Phage Display–Mediated Discovery of Novel Tyrosinase-Targeting Tetrapeptide Inhibitors Reveals the Significance of N-Terminal Preference of Cysteine Residues and Their Functional Sulfur Atom


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ABSTRACT

Tyrosinase, a key copper-containing enzyme involved in melanin biosynthesis, is closely associated with hyperpigmentation disorders, cancer, and neurodegenerative diseases, and as such, it is an essential target in medicine and cosmetics. Known tyrosinase inhibitors possess adverse side effects, and there are no safety regulations; therefore, it is necessary to develop new inhibitors with fewer side effects and less toxicity. Peptides are exquisitely specific to their in vivo targets, with high potencies and relatively few off-target side effects. Thus, we systematically and comprehensively investigated the tyrosinase-inhibitory abilities of N- and C-terminal cysteine/tyrosine-containing tetrapeptides by constructing a phage-display random tetrapeptide library and conducting computational molecular docking studies on novel tyrosinase tetrapeptide inhibitors. We found that N-terminal cysteine-containing tetrapeptides exhibited the most potent tyrosinase-inhibitory abilities. The positional preference of cysteine residues at the N terminus in the tetrapeptides significantly contributed to their tyrosinase-inhibitory function. The sulfur atom in cysteine moieties of N- and C-terminal cysteine-containing tetrapeptides coordinated with copper ions, which then tightly blocked substrate-binding sites. N- and C-terminal tyrosine-containing tetrapeptides functioned as competitive inhibitors against mushroom tyrosinase by using the phenol ring of tyrosine to stack with the imidazole ring of His263, thus competing for the substrate-binding site. The N-terminal cysteine-containing tetrapeptide CRVI exhibited the strongest tyrosinase-inhibitory potency (with an IC50 of 2.7 ± 0.5 μM), which was superior to those of the known tyrosinase inhibitors (arbutin and kojic acid) and outperformed kojic acid-tripeptides, mimosine-FFY, and short-sequence oligopeptides at inhibiting mushroom tyrosinase.

Introduction

The expression and distribution of melanin primarily determine the color of hair and skin in animals. Melanin is mainly produced by melanocytes and is deposited in vesicles called melanosomes, which are translocated to keratinocytes on the skin surface by dendritic activity (Wasmeier et al., 2008). The produced melanin can absorb UV light to prevent direct DNA damage (Meredith and Riesz, 2004) and eliminate free radicals generated by UV light’s action (Herrling et al., 2008). Although melanin has a protective ability, it is not metabolized in a timely fashion, leading to hyperpigmentation that results in a darkening of the skin, production of freckles and dark spots, or even as serious an effect as the production of melanomas. Therefore, much research is being performed to identify inhibitors against melanin formation.

Melanogenesis is triggered when the skin is exposed to the UV radiation of sunlight. The pathway of melanin biosynthesis encompasses two parts: the generation of eumelanin (eumelanogenesis) and the production of pheomelanin (pheomelanogenesis) (Chang, 2009). These two pigments further combine to form mixed melanin, which is the main pigment affecting the color of skin.

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ABBREVIATIONS: ddH2O, double-distilled H2O; DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; DOPA, 3,4-dihydroxyphenylalanine; GOLD, Genetic Optimization for Ligand Docking; OD, optical density; PBS, potassium phosphate buffer; PBST, PBS with 0.05% Tween 20; PDB, Protein Data Bank; PEG, polyethylene glycol.
and hair. In eumelanogenesis, l-tyrosine is converted to 3,4-dihydroxyphenylalanine (DOPA) by tyrosinase, which then catalyzes the conversion of DOPA to dopaquinone. Dopaquinone is the main determinant in the formation of different melanin pigments. When it reacts with glutathione or cysteine, glutathionyldopa or cysteinyldopa, respectively, is generated, eventually forming the yellowish-red or cysteinyldopa, respectively, is generated, eventually forming the brownish-black-colored eumelanin. Tyrosinase is the key enzyme in melanin biosynthesis; thus, its structure and function have been investigated and applied to the development of tyrosinase inhibitors against melanogenesis.

Tyrosinase inhibitors, such as hydroquinone (Arndt and Fitzpatrick, 1965; Fitzpatrick et al., 1966), arbutin (Germanas et al., 2007), kojic acid (Mishima et al., 1988; Elsner and Maibach, 2000; Ahn et al., 2011), azelaic acid (Breathnach et al., 1989; Rigoni et al., 1989), and electron-rich phenols (Jimbow, 1991), have been tested in cosmetics and pharmaceuticals for their ability to prevent the overproduction of melanin. Although these compounds can effectively inhibit tyrosinase activity, their use is strictly limited because they cause skin allergies, inflammation, and other adverse side effects. It was reported that kojic acid in food may cause liver cancer or genotoxicity (Takizawa et al., 2003). Therefore, the Ministry of Health of Japan has banned the addition of kojic acid to food products. Hydroquinone has a good inhibitory effect on tyrosinase; however, it was found to be a potential mutagen in mammals (Barlow et al., 1999) and can cause skin allergies (Parvez et al., 2006). Additionally, oxidized hydroquinone, i.e., benzoquinone, can cause cancer and cytotoxicity of liver cells and melanoma cells (Germanas et al., 2007), and its use has been prohibited in cosmetics. Arbutin, a structural analog of hydroquinone, can be metabolized into hydroxiquinone (Blaut et al., 2006), increasing the possibility of getting cancer. Thus, much research has identified tyrosinase inhibitors synthesized in the laboratory (Kubo et al., 2000; Shino et al., 2001; Jun et al., 2007; Jirawattanapong et al., 2009; Delogu et al., 2010; Yi et al., 2010; Cha et al., 2011; Tajima et al., 2011; Song et al., 2012; Hamidian, 2013; Hamidian et al., 2013; Zhu et al., 2013) and extracted from plants (Piao et al., 2009; Sung et al., 2009; Chang et al., 2011; Liang et al., 2012; Zheng et al., 2012; Sarkhail et al., 2013). Some attention has been drawn to applying peptide sequences for tyrosinase inhibition; however, most of them are fragments isolated from known proteins and peptide-derived compounds. There is no comprehensive study of tyrosinase inhibition by peptide sequences.

Our previous study of peptides in tyrosinase inhibition found that the tripeptide CRY showed the most profound inhibitory potencies against tyrosinase (Hsiao et al., 2014). Additionally, the structure of Streptomyces castaneoglobisporus tyrosinase complexed with a caddie protein (PDB ID: 1WX2) (Matoba et al., 2006) revealed that the tetrapeptide VSHY loop of the caddie protein makes considerable contributions to interface binding with tyrosinase (Fig. 1A). Based on this information, this study is the first to systematically and comprehensively investigate N- and C-terminal cysteine/tyrosine-containing tetrapeptides for their ability to inhibit mushroom tyrosinase. In this study, we comprehensively and successfully screened tetrapeptides with potential tyrosinase-binding capabilities by constructing a random phage-displayed tetrapeptide library. Tetrapeptides with potential tyrosinase-binding affinity were further subjected to tyrosinase-inhibitory assays to determine their biologic activities as the 50% inhibitory concentration (IC50). Functional variations among these N- and C-terminal cysteine/tyrosine-containing tetrapeptides were analyzed by molecular docking to elucidate their molecular interactions with tyrosinase. The N-terminal cysteine-containing tetrapeptide CRVI, which showed the strongest tyrosinase inhibitory potency (with an IC50 of 2.7 ± 0.5 µM), outperformed known tyrosinase inhibitors (arbutin and kojic acid) in inhibiting mushroom tyrosinase.

### Materials and Methods

Molecular docking studies were carried out using Genetic Optimization for Ligand Docking (GOLD; Cambridge Crystallographic Data Center, Cambridge, UK). All of the calculations were performed at the National Center for High-Performance Computing (Hsinchu City, Taiwan). Mushroom tyrosinase, l-tyrosine, arbutin, kojic acid, and potassium phosphate buffer (PBS) (67 mM, pH 6.8) were purchased from Sigma-Aldrich (St. Louis, MO). Moreover, cysteine was purchased from Acros Organics (Geel, Belgium). All peptides of this study were custom synthesized by Kelowna International Scientific (New Taipei City, Taiwan) with purities of >95% (http://www.kelowna.com.tw/en/omsService.php).

#### Library Primer Design.

DNA primers encoding for four tetrapeptide libraries, XXXxC, XXcXX, XcXXY, and XXcXX, were synthesized by Integrated DNA Technologies (Coralville, IA). The four tetrapeptide library primers shown in Fig. 1B are listed as follows: primer XcXXcX, 5′-GCC GCC GCT GAT GAA CCT GAG CAG GNM NNM NNA CGG CAT CTA CCT AAC CAC CC-3′; primer cXXcXX, 5′-GCC GCC GCT GAT GAA CCT GAN NNM NNM NMT GTA CCA GAT CCA CTA GAG CC-3′; primer XcXXcXX, 5′-GCC GCC GCT GAT GAA CCT GAA CTAM TNN NNM NMM CCG GAT CCA CTA CCT AAC CAC CC-3′; and primer XXcXXcXX, 5′-GCC GCC GCT GAA CCT GAN NNM NNM NMA TAA CCG GAT CCA CTA CCT AAC CAC CC-3′, where M = A or C at 50% each and N = A, G, T, or C at 25% each.

#### Phage-Display Library Construction.

The phagemid pCAN-TABSE (GE Healthcare, Piscataway, NJ) template was used for construction of the tetrapeptide library. DNA primers coding the tetrapeptide libraries were inserted at the site where an EcoRI restriction site and a TAA stop codon were previously constructed in the template. The EcoRI restriction site was designed to ensure successful primer insertion, and the TAA stop codons were designed to ensure that only the inserted peptide could be fused with the pIII protein and displayed on the phage surface. The phage display libraries were constructed by the oligonucleotide-directed mutagenesis method according to published protocols, with minor modifications (Kunkel et al., 1987). In brief, pCAN-TABSE template DNA was transformed into the Esherichia coli (E. coli) CJ236 strain (dut-, ung-, camR) (New England BioLabs, Ipswich, MA), and a single colony was cultured and rescued by the M13KO7 helper phage (GE Healthcare). After overnight culture in the presence of 100 µg/ml ampicillin, 70 µg/ml kanamycin, and 10 µg/ml uridine in the medium, amplified phage particles were harvested and precipitated with 4% polyethylene glycol (PEG) 8000 and 3% NaCl (w/v) and finally dissolved in PBS. Next, the dU single-stranded DNA from the phage particles was extracted with the QIAprep M13 kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions for subsequent library construction.

The primer was treated with T4 polynucleotide kinase (New England Biolabs) for 5 hours at 37°C for 2 hours. The dU single-stranded DNA template was annealed with a library primer for extension and ligation with T7 DNA polymerase and T4 DNA ligase (New England Biolabs) at 20°C overnight to generate covalently closed circular, double-stranded DNA. Next, extended and ligated covalently closed circular, double-stranded DNA was desalted and purified with
a spin column (Ultra-0.5; Millipore Amicon; Billerica, MA) and transformed into *E. coli* ER2738 by electroporation. After being rescued by the helper phage, recombinant library phage was precipitated with PEG and NaCl as described earlier and resuspended in PBS with 5% bovine serum albumin for the following panning process.

**Biopanning.** The panning procedure was carried out according to previous studies, with minor modifications (Lee et al., 2007, 2013; Leu et al., 2010). In brief, microtiter plates precoated with tyrosinase (1 µg/well) at 4°C overnight were blocked with 5% skim milk in PBST (PBS with 0.05% Tween 20) for 1 hour at 37°C. Then, 10^12 plaque-forming...
Supernatant from each well of the culture plates was mixed with 50 µl of 0.1 M HCl/glycine (pH 2.2) and neutralized with 2 M Tris-base buffer. Eluted phages were used to infect E. coli ER2738 strain in the log phase for amplification, and they were recovered with 4% PEG 8000 and 3% NaCl for the next round of panning. In this study, the panning procedure was repeated in wells four times.

Single-colony analysis. To prepare single-phage clones, individual colonies were randomly picked from the final panning cycle and cultured in 96-well deep-well culture plates. Each well contained 1 ml of 2YT with 100 µg/ml ampicillin for individual clone growth. Culture plates were incubated at 37°C with vigorous shaking for 4–6 hours before adding 20 µl of the helper phage (10^12 plaque-forming units/ml). Plates were then incubated at 37°C for another 2 hours at vigorous shaking. Next, kanamycin was added at a final concentration of 70 µg/ml and incubated at 37°C overnight with vigorous shaking.

Microtiter plates were precoated with tyrosinase (1 µg/well) and blocked with 5% skim milk. After being spun, 50 µl of the phage supernatant from each well of the culture plates was mixed with 50 µl of 5% skim milk and added to a corresponding well of a microtiter plate at 37°C for 1 hour of incubation. After washing with PBST, the bound phage particles were measured with horseradish peroxidase plate at 37°C for 1 hour of incubation. After washing with PBST, the bound phage was detected as described earlier. Next, kanamycin was added at a final concentration of 70 µg/ml and incubated at 37°C overnight with vigorous shaking.

Microtiter plates were precoated with tyrosinase (1 µg/well) and blocked with 5% skim milk. After being spun, 50 µl of the phage supernatant from each well of the culture plates was mixed with 50 µl of 5% skim milk and added to a corresponding well of a microtiter plate at 37°C for 1 hour of incubation. After washing with PBST, the bound phage particles were measured with horseradish peroxidase–labeled mouse anti-M13 antibody (Gen HealthCare). For the other side, to measure peptide expression levels, the antibody was used to capture the peptide fused E-tag on the phage surface for detection. Microtiter plates were precoated with a polyclonal goat anti–E-tag antibody (1 µg/well; Novus Biologicals, Littleton, CO) with the same manipulation.

To generate peptide-expressing phage with mutagenesis, the protocol was according to the instructions of the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Next, phage enzyme-linked immunosorbent assay was performed under the conditions as described earlier. For the competitive inhibition, the assay protocol was according to a previous report with minor modification (Lee et al., 2013). In brief, microtiter plates were coated with tyrosinase (1 µg/well) and blocked with 5% skim milk. After washing, each phage sample (10^7 particles) was incubated first with various concentrations of soluble tyrosinase diluted in PBS (from 0.1 to 50 µg/ml). Next, the mixtures were added to the wells to react with coated tyrosinase. Then, bound phage was detected as described earlier.

Sequencing. Single-phage analysis with measurable binding strengths was ranked. Around 40 of the top-ranked phage clones were selected for sequencing under selection criteria of tyrosinase-binding values (Tyr, phage clone binding to tyrosinase) of ≥0.5, peptide expression levels of ≥1.0 (E, E-tag–fused peptide on the phage surface captured by the antibody for detection), and a Try/E ratio of ≥0.5. For sequencing, the M13 reverse sequencing primer (−48: 5′-AGCGGATAACATTTCCACACAGG-3′) was used for the peptide gene analyses.

Peptide synthesis. To verify a peptide’s activity in advance, three peptide sequences were selected from each library for synthesis. Selection was based on three principles as follows: 1) it was a repeated sequence; 2) its Tyr/E value had the best ranking; and 3) it was based on the amino acid property of the X1 site, with the first priority of a positively charged amino acid (His, Arg, and Lys), the second of a polar amino acid (Asn, Glu, Thr, and Ser), and the last of an amino acid with a benzene ring (Trp, Phe, and Tyr). Peptide synthesis was performed by Kelowna International Scientific Inc. as described in Materials and Methods.

Quantification of mushroom tyrosinase-inhibitory ability of tetrapeptides in terms of the IC_{50}. The inhibitory abilities of tetrapeptides (CNHT, CRVI, CFNL, KARC, RAQC, DALC, YHSR, YFSR, YTNS, VQHY, PNAY, and RWYY) were quantified as the IC_{50} by a mushroom tyrosinase-inhibitory assay according to a previous method (Takahashi et al., 2012). In brief, 25 µl of the desired tetrapeptide solution (with the exception of the cysteine-containing tetrapeptides—dissolved in 40 µM reducing agent (dithiothreitol) solution; all the tetrapeptides were prepared by dissolving in double-distilled H_{2}O (ddH_{2}O), 80 µl of 67 mM potassium phosphate buffer (NaH_{2}PO_{4}, Na_{2}HPO_{4}, pH6.8), and 125 µl of 5 mM L-tyrosine were mixed, added to each well of a 96-well microtiter plate, and incubated at 25°C for 5 minutes. Subsequently, 20 µl of a 1250 U/ml mushroom tyrosinase solution was added to each well to a final volume of 250 µl and incubated at 25°C for another 5 minutes. Meanwhile, kojic acid and arbutin were used as positive controls at the same concentrations and conditions. The amount of dopachrome produced was determined against a blank (solution without tyrosinase) and recorded with a spectrophotometer (Varian Cary-50 Bio UV-Visible spectrophotometer; Varian BV, Houten, The Netherlands) at 475 nm every 10 seconds for 10 minutes. The reaction correlating with the amount of dopachrome produced was determined by a previously described method (Takahashi et al., 2012). Tyrosinase activity was calculated with the following equation:

\[
\text{tyrosinase activity}\% = \frac{(S - B)}{(C)} \times 100.
\]

where S is the optical density at 475 nm (OD_{475}) absorbance of the test compound, B is the OD_{475} absorbance of the blank, and C is the OD_{475} absorbance of the control. IC_{50} values of tyrosine/cysteine-containing tetrapeptides were accordingly determined by dose-dependent inhibition in experiments performed in triplicate.

Molecular modeling. Models of tetrapeptides in complex with tyrosinase were built by a computational analysis and molecular docking. The GOLD molecular docking program (Cambridge Crystallographic Data Center, version 5.2) with the Goldscore scoring function was used to predict binding poses of tetrapeptides in the active site of mushroom tyrosinase. All water and cofactor molecules were removed before docking, with the exception of two copper ions. The three-dimensional structures of the desired tetrapeptides were generated and optimized using Discovery Studio v.4.0 (Accelrys Software, San Diego, CA) and further docked against the structure of mushroom tyrosinase (PDB ID: 2Y9K) with the flexible docking option turned on. Initially, 100 independent genetic algorithm cycles of computation were performed with ligand torsion angles varying between −180° and 180°. The search efficiency was set to 200% to exhaustively explore the docking conformational space. Default settings were used for all other parameters. Finally, from the 100 docking conformations of each tetrapeptide, the top one with the highest GOLD fitness score was selected to disclose its binding in the mushroom tyrosinase-active site using Goldscore.

Molecular models of the tetrapeptide-tyrosinase complexes were presented using PyMOL software (http://www.pymol.org).

A model of the mushroom tyrosinase in complex with N-terminal cysteine-containing tetrapeptides, CNHT (chain B), CRVI (chain C), and CFNL (chain D), is provided in the Supplemental Material. A model of the mushroom tyrosinase in complex with C-terminal cysteine-containing tetrapeptides, KARC (chain B), RAQC (chain C), and DALC (chain D), is provided in the Supplemental Material. A model of the mushroom tyrosinase in complex with CRVI (chain B) is provided in the Supplemental Material. A model of the mushroom tyrosinase in complex with VQHY (chain B), PNAY (chain C), and RWYY (chain D), is provided in the Supplemental Material. A model of the mushroom tyrosinase in complex with RWYY (chain B) is provided in the Supplemental Material.

Results

The crystal structure of tyrosinase in complex with a caddie protein, ORF378 (PDB ID: 1WX2) (Matoba et al., 2006), revealed that the VSHY loop structure retrieved from the

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caddie protein made a considerable contribution to the interaction with the active site of tyrosinase from *S. castaneoglobisporus*. In particular, the structural characteristic of the tyrosine residue in the VSHY peptide sequence was accommodated in the substrate-binding pocket of tyrosinase, and was suggested to function as a competitive inhibitor of the substrate tyrosine. Herein, we therefore synthesized the VSHY tetrapeptide and tested its ability to inhibit tyrosinase. The results showed that VSHY (in ddH$_2$O) was more effective than arbutin (in ddH$_2$O) at inhibiting tyrosinase; their respective IC$_{50}$ values were 171.2 and 922.6 μM. To optimize the inhibitory potency of VSHY, we initially expressed the caddie protein on the phage surface by a phage display method. However, little or no associated binding affinity was observed among those constructed caddie proteins displayed on the phage surface that interacted with tyrosinase. As a result, we straightforwardly only expressed the VSHY tetrapeptide on the phage surface, and its binding with tyrosinase was detectable (Fig. 1A). Using this platform, we comprehensively screened around 30,000 tetrapeptides, in which the N or C terminus contained cysteine or tyrosine residues, for their ability to inhibit mushroom tyrosinase.

**Library Construction, Biopanning, and Single-Colony Analysis.** To isolate specific tetrapeptides against tyrosinase, phage-display tetrapeptide libraries were constructed for panning. As described earlier, the complex’s structure revealed the importance of the VSHY sequence with the caddie protein interacting with the tyrosine and the tyrosine on the VSHY loop being the key residue in this binding interface (Fig. 1A). Therefore, two libraries with mutagenesis of XX$_{X_3}$X$_{X_3}$ and X$_3$X$_2$X$_1$Y were used to select tyrosinase tetrapeptide inhibitors. On the other hand, according to our previous findings (Hsiao et al., 2014), cysteine in the tripeptide sequence showed superior inhibitory activity against tyrosinase. The N- and C-terminal tyrosines were replaced with cysteine in the two libraries with CX$_1$X$_2$X$_3$ and X$_3$X$_2$X$_1$C mutagenesis. The constructed library DNA was transformed into *E. coli* and rescued by the helper phage, and the sizes of the four CX$_1$X$_2$X$_3$, X$_3$X$_2$X$_1$C, YY$_{X_3}$X$_{X_3}$, and X$_3$X$_2$X$_1$Y libraries were calculated to be $6.2 \times 10^6$, $2.2 \times 10^7$, $1.2 \times 10^7$, and $2 \times 10^7$, respectively. The complexities of the four libraries were all beyond the variations that could be covered. To validate the quality of the four constructed libraries, 15 colonies of each library were randomly selected to confirm mutagenesis. Successful mutagenesis ratios of the four libraries were 87–100%, and none of the sequences was redundant (data not shown). The four libraries were proven to have good quality for subsequent panning rounds. After panning, as shown in Fig. 2A, the eluted phage numbers of the four libraries had all significantly increased. The increased phage reached 20–700-fold, compared with the number after the first round of panning (Fig. 2A). These results indicated that specific phage clones were enriched after the panning process. Similar indications were also found in the following single-colony analyses. Figure 2B shows the five variants which were selected from each library for tyrosinase and anti-E-tag binding by the phage enzyme-linked immunosorbent assay. The tyrosinase- and E-tag–binding strengths were compared with the positive control of the VSHY peptide–expressing phage and negative control of the wild-type M13 phage. The results indicated that the panning process should be successful because almost selected clones all had better or comparable binding signals against tyrosinase than the VSHY peptide–expressing phage. Next, positive clones of each library were ranked by their signal intensity according to the selection criteria (see Materials and Methods), and around 40 top clones were selected for sequencing (Supplemental Tables 2, 3, 4, and 5). Further, three interesting tetrapeptides from each library were selected for peptide synthesis and a subsequent tyrosinase-inhibitory assay.

**The Potency of Mushroom Tyrosinase Inhibition by the Tetrapeptides.** Based on the results of sequencing and values of the E-tag, three tetrapeptides were selected from each tetrapeptide library: CNHT, CRVI, and CFNL from CX$_1$X$_2$X$_3$; KARC, RAQC, and Dalc from X$_3$X$_2$X$_1$C; YHSR, YTNS, and YFSR from YY$_{X_3}$X$_{X_3}$; and RWWY, PNAY, and VQHY from X$_3$X$_2$X$_1$Y. Figure 3A shows results of the mushroom tyrosinase-inhibitory assay to determine IC$_{50}$ values of the positive control VSHY tetrapeptide and two known tyrosinase inhibitors (arbutin and kojic acid). After treatment with a 2-fold dilution of the peptide or tyrosinase inhibitors, the tyrosinase-inhibitory activity showed different degrees of decrease. The positive control tetrapeptide (VSHY) and arbutin and kojic acid in ddH$_2$O were determined to have IC$_{50}$ values of around 171.2, 922.6, and 84.4 μM, respectively.

The tyrosinase-inhibitory capabilities of the tetrapeptides of interest were quantified as IC$_{50}$ values and compared with those of the positive controls, VSHY, arbutin, and kojic acid (Fig. 3, B and C). In particular, the cysteine-containing tetrapeptides were treated with reducing agent (dithiothreitol) to avoid intermolecular disulfide bond–mediated dimer formation. The results show that the N-terminal cysteine-containing tetrapeptides exhibited the most outstanding tyrosinase-inhibitory ability; their IC$_{50}$ values were all <10 μM. In particular, the CRVI tetripeptide showed the strongest tyrosinase-inhibitory potency (with an IC$_{50}$ of 2.7 ± 0.7 μM). C-terminal cysteine-containing tetrapeptides displayed moderate tyrosinase-inhibitory activities, with IC$_{50}$ values of about 10–50 μM. On the contrary, C- and N-terminal tyrosine-containing tetrapeptides were less potent at tyrosinase inhibition; their IC$_{50}$ values were in the range of near 100–1000 μM. It is noteworthy that the RWWY tetrapeptide showed profound inhibitory ability among N- and C-terminal tyrosine-containing tetrapeptides.

To validate whether tetrapeptide structure will affect the binding to tyrosinase, we used a site-directed mutagenesis method to change CRVI to CAVI, and used the substitution to negative charge-bearing CEVI and CDVI to further test the binding strength of peptide-expressing phage to tyrosinase (Supplemental Fig. 3A). As shown in the results, the binding signal of CAVI was reduced to lower than 50%, and CEVI and CDVI did not show any binding response, which means that the structure of a cysteine-containing peptide may influence its binding ability and correlates to the panning results. Because phage display technology is used in screening according to affinity, weak binding and nonspecific binding can be excluded; therefore, we found that, after panning, unexpected peptide sequences such as CAVI, CEVI, and CDVI did not appear in the randomly screened clone sequences (Supplemental Table 2).

In addition, we chose two peptide-expressing phages bearing peptides CRVI and Dalc, respectively, from Fig. 3B and used competition assay to test their tyrosinase binding ability. As shown in Fig. 3B, CRVI excels Dalc in tyrosinase inhibition ability, and after using different concentrations of free-form tetrapeptide...
tyrosinase to carry out competition, CRVI has a higher binding affinity (lower IC₅₀ values) than DALC (Supplemental Fig. 3B). This result shows that cysteine-containing peptides with different terminals will generate different binding strengths due to structure variation and result in different inhibition outcomes.

**Molecular Modeling of Tetrapeptides Complexed with Mushroom Tyrosinase.** N and C termini of tetrapeptides containing cysteine or tyrosine residues showed divergent tyrosinase-inhibitory potencies. To further clarify their functional variations, residual interactions of these tetrapeptides with mushroom tyrosinase were analyzed by molecular modeling. Models of tetrapeptides complexed with mushroom tyrosinase are shown in Figs. 4 and 5 and Supplemental Figs. 1 and 2. Their molecular interactions with tyrosinase are summarized in Table 1 and Supplemental Table 1. The results demonstrate that both N- and C-terminal cysteine/tyrosine-containing tetrapeptides well fit in the active site of mushroom tyrosinase, and the terminal cysteine and tyrosine residues of these tetrapeptides made close contact with the dinuclear copper ions (Fig. 4; Supplemental Fig. 1). N-terminal cysteine-containing tetrapeptides commonly interacted with active site residues Val248 and Phe264 (hydrophobic interactions), Glu256 and Arg268 (electrostatic interactions), and Asn260 (hydrogen bonding), whereas the P2 and P4 positions of all C-terminal cysteine-containing tetrapeptides frequently interacted with the active site residues Asn260 (by hydrogen bonding) and Phe264 (by a hydrophobic interaction) (Table 1). Detailed molecular interactions of the CRVI tetrapeptide with tyrosinase are presented in Fig. 5 and Table 1. The amide

**Fig. 2.** Eluted phage titers after each round of panning. (A) Panning cycles were processed four times. Each time the eluted phage was titered and amplified for the next round of panning. (B) The black and gray histograms show the binding strengths of the phage displaying tetrapeptide variants for immobilizing mushroom tyrosinase and anti–E-tag antibody, as measured with an enzyme-linked immunosorbent assay. Black histograms show tyrosinase binding values, and gray histograms show peptide expression levels. The VSHY peptide expressing phage was the positive control, and the negative control wild-type M13 phage contained no displayed peptide.
group of the cysteine residue of CRVI interacted with Glu256 (by electrostatic interaction) and Asn260 (by hydrogen bonding). The side chain of the arginine residue was electrostatically attracted to the carboxyl group of Glu322 and formed a hydrogen bond with Ala246. On top of that, the valine residue of CRVI displayed hydrophobic interactions with Val248 and Phe264. The C-terminal carboxyl group and side chain of the isoleucine residue electrostatically interacted with Arg268 and made individual hydrophobic contacts with Val283 and Pro284.

The binding poses of tyrosine-containing tetrapeptides in the active site overlapped and are shown in Supplemental Fig. 1. In N-terminal tyrosine-containing tetrapeptides, the P1 position [tyrosine] was deep in the substrate binding pocket, and π–π interactions were observed within the phenol ring of tyrosine and the imidazole ring of His263 (Supplemental Table 1). The P2 position (H/Y/T) hydrophobically interacted with Phe264. In addition, hydrophobic contacts were also observed in the P2 position of YFSR and YTNS with Val248. Moreover, the arginine residue in the P4 position of YFSR electrostatically interacted with Glu322, whereas in C-terminal tyrosine-containing tetrapeptides, the C-terminal P4 position [tyrosine], situated near the copper ions, was aromatically stacked with the imidazole ring of His263. The P3 positions of RWWY and VQHY contributed hydrophobic contacts with Val283 and Pro284. The P2 positions of VQHY and PNAY interacted with Arg268 by hydrogen bonding, whereas the tryptophan residue in the P2 position of RWWY interacted with Phe264 and Arg268 by hydrophobic and cation–π interactions. Only the P1 position of RWWY displayed an electrostatic interaction with Glu189 in the active site of tyrosinase (details shown in Supplemental Figs. 1 and 2 and Supplemental Table 1).

**Discussion**

To investigate enzyme-substrate interactions and further look for possible tetrapeptide inhibitors, phage-display technology is a powerful tool with simple manufacture and high complexity expression. After three to five rounds of panning, clones with specific binding ability can be enriched out. According to our previous experience, more than 10-fold increases in eluted
Phage numbers can obtain specific binders (Lee et al., 2013). After panning, eluted phage numbers of the four libraries all showed significant increases, with the X3X2X1C library being highest. It is interesting that the CRVI tetrapeptide with the strongest tyrosinase-inhibitory potency within the CX1X2X3 library only showed an increase of around 20-fold. Because the tetrapeptide expression fused to the g3 structural protein on the phage surface, the structure of terminal cysteine-containing tetrapeptide libraries may have affected peptide display and interfered with the panning process. Compared with cysteine-containing tetrapeptides, most peptides did not have good tyrosinase-inhibitory ability, although the YX1X2X3 and X3X2X1Y libraries had well eluted phage number enrichment. The results corresponded to our previous findings about tyrosinase dipeptide inhibitors: that cysteine residues located at the N or C terminus show better tyrosinase-inhibitory abilities. The cysteine moiety of N- or C-terminal cysteine-containing tetrapeptides coordinates with copper ions which then tightly block the substrate-binding site. Therefore, a tetrapeptide inhibitor also reveals the significance of the N-terminal preference of cysteine residues better than that of the C terminus of cysteine residues (Fig. 3B).

We quantified the tyrosinase-inhibitory potency of N- and C-terminal cysteine/tyrosine-containing tetrapeptides in terms of the IC50 (Fig. 3, B and C). The results showed that N-terminal cysteine-containing tetrapeptides exhibited the most potent tyrosinase-inhibitory abilities. The tetrapeptide CRVI showed the strongest tyrosinase-inhibitory potency (IC50 = 2.7 μM); however, the IC50 values of CRVI turned out to be 2311.6 μM when the cysteine residue was replaced by a serine residue (SRVI), indicating that the sulfhydryl (–SH) group of the cysteine residue makes a more considerable contribution than the hydroxyl group (–OH) of the serine residue to the tyrosinase inhibition. In contrast, the C-terminal cysteine-containing tetrapeptides, KARC, RAQC, and DALC, exhibited less-potent inhibitory capacities than those of N-terminal cysteine-containing tetrapeptides. This implies that the positional preference of cysteine residues at the N terminus in tetrapeptides significantly contributes to their function of inhibiting tyrosinase. On the other hand, few functional variations were observed between the less-potent N- and C-terminal cysteine-containing tetrapeptides, which indicates that the selectivity for the N or C terminus by tyrosine residues is not determinately associated with the tyrosinase-inhibitory function of the tetrapeptides. In addition, the C-terminal tyrosine-containing tetrapeptide VQHY (IC50 = 195.7 μM) showed tyrosinase-inhibitory effectiveness comparable to that of the control VSHY tetrapeptide, retrieved from the caddie protein (IC50 = 171.2 μM), giving strong confidence to our rationale. Moreover, RWWY (IC50 = 82.4 μM), the most potent among all N- and C-terminal tyrosine-containing tetrapeptides in tyrosinase inhibition, corroborates our previous finding that the structure of a natural compound, A5, resembled that of WY, which exhibits effective tyrosinase-inhibitory ability.

In addition, the IC50 value of cysteine along the amino acid chain has been determined and compared with those of cysteine-containing tetrapeptides (Fig. 3B). Although the single cysteine shows comparable tyrosinase inhibitory potency (IC50 = 9.7 μM), it has been reported that the cysteine-alone amino acid indeed reacts with dopaquinone instead of interacting straightforwardly with the active site of tyrosinase to block the melanin formation (Slominski et al., 2004). However, whether the cysteine-containing tetrapeptides undergo the same inhibition pathway is controversial because of their large molecular sizes and distinct...
chemical properties compared with that of the cysteine-alone residue.

The secondary structural contents of N- and C-terminal cysteine/tyrosine-containing tetrapeptides were estimated by circular dichroism. However, only random coil conformations were observed in all examined tetrapeptides, indicating that their functional deviations in inhibiting tyrosinase were not determined by secondary structural conformations. To further elucidate functional variations of N- and C-terminal cysteine/tyrosine-containing tetrapeptides, their molecular interactions with the active site of tyrosinase were further analyzed by molecular modeling. The docking models demonstrated that the cysteine moiety of N- and C-terminal cysteine-containing tetrapeptides made close contact with the dinuclear copper ions, suggesting that the sulfur atom of the thiol group in the cysteine residue may coordinate with the copper ions that tightly block the substrate-binding site (Fig. 4). The positional preference of the cysteine residue in the terminus of tetrapeptides was elucidated—when the cysteine residue is located in the N terminus of the tetrapeptides, its free amide group is capable of causing electrostatic interactions with Glu256 and hydrogen bonding with Asn260, and the free carboxyl group of the C terminus is electrostatically attracted to Arg268 (Table 1). When a cysteine was situated in the C terminus of the tetrapeptides, its carboxyl group only contributed to hydrogen bonding with Arg260, and the NH₂ group of the N terminus showed no molecular interactions with the active residues. This implies that the terminal effect may play a significant role in binding with tyrosinase, leading to functional variations in inhibiting tyrosinase between N- and C-terminal cysteine-containing tetrapeptides. In addition, lysine and arginine moieties in the P1 position (K/R) of KARC
TABLE 1
Molecular interactions between each tetrapeptide (CNHT, CRVI, CFNL, KARC, RAQC, and DALC) and active site residues of mushroom tyrosinase

The numbers 1, 2, 3, 4, 5, and 6 in this table stand for electrostatic interactions, hydrogen bonding, and hydrophobic $\pi$-$\pi$, cation-$\pi$, and anion-$\pi$ interactions, respectively. The N terminus NH$_2$, amide, side chain, and C-terminal carboxyl groups of tetrapeptides are abbreviated as n, a, s, and c, respectively.

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and RAQC electrostatically interacted with the active site residue Glu189 (Fig. 4); however, the P1 position (D) of DALC was tilted up far from Glu189. This difference may have caused their functional divergences in inhibiting tyrosinase, and indicates that the active Glu189 residue is important for tyrosinase binding and/or inhibition. Moreover, it was reported that good tyrosinase-inhibitory peptides preferably contain arginine in combination with valine, alanine, and/or leucine (Schurink et al., 2007); this was also observed in our screened tetrapeptides, CRVI, KARC, and RAQC.

Molecular models of the active site of tyrosinase of C- and N-terminal tyrosine-containing tetrapeptides revealed that tyrosine residues of tyrosine-containing tetrapeptides were most likely to interact with copper ions as well, regardless of being located at the N or C terminus. Based on this result and the similar tyrosinase-inhibitory potencies of these terminal tyrosine-containing tetrapeptides, we propose that there is no N- or C-terminal preference of tyrosine residues in these tetrapeptides for tyrosinase inhibition. Additionally, it was reported that the side chain of the Tyr98 residue of VSHY within the caddie protein (PDB ID: 1WX2) is accommodated in the substrate-binding pocket of tyrosinase, and its phenol ring is stacked with the imidazole ring of His194, a CuI ligand in the tyrosinase active site; therefore, it was suggested to be a competitive inhibitor. A similar phenomenon was observed in our docked models—the tyrosine moieties of these N- and C-terminal tyrosine-containing tetrapeptides were all aligned with similar orientations in the active site of tyrosinase, and their phenol rings perfectly stacked with the imidazole ring of His263 (PDB ID: 2Y9X) (Supplemental Figs. 1 and 2; Supplemental Table 1). Accordingly, we propose that the tyrosine residues of these tyrosine-containing tetrapeptides may also act as competitive inhibitors against mushroom tyrosinase.

The active site properties of tyrosinase and the functional moieties of the CRVI tetrapeptide reached perfect complementarity (Fig. 5), leading to the strongest inhibitory potency of CRVI. The active site of mushroom tyrosinase can be divided into three regions: 1) a substrate-binding pocket (six histidine residues along with the dinuclear copper ions) surrounded by charged residues such as Glu256 and Glu322 residues; 2) a hydrophobic region, consisting of Val248, Phe264, Val283, and Pro284 residues; and 3) a solvent-exposed region, composed of Glu189 and Arg268 residues. The cysteine moiety of CRVI fit well in the substrate-binding pocket, and its NH2 group interacted with both Glu322 by electrostatic interaction and Glu256 by hydrogen bonding, fixing the N terminus of CRVI (Fig. 5B). The arginine moiety interacted with Glu322. Regarding the following valine and C-terminal isoleucine moieties, their side chains fulfill the hydrophobic complementarities with the hydrophobic region of the active site. Moreover, the C-terminal carboxyl group of isoleucine electrostatically interacts with the side chain of Arg268, stabilizing the C terminus of CRVI.

Known tyrosinase inhibitors, such as hydroquinone (Arndt and Fitzpatrick, 1965; Fitzpatrick et al., 1966), arbutin (Germanas et al., 2007), and kojic acid (Mishima et al., 1988; Elsner and Maibach, 2000; Ahn et al., 2011), are not completely adequately used in medicine and cosmetics today, because they are thought to be severely carcinogenic and can damage the structural architecture of several different tissues (Fujimoto et al., 1999; Philips et al., 2004; McGregor, 2007; Charlin et al., 2008). Meanwhile, peptides are thought to bind with exquisite specificity to their in vivo targets, resulting in exceptionally high potencies of action and relatively few off-target side effects (Craik et al., 2013). Thus, several studies made efforts to find novel proteins and peptides from natural resources, such as silk (Kato et al., 1998), milk (Nakajima et al., 1996; Chen et al., 2006), honey (Oszmianski, 1990; Ates and Cokmus, 2001), wheat (Okot-Kotber et al., 2001), and the housefly (Daquinag et al., 1995, 1999), for tyrosinase inhibition. On top of that, dipeptides (Girelli et al., 2004), kojic acid tripeptides (Noh et al., 2007), mimosine tetrapeptides (Upadhyay et al., 2011), cyclic peptides (Morita et al., 1994), short-sequance oligopeptides (Abu Ubeid et al., 2009), and octameric peptides (Schurink et al., 2007) were also investigated for their tyrosinase-inhibitory abilities. Herein without precedent, we systematically and comprehensively investigated C- and N-terminal cysteine/tyrosine-containing tetrapeptides for tyrosinase inhibition and found that the CRVI tetrapeptide showed the strongest mushroom tyrosinase-inhibitory potency. The inhibitory ability of CRVI (IC50 = 2.7 μM) was superior to that of known tyrosinase inhibitors, including arbutin (IC50 = 922.6 μM) and kojic acid (IC50 = 84.4 μM). Additionally, the CRVI tetrapeptide was not only comparable to kojic acid tripeptides (kojic acid–FWF, IC50 = 6.17 μM) and kojic acid–FWM, IC50 = 4.48 μM), but also outperformed the mimosine–FFY (IC50 = 18.3 μM) and short-sequance oligopeptides (with IC50 values of 40 μM to 8 mM) in inhibiting mushroom tyrosinase.

Conclusions

In this study, we systematically and comprehensively investigated the abilities of N- and C-terminal cysteine/tyrosine-containing tetrapeptides to inhibit mushroom tyrosinase. N-terminal cysteine-containing tetrapeptides showed the most potent tyrosinase-inhibitory abilities, and the CRVI tetrapeptide exhibited the strongest tyrosinase-inhibitory potency (with an IC50 of 2.7 ± 0.5 μM). The positional preference of cysteine residues at the N terminus in tetrapeptides significantly contributes to its function of tyrosinase inhibition. The terminal effect has an essential role in binding with tyrosinase, leading to functional variations between N- and C-terminal cysteine-containing tetrapeptides in inhibiting tyrosinase. The sulfur atom of the thiol group in the cysteine residue of N- and C-terminal cysteine-containing tetrapeptides coordinates with copper ions that tightly block the substrate-binding site. N- and C-terminal tyrosine-containing tetrapeptides function as competitive inhibitors against mushroom tyrosinase using the phenol rings of tyrosine moieties to stack with the imidazole ring of His263 and occupy the substrate-binding site. The tyrosinase-inhibitory ability of CRVI was superior to those of known tyrosinase inhibitors (arbutin and kojic acid) and outperformed kojic acid tripeptides, mimosine–FFY, and short-sequance oligopeptides (with IC50 values of 40 μM to 8 mM) in inhibiting mushroom tyrosinase.

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