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# Lysozyme Modification by the Fenton Reaction and Gamma Radiation

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A comparative study was performed on lysozyme modification after exposure to Fenton reagent ($Fe(II)/H_2O_2$) or hydroxyl radicals produced by γ radiation. The conditions were adjusted to obtain, with both systems, a 50% loss of activity of the modified ensemble. γ radiation modified almost all types of amino acid residues in the enzyme, with little specificity. The modification order was Tyr > Met = Cys > Lys > Ile + Leu > Gly > Pro = Phe > Thr + Ala > Trp = Ser > Arg > Asp + Glu, with 42 mol of modified residues per initial mole of native enzyme. In contrast, when the enzyme was exposed to the Fenton reaction, only some types of amino acids were modified. Furthermore, a smaller number of residues (13.5) were damaged per initial mole of enzyme. The order of the modified residues was Tyr > Cys > Trp > Met > His > Ile + Leu > Val > Arg. These results demonstrate that the modifications elicited by these two free radical sources follow different mechanisms. An intramolecular free radical chain reaction is proposed to play a dominant role in the oxidative modification of the protein promoted by γ radiation.

*Keywords*: Lysozyme; γ radiation; Fenton reaction; Hydroxyl radical

## INTRODUCTION

Reactive oxygen species (ROS), such as singlet oxygen, ozone, superoxide anion, and alkoxyl, peroxyl, and hydroxyl radicals are important intermediates in cellular damage, and are likely to participate in the pathogenesis of a number of diseases, as well as in physiological processes such as aging, ischemia-reperfusion injury, and protein turnover.[1]

Traditionally, the damage associated with ROS has been mainly attributed to lipid peroxidation and membrane damage.[2] However, in recent years, the importance of the damage to proteins and their relation with some physiological disorders, has become apparent with the development of techniques that can detect and quantify various kinds of free radical-mediated protein modifications.[1,3]

The sensitivity of different proteins toward a given ROS depends on factors such as the site where the ROS are generated, amino acid composition of the protein, localization of the amino acids directly related to the biological activity of the protein, and the presence of scavengers or substances that can repair the damage etc. There is little kinetic information to offer an explanation of the observed differences in protein behavior. The reactivity of a given amino acid toward ROS is generally very different for the free moiety and for the residues bound to different proteins.[4–7] As a rule, the exposition to the solvent increases the reactivity of the given residue against singlet oxygen and other ROS.[8,9]

Ames *et al.*[10] have estimated that free radicals are responsible for $10^4$–$10^5$ DNA base modification per cell per day. To achieve such massive DNA damage,

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individuals would have to be exposed to levels of ionizing radiation that are several orders of magnitude greater than those present in normal environments. It is therefore unlikely that radiation is the major source of oxygen free radicals *in vivo*. The role of transition metals, and also ozone and nitric oxides, in free radical generation, is also likely to be biologically relevant.[1] These processes can lead to the production of the very harmful hydroxyl radical.

The free $HO^{\cdot}$ radical reacts with virtually all biological molecules at or near diffusion-controlled rates. Thus, when this radical is produced biochemically or in a biological system, it should not be regarded as freely diffusible.[11] The damage inflicted to a system by this radical will be determined by the locus of its generation ("site specific" processes).

In this work, we study the effect of the Fenton reaction, which has been frequently invoked as a mechanism of hydroxyl radical production in biological systems,[12] and the effect of hydroxyl radicals generated by γ radiation on the enzyme lysozyme [EC 3.2.1.17]. This enzyme is a good protein model because of its high structural stability, easily measurable enzymatic activity, and the fact that its structure has been determined by X-ray crystallography.[13] A comparison of the data will allow us to establish the type and efficiency of the damage produced by randomly distributed hydroxyl radicals (γ radiation) and the active species generated by the Fenton reaction. The possible differences could be due to site-specific generation of hydroxyl radicals,[1,11] and/or to the presence of other active ROS. In particular, evidence has been presented for reactions that lead to homolytic cleavage of $H_2O_2$ and the production of a highly reactive intermediate, which is not identical to the "free" hydroxyl radical.[11,14]

## MATERIAL AND METHODS

Hen egg-white lysozyme grade I, *micrococcus lysodeikticus*, urea, guanidine hydrochloride grade I, and 4-methylumbelliferyl-tetra-*N*-acetyl-β-chitotetraoside (MLF), were obtained from Sigma Chemicals. All other reagents were of analytical grade.

### Spectroscopic Measurements

Absorption spectra and their second derivatives were obtained in a Hewlett Packard 8453 spectrophotometer. Fluorescence emission was measured with a 650 10S Perkin–Elmer fluorescence spectrophotometer.

### Enzymatic Activity

The activity of lysozyme was assayed by measuring the lysis of *micrococcus lysodeikticus*.[15] *Micrococcus lysodeikticus*, 0.2 mg/ml, was suspended in 3 ml of 0.05 M phosphate buffer pH 7.0. The reaction was started by adding 10 μl of an enzyme solution.

For the determination of $K_M$ and $V_{max}$, the activity of the enzyme was measured by a fluorometric assay using the synthetic substrate MLF,[16,17] due to a better reproducibility of the results. The activity of the enzyme was evaluated from the fluorescence increase at 445 nm ($\lambda_{ex} = 320$ nm) when the substrate (3 ml of 5 μM MLF in 0.05 M citrate buffer pH 5.2) was hydrolyzed by lysozyme. The reaction was started by adding 700 μl of the enzyme solution.

The conditions in the inactivation experiments were adjusted in such a way that the enzyme ensemble retains nearly 50% of its initial activity after being exposed to the Fenton reaction or to γ radiation under the conditions described below.

### Fenton Reaction

Hydrogen peroxide was added to solutions containing 10.7 μM lysozyme and 20 μM $FeSO_4$ in HPLC grade water (5 ml) until a final 0.1 mM analytical concentration. The enzyme loss of activity was almost instantaneous.

### Gamma Radiolysis

Solutions containing 10.7 μM lysozyme in HPLC grade water (5 ml) and saturated with $N_2O$(g) were irradiated under steady state conditions during 25 min in a $^{60}Co$ source yielding 2.7 Gy/min (Comisión Childena de EnergÍa Nuclear, La Reina, Santiago, Chile).

### Amino Acid Analysis

Lysozyme was hydrolyzed with 6N HCl and derivatized with phenylisothiocyanate in Waters Pico-Tag work station. HPLC analyses were performed using a 15 cm reverse phase Waters Pico-Tag column with a 440 Waters Absorbance Detector, a 680 Waters Automated Gradient Controller (Eluent A: 1.9% (w/v) sodium acetate aqueous solution; Eluent B: acetonitrile/water: 60/40 (v/v)) and a Waters 746 Data Module. Trp was evaluated by the second derivative of the absorbance spectra (288 nm) in 0.1 M KOH.

### Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (7.5% acrylamide and 0.2% bis acrylamide)

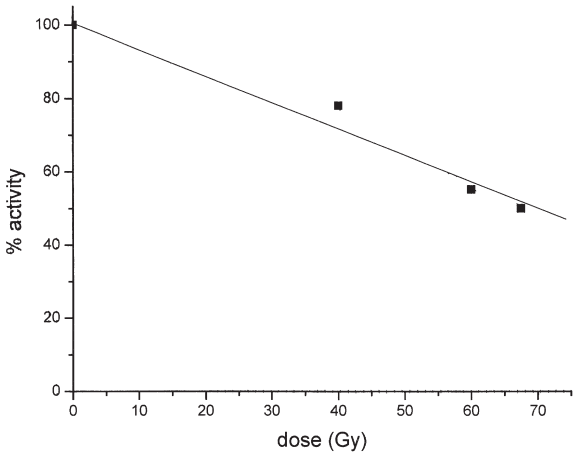


FIGURE 1 Kinetics of activity loss of lysozyme (10.7 μM) exposed to a dose rate of 2.7 Gy/min.

was performed according to the procedure described by Laemmli[18] in a LKB 2117 Multiphor gel apparatus. Protein samples were dissolved (3 mg/ml) in 0.2 M phosphate buffer, pH 7.1 containing 1% (v/v) 2-mercaptoethanol and 1% (w/v) SDS, and incubated at 37°C for 8 h.

In order to obtain the protein concentration required for SDS-PAGE, the concentration of all the reagents in the Fenton reaction and the protein concentration were increased 20-fold. Unfortunately, for the γ radiation process, the protein concentration could not be increased in such proportion. At this protein concentration, extremely long irradiation times were required to obtain a 50% inactivation with the dose rate provided by the $^{60}$Co source used in the present work.

## RESULTS AND DISCUSSION

The enzymatic activity of lysozyme decreases when the enzyme (10.7 μM) is exposed to the Fenton reaction or to γ radiation. The decrease of activity in the γ radiation experiments was proportional to the reaction time. Typical results are shown in Fig. 1. On the other hand, inactivation by the Fenton reagent was almost instantaneous. The conditions (time of exposition in the radiolysis and reagent concentration in the Fenton inactivation), were adjusted to obtain a 50% activity loss, measured at a fixed (0.2 mg/ml) *micrococcus lysodeikticus* concentration. This substrate concentration is lower than that required to reach the enzyme saturation stage ($V_{max}$ condition). The conditions necessary to reach the 50% inactivation of the enzyme ensemble activity were 20 μM Fe(II) and 0.1 mM $H_2O_2$ in the Fenton reaction, and a dose of 67.5 Gy in the radiolysis experiments. Under the employed conditions, neither Fe(II) nor $H_2O_2$ alone produced any

TABLE I Kinetic parameters $K_M$ and $V_{max}$ for native and modified lysozymes

| Enzyme | $K_M$ (μM) | $V_{max}$ (μM/min) | $r$* |
|---|---|---|---|
| Native lysozyme | 2.31 | 595 | 0.997 |
| Lysozyme–Fenton | 2.53 | 302 | 0.990 |
| Lysozyme–γ radiation | 3.0 | 362 | 0.997 |

*Correlation coefficient for the linear fit of the Lineweaver–Burk plot.

inactivation. Furthermore, the effect of the Fenton reagent was almost negligible, even after 1 h of incubation, when phosphate buffer (50 mM, pH 7.0) was used as the reaction medium.

The characteristics of the ensembles partially modified by their exposure to the Fenton reagent or γ radiation were assessed by their resistance to denaturation (thermal or pH driven), Michaelis–Menten parameters, and amino acid composition.

The effect of temperature on the enzymatic activity on both Fenton and γ radiation-modified lysozymes were similar to that of the native enzyme (data not shown). When the influence of pH was studied, a lower activity in basic media was observed for the Fenton-modified enzyme (not shown). Both Fenton and γ radiation modified enzymes showed a nearly Michaelis–Menten behavior. The $K_M$ and $V_{max}$ values, obtained from Lineweaver–Burk plots employing MLF as substrate, are shown in Table I. The $K_M$ values obtained, that correspond to their average over all the partially modified ensembles, do not indicate a significant change in the affinity of the enzyme for the substrate. Nevertheless, a more detailed inspection of the Lineweaver–Burk plot corresponding to the enzyme pre-exposed to the Fenton reaction, which has a lower correlation coefficient (Table I), indicates that it is best fitted to a polynomial ($r = 0.997$), as shown in Fig. 2. This

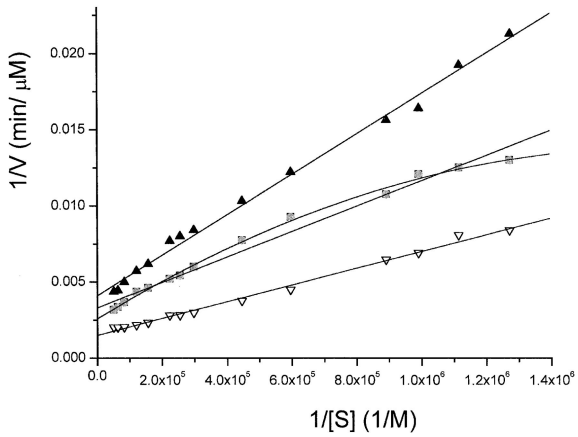


FIGURE 2 Lineweaver–Burk plot for native, 10.7 μM (▽), Fenton modified, 10.7 μM (■) and γ radiation modified, 7.1 μM (▲) lysozyme using MLF as substrate. Both linear and polynomial fits are shown for the data of the Fenton modified enzyme. Data are shown at different enzyme concentrations for the two sets of modified enzyme in order to improve the clarity of the representation.

TABLE II Amino acid analysis of Fenton and γ radiation modified lysozymes

| | | Lysozyme–Fenton | | | Lysozyme – γ radiation | | |
|---|---|---|---|---|---|---|---|
| Amino acid | Native lysozyme | Damage% | Damaged amino acids | Remaining amino acids | Damage% | Damaged amino acids | Remaining amino acids |
| Asp + Glu | 26 | 5 | 1.3 | 24.7 | 8 | 2.08 | 23.92 |
| Ser | 10 | 4 | 0.4 | 9.6 | 23 | 2.3 | 7.7 |
| Gly | 12 | 0 | 0 | 12 | 41 | 4.92 | 7.08 |
| Arg | 11 | 10 | 1.1 | 9.9 | 20 | 2.2 | 8.8 |
| Thr + Ala | 19 | 0 | 0 | 19 | 30 | 5.7 | 13.3 |
| Pro | 2 | 0 | 0 | 2 | 32 | 0.64 | 1.36 |
| Tyr | 3 | 80 | 2.4 | 0.6 | 73 | 2.19 | 0.81 |
| Val | 6 | 12 | 0.72 | 5.28 | 46 | 2.76 | 3.24 |
| Met | 2 | 24 | 0.48 | 1.52 | 64 | 1.28 | 0.72 |
| Cys | 8 | 32 | 2.56 | 5.44 | 63 | 5.04 | 2.96 |
| Leu + Ile | 14 | 15 | 2.1 | 11.9 | 48 | 6.72 | 7.28 |
| Phe | 3 | 0 | 0 | 3 | 32 | 0.96 | 2.04 |
| Lys | 6 | 8 | 0.48 | 5.5 | 62 | 3.72 | 2.28 |
| Trp* | 6 | 29 | 1.74 | 4.26 | 26 | 1.56 | 4.44 |
| His | 1 | 19 | 0.19 | 0.81 | 0 | 0 | 1 |
| Total | 129 | | 13.5 | | | 42 | |

*Second derivative method.

behavior could indicate the presence of two or more populations of active molecules, which have been affected in different ways by the Fenton reaction. In particular, the first linear part of the curve would reflect an enzyme population with a lower affinity for the substrate ($K_M = 3.9$ mM). Activity from protein molecules having $K_M$ values higher than the native enzyme have also been reported in the inactivation of lysozyme promoted by photochemically generated singlet oxygen.[19]

The effect of Fenton and γ radiation modifications on the different amino acid residues of lysozyme was quantified by HPLC amino acid analysis. The results obtained are shown in Table II. The method requires acid hydrolysis of the protein, which promotes Trp oxidation. As a first attempt to quantify the effect on the Trp residues, the fluorescence of γ-irradiated and Fenton-modified enzymes were determined. The native and partially inactivated enzyme solutions were treated with 6 M guanidine hydrochloride to assure that Trp residues were similarly exposed to the solvent in the native and oxidized proteins. Under these conditions, it is possible to minimize the differences in the fluorescence quantum yield of the six Trp residues of lysozyme due to their different microenvironment in the protein structure. Despite exhaustive dilution of the Fenton-modified enzyme solution with 6 M guanidine hydrochloride, the filter effect due to the absorbance of both Fe(II) and $H_2O_2$ (Fig. 3a) at the excitation wavelength (295 nm)




FIGURE 3 (A) Absorption spectra of 5-fold diluted solutions of: lysozyme (—), lysozyme and Fe(II) (···), lysozyme and $H_2O_2$ (———), lysozyme and (Fe(II) + $H_2O_2$) (– –); (B) second derivative of the absorption spectra of solutions in 0.1 M KOH of: lysozyme (—) and lysozyme and (Fe(II) + $H_2O_2$) (– –). The curves for lysozyme and Fe(II) and lysozyme and $H_2O_2$ are practically indistinguishable from that of lysozyme.

interfered with the determination. With the aim to minimize Tyr residue interference in the evaluation of the number of intact Trp residues in the enzymes, KOH (final concentration 0.1 M) was added to the protein solutions. Under this strongly alkaline condition, all the Tyr residues are transformed into tyrosinate ions characterized by an unstructured absorption band, allowing excellent resolution of the fine structure of Trp residues by second-derivative absorption spectroscopy.[20–22] Solutions with a unique Trp concentration but with increasing concentrations of Tyr, give exactly the same second-derivative absorption spectra (data not shown). Figure 3b shows the second-derivative absorption spectra for the Fenton-modified enzyme and for the native lysozyme with and without $H_2O_2$ or Fe(II). The value obtained (Table II) for the γ radiation damage on the Trp residues (26%) agrees remarkably well with that determined by fluorescence measurements (data not shown). This agreement was due to the lack of important filter effects in the γ radiation system. This contrasts with the Fenton reaction mixture, where a considerably higher value for the Trp damage was calculated by fluorescence measurements, relative to that obtained by second-derivative absorption spectroscopy. The Trp damage produced by γ radiation in this work is similar to that described by Franzini *et al.*[23] (with a 40 Gy dose, condition for a 50% inactivation).

The results shown in Table II indicate that γ radiation produces a higher and less selective effect than the Fenton reaction, with modification of almost all types of amino acid residue in lysozyme, with the only exception of His. Davies *et al.*[4] reported that all types of amino acids were modified in bovine serum albumin (BSA) after γ irradiation.

Irradiation of the protein solution can reduce the enzyme activity by its direct modification by the γ radiation and/or through the reactions of hydroxyl radicals generated from the cleavage of water molecules. However, at the low protein concentrations employed in the present work (below 0.15 mg/ml), it is generally accepted that most of the damage arises from water-derived free radicals.[3,24] Furthermore, the kinetics obtained in the present work are not compatible with a significant contribution of direct radiation damage. At low protein concentrations, the damage inflicted by the radiation must follow first order kinetics. The date obtained in the present work is best fit to zero order kinetics (Fig. 1), as expected from free radical damage under conditions of total trapping of the produced radicals. In agreement with these considerations, the rate of the inactivation process was nearly independent of the protein concentration when it was changed from 3.45 to 10.7 μM. Similar considerations apply regarding the Trp fluorescence loss. The rate of the process only varied 25% when the protein concentration increases by 300%. These data are compatible with the damage promoted by free radicals generated in the radiolysis of water. The γ radiolysis ($^{60}$Co source) of water can be represented by:[1]

$$H_2O \xrightarrow{\gamma-\text{radiation}} HO^\bullet + e^-_{aq} + H^+ \quad (1)$$

$$H_2O \xrightarrow{\gamma\text{-radiation}} HO^\bullet + H^\bullet \quad (1')$$

$$O_2 + e^-_{aq} \rightarrow O_2^{\bullet -} \quad (2)$$

$$H^\bullet + O_2 \rightarrow HO_2^\bullet \quad (3)$$

$$HO_2^\bullet \rightarrow H^+ + O_2^{\bullet -} \quad (4)$$

The generation of $H^\bullet$ (Eq. (1′)) represents less than 10% of the yield of $HO^\bullet + e^-_{aq}$.

In our system, the aqueous solution was saturated with nitrous oxide ($N_2O$) before irradiation in order to remove the $e^-_{aq}$ and prevent the generation of other ROS different to $HO^\bullet$:[25]

$$e^-_{aq} + N_2O \rightarrow N_2 + O^- \quad (5)$$

$$O^- + H^+ \rightarrow HO^\bullet \quad (6)$$

The dose rate of the $^{60}$Co source used in this study was 2.7 Gy/min, and the exposition time was 25 min (to obtain 50% inactivation). Oxygen radical yields are based on known *G* values in water since the low protein concentrations employed do not significantly affect the radical yields.[3,24] Under 100% $N_2O$, the $HO^\bullet$ generation is 0.6 μM/J. In our conditions, lysozyme (10.7 μM) was 50% inactivated with a dose of 4.0 mol of hydroxyl radicals per initial mole of lysozyme. This result is compatible with that reported by Franzini *et al.*[23] with a 19.8 Gy/min $^{60}$Co source (6.5 mol $HO^\bullet$ mole of inactivated lysozyme).

Hydroxyl radicals are randomly generated in the bulk solution and react with most types of amino acid residues, without showing a clear specificity. Of note is the high number (42) of amino acid residues modified per initial lysozyme molecule, when the total enzyme solution retains half of the original activity. According to these results, as a mean value, each $HO^\bullet$ would trigger the modification of approximately 10 amino acid residues. This implies that the $HO^\bullet$ initiates a chain reaction in the protein. This chain reaction and a high efficiency of capture of the initial radicals by the protein are favored by:

i) The initial hydroxyl radicals have protein as their only target. This leads to a *ca.* 100% efficiency of capture.

ii) The radicals formed in the protein from the initial hydroxyl radicals cannot be trapped by other species (such as HOOH in Fenton's).
iii) The low rate of radical production disfavors bimolecular termination reactions between two protein-derived radicals, favoring the occurrence of inter- and intra-molecular chain reactions at the protein level.

Lysozyme's three-dimensional structure is known from X-ray diffraction.[14] A peculiarity of this protein is the presence at the surface of normally-buried hydrophobic residues such as Val-2, Phe-3, Leu-17, Phe-34, Leu-75, and Trp-123. Furthermore, the active site consists of a great cleft that allows water access, and residues such as Trp-62, Trp-63, Ile-98, Trp-108, and Val-109 could also be accessible to the radicals.

Lysozyme is characterized by a high structural stability.[26,27] There is no inactivation when lysozyme is irradiated in the presence of *tert*-butanol,[28] and it is necessary to increase the temperature to 52°C to unfold most lysozyme molecules.[29] The Trp residues were completely exposed to the solvent when the enzyme was dissolved in 6 M guanidine hydrochloride, but not if the enzyme was either dissolved in 8 M urea or when the four disulfide bridges were reduced and S-carboxymethylated.[8] The enzymatic activity was lost in urea and in guanidine hydrochloride denaturing media, but it was almost instantaneously restored when an aliquot of these solutions was added to the normal assay buffer (0.07 M phosphate buffer pH 7.0, 0.017 M NaCl.[8] The high enzyme stability makes possible that a number of non-essential amino acid residues could be modified without an appreciable effect in the enzymatic activity.

Traditionally, $HO^{\bullet}$ is invoked as the primary intermediate in Fenton-type oxidations:[1,30–32]

$$Fe^{2+} + HOOH \rightarrow HO^{\bullet} + OH^{-} + Fe^{3+} \qquad (7)$$

However, other intermediates have been proposed for related reactions involving chelated iron.[30,31] Wink *et al.*[12] postulated two key oxidizing intermediates, neither of which is the free $HO^{\bullet}$. One would be an iron complex formed via direct reaction of $H_2O_2$ and Fe(II), and the second would be a Fe(IV) oxo complex.

Independent of what exactly is the oxidizing species, the behavior of our system is compatible with a site-specific Fenton oxidation.[13,33] We first add the iron to the enzyme solution, allowing the interaction at the binding site in the protein, and then the reaction is triggered by the addition of $H_2O_2$ to obtain a 50% inactivation. The number of amino acid residues modified per initial lysozyme molecule (13.5 residues), was less than a third of the number modified in the γ radiation experiments. Furthermore, Gly, Thr, Ala, Phe residues were unaffected and His damage was less than 20%. Despite the lower number of modified amino acids, the same 50% inactivation was achieved, suggesting that the amino acids modified in this system were more related to the enzyme activity than those affected by γ radiation. In the Fenton system, it is more difficult to evaluate stoichiometric coefficients, such as the number of residues modified per radical produced. In our conditions, 9.34 mol $H_2O_2$/mol lysozyme was required to obtain a 50% inactivation. If it is assumed that the generation of the oxidizing species is determined by the $H_2O_2$ concentration, it can be calculated that each oxidizing species would trigger the modification of approximately 1.4 amino acid residues. This is compatible with a site-specific Fenton reaction. The lack of chain reaction in this system could be related to both the presence of alternative substrates (i.e. HOOH) and to a much higher radical input, that favors bimolecular termination reactions.

The above considerations can overestimate the number of radicals that have reacted with the protein. In fact, it can be considered that the occurrence of Haber–Weiss cycle, such as:

$$HO^{\bullet} + HOOH \rightarrow H_2O + O_2^{\bullet -} + H^{+} \qquad (8)$$

$$O_2^{\bullet -} + Fe^{3+} \rightarrow Fe^{2+} + O_2 \qquad (9)$$

could notably reduce the number of hydroxyl radicals that reacted with the protein. This will increase the number of amino acid residues modified as a consequence of an initial protein–hydroxyl radical interaction. Furthermore, it must be considered that the reaction of a protein-derived radical with $H_2O_2$ to generate a superoxide anion, followed by Reaction (9), can also contribute to a decrease in the number of hydroxyl radicals able to interact with the enzyme. Our results differ with those reported by Sellak *et al.*[31] who observed only a reversible inactivation when lysozyme was exposed to Fe(II) + $H_2O_2$. Furthermore, in this previous work, no modification with respect to the control was found on the band that appeared on SDS-PAGE. This result also contrasts with those found in the present work. Figure 4 shows SDS-PAGE of lysozyme exposed to Fenton reaction (50% inactivation) and of native lysozyme. A decrease in the intensity of the band corresponding to native lysozyme was observed for the Fenton modified enzyme, and new bands appear corresponding to aggregated forms of the protein. Also, to a much lesser extent, other bands corresponding to lower molecular weight fractions appear. In this regard, it must be noted that in the presence of phosphate (50 mM), we did not observe any effect (see above). In spite of the fact that the buffer concentration used by Sellak *et al.*[30] was

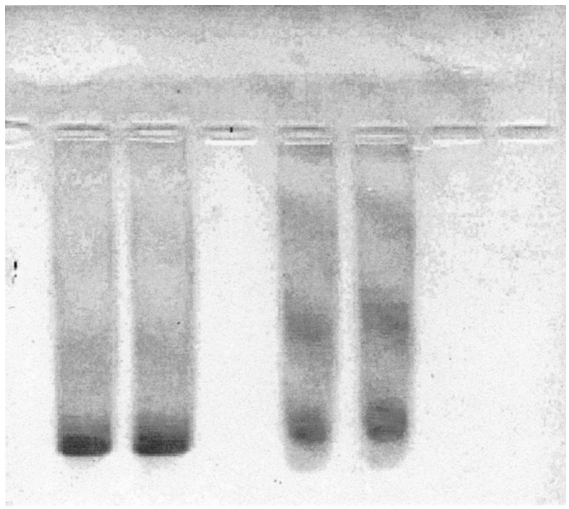


FIGURE 4 Sodium dodecyl sulfate polyacrylamide gel electrophoresis of native lysozyme (A and B) and of Fenton modified lysozyme (C and D).

considerably lower (10 mM), an inhibitory effect of phosphate under their experimental conditions cannot *a priori* be disregarded.

Unfortunately, the amount of γ-radiation-modified lysozyme needed for SDS-PAGE could not be obtained (see "Materials and methods"). Franzini *et al.*[23] and also Sellak *et al.*[31] reported that after γ irradiation, aggregation of lysozyme occurs and also two new bands of lower molecular weight than that of the native enzyme, appear.

When the effect on lysozyme of both the Fenton reaction and γ radiation are compared, consideration must be given to the fact that the $HO^{\bullet}$ radicals produced by γ radiolysis can initiate chain reactions in the protein. Lissi and Clavero[34] have previously reported the occurrence of short chain reactions in the alkylperoxyl radicals-driven inactivation of lysozyme, and Lissi *et al.*[35] have also postulated this mechanism for the inactivation of horseradish peroxidase (HRP) by the same type of radicals.

Traditionally, when γ radiolysis is the source of the $HO^{\bullet}$ radicals, the damage observed at the protein level is interpreted as an effect of the direct reaction of this radical with the protein. Under the conditions used in this work, it must be considered that even though all the hydroxyl radicals produced by γ radiolysis probably react with amino acid residues of the protein, almost all of them initiate intramolecular chain reactions, responsible for a much higher protein damage. This chain can explain both, the very high number of amino acids damaged by hydroxyl radicals produced and the very high number of amino acids modified by inactivated enzyme. The differences observed with the reaction promoted by the Fenton reagent can also be due to the different relevance of the intraprotein chains. The occurrence of these chains could be a general feature of free radical protein modifications carried out in the absence of free radical substrates that can act as inhibitors and/or could transfer the damage to other molecules.

### *Acknowledgments*

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