAVRCNIPYMGYENLV GTVEAAGIRGHSAWGLLVLGTLDDGWT TKEAMVACRQLGLGYSLHAVTETWYW DA SNVTEMVLSGVKCAGHEMSLNHCQHHG TSLNCRKTGTRFAAGVICSETASDLLLHA PLVQETAYIEDRPLHMLYCAAEENCLSSS ARLANWPYGHRRLLRFSSQIHNNGRADF RPKAGRHSWVWHECHRHYHSMDVFTH YDILTPNGTKVAEGHKASFCLEDTECEE
Acidic mammalian chitinase	A0A093BFV9	*Chaetura pelagica (Chimney swift)*	352	39.104	GTANVLTCYFTNWAQYRPGLGKFMPEN IDPFLCNHLIYAFANMNNNEITTYEWND ETLYKSFNGLKNQNRNLKTLLAIGGWNF GTAKFSTMVSTPQNRRTFINSVIRFLRKH NFDGLDLDWECPGSRGSPPQAKTLFTVL VKEMVAAFEQEARQSNRPRLMVTAAVA AGLSTIQAGYEIAELGKYLDYIHVMTYD FHGSGDGRTGENSPLHSGGNPQLSVEYA MKYWRDNGAPAKKLLVGFPTYGRTFTL QNPSNTAVGAPASGPGPAGTYTQEAGLL AYYEICSFLNSGATQAWDAPEDVPYAYK

					GSEWVGYDNVKSFNIKVDWLKKNNFGG AMVWTVDLDDFTGTFCNQG
NK-lysin	A0A0H5A QZ5	*Coturnix japonica (Japanese quail)*	137	14.663	MAAAIIVMMAMGAVLQVVVTEPPHDDQ RDVAAGSPWEQQWQLLQDGSAVWDEG DAMGPGKMKCSACVKLVKKLQKIVGD DPDEEAIGTALGQVCGTKRILKGICRQLG KKLRQQLSDALQDDSDPRSVCTTLGLCK G
Mucin - 5AC	R7VT28	*Columba livia (Rock dove)*	16544	18.104	MGTGGGIRIPLWISILALAFIQIKVQAQDV DPQTKSNYVSPSILQRQKRVPPSSKSQEV TIIPPFQNTLKLKAGNPSHNGRVCSTWGN FHFKTFDGDIFYFPGICNYIFASNCKSPYE DFNIQIRRTMVENATIITNVIMKLDGIVIE LTRGSVLLDGKLVQMPYSHMGVLIEKSN NYLKVSAKLGLTFLWNEEDALLVELDK KYANQTCGLCGDFNGIPISNEFISENTKL TPIQFGNRQKMDGPTEQCDDPIPPTLLVN CSAEFASICETVLTSKAFTSCNVLVNVQD YIETCIQDLCHCDSSMADFCMCNTFAEY SRQCAHAGGQPLNWRTSELCPKLCPFN MQYQECGSPCSDTCSNPERSALCEDHCT DGCVCPPGKLISCSFLIAEAARTYICAGC VPRKECHCTYEGEIYAPGASFSSKCRSCT CTGGEWSCVSQSCLGTCSIEGGSHISTFD EKFYSFFGDCSYVLTKLCDSNEFTVLGDI QKCGLTDTETCLKGIAISLSGGQTNIVIQP SGSVFVNMIYTQLPFSAANVTIFRPSSFFII LQTTFGLQLQVQLVPLMQLFIDLDPSHK GRTCGLCGNFNDMQTDDFKTTSGVIEGT SAAFGNTWKTRADCPDAKNTFENPCTVS IQNDQYAQHWCGLLSDTMGPFAECHST VNPEVYEKNCMFDTCNCEKSEDCMCAA LSSYVRACAAKGVLLTGWRSKACTKYT TLCPKSLKYMDNVDACQPTCRSLSEPDV TCSIKFVPVDGCTCINGTYMDESGKCVP ASSCPCYYKGMPLSSGEVVHDNGVVCT CTYGKLSCIGEKPEPVCVPPMVYIDCGN VTTDVVGAGCQKSCQTLDMECYRTHCV SGCVCPHNQVLDGKGSCIAPEDCPCIHN GNSYSPGESIRVGCNNCTCRNRKWHCSQ EPCLETCSVYGDGHYTTFDGKRFDFEGD CEYVLIQNYCGQQGVNQGTFRVITENIPC GTTGTTCSKSIKVFLGNYELVLSDGHSD VIQRTPGGKMPFQIRSMGIYLVVDTTVG LILMWDKKTSIFIKLSPSFQGNVCGLCGN YDGNGNNDFTTRSQSVVGNVLEFANSW KVSSSCPNASPTKDPCTANPYRKAWAQK QCSIITSEVFAKCHSQVEPNEYYQACVDD ACACDTGGDCECFCTAVAAYAQACNEL DICISWRTPSICPLFCDYYNPQGECEWHY

					KPCGAPCMKTCNNPSGNCLHELRGLEGC YPHCPKNKPYFDEETMTCVSNCGCFENG KNYKPGMQMPSKQNCQSCECTNYGKKC KYDEHECVCVYEGQKYNYEDVIYNTTD GTGGCIVATCGSNGTLQRV VYECPISTTPMTATTFHFSTTPPATTSTEN TSFSVTTTSPAITTSESTTFIPSATKEETTT TTEMTKPVTTSPSTTSLCTKEECYWSMW YDASYPGSGYNDGDFDTIQNIEKKGYKV CDNRKEVQCRAVRFPNTPYPLLEQNITC NKEEGLICYNKDQLPPICYNYKIRFKCCK NVRVPCHTTAAPHTTRISTSTSISSTTPST SPTEESTTPHLQTKHKTTTTSMTQPETQY IHTRTTTQPITQTQTETTTSISMPSVSSTTT HSTTACEPEVCSWSEWIDVDVPSSGPNQ GDFETYQRIRAAGMEVCQHPKEIECQAE DYPEVPIQNVGQVV

Deleted in malignant brain tumors 1 (DMBT1), a scavenger receptor cystine rich (SRCR) (also known as glycoprotein-340 or salivary agglutinin) was found to give the shortest sequence (115). The role of DMBT1 is to innate immunity with several factors of protection such as secretory IgA, surfactant protein D and A, and also binding to the bacteria directly (Müller et al., 2013). Besides that, DMBT1 also binds to bovine and human lactoferrin, an 80 kDa iron binding protein belongs to the transferrin family (Ligtenberg et al., 2010). According to Madsen et al. (2013), DMBT1 plays an important role to innate defense in the gastrointestinal and respiratory tracts against bacteria and viruses through direct interaction with microorganism. It also binds with other microorganisms such as *Helicobacter pylori*, several strains of streptococcus and viruses including Human Immunodeficiency Virus Type 1 (HIV) and influenza A virus as well as taking part in cell differentiation and growth. Other than that, the content of DMBT1 can be a biomarker in diagnosis of acute respiratory distress syndrome (ARDS) and predictions of severity disease (Ren et al., 2016). Lack of DMBT1 was found first in brain tumors, but over-expression of this protein has been discovered in pancreatic cancers (Cheung et al., 2008)

Besides DMBT1, lysyl oxidase 3 protein with 527 amino acids also can be found in EBN. Lysyl oxidase 3 (LOX) is a family of copper-dependent oxido-deaminase capable of modifying the side chains of lysyl residues in collagen and elastin, leading to the spontaneous formation of inter-polypeptide chain cross-links derived from non-reducible aldehyde (Herchenhan et al., 2015). Initially this enzyme catalyse the formation of covalent collagen cross-links that is an essential process for fibril stabilization. Not only collagen and elastin, lysyl oxidase also regulate the interaction between the different substrates rather than modification of its intrinsic enzymatic capacity (Rosell-García et al., 2019). Collagen plays an important role to provide mechanical stability in tissues and structures such as skin, blood vessels, bones and tendons (Herchenhan et al., 2015). This proved that EBN is a bone and chondro-protective agents against osteoporosis and osteoarthritis (Kong et al., 2016) because of LOX present in EBN. Wong et al. (2018) also reported the presence of lysyl oxidase in EBN.

Acidic mammalian chitinase (AMCase) was also found in EBN. This enzyme is characterized by an acidic isoelectric point, Therefore, it is known as acidic mammalian chitinase which is able to degrade artificial chitin-like substrates as well as chitin from crab shell and chitin as present in the fungal cell wall (Boot et al., 2001). According to Wong et al. (2018), AMCase can be found in many species such as human, mouse and bird. Identification of AMCase in EBN in

this study is consistent with the finding by Wong et al. (2018) that AMCase-like was the major protein identified, suggesting its abundance in EBN using shotgun and immunoprecipitated products by anti-EBN antibodies methods. According to Ohno et al. (2016), AMCase can act as a digestive enzyme as well as part of the host defense against chitin-containing pathogens in the mouse gastrointestinal tract and as a protease resistant major glucosidase to produce N-acetylglucosamine. Kong et al. (2016) also reported that acidic mammalian chitinase-like protein was found in EBN that was able to promote healthy stomach by preventing gastric caused pathogens by releasing the fragments from EBN. AMCase protein also discovered by Liu et al. (2012) in EBN using liquid-phase isoelectric focusing matched with two-dimensional electrophoresis technique.

With 137 amino acids sequence and 14.663 kDa mass, NK-lysin is a cationic peptide with antibacterial activity that was originally isolated from porcine intestinal tissue. The protein sequences of NK-lysin are rich in amino acid with positive charges and include maintained cysteine residues that form intrachain disulfide bonds (Wang et al., 2006). It is a member of the saposin-like protein family, and exhibits potent antitumor activity and has antimicrobial activity (Lee et. al, 2014). The antitumor and antimicrobial properties of the NK-lysin protein have the same effect with the other saposin-like proteins that able to form pores in the cell membrane due to its α-helical structure (Zhang et al., 2000). This statement supported by (Lee et al., 2014) that antibacterial activity is found in all peptides corresponding to each helical region of NK-lysin. This findings is similar reported by (Syarmila et. al, 2018) which glycopeptides in EBN both in crude and hydrolysate demonstrated functional bioactivities such as antimicrobial, antioxidant and ACE-inhibitory components.

Last but not least, mucin-5AC is also identified as a major structural protein in crude EBN. Under simulated gastro-intestinal condition, EBN was released peptides high likely to mucin using de novo sequencing (Kong et al., 2016). Wong et al., (2018) also identified mucin-5AC like in EBN by monoclonal antibodies method. Mucin is a family of polydisperse molecules containing high molecular mass and a high proportion of covalently bound oligosaccharide side chains that afford high resistance to the effects of acid and digestive enzymes. Mucin also contains a few compounds that have good functions such as bicarbonate ions, epidermal growth factor, trefoil peptides, bactericidal factors, protease inhibitors and surface-active lipids (Miner-Williams et. al, 2009). Thus, structural proteins may enhance the properties in EBN. To date, only this study discovered DMBT1 and NK-lysin proteins in EBN.

3.3. The potential of edible bird's nest as precursor for bioactive peptides

The potential of the five EBN proteins as precursor for bioactive peptides were analyzed using BIOPEP software. BIOPEP gave values of A, ∑A, B and also lists of potential bioactive peptides from each protein sequence. From the BIOPEP analysis, A and B values for major bioactivity was detected from EBN proteins. The A value gave the frequency of encrypted bioactive peptides occurring in a particular protein (Dziuba *et al.* 2004). BIOPEP contains 48 major classes of peptide bioactivity databases. However, BIOPEP analysis of EBN protein sequences showed only 29 subclasses of potential bioactivity were present for EBN. Among the 29 subclasses of bioactivities, 6 of these are the major bioactivities present in EBN protein sequences as stated in Table 2, which were angiotensin I-converting enzyme (ACE) inhibitor, dipeptidyl peptidase (DPP) IV inhibitor, dipeptidyl peptidase (DPP) III inhibitor, antioxidative, stimulating and renin inhibitor. The other 23 bioactivities present in at least any one of the EBN protein sequences were activating ubiquitin-mediated proteolysis, bacterial permease ligand, hypotensive, alpha-glucosidase inhibitor, antiamnestic, antibacterial, antithrombotic,

chemotactic, immunomodulating, immunostimulating, inhibitor, opioid and others.

Table 2. Number of potential bioactive peptides and potential biological activity (B) of identified proteins using BIOPEP

Proteins	UniProt Accession number	Activity					
		ACE inhibitor	Dipeptidyl peptidase IV inhibitor	Dipeptidyl peptidase III inhibitor	Antioxidative	Stimulating	Renin inhibitor
Deleted in malignant brain tumors 1	A0A093BE17	23 (0.0209)	51 (0.0002)	7	10	4	3
Lysyl oxidase 3	A0A093BIT2	108 (0.0093)	161 (0.0004)	24	33	9	7
Acidic mammalian chitinase	A0A093BFV9	94 (0.0198)	141 (0.0005)	22	19	5	9
NK-lysin	A0A0H5AQZ5	42 (0.0157)	53 (0.0003)	3	7	7	-
Mucin -5AC	R7VT28	166 (0.0117)	229 (0.0004)	30	47	11	12

Based on the A values, the predominant bioactivity for all EBN proteins are DPP IV inhibitor and ACE inhibitor. This finding is consistent with previous studies on *in-vitro* hydrolysis of EBN releasing ACE inhibitory and antioxidative activity (Amiza *et al.* 2014; Ghassem *et al.* 2017).

DPP IV inhibitor is one of the main peptides bioactivities in EBN proteins. DPP-IV (EC 3.4.1.4.5), a serine protease cleaves dipeptides of X-Pro or X-Ala from N terminal (Hildebrandt *et al.* 2000). Inhibition of DPP IV activity has a positive effect on type 2 diabetes (Agirbasli & Cavas 2017). Diabetes is a chronic metabolic disorder resulted in high blood sugar levels over a prolonged period. In recent years, diabetes has become one of the leading causes of death worldwide. According to the International Diabetes Federation (IDF), in 2017, about 425 million people were living with diabetes globally. However, synthetic DPP-IV drugs are reported to have some adverse effects such as gastrointestinal adverse effects, allergic reactions, skin-related side effects and musculoskeletal disorders (Liu *et al.* 2019). Many DPP-IV inhibitory peptides have been discovered in various food protein hydrolysates, including milk proteins (hua *et al.* 2011), rice bran (Hatanaka *et al.* 2012) and oat (Bleakley *et al*. 2017).

ACE inhibitor is the other main peptide bioactivity in EBN proteins. ACE plays an important role in regulating blood pressure in the renin angiotensin system (RAS) and kallikrein-kinin system. In RAS, ACE converts angiotensin I to an active vasoconstrictor angiotensin II, resulting in blood pressure increase. ACE activity inhibition is mainly used to prevent hypertension (Shahidi & Zhong 2008). ACE inhibitors such as captopril is widely used as pharmaceutical drugs for treatment of cardiovascular diseases. However, they often cause side effects such as coughing, skin rashes, and taste disturbances (Lee & Hur, 2017). Natural ACE inhibitory peptides are a natural alternative to synthetic drugs. ACE-inhibitory peptides usually contained hydrophobic (proline) and aliphatic amino acids (isoleucine and leucine) at the N-terminal (Lee & Hur, 2017).

BIOPEP analysis shows that the highest value of ∑A value is that of mucin-5AC like

gave the lowest ∑A value with 1.1241. According to Wong *et al.* (2018), mucin-5AC like was one of major protein found in EBN and it consists of polydisperse molecules with high molecular mass and a high proportion of covalently bound oligosaccharide side chains. The function of mucin-5AC like is to protect the delicate epithelial surfaces of the mucosa is primarily due to the polymerization of mucin monomers to form viscoelastic gels (Miner-Williams *et al.*, 2009).

Table 3 shows that taking into consideration both A and B values, the most potent bioactivity in EBN proteins is ACE inhibitor, followed by DPP IV inhibitor. Although DPP IV inhibitor has the highest A value, however, its B value is much lower than that of ACE inhibitor. On the other hand, although ACE inhibitor has lower A value than DPP IV inhibitor, its B value is much higher.

Table 3. The frequency of occurrence of peptides with a given activity (A) in selected protein sequences

Proteins	**UniProt Accession number**	**Number of Activities**	**∑A**	**A_1**	**A_2**
				ACE Inhibitor	DPP IV Inhibitor
Deleted in malignant brain tumors 1	A0A093BE17	10	1.1653	0.2435	0.5913
Lysyl oxidase 3	A0A093BIT2	17	1.3018	0.4099	0.6262
Acidic mammalian chitinase	A0A093BFV9	17	1.4743	0.4688	0.6705
NK-lysin	A0A0H5AQZ5	16	1.2263	0.4453	0.5036
Mucin -5AC	R7VT28	19	1.1675	0.3482	0.5719

The third highest value of A is given by DPP III activity; however, its B value is lower than renin inhibitor, alpha-glucosidase inhibitor and regulating. DPP III is a member of the M49 family of zinc dependent metallopeptidases and cleaves dipeptides sequentially from the N-terminus of various bioactive peptide substrates (Kumar et al., 2016). Diaz et al., (2018) reported that the main function of DPP III is to hydrolyze peptides ranging from 3 to 10 amino acids in length from their N-terminal in dipeptides and free amino acids and the activity of DPP III enable inhibited by synthetic hermophin-like peptidases in rats and humans. Furthermore, for human DPP III, high resolution crystal structures of the protein in complexes with opioid peptides (Met-and Leu-enkephalin, and endomorphin2), angiotensin-II and the peptide inhibitor have been reported (Kumar et al., 2016). This shows that differences in the binding modes allow a distinction between real substrates and inhibitory peptides. To date, no scientific research has been reported on DPP III in EBN.

Antioxidative is one of the major activities found in EBN but the B value is the lowest among the 10 activities. Ghassem *et al.* (2017) reported from Swiss-Prot and NCBI database found 13 antioxidant peptides sequences from EBN peptides. The peptides have superior antioxidant properties with high ORAC value and acted as a bioavailable free-radical scavenger. Meanwhile, antioxidative protein reported by Ghassem *et al.* (2017) that were ovotransferrin, cyctochrome b and glycosyl-transferase did not show any similarity with the antioxidative proteins in this study. This may suggest that the antioxidant peptides sequences reported by Ghassem *et al.* (2017) were derived from other EBN proteins, different from the types of EBN proteins used in this study.

3.4. *In silico* proteolysis of EBN proteins

Enzymatic hydrolysis is the most common approach to release biologically active peptide (Meng et al., 2018). In the BIOPEP database, there were 33 types of enzymes and all these enzymes were chosen for the *in silico* proteolysis of ACE inhibitory peptides. Most of potential bioactive peptides were dipeptides or tripeptides and the length of bioactive peptides was less than ten amino acids (Huang et al., 2015). Although there were 33 types of proteases in BIOPEP but only 5 proteases have been selected as they demonstrated the action of enzyme to produce the most potent of ACE-inhibitory and possess the highest degree of hydrolysis (DH) as specified in Table 4. According to Hall et al. (2018), DH is defined as the ratio of the number of peptide bonds cleaved to the total number of peptide bonds per unit weight (stated in percentage). The range of DH was between 36.15% to 72.02% for five EBN proteins with five selected enzymes meanwhile pepsin conveyed the highest DH compared to other enzymes that was in range 59.48% to 72.02%. In addition, pepsin also gave the highest value of DH and ACE inhibitory activity in tuna frame protein hydrolysate compared to other enzymes include papain and trypsin (Lee et al., 2010).

Table 4. The predicted efficiency of release of bioactive fragments from selected edible bird's nest protein by *in silico* proteolysis

Protein	Enzymes	DH_t (%)	ACE Inhibitor		DPP IV Inhibitor	
			A_E	W	A_E	W
Deleted in malignant brain tumors 1	Pepsin	59.48	0.0256	0.0966	0.0171	0.1819
	Stem bromelain	52.59	0.0513	0.1936	0.1026	0.1792
	Pancreatic elastase	52.59	0.0342	0.1291	0.0940	0.1642
	Leucocyte elastase II	42.24	0.0342	0.1291	0.0940	0.1642
	Ficin	45.69	0.0513	0.1936	0.0855	0.1493
Lysyl oxidase 3	Pepsin	60.33	0.0522	0.1388	0.1013	0.1703
	Stem bromelain	51.66	0.0350	0.0880	0.0792	0.1332
	Pancreatic elastase	48.71	0.0442	0.1111	0.0902	0.1516
	Leucocyte elastase II	37.82	0.0368	0.0925	0.0681	0.1145
	Ficin	44.65	0.0424	0.1066	0.0663	0.1115
Acidic mammalian chitinase	Pepsin	72.02	0.0663	0.1455	0.0994	0.1544
	Stem bromelain	51.80	0.0387	0.0849	0.0746	0.1159
	Pancreatic elastase	50.69	0.0304	0.0667	0.0801	0.1245
	Leucocyte elastase II	36.57	0.0249	0.0546	0.0691	0.1074
	Ficin	42.11	0.0580	0.1272	0.0663	0.1030
NK-lysin	Pepsin	70.71	0.0496	0.1147	0.0709	0.1470
	Stem bromelain	47.86	0.0709	0.1639	0.0709	0.1470
	Pancreatic elastase	48.57	0.0496	0.1147	0.0496	0.1028
	Leucocyte elastase II	39.29	0.0355	0.0821	0.0213	0.0442
	Ficin	35.00	0.0355	0.0821	0.0355	0.0736
Mucin -5AC	Pepsin	63.91	0.0480	0.1436	0.0767	0.1409
	Stem bromelain	45.11	0.0427	0.1277	0.0603	0.1107
	Pancreatic elastase	47.92	0.0410	0.1226	0.0714	0.1311
	Leucocyte elastase II	37.49	0.0176	0.0526	0.0480	0.0882
	Ficin	36.15	0.0427	0.1277	0.0615	0.1129

Table 4 shows that the degree of hydrolysis (DH) is not proportional to the release of bioactive peptides as in lysyl oxidase 3 protein that has 37.82% of DH with leucocyte elastase II enzyme exhibit A_E and W value of ACE inhibitor 0.0368 and 0.0925 respectively while the same enzyme in NK-lysin protein has 39.29% of DH with A_E and W value 0.0355 and 0.0821 respectively. Most of the enzymes action in the same protein sequence in Table 4 shows

the release frequency (A_E) of ACE inhibitory peptides lower than DPP IV inhibitory peptides and similar action goes to relative release frequency (W) of peptides except for some proteins such as DMBT1 treated by pepsin and NK-lysin treated by leucocyte elastase II also in mucin-5AC treated by pepsin, stem bromelain and ficin.

Every enzyme showed the specific cleavage sites and has different potential to release bioactive peptides from proteins (vu et al., 2019). In this study, pepsin demonstrated the most effective enzyme to produce ACE inhibitory and DPP IV peptides from EBN proteins.

Table 5. Bioactive peptides predicted to be released from edible bird's nest protein based on *in silico* proteolysis

Enzyme	ACE inhibitors	DPP-IV inhibitors
Pepsin	**145**	**239**
	CF (2), HG (1), HK (1), HL (1), HY (1), IA (4), IE (9), IF (4), IG (4), IL (7), 1P (1), IY (5), PG (7), PL (1), PP (1), PG (4), PT (4), RA (3), RF (4), RG (2), RP (1), RY (1), SF (5), SG (7), ST (7), SY (2), VE (7), VF (4), VG (12), VK (5), VM (2), VP (2), VR (1), VY (6), WA (4), WG (4), WL (1), IPY (1), IRA (1)	HA (3), HE (3), HF (2), HL (1), HT (1), HY (2), IA (4), IL (7), IM (1), IN (1), IP (1), IQ (12), IR (2), PA (4), PF (4), PG (7), PK (1), PL (5), PM (1), PN (4), PP (1), PQ (4), PS (2), PT (4), PY (4), RA (2), RG (2), RK (3), RL (1), RM (1), RN (3), RP (1), SF (5), SH (2), SK (3), SL (5), SY (2), VA (8), VD (9), VE (7), VF (4), VG (12), VK (5), VL (19), VM (2), VN (7), VP (2), VQ (7), VR (1), VS (1), VT (12), VY (6), WA (3), WD (4), WG (4), WK (1), WL (1), WN (3), WQ (1), WT (1), WY (2), VPL (1), WRD (1), WRT (1)
Stem bromelain	**175**	**195**
	CF (1), DA (3), DF (6), DG (12), EA (3), EF (2), EG (5), EV (3), HG (1), IA (2), IF (1), IG (3), IL (5), KA (4), KF (3), KG (5), KL (8), KR (2), MG (6)L NH (3), NG (5), PG (7), PL (4), PR (2), PT (5), QG (2), WA (2), WG (3), YA (1), YG (3), YL (2), YV (2), YPG (1)	EG (5), ES (2), ET (6), EV (3), HA (5), HF (2), HR (1), HS (5), HV (2), IA (2), IL (5), IR (5), KA (4), KF (3), KG (5), KR (2), KS (4), KT (5), KV (2), MA (2), MG (6), MV (5), NA (1), NF (3), NF (3), NG (5), NL (1), NR (3), NT (2), NV (9), PA (6), PF (2), PG (7), PL (2), PS (9), PT (5), PV (3), QA (4), QG (2), QL (5), QS (5), QT (5), QV (5), WA (2), WG (3), WR (2), WS (2), WT (2), WV (1), YA (1), YF (1), YG (3), YL (2), YS (3), YT (2), YV (2)
Pancreatic elastase	**116**	**218**
	DA (4), DG (10), DY (2), EA (3), EG (8), EI (3), EV (3), EY (2), FG (3), FY (1), HL (1), HY (2), KA (3), KG (6), KL (10), KY (3), MG (6), NG (3), NY (6), PG (7), PL (5), PT (4), QG (2), RA (4), RG (4), RL (3), RY (1), WA (2), WG (3), WL (1), MKY (1)	EG (8), EI (3), ES (2), ET (7), EV (3), EY (2), FA (4), FL (6), HA (4), HI (1), HL (1), HS (7), HT (1), HV (4), HY (2), KA (3), KG (6), KI (2), KS (5), KT (2), KV (6), KY (3), MA (2), MG (6), MV (5), NG (3), NT (3), NV (9), NY (9), PA (5), PG (7), PI (4), PL (5), PS (9), PT (4), PV (2), PY (5), QA (6), QG (2), QI (4), QL (3), QS (4), QT (5), QV (2), QY (2), RA (4), RG (4), RG (4), RI (4), RL (3), WA (2), WG (3), WI (1), WL (1), WS (3), WT (2), WV (1), WY (1), WRS (1), WRT (1),
Leucocyte elastase II	**65**	**158**
	DA (2), EA (3), EV (2), GA 950, GI (1), GL (6), GS (3), GT (10), GV (3), HL (1), KA (3), KL (6), PL (4), PT (3), RA (3), WA (1), WL (1), YA (3), YL (3), YV (2)	ES (1), ET (7), EV (2), FA (4), FL (6), GA (5), GI (1), GL (6), GV (3), HA (4), HI (1), HL (1), HS (3), HT (1), HV (3), KA (3), KS (5), KT (2), KV (5), MA (2), MV (5), NT (1), NV (6), PA (4), PI (3), PL (4), PS (9), PT (3), PV (2), QA (5), QI (4), QL (2), QS (4), QT (4), QV (3), RA (3), RI (3), RL (3), WA (1), WI (1), WL (1), WS (2), WT (1), WV (1), YA (3), YI (4), YL (3), YS (1), YT (3), YV (2), WRT (1)

Ficin	**123**	**180**
	AF (3), AG (4), AR (3), AY (2), CF (1), DF (5), DG (11), DY (2), EF (1), EG (5), EK (3), IF (2), IG (1), IL (4), IY (2), MG (5), NF (4), NG (7), NK (2), NY (6), PG (7), PH (1), PL (3), PR (1), QG (3), QK (4), TF (7), TG (8), VF (2), VG (4), VK (3), VR (2), VY (2), WG (1), AVL (1), IVR (1)	AF (3), AG (4), AL (3), AS (5), AY (2), EG (5), EK (3), ES (2), IH (2), IL (4), IR (5), MG (5), ML (1), NF (4), NG (7), NH (1), NL (1), NR (3), NY (6), PF (2), PG (7), PH (1), PK (2), PL (3), PS (6), PY (3), QG 93), QL (3), QS (5), TF (7), TG (8), TH (2), TK (3), TL (5), TR (5), TS (9), TY (4), VF (2), VG (4), VK (3), VL (8), VR (2), VS (4), VY (2), WG (1), WH (1), WK (1), WR (3), WS (3), APG (1), VPL (1)

Table 5 shows the predicted of identified ACE inhibitor dipeptides and tripeptides to be released from EBN proteins by proteolysis. Besides that, novel amino acid sequences could be released from the parent peptide during the process of digestion and may exert toxic, allergenic or other potent biological activities when absorbed (Garcia-vaquero et al., 2019). Enzymatic hydrolysis by *in silico* method resulting all the peptides to be compared with the BIOPEP database whether it has been reported or not previously.

After gastrointestinal digestion simulation, there were 10 tripeptides has been released. However, all these 10 tripeptides have been reported previously as ACE and DPP IV inhibitory. From these 10 tripeptides, 5 tripeptides were ACE inhibitor peptides and the remaining were DPP IV peptides. The sequence of IVR that had ACE inhibitory activity property showed the lowest IC_{50} value (0.81 µM) (Rawendra et al., 2014) compared to IPY, IRA and MKY that possess the IC_{50} value 206 µM (Vukic et al., 2017), 1.11 µM and 0.86 µM (Wu et al., 2006) respectively. While the sequence of YPG has been reported to have opioid inhibitory activity in wheat with EC_{50} value 5.4 mM and 1.8 mM for both µ and κ opioid receptor (Garg et al., 2018) and no study was reported the sequence has ACE inhibitory activity to date. An ACE inhibitory peptides that contain amino acid sequences and the activities expressed as the concentration of the necessary to inhibit the activity by 50% (IC_{50}) (Vukic et al., 2017).

Meanwhile for DPP IV inhibitory peptides were showed by the sequence of tripeptides WRD, WRT and WRS that have IC_{50} value 376 µM, 536 µM and 483 µM respectively (Lan et al., 2014). The other tripeptide sequence of APG in dietary proteins that have IC_{50} value 4000 µM (Lacroix & Li-chan, 2012) and IC_{50} value for VPL tripeptide sequence was 15.8 µM (Nongonierma & Fitzgerald, 2014).

Although all the predicted most potential peptides has been reported previously in literature, this results can be a platform for better understanding of protein sequences for searching potential bioactive peptides in food proteins in comparison to traditional approaches conducted in laboratory (Huang et al., 2015). Further *in vitro* and *in vivo* studies is needed to have a better understanding of bioactive peptides from EBN.

4. Conclusions

Profiling of EBN proteomic profiles found 5 proteins as potential precursors for bioactive peptides which were deleted in malignant brain tumors 1, lysyl oxidase 3, acidic mammalian chitinase, NK-lysin and mucin-5AC. BIOPEP analysis of these proteins found that the main bioactivity was those of ACE inhibitory and DPP IV inhibitory activity. Besides, it was predicted that pepsin gave the greatest action in proteolysis of the most EBN proteins with gave the highest degree of hydrolysis and generated the highest DPP IV inhibitory peptides while stem bromelain produced the highest ACE inhibitory peptides. *In silico* proteolysis, a total of 10 tripeptides were discovered to give the most potent of ACE inhibitory and DPP IV activity and sequence of IVR showed the lowest IC_{50} value and YPG was selected to be the novel peptide. This study shows that EBN proteins is

a favorable precursor in producing the bioactive peptides.

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Acknowledgment

The authors acknowledge the financial support from the Ministry of Higher Education Malaysia through the Fundamental Research Grant Scheme (FRGS/1/ 2018/ WAB01/ UMT/02/4) to carry out this research work.