Antioxidant activities of the synthesized thiol-contained peptides derived from computer-aided pepsin hydrolysis of yam tuber storage protein, dioscorin

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ABSTRACT

Our previous report showed that yam dioscorin and its peptic hydrolysates exhibit radical scavenging activities; however, the functions of these peptic hydrolysates are still under investigation. In this study, the thiol-containing peptides derived from computer-aided simulation of pepsin hydrolysis of dioscorin, namely, KTCGNGME (diotide1), PPCSE (diotide2), CDDRVIRTPLT (diotide3), KTCGY (diotide4), and PPCTE (diotide5) were synthesized to compare their antioxidant activities with GSH and/or carnosine by examining hydroxyl radical scavenging activity by electron spin resonance spectrometry, anti-low-density lipoprotein peroxidation, anti-AAPH-induced hemolysis, and oxygen radical absorbance capacity activity. We found that while all the synthesized diotides showed antioxidant activity, diotide4 exhibited the highest levels. Moreover, all diotides (100 μM) showed protective effects against methyglyoxal-induced human umbilical vein endothelial cell death. These results suggest that thiol-containing diotides derived from dioscorin hydrolysis exhibit antioxidant activities and reveal the benefits of yam tuber as an antioxidant-rich food.

1. Introduction

Reactive oxygen species (ROS) and free radical-mediated reactions are associated with aging and various diseases such as cardiovascular diseases and neurodegenerative diseases (Ames, 1983; Gey, 1990). Many epidemiological studies indicate an association between people who have a diet rich in fresh fruits and vegetables and a decreased risk of cardiovascular disease (Gey, 1990). The problems associated with age-related diseases, such as neurodegenerative diseases (e.g., Alzheimer’s, Parkinson’s, and Huntington’s disease), have recently been emphasized, while, the intricate causes of the aging process are still a matter of extensive speculation, and many theories have been proposed as to what promotes aging. The free radical theory of aging would suggest that understanding the role of ROS is a prerequisite to understanding the aging process (Beckman & Ames, 1998). Several reports have focused on the antioxidant activities of extracts or constituents of fruits, vegetables, and plants, or herbal medicines such as wild

bitter gourd extracts (Lu, Chia, Liu, & Hou, 2012; Lu et al., 2012), anthocyanin (Espin, Soler-Rivas, Wichers, & Viguera-Garcia, 2000), and geraniin from Phyllanthus urinaria (Lin, Wang, Wu, & Hou, 2008).

Many peptides of animal or plant proteins released in vitro or in vivo are bioactive and have regulatory functions in humans beyond being involved in nutrition alone (Erdmann, Cheung, & Schröder, 2008; Fitzgerald, Gallagher, Tisdemir, & Hayes, 2011; Hartmann & Meisel, 2007), such as antioxidant activity, anti-inflammatory activity, and anti-atherosclerotic activity (Matsui et al., 2010). One such peptide, reduced glutathione (GSH), is a tripeptide which plays critical, protective, and physiological functions in cells (Sies, 1999). Carnosine, another type of peptide, is a dipeptide of β-alanyl-L-histidine, which is found in long-lived mammalian tissues at relatively high concentrations (up to 20 mM). It is also known to exhibit antioxidant activities (Kohen, Yamamoto, Cundy, & Ames, 1988) and delay aging in cultured cells (McFarland & Holliday, 1994).

Yams (Dioscorea spp., Dioscoreaceae) are an important tuber crop in Africa, Asia, and Middle and South America and are also a staple food in the Caribbean. Conlan et al. (1995) first reported 2 classes of cDNA clones encoding the major tuber storage proteins, dioscorin A and dioscorin B, in D. cayenensis. The protein coding re-
gions from the 2 classes have 84.1% similarity, and the deduced amino acid sequences are 69.6% similar with respect to each other. Yam dioscorin and its peptic hydrolysates have been reported to exhibit several biological activities in vitro and in vivo (Lu, Chia et al., 2012), including antioxidant activities (Hou et al., 2001; Liu, Liang, Cheng, Liu, & Hou, 2006). In this study, we used a computer-aided simulation of pepsin hydrolysis of yam dioscorin to generate hydrolysate peptides, among which five thiol-contained peptides (diotide1, diotide2, diotide3, diotide4, and diotide5) were then selected, synthesized and tested for antioxidant activity. In addition, we also evaluated the peptides’ protective effects against methylglyoxal (MGO)-induced human umbilical vein endothelial cell (HUVEC) death in comparison to GSH and/or carnosine. The results showed that thiol-containing diotides derived from dicoscorin hydrolysis exhibit antioxidant activities indicating that yams could be used to develop functional antioxidant-rich foods.

2. Materials and methods

2.1. Materials

2,2'-azo-bis(2-amidinopropane)dihydrochloride (AAPH), carnosine, copper (II) sulfate (penta hydrate), 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), ferrous sulfate, GSH, ethylenediaminetetraacetic acid (EDTA), human low-density lipoprotein (LDL), MGO, and sulfonohydamine B (SRB) were purchased from Sigma Chemical Co. (St. Louis, MO). Hydrogen peroxide (33%) was obtained from Wako Pure Chemical Industry (Osaka, Japan). The 5 peptides, Lys-Thr-Cys-Gly-Asn-Gly-Met-Glu (KTCGNGME, diotide1), Pro-Cys-Ser-Glu (PPCSE, diotide2), Cys-Asp-Arg-Val-Ile-Arg-Thr-Pro-Leu-Thr (CDVRIRPTLT, diotide3), Lys-Thr-Cys-Gly-Tyr (KTCGY, diotide4), and Pro-Pro-Cys-Thr-Glu (PPCTE, diotide5) used in this study were synthesized by Shanghai Hanhong Chemical Co., Ltd. (Shanghai, China). The purity of each peptide was determined to be greater than 95% by performing HPLC chromotography and mass spectroscopy.

2.2. Scavenging activity of diotides against hydroxyl radicals determined using electron spin resonance (ESR) spectrometry

The hydroxyl radical was generated by the Fenton reaction according to the methods described previously (Kohno, Masumizu, & Mori, 1995) and detected using an ESR spectrometer. The mixture contained 100 μM of each diotide (dissolved in distilled water), 5 mM 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and 0.05 mM ferrous sulfate. After mixing, the solution was transferred to an ESR quartz cell and placed on the cavity of the ESR spectrometer, before hydrogen peroxide was added to a final concentration of 0.25 mM in a total volume of 500 μl. Deionized water was used instead of the sample solution for blank experiments, and the same concentrations of carnosine were used as positive controls. After 40 s, the signal intensities of the DMPO-OH spin adduct were measured and expressed as the intensity relative to blank (%). The ESR spectra were recorded at ambient temperature (298 K) on a Bruker EMX-6/1 spectrometer equipped with SimFonia software version 10. The conditions of ESR spectrometry were as follows: center field, 345.4 ± 5.0 mT; microwave power, 8 mW (9.416 GHz); modulation amplitude, 5 G; modulation frequency, 100 kHz; time constant, 0.6 s; and scan time, 1.5 min.

2.3. Protective effects of diotides against Cu2+-induced LDL peroxidation

LDL was dialyzed against 10 mM phosphate buffer (pH 7.4) containing 0.05 M NaCl, overnight at 4 °C so as to remove the EDTA in the commercial preparation. Dialyzed LDL was then incubated at 37 °C in 10 mM phosphate buffer (pH 7.4) containing 10 μM CuSO4 and 50 μM of each diotide for 4 h. The peroxidation reaction was then arrested by adding 1 mM EDTA. GSH was used as the positive control and EDTA additions before copper was used as the blank. The protective effects of diotides against Cu2+-induced LDL peroxidation were measured by analyzing the absorbance at 234 nm every 30 min for determining conjugated diene formations (Abuja, 1999). These effects were also determined by checking the mobility on an 1.0% agarose gel after electrophoresis and staining with Sudan Black B (Yang, Cheng, Lin, Liu, & Hou, 2004). Mobility of the blank was assigned a value of 100%, and the results from all other samples were used to determine the relative mobility compared to the control.

2.4. Protective effects of diotides against AAPH-mediated hemolysis

The free-radical chain oxidation of rat red blood cells (RBC) was through AAPH-mediated hemolysis (Miki, Tamai, Mino, Yamamoto, & Niki, 1987). Rat blood was placed into heparinized tubes and centrifuged at 1000g for 10 min. After washing with 0.15 M NaCl thrice, the packed RBCs were harvested by centrifugation at 1000g for 10 min. Each diotide (50 μM or 100 μM) was mixed with 25 μl of the 20% RBC suspension (V/V, in 10 mM PBS) and 100 μl of 300 mM AAPH solution and incubated at 37 °C for 0, 1, 2, 2.5, 3, 3.25, 3.5, or 4 h with gentle shaking. Each mixture was then centrifuged at 1000g for 10 min, after which the supernatant was collected and its absorbance was measured at 536 nm. Deionized water was used instead of AAPH solution or the sample solution, as the blank or the control, respectively. GSH and carnosine were used for comparisons. The protective activity (%) of each diotide against AAPH-mediated hemolysis was calculated on the basis of the area under curve (AUC) as follows: (AUCcontrol − AUCsample) × 100.

2.5. Effects of diotides on oxygen radical absorbance capacity (ORAC)

The ORAC of each diotide was determined using the OxiSelect™ Assay Kit (STA-345, Cell Biolabs Inc., San Diego, CA), as per the manufacturer’s instructions, in which AAPH acts as a peroxyl radical initiator to quench fluorescein over time. The diotides present in the assay system block peroxyl radical-mediated fluorescein oxidation until the antioxidants in the sample are depleted. Each diotide at a concentration of 2, 5, or 10 μM was used to determine ORAC activity, and both GSH and carnosine were used for comparison. The area under curve (AUC) of each Trolox concentration (2.5, 5, 10, 20, 40, and 60 μM) was used to plot the standard curve for ORAC activity, and each diotide was calculated and expressed as Trolox equivalents (TE) (Huang, Ou, & Prior, 2005).

2.6. Protective effects of diotides against methylglyoxal (MGO)-induced human umbilical vein endothelial cell (HUVEC) death

The human umbilical vein endothelial cells (HUVEC, BCRC H-UV001) were purchased from the Bioresource Collection and Research Center (BCRC) of the Food Industry Research and Development Institute (Hsinchu, Taiwan) and cultured in M199 medium containing 10% fetal bovine serum, 25 U/ml heparin, and penicillin-streptomycin solution (10,000 units/ml of penicillin and 10 mg/ml of streptomycin). Briefly, the cells were seeded onto a 96-well microtitr plate (1 × 104 cells/well), and 50 μM or 100 μM of each diotide was added and incubated at 37 °C in a humidified atmosphere with 5% CO2 for 24 h. Cells were then washed with PBS, and then, a medium with or without 550 μM of MGO was added and incubated for another 24 h at 37 °C. The SRB dyes were used for cell viability assays. After washing, the cells were fixed with 10% trichloracetic acid at 4 °C for 1 h, washed with distilled water,
and then stained with 0.4% SRB (dissolved in 1% acetic acid) for 30 min. After being washed 4 times with 1% acetic acid to remove the unbound dye in each well, and then air-dried, the bound dyes were dissolved in 500 µl of a 10 mM Tris–HCl buffer (pH 7.9). The medium with and without MGO were the blank and the control (MGO group), respectively. The absorbance was measured at 540 nm using an ELISA reader (Sunrise, TECAN, Männedorf, Switzerland) and is expressed in percentages relative to the blank (%).

2.7. Statistical analyses

The values are presented as mean ± SD and were analyzed using one-way ANOVA, followed by a post hoc Tukey’s test for multiple mean comparisons. A P value of <0.05 was considered statistically significant. The statistical analysis was performed using GraphPad Prism Software 5.0.

3. Results

3.1. Scavenging activity of diotides against hydroxyl radicals determined by electron spin resonance (ESR) spectrometry

Dioscorin A (UniProtKB/Trembl: Q9M519) and dioscorin B (UniProtKB/Trembl: Q9M519) were hydrolyzed by computer-aided simulation of pepsin hydrolysisa of yam tuber storage protein, dioscorin.b,c

Five of the thiol-containing diotides obtained (Table 1) were then synthesized and tested using a series of antioxidant activity assays.

The hydroxyl radical, which is generated by Fe²⁺-catalyzed formation, is trapped by DMPO to form the DMPO–OH adducts. The intensities of the DMPO–OH spin signal (a 1:2:2:1 quartet spin signal) obtained using ESR spectrometry were used to evaluate the scavenging activities of 100 µM diotides (diotide1 to diotide5, respectively; Fig. 1B to Fig. 1F) and compared to carnosine (diotide4, and diotide5 against hydroxyl radicals at 100 µM were 54.44%, 18.84%, 13.28%, 66.20%, and 41.54%, respectively, compared to 2.64% of carnosine. Our results indicate that the intensity of the DMPO–OH adduct is decreased by adding diotides and show significantly different results compared to carnosine (diotide1, diotide4, and diotide5, P < 0.001; diotide2, P < 0.01; diotide3, P < 0.05) (Fig. 1H). All the diotides showed higher hydroxyl radical scavenging capacities than carnosine, even at similar concentrations. However, the diotide scavenging properties were 100-folds Ellless than that of GSH (1 µM showed 78.93% scavenging activities against hydroxyl radicals, data not shown).

3.2. Protective effects of diotides against Cu²⁺-induced LDL peroxidation

The protective effect of diotides against LDL peroxidation was investigated using conjugated diene formation (Fig. 2A) or relative mobility of oxidized LDL on agarose gel (Fig. 2B and C). From the results (Fig. 2A), we concluded that the conjugated diene formation increased (A_{234nm}) with the increasing time of the control; however, except for diotide2, the conjugated diene formation was retarded after 50 µM of the diotides (diotide1, 3 and 5) were added. The retardation order for conjugated diene formation was GSH > diotide1 > diotide3 > diotide5 > diotide4 > diotide2. On the other hand, from the gel electrophoresis results shown in Fig. 2B, we concluded that the oxidized LDL in the control (lane C) mobilizes faster than non-oxidized LDL in the blank (lane B), and the addition of diotides can reduce the mobility more closely to that of the blank, which means that it slows the degree of LDL peroxidation. The quantitative data of the relative mobility in each diotide addition also showed that the significant difference to the control (diotide1 and diotide3, P < 0.001; diotide4 and diotide5, P < 0.01; diotide2, P < 0.05) (Fig. 2C) and the protective capacities of diotide1 and diotide3 were better than those of GSH against LDL peroxidation.

3.3. Protective effects of diotides against AAPH-mediated hemolysis

The free-radical chain oxidation of rat red blood cells (RBC) was performed using AAPH radicals (Miki et al., 1987), and 50 or 100 µM of each diotide. GSH and carnosine were used for comparisons. Fig. 3A shows the representative results of protective activity of 100 µM diotide against AAPH-mediated hemolysis. It was found that the hemolysis in rat RBCs dramatically increased (expressed as A_{536nm} after 4-h reactions in the presence of AAPH radicals (the blank circles represent the control groups), and little or no hemolysis was observed in the absence of AAPH radicals during reactions (as blank groups; filled circle). The delayed increases at 536 nm induced stronger protective activity against AAPH-mediated hemolysis (Fig. 3A). The protective activity (%) of each diotide at 50 or 100 µM was calculated on the basis of the area under curve (AUC). The values are presented as mean ± SD and were analyzed using one-way ANOVA, followed by a post hoc Tukey’s test for multiple mean comparisons. A P value of <0.05 was considered statistically significant. The statistical analysis was performed using GraphPad Prism Software 5.0.

3.4. Effects of diotides on ORAC activity

AAPH acts as peroxyl radical initiator to quench fluorescein over time, and AUC of each Trolox (2.5, 5, 10, 20, 40, and 60 µM) was used to plot the standard curve for determining the ORAC. Each diotide was analyzed and expressed as Trolox equivalents (TE)
(Huang et al., 2005). Fig. 4A shows the standard curve of Trolox and its corresponding net AUC, which was calculated from each curve (insert, Fig. 4A). It is clear that a high concentration of Trolox in the assay system can slowly decay fluorescent intensity. The fluorescent intensity at lower concentrations of Trolox, such as at 2.5 and 5 μM, rapidly decreases over 10 min. Fig. 4B shows the effects of each diotide (5 μM) in delaying fluorescent decays, which are expressed as μM TE. In this ORAC activity assay, 5 μM of diotide1, diotide4, and GSH had 21.83 ± 0.83 μM TE, 54.18 ± 5.42 μM TE, and 14.87 ± 0.08 μM TE, respectively. We found that diotide1 and diotide4 showed significantly higher (P < 0.001) levels of decay, whereas diotide2, diotide3, diotide5 and carnosine had significantly lower (P < 0.001) levels of decay, unlike that observed in the case of GSH (Fig. 4B).

3.5. Effects of diotides on protection against methylglyoxal (MGO)-induced human umbilical vein endothelial cell (HUVEC) death

Diotide did not show any cell toxicity toward HUVECs at 50 or 100 μM (Fig. 5A). The cells are pretreated with each diotide at 37 °C in a humidified atmosphere with 5% CO₂ for 24 h, washed with PBS, and a medium with or without 550 μM of MGO was added and incubated for another 24 h. It is clear that the cell viability of HUVECs dramatically decreased to about 66.61% (Fig. 5B); however, pretreatment with each diotide (especially at concentrations of 100 μM) could recover the cell viability up to 80% to 90%, and were significantly different compared to the control (MGO group, diotide1, diotide2, diotide3, diotide4, P < 0.001; diotide5, P < 0.01, Fig. 5B). Pretreatment of diotide4 at 50 or 100 μM could recover cell viability up to 82.32 ± 2.01% and 92.94 ± 1.37%, respectively.

4. Discussion

Many peptides of animal or plant proteins released in vitro or in vivo are bioactive and have regulatory functions in humans beyond being involved in nutrition alone (Hartmann & Meisel, 2007; Erdmann et al., 2008; Fitzgerald et al., 2011), including anti-hypertensive activities (Wang et al., 2010) and anti-atherosclerotic activity (Matsui et al., 2010). A number of peptides have now been shown to have antioxidant activities. A 9 kDa defensin protein and its 4 synthesized tryptic peptides—GFR, GPCSR, CFCTKPC, and MCE-SASSK—show antioxidant activities in vitro, among which, the 3 Cys residue-containing peptide (CFCTKPC) showed better total antioxidant effects, anti-lipid peroxidation activity, and DPPH radical scavenging activity than GSH did (Huang et al., 2012). A dipeptide of MY isolated from sardine muscle (Erdmann, Grosser,
Schipporeit, & Schröder, 2006) was reported to activate heme oxygenase-1 and ferritin expression, which can reduce ROS damage of the endothelial cells; MHIRL, YVEEL, and WYSLAMASDI from Corolase PP hydrolysates of α-lactalbumin and β-lactoglobulin have also been reported to have antioxidant activities by ORAC activity (Hernández-Ledesma, Dávalos, Bartolomé, & Amigo, 2005).

Thiol-containing molecules are generally acceptable as free radical scavengers, including Cys, 2-mercaptoethanol, dithiothreitol, and GSH. We also reported that yam dioscorin can act as a dehydroascorbate reductase by reducing dehydroascorbate to generate ascorbate, in the absence of GSH, through intermolecular thiol-disulfide interchange using thiol-labeling staining (Hou, Chen, & Lin, 1999). Therefore, 5 thiol-containing peptides—KTCGNGME (diotide1), PPCSE (diotide2), CDDRVIRTPLT (diotide3), KTGCY (diotide4), and PPCTE (diotide5)—were selected, synthesized, and used in this study. The results reveal that there are very different antioxidant behaviors among diotides with the same Cys residue in each diotide. It is noted that the penta-peptide of Pro-Pro-Cys-Thr-Glu (PPCTE, diotide5); however, diotide2 was one of the least efficient among the diotides in the anti-LDL peroxidation assay (Fig. 2A and 2B), anti-AAPH-mediated hemolysis assay (Fig. 3), and in AAPH-mediated ORAC activity (Fig. 4). The penta-peptide of Lys-Thr-Cys-Gly-Tyr (KTCGY, diotide4) is the best one among the diotides for the hydroxyl radical scavenging activity (Fig. 1), anti-AAPH-mediated hemolysis assay (Fig. 3), and in AAPH-mediated ORAC activity (Fig. 4).

**Fig. 2.** The protective effects of 50 μM diotides against Cu²⁺-induced LDL peroxidation were measured (A) by absorbance at 234 nm every 30 min for conjugated diene formations; or (B) by mobility on the 1.0% agarose gel after electrophoresis and being stained with Sudan Black B (arrow indicated). The migration direction is run to the anode. (C) Mobility of the blank was assigned a value of 100% and others were calculated on the relative mobility. Values are presented as means ± SD and analyzed using one-way ANOVA, followed by the post hoc Tukey’s test for multiple mean comparisons. *P < 0.001, the blank compared to the control; **P < 0.05, ***P < 0.01, and ****P < 0.001, each treatment compared to the control.
The AAPH-induced hemolysis and AAPH-mediated ORAC activity can be classified as hydrogen atom transfer reactions, which quantify the hydrogen atom donating capacity of the tested compounds (Huang et al., 2005). The calculated pI of PPCSE, PPCTE, and KTCGY, was 4.6, 4.6, and 8.2 respectively (http://www.web.expasy.org/proteomics/). It is proposed that under the tested conditions, KTCGY (diotide4) had a net positive charge that may easily force hydrogen atom transfer (dissociation) from Cys residues, unlike that observed in the case of the other 2 penta-peptides. This together with Tyr-phenolic group dissociation may have contributed to much better anti-AAPH-mediated hemolysis (Fig. 3) and AAPH-mediated ORAC activity (Fig. 4). The lowest ORAC activities of diotide2, diotide5, and diotide3 were calculated to be 0.436, 0.534, and 0.754 μM TE/μM diotide, respectively, and the highest ORAC activities of diotide1 and diotide4 had calculated values of 4.37, and 10.84 μM TE/μM diotide (Fig. 4), respectively, unlike those observed for carnosine (0.906 μM TE/μM), and GSH (2.97 μM TE/μM) in the AAPH-mediated ORAC activity assay (Fig. 4) and the AAPH-mediated hemolysis assay (Fig. 3).

Diotide1 (KTCGNGME) of the first 4 residues are the same as diotide4, and its antioxidant behaviors are generally second after diotide4 and better than GSH in these assays, which may partly explain the capacity of the hydrogen atom donation from Met and Cys residues as mentioned above. It is noted that diotide1 exhibits the best antioxidant capacity in anti-LDL peroxidation (Fig. 2A and B). Fig. 2 shows the different evaluation methods for determining LDL peroxidation, one for conjugated diene formations from Cu²⁺-induced lipid peroxidation (Fig. 2A) and the other for the relative mobility from a net increase in negative charges and a faster anodic mobility by side chain modifications of Lys residues in apoB (Fig. 2B) (Yan, Droy-Lefaix, & Packer, 1995). It is postulated
that the better metal ion chelating capacity and a proper hydrophobicity of diotide1 may be important for anti-LDL peroxidation.

The putative hydroxyl radical is an extremely reactive and short-lived species that can modify DNA, proteins, and lipids. Hydroxyl radical scavenging activities can be classified as electron transfer reactions that measure the reducing capacity of the tested sample. Diotide4 and diotide1 are the first 2 potent scavengers against hydroxyl radicals and are much better than carnosine (Fig. 1). We propose that the molecular reducing power of the Cys residue and a metal ion chelating capacity other than His residue (in carnosine) may be contributing to the hydroxyl radical scavenging activities.

Glycation is the non-enzymatic modification of proteins and their metabolized intermediates, such as glyoxal or MGO, by using reducing sugars. It occurs both in vitro and in vivo in a process called the Maillard reaction and leads to the irreversible formation of advanced glycation end products, which may alter protein functions and protein degradation (Kikuchia et al., 2003). A major reactive dicarbonyl species in the human body is MGO, which increases in patients with chronic diseases of diabetes and renal failure. Our results show that diotides have protective abilities against MGO-mediated cell death as they were able to increase the cell survival from 66.61% up to 80 to 90% (Fig. 5). The pretreatments of diotide4 at 100 μM could recover cell viability up to 92.94%.

In conclusion, thiol-containing diotides from computer-aided pepsin hydrolysis of yam dioscorin show antioxidant activities, hydroxyl radical scavenging activities, anti-LPL peroxidation properties, anti-AAPH-induced hemolysis activity, oxygen radical absorbance capacity, and protective abilities against MGO-induced cell death in a HUVEC cell model. Further evaluation of these results using animal experiments are required; however, they provide some evidence that in addition to their nutritional functions, thiol-containing diotides derived from yam may act as antioxidants. This would suggest that yams should be promoted as a functional antioxidant-rich food.

Fig. 4. (A) The area under curve (AUC) of each Trolox (2.5, 5, 10, 20, 40, and 60 μM) was used to plot the standard curve for the ORAC activity; (B) The ORAC activity of 5 μM diotide is calculated and expressed as Trolox equivalents (μM TE). Values are presented as means ± SD and analyzed using one-way ANOVA, followed by the post hoc Tukey’s test for multiple mean comparisons. ***P < 0.001, each treatment compared to GSH group.