Methionine Oxidation and Proline *cis-trans* Isomerization in α_{s1} -Casein on Emulsification with Oxidized Lipid

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Received August 26, 1986

An emulsion was prepared by homogenizing soybean oil with an α_{s1} -casein solution. The α_{s1} -casein adsorbed to the oil/water interface in the emulsion was digested with trypsin and then analyzed by reversed-phase high-performance liquid chromatography. Two peaks, T-1 and T-2E, due to the same fragment ($^{194}\text{Thr-Thr-Met-Pro-Leu-Trp}$) were found. Amino acid analysis showed that the ^{196}Met of T-1 had been oxidized to methionine sulfoxide, and the NMR spectrum indicated that the $^{196}\text{Met-Pro}$ bond of T-1 had been subjected to *cis-trans* isomerization. The isomerization of the same peptide bond was also observed when this peptide fragment was oxidized by chloramine-T in solution, suggesting that *cis-trans* isomerization of ^{197}Pro was induced by ^{196}Met oxidation.

Physical interactions between proteins and lipids, and especially hydrophobic interactions, play important roles in the formation of protein-stabilized food emulsions. However, chemical interactions of proteins with lipids could also occur in such protein-lipid mixtures. If a lipid contains peroxide, for example, chemical interaction between proteins and lipid hydroperoxide occurs, resulting in deterioration of the proteins. Various reactions of proteins with lipid hydroperoxide are known;¹⁾ protein-protein cross-links, protein scission, protein-lipid adducts and amino acid damage. Production of methionine sulfoxide (Met(O)) during amino acid damage has been well studied in particular. Some proteins have been reported to lose their biological activity when methionine residues are oxidized *in vitro*,²⁾ but the influence of methionine oxidation upon protein structures and the mechanism of the inactivation of proteins on methionine oxidation have not been elucidated.

Recently, we analyzed the topography of α_{s1} -casein adsorbed to soybean oil droplets by

applying the protease digestion method.³⁾ The proteolytic products of α_{s1} -casein in a soybean oil emulsion were analyzed by high-performance liquid chromatography (HPLC). During this study we found two peaks due to the same fragment, $^{194}\text{Thr-Thr-Met-Pro-Leu-Trp}$, in the HPLC pattern. In this work, the structures of the peptides corresponding to these two peaks were studied. Oxidation of Met and *cis-trans* isomerization of Pro occurring in α_{s1} -casein on emulsification with soybean oil are demonstrated.

MATERIALS AND METHODS

Preparation of α_{s1} -casein. Bovine α_{s1} -casein (variant B) was fractionated from fresh raw skim milk by the procedure of Zittle *et al.*⁴⁾ and purified by chromatography on DEAE-Cellulofine AM (Seikagaku Kogyo Co., Ltd.).

Emulsification. A 1% α_{s1} -casein solution, adjusted to pH 7.0 with NaOH, was homogenized with 20% (w/w) of an oil with a Polytron PT-20 (Kinematica GmbH) at 19,500 rpm for 8 min at 30°C. Soybean oil (Sanko Seiyaku Kogyo Co., Ltd.) or tricaprylin (Sigma Chemical Co.) was

used as the oil. After centrifuging an emulsion at $12,000 \times g$ for 10 min, the aqueous portion was removed by aspiration. To the floating cream was added 0.1 M $\text{NH}_4\text{OH}/\text{HCOOH}$ buffer, pH 8.5. The diluted cream was gently mixed and then centrifuged at $12,000 \times g$ for 10 min at 20°C , a washed cream being obtained by repeating this step twice with dilution in the same buffer.

Determination of α_{s1} -casein adsorbed to oil globules. To a given volume of the diluted cream were added 1/3 volumes of solutions of 6% sodium dodecyl sulfate and 10% glycerol. The mixture was heated at 100°C for 3 min and then centrifuged at $12,000 \times g$ for 30 min at 10°C . The protein in the aqueous portion was determined by the method of Lowry *et al.*⁵⁾

Tryptic digestion of α_{s1} -casein adsorbed to oil globules. To a portion of the emulsion was added L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corporation) at an enzyme/substrate ratio of 1/100 (w/w). The digestion was stopped by adding soybean trypsin inhibitor (Sigma Chemical Co.) at an enzyme/inhibitor ratio of 1/100 (w/w). The peptides in the emulsion were recovered by extracting the oil with a methanol-chloroform mixture (1:2, v/v).

High performance liquid chromatography (HPLC). The tryptic peptides were chromatographed with a TRI ROTAR SR-2 (Japan Spectroscopic Co., Ltd.) on Hibar LiChrosorb RP-18 (Kanto Kagaku Co., Ltd.), using a linear gradient of from 0.1% (v/v) trifluoroacetic acid (sequanal grade, Wako Pure Chemical Industries) to 95% (v/v) acetonitrile (chromatography grade, Wako Pure Chemical Industries).

Amino acid analysis. The peptides purified by HPLC were hydrolyzed in 6 N HCl at 110°C for 20 hr *in vacuo* or with carboxypeptidase Y (Oriental Yeast Co., Ltd.) in 0.1 M $\text{NH}_4\text{OH}/\text{HCOOH}$ (pH 6.5) at 37°C . For the detection of methionine sulfoxide, two different methods were adopted. One comprised HCl hydrolysis after carboxymethylation and performic acid oxidation of the peptides.⁶⁾ The other comprised hydrolysis in 3 N *p*-toluenesulfonic acid (*p*-TOS) at 110°C for 20 hr *in vacuo*.^{7,8)} Amino acid analysis of each hydrolysate was carried out with a Hitachi 835 amino acid analyzer.

Thiobarbituric acid (TBA) test.⁹⁾ The sample oil was dissolved in methanol. For the TBA test, 0.1 ml of the sample methanol solution, 2 ml of 0.02 M phosphate buffer (pH 6.0) and 2 ml of a 1% TBA solution were mixed. The reaction mixture was heated for 15 min in a boiling water bath, before the absorbance at 535 nm of the colored aqueous layer was measured.

Nuclear magnetic resonance (NMR). ^1H -NMR was re-

corded at 270 MHz with a Bruker WH-270 at 25°C . The peptide samples for NMR investigation were dissolved in deuterium oxide (>99.85%) purchased from the Commissariat a l'Energie Atomique, France. Chemical shifts are expressed as parts per million (ppm) from the internal standard, sodium 3-trimethylsilyl-2,2,3,3- $^2\text{H}_4$ propionate (TSP).

Oxidation of methionine residues in a peptide. A peptide purified by HPLC was oxidized with chloramine-T according to the method of Shechter *et al.*¹⁰⁾ The peptide (0.3 mg) was dissolved in 0.75 ml of 0.1 M tris(hydroxymethyl)-aminomethane-HCl buffer (pH 8.5) and then 0.75 ml of a 0.01 M chloramine-T aqueous solution was added. The reaction was allowed to proceed for 20 min at room temperature, before the mixture was subjected to HPLC. The fraction corresponding to the peptide was collected, lyophilized and submitted to NMR measurement.

RESULTS

Tryptic digestion of α_{s1} -casein in solution and emulsion systems

Trypsin was added to α_{s1} -casein dissolved in an aqueous solution (the solution system) and to that adsorbed to oil globules (the emulsion system). Figure 1 shows the HPLC patterns of the tryptic peptides produced in the solution (a) and emulsion (b) systems. The amino acid compositions and C-terminal groups of the peptides separated by HPLC were analyzed by means of HCl hydrolysis and carboxypeptidase Y digestion, respectively. The peptides were identified by comparing their amino acid compositions and C-terminal sequences with those of the peptides which were expected to be derived from α_{s1} -casein in accordance with the substrate specificity of trypsin. Both T-1 and T-2E in the emulsion system were identified as $^{194}\text{Thr-Thr-Met-Pro-Leu-Trp}$, although they had quite different retention times (Fig. 1b). T-2S in the solution system had the same retention time as T-2E, but a peptide corresponding to T-1 was not observed in the solution system.

Effect of the oil used for emulsification on the HPLC patterns of the tryptic peptides

Soybean oil stored for 2 and 10 months, and tricaprylin were used as the oils for emulsifi-

FIG. 1. HPLC Patterns of Peptides Derived from α_{s1} -Casein with Trypsin in the Solution System (a) and in the Emulsion System (b).

T-1, 2E and 2S were identified as ^{194}Thr - ^{199}Trp by amino acid analysis after HCl hydrolysis and carboxypeptidase Y digestion. HPLC was performed on LiChrosorb RP-18, and the elution system was a linear gradient of from 0.1% trifluoroacetic acid to 95% acetonitrile.

cation. When soybean oil stored for 2 months was used, both T-1 and T-2E were observed in the tryptic digest (Fig. 1b). The digest prepared from the emulsion with soybean oil stored for 10 months contained only T-1 (data not shown). On the other hand, the digest of the emulsion with tricaprylin contained only T-2E (data not shown).

Determination of the TBA test values of the oils

A TBA test was carried out in order to determine the degrees of oxidation of the oils used for emulsification. No thiobarbituric acid-reactive substances (TBARS) were observed in tricaprylin. Soybean oil stored for 10 months contained 140 times more TBARS than that stored for 2 months.

Detection of Met(O)

The amino acid compositions of T-1, T-2E and T-2S were examined after hydrolysis by two different methods (see MATERIALS AND METHODS), and the results are shown in Table I. The results indicate that ^{196}Met of T-1 had

TABLE I. AMINO ACID ANALYSIS OF T-1 AND T-2 BY TWO DIFFERENT PROCEDURES

Sample procedure	T-1		T-2E		T-2S
	I	II	I	II	II
Met (O)	—	0.9	—	—	—
Met (O ₂)	0.9	—	—	—	—
Thr	2.0	1.9	1.8	1.9	1.8
Homoserine	—	—	0.5	—	—
Pro	1.0	1.1	1.0	1.2	1.1
Met	—	—	—	0.9	0.7
Leu	1.1	1.2	1.1	1.2	1.4

Procedure I: Samples were hydrolyzed with 6N HCl after carboxymethylation and performic acid oxidation. Met was converted mainly into homoserine, and Met (O) into methionine sulfone (Met (O₂)) during this treatment.

Procedure II: Samples were hydrolyzed with 3N *p*-TOS. Trp was not determined.

been oxidized and changed to Met(O), while ^{196}Met of T-2E and T-2S had not been modified.

$^1\text{H-NMR}$ of T-1, T-2E and T-2S

$^1\text{H-NMR}$ was used to investigate the molec-

FIG. 2. ^1H -NMR Spectra of T-2S (a) and T-1 (b) in D_2O at 24°C .

The peptide concentration was $\approx 1\text{ mM}$. The names of atoms are those given in the IUPAC-IUB recommendations of 1974.¹¹⁾ The assignments are

ular structures of the peptides, T-1, T-2E and T-2S (Figs. 2a and b). The T-1 samples showed a downfield shift of the $\epsilon\text{-CH}_3$ resonances derived from $^{16}\text{Met}(\text{O})$ and the splitting of the CH_2 resonances from ^{17}Pro into two. Judging from the chemical shifts of CH_3 resonances from $\text{Met}(\text{O})$, the oxidation of the thioether group to sulfoxide and/or sulfonate was suggested and was confirmed as mentioned above. At the same time, the co-existence of *cis* and

trans forms of the peptide bond of Met-Pro was suggested from the splitting of the ^{17}Pro resonances in T-1. On the other hand, the Met-Pro bonds of T-2S and T-2E were found to be of only the *trans* form (data not shown). To gain insight into the effect of Met oxidation, T-2S was oxidized mildly with an oxidizing agent, chloramine-T, in solution, and the oxidized sample was designated as T-2S- $\text{Met}(\text{O})$. The ^1H -NMR spectrum of this sample re-

vealed the *cis-trans* isomerization of Pro (data not shown).

DISCUSSION

Tryptic digests of α_{s1} -casein were analyzed by reversed-phase HPLC. The reversed-phase separation was based upon hydrophobic interactions between the sample molecules and the stationary phase. The retention times of peptides are related to their amino acid compositions,^{12,13} T-1 and T-2E (Fig. 1) being shown to have the same sequences and being identified as ¹⁹⁴Thr-Thr-Met-Pro-Leu-Trp by amino acid analysis after HCl hydrolysis and carboxypeptidase Y digestion. However, amino acid analysis, with HCl hydrolysis after carboxymethylation and performic acid oxidation or *p*-TOS hydrolysis, revealed that ¹⁹⁶Met of T-1 had been oxidized to Met(O), while that of T-2E had not. T-1 was eluted from the reversed-phase column faster than T-2E, because the hydrophobicity of Met(O) was lower than that of Met. Met in proteins is known to be easily oxidized to Met(O) in the presence of lipid containing hydroperoxide.¹⁴ The present results suggest that peroxides formed in soybean oil during storage oxidized ¹⁹⁶Met in the α_{s1} -casein molecules adsorbed to the emulsified oil droplets.

¹H-NMR revealed that the *cis-trans* isomerization of Pro, as well as the oxidation of Met, had occurred in α_{s1} -casein during emulsification. Either denaturation of α_{s1} -casein during emulsification or oxidation of ¹⁹⁶Met is considered to have caused the *cis-trans* isomerization of ¹⁹⁷Pro. The Met-Pro bond of T-2S was only of the *trans* form, while that of T-2S-Met(O), which was oxidized in the solution, underwent *cis-trans* isomerization of the Pro. On the other hand, the Met-Pro bond of T-2E in the emulsion system was only of the *trans* form. These results indicate

that the isomerization cannot be attributed to denaturation of the α_{s1} -casein during emulsification, but to the oxidation of ¹⁹⁶Met, whether in the solution or the emulsion system.

The reversed-phase HPLC analysis showed that the addition of an oxygen atom to the sulfur atom in ¹⁹⁶Met decreased the hydrophobicity of the peptides containing ¹⁹⁶Met. The peptide bond of ¹⁹⁶Met-Pro may be fixed in the *trans* form by some structural factor in the native α_{s1} -casein. If so, the oxidation of ¹⁹⁶Met destroys this factor, through which the *cis-trans* isomerization of ¹⁹⁷Pro may occur. Further detailed studies should be performed to elucidate the mechanism for this isomerization more precisely.

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