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ESR AND SPIN-TRAPPING STUDY OF FREE RADICALS IN γ-IRRADIATED SOLID LYSOZYME

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Free radicals produced by γ-irradiation of solid lysozyme were investigated by a technique combining ESR, spin-trapping and enzymatic digestion. MNP and DMPO were used as spin-trapping reagents. The solid lysozyme was first γ-irradiated and then dissolved in an aqueous solution containing the spin-trapping reagent to stabilize free radicals. The spin adducts of lysozyme were digested to oligopeptides to get ESR spectra having a well-resolved hyperfine structure. The ESR spectra obtained showed that carbon-centered radicals, –ĊH–, at the side chains of amino acids, and thiyl radicals, $-CH_2-S\cdot$, at disulfide bridges were produced in γ-irradiated solid lysozyme.

KEY WORDS: ESR, spin-trapping, lysozyme, free radicals.

Abbreviations used: ESR, electron spin resonance; MNP, 2-methyl-2-nitrosopropane; DMPO, 5,5-dimethyl-1-pyrroline-1-oxide.

INTRODUCTION

Analysis of radiation damage in solid protein is of basic importance in the application of radiation sterilization to high-protein foods.[1–3] In early investigations, ESR spectroscopy in the solid state revealed data concerning primary and secondary free radicals produced by irradiation. ESR spectra obtained from irradiated proteins containing cystein residues are generally well-known as having unsymmetrical, broad hyperfine structures due to α-carbon radicals and thiyl radicals.[4–6] More recently, the spin-trapping technique has been widely utilized to identify the chemical structures of free radical intermediates produced in various biological systems.[7] This method was also applied to analyze the radical structures produced in γ-irradiated amino acids, N-acetyl amino acids and diepetides as model compounds of protein. Decarboxylated and deaminated radicals, as well as deprotonated radicals at α-carbon sites of the peptide (α-carbon radicals), have been identified.[8,9] However, this technique was inapplicable to an intact protein until we presented an improved method for its application to large molecules such as proteins and DNA.[10–13]

In this communication, in order to obtain information on radiation-induced free radicals in an intact solid protein, the spin-trapping method, improved by combination with enzymatic digestion, was applied to lysozyme as a model of proteins. Lysozyme is made up of a single polypeptide chain of 129 amino acids, cross-linked by four disulfide bridges and contains no free –SH groups.

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MATERIALS AND METHODS

Chemicals

Lysozyme powder was purchasd from Seikagaku Kogyo Company Ltd., Japan. Protease of *S. aureus* and glutathione were purchased from Sigma Chemical Company. MNP and DMPO were obtained from Aldrich Chemical Company. DMPO was purified by activated charcoal.[14]

γ-Irradiation and Spin-Trapping

A few hundred mg of solid lysozyme was put into a cylindrical quartz tube (0.4 cm i.d. and 10.4 cm long) designed for ESR measurements in the solid state. After air in the tube was replaced by Ar gas, the tube was exposed to γ-rays from a ^{60}Co source at a dose of 80 kGy at room temperature. After being analyzed by ESR spectroscopy in the solid state, the irradiated lysozyme was subsequently investigated by spin-trapping. The free radicals formed in solid lysozyme were spin-trapped by dissolving 20 mg of irradiated poweder into 2 ml of aqueous solution containing 20 mM MNP or 110 mM DMPO. The solution was then dialysed against 10 mM Tris-HCl buffer (pH 7.5). The spin-adducts were incubated with protease (0.1 mg/ml) at 37°C for 3 h for digestion and transferred to an aqueous quartz flat cell (6 × 1 × 0.025 cm) designed for the analysis of aqueous samples by ESR spectroscopy.

ESR Observation

ESR spectroscopy was employed to examine the free radicals of the solid lysozyme as well as the nitroxide free radicals of the digested spin-adducts. ESR measurements were made at room temperature with a JES-FE-3X X-band spectrometer. ESR spectra were recorded as first derivatives of the absorbance curve. The ESR scan for the solid sample was traced using a cylindrical quartz tube at 100 kHz of field modulation with an amplitude of 0.4 mT and the microwave power level was maintained at 1 mW to protect the ESR spectra from saturation. In the case of the digested spin-adducts, the ESR spectra were traced using an aqueous flat cell at 100 kHz of field modulation with an amplitude of 0.02 mT and the microwave power was maintained at 20 mW.

RESULTS AND DISCUSSION

ESR Spectrum of γ-Irradiated Solid Lysozyme

The ESR spectrum of γ-irradiated solid lysozyme is shown in Figure 1. This spectrum is markedly unsymmetrical, and has a doublet signal centered at a g-value of 2.003 (indicated by the arrow) and a broad signal which appears at low-field with a g-value between 2.020 and 2.080. In previous ESR studies concerning a large number of model proteins, numerous investigations showed that the doublet centered at the g-value of 2.003 was due to the radicals at the α-carbon of the peptide (–NH-C·(R)–CO–, R; amino acid side chains) and that the broad signal in the low-field originated from secondary thiyl radicals (–CH_2–S·) which were produced at the side chains according to the following reactions.[6]

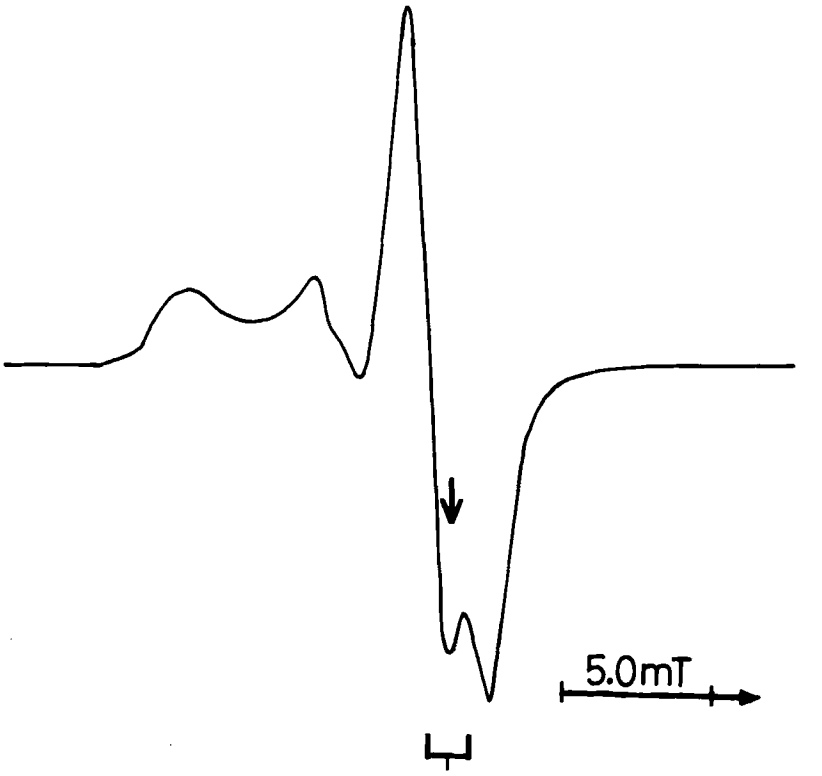

FIGURE 1 First-derivative ESR spectrum obtained after solid lysozyme was exposed to 80 kGy of γ-rays at room temperature. The arrow indicates the absorption at g = 2.003. The stick diagram indicates the positions of hyperfine lines due to carbon-centered radicals.

$$e^- + -S-S- \rightarrow -S^- -S- \qquad \text{(primary thiyl radical)}$$

$$-S^- -S- \xrightarrow{R^+ \; R\cdot} 2\,-CH_2-S\cdot \qquad \text{(secondary thiyl radical)}$$

Using the technique combining spin-trapping with enzymatic digestion, we re-examined the samples to clarify whether these radicals were produced by γ-irradaition of the solid lysozyme.

Spin-Trapping of Free Radicals Produced in γ-Irradiated Solid Lysozyme by MNP

The ESR spectrum derived from radicals trapped by MNP is shown in Figure 2a. The spectrum showed a triplet hyperfine structure consisting of a broad signal. No further hyperfine structures were observed. Since this line broadening may be due to the slow tumbling of the nitroxide radical, we digested the spin-adducts of lysozyme into those of oligopeptides by protease to get an ESR spectrum with a more resolved hyperfine structure. The resulting ESR spectrum is shown in Figure 2b. This spectrum, as shown

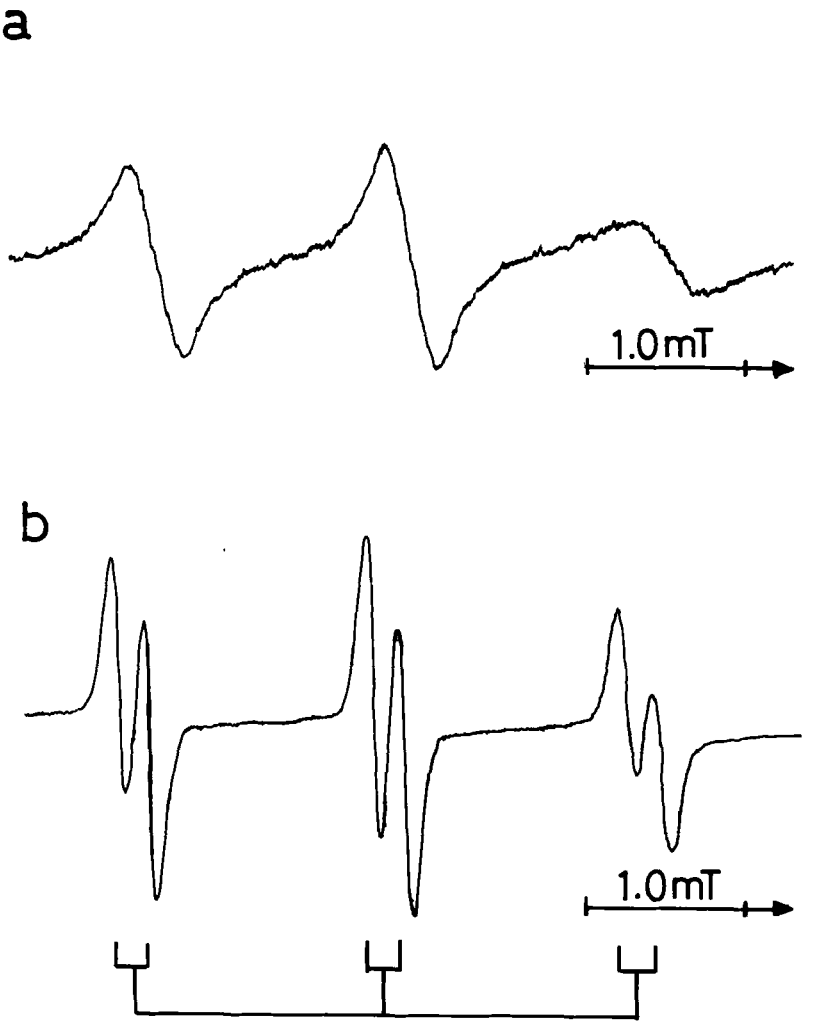

FIGURE 2 (a) ESR spectrum of spin-trapped radicals from solid lysozyme γ-irradiated at room temperature and subsequently dissolved in aqueous solution containing MNP. (b) ESR spectrum after being digested by protease at 37°C for 3 h. The stick diagram indicates the positions of the hyperfine lines.

by the stick diagram, has a primary nitrogen splitting of 1.59 mT which further splits into a 0.2 mT doublet probably arising from the interaction of the spin with a β-proton. A secondary doublet ranging from 0.16 mT to 0.36 mT was sometimes observed in photolyzed aqueous solutions containing H_2O_2, MNP and amino acids such as isoleucine, serine and asparaticacid, and was assigned to the radicals produced by H-abstraction from a side chain of the amino acid.[15] Therefore, the secondary doublet of 0.2 mT observed in Figure 2b can be assigned to radicals produced by the loss of a proton from the side chains of these amino acids which are involved in lysozyme.

In this spin-trapping experiment, the α-carbon radicals and the thiyl radicals were

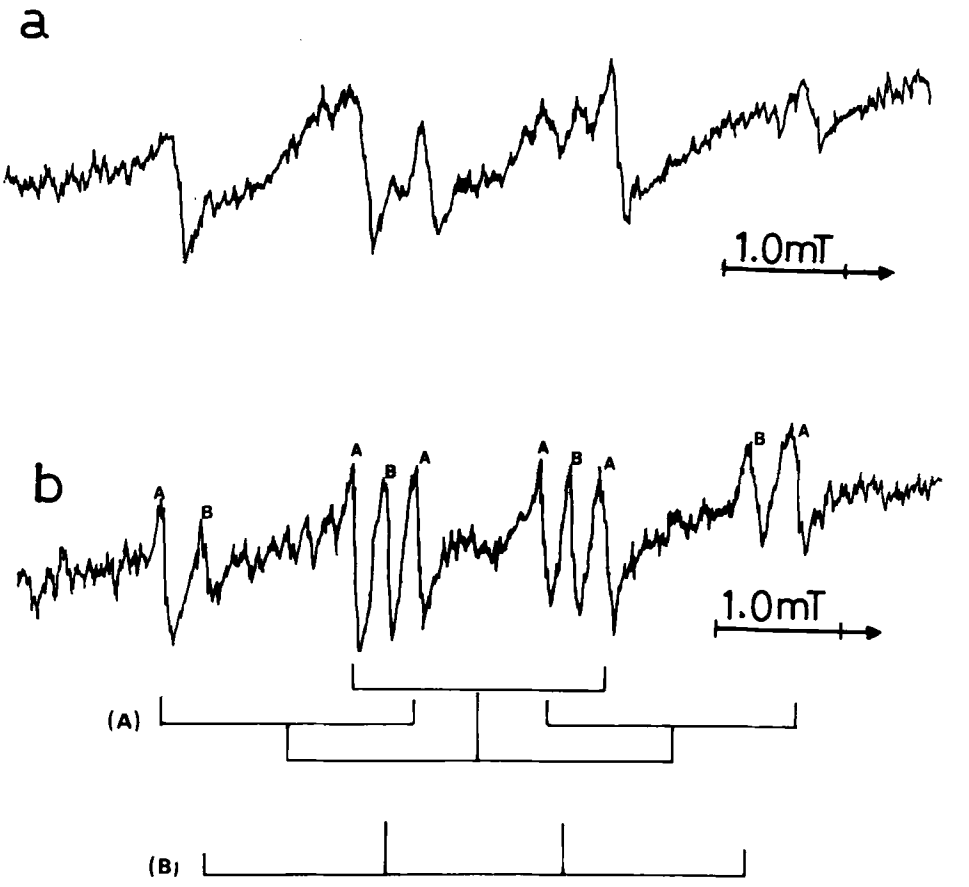

FIGURE 3 (a) ESR spectrum of spin-trapped radicals from solid lysozyme γ-irradiated at room temperature and subsequently dissolved in an aqueous solution containing DMPO. (b) ESR spectrum after being digested by protease at 37°C for 3 h. The stick diagrams indicate the hyperfine structures of the signals (A) and (B).

not identified, though it has been reported that these radicals were produced in the solid state.[1-6] This discrepancy may be explained by the instability of the spin-adducts or the inefficiency of spin-trapping of these radicals. Indeed, even in the simple model experiments such as UV photolysis of aqueous solutions containing amino acids and H_2O_2, the α-carbon radicals could not be identified except for glycine and alanine[15] and it has also been reported that the MNP-thiyl radical adduct is unstable.[16]

Spin-Trapping of Free Radicals Produced in γ-Irradiated Solid Lysozyme by DMPO

DMPO is reported to be able to trap thiyl radicals, alkoxyl radicals and peroxyl radicals. The ESR spectrum of each adduct gives a characteristic hyperfine structure,[17,18] although this trapping reagent is not suitable for the exact analysis of the structures of radicals with the electron centered on carbon atoms. This compound

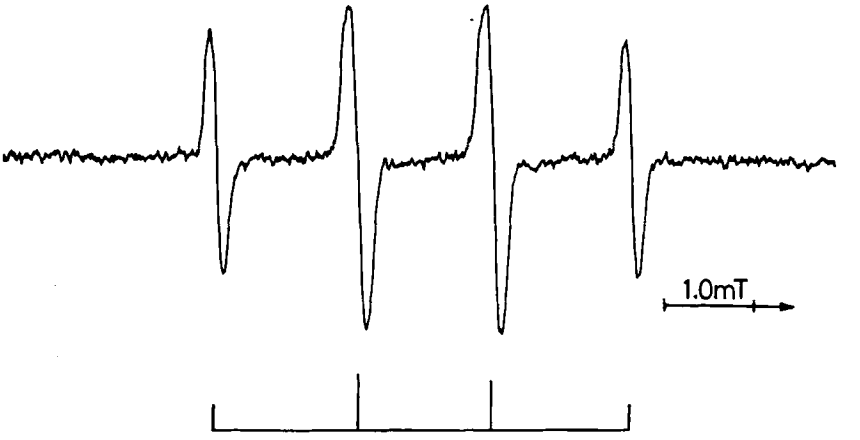

FIGURE 4 ESR spectrum of spin-trapped radicals from UV photolysis of an aqueous solution containing glutathione (100 mM), DMPO (100 mM) and H_2O_2 (10 mM). The stick diagram indicates the positions and amplitudes of the hyperfine lines.

was, therefore, employed in the next spin-trapping study of radicals produced in γ-irradiated solid lysozyme. Figure 3a shows an ESR spectrum obtained by dissolving γ-irradiated solid lysozyme into 100 mM DMPO solution. This signal exhibited a broad line-shape characteristic of slow tumbling nitroxide radicals, as in the case of MNP. Figure 3b is the ESR spectrum obtained by converting the spin-adducts of lysozyme to those of oligopeptides by protease digestion. This spectrum consists of two signals, labeled A and B.

The ESR signal labeled A showed a primary triplet splitting of 1.57 mT due to a nitrogen of the nitroxide group and a secondary doublet splitting of 2.08 mT due to a proton at the β-position. Davis and Slater have reported that the adducts between carbon-centerd radicals and DMPO produce β-proton splittings on order of 2.0 mT, whereas adducts between alkoxyl radicals or peroxyl radicals and DMPO give ESR spectra having β-proton splittings of 0.6–0.9 mT or 0.9–1.2 mT, respectively.[17] Therefore, ESR spectrum A can be associated to the carbon-centered radicals produced at α-carbons of the peptide and/or at side-chains of amino acids.

The ESR spectrum labeled B showed 1:2:2:1 quartet hyperfine splitting due to a nitrogen and a proton at the β-position. This ESR spectrum was identical to that of the DMPO-thiyl radical adduct reported by Eling *et al.*[18] To confirm this assignment, UV photolysis of aqueous solutions containing reduced glutathione (100 mM), H_2O_2 (10 mM) and DMPO (100 mM) was carried out. In this experiment, OH radicals generated by the photolysis of H_2O_2 attack the –SH group of glutathione to produce –S·, resulting in the formation of a DMPO-thiyl radical adduct. The ESR spectrum of this adduct is shown in Figure 4. The ESR parameters obtained from this spectrum were identical to those obtained from ESR spectrum B in Figure 3b. ESR spectra having hyperfine splittings of 0.6–0.9 mT or 0.9–1.2 mT due to a proton at the β-position were not observed. This fact indicates that neither alkoxyl nor peroxyl radicals were trapped in the present system.

CONCLUSIONS

We have demonstrated that carbon-centered radicals at side-chains of amino acid residues such as Ile, Ser and Asp, and thiyl radicals at cystein residues could have been produced by γ-radiolysis of solid lysozyme. However, alkoxyl and peroxyl radicals were not detected.

Acknowledgments

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