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Identification and enhancement of antioxidant P1-peptide isolated from *Ganoderma lucidum* hydrolysate

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ABSTRACT

Ganoderma lucidum is an edible fungus which typically used in functional food products because of the potential enhancement of health. Many hydrolysates from G. *lucidum* exhibit various biological functions. Using LC-MS/MS, the peptide sequence was predicted based on the *de novo* sequencing algorithm and P1-peptide (VDLPTCKGF) was identified. The peptide was modified by shortening C-terminal residue one at a time until three residues remained, and chemically synthesized all modified peptides by SPPS. Seven-synthetic peptides were purified and molecularly evaluated by HPLC and LC-MS. The antioxidant capacity of these peptides was evaluated, while also the intracellular-ROS was detected to validate the antioxidant activity. This study showed that 3 of 7 synthetic peptides had *in vitro* antioxidant activity and suppressed intracellular-ROS. Among all modified peptides, VDLPTC exhibited the highest antioxidant capacity and revealed intracellular-ROS suppression. This evidence reflects their radical-scavenging activities and provides the rationale for the development of novel functional food ingredients.

KEYWORDS

Antioxidant peptide; *Ganoderma lucidum*; functional ingredient; peptides; LC-MS/MS

1. Introduction

Oxidative stress is an imbalance between the reactive oxygen species (ROS) and antioxidant defenses. Generally, ROS play important roles in human physiological and pathophysiological processes. However, at high concentrations, ROS promptly react with biological components within cells including proteins, lipids, and genetic material (DNA), often inducing damage or functional alterations. At the cellular level, ROS control cellular machinery including cell growth, apoptosis, and cell signaling. Excessive ROS can induce oxidative stress followed by various diseases such as Alzheimer's, Parkinson's, and cardiovascular diseases (Dhalla, Temsah, and Netticadan 2000; Qin et al. 2006; Zhang, Dawson, and Dawson 2000). Moreover, oxidative stress is directly associated with the aging process and programmed cell death in biological systems (Krause 2007). The high levels of oxidative stress affect human health by causing illnesses and

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diseases, public health researchers have shown that consumption of foods rich in antioxidants is beneficial as it potentially decreases ROS-related diseases (Wannamethee et al. 2006).

Lingzhi, also known as *Ganoderma lucidum*, is an edible fungus which has a variety of potential health benefits. The specific bitter taste of *G. lucidum* is utilized as a functional ingredient to improve the sensory characteristics of foods and also to increase the biological activity of alcoholic beverages, including promotion of antioxidative benefits and delaying of the aging process (Veljović et al. 2019). Additionally, bioactive compounds extracted from *G. lucidum* including triterpenoids, polysaccharides, sterols, steroids, fatty acids, and proteins/peptides yield various health benefits such as tumor suppression, pathogenic bacterial inhibition, and promotion of antioxidative reactions (Batra, Sharma, and Khajuria 2013). Therefore the novel potent antioxidants derived benefits from Lingzhi have promising potential as functional food ingredients.

Protein from food sources contains a great number of bioactive peptides, while the proteins in their native form by itself show lack of bioactivity. As shown in a previous study, fragmentation of food proteins by enzymatic hydrolysis into smaller sized proteins or peptides (protein hydrolysate) result in specific health benefit functionalities. This includes antithrombotic peptides (Leu-Ser-Phe-Met-Ala-Ile-Pro-Pro-Lys) which was a partial sequence obtained from milk proteins (Fiat et al. 1993) and antioxidant peptides which was a partial sequence obtained from ovotransferrin proteins (Shen et al. 2010). Moreover, the tripeptide (Trp-Asn-Ile) which was fragmented from ovotransferin exhibits higher antioxidant capacities than the ovotransferrin protein (Shen et al. 2010).

There are limited studies about antioxidant peptides from *G. lucidum* as a source of functional food ingredients. Hence, in this study we investigated enzymatic hydrolyzed proteins derived from *G. lucidum* and explored its potential benefit as a source of antioxidative peptides. We synthesized the protein hydrolysate to resemble that found in the digestive system in order to identify the novel antioxidant peptides. The potential antioxidant properties of specific peptide sequences can be potentially enhanced, and therefore its economic value as a functional food ingredient in the food industry was explored.

2. Materials and methods

2.1. Materials

The materials and chemicals used in this research are as follows: Raw material, Lingzhi was obtained from agricultural farm located in Yala, Thailnad (July 2019), Pepsin from porcine source, Trysin from bovine pancreas, Bovine serum albumin (BSA), 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH),

2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS), LPS (originating from *Escherichia coli* 055:B5) were purchased from Sigma Co. (St. Louis, MO, USA). UV-Vis spectroscopy used in this study was Synergy H1 microplate reader (BioTek, HT, USA). Sep-Pak C-18 cartridges SPE was purchased from Waters Corporation (Milford, MA. USA), Ultra-filtration centrifugal tubes (Vivaspin 20, MWCO; 3000 Da) were from GE Healthcare Bio-Sciences AB (Uppsala, Sweden) ROS assay kits were DCFDA/H_2DCFDA – Cellular ROS Assay Kit (ab113851) purchased from Abcam. All other chemicals used in the experiments were of analytical grade. LC-MS/MS for peptide identifaction was Impact II (Bruker Daltonics Ltd., Germany). All solvents for LC-MS including LC-MS waters, Acetonitrile (ultra LC-MS) were purchased from J.T. Baker (Fisher Scientific, Loughborough, UK).

2.2. Methods

2.2.1. Identification antioxidant peptide from G. lucidum by de novo sequencing

The antioxidant peptide was obtained from G. *lucidum* protein hydrolysate. The dried G. *lucidum* was extracted by modified Pressurized Hot Water Extraction (PHWE) method (Sereewatthanawut et al. 2008). Briefly, the 100 g. of G. *lucidum* was mixed with 400 mL of water and put into autoclave with standard conditions (121°C, 15 psi. 15 minutes). The extracted G. *lucidum* was enzymatically hydrolyzed with Pepsin (1:20) and Trypsin (1:100). Then, the hydrolyzed protein was centrifuged at 10,000 g for 30 min at 8°C. The supernatant was fractionated through Vivaspin-20, with molecular weights cut off (MWCO) of 3000 Dalton (Da). A total of 3 mg of peptide was loaded on equilibrated Sep-Pak C_{18} column and the peptide was eluted through 40–35% Acetonitrile/Water. The supernatant was concentrated by rotatory evaporation. The dried hydrolysate was reconstituted in 0.1% formic acid and subjected to tandem mass spectroscopy. LC-MS/MS separated the hydrolysate by a Linear gradient of 5–60% B in 60 min at constant flow rate 0.3 µL/min (A: 0.1% formic acid/water, B: 0.1% formic acid/80% acetonitrile (ACN)). Mass spectra of the hydrolysate were fully acquired using a data-dependent acquisition mode (DDA) and collected at +2, +3 and +4 charge state. A *m/z* range from 400 to 1200 was observed. The raw mass spectra were analyzed by PeakX studio 10.0 program. Further *de novo* peptide sequencing of the highest peptide ion intensity was performed with default parameters. LC-MS run was analyzed with no specific digestion enzyme. Mass error tolerance for MS and MS/MS was 120 ppm and 0.05 Da, respectively. CID-Fragmentation series (a, b, c, y, z, b-H_2O, y-H_2O, and y-NH_3) which emphasized on b and y-ion series were used to construct peptide sequence (Ma et al. 2003). The acceptable *de novo* peptide sequences were achieved by filtering average local confidence (ALC) to ≥85%.

2.2.2. Peptide synthesis by solid phase peptide synthesis (SPPS) approach

Synthetic peptides used in this study were generated by using solid-phase peptide synthesis (SPPS) following standard protocol (Coin, Beyermann, and Bienert 2007). Briefly, the synthetic peptide was established stepwise on solid support, Rink amide resin AM (100–200 mesh). The resin was incubated in N-methylpyrrolidinone (NMP) for 6 h. The Fmoc-protection group removal was carried out through treatment with 20% of piperidine in DMF. The amino acid with N-protection group as building blocks was used including Fmoc-Val -OH, Fmoc-Asp(OtBu)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Thr(tBu)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Lys-OH, Fmoc-Gly-OH, and Fmoc-Phe-OH. The amino acid residues were mixed with coupling reagent with HATU as coupling reagent. Final synthetic peptides were deprotected and cleaved from the resin by cleavage cocktail containing trifluoroacetic acid (TFA)/ethanedithiol (EDT)/Triisopropylsilane (TIPS)/water = 93:2.5:2.5:2. The synthetic peptide was precipitated with cold diethyl ether in ratio of 1:10. The crude peptide was reconstituted in 0.1% formic acid/water and subjected to RP-HPLC and LC-MS to evaluate peptide purity and observed peptide mass. The RP-HPLC analysis was performed on an Inertsil ODS-3 (4.6 x 250 mm) analytical column with a linear gradient applied from 5% to 95% solvent B (0.05% trifluoroacetic in 100% acetonitrile) and solvent A (0.065% trifluoroacetic in 100% water) with a gradient time of 40 min. The flow rate was 1.0 mL/min at room temperature. Mass measurement error calculation of synthetic peptides was conducted by following equation (Brenton and Godfrey 2010).

$$\text{Mass measurement error}(\%) = \Delta\text{mass}/(\text{Theoeical mass}) \times 100 \quad (1)$$

Where, Δmass defined as different value of observed mass from LC-MS and theoretical mass.

2.2.3. In vitro radical scavenging assay of the peptides by DPPH, ABTS, and FRAP assays

For DPPH scavenging activity, 10 µL of the peptides (1 µg/µL) was mixed with 200 µL of DPPH solution (0.2 mM in methanol). The control sample is composed of 20 µL of methanol and 200 µL of DPPH (0.2 mM). The DPPH scavenging activity of the samples was expressed as the Ascorbic acid Equivalent Antioxidant Capacity (AsEAC) and percentage of inhibition by Equation 2. For ABTS scavenging activity, ABTS radical solution (7 mM ABTS stock solution with 2.45 mM potassium persulfate) was diluted in 5 mM PBS pH 7.4, to an absorbance of 0.50 at 734 nm before performing the assay. T volume of 10 µL of the peptides (1 µg/µL) was mixed with 200 µL of ABTS solution. The reaction was incubated at room temperature in the dark for 10 min. Absorbance of the mixture was evaluated at 734 nm using a UV-Vis spectrophotometer. The ABTS scavenging activity of the

samples was expressed as the Gallic Equivalent Antioxidant Capacity (GaEAC) and percentage of inhibition was calculated by Equation 2. For FRAP scavenging activity, the assay was performed following previous method (Benzie and Strain 1996) with minor modifications. Briefly, the working solution (10 mM TPTZ/20 mM $FeCl_3$ in 300 mM sodium acetate) was freshly prepared. A volume of 10 µL of the peptides (1 µg/µL) was mixed in 200 µL of working solution. The reaction was incubated at room temperature in the dark for 30 min. Absorbance of the mixture was evaluated at 593 nm using a UV-Vis spectrophotometer. The FRAP scavenging activity of the samples was expressed as $FeSO_4$ molar equivalents. The relative antioxidant capacity (DPPH, ABTS, and FRAP) was calculated by following equation.

$$\%\text{inhibition} = \frac{\left(Abs_{sample} - Abs_{blank}\right)}{Abs_{blank}} \times 100 \quad (2)$$

where Abs_{sample} is the absorbance of the reaction with the test sample and Abs_{blank} denotes the absorbance of the reaction without the test sample.

2.2.4. Cell-based intracellular ROS assay

Cercopithecus aethiops kidney normal cell lines (Vero ATCC ® CCL-81) were used to explore the antioxidant effect when treated with the peptides. The cells were cultured in DMEM containing 10% FBS and 100 IU/mL of Penicillin in humidified atmosphere containing 5% CO_2 at 37°C. Briefly, the cells were seeded at a density of 2×10^4 cells per well in a 96-well plate. The cells were treated with 1 µg/mL of LPS for 6 h to induce oxidative stress, then, the peptides were added into the cells (final concentration = 100 µg/mL). Cellular ROS Assay Kit was used to determine ROS within the cells. Procedures were followed according to the manufacturer's instructions. Briefly, stock ROS staining solution was prepared by adding 40 µL of DMSO to ROS Dye and the working ROS staining solution was prepared by using 4 uL of stock-staining solution which was freshly diluted with 2 mL of assay buffer. Intracellular ROS was detected by adding 100 uL of working ROS staining solution into the cells for 1 h. The ROS was detected by fluorescence intensities at 535 nm ($\lambda_{excited}$ at 485 nm and $\lambda_{emitted}$ at 535 nm) by UV-Vis spectroscopy. The experiment was performed in triplicate (n = 3). The intracellular ROS was expressed as %relative ROS abundance normalized to the fluorescence of the negative control (untreated cells; buffer solution) by the following equation.

$$\text{Relative ROS abundance}(\%) = \frac{\left(Abs_{sample} - Abs_{control}\right)}{Abs_{control}} \times 100 \quad (3)$$

where Abs_{sample} is the absorbance of the reaction with the test sample and Abs_{blank} denotes the absorbance of the reaction without the test sample (buffer in each assay was added instead of the test sample).

2.2.5. Statistical analysis

All experiments were carried out with at least three independent replicates (n = 3), and all data were expressed as means ± standard deviation. The significance in differences was determined by Duncan's multiple range test ($p < .05$).

3. Results and discussion

3.1. Purification of antioxidant peptide from G. lucidum hydrolysate

The protein content was extracted using a modified Pressurized hot water extraction (PHWE) method, yielding 2.01 ± 0.04 mg of protein per 100 mg of dried *G. lucidum*. The protein content after hydrolysis by pepsin and trypsin was 2.76 ± 0.08 mg per 100 mg of dried Lingzhi, increasing 1.37-fold after the enzymatic process. Although the protein content of *G. lucidum* was previously reported to be approximately 7–8 mg per 100 mg of its dry weight by using AOAC protein determination, the method involved cationic groups of basic amino acids, which may affect the accuracy of the protein concentration (Mau, Lin, and Chen 2001). Therefore, in this study we used the Bradford protein assay which utilizes a different protein determination mechanism with Bovine Serum Albumin as the reference protein (Compton and Jones 1985). In addition, rigid textural structure of *G. lucidum* may trap protein inside its structure. The protein content in the solution may be lower than the protein content in *G. lucidum.*

The chromatography methods used in this study are also mostly used for protein hydrolysates preparation because of the simple, economical, and robust approach (Insuasty Cepeda et al. 2019). Numerous functional peptides are found which contain less than 20 amino acid residues (Pihlanto-Leppälä 2000). Therefore, the lower 3-kDa molecular weight cutoff fractions of peptides were assumed to have the greatest potential bioactivity (Pihlanto-Leppälä 2000). The hydrolysate after enzymatic hydrolysis was dissolved in water. The dissolved hydrolyzate was promptly loaded onto the RP-stationary phase, and the non-retained impurities such as salts from HCl, NaOH, and trace metals which were added during the enzymatic digestion were eluted out by washing with water. The bioactive functional peptide of interest was then eluted by washing with a 40–35% Acetonitrile/Water solution. The eluted fractions were used for peptide identification by LC-MS/MS.

3.2. Antioxidant peptide identification by using a de novo sequencing algorithm

A *de novo* sequencing algorithm was used to predict the sequence originating from parent ion peak. The peak parent peptide ion $(M + 2H)^{2+}$ was 490.2496. The peptide sequence was fragmented by collision-induced dissociation into daughter peptide ions including the y-ion series (y_2 = 112.058, y_4 = 227.610, y_5 = 278.133, y_6 = 326.660, y_7 = 383.202, and y_8 = 440.715) and b-ion series (b_2 = 108.055, b_3 = 164.597, b_4 = 213.123, b_5 = 263.647, and b_8 = 407.710) (Fig. 1). The peptide sequence was assigned to the MS/MS spectrum based on the difference of their mass values for a series of successive peptide b- and y-ion series. The sequence was identified as Valine-Aspartic acid-Leucine-Proline-Threonine-Cysteine-Lysine-Glycine-Phenylalanine (VDLPTCKGF) which contains nine types of amino acid residues. This antioxidant peptide identification is in agreement with previous reports that most short peptides containing 2–10 amino acid residues exhibit antioxidant activities in contrast to native proteins or high-molecular-weight polypeptides (Li et al. 2007). Spectrum-based sequences obtained by *de novo* sequencing account for more than 85% when combining both b- and y-ions as illustrated in Figure 1. However, this sequence was obtained by matching MS and MS/MS spectra alone. Database-assisted sequence matching for *de novo* peptide sequencing is more reliable and can be used for species with a fully decoded genome database (Gorshkov et al. 2016). However, *G. lucidum* not being a model organism, it cannot be matched with the database. Therefore, alternative strategies are needed to predict the best matches with the MS/MS spectrum.

3.3. Solid phase peptide synthesis (SPPS)

The peptide sequence VDLPTCKGF (P1), obtained from LC-MS/MS, along with its fragments VDLPTCKG (P1C-1), VDLPTCK (P1C-2), VDLPTC (P1C-3), VDLPT (P1C-4), VDLP (P1C-5), and VDL (P1C-6) were chemically

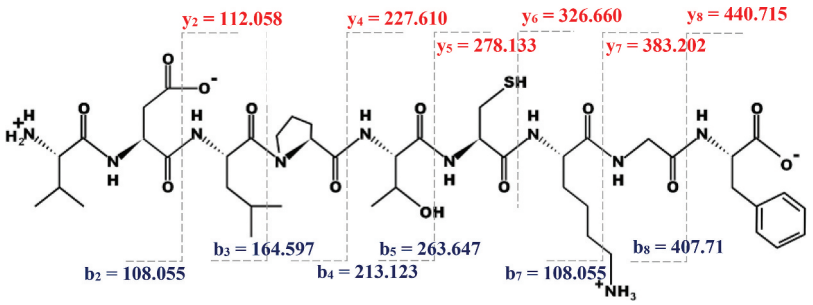

Figure 1. Fragmentation pattern of peptides with b- and -y ion intermixed spectrum, used to construct the peptide sequence VDLPTCKGF. The dashed vertical line represents the fragmentation sites. Fragments extended from amino termini are depicted as b-ions while from the carbonyl termini are depicted as y-ions.

synthesized by SPPS. Peptide purity and mass deviation are two crucial quality control parameters used to determine whether the peptides were successfully synthesized. Purity analysis and molecular mass measurement of the crude synthesized peptides were determined by using HPLC and mass spectrometry (LC-MS), respectively, as shown in Table 1.

A 220 nm portion of the HPLC chromatogram was used in the peptide purity calculation, which is defined as the ratio of the integrated peak area corresponding to the peptide of interest to the total area of all peaks. The purity of the synthesized peptides was found to be in the range of 92.1–96.9%. The peak position of the LC-MS spectrum was used to confirm the molecular mass of the peptide of interest. Molecular mass deviation of the synthesized peptides was 0.016–0.083%. The structures of the synthetic peptide ions measured by the LC-MS chromatograms are illustrated in Figure 2.

The $[M + 2H]^{2+}$ molecular ion peaks at 490.5, 416.9, and 388.4 correspond to the structures shown in Figure 2A–C respectively, while the $[M + H]^{1+}$ peaks at 647.3, 544.3, 443.2, and 346.1 correspond to structures shown in Figure 2D–G respectively, indicating that the peptides had been successfully synthesized.

Although the synthesized peptides exhibited 92.1–96.9% purity, in general, synthetic peptides of over 80% purity are regarded as appropriate for antioxidant assays, enzyme kinetic reactions, and semi-quantitative applications (Yao et al. 2019). Considering the quality control, purity, and molecular mass, we can infer that the peptides were successfully synthesized.

3.4. *In vitro radical scavenging assay of the peptides by DPPH, ABTS, and FRAP*

The antioxidant capacity of 10 µg of peptides was tested by using three different methods: DPPH, ABTS, and FRAP-based antioxidant assays. The free radical scavenging activity of antioxidants was expressed in terms of relative antioxidant and equivalent antioxidant capacities as shown in Table 2.

P1, P1C-1, P1C-2, and P1C-3 synthetic peptides exhibited antioxidant abilities. Relative antioxidant activity abilities were measured by using three assays; DPPH, ABTS, and FRAP. The correlation between the relative and equivalent antioxidant activity indicates that they can be used to validate the

Table 1. Purity and molecular mass of peptides synthesized by using the SPPS method.

Peptide code; Sequence	Theo. mass[a] (Da)	Obser. Mass[b] (Da)	Mass error[c] (%)	Purity (%)
P1; VDLPTCKGF	979.16	979.00	0.016	95.6
P1C-1; VDLPTCKG	831.98	831.80	0.021	93.3
P1C-2; VDLPTCK	774.93	774.80	0.017	96.9
P1C-3; VDLPTC	646.76	646.30	0.071	92.6
P1C-4; VDLPT	543.61	543.30	0.057	92.1
P1C-5; VDLP	442.51	442.20	0.070	95.9
P1C-6; VDL	345.39	345.10	0.083	95.5

[a]Molecular mass which calculated monoisotopic masses by atom composition.
[b]Molecular mass which observed monoisotopic masses by LC-MS.
[c]Percentage of mass measurement error of the theoretical masses and the observed masses.

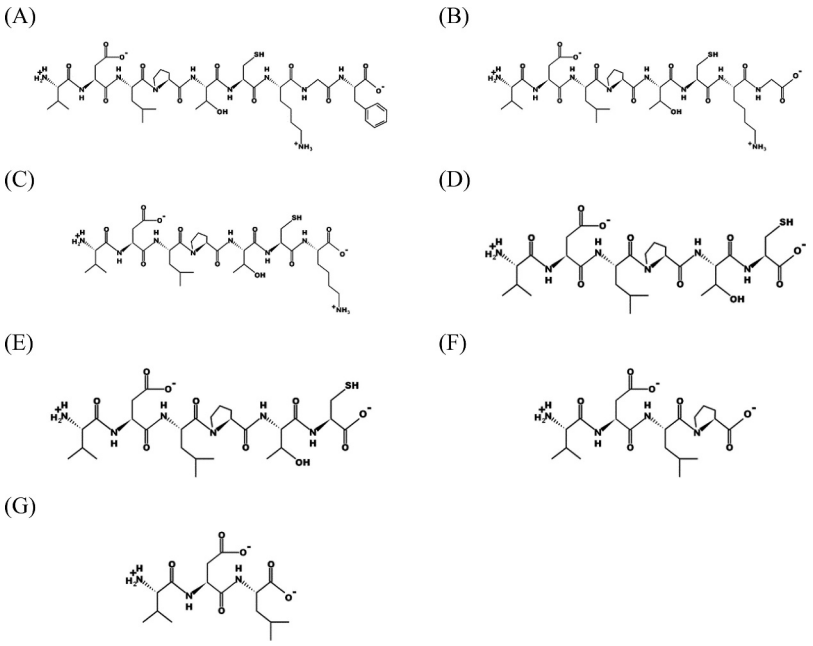

Figure 2. Synthetic peptide structures of (A) P1, (B) P1C-1, (C) P1C-2, (D) P1C-3, (E) P1C-4, (F) P1C-5, and (G) P1C-6.

antioxidant abilities of these peptides in comparison with well-known standards including ascorbic acid, gallic acid, and $FeSO_4$ (Abramovič et al. 2018). The relative antioxidant activity of the P1 peptide was shown to be 25.458% in DPPH, 72.612% in ABTS, and 9.615% in FRAP-based assays. The antioxidant activity of the P1 peptide, which is linearly correlated with the 99% confidence R-square ($R^2 > 0.99$) interval for all assays, was found to be 0.118 (equivalent to 1 mg of ascorbic acid), 0.257 (equivalent to 1 mg of gallic acid), and equivalent to 0.274 mM $FeSO_4$). The amino acid residues of P1 shortened at the C-terminal region significantly affects antioxidant activity as shown in Table 2. Interestingly, P1C-3 exhibited the strongest antioxidant activity compared to the others. The relative antioxidant activity of P1C-3 peptide is higher than that of P1 by 2.43-, 1.22-, 117.09-fold in DPPH, ABTS, and FRAP assays, respectively.

Because the radical scavenging activity was evaluated through the use of DPPH and ABTS, there were differences stemming from solubility issues of radicals and their dispersion in each solvent. Typically, DPPH can be completely dissolved in alcoholic solvents and is capable of accepting electrons as well as hydrogen ions whereas ABTS can be completely solubilized in water and other organic media. Hence, the antioxidant activity of DPPH and ABTS-based assays can be measured for both hydrophilic and lipophilic compounds (Shalaby and Shanab 2013). FRAP is another means to determine antioxidant activity by monitoring ferric ion reduction to ferrous ions in the presence of an antioxidant or an electron-

Table 2. Antioxidant capacity determination of synthetic peptides by DPPH, ABTS, and FRAP assays.

	Relative Antioxidant Capacity			Equivalent Antioxidant Capacity*		
Code	DPPH (%)	ABTS (%)	FRAP (%)	Ascorbic acid (mg)	Gallic acid (mg)	$FeSO_4$ (mM)
P1	25.46 ± 0.14[a]	72.61 ± 0.43[a]	9.62 ± 0.24[a]	0.12 ± 0.021	0.26 ± 0.008	0.27 ± 0.011
P1C-1	16.03 ± 0.37[b]	66.02 ± 0.49[b]	6.04 ± 0.13[b]	0.09 ± 0.004	0.23 ± 0.013	0.19 ± 0.006
P1C-2	17.57 ± 0.05 [c]	68.98 ± 0.31 [c]	10.44 ± 0.26 [c]	0.12 ± 0.016	0.24 ± 0.019	0.26 ± 0.014
P1C-3	61.89 ± 0.12[d]	88.67 ± 0.17[d]	707.69 ± 0.19[d]	0.33 ± 0.019	0.32 ± 0.007	11.95 ± 0.019
P1C-4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P1C-5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
P1C-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

* Results are expressed as mean values ± standard deviation (SD) (n = 3). Values in the relative antioxidant capacity column followed by a different letter superscript (a-d) are significantly different ($p < 0.05$). The n.d. stands for not detected.

donating reductant (Huang, Ou, and Prior 2005). The P1, P1C-1, P1C-2, and P1C-3 peptides have electron-donating capabilities which reduce ferric to ferrous ions, which are related to the radical scavenging abilities in DPPH and ABTS.

Our findings revealed that the synthetic peptides P1C-1 and P1C-2 have slightly lower free radical scavenging activity in aqueous solutions. In contrast, P1C-3 shows higher radical scavenging activity in ABTS-based assay than in alcoholic solvents. This indicates that these peptides have specific conformation in aqueous solutions which facilitate electron as well as hydrogen accepting abilities to clear out free radical molecules. Moreover, the P1C-3 peptide being 640 Da in size may influence its antioxidation properties of the molecule. Hydrophobic amino acids and cysteine residues are crucial factors to enhance the antioxidant abilities of antioxidant peptides (Ren et al. 2008). Our findings show that loss of the cysteine-residue (P1C-4) at the C-terminal of peptides may reflect absence of antioxidant abilities.

3.5. Intracellular reactive oxygen species (ROS) estimation

A ROS detection kit was used to investigate ROS generation after exposure to synthetic peptides (P1, P1C-1, P1C-2, P1C-3, P1C-4, P1C-5, P1C-6, and P1C-7) in LPS-induced oxidative stress in Vero cells. The assay kit contains non-fluorescent 2′,7′-dichlorofluorescin diacetate which diffuses into the cells. The DCFDA molecule can be oxidized by hydroxyl, peroxyl, and other ROS within the cells. The fluorescent intensities at 535 nm can be directly related to the ROS in the cells.

Scavenging activities evaluated by DPPH, ABTS, and FRAP have generally been used to access antioxidant properties. However, these assays are used only to evaluate *in vitro* experiments, which may not be completely realistic. Therefore, an intracellular ROS estimation by using a cell-based assay was conducted in order to explore the antioxidant abilities closer to the realistic situation in living organisms.

The intracellular ROS determination revealed that cells treated with synthetic peptides (P1, P1C-1, P1C-2, P1C-3, P1C-4, P1C-5, P1C-6, and P1C-7) had changes in ROS content as illustrated in Figure 3.

The presence of synthetic peptides P1, P1C-1, P1C-2, and P1C-3 inhibited ROS generation of LPS-induced oxidative stress in Vero cells. However, P1C-4, P1C-5, P1C-6, and P1C-7 did not have any effect on ROS generation. The P1C-3 peptide exhibited the greatest ROS suppression at 19.59 ± 0.63% compared to the control experiments (buffer solution).

Therefore cell-based assays confirmed the radical scavenging activity of P1C-3 peptides. The shortening peptide approach lowers production cost and time, because it uses less amino acids and enables faster synthesis times, respectively. Hence, P1C-3 may have potential applications in the production of functional food ingredients.

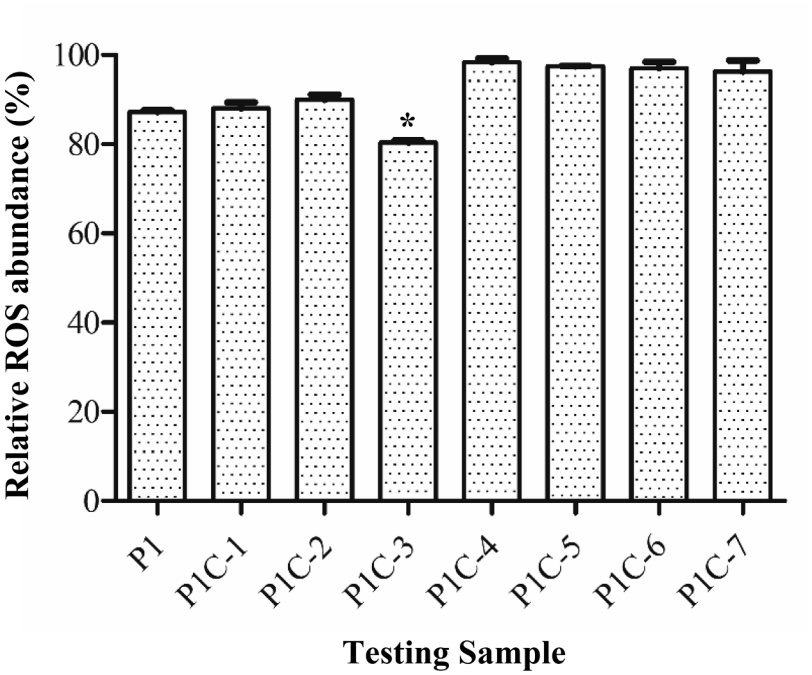

Figure 3. Intracellular ROS determination in LPS-induced oxidative stress in Vero cells. Relative differences of intracellular ROS depend on the type of synthetic peptide. The P1C-3 treatment group revealed the lowest relative intracellular ROS (p-value<0.05). (*; significant difference (p-value < 0.05).

4. Conclusions

By using a *de novo* algorithm and by utilizing LC-MS/MS, we successfully identified a novel antioxidant peptide, VDLPTCKGF, from *G. lucidum* protein hydrolysate. Modification of antioxidant peptides by shortening amino acids at the C-terminal leaves the remaining VDLPTC with enhanced antioxidant capacity and reduced intracellular ROS in comparison to the original larger peptide. This modified peptide could become a valuable bioactive peptide in the food industry as a novel functional food ingredient with antioxidant properties.

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References

Abramovič, H., B. Grobin, N. Poklar Ulrih, and B. Cigić. 2018. Relevance and standardization of *In Vitro* antioxidant assays: ABTS, DPPH, and folin–ciocalteu. *J. Chem.* 2018:4608405. doi:10.1155/2018/4608405.

Batra, P., A. K. Sharma, and R. Khajuria. 2013. Probing lingzhi or reishi medicinal mushroom *Ganoderma lucidum* (Higher Basidiomycetes): A bitter mushroom with amazing health benefits. *Int. J Med Mushrooms*. 15 (2):127–143.

Benzie, I. F., and J. J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* 239 (1):70–76. doi:10.1006/abio.1996.0292.

Brenton, A. G., and A. R. Godfrey. 2010. Accurate mass measurement: Terminology and treatment of data. *J. Am. Soc. Mass Spectrom.* 21 (11):1821–1835.

Coin, I., M. Beyermann, and M. Bienert. 2007. Solid-phase peptide synthesis: From standard procedures to the synthesis of difficult sequences. *Nat Protoc* 2 (12):3247–3256. doi:10.1038/nprot.2007.454.

Compton, S. J., and C. G. Jones. 1985. Mechanism of dye response and interference in the Bradford protein assay. *Anal. Biochem.* 151 (2):369–374. doi:10.1016/0003-2697(85)90190-3.

Dhalla, N. S., R. M. Temsah, and T. Netticadan. 2000. Role of oxidative stress in cardiovascular diseases. *J. Hypertens.* 18 (6):655–673. doi:10.1097/00004872-200018060-00002.

Fiat, A. M., D. Migliore-Samour, P. Jolles, L. Drouet, C. Bal Dit Sollier, and J. Caen. 1993. Biologically active peptides from milk proteins with emphasis on two examples concerning antithrombotic and immunomodulating activities. *J. Dairy Sci.* 76 (1):301–310. doi:10.3168/jds.S0022-0302(93)77351-8.

Gorshkov, V., S. Y. K. Hotta, T. Verano-Braga, and F. Kjeldsen. 2016. Peptide *de novo* sequencing of mixture tandem mass spectra. *PROTEOMICS* 16 (18):2470–2479. doi:10.1002/pmic.201500549.

Huang, D., B. Ou, and R. L. Prior. 2005. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* 53 (6):1841–1856. doi:10.1021/jf030723c.

Insuasty Cepeda, D. S., H. M. Pineda Castaneda, A. V. Rodriguez Mayor, J. E. Garcia Castaneda, M. Maldonado Villamil, R. Fierro Medina, and Z. J. Rivera Monroy. 2019. Synthetic peptide purification via solid-phase extraction with gradient elution: A simple, economical, fast, and efficient methodology. *Molecules* 24:7. doi:10.3390/molecules24071215.

Krause, K. H. 2007. Aging: A revisited theory based on free radicals generated by NOX family NADPH oxidases. *Exp. Gerontol.* 42 (4):256–262. doi:10.1016/j.exger.2006.10.011.

Li, B., F. Chen, X. Wang, B. Ji, and Y. Wu. 2007. Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and electrospray ionization–mass spectrometry. *Food Chem.* 102 (4):1135–1143. doi:10.1016/j.foodchem.2006.07.002.

Ma, B., K. Zhang, C. Hendrie, C. Liang, M. Li, A. Doherty-Kirby, and G. Lajoie. 2003. PEAKS: Powerful software for peptide de novo sequencing by tandem mass spectrometry. *Rapid Commun. Mass Spectrom.* 17 (20):2337–2342. doi:10.1002/rcm.1196.

Mau, J.-L., H.-C. Lin, and -C.-C. Chen. 2001. Non-volatile components of several medicinal mushrooms. *Food Res. Int.* 34 (6):521–526. doi:10.1016/S0963-9969(01)00067-9.

Pihlanto-Leppälä, A. 2000. Bioactive peptides derived from bovine whey proteins: Opioid and ace-inhibitory peptides. *Trends Food Sci. Tech.* 11 (9):347–356. doi:10.1016/S0924-2244(01)00003-6.

Qin, B., L. Cartier, M. Dubois-Dauphin, B. Li, L. Serrander, and K. H. Krause. 2006. A key role for the microglial NADPH oxidase in APP-dependent killing of neurons. *Neurobiol. Aging* 27 (11):1577–1587. doi:10.1016/j.neurobiolaging.2005.09.036.

Ren, J., M. Zhao, J. Shi, J. Wang, Y. Jiang, C. Cui, Y. Kakuda, and S. J. Xue. 2008. Purification and identification of antioxidant peptides from grass carp muscle hydrolysates by consecutive chromatography and electrospray ionization-mass spectrometry. *Food Chem.* 108 (2):727–736. doi:10.1016/j.foodchem.2007.11.010.

Sereewatthanawut, I., S. Prapintip, K. Watchiraruji, M. Goto, M. Sasaki, and A. Shotipruk. 2008. Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis. *Bioresour. Technol.* 99 (3):555–561. doi:10.1016/j.biortech.2006.12.030.

Shalaby, E. A., and S. M. M. Shanab. 2013. Comparison of DPPH and ABTS assays for determining antioxidant potential of water and methanol extracts of *Spirulina platensis*. *Indian J. Geo-Marine Sci.* 42:5.

Shen, S., B. Chahal, K. Majumder, S. J. You, and J. Wu. 2010. Identification of novel antioxidative peptides derived from a thermolytic hydrolysate of ovotransferrin by LC-MS/MS. *J. Agric. Food Chem.* 58 (13):7664–7672. doi:10.1021/jf101323y.

Veljović, S., Nikićević, N., Nikšić, M. 2019. Medicinal fungus *Ganoderma lucidum* as raw material for alcohol beverage production. *Alcoholic Beverages.*7:161-197. doi:10.1016/B978-0-12-815269-0.00006-4.

Wannamethee, S. G., G. D. Lowe, A. Rumley, K. R. Bruckdorfer, and P. H. Whincup. 2006. Associations of vitamin C status, fruit and vegetable intakes, and markers of inflammation and hemostasis. *Am. J. Clin. Nutr.* 83 (3):567–574. quiz 726-567. doi:10.1093/ajcn.83.3.567.

Yao, G-L., W. He, W. Y-G, J. Chen, H. X-W, and J. Yu. 2019. Purification of angiotensin-i-converting enzyme inhibitory peptides derived from *Camellia oleifera* abel seed meal hydrolysate. *J Food Qual* 2019:1–9.

Zhang, Y., V. L. Dawson, and T. M. Dawson. 2000. Oxidative stress and genetics in the pathogenesis of Parkinson's disease. *Neurobiol. Dis.* 7 (4):240–250. doi:10.1006/nbdi.2000.0319.