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Antioxidative Properties of Tripeptide Libraries Prepared by the Combinatorial Chemistry

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Two series of combinatorial tripeptide libraries were constructed, based on an antioxidative peptide isolated from a soybean protein hydrolysate. One was a library of 108 peptides containing either His or Tyr residues. Another was a library of 114 peptides related to Pro−His−His, which had been identified as an active core of the antioxidative peptide. The antioxidative properties of these libraries were examined by several methods, such as the antioxidative activity against the peroxidation of linoleic acid, the reducing activity, the radical scavenging activity, and the peroxynitrite scavenging activity. Two Tyr-containg tripeptides showed higher activities than those of two His-containing tripeptides in the peroxidation of linoleic acid. Tyr−His−Tyr showed a strong synergistic effects with phenolic antioxidants. However, the tripeptide had only marginal reducing activity and a moderate peroxynitrite scavenging activity. Cysteine-containing tripeptides showed the strong peroxynitrite scavenging activity. Change of either the N-terminus or C-terminus of Pro−His−His to other amino acid residues did not significantly alter their antioxidative activity. Tripeptides containing Trp or Tyr residues at the C-terminus had strong radical scavenging activities, but very weak peroxynitrite scavenging activity. The present results allow us to understand why protein digests have such a variety of antioxidative properties.

KEYWORDS: Antioxidative peptide; combinatorial chemistry; peptide library, antioxidant; radical scavenger

INTRODUCTION

The antioxidative activities of peptides generated from the digestion of various proteins have been reported (*1*−*6*). We isolated six antioxidative peptides from the proteolytic digest of a soybean protein. (*7*). The peptides were composed of 5−16 amino acid residues, including hydrophobic amino acids, Val or Leu, at the N-terminal positions, and Pro, His, or Tyr in the sequences. On the basis of the smallest peptide, Leu−Leu−Pro−His−His (LLPHH), 28 structurally related peptides were synthesized, and their antioxidative activities against the peroxidation of linoleic acid were compared in an aqueous system. Pro−His−His (PHH) was found to be the most active among the peptides tested (*8*). Further study with 22 synthetic peptides containing His residues demonstrated that His-containing peptides can act as a metal-ion chelator, an active-oxygen quencher, and a hydroxyradical scavenger (*9*). The results obtained so far indicate that the overall antioxidative activities of peptides are attributed to the cooperative effects of these properties. However, there is still little information concerning antioxidative properties of peptides.

In this study, we constructed two series of combinatorial tripeptide libraries to explore antioxidative properties of peptides; one was composed of 108 peptides containing either two His or Tyr residues in the peptides and the other was composed of 114 peptides structurally related to Pro−His−His. The antioxidative activities of the libraries were examined by several methods, including the antioxidative activity against the peroxidation of linoleic acid, the reducing activity, the radical scavenging activity, and the peroxynitrite scavenging activity.

MATERIALS AND METHODS

Materials. Linoleic acid (∼99%) and myoglobin (from horse skeletal muscle: 95−100%) were purchased from Sigma Chemical (St. Louis, MO), and D-δ-tocopherol (∼86%) was from Eisai (Tokyo, Japan). Butylated hydroxyanisole (BHA) was from Tokyo Kasei (Tokyo, Japan). DL-α-Tocopherol (∼96%), 2,4,6-tripyridyl-s-triazine (TPTZ), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), and diethylenetriaminepentaacetic acid (DTPA) were from Nacalai Tesque (Kyoto, Japan). Dichlorodihydrofluorescein (DCDHF) was from Molecular Probes (Eugene, OR). Amino acid derivatives, coupling reagents, and resins for peptide-assembly were SynProPep

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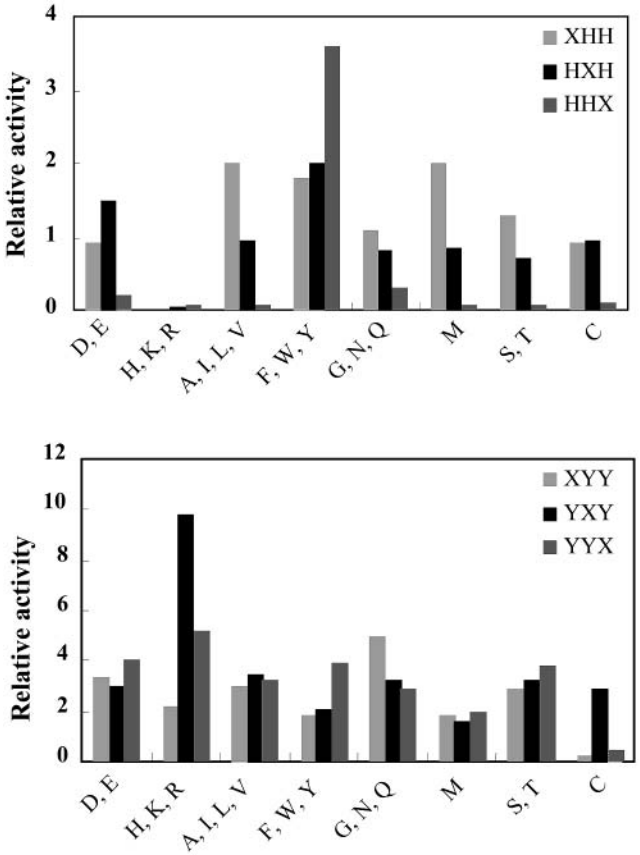

Figure 1. Antioxidative activity of two His- or two Tyr-containing tripeptide libraries in an aqueous autoxidation system of linoleic acid. The peptide library was constructed by incorporating amino acids, which were classified into eight categories, depending on side chain groups, in the position X. The concentration of each peptide were 40 μM (final concentration). The activity was measured by the ferric thiocyanate method. The results are shown as relative activities by adjusting the control to 1.0, and are the averages of three independent experiments.

reagents (Shimadzu Scientific Research Inc., Kyoto, Japan). All other reagents were of analytical grade from Nacalai Tesque or Wako Pure Chemical (Osaka, Japan).

Peptide Synthesis. Peptides were prepared by the solid-phase synthesis with the fluorenylmethoxycarbonyl (Fmoc)-strategy, using an automated simultaneous multiple peptide synthesizer (model PSSM-8, Shimadzu) as described previously (*10*), manual multiple synthesis using multiple LibraTube (HiPep Laboratories, Kyoto, Japan) or Module SRM96 (HiPep Laboraories) (*11*, *12*). After cleavage from resin, high quality peptides were obtained. The quality and identity of the resulting peptides were confirmed by reversed phase HPLC, with a linear gradient elution with 0.01 M HCl and acetonitrile at a flow rate of 1.0 mL/min and monitored at 210 nm., and by mass spectrometry on a Shimadzu-Kratos Kompact III (Manchester, UK).

Antioxidative Activity in an Aqueous Autoxidation System. For oxidation, 1.0 mL of 0.1 M sodium phosphate buffer (pH 7.0), 0.5 mL of distilled water, and 1.0 mL of 50 mM linoleic acid in ethanol (99.5%) were mixed in a glass test tube (5 mL volume). Test samples were added with the aforementioned buffer or ethanol, and the total volume was fixed uniformly. The tubes were tightly sealed with silicon rubber caps and kept at 60 °C in the dark. Aliquots of the reaction mixtures were withdrawn at intervals with a microsyringe for the measurement of antioxidative activity by the ferric thiocyanate method (*13*).

To the reaction mixture (50 μL) was added 75% ethanol (2.35 mL), 30% ammonium thiocyanate (50 μL), and 20 mM ferrous chloride solution in 3.5% HCl (50 μL). After 3 min, the absorbance of the colored solution was measured at 500 nm in a 1-cm cuvette with a Jasco model Ubest 30 spectrophotometer (Tokyo, Japan). The number of days taken to attain the absorbance of 0.3 was defined as the

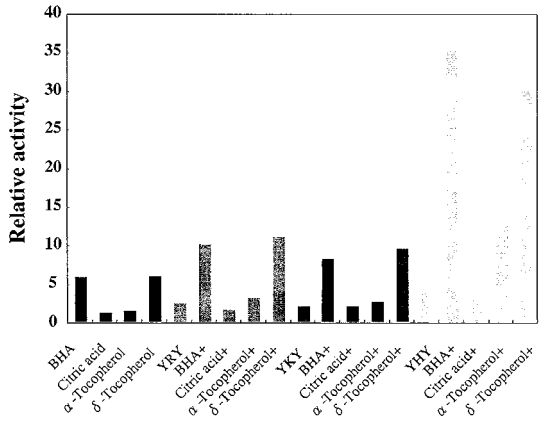

Figure 2. Synergistic antioxidative activity of YRY, YKY, and YHY, with nonpeptidic antioxidants in an aqueous autoxidation system of linoleic acid. The peptide concentrations were 40 μM (final concentration). BHA (100 μM, final concentration), citric acid (100 μM), α-tocopherol (10 μM), or δ-tocopherol (10 μM) were mixed with the tripeptides. The activity was measured by the ferric thiocyanate method. The results are shown as relative activities by adjusting the control to 1.0 and are the averages of three independent experiments.

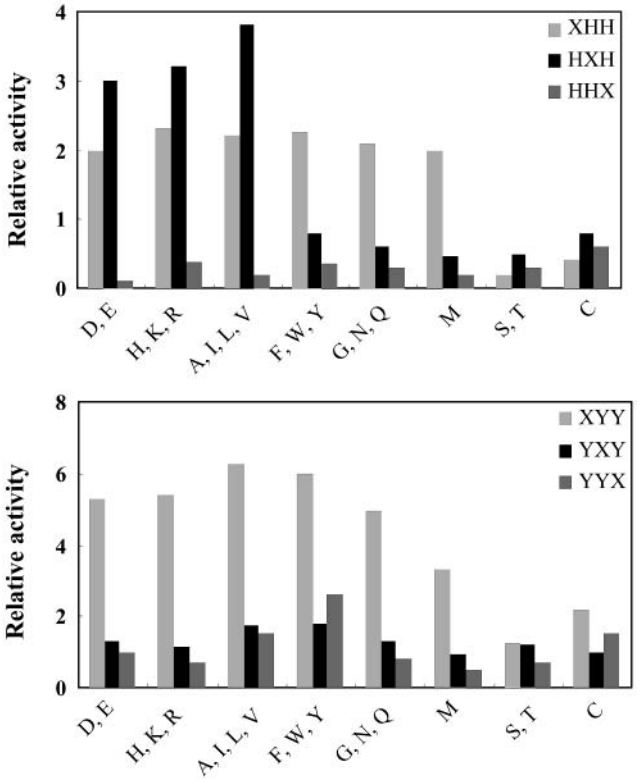

Figure 3. Reducing activity of two His- or two Tyr-containing tripeptide libraries. The peptide library was constructed as described in Figure 1. The final concentration of each peptide was 40 μM. The activity was measured by the colored ferrous-TPTZ. The results are shown as relative activities by adjusting the control to 1.0, and are the averages of three independent experiments.

induction period. The relative antioxidative activity was calculated by dividing the induction period of test samples by that of the control. All tests and analyses were run in triplicate and averaged.

Reducing Activity. The reducing activity was measured by the colored ferrous−TPTZ complex formed upon reduction of ferric ions (*14*). The TPTZ solution was prepared by mixing 25 mL of 0.3 M sodium acetate buffer (pH 3.6), 2.5 mL of 10 mM TPTZ in 40 mM HCl, and 2.5 mL of 20 mM $FeCl_3$ in water. Samples (20 μL) were

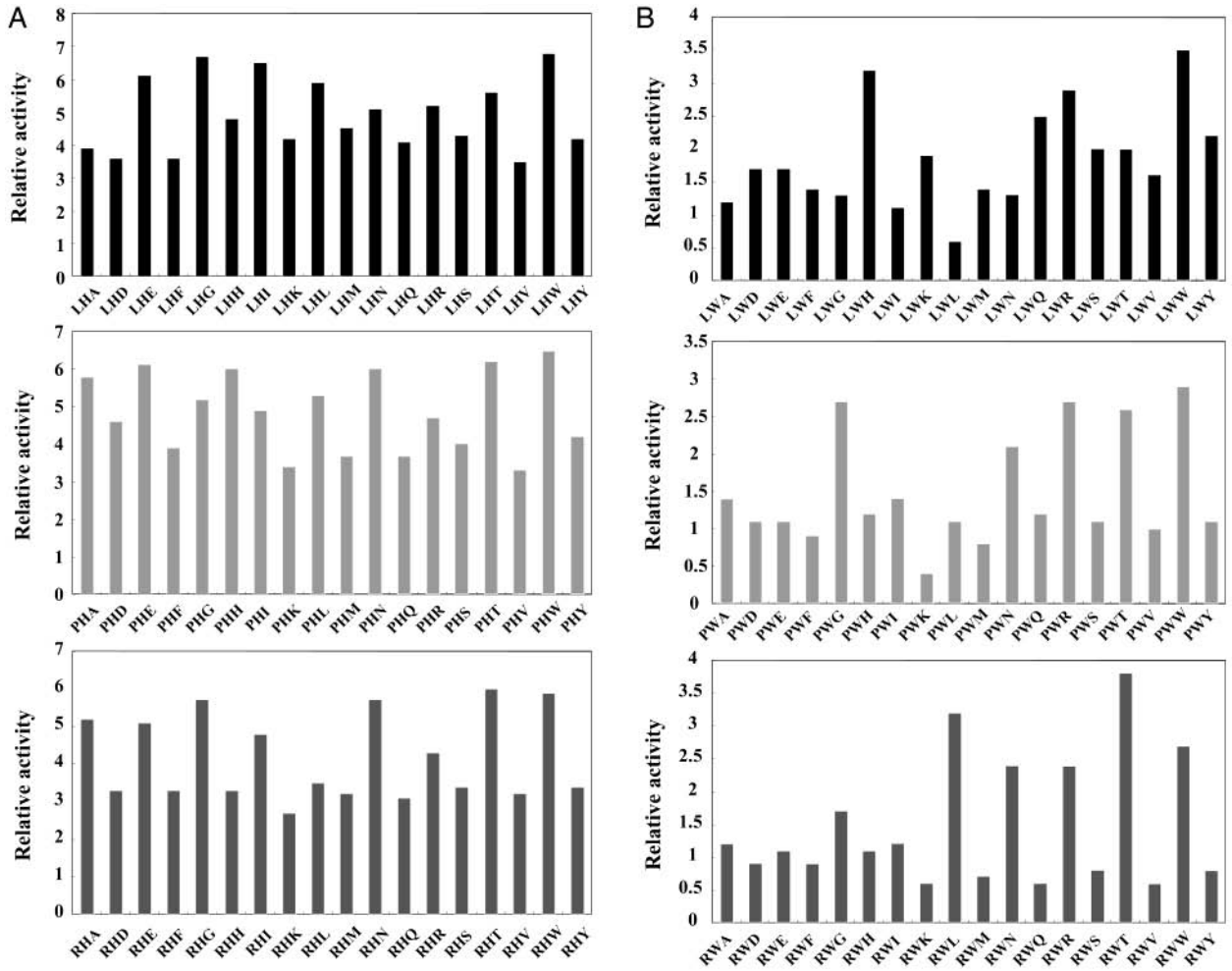

Figure 4. Antioxidative activity of PHH and its structurally related peptides in an aqueous autoxidation system of linoleic acid. The final concentration of each peptide was 40 μM. The activity was measured by the ferric thiocyanate method. The results are shown as relative activities by adjusting the control to 1.0, and are the averages of three independent experiments.

added to the TPTZ solution (150 μL) in a 96-well microtiter plate. After incubating at room temperature for 60 min, the absorbance of the reaction mixture was measured at 593 nm with a titer plate reader (SJeia autoreader model ER-8000, Tokyo, Japan). Aqueous Fe^{2+} solutions in the range of 10−1000 μM were used for calibration.

Radical Scavenging Activity. The radical scavenging activity was measured by spectrophotometric changes of the ABTS radical cation, using Trolox as a standard (*15*). Samples (20 μL) were mixed with 125 μL of PBS (138 mM NaCl in 5 mM sodium phosphate buffer, pH 7.5), 25 μL of 24.5 μM metmyoglobin (MetMb), and 50 μL of 735 μM ABTS in a 96-well microtiter plate. After the addition of 25 μL of 735 μM H_2O_2, the absorbance at 734 nm was recorded with a titer plate reader. Solutions of known Trolox concentration were used for calibration. The relative activity was calculated by using the Trolox calibration curve and converted to the Trolox equivalent antioxidant capacity (TEAC) value. The TEAC is equal to the micromolar concentration of a Trolox solution having the antioxidant capacity equivalent to a 100 μM solution of the substance under investigation.

Peroxynitrite-Scavenging Activity. Peroxynitrite-scavenging activities of tripeptides were determined by their abilities to inhibit the formation of dichlorofluorescein (DCF) from DCDHF induced by peroxynitrite (*16*). Peroxynitrite was prepared as previously described (*17*). Hydrogen peroxide (0.7 M) in 0.6 M HCl (5 mL) was mixed with 5 mL of 0.6 M KNO_2 on an ice bath for 1 s. The reaction was quenched with 5 mL of ice-cold 1.2 M NaOH, and the reaction mixture was then left overnight at −20 °C. The resulting yellow liquid layer on the top of the frozen mixture was collected for the experiment. The concentration of peroxynitrite was determined spectrophotometrically at 302 nm, using a molar absorption coefficient of $1670 M^{-1}cm^{-1}$.

To 1.0 mL of 0.1 mM DTPA in 0.1 M sodium phosphate buffer (pH 7.4) in a 3-mL glass cuvette, 0.5 mL of sample solution and 0.5 mL of 0.1 mM DCDHF were added at 37 °C. By the addition of 50 μL of 2.5 μM peroxynitrite solution, the reaction was initiated and monitored for 3 min by measuring the absorbance at 500 nm. The final concentration of sample peptides in the reaction mixture was adjusted to 80 μM.

RESULTS

Antioxidative Activity in an Aqueous Autoxidation System. Antioxidative activities of the tripeptide library were examined against the peroxidation of linoleic acid (**Figure 1**). The peptide library, consisting of two His or two Tyr residues, was constructed by incorporating various amino acids, which were classified into eight categories depending on side chain groups, to the position X. The peptides containing two Tyr residues had higher activities than the corresponding peptides containing two His residues at the concentration of 40 μM. Among the tested peptides, Tyr−(His, Lys, Arg)−Tyr showed the highest antioxidant activity.

Further, a sub-library consisting of Tyr−His−Tyr (YHY), Tyr−Lys−Tyr (YKY) and Tyr−Arg−Tyr (YRY) were prepared to define the most active peptide. Thus, YHY showed the highest activity (**Figure 2**). The peptide showed strong synergestic effects in the presence of BHA and δ-tocopherol, whereas neither citric acid nor α-tocopherol had an effect. In particular,

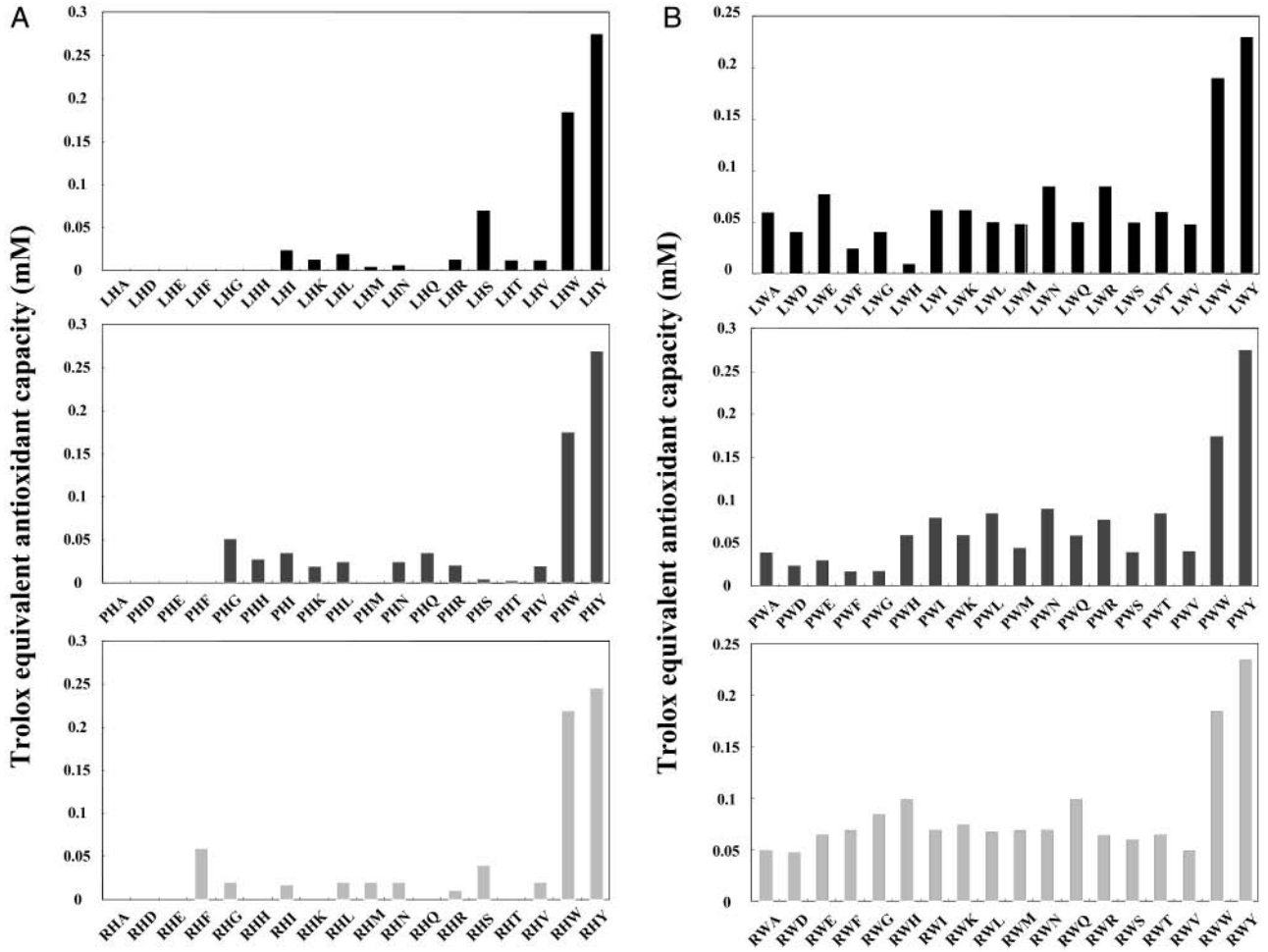

Figure 5. Radical scavenging activities of PHH and its structurally related peptides. The final concentration of each peptide was 8.2 μM. The relative activity was calculated by using a Trolox calibration curve, and converted to the Trolox equivalent antioxidant capacity (TEAC) value. The results are the avarages of three independent experiments.

YHY exerted more than 30-fold larger synergistic effect than did the control. The antioxidative activities of YRY and YKY did not show increased synergestic actions in the presence of the nonpeptidic antioxidants. The above three tripeptides were not synergistic with each other.

Reducing Activity. The reducing activity of tripeptides was examined using the colored ferrous−TPTZ complex (**Figure 3**). Upon reduction of ferric ion to ferrous ion in the test solutions, the colored complex forms and the reducing activity can be measured by the absorbance at 593 nm. Peptides showed different patterns in the activities from the antioxidative activities against the peroxidation of linoleic acid. Although Y(H, K, R)Y exerted the highest activity in the peroxidation of linoleic acid system, it showed only moderate activity. XYY had higher reducing activity than other peptides. The activities were comparable to those of BHA, α-tocopherol, and δ-tocopherol (data not shown).

Antioxidative Activities of PHH Related Peptides. On the basis of PHH, we constructed 108 structurally related peptides and measured their antioxidative activities against the peroxidation of linoleic acid in an aqueous system. The replacement of C-terminal His residue of PHH with other amino acid residues did not show a significant change in their activity (**Figure 4a**). On the other hand, the replacement of the central residue of PHX with Trp largely decreased the activities (**Figure 4b**). The replacement of the N-terminal Pro residue of PHX or PWX with Leu or Arg residue did not affect the activities. These results indicate that N- and C-terminal residues of PHH are not as important as the His residue in the middle position. This is supported by the result that the substitution of the second L-His with D-His diminished the activity (*8*).

Radical Scavenging Activity. A spectrophotometric technique was used to measure the relative abilities for scavenging the ABTS radical cations for comparison with the potency of Trolox. Tripeptides gave quite different patterns in the radical scavenging activities from those of their antioxidative activity against the peroxidation of linoleic acid (**Figure 5**). It is apparent that the peptides containing Trp or Tyr residue at the C-terminus have high activities. The replacement of His residue in the middle of PHX with Trp slightly increased the activities. The synergistic effect of peptides with nonpeptidic antioxidants was not observed in the radical scavenging activity (data not shown).

Figures 6 and **7** show the time courses of the spectral changes corresponding to the ABTS radical cations. Trolox inhibited the formation of the radical cation as well as scavenging the radical, as indicated by an induction time in the absorbance curve. On the other hand, tripeptides did not inhibit the formation, but clearly scavenged the ABTS radical cation, as shown by a post-addition assay (**Figures 6b** and **7b**). That is, the absorbance of the radical cations decreased upon addition of active peptides or Trolox. PHH could not scavenge the radical cations. The ABTS assay is very useful for the assessment of total antioxidant capacity; however, one has to consider that the assay does not distinguish between a scavenger effect and

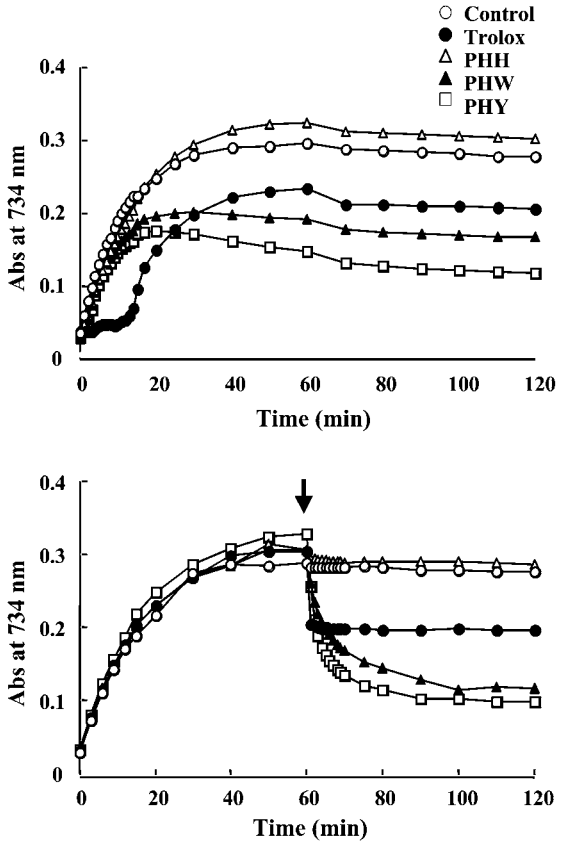

Figure 6. Time course of ABTS radical cation formation in the presence of various tripeptides and Trolox. The reaction was followed by measuring the absorbance at 734 nm for 120 min. ○ = Control, ● = Trolox, △ = PHH, ▲ = PHW, □ = PHY. The final concentration of each sample was 8.2 μM. The results are the averages of three independent experiments. (a) The reaction was started in the presence of samples. (b) The samples were added to the reaction mixture when the formation of the ABTS radical cations reached a maximum.

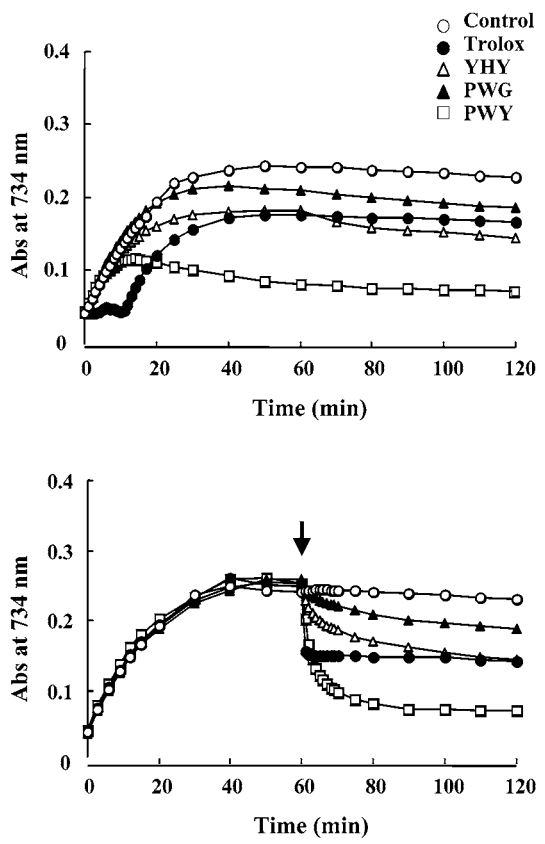

Figure 7. Time course of ABTS radical cation formation in the presence of various tripeptides and Trolox. The reaction was followed by measuring the absorbance at 734 nm over 120 min. ○ = Control, ● = Trolox, △ = YHY, ▲ = PWG, □ = PWY. The final concentration of each sample was 8.2 μM. The results are the averages of three independent experiments. (a) The reaction was started in the presence of samples. (b) The samples were added to the reaction mixture when the formation of the ABTS radical cations reached a maximum.

a decrease in rate of ABTS radical formation. This disadvantage could be overcome by the post-addition assay (*18*) as shown in this study.

Peroxynitrite-Scavenging Activity. Among the tripeptides tested, cysteinyl resdidue-containing tripeptides except for Tyr−Cys−Tyr (YCY) had high activities (**Figure 8**). Interestingly, the tripeptides containing Trp or Tyr at the C-terminus showed none or only weak activity in the peroxynitrite-scavenging and differed from the scavenging activity of the ABTS radical cations (**Figure 9**).

DISCUSSION

Many proteins have been shown to have antioxidative activities upon hydrolysis (*1*−*7*). To gain insight into the antioxidative mechanism of protein hydrolysates, the antioxidative properties either of amino acids or peptides have been investigated (*19*−*21*). Several amino acids, such as Tyr, Met, His, Lys, and Trp, are generally accepted to be antioxidative and exhibit higher antioxidative activities when incorporated into peptides (*22*−*25*). However, neither the structure−activity relationship nor the antioxidant mechanism of peptides is fully understood. In this study, therefore, we attempted to accumulate knowledge on this matter by means of combinatorial chemical peptide library construction.

By screening 40 peptides structurally related to LLPHH, which is an antioxidative peptide isolated from soybean protein digests, PHH was identified as the active center. On the basis of this result, we have prepared libraries consisting of 222 tripeptides to explore the antioxidative properties. The characteristic feature of the libraries is that they were designed based on PHH in terms of size and amino acid composition. The antioxidative properties of peptides varied depending on their structure and the assay system. In an aqueous autoxidation system of linoleic acid, PHH did not show higher activity than other structurally related tripeptides; that is, the replacement of N- and/or C-terminal amino acids did not affect the activity greatly. On the other hand, a His residue in the center was found to play an important role in the activity. YHY had a strong synergestic effect with some phenolic antioxidants as well as an antioxidative activity. This is an important aspect to understanding the synergestic effect of protein hydrolysates and for the application of peptidic antioxidants.

The tetrapeptide, Asp−Thr−His−Lys (DTHK), was isolated from bovine serum albumin hydrolysates as a peptidic synergist to increase the antioxidative activity of α-tocopherol and sodium ascorbate (*26*). Although the precise mechanism of the synergistic effect is not clear, His residue was supposed to play an important role. It is interesting to note that a homologous tetrapeptide, Asp−Ala−His−Lys (DAHK), derived from the N-terminus of human serum albumin, attenuated copper-induced oxidative DNA double strand breaks and telomere shortening (*27*). Possible mechanisms for the activity were proposed to be the chelation of copper ions and/or the formation of DAHK−

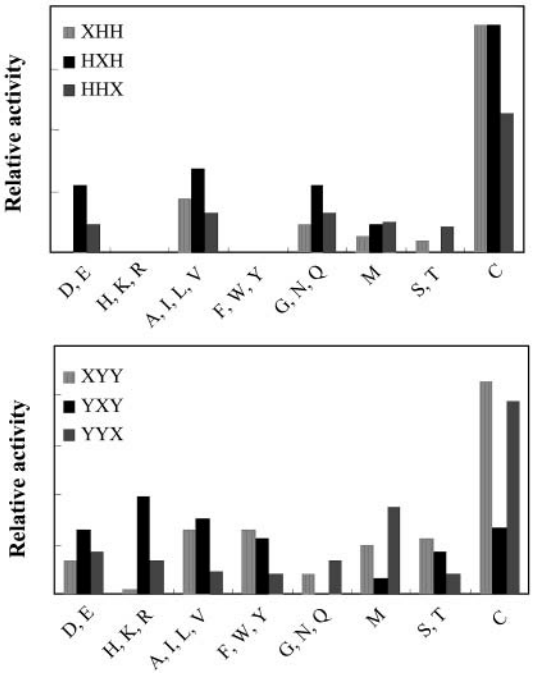

Figure 8. Peroxynitrite scavenging activity of two His- or two Tyr-containing tripeptide libraries. The peptide library was constructed as described in **Figure 1**. The final concentration of each peptide was 80 μM. The relative activity was expressed by inhibition rate of DCF formation from DCDHF induced by peroxynitrite.

copper−peroxide complexes. Other aspects need to be considered regarding the synergestic effect of YHY; that is, oxidized Tyr and Trp residues could be re-reduced by conventional antioxidants such as tocopherol.

The tripeptides containing Trp or Tyr residue at the C-terminus showed high radical scavenging activities. However, these amino acid residues positioned at the C-termini disturbed the peroxyntrite scavenging acivity, indicating that these scavenging activities were attributed to different mechanisms. Although peroxynitrite, the reaction product between nitric oxide and superoxide, is not a free radical, it can produce some of the strongest oxidants such as the hydroxy radical, nitronium ion, and nitrogen dioxide (*17*). This may cause the characteristic reactivity of peroxynitrite, because there is a wide variation in the magnitude of the rate constants for attack by reactive species on amino acid side chains. It appears that cysteine residues in tripeptides are the prime target for peroxynitrite, though Trp and Tyr residues are also known to be modified with the oxidant.

The positional selectivity and rates of radical attack on peptides and proteins accounted for the presence of the deactivating protonated amino group and the radical stabilizing groups on side chains (*28*). Site-specific oxidation can also arise from the binding of a metal ion or other initiating species at particular site on peptides (*29*). Selective modification of His, Pro, Met, Cys, Arg, Lys, and Trp residues has been observed with various peptides and proteins. These distinct properties of peptides relate with their antioxidative activities. The antioxidative activities of Trp and Tyr may be explained by the special capability of phenolic and indolic groups to serve as hydrogen donors. The phenoxyl and indoyl radicals are much more stable and have longer lifetimes than simple peroxy radicals, so any reverse reaction or the propagation of the radical-mediated peroxidizing chain reaction are inhibited. The stability of Trp-containing peptides in the presence of an L-ascorbic acid-ferric ion system depends on neighboring amino acid residues (*30*).

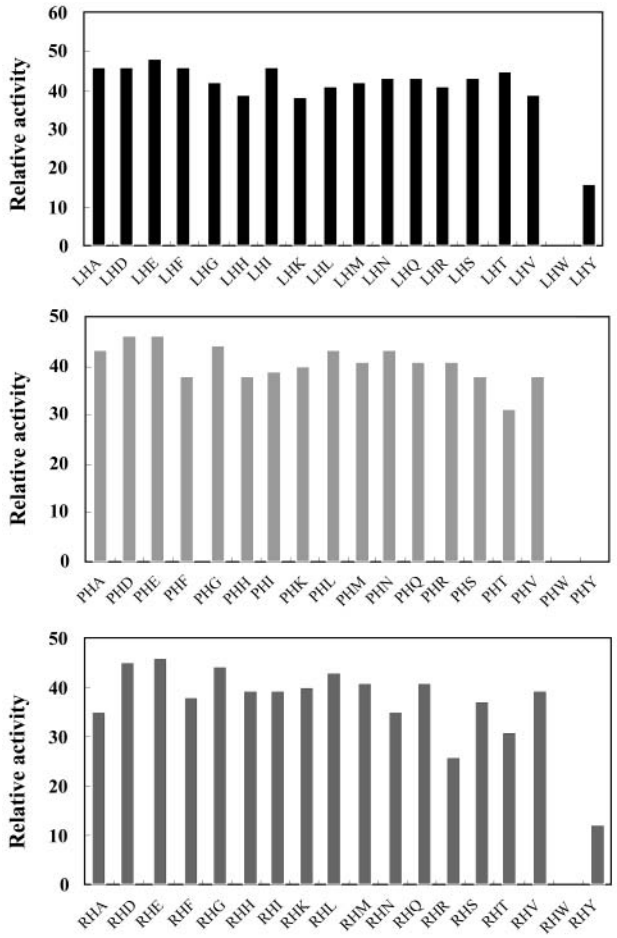

Figure 9. Peroxynitrite scavenging activity of PHH and its structurally related peptides. The final concentration of each peptide was 80 μM.

In a previous paper, we demonstrated that His-containing peptides acted as a metal-ion chelator, an active-oxygen quencher, and a hydroxyradical scavenger and proposed that these properties cooperated to produce the antioxidative activity (*9*). The present study also showed that each tripeptide had a distinct activity in different assay systems. The difference in these activities is worth investigating further, to understand the antioxidative properties of peptides more thoroughly. Besides antioxidative peptides derived from soybean proteins, the amino acid sequences of several antioxidative peptides have been reported: Ala−His−Lys, Val−His−His, and Val−His−His−Ala−Asn−Glu−Asn from egg-white albumin (*4*); Pro−Ser−His−Asp−Ala−His−Pro−Glu, Val−Asp−His−Asp−His−Pro−Glu, Pro−Lys−Ala−Val−His−Glu, Pro−Ala−Gly−Tyr, Pro−His−His−Ala−Asp−Ser, and Val−Asp−Tyr−Pro from tuna cooking juice (*31*); Ala−Arg−His−Pro−His−Pro−His−Leu−Ser−Phe−Met from fermented milk (*32*); Gly−Glu−Hyp−Gly−Pro−Hyp−Gly−Pro−Hyp−Gly−Pro−Hyp−Gly−Pro−Hyp−Gly, and Gly−Pro−Hyp−Gly−Pro−Hyp−Gly−Pro−Hyp−Gly−Pro−Hyp−Gly from Alaska pollack skin (*33*); and Leu−Met−Ser−Tyr−Met−Trp−Ser−Thr−Ser−Met, and Leu−Glu−Leu−His−Lys−Leu−Arg−Ser−Ser−His−Trp−Phe−Ser−Arg−Arg from lecithin-free egg yolk (*34*). It is interesting to note that all of these peptides contain one or more residues of His, Pro, Tyr, and Trp. Furthermore, it may be possible to modulate their activities by enzymatic trimming by referring to the present results.

In conclusion, the antioxidative properties of the combinatorial tripeptide libraries were investigated. Several unique tripeptides could be identified such as Tyr−His−Tyr, Xaa−

Xaa−Trp/Tyr, and Xaa−Xaa−Cys(SH) that had a strong synergistic effect with phenolic antioxidants, a high radical scavenging activity, and a high peroxynitrite scavenging activity, respectively.

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