Short-Sequence Oligopeptides with Inhibitory Activity against Mushroom and Human Tyrosinase

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Cutaneous hyperpigmentation is a common disorder due to excess melanin production by the enzyme tyrosinase. The gold standard for treatment is hydroquinone (HQ), which reduces pigmentation through its toxicity to melanocytes rather than via tyrosinase inhibition. We screened an internal library for oligopeptides that inhibited both mushroom and human tyrosinase but showed no cytotoxicity to human melanocytes. We identified two highly active inhibitory sequences, P3 and P4, of 8- and 10-amino-acid-length, respectively. Mushroom tyrosinase inhibition was dose-dependent with IC_{50} (half-maximal inhibitory concentration) values of 123 and 40 μM, respectively, compared with 680 μM for HQ. Other oligopeptides showed weaker or no inhibitory activity. Kinetic studies showed that P3 and P4 are competitive inhibitors of mushroom tyrosinase. At 100 μM, P3 and P4 inhibited human tyrosinase by 25–35%. This inhibition partially depended on whether L-dopa or L-tyrosine was the substrate, suggesting that tyrosinase may contain contains two distinct catalytic sites. Treatment of melanocytes with 100 μM P3 or P4 for 7 days led to a 27 or 43% reduction in melanin content. This inhibition was independent of cell proliferation and cytotoxic effects. Our data suggest that peptide-mediated inhibition of melanogenesis is due to reduction in tyrosinase activity.

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INTRODUCTION

Melanin, the end product of melanogenesis, determines the color of human skin, hair, and eyes and is synthesized within unique membrane-bound organelles called melanosomes. The type and amount of melanin and its distribution pattern in the surrounding keratinocytes determine the actual color of skin (Parvez *et al.*, 2006). Melanogenesis is regulated by various environmental, hormonal, and genetic factors, such as UV exposure, α-melanocyte-stimulating hormone, melanocortin 1 receptor, and agouti-related protein (Kadekaro *et al.*, 2003; Petit and Pierard, 2003). The functions of melanin include protection of skin from UV radiation, the inhibition of photocarcinogenesis, and the synthesis of vitamin D3 (Lindquist, 1973). Hyperpigmentation is a very common and usually harmless disorder in which patches of skin become darker in color than the normal surrounding skin. This darkening occurs when an excess of melanin is formed and deposited in the skin. The most common pigmentation disorders for which patients seek treatment are melasma and post-inflammatory hyperpigmentation (PIH) (Lynde *et al.*, 2006). These conditions may have a major impact on a person's psychological and social well-being, contributing to lower productivity, social functioning, and self-esteem (Finlay, 1997).

Melasma is an acquired pigmentary disorder that occurs in women of all racial and ethnic groups (Rendon *et al.*, 2006). Although the cause of melasma is unknown, factors include genetic predisposition, UV light exposure, and estrogen (Johnston *et al.*, 1998). PIH represents a pathophysiological response to cutaneous inflammation, such as acne, atopic dermatitis, lichen planus, chronic dermatitis, and phototoxic eruptions (allergic reactions to drugs coupled with exposure to sunlight) (Lacz *et al.*, 2004). Tyrosinase is a multifunctional copper-containing oxidase, and is considered the key enzyme orchestrating mammalian melanogenesis (Sanchez-Ferrer *et al.*, 1995). This key enzyme catalyzes two distinct reactions in the biosynthesis pathway of melanin: the hydroxylation of a monophenol (tyrosine) and the conversion of an o-diphenol (dopa) to the corresponding o-quinone (dopaquinone). A multitude of different tyrosinase inhibitors are used today to treat epidermal hyperpigmentation conditions. Skin-whitening agents in the cosmetics industry include hydroquinone (Jimbow *et al.*, 1974), arbutin (Maeda and Fukuda, 1996), kojic acid (Cabanes *et al.*, 1994), vitamin C (Kojima *et al.*, 1995), retinol (Pathak *et al.*, 1986), azelaic acid (Schallreuter and Wood, 1990), and other botanicals.

The most popular depigmenting agent, hydroquinone (dihydroxybenzene; HQ), has been in use since 1961. HQ, with formulations of 2–4%, has been widely used for melasma treatment and reports of its clinical efficacy have been well documented (Ennes *et al.*, 2000). HQ inhibits the conversion of dopa to melanin by inhibiting the activity of tyrosinase (Palumbo *et al.*, 1991). In addition to tyrosinase

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Abbreviations: [E], enzyme concentration; HQ, hydroquinone; [I], inhibitor; PIH, post-inflammatory hyperpigmentation; [S], substrate concentration

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inhibition, previous studies suggested that HQ exerts its effect on melanogenesis through degradation of melanosomes and destruction of melanocytes. It was also found that alteration of melanosome function, depletion of glutathione, generation of reactive oxygen species, and subsequent oxidative damage of membrane lipids and proteins may play a role in the depigmenting effect of HQ (Briganti *et al.*, 2003). HQ is considered to be cytotoxic to melanocytes, potentially mutagenic to mammalian cells (Curto *et al.*, 1999), and causes skin irritation (Parvez *et al.*, 2006). Owing to the long-term risk of mutagenesis (DeCaprio, 1999), the use of HQ in cosmetics has been banned by the European Committee (24th Dir. 2000/6/EC) and formulations of 4% are available only by prescription in selected other countries (Solano *et al.*, 2006).

This has led to the use of alternative agents such as kojic acid and arbutin. However, these agents failed to show efficacy *in vivo*, as clinical trials have been disappointing to date (Curto *et al.*, 1999). The efficacy of these products appears to be limited due to their adverse effects, poor skin penetration, and low formulation stability (Hermanns *et al.*, 2000). More recently, investigators have turned their attention to short-sequence peptides as potential therapeutic candidates for the treatment of skin disorders. The most widely studied and popular signal peptide is the sequence lysine–threonine–threonine–lysine–serine (KTTKS) found in type-I procollagen (Katayama *et al.*, 1993). This pentapeptide has been shown to stimulate feedback regulation of new collagen synthesis and to result in an increased production of extracellular matrix proteins such as types I and II collagen and fibronectin, significantly improving the appearance of photoaged human facial skin (Robinson *et al.*, 2005; Lupo and Cole, 2007).

In this study, we screened an internal oligopeptide library for inhibitory activity against tyrosinase and the absence of melanocyte toxicity. We discovered several short-sequence peptides that met both these criteria.

Table 1. Oligopeptide inhibitory profile against mushroom tyrosinase

Oligopeptide	Sequence	IC_{50} [M][1]
P1	SFLLRN	8 mM
P2	KFEKKFEK	3.6 mM
P3	RADSRADC	123 μM
P4	YRSRKYSSWY	40 μM
P5	PLG	2.73 mM
P6	VLLK	No effect
P7	KFEFKFEF	No effect

[1]IC_{50} [M] is the concentration at 50% inhibition of mushroom tyrosinase in molar.

RESULTS

Inhibition of mushroom tyrosinase by oligopeptides

As the rate-limiting enzyme in melanin production, tyrosinase was targeted during the screening of seven potential hypopigmenting agents (Table 1). Of these oligopeptides, two showed substantial inhibitory effects on mushroom and human tyrosinase activity, whereas the other peptides had either poor or no inhibitory activity on tyrosinase. Dose–response curves were obtained after an incubation time of 120 minutes (Figure 1). Peptide P6 had no effect on tyrosinase activity, whereas peptides P1, P2, P5, and P7 showed an inhibitory effect at high concentrations. On the other hand, peptides P3 and P4 showed very impressive inhibition at relatively low concentrations. The IC_{50} (half-maximal inhibitory concentration) for P3 and P4 was 123 and 40 μM, respectively, compared with 680 μM for HQ. Figure 1 also illustrates that the inhibitory activity of the peptides was dose-dependent, with P4 showing inhibitory activity at concentrations as low as 50 μM. On the basis of the above results, we focused our attention on peptides P3 and P4. Figure 2 shows the time–response curves and indicates that P3 and P4 exhibited similar inhibitory activity as HQ. Moreover,

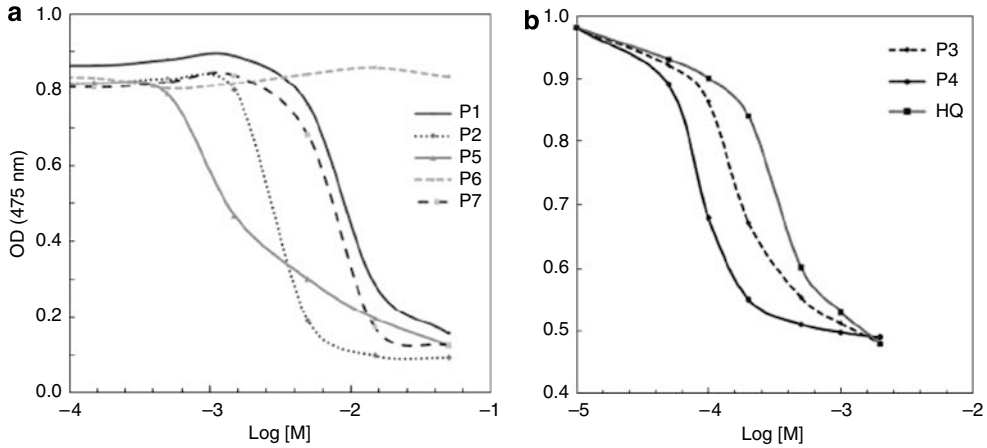

Figure 1. Dose–response curves for all seven peptides and HQ were drawn at 120 minutes post incubation with varying concentrations. The experiment was conducted in a 96-well flat-bottom plate. Each well contained 80 μl of 0.067 M potassium phosphate buffer (pH 6.8), 40 μl of 5 mg ml^{-1} L-tyrosine dissolved in 0.067 M potassium phosphate buffer (pH 6.8), 40 μl of the different concentrations of inhibitor in 5% DMSO solution, and 40 μl of 480 U ml^{-1} mushroom tyrosinase solution. The assay mixture was incubated at 37 °C and spectrophotometric readings at 475 nm were taken every 10 minutes. (**a**) P1, P2, P5, P6, and P7; (**b**) HQ, P3, and P4.

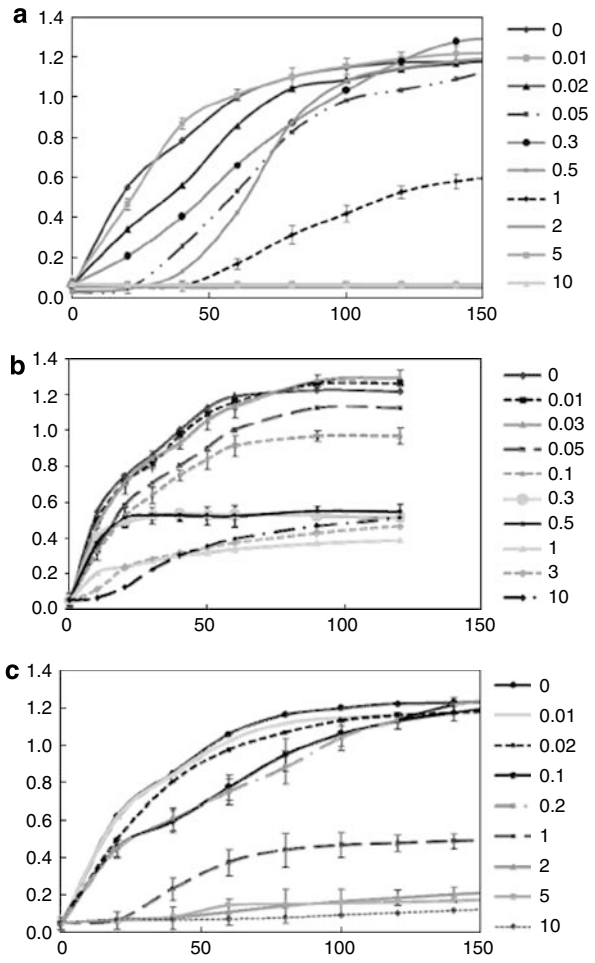

Figure 2. Time-response curves for peptides P3, P4, and HQ. The final volume of each well was 200 µl, containing 1 mg ml^{-1} L-tyrosine, 96 U ml^{-1} of mushroom tyrosinase, and varying concentrations of inhibitors, ranging from 10 µM to 10 mM. The 96-well plate was incubated at 37 °C and the spectrophotometric readings taken every 10 minutes. Each point on the graph is an average of five independent trials, displayed with error bars. (**a**) P3; (**b**) P4; and (**c**) HQ.

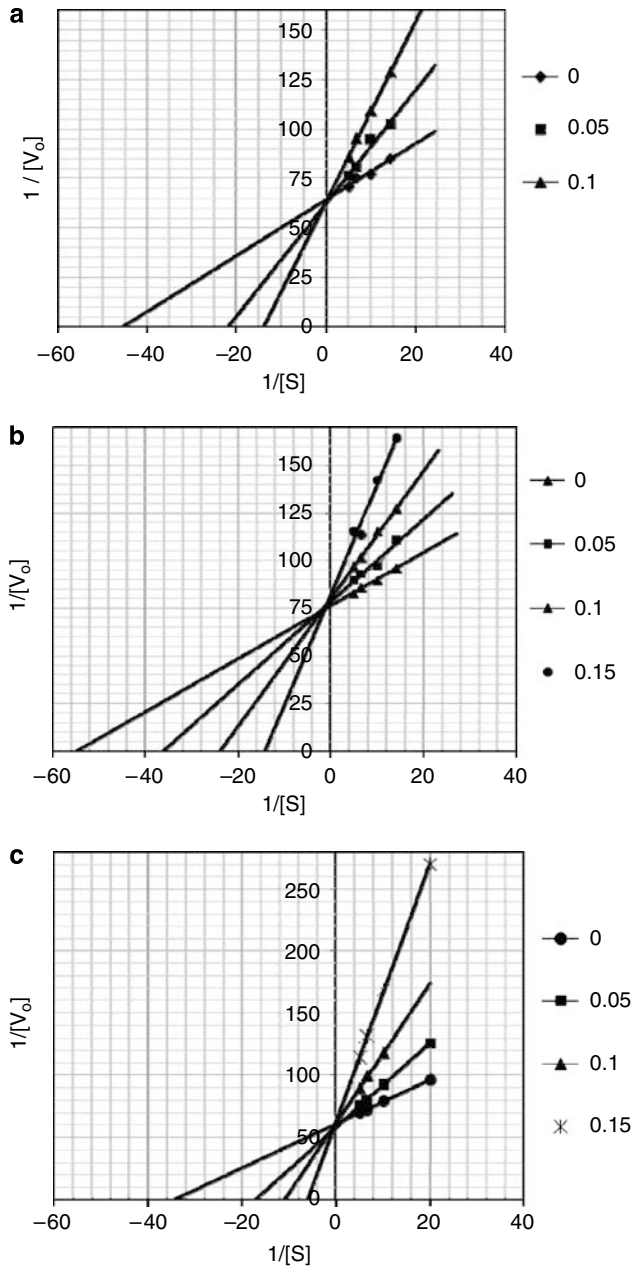

Figure 3. Lineweaver–Burke plots. (**a**) P3 (**b**) P4 and (**c**) HQ. The concentration of mushroom tyrosinase [E] was 24 U ml^{-1}. The series of substrate concentrations [S] were 0.05 (or 0.07 for peptide inhibitors), 0.1, 0.15, and 0.2 mg ml^{-1}. The series of inhibitor [I] were 0, 0.01, and 0.02 mM. The results were analyzed according to the Lineweaver–Burke plot method that allows the determination of the Michaelis constant (K_m) and maximum velocity (V_m). All inhibitors showed a competitive inhibition pattern.

peptide P4 showed a more potent inhibitory effect at concentrations as low as 50 µM.

Kinetics of mushroom tyrosinase inhibition by oligopeptides

We next determined the kinetics of the inhibition for P3, P4, and HQ using a standard assay (Pomerantz, 1966). The inhibition mechanisms of the two peptides P3 and P4, as well as that of HQ, are all competitive as shown by Lineweaver–Burke plots in Figure 3. The double reciprocal plots illustrate that V_{max} was independent of substrate concentration for all three inhibitors, whereas the K_m was altered. The inhibition constant (K_i) for HQ, P3, and P4 was determined to be 0.053, 0.048, and 0.070 mM, respectively.

Inhibition of human tyrosinase by oligopeptides

We also examined the inhibitory activity of P3 and P4 relative to HQ against human tyrosinase under two conditions: the first using L-dopa as the substrate and the second using L-tyrosine as the substrate with L-dopa as the cofactor. In the latter, L-dopa acts as a hydrogen donor helping eliminate the lag period observed during tyrosine hydroxylation (Pomerantz and Warner, 1967). Figure 4 shows dose-dependent inhibition for P3, P4, and HQ. It can be seen that P3 and P4 are more potent than HQ at concentrations as low as 30 µM. At 100 µM, human tyrosinase activity was reduced by 35% for P4 and by 25% for P3. With L-dopa as the

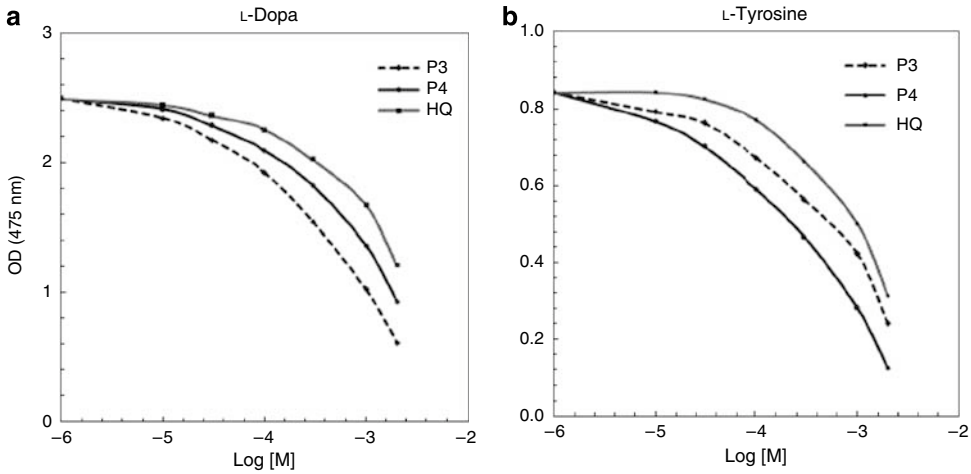

Figure 4. Dose–response curves with human tyrosinase. Melanocytes were lysed using 1% Triton X-100 in 0.067 M potassium phosphate buffer (pH 6.8). Cells were then sonicated twice at 20% intensity for 30 seconds. Lysates were clarified by centrifugation at 10,000 *g* for 10 minutes after measuring protein content, an equal amount of 40 µg was added to each well and adjusted with lysis buffer to reach 150 µl. Each triplicate of wells received different concentrations (0–2 mM) of oligopeptide or HQ. (**a**) Using L-dopa as the substrate. L-Dopa was dissolved in lysis buffer and added to each well at a final concentration of 2.5 mM. The plate was incubated at 37 °C for 120 minutes, and spectrophotometric readings at 475 nm were taken at 10 minutes intervals. (**b**) Using L-tyrosine as the substrate with L-dopa at a final concentration of 0.005% as a cofactor.

substrate, P3 was more potent than P4 at inhibiting the enzyme. On the other hand, when L-tyrosine was used as the substrate, P4 showed stronger inhibition than P3. These data support the hypothesis that tyrosinase has two distinct catalytic sites for L-dopa and L-tyrosine (Olivares *et al.*, 2001).

Effects of HQ, P3, and P4 on melanin content of melanocytes

To determine the effect of the oligopeptides on melanogenesis, we incubated melanocytes with HQ, P3, or P4 for 1 week and then measured melanin content. At a concentration of 10 µM, HQ, P3, and P4 reduced melanin content by 7 ± 2, 22 ± 1, and 40 ± 1%, respectively (Figure 5). At 100 µM, P3 and P4 reduced melanin content by 27 ± 2 and 43 ± 2%, respectively, whereas HQ was found to be 100% toxic to melanocytes.

Cytotoxic and proliferative effects of HQ, P3, and P4 on melanocytes

To exclude the possibility that P3 and P4 were reducing melanin content by inhibiting cell proliferation or inducing cytotoxicity, melanocytes were incubated in the presence or absence of various concentrations of HQ, P3, or P4, and proliferation was assessed at various time points using the WST-1 Cell Proliferation Kit (Roche, Palo Alto, CA). Figure 6 shows that treatment with 100 µM or greater of HQ completely abolished WST-1 activity (from 0.45 ± 0.02 at day 1 down to 0.0 at day 5). These data are consistent with our above findings and imply that HQ is cytotoxic to melanocytes at these concentrations. Unlike HQ, 10 µM, 100 µM, or 1 mM P3 did not alter melanocyte proliferation rates relative to the control group. Although no effect on melanocytes proliferation was observed for P4 at 10 and 100 µM after 5 days of incubation, proliferation was inhibited by 10 ± 2% at a concentration of 1 mM.

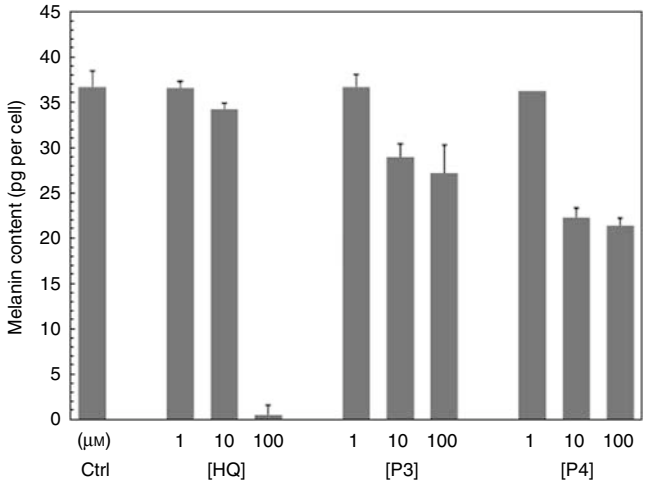

Figure 5. The effects of peptides and HQ on the melanin content of melanocytes, expressed in pg per cell. The tested concentrations were 1, 10, and 100 µM. Melanocytes were incubated with test samples for a week. Cells were lysed and incubated O/N in 1 M NaOH. Melanin content was determined by comparing the absorbance results with a standard curve generated from synthetic melanin.

Table 2 further supports our findings regarding the toxicity of HQ on melanocytes compared to peptides P3 and P4. After a 24-hour incubation of melanocytes with 10 µM HQ, viability staining with trypan blue indicated that 27% of cells were dead. This was one order of magnitude greater than that for P4. The toxic effects of HQ were dose-dependent and at concentrations of 30 and 100 µM, 41 and 98% of melanocytes were no longer viable, respectively. The highest rate of cell death for P4 was 18% after incubating the melanocytes with 1 mM of P4 for 6 days. At concentrations >100 µM for HQ,

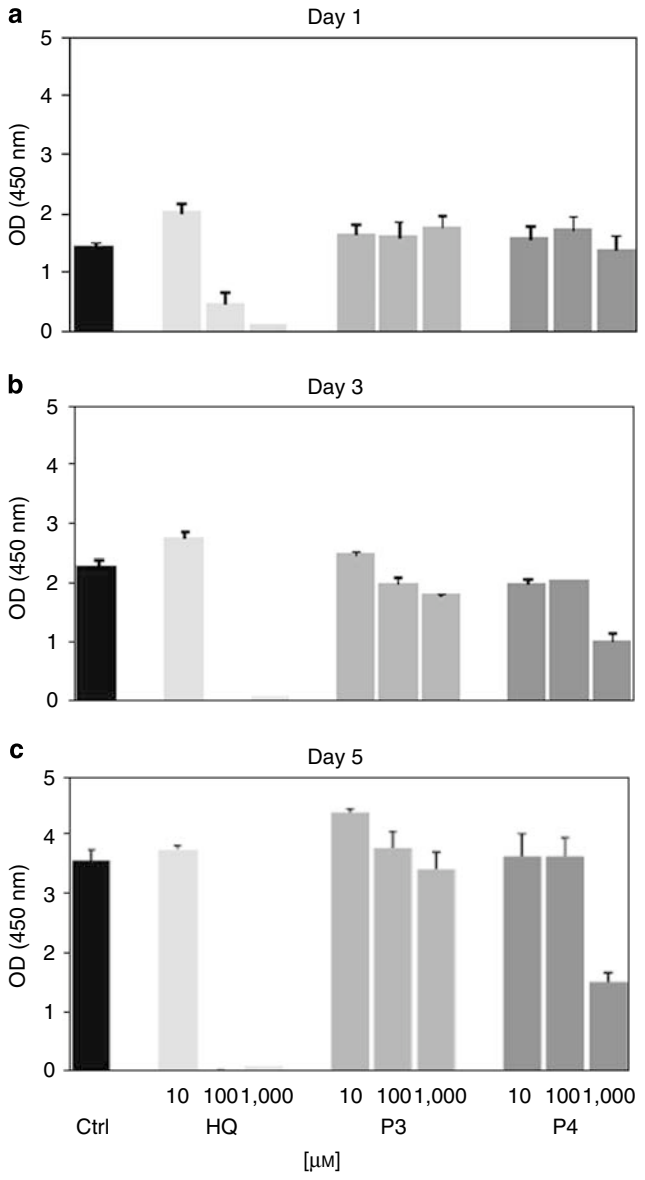

Figure 6. Proliferation of human melanocytes treated with HQ, P3, and P4. A total of 25,000 human melanocytes per well were incubated at 37 °C in the presence of 10, 100, 1,000 μM HQ, P3, and P4 in Medium 254 for (**a**) 1, (**b**) 3, and (**c**) 5 days. After the incubation, 10 μl WST-1 was added to each well and absorbance was read at 450 nm after 4 hours incubation at 37 °C.

there was 100% cell death starting within 24 hours of exposure. P3 was found to be considerably less toxic to melanocytes even at concentrations as high as 1 mM (2% cell death after 6 days).

DISCUSSION

In this study, we screened an internal library for oligopeptides with inhibitory activity against mushroom and human tyrosinase. Even though the amino-acid sequence similarity between mushroom and human tyrosinase is only 23%, our initial experiments were performed using mushroom tyrosinase as earlier studies suggested a strong correlation of efficacy with agents tested against the human form (Sugimoto *et al.*, 2003). The peptide dose- and time-response curves showed that P3 and P4 were 6- and 17-fold more potent inhibitors of mushroom tyrosinase than HQ (IC_{50} values presented in Table 1). On the basis of this initial screen, we decided to further evaluate and characterize the effects of P3 and P4.

Table 2. The toxic effect of HQ, P3, and P4

	% Cell death[1]								
	Day 1			Day 3			Day 6		
Concentration [μM]	P3	P4	HQ	P3	P4	HQ	P3	P4	HQ
1	0	2	10	0	3	12	0	3	10
10	0	3	27	0	6	29	0	5	48
30	0	3	41	0	4	53	0	5	76
100	0	4	98	0	4	100	0	7	100
300	0	6	100	0	11	100	1	9	100
1,000	0	10	100	0	15	100	2	18	100

HQ, hydroquinone.
[1]Human melanocytes were incubated with HQ, P3, or P4 at the above concentrations for 1, 3, or 6 days. The mean percentage cell death for at least three independent runs is shown.

To determine the mode of inhibition, we determined Lineweaver–Burk plots of P3 and P4 and compared the kinetics to those of HQ. These studies showed that similar to HQ, P3 and P4 were competitive inhibitors of tyrosinase. Peptides P3 and P4 were also tested against fresh extracts of human tyrosinase. At a concentration of 100 μM, the peptides were able to inhibit human tyrosinase activity by 25–35%. Although the potency against human tyrosinase appeared reduced relative to that observed with mushroom tyrosinase, several experimental conditions may account for this discrepancy. First, the assay was carried out on extracts from lysed melanocytes and not purified human tyrosinase. Although protease inhibitors were included to reduce degradation of human tyrosinase, we did not optimize these conditions. Second, the catalytic site of the extracted human tyrosinase can exist in different redox states than the purified form.

We observed a lengthy lag period for tyrosine oxidation. The oxidation of L-tyrosine by tyrosinase has previously been characterized by a lag period that is affected by enzyme and substrate concentration, pH, and hydrogen donors such as L-dopa (Pomerantz and Warner, 1967). Therefore, when L-tyrosine was the substrate, L-dopa was added as a cofactor at low micromolar levels to eliminate this lag period. The ability to act as substrate requires a free electron donor group, whereas competitive inhibition, on the contrary, requires the presence of powerful electron acceptor groups (Passi and Nazzaro-Porro, 1981). These experiments also unexpectedly showed that P3 inhibited human tyrosinase more potently in the presence of L-dopa than L-tyrosine, whereas P4 showed greater inhibition in the presence of L-tyrosine as the substrate. Hearing and Ekel (1976) proposed that there are two catalytic sites on tyrosinase, one for tyrosine hydroxylase

and another site specific for dopa oxidase activity. Our findings with P3 and P4 further support the hypothesis of separate catalytic sites for L-dopa and L-tyrosine (Schallreuter *et al.*, 2008).

Despite the fact that little is known about the effect of short-sequence peptides on melanogenesis and tyrosinase inhibition, there is great potential for these agents to replace more toxic drugs such as HQ, as they comprise naturally occurring amino acids that are easily metabolized in skin. Therefore, risks of systemic absorption and liver-related mutagenesis may be avoided during the chronic treatment of hyperpigmentation disorders. Some amino acids such as L-alanine, glycine, L-isoleucine, and L-leucine have previously been shown to possess beneficial effects toward disruption of melanogenesis (Ishikawa *et al.*, 2007). Ishikawa *et al.* reported that a combination of these four amino acids had an additive effect on hypopigmentation similar to that of kojic acid. Schurink *et al.* (2007) proposed that the peptide should contain one or more arginine residues for strong tyrosinase inhibitory and binding activity. Moreover, their studies indicated that tyrosinase inhibition is optimal when arginine and/or phenylalanine is/are combined with hydrophobic aliphatic residues such as valine, alanine, or leucine. Besides hydrophobic residues, peptides containing the polar, uncharged residues cysteine and serine also showed significant inhibitory activity.

The amino acid tyrosine contains a phenol group, which makes it unique in that it can serve as a substrate or a competitive inhibitor. Oligopeptide P3 contains the combination Arg–Ala in positions 1/2 and 5/6, as well as the amino acids serine and cysteine at positions 4 and 8, respectively. The presence of these key residues may explain the strong inhibitory activity observed for P3. For P4, the presence of tyrosine at positions 1 and 10, arginine at positions 2 and 4, and serine at 3, 7, and 8 may explain the robust inhibition observed for this oligopeptide.

One of the main advantages of oligopeptides P3 and P4 is the relative absence of melanocyte cytotoxicity (Table 2). Whereas HQ was 100% toxic after a 24-hour treatment at 100 μM, P3 showed no toxic effects after 6 days of treatment. P4 also showed minimal (7%; Table 2) toxicity after the same incubation period. Treatment with 1 mM P3 resulted in a 2% toxicity rate (Table 2) and no effect on melanocyte proliferation (Figure 6). At a concentration 25-fold higher than its IC_{50} value, P4 began to show mild (18%; Table 2) melanocyte toxicity after a prolonged incubation of 6 days. We also confirmed these findings using proliferation assays, which showed that HQ was toxic to melanocytes at concentrations as low as 30 μM (data now shown), whereas mild reduction of proliferation was first noted only after 3 days of incubation with 1 mM P4 (Figure 6). Lower concentrations did not show any effect on melanocyte proliferation even after prolonged incubation for 5 days, whereas HQ dramatically reduced melanocyte proliferation within 24 hours at concentrations as low as 30 (data not shown) and 100 μM (Figure 6).

In conclusion, our findings suggest that oligopeptides P3 and P4 may be ideal tyrosinase inhibitors due to their potent enzymatic inhibitory activity, relative absence of melanocyte toxicity, and minimal effects on melanocyte proliferation rates. Further studies are under way to optimize cell penetration and oligopeptide stability for transdermal applications. We believe these oligopeptides may hold significant therapeutic potential for the treatment of skin hyperpigmentation disorders. As both oligopeptides showed substantially improved potency against tyrosinase with minimal melanocyte toxicity, clinical use of these agents may obviate the need for HQ, which derives its primary skin-lightening capability through its cytotoxic activity.

MATERIALS AND METHODS

Materials

Mushroom tyrosinase, L-tyrosine, HQ, and L-dopa were purchased from Sigma-Aldrich (St Louis, MO). The peptides (Table 1) were synthesized by NeoMPS (San Diego, CA) using solid-phase 9*H*-(f)luoren-9-yl(m)eth(o)xy(c)arbonyl chemistry.

Enzymatic assay of mushroom tyrosinase

Tyrosinase inhibition activity was determined *in vitro* using L-tyrosine as the substrate using a modified method from Piao *et al.* (2002). The concentration of enzyme, substrate, and inhibitor was denoted as [E], [S], and [I], respectively. The experiment was conducted in a 96-well flat-bottom plate. Each well contained 80 μl of 0.067 M potassium phosphate buffer (pH 6.8), 40 μl of 5 mg ml^{-1} L-tyrosine dissolved in 0.067 M potassium phosphate buffer (pH 6.8), 40 μl of the different concentrations of inhibitor in 5% DMSO solution, and 40 μl of 480 U per ml mushroom tyrosinase solution. The final volume of each well was 200 μl, containing 1 mg ml^{-1} [S], 96 U ml^{-1} [E], and varying concentrations of [I], ranging from 1 μM to 10 mM. For the negative control wells, the inhibitors were substituted with 5% DMSO solution and adjusted to the total volume of 200 μl. HQ served as the positive control in our experiments. HQ was tested in parallel with the oligopeptides at the same concentrations and same conditions. The assay mixture was incubated at 37 °C. The optical density of the reaction mixtures, which correlates with the amount of dopachrome produced, was measured using a spectrophotometer (Varioskan, Thermo Electron Corporation, Waltham, MA) at 475 nm and at different time periods.

Kinetic studies

The Lineweaver–Burk plot method was used to determine the reaction kinetics (Burk *et al.*, 1934). Experiments were conducted using the same protocol used for measuring mushroom tyrosinase activity except for changes in concentrations of [E], [S], and [I]. [E] was 24 U ml^{-1}. The series of [S] were 0.05 (or 0.07 for peptide inhibitors), 0.1, 0.15, and 0.2 mg ml^{-1}. The series of [I] were 0, 0.01, and 0.02 mM. The results were analyzed according to the Lineweaver–Burk plot method that facilitated the determination of the Michaelis constant (K_m) and maximum velocity (V_{max}).

Melanin content measurement

Melanin content of the melanocytes was measured according to the method of Naeyaert *et al.* (1991). Human melanocytes were cultured in 24-well plates and treated with individual test samples for 7 days. P3, P4, and HQ were tested at 10 and 100 μM each, all in triplicates. Media were changed every other day and fresh oligopeptide or HQ was added. After 7 days, the media were discarded and the wells

were washed with phosphate-buffered saline. Cells were detached by short incubation in trypsin/EDTA (0.25%/0.1% in phosphate-buffered saline). An aliquot was used for cell counting and the remaining cells were sonicated and incubated overnight in dark in 500 µl of 1 M NaOH at 37 °C. Melanin concentrations were calculated by comparison of the optical density at 475 nm of unknown samples with a standard curve obtained with synthetic melanin (Sigma-Aldrich). Melanin content was expressed as pg per cell.

Assay of human tyrosinase activity

The following methods are modifications of protocols adopted from Nagata *et al.* (2004) and Cheng *et al.* (2007). Human melanocytes were cultured in 100 mm dishes using Medium 254 (Invitrogen, Carlsbad, CA), incubated at 37 °C in a humidified 5% CO_2 chamber. At 80–90% confluence, cells were harvested and lysed using 1% Triton X-100 in 0.067 M potassium phosphate buffer (pH 6.8). Cells were then sonicated twice at 20% intensity for 30 seconds (on ice). Lysates were clarified by centrifugation at 10,000 *g* for 10 minutes. Protein content was determined using a Bio-Rad protein assay kit (Richmond, CA). Lysates were added to a 96-well flat-bottom plate containing equal amounts of protein (40 µg), and adjusted with lysis buffer to reach 150 µl in each well. L-Dopa was dissolved in lysis buffer and added to each well at a final concentration of 2.5 mM. Each triplicate of wells received different concentrations (0–2 mM) of oligopeptide or HQ. The plate was incubated at 37 °C for 120 minutes, and spectrophotometric readings at 475 nm (Varioskan, Thermo Electron Corporation) were taken at 10 minutes intervals.

In certain experiments, L-tyrosine was substituted for L-dopa, and used at a final concentration 5 mM in lysis buffer. L-Dopa was also added at a final concentration of 0.005% to serve as a cofactor. Several control wells were used in the experiment. Certain wells served as controls and contained 40 µg of protein extract in lysis buffer, and substrate at varying compositions but without peptide inhibitors. Other control wells contained lysis buffer, substrate, peptide inhibitors but no protein extract (no enzyme).

WST assay for melanocyte proliferation

Melanocyte proliferation was determined using the WST-1 Cell Proliferation Kit (Roche). Cells were plated at 2.5×10^4/well in 96-well plates in a humidified atmosphere with 5% CO_2 at 37 °C. Twenty-four hours after plating, test samples were added and cultures were incubated for an additional 1, 3, or 5 days. Fresh medium and test samples were replaced every other day. At the end of the treatment period, 10 µl WST-1 mixture was added to each well containing 100 µl fresh medium. The plates were placed at 37 °C for 4 hours in the dark and the absorbance at 450 nm was read using the HTS 7000 Plus Bio Assay reader (Perkin-Elmer, Waltham, MA). Three replicate wells were measured for each group to be tested. Wells containing medium but no cells served as background controls.

Evaluation of the cytotoxic effect of P3, P4, and HQ on melanocytes

Melanocytes were cultured in three 6-well plates at a density of 4×10^5 cells per well for 1, 3, or 6 days in 2 ml of Medium 254 containing different concentrations of peptides or HQ (0, 10, 30, 100, 300, or 1,000 µM). The plates were incubated at 37 °C in a humidified 5% CO_2 chamber. Media and inhibitors were changed every 48 hours. After 24 hours, the plate labeled "Day-1" was harvested and the cells were counted in each well using a hemacytometer. The control well contained 2 ml of media without any inhibitor. The toxicity of the test sample was measured according to the following formula:

$$\frac{[1 - (\text{number of cells in control well}) - (\text{number of live cells in test sample well})]}{\text{number of cells in control well}} \times 100\%$$

The same procedure was repeated after 72 hours of incubation for the plate labeled "Day-3" and after 144 hours for the "Day-6" plate.

Statistical analysis

Each experiment was performed in triplicates and run a minimum of five independent times. The results were averaged, and standard error of the mean was calculated for all conditions using Microsoft Excel.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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