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Effect of *in vitro* Gastrointestinal Digestion on Bioactivity of Poultry Protein Hydrolysate

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ABSTRACT

In vitro simulated gastrointestinal digestion (GID) was performed to evaluate changes in bioactive properties of Poultry protein hydrolysate HCP Premium P150 (PPH) showing strong antioxidant (448.2±37.0 µM TE/g of protein) and moderate Angiotensin-I converting enzyme inhibitory activity (IC_{50} 0.617±0.022 mg/ml). Antioxidant and ACE-inhibitory activity were measured with use of ORAC assay and FRET-substrate methods, correspondingly. Gastric digestion (GD) increased ACE inhibitory activity 2.23 times and didn't change antioxidant activity of PPH significantly. The subsequent intestinal digestion increased antioxidant activity 1.29 times and didn't change ACE-inhibitory activity significantly. New potent ACE-inhibitory peptides: APGAPGPVG (IC_{50} 16.2±3.8 µM), PDLVF (IC_{50} 84.9±6.3 µM) and an antioxidant dipeptide WG (2.29±0.04 µM TE/µM) were identified in the digested PPH. The digested PPH proved to be a rich source of antioxidant and ACE inhibiting molecules and could be a potential new food ingredient used for prevention or treatment of socially significant diseases.

Keywords: Antioxidant hydrolysate, ACE-inhibitory hydrolysate, *in vitro* gastrointestinal digestion, chicken hydrolysate

INTRODUCTION

The growth of common diseases such as cardiovascular disorders, strokes, diabetes, cancer and atherosclerosis has become a serious global problem. In the last decade, the researches paid considerable attention to the use of certain food components, especially bioactive peptides, to prevent socially significant diseases or reduce their negative effects. The use of biopeptides and bioactive hydrolysates as functional food ingredients is a promising solution for some health problems. In contrast with synthetic drugs, peptides display very few side effects in humans due to their natural origin. Bioactive peptides may be released during in vivo digestion or food processing steps, i.e. fermentation, ripening, *in vitro* enzymatic hydrolysis[1]. Recently, dairy proteins have been the most studied source of bioactive peptides[2]. However, other proteins of animal origin, such as collagens of skins, muscle myosins, and sarcoplasmic proteins have also been shown to have varying potential biological effects.

Chicken is a significant source of food and plays an important role in the economy of many countries. Its processing industry produces huge amounts of waste which are usually discarded or rarely processed into hydrolysates and used as food-producing animal protein supplements. Such hydrolysates are perspective sources of molecules with bioactive properties such as ACE-inhibitory or antioxidant peptides and may be used for prevention or treatment of cardiovascular or cancer diseases, organism aging, and degenerative diseases[3]. The hydrolysate of *Gallus Gallus* collagen and muscle proteins, HCP P150 (PPH), was previously designed in the course of work on poultry meat-and-bone residues bioconversion with the use of an enzyme composition containing Alcalase, Protamex, Neutrase, and Flavourzyme[4]. The PPH has shown strong antioxidant effect *in vitro*.

After oral administration, during passage through the gastro-intestinal tract, peptide profiles of hydrolysates and consequently their bioactive properties may suffer serious changes before reaching the target organs. Resistance of peptides to proteolysis by gastrointestinal, brush border and serum peptidases is important. In our previous studies[5], Nikolaev et al. observed a mild antioxidant effect of PPH in the central nervous system *in vivo*, in Wistar-Kyoto rats. However, the bioactive peptides profile in PPH remains unclear, as well as the peptide degradation processes during the passage through the digestive tract.

The objective of this study was to evaluate the stability of antioxidant and ACE-inhibitory properties of hydrolysate HCP Premium 150 (PPH) (Proliver, Belgium) when exposed to digestive enzymes, and on the base of amino acid descriptors of antioxidant and ACE inhibitory activities to analyze the hydrolysate components responsible for bioactivity.

MATERIALS AND METHODS

Materials

Poultry hydrolysate HCP Premium (PPH) (Proliver, Belgium);
Trypsin from hog pancreas, 11909 U/Mg (93614 Fluka BioChemika);
Pepsin from porcine gastric mucosa, 3200-4500 units/mg protein (P 6887 Sigma);
α-Chymotrypsin from bovine pancreas, 60 U/mg protein (Sigma C 4129);
Abz-FRK(Dnp)-OH (A4980 Sigma);
Abz-LFK(Dnp)-OH (A5855 Sigma);
Peptides H-His-Tyr-Val-Pro-Val-OH, H-Pro-Asp-Leu-Val-Phe-OH, H-Ala-Pro-Gly-Ala-Pro-Gly-Pro-Val-Gly-OH, H-Trp-Gly-OH, H-Met-Lys-OH, and H-Met-Met-OH with purity >98% were purchased from Bachem Budendorf, Switzerland.

Evaluation of in vitro hypotensive activity

ACE-inhibitory activity was measured according to the method of Carmona et al.[6] with use of Abz-FRK(Dnp)P-OH and Abz-LFK(Dnp)P as FRET substrates for hydrolysates and peptides, correspondingly. ACE from rabbit lung (Sigma) was mixed with serial dilutions of hydrolysates or peptide solutions in wells of 96-well black microplate (Greiner bio) with a final ACE concentration of 0.01 U/ml. Microplate was incubated and stirred for 30 min at 37°C. After incubation, FRET substrates in concentration 10μM were added to each well. Total volume of reaction solution was 200μL. Fluorescence intensity was monitored using Synergy 2 (BioTek) spectrophotometer-fluorimeter for 30 min at the following working parameters of the reader: excitation at 320 nm, emission filter at 420 nm, reading interval 45s, temperature 37 °C, and constant stirring. ACE residual activity was calculated as follows:

$$\text{ACE residual activity (\%)} = A_{inhibitor} \times 100/A_{control}$$

The half maximal inhibitory concentration (IC_{50}) was used to express the potency of the sample to inhibit ACE activity. The IC_{50} value was expressed in mg of protein/ml for hydrolysates and in μM for peptides.

Determination of antioxidant capacity of the hydrolyzates by ORAC-FL assay

ORAC assay was based on the method of Moor *et al.*[7] modified by Koroleva *et al.*[8] Trolox solutions (5–75 μM) were used for calibration. Fluorescence intensity was monitored for 1 hour under the following working parameters of the reader: excitation at 485 nm, emission filter at 528 nm, reading interval 60 s. The final ORAC-FL values of hydrolysates were calculated by using the linear regression equation between the trolox concentration

and the net area (net AUC) of the fluorescein decay curve (net AUC=Rf [trolox]). The ORAC values were expressed in μMTE/g for the hydrolysates and in μMTE/ μmol for peptides.

Simulated gastric juice

The simulated gastric acid solution was prepared on the base of USP 26 standard and contained HCl (0.23 M) and NaCl (0.034 M). The simulated gastric enzyme solution was prepared by dissolving porcine gastric mucosa pepsin (activity 3411 U/mg of protein calculated using hemoglobin as substrate, Sigma) in the described salt mixture to reach the final concentration of 8000U/mL pepsin.

Simulated pancreatic juice

The simulated pancreatic juice was prepared on the base of USP 26 and Pitino *et al*[9]. Pancreatic juice solution contained NaCl (0.15 M) and KH_2PO_4 (0.05 M). Trypsin from hog pancreas (activity 11,909 U/mg of protein calculated using BAEE as substrate, Fluka BioChemika), a-chymotrypsin from bovine pancrease (activity 40 U/mg of protein calculated using BTEE as substrate, Novozymes, Belgium) were added to pancreatic juice so that the final enzyme concentrations were: 11 U/mL for Hog trypsin and 24 U/mL for bovine a-chymotrypsin.

Simulated gastrointestinal digestion

Chicken hydrolysate was dissolved in the gastric juice to reach protein concentration of 20 mg/ml. pH was adjusted to 1.8. The mixture was incubated at 37°C for 1 hour with constant stirring. Upon completion of the hydrolysis, the pH of the solution was adjusted to 6.8 pH to inactivate the enzyme.

20 ml of the neutralized mixture after gastric hydrolysis was quantitatively transferred into a flask containing 10 ml of intestinal juice. pH was adjusted to 6.8 and solutions of trypsin and chymotrypsin were added and incubated at 37 °C for 2 hours. The inactivation of enzyme preparations after intestinal hydrolysis was carried out in a water bath at 80 °C for 15 minutes.

Determination of molecular mass distribution

Molecular mass distribution was analyzed using a BioSep S 2000 column (7.8×300 mm) from Phenomenex (Torrance, Ca, USA) installed into the ProStar HPLC chromatographic device (Varian Inc., USA). Protein and peptide kits from GE Healthcare (USA), Sigma (USA) and Serva (Germany) were used as standards for calibration. The hydrolysates were filtered through 0.45 μm hydrophilic filters (Sartorius AG, Germany), and were injected (20 μL) into the system with subsequent elution with 50 mM potassium phosphate buffer, pH 6.9, at a flow rate of 1 ml/min. The UV-Vis detector was set at 214 nm.

Peptide profile analysis

Peptide profile analysis was performed by means of high performance reversed-phase chromatography with electrospray ionization. The components were identified by mass spectrometry of ion cyclotron resonance with Fourier transform. Fractions were sub-fractioned in a liquid chromatographic system Agilent 1100 (Agilent Technogies, Paolo Alto, CA, USA) on a capillary column (inner diameter 75 μm x length 12 cm) containing Reprosil-Pur Basic C18 media, 3 μm (Dr. Maisch HPLC GmbH, Ammerbuch-Entringen, Germany).

The detectors of the chromatographic system consisted of a mass spectrometer 7-Tesla Finnigan LTQ-FT Ultra (Thermo Electron, Germany) equipped with a nanoelectrospray. The Xcalibur software package (Thermo Finnigan, USA) was used for experimental data registration.

The Mascot Daemon 2.2.2 (Matrix Science, UK) software was used for automatic searching of the UniProtKB_SwissProt sprot_v.57.9 database. Acceptable level of significance was set at 95% probability. The peptides were considered reliably identified if Mascot score was at least 24.

Statistic analysis

The measurement of antioxidant capacity and ACE inhibition concentration values was performed in 4 replicates. The results are given as mean ± sd.

RESULTS AND DISCUSSION

Changes of MW distribution after simulated digestion of PPH

It was previously shown that the hydrolysate of collagen and muscle proteins, HCP Premium

150 (PPH), had a pronounced antioxidant activity *in vitro*[4,5] and a moderate antioxidant effect *in vivo*[5]. However, the bioactive peptides profile in PPH remains unclear, as well as the peptide degradation processes during the passage through the digestive tract.

An *in vitro* model was designed on the basis of USP 26 protocol to assess the changes of hydrolysate bioactivity properties during digestion by the enzymes of the gastrointestinal tract (GIT). The *in vitro* model included consecutive processing of PPH first by artificial gastric (GD), and then by intestinal juice (GID), to obtain experimental digestive protein hydrolysates, DPHexp-GD and DPHexp-GID. Molecular weight distribution analysis was performed for PPH, DPHexp-GD, and DPHexp-GID. After GID, the PPH fraction containing peptides >3 kDa was reduced from 45.54% to 36.03% (Table 1). The HPLC profiles of PPH before and after the GID are similar, but the digestion results in a decrease of the peak intensity with MW of 3800 Da, while the intensity of the peak with MW 1500 Da increases (Fig 1). Also a new peak with MW 170 Da, corresponding to the MW of di - and tripeptides appears in the DPHexp-GID sample. Specificity of trypsin and chymotrypsin is wider than that of pepsin[10,11], which allows to obtain fractions with a lower MW.

In vitro analysis of PPH bioactivity after processing by gastrointestinal enzymes

Comparison of bioactive properties in PPH, DPHexp-GD, and DPHexp-GID showed a statically significant ($P<0.05$) increase of ACE inhibitory activity (IC_{50} of PPH 0.617±0.022 mg/ml) after gastric digestion (IC_{50} of DPHexp-GD 0.270±0.016 mg/ml), while antioxidant activity was not influenced (Fig 2). Subsequent intestinal digestion (GID) increased antioxidant activity 1.29 times ($P<0.05$), from 486.5±25.8 μM TE/g of protein for DPHexp-GD to 628.1±32.8 μM TE/g of protein for DPHexp-GID (Fig 2). After GID, the ACE-inhibitory activity didn't change significantly compared to GD. This is probably due to the appearance of a peak with MW 170 Da, corresponding to the di- and tripeptides. It appears that dipeptides which are more abundant in DPHexp-GID (MW 170 Da) form weaker contacts with the active site of the enzyme, while tripeptides and longer molecules inhibit ACE more effectively[12]. Meanwhile, a dipeptide sequence is probably sufficient for effective quenching of free radical reactions that may cause an increase in AOE after GID (Fig 2).

Similar results were observed in report of Senphan and Benjakul[13] when ABTS radical scavenging activity and chelating activity of sea bass skin hydrolysate was slightly increased during pepsin digestion, but extremely increased within the first 20 min under duodenal conditions. The ACE inhibitory properties of the sardine and tuna by-products hydrolysates were greatly improved by *in vitro* gastric digestion, and were barely affected by further intestinal digestion[14], which is in good agreement with results observed in our work. However, Ketnawa *et al.*[15] found that the ABTS radical scavenging activity observed after GID Gelatin hydrolysates from farmed Giant Catfish skin varied significantly depending on the enzyme used for gelatin hydrolysis, but generally increased. The antioxidant activity of porcine trypsin hydrolysate mainly increased during the gastric digestion. In contrast, the ABTS radical scavenging activity of Izyme AL® hydrolysate slightly increased during gastric digestion and significantly increased during the intestinal digestion[15]. Espejo-Carpio *et al.*[16] reported that intestinal digestion altered the peptide profiles and bioactivities of goat milk hydrolysates, whereas the effect of the gastric digestion was negligible. They observed a decrease of ACE inhibitory activity after GID when peptides were produced with trypsin, and an increase of antioxidant activity when either trypsin or subtilisin were used for raw material hydrolysis[16]. It indicates that effects of GID on bioactivity are associated not only with specificity of digestive enzymes and MW decrease of peptides[17,18], but also with sequences and conformation of peptides in hydrolysates, which is related with the specificity of enzymes used in raw material hydrolysis.

Peptide profile of hydrolysates

The peptide profile of the PPH and DPHexp-GID hydrolysates was established by high performance reversed-phase chromatography with electrospray ionization. Identification of the components was performed by mass spectrometry of ion cyclotron resonance with Fourier transform. The number of identified peptides was 689 and 450 for PPH and DPHexp-GID, respectively. The minimum peptide size that can be identified by the ESI-MS-

MS method is 5 amino acid residues, while peptides with high bioactivity potential with a size of 2-4 amino acid residues are not taken into account. To model the di-, tri-, and tetrapeptide formation during gastro-intestinal hydrolysis of PPH, an *in silico* digestion of PPH was performed using ExPASy database[19], taking into account the specificity of pepsin, trypsin and chymotrypsin. The resulting hydrolysate, DPHmod, was a mixture of 722 peptides with length of 2-19 amino acid residues. The comparison of the peptide profiles showed that after *in vitro* simulated digestion, the peptides of sarcoplasmic origin prevailed in DPHexp-GID, while in the original PPH and its *in silico* digested form (DPHmod) the peptides of myofibrillar proteins prevailed. The difference is probably due to the identification limits of ESI-MS-MS method. In DPHexp-GID, 33 peptides were similar to those identified in the original PPH: 9 formed during digestion from longer peptides, 13 peptides remained undigested, and 11 peptides might form either way. 30 peptides found in DPHmod were identical to those in the original PPH and 7 peptides identical to those in DPHexp-GID.

Table 1: Molecular weight distribution of the hydrolysates

Sample	Content of the fraction, %		
	<3 kDa	3-10 kDa	>10 kDa
PPH	54.46	41.20	4.34
DPHexp-GD	59.96	35.13	4.91
DPHexp-GID	63.97	34.19	1.84

In silico analysis of PPH, DPHexp-GID, and DPHmod peptide profiles showed the presence of highly active ACE-inhibitory peptides WVPSV (IC_{50} 0.37 µM), VVYPW (IC_{50} 0.36 µM) in DPHexp-GID and less active peptides DMIPAQK (IC_{50} 45.0 µM) and EKSYELP (IC_{50} 14.41 µM) in PPH. Also, an antioxidant peptide WDDMEK was identified in both DPHexp-GID and PPH. Only the dipeptides[20] with biological activity were predicted in DPHmod (Table2).

New bioactive peptides

It seems likely that aside from a few peptides with annotated biological activity, high antioxidant and ACE-inhibitory activity of the

Table 2: Bioactive peptides with previously reported properties identified in DPHexp-GID and predicted in DPHmod

Hydrolysate	ACE-inhibitory peptides	Antioxidant peptides
PPH	DMIPAQK, EKSYELP[21, 22]	WDDMEK[33]
DPHexp-GID	WVPSV, VVYPW[23, 24]	
DPHmod	AY, AI, PQ, GY, LF, VG, IG, GA, GL, GH, GR, GK, GT, GE, SG, GD, VR, QK, NY, SY, KY, AR, EI, IE, EV, VE, TQ, AH, EK, WG[25-32]	AY, AH[34, 35]

Table 3: Characteristics of the synthesized bioactive peptides identified in DPHexp-GID and predicted in DPHmod

Peptide	IC_{50} for C-domain of ACE, µM	Antioxidant activity (ORAC) µM TE/µM	Precursor protein	Precursor hydrolysate
APGAPGPVG	16.2 ± 3.8	0.000759 ± 0.00001	Collagen alpha-1(I) chain	DPHexp-GID
HYVPV	998.2 ± 125.2	0.36 ± 0.09	Collagen alpha-3(VI) chain	DPHexp-GID
PDLVF	84.9 ± 6.3	0.0023 ± 0.0003	Cytochrome P450 26A1	DPHexp-GID
WG	729.4 ± 45.6	2.29 ± 0.04	Hemoglobin subunit alpha-â	DPHmod
MK	6498.6 ± 626.0	0.30 ± 0.01	α- Actin	DPHmod
MM	547.5 ± 51.3	0.64 ± 0.03	α- Actin	DPHmod

digested PPH are determined by a number of other peptides with previously unstudied properties.

The amino acid side chains mostly determine the bioactivity of the peptides. Sagardia et al. established a QSAR model for oligopeptides and showed that the most important factors for ACE inhibitory activity are: the presence of aliphatic bulky side chain amino acid residues such as I, L, V, A, G for C1 terminal position; T, R, V for C2; polar and charged amino acids as D, N, K, and not hydrophobic amino acids L, M, F for C3; W, Y, C, M for C4; aliphatic amino acids V, I, A for C5 position[36]. Also their work[36] has shown that C1 and C4 terminal position are the most important positions for ACE inhibition potency, which is in agreement with an earlier work of Wu et al[37]. In a number of papers the preference of hydrophobic amino acids I, L, V and aromatic amino acids W, F, Y was established for ACE-inhibitory activity of di- and tripeptides[38-41]. It has also been reported that P at C1 and C2 positions is important both for ACE-inhibition and for resisting the proteolysis by gastrointestinal enzymes and serum peptidases[11,12,39,41]. Many agree that the presence of highly hydrophobic amino acids and especially aromatic amino acids in peptides, such as W, Y, M, L, T, and F, have a great importance for antioxidant properties of di- and tripeptides as well as longer peptides, especially for C3 position in the case of peroxyl radical scavenging activity (ORAC)[5,42,43]. The electrons in the resulting radicals are delocalized by the peptide bond or functional groups of the side chain, i.e. tryptophan aromatic ring, making these radicals more stable and less reactive[44].

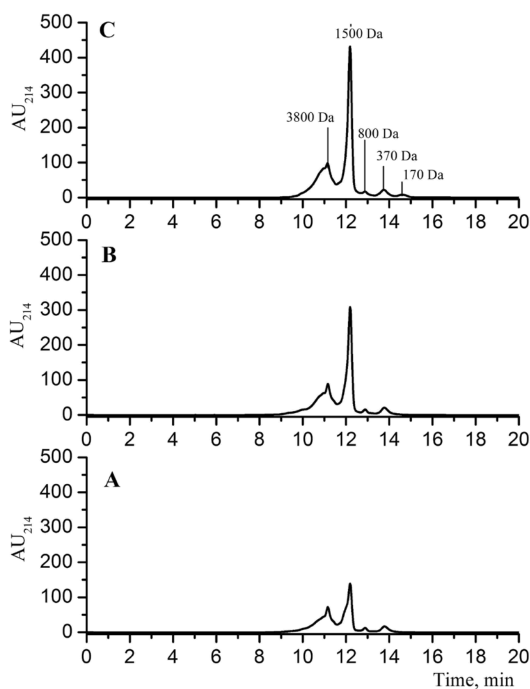
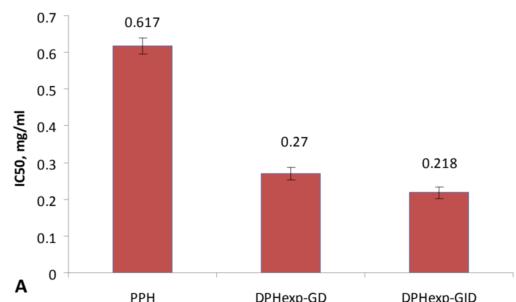


Fig. 1: HPLC chromatogram of PPH (A), DPHexp-GD (B), DPH exp-GID (C)

70 peptides with high biological activity potential were selected based on the reported descriptors, of which 6 were synthesized for subsequent verification of the effects *in vitro*. The ability of each peptide to inhibit the C-domain of ACE, responsible for the regulation of blood pressure, was evaluated, as well as its antioxidant activity against the peroxyl radical. Characteristics of these peptides are shown in table 3.

APGAPGPVG peptide (IC_{50} 16.2±3.8 μM) of collagen origin, characterized by a high content of P and the presence of V in the C-2 position, displayed



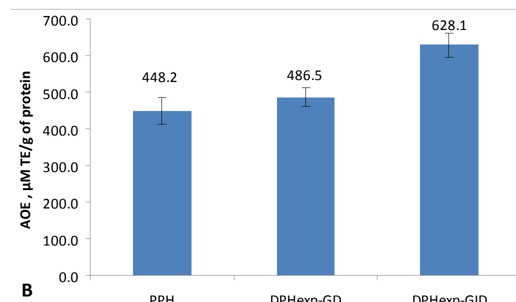


Fig. 2: Bioactivity of PPH and its digested forms, DPHexp-GD and DPHexp-GID: A) ACE-inhibitory activity, B) antioxidant activity

the greatest ACE inhibitory activity comparable with the well-known ACE inhibitory peptides, IPP (IC_{50} 5 µM) and VPP (IC_{50} 9 µM), as well as other peptides of collagen origin: IAPG (IC_{50} 11.4 µM), GPV (IC_{50} 4.7 µM)[45-47]. PDLVF peptide (IC_{50} 84.9±6.3 µM) also containing V in the C-2 position and P in N-1 position, showed a moderate ability to inhibit ACE.

HYVPV peptide (IC_{50} 998.2±125.2 µM) showed a very weak ability to inhibit ACE, as well as dipeptides, but had the highest peroxyl radical scavenging activity among the oligopeptides, probably due to the presence of H and Y residues[5]. None of the selected dipeptides expressed ACE-inhibitory activity, which is probably related to the structural features of the active site of the enzyme and the mechanism of the peptide bond hydrolysis by an ACE molecule[12,19].

The highest antioxidant activity was observed for the WG dipeptide (AOC 2.29±0.04 µM TE/µM), which is associated with the presence of W - the most redox-active amino acid after cystein[48]. Dipeptides containing M also showed medium antioxidant activity against peroxyl radical: 0.30±0.01 µM TE/µM and 0.64±0.03 µM TE/µM for MK and MM, respectively. Antioxidant activity of APGAPGPVG and PDLVF was very weak.

CONCLUSION

The simulated gastrointestinal digestion of the poultry protein hydrolysate PPH improved its bioactivity significantly. Analysis of the peptide profiles of *in vitro* and *in silico* gastro-intestinal digests, DPHexp-GID and DPHmod, revealed the presence of previously reported and newly discovered peptides with high ACE-inhibitory (APGAPGPVG with IC_{50} 16.2±3.8 µM and PDLVF with IC_{50} 84.9±6.3 µM) and antioxidant activity (WG, 2.29±0.04 µM TE/µM). Results of antioxidant capacity analysis of DPHexp-GID *in vitro* is in good agreement with the mild antioxidant effect in central nervous system previously discovered for Wistar rats with feed rations containing different protein ingredients (casein, PPH and protein-free diet)[5]. Both direct radical scavenging and indirect mechanisms (i.e. activation of intrinsic antioxidant defense system) could be involved in the observed antioxidant effect of PPH *in vivo*. Since the antihypertensive effects observed *in vitro* often disagree with *in vivo* tests, the ACE-inhibitory effects of hydrolysates and identified peptides should be further verified *in vivo*. Generally, PPH has great perspectives as a functional supplement in food industry.

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