Rice (Oryza sativa) is consumed as a staple food globally, with an estimated production of 645 million tons per year. The demand for rice in Asia is predicted to increase by 70% over the next 30 years, driven primarily by population growth.1 Whole rice grains comprise 35% outside husk (hull), 60% inner starchy endosperm, and 5% bran layer over the endosperm.2 Most of the rice bran is produced by postproduction milling and is ultimately discarded as waste. However, rice bran contains protein, fiber, minerals, and physiologically active phytochemicals such as γ-oryzanol, ferulic acid, and tocopherols.1 Therefore, the utilization of rice bran biomass warrants investigation.

Human skin is repeatedly exposed to environmental DNA-damaging agents, such as ultraviolet radiation (UVR),3,4 and therefore requires numerous endogenous protective systems.5,6 Among these, skin pigmentation follows melanin deposition in the expression epidermis, and among melanin subtypes, eumelanin and pheomelanin are induced by UVR to provide a physical barrier that scatters UVR and an absorbent eumelanin and pheomelanin to provide a physical barrier that scatters UVR and an absorbent...
reaction of mushroom tyrosinase, with a half-maximal inhibitory concentration (IC$_{50}$) of 102 μM, similar to that of P4.23 TH10 is a peptide comprising 10 amino acids with a C-terminal tyrosine residue, and docking simulation analysis of various TH10 variants revealed that the C-terminal tyrosine residue is essential for inhibitory activities and functions as a substrate analogue in the tyrosinase active site.23 Accordingly, approximately half of the known tyrosinase inhibitory peptides carry C-terminal tyrosine residues and appear to have similar mechanisms of action.

In the present study, a technique for producing tyrosinase inhibitory peptides from rice bran protein (RBP) hydrolysates is demonstrated. Enzymatic hydrolysis is a predominant approach for producing bioactive peptides from food sources such as milk, soybean, wheat, rice, and barley proteins.24 Accordingly, the present simultaneous treatment with chymotrypsin and trypsin efficiently produced the above-mentioned “tyrosine-type” tyrosinase inhibitory peptides. This is the first report showing the production of tyrosinase inhibitory peptides from a food protein source.

■ RESULTS AND DISCUSSION

Tyrosinase Inhibition by RBP Hydrolysates. Previously, it was demonstrated that the C-terminal tyrosine residue plays an important role in the tyrosinase inhibitory effects of peptides.23 Accordingly, the focus was on the substrate specificity of the proteinase chymotrypsin to produce inhibitory peptides. Chymotrypsin preferentially cleaves peptide bonds at the carboxyl sides of large hydrophobic and aromatic amino acids, such as phenylalanine, tyrosine, and tryptophan, leading to the production of peptides with a C-terminal tyrosine residue. However, the frequency of these aromatic residues in general protein sequences leads to a tendency for longer average lengths of chymotrypsin-generated peptides. Thus, because most previously characterized inhibitory peptides have short sequences of 2–10 amino acid residues,18–21 the proteinase trypsin was used in addition. These two proteinases are optimally catalytic under similar physical conditions of 37–50 °C and pH 6–9.

The high absorbance of RBP suspensions at 470 nm precluded the determinations of tyrosinase inhibitory activity. Thus, after hydrolysis by double digestion with chymotrypsin and trypsin, P-6 gel column chromatography was performed and inhibitory activities of each eluted fraction were confirmed. Fractions from nonhydrolyzed RBP suspensions showed no significant inhibition against monophenolase and diphenolase reactions of tyrosinase (Figure S1, Supporting Information). Slight inhibition of the diphenolase reaction likely reflected the presence of nonprotein compounds that lack significant absorbance at 210 nm. Chromatograms for RBP hydrolysates that were produced by double digestion with chymotrypsin and trypsin indicated the moderate digestion of RBP (Figure 1A), with peptide molecular weights from the main peak of 1000–3000 Da, based on elution times of the standard protein (data not shown). However, a significant inhibition of both tyrosinase reactions was observed in fractions containing low-molecular-weight peptides of <1500 Da (Figure 1B and C). These results indicated that tyrosinase inhibitory peptides are produced following the treatment of RBP with chymotrypsin and trypsin. In further experiments, RBP suspensions were digested using the proteinase thermolysin, and P-6 gel column chromatography was performed. Under these conditions, RBP was effectively digested, and fractions containing digested peptides showed significant inhibition against both tyrosinase reactions (Figure S2, Supporting Information), indicating the presence of “tyrosine-type” and other types of inhibitory peptides. However, the inhibitory activities of eluted fractions were significantly weaker than those of chymotrypsin and trypsin digests, particularly in fractions that contained low molecular weight peptides. These data indicate that the double digestion of RBP by chymotrypsin and trypsin is an efficient technique for producing tyrosinase inhibitory peptides.

Isolation of Tyrosinase Inhibitory Peptides. Following P-6 gel column chromatography of RBP hydrolysates, eluted fractions 69–84 were combined, RBP hydrolysis and P-6 gel column chromatography steps were repeated, and a total of 4.0
g of RBP powder was treated in the same manner. Subsequently, the combined active fraction was applied to a Superdex Peptide 10/300 GL column chromatograph. The resulting chromatogram showed a broad range of peptide molecular weights (500–7000 Da; Figure 2A), indicating greater precision of peptide separation. Subsequently, fractions 7–9 significantly inhibited the monophenolase reaction of tyrosinase (Figure 2B). However, inhibitory activity against the diphenolase reaction was markedly reduced (data not shown). In further experiments, fraction 9 was subjected to RP-HPLC on a Cadenza CD-C18 column, and several peaks were separated (Figure 3A). Fractions from peaks C, D, E, F, G, H, I, and J showed significant inhibitory activity against the monophenolase reaction (Figure 3B), and peptides from active fractions were further purified by subsequent rechromatography using a Cadenza CD-C18 column.

Identification of Isolated Peptides and Tyrosinase Inhibitory Activities. Molecular weights of isolated peptides were analyzed using MALDI-TOF/MS, and amino acid sequences were determined in subsequent MS/MS analysis. Six peptides from fractions E, F, G, H, and J were identified, namely, His–Gly–Gly–Glu–Gly–Gly–Arg–Pro–Tyr (HGGEGGRPY, from peak E), Leu–Gln–Pro–Ser–His–Tyr (LQPSHY, from peak G), His–Pro–Thr–Ser–Glu–Val–Tyr (HPTSEVY, from peak H), Trp–Glu–Ala–Gly–Gln–Asp–Gln–Arg (WEAGQDQR, from peak F), Trp–Glu–Glu–Pro–Glu–Asp–Glu–Arg (WEEPEDEER, from peak H), and Asp–Thr–Val–His–Gly–Gln–Trp (DTVHGQW, from peak J), and were designated CT-1, -2, -3, -4, -5, and -6, respectively. All of these peptides have short amino acid sequences, reflecting the activities of the proteinases chymotrypsin and trypsin. Peptides CT-1 and CT-2 are separate partial nine- and six-amino acid sequences of the same uncharacterized protein (gene name: Os03g0793700), respectively, and peptide CT-3 is a partial seven-amino acid sequence of another uncharacterized protein (gene name: Os03t0197300). Peptides CT-4, -5, and -6 are partial sequences of an uncharacterized protein (gene name: Os11t0582400), the 11-S plant seed storage protein family protein (gene name: Os03t0336100), and another uncharacterized protein (gene name: Os02g0601300), respectively. Three of these six peptides carry C-terminal tyrosine residues.

Inhibitory Effects of Isolated Peptides against Tyrosinase Activity and Melanin Contents in Mouse B16 Melanoma Cells. Tyrosinase inhibitory effects of isolated peptides were determined (Figure 4). In these experiments, peptides CT-4, -5, and -6 showed no inhibitory effect (data not shown), whereas peptides CT-1, -2, and -3 significantly inhibited the monophenolase reaction of tyrosinase (Figure 4A). In particular, the IC50 of peptide CT-2 was 156 μM. However, peptides CT-1 and CT-3 were not sufficiently inhibitory to determine IC50 values. Moreover, all isolated peptides were poorly inhibitory against the diphenolase reaction of tyrosinase (Figure 4B). There is little information on peptides with strong inhibitory activities against the diphenolase reaction. The threshold of inhibitory concentrations against the diphenolase reaction may be higher than those against the monophenolase reaction. In the previous study, an inhibition mechanism for “tyrosine-type” tyrosinase inhibitory peptides such as TH10 was proposed. Moreover, docking simulation analyses suggested that these peptides act as substrate analogues and prevent L-tyrosine from approaching the active site of tyrosinase. In the present study, the binding mode of the peptide CT-2 was also investigated via using a simulation analysis with the mushroom tyrosinase structure. These experiments showed that the C-terminal tyrosine residue of CT-2 binds the copper-containing active site of tyrosinase in a similar manner to TH10 (C-terminal short sequence of TH10; Figure S3, Supporting Information), suggesting that CT-2 has high affinity for the active site of tyrosinase and acts as a substrate analogue. On the other hand, it remains unknown why CT-2 had a greater inhibitory effect than the other isolated peptides. As indicated in the previous report, whereas the C-terminal tyrosine residue in “tyrosine-type” tyrosinase inhibitory peptides is the greatest contributor to inhibitory activities, adjacent amino acid residues may also play significant roles. Furthermore, because most previously characterized inhibitory peptides have short sequences, as described above, long sequence structures may cause steric hindrance of the interactions between the two.

Among previously reported tyrosinase inhibitors, many have not been shown to inhibit melanin production in melanoma cells. Therefore, assays of melanin contents and cell viability were performed to determine the effects of peptides CT-1, -2, and -3 on melanogenesis and to evaluate their cytotoxicity. Peptides CT-1 and -3 increased melanin production in mouse B16 melanoma cells, whereas peptide CT-2 inhibited melanin production.
production by >50% at 500 μM and was as effective as the arbutin positive control (Figure 5A). Furthermore, none of the present isolated peptides were cytotoxic in mouse B16 melanoma cells, and significant increases in cell growth were observed following peptide treatments, particularly in the presence of peptide CT-2 (Figure 5B). However, effective concentrations of CT-2 (micromolar) were much higher than those of epidermal growth factor (picomolar), which is a well-known growth factor that stimulates cell growth, proliferation, and differentiation.\textsuperscript{25} Hence, although CT-2 is not applicable as a growth factor, the present cytotoxicity data indicate that CT-2 can be safely applied as a skin-whitening agent. Overall, these data demonstrate that CT-2 inhibits mushroom tyrosinase activity and potently inhibits melanogenesis in mouse B16 melanoma cells without causing cytotoxicity.

In contrast, peptides CT-1 and -3 did not inhibit melanin production in mouse B16 melanoma cells, suggesting that these peptides do not inhibit mammalian tyrosinase. These observations may reflect differences in active site structures between mammalian and mushroom tyrosinases, which are well conserved but differ in some active site amino acid residues.\textsuperscript{26,27} Furthermore, PeptideCutter analysis (http://web.expasy.org/peptide_cutter/) indicated that CT-2 has almost no cleavage sites for major proteinases, except for pepsin and proteinase K, which readily lose specificity under certain conditions. In contrast, CT-1 and -3 carry cleavage sites for other major proteinases such as Arg-C and glutamyl endopeptidases. This property may affect stability and the ensuing inhibition of melanogenesis after absorption into cells. Alternatively, appropriate hydrophobicity and molecular weight are considered requirements of skin permeability,\textsuperscript{28,29} and whereas identified peptides have similar hydrophobicity, the molecular weight of CT-2 (743.8) is lower than those of CT-1 (928.9) and CT-3 (831.8). Previously, Bos and co-workers proposed a “500 Da rule” for skin penetration of chemical compounds and drugs.\textsuperscript{28} Hence, if this rule is applicable to peptide drugs, low molecular weight CT-2 may be a more efficient drug candidate.

A total of 4.0 g of RBP powder was used, and fraction 9, which was separated by Superdex Peptide 10/300 GL column chromatography, contained 19.2 mg of dry weight of peptide (data not shown). As calculated from the eluted peak area in the subsequent RP-HPLC separation, peak G (3.84% of total peak area) contained 0.73 mg of CT-2, indicating that 4.0 g of RBP powder contained at least 0.73 mg of CT-2.

Figure 3. Separation of tyrosinase inhibitory peptides by RP-HPLC using a Cadenza CD-C18 column. (A) Chromatogram of eluted peptides; numbers of peak fractions are shown above each peak. (B) Inhibitory activity of each fraction against the monophenolase reaction of tyrosinase; all procedures are described below.
In conclusion, in this study it was shown that RBP is a potent source of tyrosinase inhibitory peptides, and a technique was developed for isolating these peptides from RBP. Specifically, simultaneous treatments of RBP with chymotrypsin and trypsin led to efficient production of tyrosinase inhibitory peptides. Among identified peptides, CT-1, -2, and -3 significantly inhibited the monophenolase reaction of tyrosinase, and CT-2 potently inhibited melanogenesis in mouse B16 melanoma cells without causing cytotoxicity. This is the first report to show production of tyrosinase inhibitory peptides from a food protein source. Furthermore, the present data (Figures 1, 2, and 3) show that double digestion of RBP hydrolysates with chymotrypsin and trypsin increased the concentrations of tyrosinase inhibitory peptides, as indicated by numerous peptide peaks with inhibitory activity in chromatograms. Hence, the present data warrant further investigations of this technique for wide application to other natural protein sources.

**■ EXPERIMENTAL SECTION**

**Materials.** RBP 55 (protein contents, 55%) was kindly provided by Tsuno Food Industrial Co., Ltd. Trypsin (trypsin from porcine pancreas, lyophilized powder, 36 USP trypsin units/mg protein, catalog number: C4129) and chymotrypsin (α-chymotrypsin from bovine pancreas, lyophilized powder, 60 BTEE units/mg protein, catalog number: C4129) were purchased from Wako Pure Chemicals Ltd. (Osaka, Japan) and Sigma–Aldrich (St. Louis, MO, USA), respectively. Mushroom tyrosinase (EC 1.14.18.1) (lyophilized powder, catalogue number: T3824), t-tyrosine (catalogue number: T3754), and l-dopa (catalogue number: D9628) were also purchased from Sigma–Aldrich. The specific activity of the tyrosinase was 1320 U/mg (one unit = ΔA280 of 0.001 per min at pH 6.5 at 25 °C in a 3 mL reaction mixture containing l-tyrosine). All other reagents were of analytical grade and were purchased from Wako Pure Chemicals Ltd.

**Preparation of RBP Hydrolysate.** RBP powder (1.0 g) was suspended in ultrapure water (30 mL), and the pH was adjusted to 6.5 using NaOH prior to homogenization in a Polytron homogenizer (Kinematica; Bohemia, NY, USA). After dialysis against ultrapure water using Spectra/Por 1 dialysis tubing (molecular weight cutoff, 6–8 kDa; Spectrum Laboratories, Inc., Rancho Dominguez, CA, USA), RBP solution was hydrolyzed by incubating with 20 mg of trypsin and 20 mg of chymotrypsin at 50 °C for 6 h. These enzymes were inactivated by incubating at 90 °C for 10 min, and after centrifugation at 12000g for 30 min at 4 °C, the clear supernatant was freeze-dried and used in subsequent isolation steps. Because trypsin from Wako Pure Chemicals Ltd. and chymotrypsin from Sigma–Aldrich have similar optimum pH (6–9) and temperature (37–50 °C), these conditions were used in all experiments with these proteases.

**Isolation of Tyrosinase Inhibitory Peptides.** Dried RBP hydrolysate (656 mg) was dissolved in ultrapure water (15 mL) and was applied to a Bio-Gel P-6 gel column (2.5 × 110 cm; Bio-Rad; Hercules, CA, USA) that had been equilibrated using ultrapure water. Hydrolyzed peptides were eluted using ultrapure water (150 mL), and 6 mL fractions were collected every 6 min. Peptides in each fraction were freeze-dried and dissolved into small volumes of ultrapure water (300 mg/mL peptide concentration), and inhibitory activities against tyrosinase were determined. RBP hydrolysis and P-6 gel isolation steps were repeated, and a total of 4.0 g of RBP powder was treated as described above. Subsequently, all fractions containing tyrosinase inhibitory peptides were combined and applied to a Superdex Peptide 10/300 GL column (GE Healthcare; Uppsala, Sweden) that had been equilibrated with ultrapure water. Peptides were eluted using ultrapure water (25 mL) with an AKTA purifier (GE Healthcare), and 1 mL fractions were collected every 2 min. Peptides in each fraction were freeze-dried and dissolved in ultrapure water (30 mg/mL peptide concentration). Active fractions were combined and freeze-dried again, and the peptides were dissolved in 0.1% TFA in H2O and were further separated using RP-HPLC with a Cadenza CD-C18 column (150 × 10 mm, 3 μm; Imtakt Co., Kyoto, Japan). The HPLC system was further investigated.
Peptide Synthesis. Peptides were synthesized using the Fmoc solid-phase method with a PSSM-8 automated peptide synthesizer (Shimadzu) and were purified to >98% purity using RP-HPLC with a Cadenza CD-C18 column. Molecular masses of purified peptides were confirmed using MALDI-TOF/MS data with an Autotof III TOF/TOF (Bruker). Stock solutions of peptides were prepared in 10% (v/v) DMSO.

Enzyme Assays. Tyrosinase catalyzes the two-step oxidative reaction from L-tyrosine to L-dopaquinone. Specifically, during the initial monophenolase reaction, tyrosinase hydroxylates L-tyrosine into L-dopa in the presence of oxygen, and in the subsequent diphenolase reaction, L-dopa is oxidized into L-dopaquinone, which spontaneously decays into dopachrome. Dopachrome is a brown compound with a peak absorbance at 475 nm (molar extinction coefficient, ε = 3700 M⁻¹ cm⁻¹).

Monophenolase activity was measured using a method similar to that reported by Chen and co-workers. In brief, 100 μL of 2.0 mM L-tyrosine, 45 μL of 0.1 M phosphate buffer (pH 6.5), and 5 μL of a peptide inhibitor solution or ultrapure water were placed in a 96-well plate and were preincubated at 30 °C for 10 min. After addition of 50 μL of 1320 units/mL mushroom tyrosinase to the mixture, the plates were incubated at 30 °C and were monitored at 475 nm using a microplate reader. Lag times were estimated by extrapolation of the linear portion of the L-dopa chrome accumulation curve to the abscissa axis. The remaining monophenolase activity (%) after treatment with the peptide inhibitor was calculated as follows: \( A = (C/D) \times 100 \), where \( A \) represents the reciprocal of lag time (s) in the presence of peptide inhibitor, and \( B \) represents the reciprocal of the lag time (s) without peptide inhibitor (ultrapure water). IC₅₀ values were manually estimated from inhibition curves.

Diphenolase activity was determined using a similar method to that reported by Chen and co-workers. In brief, 100 μL of 2.0 mM L-dopa, 45 μL of 0.1 M phosphate buffer (pH 6.5), and 5 μL of a peptide inhibitor solution or ultrapure water were placed in a 96-well plate and preincubated at 30 °C for 10 min. After addition of 50 μL of 1320 units/mL mushroom tyrosinase to the mixture, the reaction proceeded at 30 °C and was monitored at 475 nm using a microplate reader. The remaining diphenolase activity (%) after peptide inhibitor treatment was calculated as follows: \( B = (C/D) \times 100 \), where \( B \) represents the absorbance change per min at 475 nm (ΔABS₄₇₅/min) in the presence of peptide inhibitor, and \( D \) represents the reaction rate (ΔABS₄₇₅/min) without peptide inhibitor (ultrapure water). The tyrosinase inhibitor arbutin was used as a positive control. All measurements were performed three times.

Cell Viability Assay. Mouse B16 melanoma cells were maintained in Eagle’s minimum essential medium with Earle’s salts supplemented with 10% (v/v) fetal bovine serum and 100 units/mL penicillin (Invitrogen; Carlsbad, CA, USA) at 37 °C in a 5% CO₂ atmosphere. Cell viability was determined using an MTT-based assay 3 days after treatment with peptides; cells were treated with each peptide at indicated concentrations. Arbutin was used as a positive control. Data are presented as means of three independent experiments ± standard deviations (SD).

Figure 5. Effects of isolated peptides CT-1, -2, and -3 on melanin contents and cell viability in mouse B16 melanoma cells. (A) Relative melanin contents 3 days after treatment with peptides. (B) Cell viability was determined using MTT-based assays 3 days after treatment with peptides; cells were treated with each peptide at indicated concentrations. Arbutin was used as a positive control. Data are presented as means of three independent experiments ± standard deviations (SD).
with 5% or 10% (v/v) fetal bovine serum (FBS; Moregate Biotech; Bulimba, Australia) and 1% penicillin–streptomycin (10 000 U/mL; Thermo Fisher Scientific; Waltham, MA, USA) in 5% CO2 at 37 °C. Cells were seeded at 1.0 × 104 cells/well in 200 μL of medium in 96-well culture plates and were incubated for 24 h. Medium was replaced with 200 μL of fresh medium containing 5% FBS and various concentrations (125, 250, or 500 μM) of each identified tyrosinase inhibitory peptide. Cells were maintained under these conditions for 3 days, and viability was then assayed using a Premix WST-1 cell proliferation assay system (Takara Bio Inc.; Shiga, Japan) according to the manufacturer’s instructions. As described above, each peptide solution contains DMSO. Therefore, cells under the same DMSO conditions (and without peptides) were used as a background control in each assay. All assays were performed three times.

**Measurements of Melanin Contents.** Melanin contents were measured using a previously described method54 with some modifications. In brief, mouse B16 melanoma cells were seeded at 1.5 × 104 cells/well in 3 mL of medium in six-well culture plates and were incubated for 24 h. Subsequently, media was replaced with 3 mL of fresh medium containing 5% FBS and various concentrations (125, 250, or 500 μM) of identified tyrosinase inhibitory peptides. Cells were maintained under these conditions for 3 days and were recovered by treating with 750 μL of 0.25% trypsin solution containing 1 mM EDTA. Recovered cells were washed with PBS and were lysed with 225 μL of 1 M NaOH for 1 h at 60 °C, and absorbance was measured at 420 nm. The tyrosinase inhibitor arbutin was used as a positive control, and the cells under the same DMSO conditions without peptides were used as a background control in each assay. All measurements were performed three times.

**ASSOCIATED CONTENT**

3 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.6b00449.

P-6 gel column chromatography for tyrosinase inhibitory peptides following the treatment of RBP with or without thermolysin; docking simulation analyses (PDF)

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**Notes**

The authors declare no competing financial interest.

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