Isolation of an Active Peptide Fragment from Human Serum Albumin and Its Synergism with α-Tocopherol

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ABSTRACT: An active peptide was isolated from hydrolysates of human serum albumin. This peptide was initially isolated by gel permeation chromatography and subsequent reversed-phase high-performance liquid chromatography. This active peptide, composed of 10 amino acid residues, was further hydrolyzed with a lysyl endopeptidase to give two peptide fragments. Only one fragment, identified as the tetrapeptide Leu-Gln-His-Lys, was found to have activity comparable to the original peptide and corresponded to the amino acid residues 103–106 of human serum albumin. Among these four amino acid residues, the His-Lys sequence seemed to be important in the occurrence of potent activity by comparison of the structural similarity with another active tetrapeptide, Asp-Thr-His-Lys, which had been previously isolated from bovine serum albumin hydrolysates. In addition, the active fragment showed potent synergism by preventing consumption of α-tocopherol during the autoxidation of linoleic acid.

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KEY WORDS: Antioxidant, human serum albumin, peptide synergist, synergistic effect, tocopherol.

α-Tocopherol, one of the most reliable natural antioxidants, has become widely used as a food additive since interest in utilization of natural antioxidants has increased. Once absorbed by the body, α-tocopherol is also assumed to function as an effective antioxidant in various tissues in which lipid peroxidation occurs (1–3). It is also known that the antioxidant activity of α-tocopherol is strongly enhanced by some substances, such as ascorbic acid (4,5) and nitrogen compounds, such as proteins, peptides, and amino acids (6–15).

A large number of peptides, originating from various materials, have been found to exhibit synergisms with antioxidative substances such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and α-tocopherol (11–14). In a previous paper (15), an active peptide synergist was successfully isolated from bovine serum albumin hydrolysates and was identified as the tetrapeptide Asp-Thr-His-Lys. The present study intends to isolate an active peptide from human serum albumin (HSA) and to find out the important amino acid sequence in the active peptide by comparing the structural similarity of these active peptides. In addition, the effect of the active peptide on the behavior of α-tocopherol is also investigated.

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MATERIALS AND METHODS

HSA (lyophilized, substantially free from fatty acids) and porcine pepsin (lyophilized and crystallized) were purchased from Sigma Chemical Co. (St. Louis, MO). α-Tocopherol, 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMHC), linoleic acid, 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS), and lysyl endopeptidase (E.C. 3.4.21.50) were purchased from Wako Pure Chemical Ind. (Osaka, Japan). All other reagents used in this experiment were of analytical grade.

Determination of α-tocopherol. α-Tocopherol was determined by normal-phase high-performance liquid chromatography (HPLC) with PMHC as an internal standard. An aliquot (100 μL) of the sample solution and 1 μg of PMHC were mixed with 2 mL of 5% NaCl solution. The sample mixture was extracted twice with 2 mL ether. The ether extract was dehydrated with anhydrous sodium sulfate and then evaporated in a centrifugal evaporator (CVE-200D, Tokyo Rikakikai Co. Ltd., Tokyo, Japan). The dried sample was dissolved with 0.5 mL of 0.5% isopropanol in *n*-hexane prior to the HPLC analysis. Analytical conditions of the HPLC were: Column, Shim-pack CLC-SIL (M) (4.6 × 250 mm, Shimadzu Co., Kyoto, Japan); detection wavelength, 298 nm; mobile phase, 0.5% isopropanol in *n*-hexane; sample volume, 10–50 μL. Each reported result is the mean of duplicate measurements.

Preparation of S-carboxymethylated HSA and its hydrolysis by pepsin. HSA was reduced with dithiothreitol and subsequently S-carboxymethylated with iodoacetic acid. The S-carboxymethylated HSA (CM-HSA) was exhaustively dialyzed against distilled water (Cellophane tubing-seamless, 36/32 inch for dialysis, Wako Pure Chemical Ind.) and then lyophilized. The lyophilized CM-HSA (50 mg), dissolved in 10 mL of 0.02 N HCl, was heated in a test tube in boiling water for 15 min, and then the cooled CM-HSA solution (0.5%), pH 2.5, was hydrolyzed with pepsin at 37°C for 9 h at an enzyme-to-CM-HSA ratio of 1 to 100 (w/w). The proteolytic reaction was stopped by heating at 100°C and yielded the CM-HSA hydrolysates.

Separation of CM-HSA hydrolysates by Sephadex G-25 chromatography. The CM-HSA hydrolysates (about 50 mg)

were applied to a Sephadex G-25 column (superfine, 2.0 × 120 cm) and eluted with 0.001 M HCl at a flow rate of 28 mL/h. The amount of peptide eluted in each fraction was monitored at 254 nm (14). The concentration of peptide was also estimated by the determination of free amino groups in the peptide, according to the TNBS method (17) with L-leucine as a standard.

Assessment of antioxidant activity. Each sample was added to an oxidation model (15,16) that contained 0.1 mmol linoleic acid in 2.0 mL ethanol, 2.0 mL of 0.05 M phosphate buffer (pH 7.0), and 1.0 mL distilled water. The oxidation model also contained 50 μg α-tocopherol when the synergistic effect of the sample with α-tocopherol was assayed. The initial volume of an oxidation mixture was adjusted to 5.0 mL by diminishing the distilled water according to added sample volume. The oxidation of linoleic acid was carried out in a test tube (20 × 125 mm) with a screw cap at 60°C in the dark. The extent of oxidation was periodically measured by the ferric thiocyanate method (16). The potency of antioxidant activity of the sample was evaluated by the induction period, which indicated days for the peroxide value (PV) to reach 100 meq/kg and was expressed as the mean of duplicate measurements.

Isolation of active peptide and its fragments by HPLC. The peptide fractions obtained by Sephadex G-25 were separated by a reversed-phase HPLC on a Wakosil 5C-200 column (4.6 × 250 mm, Wako Pure Chemical Ind.). The active peptide obtained by HPLC was again hydrolyzed with lysyl endopeptidase, and the resulting peptide fragments were also separated by HPLC on a TSK gel ODS-80 Ts column (4.6 × 250 mm, Tosoh Co., Tokyo, Japan). The peptides and fragments were eluted with a linear gradient of acetonitrile in water, containing 0.1% trifluoroacetic acid, at a flow rate of 0.5 mL/min and monitored at 220 nm.

Amino acid analysis and sequence determination of peptides. The peptides obtained by HPLC were hydrolyzed in 6 N HCl *in vacuo* at 110°C for 24 h. Amino acid analysis was performed on an automatic amino acid analyzer Model AA01 (Mitsubishi Kasei Co., Tokyo, Japan). The amino-terminal amino acid sequence of the peptide was determined by automated Edman degradation with a model 476A protein sequencer (Applied Biosystems, Foster, CA).

RESULTS

Figure 1 shows the elution profile of the CM-HSA hydrolysates and the effect of each fraction (0.5 mL) on the antioxidant activity of α-tocopherol. Potent activity was observed around fractions 23–42 (fraction A), where the induction periods were more than 10 d, while the induction period of α-tocopherol without the CM-HSA hydrolysates was only about 2 d. However, the pooled fraction A (0.5 mL) did not show any antioxidant activity when it was added to the oxidation system in the absence of α-tocopherol (data not shown). This result indicates that peptides in fraction A acted as synergists for α-tocopherol, as observed in the hydrolysates from various proteins (7, 9, 11–15). Therefore, fraction A was used for isolation of an active peptide synergist for α-tocopherol.

The lyophilized fraction A was separated by reversed-phase HPLC in a Wakosil 5C-200 column. Figure 2 shows the typical elution profiles of peptides in fraction A. Observed peaks (fractions 1–12) were repeatedly recovered and were then lyophilized. Each fraction was first dissolved in 1–2 mL water, and the concentration of peptide was estimated by the TNBS method (17). Each fraction, corresponding to 15 nmol of peptide, was examined for its stimulating effect toward α-tocopherol (Fig. 3). Although fractions 1, 5, and 9 showed stronger activity among the fractions obtained, an active pep-

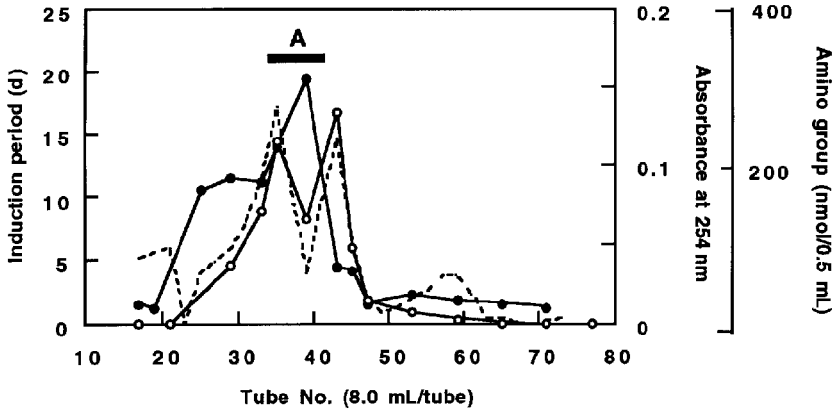

FIG. 1. Separation of carboxymethylated human serum albumin hydrolysates by chromatography on a Sephadex G-25 column, and effect of each fraction on antioxidant activity of α-tocopherol. Each fraction (0.5 mL) was examined for its stimulating effect toward α-tocopherol (50 μg). Conditions are detailed in the Materials and Methods section. Induction period (●) was the mean of duplicate measurements. Peptide elution was monitored at 254 nm (----), and its concentration was estimated by the determination of free amino groups in the peptide (○).

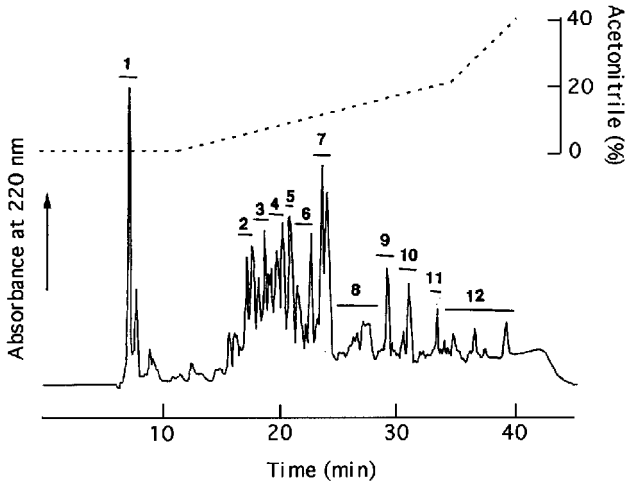

FIG. 2. High-performance liquid chromatography (HPLC) chromatogram of active peptide fractions obtained by Sephadex G-25 chromatography. Column, Wakosil 5C-200 column (4.6 × 250 mm; Wako Pure Chemical Ind., Osaka, Japan); flow rate, 0.5 mL/min. The starting solvent was 0.1% trifluoroacetic acid in water, and a gradient of acetonitrile with 0.1% trifluoroacetic acid was employed as indicated (----).

tide (designated as HSA-H-5) was isolated only from fraction 5. Because this active peptide HSA-H-5 comprised 10 amino acid residues, as will be mentioned later, it was again hydrolyzed with a lysyl endopeptidase to obtain smaller peptides. About 1 mg of HSA-H-5 was treated with 10 μg of lysyl endopeptidase in 1.0 mL of 0.1 M Tris-HCl buffer (pH 9.0) at 37°C for 3 h (15).

Figure 4 shows the separation of the peptide fragments that originated from peptide HSA-H-5 after separation in a TSK gel ODS-80 Ts column. Two predominant peaks were formed by the hydrolysis with lysyl endopeptidase, while the original

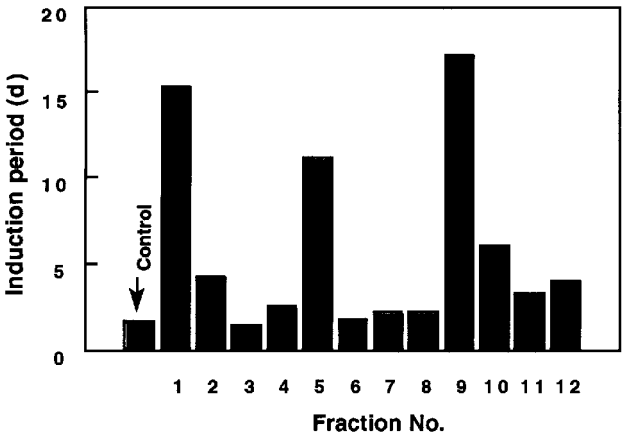

FIG. 3. Effect of peptide fractions obtained by HPLC on antioxidant activity of α-tocopherol. Each fraction, corresponding to 15 nmol of peptide, was examined for its stimulating effect toward α-tocopherol (50 μg). Conditions are detailed in the Materials and Methods section. Control: α-tocopherol without peptide fractions. Induction period was the mean of duplicate measurements. For abbreviation see Figure 2.

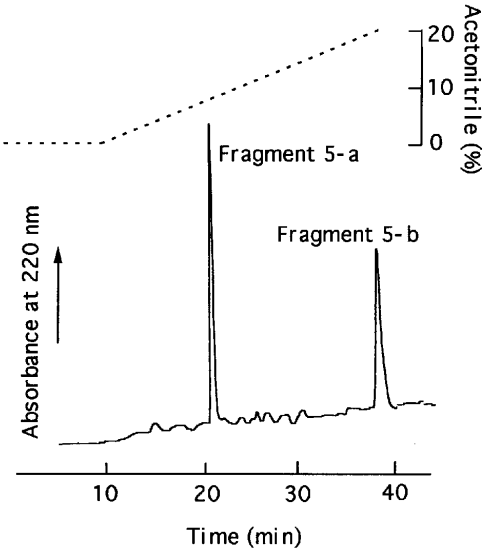

FIG. 4. HPLC chromatogram of an active peptide, HSA-H-5, after treatment with lysyl endopeptidase at 37°C for 3 h. Two peaks observed were recovered, and their effects on the antioxidant activity of α-tocopherol were examined. Only fragment 5-a showed the potent activity comparable to the original peptide, HSA-H-5. Column: TSK gel ODS-80 Ts column (4.6 × 250 mm; Tosoh Co., Tokyo, Japan); flow rate, 0.5 mL/min. The starting solvent was 0.1% trifluoroacetic acid in water, and a gradient of acetonitrile with 0.1% trifluoroacetic acid was employed as indicated (-----).

peak rapidly disappeared. This suggests that HSA-H-5 was composed of at least two peptide fragments, 5-a and b. The synergism of each fragment with α-tocopherol was examined as described above, and the synergism of fragment 5-a was comparable to that of the original peptide, HSA-H-5, whereas fragment 5-b had little effect on the antioxidant activity of α-tocopherol (data not shown). This result suggested that fragment 5-a was the active site involved in the synergism of peptide HSA-H-5 toward α-tocopherol.

Table 1 shows the amino acid composition of fragments 5-a and 5-b and of the original HSA-H-5. The total amino acid composition of fragments 5-a and 5-b were in good agreement with that of the original. Moreover, the sequences of their amino acid residues were determined and compared with the primary structure of HSA (18). As shown in Figure 5, HSA-H-5 and its fragments 5-a and 5-b coincide with amino acid residues 103–112, 103–106, and 107–112 of HSA, respectively. The cleavage of the peptide bond between residues 106 and 107 of HSA-H-5 by lysyl endopeptidase is quite reasonable, because this enzyme hydrolyzes a protein only at lysyl residues to give a limited number of peptide fragments (19). Therefore, the structures of peptide HSA-H-5 and its fragments 5-a and 5-b are the proposed ones in Figure 5.

Figure 6 shows the relationship between the loss of α-tocopherol and the increase of PV during the autoxidation of linoleic acid with or without addition of the active peptide fragment 5-a. In the combined use of α-tocopherol and fragment 5-a, α-tocopherol remained for more than 5 d and thus strongly inhibited the autoxidation of linoleic acid, whereas

TABLE 1
Amino Acid Composition of HSA-H-5 and Its Fragments 5-a and b

	HSA-H-5	Fragment 5-a	Fragment 5-b
Asp	3.8[a] (4)[b]	0.2[a] (0)	3.6[c] (4)
Ser	0.2 (0)	0.1 (0)	0.1 (0)
Pro	1.0 (1)		1.1 (1)
Gln	1.1 (1)	1.0 (1)	
Gly	0.2 (0)	0.2 (0)	0.2 (0)
Ala	0.2 (0)	0.3 (0)	
Val	0.1 (0)	0.2 (0)	
Leu	2.3 (2)	1.2 (1)	1.0 (1)
Tyr	0.3 (0)		
Lys	1.0 (1)	1.0 (1)	
His	0.9 (1)	0.8 (1)	
Total	10	4	6

[a]Mole ratio to 1 mole of Lys.
[b]Number in parentheses indicates an approximate integer.
[c]Mole ratio to 1 mole of Leu.

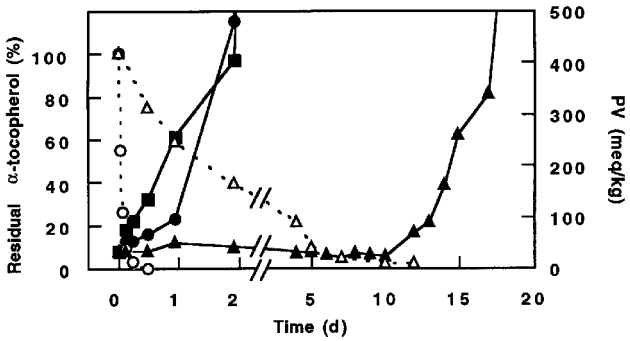

FIG. 6. Effect of an active peptide fragment, 5-a, on loss of α-tocopherol during autoxidation of linoleic acid. Autoxidation of linoleic acid was carried out in an ethanolic phosphate buffer (pH 7.0) at 60°C with and without α-tocopherol and fragment 5-a. Conditions are detailed in the Materials and Methods section. (■) Control (without α-tocopherol and fragment 5-a); (●, ○) with α-tocopherol; (▲, △) with α-tocopherol and fragment 5-a. Solid and open symbols show peroxide value (PV) and residual α-tocopherol (%), respectively. Each point was the mean of duplicate measurements.

α-tocopherol without fragment 5-a quickly disappeared (about 12 h). In addition, the PV of linoleic acid increased after disappearance of α-tocopherol. These results suggest that fragment 5-a showed potent activity, not by degrading the peroxides but by preventing the consumption of α-tocopherol during the autoxidation of linoleic acid.

DISCUSSION

Various kinds of peptides have been reported to exhibit synergism with α-tocopherol when their own antioxidant activity was not as strong as the conventional antioxidants such as BHA and BHT (11–15). Naturally, the peptide's antioxidative and synergistic activity varied according to the oxidation conditions, such as temperature, medium, and lipid used for the experiment. In this study, the synergism of peptides from HSA with α-tocopherol was evaluated in an ethanolic phosphate buffer system of linoleic acid at 60°C. This system was available for searching potent antioxidant substances from large numbers of samples (6,7,10–16). However, this system has the disadvantage that the results do not always represent real foods and biological systems. Therefore, the active peptide obtained in this study should be reexamined by using other mimics of real lipid systems in foods and biological systems, such as bulk oils, phospholipid liposomes, isolated cell membranes, and lipid emulsions. In a preliminary experiment, isolation and identification of an active peptide was tried with an intact HSA but could not be successfully performed. This was supposedly caused primarily by interference of the disulfide bridges in the HSA molecule. In this study, the CM-HSA was employed as the starting protein instead of the intact HSA, thereby allowing the isolation of the active tetrapeptide from HSA.

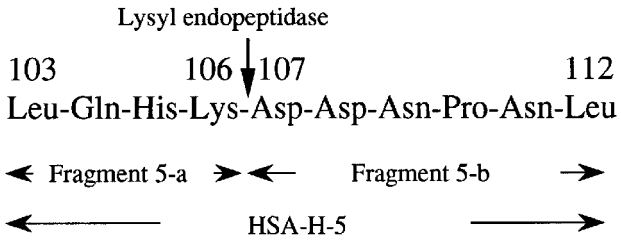

FIG. 5. Amino acid sequence of an active peptide, HSA-H-5, and its fragments 5-a and 5-b. Amino acid sequence of HSA-H-5 coincided with human serum albumin (HSA) residues 103–112. Lysyl endopeptidase hydrolyzed HSA-H-5 at the peptide bond as indicated by the vertical arrow.

In a previous study (15), another active tetrapeptide had been obtained from bovine serum albumin hydrolysates by following similar procedures, and this peptide, Asp-Thr-His-Lys, showed potent synergism similar to the tetrapeptide Leu-Gln-His-Lys obtained in this study. Both active tetrapeptides contain the His-Lys carboxyl terminal sequence as a common structural unit. On the basis of this, we suggest that the His-Lys sequence may play a primary role in the activity of these peptides. The previous paper (15) also indicated that the octapeptides Ser-Glu-Ile-Ala-*His*-Arg-Phe-*Lys* and Asp-Leu-Gly-Glu-Glu-*His*-Phe-*Lys*, in which the histidine and lysine residues are isolated from each other, did not significantly stimulate the antioxidant activity of α-tocopherol. That also suggests the importance of the His-Lys sequence in the active peptides. Nevertheless, because a large number of synergistic peptides have been recognized among various protein hydrolysates (7,9,11–13), it is questionable whether all of these active peptides contain the His-Lys sequence as part of their amino acid sequences. Therefore, the His-Lys sequence may be only one of several important active sites in peptides to exhibit potent synergism toward α-tocopherol rather than being indispensable. Although the structure–activity relationship will be thus presumed, it has not been sufficiently elucidated. That is now under investigation in this laboratory by using some synthetic peptides that are analogous to the active tetrapeptide Leu-Gln-His-Lys.

Babizhayev *et al.* (20) studied natural imidazole-containing compounds, such as carnosine (β-alanyl-L-histidine) and carcinine (β-alanylhistamine), and assumed that the histidine residue acted as a physiological antioxidant by scavenging free radicals or donating hydrogen ions. In a similar manner, the histidine residue, together with the lysine residue, in the active tetrapeptide of this study might regenerate α-tocopherol from α-tocopheroxyl radicals (21,22) and consequently prevent the consumption of α-tocopherol (Fig. 6). However, because carnosine has also been shown incapable of regenerating α-tocopheroxyl radicals (23,24), the real interaction between the active tetrapeptide and α-tocopherol is still obscure. On the other hand, amino acids and lower peptides are known to have synergistic interactions with tocopherols because of their metal-chelating properties (25,26). Owing to the metal-chelating activity, the active tetrapeptide might retard the consumption of α-tocopherol. These actions are presumed, and other mechanisms also might be involved.

At the present stage of the work, there are numerous problems concerning the synergism of peptides, as described above. Therefore, such study should be continued. However, this study presents an active tetrapeptide, Leu-Gln-His-Lys, and suggests the possibility that HSA and its derivative peptides will act as effective synergists with antioxidants, such as tocopherols, in inhibiting lipid oxidation.

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