Controlled enzymatic hydrolysis of Pollen protein as promising tool for production of potential bioactive peptides

Running Title: Enzymatic hydrolysis of Pollen protein

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Abstract

In the present study response surface method was used to optimize hydrolysis condition to generate potential bioactive peptides from pollen protein using pepsin (PHP) and trypsin (THP). Then PHP and THP prepared under optimized conditions were analysed by size-exclusion chromatography (SEC). The fractions possessing the maximum ACE-inhibitory, DPPH radical scavenging and ferric-reducing power were further purified by RP-HPLC. A heterogeneous composition of hydrophobic and hydrophilic peptides in both fractions was obtained. Finally, peptide sequences in active fractions of PHP and THP were identified by mass spectrometry in tandem. All the identified peptides had herbal protein originates. These were 6 to 21 amino acids in length, and Glycine and Alanine were two main hydrophobic amino acids present in their sequences. The results proved that by using controlled enzymatic hydrolysis of pollen protein is possible to generate bioactive peptides with high ACE-inhibitory and antioxidant activity in final product.

Keywords: pollen hydrolysate, ACE-inhibitory, Antioxidant activity, Mass spectrometry, bioactive peptides.

Practical application

Pollen is well-known as an interesting protein source. Compared to other types of hydrolysis, enzymatic hydrolysis of vegetable proteins has few or no undesirable side reactions or products. In this study, controlled enzymatic hydrolysis of pollen protein was applied as a suitable method to produce bioactive peptide. The results proved that by using controlled enzymatic hydrolysis of pollen protein is possible to generate bioactive peptides with high ACE-inhibitory and antioxidant activity in final product. This product can be used as functional and health promoting ingredient in different food formulations.

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1. Introduction

Bioactive peptides have been described as usually contain 2–20 amino acids in length (Himaya, Ngo, Ryu, & Kim, 2012). Enzymatic hydrolysis, fermentation, pH changes during acidification, heat treatments, and chemical treatments such as glycosylation can be used for releasing of inactive peptide content of parent protein sequence (Ryder, Bekhit, McConnell, & Carne, 2016). ACE-inhibitory, antioxidant, antimicrobial, antihypertensive, antithrombotic and immunomodulatory activities can be mentioned as the health advantages of bioactive peptides (Toldrá, Reig, Aristoy, & Mora, 2017). The peptides activity depends on their amino acid content and sequence length as well as their molecular mass (Aleman et al., 2011).

Pollen can be derived from honey bee and has been long employed in alternative medicine and food diets as well as supplementary nutrition. Nowadays, pollen is well-known as an interesting protein source. Bee pollen is the result of the flower pollens agglutination with nectar and saliva content of the honey bees. The collection of this natural product is a relatively recent development, dependent primarily on the basic concept of scraping pollen off of the bees' legs as they enter the hive (Feás, Vázquez-Tato, Estevinho, Seijas, & Iglesias, 2012). Proteins, lipids, carbohydrates, vitamins and minerals are the dominant constituents of pollen. Compared to other types of hydrolysis, enzymatic hydrolysis of vegetable proteins has few or no undesirable side reactions or products (Sun, 2011). A substantial deal of researches has been carried out on the hydrolysis of foods. The antioxidant enzymatic hydrolysate was derived from honey bee pollen by use of aminopeptidases and proteinase with entire plant origin; the product exhibited 42–46% DPPH radical scavenging activity (Marinova & Tchorbanov, 2010). Nagai et al., (2005) reported that enzymatic pollen hydrolysates showed antioxidant ability in a linoleic acid oxidation system

and scavenging abilities against active oxygen species. The objective of this study was the generation and purification of bioactive peptides in pollen hydrolysates using pepsin and trypsin enzymes to produce peptides showing high ACE-inhibitory and antioxidant activity.

2. Material and methods

2.1. Materials

Potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), angiotensin-converting enzyme (ACE, derived from rabbit lung), pepsin and trypsin, the solvents and chemicals applied for HPLC and mass spectrometry analyses were supplied from Sigma (St. Louis, MO). Abz-Gly-p-nitro-phe-pro-OH trifluoroacetate salt was provided from Bachem (Bubendorf, Switzerland). All other chemicals were of analytical grade.

2.2. Use of response surface methodology (RSM) to optimise the hydrolysis conditions

The combination of response-optimizing values was determined using the factorial randomised complete design (RCD). RSM was also employed to obtain the optimal levels of the two variables. In this regard, time of hydrolysis (1, 2.5, 4 h) and enzyme concentration (1, 1.5, 2 % w/w)¹ were addressed.

2.3. Preparation of pollen hydrolysates

Bee pollen specimens of this research were attained from an apiary in Gorgan naturally preserved area. The apiary was supervised by Iran's animal science research institute (ASRI). Sample collection was carried out in 30 different days (April-June 2017) by local beekeepers.

¹ - Per gram of protein dry weight in substrate

Hexane (1:3) was employed for defatting the pollen powders for 24 h through use of an orbital shaker (Fan, 52E TM Iran, Gostar). The remaining hexane was eliminated by oven-heating at 40°C for 24 h and the defatted samples were then transferred to ziploc bags.

Enzymatic hydrolysates were prepared by the following procedure: the defatted samples (14.5% protein) were individually transferred and dispersed in 5 different volumes of 0.1 M acetate buffer (pH 3) and 0.1 M potassium phosphate buffer (pH 7.6); homogenisation was performed using an ultrasonic homogeniser (Hielscher, UP100H). The process of digestion was initiated by pepsin and trypsin in concentrations of 1, 1.5 and 2% (respective to protein content). pH was kept at optimum value for pepsin and trypsin reaction throughout the procedure, while the temperature of shaker incubator (Vision Scientific co, LTD) was set to 37 °C. Hydrolysate aliquots were eliminated at intervals of 1, 2.5 and 4 h followed by immediate heating at 85°C for 15 min to halt the process; the samples were then cooled down to room temperature. The hydrolysates were separated by centrifugation at 4000×g (Hanil, Combi 514R; Korea) for 30 min; the supernatants were then lyophilised (Operon- FDB5503, Korea) and kept at -20°C.

2.4. Antioxidant activity

2.4.1. DPPH radical scavenging

The DPPH radical-scavenging performance of the samples was evaluated according to the method of Bersuder et al., (1998) with some small modifications. In a typical process, 100 µL of each sample was added to 125 µL of DPPH solution (0.02% in ethanol) and 500 µL of ethanol. The solution underwent vigorous shaking in a dark incubator. The absorbance of the solution was measured after 60 min, at 517 nm by spectrophotometry technique. DPPH radical-scavenging activity can be computed by following equation:

DPPH radical-scavenging activity (%) =

$$((\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance})) \times 100$$

The test concerning control was carried out with a similar method; the only exception was the use of distilled water instead of the sample. BHT was employed as positive control.

2.4.2. Ferric-reducing power

The reduction power of hydrolysates was evaluated by a method similar to the one described by Yildirim et al., (2001) with some slight modifications. In a this procedure, 70 μL of the sample was added to 70 μL of 0.2 M sodium phosphate buffer (pH 6.6) and 70 μL of potassium ferricyanide (10 mg/mL) then it was incubated for 20 min at 50 $^{\circ}\text{C}$. After that, 70 μL of TCA (100 mg/mL) was added to the solution and mixed; it then underwent 10 minutes of centrifugation at 2000 $\times$ g (Hanil, Combi 514R; Korea). Subsequently, 140 μL of the supernatant were added to 140 μL of distilled water and 28 μL of ferric chloride (1 mg/mL). After 10 min of keeping at ambient temperature, the absorbance was assessed at 700 nm.

2.5. Evaluation of ACE-inhibitory activity

A fluorescence-based method was employed to evaluate the ACE-inhibitory activities of hydrolysates as mentioned by Sentandreu and Toldrá (2006). Double distilled water and Captopril (0.1 mg/mL) were considered as negative and positive controls, respectively. ACE-inhibitory activity was reported in percentage.

2.6. Size-exclusion chromatography

The hydrolysate exhibiting the highest ACE-inhibitory and antioxidant activity was selected for deproteinisation for further analysis by size-exclusion chromatography (SEC). For hydrolysate

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3 deproteinisation, 20 mL of 0.01N HCL was used to dissolve 4 g of lyophilised hydrolysate using
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5 magnetic stirrer. 3 volumes of ethanol was then added to the solution and it was kept at 4-5°C for
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7 20 h. The supernatant were obtained by 20 minutes of centrifugation at 12000× g at 4°C (Hanil,
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9 Combi 514R; Korea). The supernatant was then lyophilised when the ethanol was removed
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11 through use of a rotary evaporator. The peptides were fractionated according to their molecular
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13 mass by a Sephadex G-25 column (2.5 × 65 cm, Amersham Biosciences, Uppsala, Sweden). 5
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15 mL of double distilled water was used to dissolve 100 mg of the freeze-dried deproteinised
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17 hydrolysate, which was then filtered by a 0.2 µm filter and eluted with filtered and degassed
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19 double distilled water in flow rate of 15 mL/h. 5 mL of sample was evaluated by UV-Vis
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21 spectrophotometer at different wavelengths of 214, 280 and 254 nm (Agilent Cary 60, Agilent
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23 Technologies, Palo Alto, CA, USA). The antioxidant and ACE-inhibitory activities of all the
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25 fractions were tested. Fractions with the highest activities were pooled together and freeze dried.
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33 **2.7. Reversed-phase high performance liquid chromatography (RP-HPLC)**

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35 Fractions obtained from SEC of hydrolysates showing high activity were again dissolved in 130
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37 µL double distilled water. 20 µL of them was then injected into an Agilent 1100 HPLC system
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39 (Agilent Technologies) by a Symmetry Prep™ C18 column (7.8 × 300 mm, 7µm) from Waters
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41 (Milford, MA). 0.1% TFA in double distilled water was the solvent A while the solvent B
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43 included acetonitrile/double distilled water (60:40, v/v) containing 0.085% of TFA. The elution
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45 was 100% solvent A for 2 min, followed by a linear gradient from 0% to 50% for solvent B in 50
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47 min at 3.5 mL/min flow rate. The collected fractions (1 mL) were evaluated at 214 nm and their
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49 ACE-inhibitory and antioxidant activities were measured. Fractions possessing excellent
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51 activities were freeze dried for further analysis.
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2.8. Peptide identification using mass spectrometry

The peptide identification was carried out by nano LC–MS/MS analysis through use of an Eksigent Nano-LC Ultra 1D Plus system (Eksigent of AB Sciex, CA) combined with a quadrupole-time-of-flight (Q-ToF) TripleTOF® 5600 system from AB Sciex Instruments (Framingham, MA) equipped with a nanoelectrospray ionisation source (nano-ESI). Adjustment of system parameters were the same as previously reported work of Mora et al.^[12] The hydrolysate fractions were again suspended using double distilled water containing 0.1% of trifluoroacetic acid (TFA) to reach to final concentration of 0.2 mg/mL; 5µl of the supernatant was injected. Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA) (<http://www.matrixscience.com>) was employed to carry out automated spectral analysis, generating peak list and the peptide identification through database search. Similar sequences which showed ACE-inhibitory and antioxidant activity were searched in BIOPEP databases.

2.9. Statistical analysis

Enzymatic hydrolysis was optimised using central composite design in response surface methodology (RSM) as implemented in Design expert 8.0.7.1 software (Stat-Ease Company, Minneapolis, Minnesota, United States). This software was also employed for analysis of variance (ANOVA) of variables. $p < 0.05$ was considered as statistically significant difference.

3. Results and Discussion

3.1. ACE-inhibitory and antioxidant activity optimisation in hydrolysates

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RSM was employed for prediction of optimal hydrolysis conditions. Table 1 lists hydrolysates antioxidant activity and ACE inhibition activity in the points marked by the central composite design in RSM. Based on the results, predicted models were applied and the optimized condition to achieve the highest antioxidant and ACE inhibition activity was determined. Validation tests to determine the adequacy of proposed optimum hydrolysis conditions to achieve the highest antioxidant and ACE-inhibitory activities of the PHP and THP in predicted time of 2.5 h and 4 h and enzyme concentration of 2% and 1%, respectively, were performed. Predicted results for DPPH, reducing power and ACE inhibition activity for PHP were 100, 0.68 and 44.3% which were close to validated data of 98.9, 0.7 and 45.6%, respectively. In THP, predicted results for DPPH, reducing power and ACE-inhibitory activity were 51.46, 0.62 and 98% which were close to validated data of 52.5, 0.69 and 96.9%, respectively. Based on the results, prediction of the responses for ACE-inhibitory and antioxidant activities in hydrolysates is possible with good accuracy. Ultimately, hydrolysates which were prepared under the predicted conditions were chosen for further identification.

3.1. Peptides fractionation and purification

3.1.1. ACE-inhibitory and antioxidant fractionation by SEC

SEC profiles of both hydrolysates are shown in Figure 1. The results showed that hydrolysis of pollen by gastrointestinal enzymes led to the production of peptides with a similar molecular weight, especially in the molecular mass of 70000 and 1420 Da. However, the molecular weight distribution of peptides between 1420 and 70000 Da (according to standards) in PHP was relatively more than THP. This is probably due to the difference in hydrolysis conditions and also the amino acids that are present in the active site of pepsin and trypsin.

As showed in Figure 2C, maximum ACE-inhibitory activity of PHP (69.94%) was reported for fractions 53 to 55 corresponding to the molecular weights smaller than 13000 Da. The highest ACE-inhibitory activity of THP (57.24%) was observed in fractions 62 to 64 (Figure 2D), corresponding to the elution of Bacitracin, a standard peptide with a molecular weight of 1420 Da. According to general believes, peptides with low molecular weights exhibit more powerful ACE-inhibitory activities (Lassoued et al., 2016). In PHP, fractions 53 to 55 (with a molecular weight lower than 13000 Da) showed higher ACE-inhibitory activity compared to fractions 62 to 64, implying that peptides possessing lower molecular weights do not always exhibit higher activities (Moayedi et al., 2016). Parameters such as peptide's size and hydrophilic–hydrophobic balance play a significant role in ACE-inhibitory activity (Nasri et al., 2013). Two *in vitro* assays were employed on SEC fractions to evaluate their antioxidant activities and results presented in Figures 2A and 2B. The, maximum DPPH radical scavenging of PHP and THP (79.08% and 41.30%) were recorded in fractions 62 to 64. The highest value for ferric reducing power assay of PHP and THP (0.608 and 0.644) were observed respectively in fractions 62 to 64 and 56 to 58 (Figures 2E and 2F) correspond to molecular weights between 1420 Da and 13000 Da. Accordingly, peptides having molecular weight smaller than 3000 Da possessed stronger antioxidant activity (Lassoued et al., 2015). Therefore, fractions 53 to 55 and 62 to 64 of PHP and fractions 62 to 64 THP were further purified.

3.1.2. Fractionation and purification of ACE-inhibitory and antioxidant fractions by RP-HPLC

Numerous peaks were observed in reversed-phase chromatographic separation of the pooled fractions attained from SEC of PHP and THP (Figure 3). Taking into account the two aromatic amino acids (hydrophilic tyrosine and hydrophobic tryptophan), the area under the

chromatograms can be classified into 3 zones. Zone 1 includes hydrophilic peptides eluted before tyrosine standard (before 13 min). Zone 2 is related to peptides with low hydrophobic behavior eluted between tyrosine and tryptophan standards (between 13 and 26 min); while zone 3 is attributed to highly hydrophobic peptides eluted after tryptophan standard (after 26 min) (Lassoued et al., 2015). Figure 3 illustrates the heterogeneous combination of peptides with hydrophobic and hydrophilic features in PHP and THP fractions. ACE-inhibitory activity of the fractions 53 to 55 of PHP was evaluated (Figure 3A). ACE-inhibitory activity of fractions 62 to 64 of PHP was also tested in Figure 3B, DPPH radical scavenging activity (Figure 3C) and reducing power (Figure 3D). Also Fractions 62 to 64 of THP were collected and assayed for their ACE-inhibitory activity (Figure 3E) and DPPH radical scavenging activity (Figure 3F). In terms of ACE-inhibitory activity, these fractions showed an activity of 60% for fractions 53 to 55 of PHP eluted in 3 and 27 min (Figure 3A), 50% for fractions 62 to 64 of PHP eluted in 37 min (Figure 3B) and 40% for fractions 62 to 64 of THP eluted in 35 min (Figure 3E). As can be observed, hydrophilic and lower hydrophobic peptides showed the highest ACE-inhibitory activity. ACE-inhibitory peptides can attach to ACE active site or inhibitor site, which can modify the protein confirmation and prevent from Angiotensin binding to the ACE active site (Wijesekara and Kim, 2010). Hydrophilic peptides cannot access the active site of ACE (Lassoued et al., 2016). Considering DPPH scavenging activity of PHP (fractions 62 to 64 of SEC), fraction eluting at 37 and 32 min showed the highest activity (90.89% and 89.72%). After that, fraction eluting at 6 and 12 min showed DPPH scavenging activities of 60% and 54.47%, respectively (see Figure 3C). Regarding DPPH scavenging capacity of THP (fractions 62 to 64), fraction eluting at 37 and 32 min showed the highest activity (88.5% and 63.38%, respectively). After that, fraction eluting at 11 and 13 min showed DPPH scavenging activity of 50.68 % (see

Figure 3C) where hydrophilic and low hydrophobic peptides showed the highest DPPH radical scavenging capacity.

The profile of ferric reducing power activity for PHP (fractions 62 to 64 of SEC) showed some peaks with ferric reducing power (see Figure 3D). Fractions eluting at 32 and 37 min were found to exhibit the highest activity. Similar profiles of antioxidant activity were observed in both assays. Indeed, many researches have reported that the most powerful radical-scavenging compounds are generally reducing compounds. Interestingly for PHP and THP (fractions 62 to 64 of SEC), fractions eluting at 32 and 37 min, which exhibited the highest ACE-inhibitory activity, also showed the highest antioxidant activity. The variety of peptides and proteins in bee products such as pollen can prove protein phosphorylation, glycosylation, methylation and deamidation in these proteins. Results of previous studies reporting the presence of several phosphorylated, glycosylated, methylated and deamidated proteins in pollen can provide a proper explanation for heterogeneous combination of peptides with hydrophobic and hydrophilic characteristics in the pollen RP-HPLC profile and multi-functionality proteins of pollen (Zhang, Wei, Wu, Hu, & Dietemann, 2012). Finally, fraction eluted in 27 min of PHP (fraction 53 to 55 of SEC) and fraction eluted 32 min of THP (fraction 62 to 64 of SEC) were selected for peptide identification.

3.2. Peptide identification of by mass spectrometry in tandem

The results of peptide sequencing showed a total of 80 peptide sequences in all samples as they are presented in Table 2. The protein of all identified peptides in PHP and THP had herbal origins. For example, histone H2A, enolase, triose-phosphate isomerase, glycosyltransferase, and auxin response factor are some of the origin plant species for identified peptides of fractions of

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PHP and THP. All the identified peptide sequences showed more than 95% homology with their proteins of origin. Furthermore, amino acid sequences of the identified peptides were characterized as well as their modifications, observed molecular masses, theoretical molecular masses and mass/charge (m/z) as it is shown in Table 2.

The biological activities of peptides are related to the composition and sequence of their amino acids and their size (Mora, Aristoy, & Toldra, 2016). ACE-inhibitory peptides are known to have less than 20 amino acids (Torkova et al., 2016). Mass spectrometry and data analysis tools features in the current research did not allow for determination of dipeptides and tripeptides. Numerous researches have presented molecular weight distribution of protein hydrolysates through use of mass spectrometry (Mora et al., 2016). The peptides identified from fraction eluted at 27 min of PHP displayed peptides with molecular weights ranging from 785 Da to 2184 Da, while those eluted at 32 min of THP exhibited peptides with molecular weights in the range of 706-1716 Da, these results are in line with the work of Moayedi et al. (2016) that reported the peptide molecular range from 1200 to 1500 Da are very effective in ACE-inhibitory activity. The identified peptides showed from 6 to 21 amino acids in length (see Table 2) which is in accordance with the study of Jin et al. (2016) which reported that main antioxidant peptides contain a number of amino acids between 3 to 15. Balti et al. (2015) and Torkova et al. (2016) also reported peptides from 3 to 15 amino acids length as ACE-inhibitory peptides. ACE-inhibitory peptides have also been described by Escudero, Mora and Toldrá (2014) containing Lysine, Proline or aromatic residues preferably in the three positions closest to the C-terminal site. Jin, Liu, Zheng, Wang, & He (2016) studied the relationship between antioxidant activity and structure of peptides obtained from natural proteins. They showed that the antioxidant

peptides mainly had more than three amino acid residues, and were dominantly composed from 3 to 6 amino acids.

Considering amino acid composition of PHP and THP peptides, glycine and alanine are the two major amino acids with hydrophobic features; this is consistent with the results reported by Nasri et al. (2013) expressing possible relationship between ACE-inhibitory activity and the number of hydrophobic amino acids within a peptide sequences. Some of the most hydrophobic peptides identified in fractions of PHP were GGVGVGMGTGVGVGL, LVLPVPAF, AERVGAGAPVY, and VAPLSLGMETAGGV. Also some of hydrophobic peptides identified in THP were EGGGYGGGDGGYGGGSGGGGW, IPVHIPEPVL, VISGLVGR and LAYVGALVR. Amino acid composition, hydrophobicity, molecular mass and sequence length of a peptide play a key role in determining its antioxidant activity (Escudero et al., 2014). The presence of charged amino acids such as asparagine, glutamine, arginine and lysine in some peptides obtained in pepsin hydrolysate such as QDQDQDQDQL, DLAGRDLTD, AIRNDEELNKL, VREIAQDF and other peptides from trypsin hydrolysate such as DHDDGVQVDDYPDK, VLGDALTDAEIK and QCADKGENYSNK could be responsible for the ferric reducing power capacity observed in fractions of pepsin and trypsin hydrolysates with enhanced electron donation capability of the peptides (Polgar, 1987). This is in accordance with the study of Escudero et al. (2014) which detected antioxidant dipeptides and tripeptides including asparagine, histidine, arginine and lysine in their sequences. Tyrosine and phenylalanine were the most abundant amino acids in the final sequence of peptides from PHP which is in accordance with the study of Khantaphant, Benjakul, & Kishimura (2011) which reported pepsin cleaves peptide bonds between hydrophobic and preferably aromatic amino acids such as tyrosine, tryptophan and phenylalanine. Also arginine or lysine was the most abundant amino acids in the final sequence

of peptides from trypsin hydrolysates which is in accordance with data reported by Polgar (1987).

Peptides identified in PHP (fraction eluted at 27 min in HPLC from fractions 53 to 55 of SEC) and THP (fractions eluted at 32 min from fractions 62 to 64 of SEC) after RP-HPLC, sharing sequences with previously identified bioactive peptides based on Biopep databases are shown respectively in Table S1 and S2 of supporting information. In PHP, about 70 peptides showed some homology with ACE-inhibitory and antioxidant peptides which had been identified before (Supporting information Table S1). For example, FRAAVPSGASTGIY contain IY at the C-terminal position. IY is an antioxidant peptide derived from soybean protein isolates such as beta-conglycinin and glycinin (Beermann, Euler, Herzberg, & Stahl, 2009). Further, NKL an ACE-inhibitory tripeptide and KL an ACE-inhibitory dipeptide found at the C-terminal sequences of AIRNDEELNKL and PQTAEESGND SGKL were also derived from Wakame (Meisel, Walsh, Murray, & Fitz Gerald, 2006). DFGERNGY contain DFG at the N-terminal position. DFG is an ACE-inhibitory tripeptide derived from enzymatic hydrolysates of cuttlefish (*Sepia officinalis*) muscle proteins (Balti et al., 2015). NF is a dipeptide found at the C-terminal sequence of VILAANANNF. NF is an ACE-inhibitory dipeptide derived from garlic (Meisel et al., 2006).

In THP, about 19 peptides showed some homology with ACE-inhibitory and antioxidant peptides which had been identified before (Supporting information Table S2). For example QCADKGENYSNK contain NK at the C-terminal position. NK is an ACE-inhibitory dipeptide derived from wakame (Meisel et al., 2006) that is also present at the C-terminal sequence of PSSNK, an ACE-inhibitory peptide derived from edible mushroom (*Agaricus bisporus*) (Liu et al., 2014).

4. Concluding

The current research indicated that hydrolysis of pollen using pepsin and trypsin in a predicted time and enzyme concentration will result in generation of peptides possessing different sizes and potential bioactivities. The analysis of hydrolyzed protein by SEC and then RP-HPLC showed that the hydrolyzed proteins contain fractions with high antioxidant and ACE-inhibitory activities. The results of peptide sequencing showed similar profiles of antioxidant activity in both assays (DPPH radical scavenging and ferric-reducing antioxidant power). Identified peptides showed a number of 6 to 21 amino acids in length. All peptides had herbal proteins origins from plant species which the pollens were collected by honey bee. Glycine and Alanine were the two main hydrophobic amino acids present in the peptide sequences identified in PHP and THP. In addition, there were sharing sequences with previously identified peptides described in BIOPEP database. The current research has demonstrated that hydrolyzed pollen protein can be used as functional ingredient in food formulations.

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References

- Alemán, A., Pérez-Santín, E., Bordenave-Juchereau, S., Arnaudin, I., GómezGuillén, M.C., & Montero, P. (2011). Squid gelatin hydrolysates with antihypertensive, anticancer and antioxidant activity. *Food Research International*, **44**, 1044–1051.
- Balti, R., Bougatef, A., Sila, A., Guillochon, D., Dhulster, P., & Nedjar-Arroume, N. (2015). Nine novel angiotensin I-converting enzyme (ACE) inhibitory peptides from cuttlefish (*Sepia officinalis*) muscle protein hydrolysates and antihypertensive effect of the potent active peptide in spontaneously hypertensive rats. *Food Chemistry*, **170**, 519-525.
- Beermann, C., Euler, M., Herzberg, J., & Stahl, B. (2009). Anti-oxidative capacity of enzymatically released peptides from soybean protein isolate. *European Food Research and Technology*, **229**, 637–644.
- Bersuder, P., Hole M., & Smith, G. (1998). Antioxidants from a heated histidine-glucose model system. I: Investigation of the antioxidant role of histidine and isolation of antioxidants by high-performance liquid chromatography. *Journal of the American Oil Chemists' Society*, **75**, 181-187
- Escudero, E., Mora, L., & Toldrá, F. (2014). Stability of ACE inhibitory ham peptides against heat treatment and in vitro digestion. *Food Chemistry*, **161**, 305-311.
- Feás, X., Vázquez-Tato, M.P., Estevinho, L., Seijas, J.A., & Iglesias, A., (2012). Organic bee pollen: botanical origin nutritional value bioactive compounds antioxidant activity and microbiological quality. *Molecules*, **17**, 835–837.

Himaya, S.W.A., Ngo, D.H., Ryu, B., & Kim, S.K. (2012). An active peptide purified from gastrointestinal enzyme hydrolysate of Pacific cod skin gelatin attenuates angiotensin-1 converting enzyme (ACE) activity and cellular oxidative stress. *Food Chemistry*, 132, 1872–1882.

Jin, D.X., Liu, X., Zheng, X., Wang, X., & He, J. (2016). Preparation of antioxidative corn protein hydrolysates, purification and evaluation of three novel corn antioxidant peptides. *Food Chemistry*, 204, 427-436.

Khantaphant, S., Benjakul, S., & Kishimura, H. (2011). Antioxidative and ACE inhibitory activities of protein hydrolysates from the muscle of brownstripe red snapper prepared using pyloric caeca and commercial proteases. *Process Biochemistry*, 46, 318–327.

Lassoued, I., Mora, L., Barkia, A., Aristoy, M-C., Nasri, M., & Toldra, F. (2016). Angiotensin I-converting enzyme inhibitory peptides FQPSF and LKYPI identified in *Bacillus subtilis*A26 hydrolysate of thornback ray muscle. *International Journal of Food Science and Technology*, 51, 1604–160.

Lassoued, I., Mora, L., Barkia, A., Aristoy, M-C., Nasri, M., & Toldra, F. (2015). Bioactive peptides identified in thornback ray skin's gelatin hydrolysates by proteases from *Bacillus subtilis* and *Bacillus amyloliquefaciens*. *Journal of Proteomics*, 128, 8–17.

Liu, R., Zheng, W., Li, J., Wang, L., Wu, H., Wang, X., & Shi, L. (2014). Rapid identification of bioactive peptides with antioxidant activity from the enzymatic hydrolysate of *Macra veneriformis* by UHPLC–Q-TOF mass spectrometry. *Food Chemistry*, 167, 484-489.

Marinova, M., & Tchorbanov, P. (2010). Preparation of antioxidant enzymatic hydrolysates from honeybee-collected pollen using plant enzymes. *Enzyme Research*, 41, 5949-50.

Meisel, H., Walsh, D. J., Murray, B., & Fitz Gerald, R. J. (2006). ACE inhibitory peptides. In Y. Mine, F. Shahidi (Eds.), *Nutraceutical proteins and peptides in health and disease*. CRC Taylor & Francis Group, Boca Raton, London, New York, p 269-315.

Moayedi, A., Mora, L., Aristoy, C-M., Hashemi, M. Safari, M., & Toldrá, M. (2016). ACE-Inhibitory and antioxidant activities of peptide fragments obtained from tomato processing by-products fermented using *Bacillus subtilis*: Effect of amino acid composition and peptides molecular mass distribution. *Applied Biochemistry and Biotechnology*, DOI 10.1007/s12010-016-2198-1.

Mora, L., Aristoy, M.C., & Toldra, F. (2016). Bioactive peptides in foods. In B. Caballero, P.M. Finglas & F. Toldra (Eds.), *Encyclopedia of Food and Health*, Oxford: Academic Press, p 395–400.

Nagai, T., Inoue, R., Suzuki, N., Myoda, T., & Nagashima, T. (2005). Antioxidative ability in a linoleic acid oxidation system and scavenging abilities against active oxygen species of enzymatic hydrolysates from pollen *Cistus ladaniferus*. *International Journal of Molecular Medicine*, 15, 259-63.

Nasri, R., Younes, I., Jridi, M., Trigui, M., Bougatef, A., Arroume, N., Dhulster, P., Nasri, M., & Châabouni, M.K., (2013). ACE inhibitory and antioxidative activities of Goby (*Zosterisessor ophiocephalus*) fish protein hydrolysates: Effect on meat lipid oxidation. *Food Research International*. 54, 552–561.

Polgár, L. (1987). Structure and function of serine proteases (Volume 16, Pages iii-v, 1-423). In A. Neuberger and, K., Brocklehurst (Eds.), *New Comprehensive Biochemistry*, Elsevier, p. 159-200.

Ryder, K., Bekhit, A. E. D., McConnell, M., & Carne, A. (2016). Towards generation of bioactive peptides from meat industry waste proteins: Generation of peptides using commercial microbial proteases, *Food Chemistry*, 208, 42-50.

Sentandreu, M. Á., & Toldrá, F. (2006). A rapid, simple and sensitive fluorescence method for the assay of angiotensin-I converting enzyme. *Food Chemistry*, 97, (3), 546–554.

Sun, D. X. (2011). Enzymatic hydrolysis of soy proteins and the hydrolysates utilization. *International Journal of Food Science and Technology*, 46, 2447-2459.

Torkova, A., Kononikhin, A., Bugrova, A., Khotchenkov, V., Tsentalovich, M., & Medvedeva, U. (2016). Effect of *in vitro* gastrointestinal digestion on bioactivity of poultry protein hydrolysate. *Current Research of Nutrition and Food Science*, 4, 77-86.

Toldrá, F., Reig, M., Aristoy, M.-C., & Mora, L., (2017). Generation of bioactive peptides during food processing. *Food Chemistry*, 267, <https://doi.org/10.1016/j.foodchem.2017.06>.

Wijesekara, I., & Kim, S. K. (2010). Angiotensin-I-Converting Enzyme (ACE) Inhibitors from Marine Resources: Prospects in the Pharmaceutical Industry. *Marine Drugs*, 8, 1080-1093.

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Yildirim, A., Mavi, A., & Kara, A. A. (2001). Determination of antioxidant and antimicrobial activities of *Rumex crispus* L. extracts. *Journal of Agricultural and Food Chemistry*, 49, (8), 4083–4089.

Zhang, H. Q., Wei, W. T., Wu, L. M., Hu, F. L., & Dietemann, V. (2012). Fast determination of royal jelly freshness by a chromogenic reaction. *Journal of Food Science*, 77, 247-252.

For Peer Review

Table1. Antioxidant activity and ACE-inhibitory tests of hydrolyzed pollen by Pepsin and Trypsin in the points determined with the central composite design in the RSM

Treatment	Enzyme concentration	Time (hr)	Reducing power		DPPH radical		ACE inhibitory	
			(Absorbance at 700 nm)		scavenging (%)		Activity (%)	
			Pepsin hydrolysates	Trypsin hydrolysate	Pepsin hydrolysates	Trypsin hydrolysate	Pepsin hydrolysates	Trypsin hydrolysate
1	2	1	0.68	0.622	34.09	26.21	34.09	34.24
2	1.5	1	0.65	0.624	96.51	53.87	35.58	35.714
3	1	1	0.75	0.618	88.58	42.95	67.74	34.24
4	2	2.5	0.68	0.664	100	52.179	44.3	54.66
5	1.5	2.5	0.61	0.667	91.71	73.12	27.93	29.23
6	1.5	2.5	0.57	0.668	93.64	72.11	28.94	23.32
7	1.5	2.5	0.61	0.665	92.71	72.21	27.93	23.25
8	1.5	2.5	0.57	0.667	94.64	71.11	27.93	21.23
9	1.5	2.5	0.61	0.665	95.71	73.22	27.93	25.27
10	1	2.5	0.57	0.664	91.02	51.48	44.22	44.23
11	2	4	0.81	0.638	71.88	69.58	91.49	85.8
12	1.5	4	0.74	0.636	66.69	79.8	58.24	73.16
13	1	4	0.73	0.626	67.24	51.46	57.67	98

Table 2. Peptides identified from PHP (fraction eluted 27 min from SEC fraction 53 to 55) and THP (fractions eluted 32 min from SEC fraction 62 to 64) by nano-liquid chromatography and mass spectrometry in tandem (Q-ToF) after RP-HPLC separation.

Name of proteins and Species	Sequence	Modification	Obs MW	Obs m/z	Theor MW	Theor m/z	Theo r z
PHP							
Actin (Fragment), <i>Genlisea aurea</i>	DLAGRDLTDY		1137.526	569.770	1137.530	569.772	2
Actin (Fragment), <i>Genlisea aurea</i>	RVAPEEHPVL		1145.615	573.815	1145.619	573.817	2
Actin (Fragment), <i>Genlisea aurea</i>	SGGTTMYPGIADR		1324.602	663.308	1324.608	663.311	2
Actin (Fragment), <i>Genlisea aurea</i>	DLAGRDLTDYL		1250.606	626.311	1250.614	626.314	2
Actin (Fragment), <i>Genlisea aurea</i>	DLAGRDLTD		974.392	488.203	974.467	488.241	2
Actin (Fragment), <i>Genlisea aurea</i>	LTEAPLNPKANREKMTQIM		2184.129	547.039	2184.139	547.042	4
Actin (Fragment), <i>Genlisea aurea</i>	TEAPLNPKANREK		1466.778	367.702	1466.784	367.703	4
Actin (Fragment), <i>Genlisea aurea</i>	DSGDGVTHTVPIYEG		1545.685	773.850	1545.695	773.855	2
Uncharacterized protein, <i>Glycine max</i>	FRAAVPSGASTG		1119.561	560.788	1119.567	560.791	2
Uncharacterized protein, <i>Glycine max</i>	FRAAVPSGASTGIY		1395.710	698.863	1395.715	698.865	2
Uncharacterized protein, <i>Glycine max</i>	RAAVPSGASTGIY		1248.613	625.314	1248.646	625.330	2
Uncharacterized protein, <i>Capsella rubella</i>	DVAPLSLGIETAGGV		1397.736	699.875	1397.740	699.877	2
Uncharacterized protein, <i>Capsella rubella</i>	DVAPLSLGIETAGGVM		1528.775	765.395	1528.781	765.398	2
Uncharacterized protein, <i>Capsella rubella</i>	IGDAAKNQVALNPQNT		1652.850	827.432	1652.848	827.431	2
Histone H2A, <i>Arabidopsis thaliana</i>	AERVGAGAPVY		1088.553	545.284	1088.561	545.288	2
Histone H2A, <i>Arabidopsis thaliana</i>	AIRNDEELNKL		1313.687	438.903	1313.694	438.905	3
Enolase, <i>Cynara cardunculus</i>	NVINGGSHAGNKL		1279.654	427.559	1279.663	427.562	3
Enolase, <i>Cynara cardunculus</i>	VNEYPIVSIEDPFDQDD		1993.916	997.965	1993.879	997.947	2
Enolase, <i>Cynara cardunculus</i>	LVLVPVPAF		854.520	428.267	854.527	428.271	2
Enolase, <i>Cynara cardunculus</i>	RAAVPSGASTGIY		1248.613	625.314	1248.646	625.330	2
Uncharacterized protein, <i>Musa acuminata</i>	VREIAQDF		976.489	489.252	976.498	489.256	2
Uncharacterized protein, <i>Populus</i>	PQTAEESGNDSGKL		1431.596	716.805	1431.648	716.831	2

trichocarpa

Triose-phosphate isomerase, *Zostera marina*

LVGGASLKPEF

1116.616

559.315

1116.618

559.316

2

FDNSQRQATKDAGVIAG

Predicted protein, *Hordeum vulgare*

L

1889.950

630.991

1889.960

630.994

3

Uncharacterized protein, *Prunus persica*

GFPHPVGT

810.409

406.212

810.402

406.208

2

Putative uncharacterized protein, *Arabidopsis lyrata*

QPPTTASSPF

Gln->pyro-
Glu@N-term

1014.444

508.229

1014.466

508.240

2

Uncharacterized protein, *Erythranthe guttata*

NLECLIVSRPL

1255.674

628.844

1255.696

628.855

2

growth-regulating factor 3, *Vigna radiata*

AGGWAGGSQVASMGGP
LAEAL

1885.940

472.492

1885.899

472.482

4

Uncharacterized protein, *Phaseolus angularis*

QDQDQDQDQL

1231.428

616.721

1231.495

616.755

2

Glycosyltransferase, *Brassica oleracea*

VNSSGGMEHQAL

1228.492

615.253

1228.551

615.283

2

Uncharacterized protein, *Arundo donax*

TTSLVPVSPPADSATVGF
ACL

2031.913

508.986

2032.019

509.012

4

Deamidated

Nuclease S1, *Triticum urartu*

HCGHVEDLGGNTIL

(N)@11

1464.760

733.388

1464.667

733.341

2

pathogenesis-related protein 1A-like, *Capsicum annuum*

PPGNYDGEKPY

1235.546

618.780

1235.546

618.780

2

Uncharacterized protein, *Arundo donax*

NVLGGSQSISSL

1160.616

581.315

1160.604

581.309

2

Uncharacterized protein, *Arundo donax*

VNLPNLQ

796.441

399.228

796.444

399.229

2

Uncharacterized protein, *Triticum aestivum*

AVPAAPSCARPF

1185.635

593.825

1185.596

593.805

2

Phytoene desaturation 1, *Zea mays*

DAPARVAS

785.349

393.682

785.403

393.709

2

Uncharacterized protein, *Lupinus angustifolius*

GSTGEIEVKPDIAL

1427.713

714.864

1427.751

714.883

2

Uncharacterized protein, *Triticum aestivum*

GAGDSVGGTDAANLL

1316.542

659.278

1316.621

659.318

2

1								
2								
3			Deamidated					
4	Ubiquitinyl hydrolase 1, <i>Brassica</i>							
5	<i>oleracea</i>	EVAQSPFKNL	(Q)@4	1132.576	567.295	1132.576	567.295	2
6								
7	Uncharacterized protein, <i>Oryza barthii</i>	PDKILVLDGL		1081.602	541.808	1081.638	541.826	2
8								
9	Uncharacterized protein, <i>Citrus</i>							
10	<i>clementina</i>	DVWDPFDGF		1096.451	549.233	1096.450	549.232	2
11								
12	Uncharacterized protein, <i>Daucus</i>							
13	<i>carota</i>	GGVGVGIGTGVGVGL		1197.580	599.797	1197.672	599.843	2
14								
15	Uncharacterized protein, <i>Setaria</i>							
16	<i>italica</i>	VSVLDLEDVVNY		1250.547	626.281	1250.603	626.309	2
17								
18	Guanosine nucleotide diphosphate							
19	dissociation inhibitor, <i>Vigna radiata</i> ;							
20	Guanosine nucleotide diphosphate							
21	dissociation inhibitor, <i>Vigna angularis</i>	ARFQGGSPY		981.458	491.737	981.467	491.741	2
22								
23	Uncharacterized protein, <i>Brassica</i>		Deamidated					
24	<i>rapa</i> ; BnaAnng08420D protein,							
25	<i>Brassica napus</i>	DFGERNGY	(N)@6	957.377	479.696	957.383	479.699	2
26								
27	Mitogen-activated protein kinase,							
28	<i>Oryza sativa</i> ; Mitogen-activated							
29	protein kinase, <i>Oryza rufipogon</i> ;							
30	Mitogen-activated protein kinase,							
31	<i>Oryza punctata</i> ; Mitogen-activated							
32	protein kinase, <i>Oryza nivara</i>	AFNPDYQ		853.356	427.685	853.361	427.688	2
33								
34	Uncharacterized protein,							
35	<i>Brachypodium distachyon</i>	ELGNLLNLQDLGL		1410.767	706.391	1410.772	706.393	2
36								
37	Uncharacterized protein, <i>Vigna</i>	VCVSDAFDVLRVSKAIIG						
38	<i>angularis</i>	AF		2108.980	704.001	2109.129	704.050	3
39								
40	BnaC08g23700D protein, <i>Brassica</i>							
41	<i>napus</i>	LVDVNVIDTDDPL		1426.773	714.394	1426.719	714.367	2
42								
43	isoflavone reductase-like protein,							
44	<i>Cucumis melo</i>	YIRPSGNTY		1069.514	535.764	1069.519	535.767	2
45								
46	Uncharacterized protein,							
47	<i>Dichanthelium oligosanthes</i>	ADLVAVVMAGEKTPTF		1647.852	824.934	1647.854	824.934	2
48								
49								
50			Deamidated					
51	Uncharacterized protein, <i>Citrus</i>							
52	<i>clementina</i>	TADIYQTATPF	(Q)@6	1227.570	614.792	1227.566	614.790	2
53								
54		THP						
55								
56								
57								
58								
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Pollen-specific protein-like, <i>Zea mays</i>	AEATTGPGGWYR		1264.582	633.298	1264.584	633.299	2
Pollen-specific protein-like, <i>Zea mays</i>	EATTGPGGWYR		1193.542	597.779	1193.547	597.781	2
Uncharacterized protein, <i>Eutrema salsugineum</i>	AGWGVMAHR	Oxidation (M)@6	1086.504	363.175	1086.503	363.175	3
Uncharacterized protein, <i>Eutrema salsugineum</i>	AGWGVMA	Oxidation (M)@6	706.306	354.161	706.311	354.163	2
Uncharacterized protein, <i>Beta vulgaris</i>	VLGDALTDAEIK		1243.577	622.796	1243.666	622.840	2
ubiquitin-conjugating enzyme E2 28-like isoform X1, <i>Nicotiana tabacum</i>	SWTQKYAMG		1070.486	536.250	1070.485	536.250	2
ubiquitin-conjugating enzyme E2 28-like isoform X1, <i>Nicotiana tabacum</i>	SWTQKYAMG	Oxidation (M)@8	1086.477	544.246	1086.480	544.247	2
Uncharacterized protein, <i>Oryza meridionalis</i>	MNMQDGQGGQDPK		1404.486	703.250	1404.576	703.295	2
BnaA01g05410D protein, <i>Brassica napus</i>	EGGGYGGGDGGYGGGS GGGGW		1716.638	859.326	1716.640	859.327	2
Alpha-expansin 9, <i>Zea mays</i>	AEPWTDGHATYYGGR		1679.734	560.919	1679.733	560.918	3
Pectinesterase (Fragment), <i>Vigna radiata</i> ; Pectinesterase, <i>Vigna radiata</i> ; Pectinesterase, <i>Vigna angularis</i> ; Pectinesterase, <i>Phaseolus angularis</i>	RVNWPGYH		1027.497	514.756	1027.499	514.757	2
Uncharacterized protein, <i>Beta vulgaris</i>	QCADKGENYSNK	Deamidated (N)@8	1356.572	679.293	1356.562	679.288	2
Uncharacterized protein, <i>Brassica oleracea</i>	CLSYIPSESK		1125.507	563.761	1125.538	563.776	2
ARM repeat superfamily protein (Fragment), <i>Ceratophyllum platyacanthum</i> ; ARM repeat superfamily protein (Fragment), <i>Ceratophyllum demersum</i>	VISGLVGR		799.395	400.705	799.492	400.753	2
cytochrome c oxidase subunit 5b-2, mitochondrial-like, <i>Gossypium hirsutum</i>	SYYNKRIV	Deamidated (N)@4	1042.505	522.260	1042.545	522.280	2
En/Spm-like transposon protein,	LASYVGALVR		1047.525	524.770	1047.608	524.811	2

1								
2								
3	<i>Arabidopsis thaliana</i>							
4								
5	LRR receptor-like serine/threonine-							
6	protein kinase RPK2, <i>Anthurium</i>							
7	<i>amnicola</i>	GVTCDGVSGR	949.451	475.733	949.429	475.722	2	
8								
9	Reticulon-like protein, <i>Lotus</i>							
10	<i>japonicas</i>	IPQVHIPEEPVL	1369.668	457.563	1369.760	457.594	3	
11								
12	Uncharacterized protein, <i>Cephalotus</i>							
13	<i>follicularis</i>	DHDDGVQVDDYPDK	1616.535	539.852	1616.659	539.894	3	
14								
15	Lycopene beta-cyclase 1 (Fragment),							
16	<i>Coriandrum sativum</i>	FFQCFF	837.378	419.696	837.352	419.683	2	
17								
18	Uncharacterized protein, <i>Glycine max</i> ;							
19	ubiquitin-conjugating enzyme E2-17							
20	kDa-like, <i>Nicotiana tabacum</i> ;							
21	Ubiquitin-conjugating enzyme E2-17							
22	kDa, <i>Glycine soja</i>	SWTQKYSMG	(M)@8	1102.472	552.243	1102.475	552.245	2
23								
24	Uncharacterized protein, <i>Glycine max</i> ;							
25	ubiquitin-conjugating enzyme E2-17							
26	kDa-like, <i>Nicotiana tabacum</i> ;							
27	Ubiquitin-conjugating enzyme E2-17							
28	kDa, <i>Glycine soja</i>	SWTQKYSMG	1086.477	544.246	1086.480	544.247	2	
29								
30								
31								
32	Auxin response factor, <i>Coffea</i>							
33	<i>canephora</i>	NPGNSVGR	(N)@4	800.382	401.198	800.378	401.196	2
34								
35	Pollen-specific protein-like, <i>Zea mays</i>	AEATTGPGGWYR	1264.582	633.298	1264.584	633.299	2	
36								
37	Pollen-specific protein-like, <i>Zea mays</i>	EATTGPGGWYR	1193.542	597.779	1193.547	597.781	2	
38								
39								
40	Uncharacterized protein, <i>Eutrema</i>							
41	<i>salsugineum</i>	AGWGVMASHR	(M)@6	1086.504	363.175	1086.503	363.175	3
42								
43								
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Fig 1. Size-exclusion chromatography (SEC) profile of PHP (A) and THP (B) at a concentration of respectively 100 mg/mL, in comparison to distribution of molecular weights of the standards on Sephadex G-25 column (c). Standard proteins used for column calibration include bovine serum albumin (70 kDa), cytochrome C (13 kDa), Bacitracin (1.42 kDa), Carnosin (0.22 kDa) and Tyrosine (0.2 kDa).

Fig. 2. DPPH radical scavenging activity for PHP (A) and THP (B), ACE-inhibitory activity for PHP (C) and THP (D) and reducing power for PHP (E) and THP (F), in different fractions obtained from size exclusion chromatography. Fractions 53 to 55 and 62 to 64 from PHP and fractions 62 to 64 from THP were selected for more purification.

Fig. 3. Reversed-phase chromatographic separation of the selected pooled fractions (53 to 55) and (62 to 64) of PHP and fractions (62 to 64) of THP obtained from size-exclusion chromatography. Fractions were automatically collected and assayed for their ACE-inhibitory activity and antioxidant activity. ACE-inhibitory activity of PHP fractions (53 to 55) (A) and fractions (62 to 64)(B) and THP fractions (62 to 64) (E), DPPH radical scavenging of PHP and THP for fractions (62 to 64) (C and F) and reducing power of PHP fractions (62 to 64) (D) of SEC. The most active fractions were selected for identification.

Fig 1.

Fig. 2.

Fig. 3

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Supporting information Table S1. Peptides identified from Fraction 27 after RP-HPLC separation of fraction (53 to 55) for PHP, sharing sequences with previously identified bioactive peptides basing on Biopep data bases.

Sequence	similar sequences in BIOPEP	Biological activity	references
DLAGRDLTDY	MDLA	ACE inhibitor from chickpea legumin	Murray and Fitz Gerald et al., 2007
RVAPEEHPVL	RVY	ACE inhibitor	Wu et al., 2006
SGGTTMYPGIADR	VL	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
SGGTTMYPGIADR	SG	ACE inhibitor	Cheung et al., 1980
DLAGRDLTDYL	DR	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
DLAGRDLTD	YL	ACE inhibitor (from bovine beta-Lg)	Lan et al., 2015
DLAGRDLTD	TDQHQDKIYP	synthetic fr. of human beta-CN, ACE inhibitor	Kohmura et al., 1989
LTEAPLNPKANREKM	TDY	Antioxidant peptide from marine bivalve (Mactra veneriformis)	Liu et al., 2014
TQIM	LT	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
TEAPLNPKANREK	LTF	ACE inhibitor from tuna muscles	Meisel et al., 2006
DSGDGVTHTVPIYEG	IMY	ACE inhibitor	Wu et al., 2006
FRAAVPSGASTG	IM	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
FRAAVPSGASTGIY	TE	ACE inhibitor	van Platerink et al., 2008
RAAVPSGASTGIY	EK	ACE inhibitor	van Platerink et al., 2008
DVAPLSLGIETAGGV	LTTLDS	antioxidative	Chang et al., 2015
DVAPLSLGIETAGGV	EG	ACE inhibitor	Cheung et al., 1980
DVAPLSLGIETAGGV	TG	ACE inhibitor	Cheung et al., 1980
DVAPLSLGIETAGGV	IY	antioxidative peptide from soybean protein isolates: beta-conglycinin and glycinin	Beermann et al., 2009
DVAPLSLGIETAGGV	RA	ACE inhibitor	Cheung et al., 1980
DVAPLSLGIETAGGV	LVEKGDVAFI	Antioxidative peptide	Liu et al., 2014
M	VMP	ACE inhibitor	Kohmura et al., 1989

1			
2			
3		dipeptidyl peptidase IV inhibitor (DPP IV	
4	VM	inhibitor)	Lan et al., 2015
5			
6	IGDAAKNQVALNPQN		
7	T	ACE inhibitor	Cheung et al., 1980
8			
9		dipeptidyl peptidase IV inhibitor (DPP IV	
10	NT	inhibitor)	Lan et al., 2015
11			
12	AERVGAGAPVY	dipeptidyl peptidase IV inhibitor (DPP IV	
13	AE	inhibitor)	Lan et al., 2015
14			
15	VY	antioxidative peptide	Cheng et al., 2010
16	AIRNDEELNKL	ACE inhibitor from wakame	Meisel et al., 2006
17			
18	KL	ACE inhibitor from wakame	Meisel et al., 2006
19			
20	NVINGGSHAGNKL	dipeptidyl peptidase IV inhibitor (DPP IV	
21	NV	inhibitor)	Lan et al., 2015
22	VNEYPIVSIEDPFDQD	dipeptidyl peptidase IV inhibitor (DPP IV	
23	D	inhibitor)	Lan et al., 2015
24			
25	VNP	ACE inhibitor from rice protein	Chen et al., 2013
26			
27	LVLPPVPAF	Antioxidant peptide from marine bivalve (Mactra	
28	LVSK	veneriformis)	Liu et al., 2014
29			
30	AF	ACE inhibitor	Cheung et al., 1980
31	VREIAQDF	ACE inhibitor	Lee et al., 2006
32			
33	DF	ACE inhibitor	Wu et al., 2006
34			
35	PQTAEESGNDGKL	synthetic fr.of human beta-CN (143-149) ACE	
36	PQTLALP	inhibitor	Kohmura et al., 1989
37			
38	PQ	ACE inhibitor	van Platerink et al., 2008
39			
40	KL	ACE inhibitor from wakame	Meisel et al., 2006
41			
42	LVGGASLKPEF	Antioxidant peptide from marine bivalve (Mactra	
43	LVSK	veneriformis)	Liu et al., 2014
44			
45		dipeptidyl peptidase IV inhibitor (DPP IV	
46	LV	inhibitor)	Lan et al., 2015
47	FDNSQRQATKDAGVI		
48	AGL	ACE inhibitor	Wu et al., 2006
49			
50	FDEQE	Antioxidant peptide from marine bivalve (Mactra	
51		veneriformis)	Liu et al., 2014
52	GL	ACE inhibitor	Cheung et al., 1980
53	GFPHPVGT	ACE inhibitor	Cheung et al., 1980
54			
55	GT	ACE inhibitor	Cheung et al., 1980
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3	QPPTTASSPF	GQP	ACE inhibitor	Wu et al., 2006
4				
5			dipeptidyl peptidase IV inhibitor (DPP IV	
6		PF	inhibitor)	Lan et al., 2015
7				
8			dipeptidyl peptidase IV inhibitor (DPP IV	
9	NLECLIVSRPL	NL	inhibitor)	Lan et al., 2015
10				
11		NLHLP	synthetic fr. of human beta-CN (123-127) ACE	Kohmura et al., 1989
12			inhibitor	
13	AGGWAGGSQVASM			
14	GPLAEAL	AG	ACE inhibitor	Cheung et al., 1980
15				
16			dipeptidyl peptidase IV inhibitor (DPP IV	
17		AL	inhibitor)	Nongonierma et al., 2013
18				
19		FAL	ACE inhibitor from micro algae	Murray and FitzGerald et al., 2007
20				
21	QDQDQDQDQL	QD	dipeptidyl peptidase IV inhibitor (DPP IV	Lan et al., 2015
22			inhibitor)	
23		QL	dipeptidyl peptidase IV inhibitor (DPP IV	
24			inhibitor)	Lan et al., 2015
25				
26		AQL	ACE inhibitor	Wu et al., 2006
27				
28	VNSSGGMEHQAL	VN	dipeptidyl peptidase IV inhibitor (DPP IV	Lan et al., 2015
29			inhibitor)	
30			dipeptidyl peptidase IV inhibitor (DPP IV	
31		AL	inhibitor)	Nongonierma et al., 2013
32				
33		FAL	ACE inhibitor from micro algae	Murray and FitzGerald et al., 2007
34				
35	TTSLVPVSPPADSATV		dipeptidyl peptidase IV inhibitor (DPP IV	
36	GFACL	TT	inhibitor)	Lan et al., 2015
37		VCLP	ACE inhibitor	Wu et al., 2006
38				
39	HCGHVEDLGGNTIL	IL	ACE inhibitor	Wu et al., 2006
40				
41	PPGNYDGEKPY	PYP	ACE inhibitor from b-CN (179-181)	Meisel et al, 2006
42				
43	NVLGGSQSISSL	NV	dipeptidyl peptidase IV inhibitor (DPP IV	Lan et al., 2015
44			inhibitor)	
45			dipeptidyl peptidase IV inhibitor (DPP IV	
46	VNLPNLQ	VN	inhibitor)	Lan et al., 2015
47		VNP	ACE inhibitor from rice protein	Chen et al., 2013
48				
49		LQ	ACE inhibitor	van Platerink et al., 2008
50				
51	AVPAAPSCARPF	AVP	ACE inhibitor from beta-CN (177-179)	Meisel et al, 2006
52				
53			dipeptidyl peptidase IV inhibitor (DPP IV	
54		PF	inhibitor)	Lan et al., 2015
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3	DAPARVAS	GDAP	ACE inhibitor	Balti et al., 2010
4				
5			dipeptidyl peptidase IV inhibitor (DPP IV	
6		AS	inhibitor)	Lan et al., 2015
7				
8	GSTGEIEVKPDIAL	GS	ACE inhibitor	Cheung et al., 1980
9				
10		AL	dipeptidyl peptidase IV inhibitor (DPP IV	
11			inhibitor)	Nongonierma et al., 2013
12	GAGDSVGGTDAANL			
13	L	GA	ACE inhibitor	Cheung et al., 1980
14				
15				data from WORLD INTELLECTUAL
16				PROPERTY ORGANIZATION
17		HLL	ACE inhibitor	www.wipo.int2008
18				
19	EVAQSPFKNL	EV	ACE inhibitoR	van Platerink et al., 2008
20				
21			dipeptidyl peptidase IV inhibitor (DPP IV	
22		NL	inhibitor)	Lan et al., 2015
23				
24				
25	PDKILVLDGL	PDLVF	ACE inhibitoR	Torkova et al., 2016
26				
27	DVWDPFDGF	GF	ACE inhibitor	Cheung et al., 1980
28				
29	GGVGVGIGTGVGVG		dipeptidyl peptidase IV inhibitor (DPP IV	
30	L	VGL	inhibitor)	Nongonierma et al., 2013
31		GL	ACE inhibitor	Cheung et al., 1980
32				
33				
34	VSVDLEDVVNY	VSW	ACE Inhibitor	Wu et al., 2006
35				
36		NY	ACE inhibitor from garlic	Meisel et al., 2006
37	ARFQGGSPY	AR	ACE inhibitor	Sentandreu and Toldra, 2007
38				
39		PYP	ACE inhibitor from b-CN (179-181)	Meisel et al., 2006
40				
41		IPY	ACE inhibitor from as2-casein	Murray and FitzGerald et al., 2007
42	DFGERNGY	DFG	ACE inhibitor	Balti et al., 2010
43				
44		DF	ACE inhibitor	Wu et al., 2006
45				
46		GY	ACE inhibitor	Cheung et al., 1980
47				
48		AF	dipeptidyl peptidase IV inhibitor (DPP IV	
49	AFNPDYQ		inhibitor)	Lan et al., 2015
50		AFL	ACE inhibitor	Wu et al., 2006
51				
52		AF	ACE inhibitor	Cheung et al., 1980
53				
54		YQ	dipeptidyl peptidase IV inhibitor (DPP IV	
55			inhibitor)	Lan et al., 2015
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3	ELGNLLNLQDLGL	GL	ACE inhibitor	Cheung et al., 1980
4				
5	VCVSDAFDVLRVSKA			
6	IIGAF	VVCVPW	ACE inhibitor	Liu et al., 2014
7				
8		VCLP	ACE inhibitor	Wu et al., 2015
9				
10	LVDVNVIDTDDPL	LV	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
11				
12				
13	YIRPSGNTY	YI	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
14				
15		TY	Antioxidative peptide	Cheng et al., 2010
16				
17	ADLVAVVMAGEKTP		dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
18	TF	AD		Lan et al., 2015
19	TADIYQTATPF	TAP	ACE inhibitor	Kohmura et al., 1989
20				
21			dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
22		PF		Lan et al., 2015
23				
24	GSGFGSGFGSGSGF	GS	ACE inhibitor	Cheung et al., 1980
25				
26		GF	ACE inhibitor	Cheung et al., 1980
27	DSCAKMPLPF	LTTLDS	Antioxidative peptide	Chang et al., 2015
28				
29		LPF	ACE inhibitor	Wu et al., 2006
30				
31			dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
32		PF		Lan et al., 2015
33				
34		LPF	ACE inhibitor	Wu et al., 2006
35	SDVENAAKMANAHE			
36	F	SDF	Antioxidative	Wu et al., 2006
37				
38	AHGQDNQTF	AH	ACE inhibitor	Van Platerink et al., 2008
39				
40		AH	Antioxidative	Chen et al., 1995
41				
42	NAGNLTEQLESIKESF	NA	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
43				
44		SF	ACE inhibitor from garlic	Meisel et al., 2006
45	ADGGAQDGAGAGAD		dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
46	GVALGGVL	AD		Lan et al., 2015
47				
48			synthetic fr. of human beta-CN (168-170) ACE inhibitor	
49		VLP		Kohmura et al., 1989
50				
51			dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
52		VL		Lan et al., 2015
53				
54		VLY	ACE inhibitor	Wu et al., 2006
55	FADKVPKTAENF	FA		Cheung et al., 1980
56			dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
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3		inhibitor)	
4			
5	NF	ACE inhibitor from garlic	Meisel et al., 2006
6			
7	TGLAAMLGAL	TGLKP	ACE inhibitor
8			Escudero et al., 2014
9	TG	ACE inhibitor	Cheung et al., 1980
10			
11	AL	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Nongonierma et al., 2013
12			
13	AHRAVACF	AH	ACE inhibitoR
14			Van Platerink et al., 2008
15	CF	ACE inhibitor from shark meat hydrolysate	Wu et al., 2008
16			
17	QGGGGDDDEAGVL	VL	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)
18			Lan et al., 2015
19	AGGLATNGLAQEAF	AG	ACE inhibitor
20			Cheung et al., 1980
21	AF	ACE inhibitor	Cheung et al., 1980
22	EAF	ACE inhibitor	Wu et al., 2006
23			
24		dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
25	YGPPGTGKTL	TL	Lan et al., 2015
26			
27	TEGLHLDD	TE	ACE inhibitor
28			Van Platerink et al., 2008
29		dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
30	AEAGVVSASILHRFY	AE	Lan et al., 2015
31			
32	RNEISVDTPQCAL	AL	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)
33			Nongonierma et al., 2013
34	FAL	ACE inhibitor from micro algae	Murray and Fitz Gerald et al., 2007
35			
36	VIDGGGSVHCALLGG		dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)
37	NL	NL	Lan et al., 2015
38			
39	NLHLP	synthetic fr. of human beta-CN (123-127) ACE inhibitor	Kohmura et al., 1989
40			
41		dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	
42	LLGHTGPF	PF	Lan et al., 2015
43			
44	VILAANANNF	VIIF	ACE inhibito
45			Balti et al., 2010
46	NF	ACE inhibitor from garlic	Meisel et al., 2006
47	GVDFAKIYTESSL	GV	ACE inhibitor
48			Cheung et al., 1980
49	ASL	ACE inhibitor	Wu et al., 2008
50			
51	SL	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Nongonierma et al., 2013
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Supporting information Table S2. Peptides identified from Fraction 32 after RP-HPLC separation of fraction (62 to 64) for THP, sharing sequences with previously identified bioactive peptides basing on Biopep data bases.

Sequence	similar sequence	biological activity	references
AEATTGPGGWYR	AE	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
EATTGPGGWYR	EA	ACE inhibitor	Cheung et al., 1980
	YRPY	ACE inhibitor from bonito bowels	Meisel et al., 2006
AGWGVMA	AGWNI	Antioxidative peptide	Cheng et al., 2010
	AGWNIP	Antioxidative peptide	Cheng et al., 2010
	AGWNIPIGT	Antioxidative peptide	Cheng et al., 2010
	AG	ACE inhibitor	Cheung et al., 1980
AGWGVMA	MA	ACE inhibitor	Cheung et al., 1980
VLGDALTDAEIK	VLP	synthetic fr. of human beta-CN (168-170) ACE inhibitor	Kohmura et al., 1989
	VLY	ACE inhibitor	Wu et al., 2006
SWTQKYAMG	SW	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Wu et al., 2006
	VSW	ACE inhibitor	Wu et al., 2006
	SWN	antioxidative peptide	Cheng et al., 2010
	MG	ACE inhibitor	Cheung et al., 1980
MNMQDGQGGQD	MN	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
PK	PK	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
EGGGYGGGDGGY	EG	ACE inhibitor	Cheung et al., 1980
GGGSGGGGW	GW	ACE inhibitor	Cheung et al., 1980
AEPWTDGHATYY			
GGR	AE	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
	LGECGGR	ACE inhibitor	Liu et al., 2014
	GR	ACE inhibitor	Cheung et al., 1980
RVNWPGYH	RVY	ACE inhibitor	Wu et al., 2006
	YH	ACE inhibitor	Wu et al., 2006
QCADKGENYSNK	PSSNK	ACE inhibitor from edible mushroom (Agaricus bisporus)	Liu et al., 2014

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	NK	ACE inhibitor from wakame	Meisel et al., 2006
CLSYIPSESK	GLTSK	antioxidative and ACE inhibitoR	Luna-Vital et al., 2015
	SK	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
VISGLVGR	IVGRPR	ACE inhibitor from thornback ray muscle (Raja clavata)	Lassoued et al., 2016
	GR	ACE inhibitor	Cheung et al., 1980
SYYNKRIV	SY	ACE inhibitor from garlic	Meisel et al., 2006
IPQVHIPEEPVL	IPQEVLP	synthetic fr. of human beta-CN (164-170) ACE inhibitor	Kohmura et al., 1989
	VLPIPQ	synthetic fr. of human beta-CN (161-166) ACE inhibitor	Kohmura et al., 1989
	VL	dipeptidyl peptidase IV inhibitor (DPP IV inhibitor)	Lan et al., 2015
DHDDGVQVDDYP			
DK	FDK	ACE inhibitor	Wu et al., 2006
FFQCF	FFL	ACE inhibitor from soy hydrolysate	Wang et al., 2005
SWTQKYSMG	SWN	antioxidative	Cheng et al., 2010
	MG	ACE inhibitor	Cheung et al., 1980
NPGNSVGR	GR	ACE inhibitor	Cheung et al., 1980