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Oxidative Modification of Proteins: Oxidation of Tryptophan and Production of Dityrosine in Purified Proteins Using Fenton's System

E. E. Dubinina[1]*, S. V. Gavrovskaya[1], E, V. Kuzmich[1], N. V. Leonova[1], M. G. Morozova[1], S. V. Kovrugina[1], and T. A. Smirnova[2]

[1]Bekhterev Psychoneurological Research Institute, ul. Bekhtereva 3, St. Petersburg, 193019 Russia; fax: (812) 567-7127; E-mail: spbinstb@infopro.spb.su
[2]Mechnikov St. Petersburg State Medical Academy, Piskarevskii pr. 47, St. Petersburg, 195067 Russia

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Abstract—Specific features of metal-catalyzed oxidation (MCO) of purified proteins (human serum albumin and human erythrocyte superoxide dismutase) were analyzed by the oxidation level of tryptophan and tyrosine. The production of dityrosine cross-links and the oxidation of tryptophan residues were recorded by fluorescence. The degree of oxidative modification of the amino acid residues of the proteins depended on the concentration of the Fenton's medium components and on the incubation time. These changes were different in different proteins. By electrophoresis and gel-permeation chromatography, changes in the superoxide dismutase structure are shown to be caused by oxidative modification of the enzyme and to be accompanied by a decrease in its activity. Findings with $OH^{\cdot}$ scavengers (mannitol and ethanol) suggest that oxidative modification of the proteins in Fenton's medium should be associated not only with hydroxyl radical but also with ferryl and perferryl ions and with the radical $CO_3^{\cdot-}$.

Key words: oxidative modification of proteins, dityrosine cross-links, superoxide dismutase, human serum albumin, hydroxyl radical, hydrogen peroxide, radical scavengers, $CO_3^{\cdot-}$

Oxidative degradation of proteins under conditions of oxidative stress is now intensively studied [1-7]. Oxidative modification of proteins, considering their multiple functions in tissues, unlike lipid peroxidation, can be selective and specific.

The main principles of oxidative modification of proteins have been formulated based on studies on the effect of ionizing radiation on purified proteins or on individual amino acids [8-11]. Under the influence of radiation, hydroxyl radical $OH^{\cdot}$ and superoxide anion radical $O_2^{\cdot-}$ are generated, which can not only degrade side chains of amino acid residues but also oxidize the very skeleton of the polypeptide chain in the region of the α-carbon atom with resulting fragmentation of the molecule.

Abbreviations: MCO) metal-catalyzed oxidation; HSA) human serum albumin; SOD) superoxide dismutase; CBC) carbonate-bicarbonate buffer; PB) phosphate buffer; SDS) sodium dodecyl sulfate; GPC) gel permeation chromatography.

* To whom correspondence should be addressed.

The generation of $OH^{\cdot}$ *in vivo* is considered associated with a metal-dependent degradation of hydrogen peroxide. In the body, the most common transition metal is iron. Oxidation of Fe^{2+} in the presence of H_2O_2 is the essence of Fenton's reaction [12]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\cdot}.$$

Oxidative modification of proteins is accompanied by disorders not only in the primary but also in the secondary and tertiary structures of proteins that results in aggregation or fragmentation of the protein molecule depending on its amino acid composition [1, 13]. Thus, one-electron oxidation of L-tyrosine generates a long-lived tyrosyl radical, which forms dityrosine cross-links on interaction with the same radical [1]. The production of dityrosine cross-links caused by interaction of tyrosyl radicals of different polypeptide chains results in the aggregation of proteins [13]:

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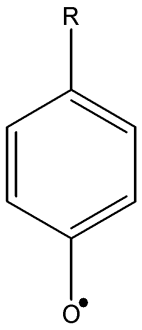
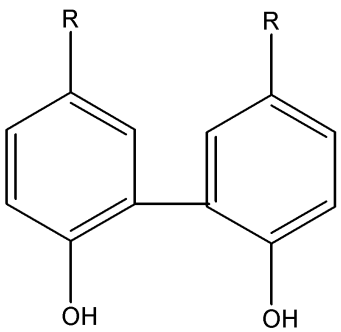


tyrosyl radical



dityrosine

Both in normalcy and in disease, protein oxidation in the body occurs with involvement of transition metal ions. This is the so-called metal-catalyzed oxidation (MCO) of proteins. According to the literature, MCO of proteins is site-specific and is characterized by interaction of H_2O_2 and transition metal ions (Fe, Cu) on a metal-binding surface of the molecule. Therefore, the metal-catalyzed reaction results in $OH^{\bullet}$ generation in a limited region, and oxidative modification is mainly induced in the adjacent amino acid residues [14, 15].

The question is constantly discussed whether biomolecules can be oxidized not only under the influence of $OH^{\bullet}$ produced during Fenton's reaction, but also under the influence of iron–oxygen complexes (ferryl and perferryl ions) generated [16-21]. Some authors think that under physiological conditions the role of $OH^{\bullet}$ in the initiation of oxidative modification of biopolymers is insignificant [16, 21]. $OH^{\bullet}$ is a short-lived radical ($t_{1/2} = 10^{-9}$ sec). The diffusion path of $OH^{\bullet}$ radical before reaction is about two molecular diameters (less than 0.001 μm); therefore, this radical can induce damage located very near the place of its generation [21, 22]. Thus, under physiological conditions, it is impossible to obtain in biological systems a high concentration of $OH^{\bullet}$, and its effect on the amino acid residues of proteins is limited to the site of its generation.

There is an opinion that in the mixture Fe^{2+}-EDTA and H_2O_2, ferryl ion is predominantly produced [16, 17, 21]:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{2+}{-}O + H_2O.$$

Ferryl ion can be produced through the stage of perferryl ion:

$$Fe^{2+} + O_2 \leftrightarrow [Fe^{2+}{-}O_2 \leftrightarrow Fe^{3+}{-}\ O_2^{\bullet-}] \leftrightarrow Fe^{3+} + O_2^{\bullet-},$$

$$Fe^{2+} + Fe^{2+}{-}O_2 \rightarrow Fe^{2+}{-}O_2{-}Fe^{2+} \rightarrow 2Fe^{2+}{-}O,$$

perferryl ion → ferryl ion.

Thus, data on the possible involvement of $OH^{\bullet}$ or of other reactive compounds in oxidation of proteins in the presence of transition metal ions under aerobic conditions are inconsistent. Therefore, we analyzed possible mechanisms of MCO of purified proteins using Fenton's system (Fe^{2+} and H_2O_2) on changing the experimental conditions and recording the oxidation levels of tyrosine and tryptophan residues.

MATERIALS AND METHODS

The following purified proteins were used: human serum albumin (HSA) and human superoxide dismutase (SOD) (Institute of Especially Pure Biopreparations, St. Petersburg, Russia), thrombin (Pharmacia, Sweden), trypsin (Sigma, USA). The purified proteins were oxidized in the presence of varied concentrations of Fe^{2+}, EDTA, and H_2O_2. The introduction of EDTA and other chelating agents into the incubation medium kept the oxidized iron in solution [23]. The level of oxidative modification of proteins was evaluated by production of dityrosine cross-links and by a decrease in the fluorescence spectrum maximum of tryptophan because of its oxidation. A Hitachi (Japan) spectrofluorimeter was used for determinations. Dityrosine was recorded at λ_{ex} = 325 nm and λ_{em} = 415 nm. The decrease in the maximum of tryptophan fluorescence spectrum was recorded at λ_{ex} = 295 nm and λ_{em} = 340 nm [1]. The production of dityrosine in the oxidized proteins was evaluated by an increase in the intensity of fluorescence at 415 nm. The control sample contained the native protein and all other components of the incubation medium except Fe^{2+}-EDTA and H_2O_2. The protein concentration in the control and experimental samples was 0.5 mg/ml.

The degree of structural changes in the oxidized proteins was analyzed by electrophoresis and by gel-permeation chromatography (GPC). Electrophoresis of the proteins was performed by the method of Laemmli [24] in polyacrylamide gradient gels in the presence of 2% SDS, at protein load of 1.25 μg per well. The protein was stained with 0.2% Coomassie R-250.

The proteins were separated by GPC using a HP-1090 device (Hewlett Packard, USA) with a column filled with Superose-12 (Pharmacia), using 0.2 M acetate buffer (pH 7.0) as eluent, at the flow rate of 0.8 ml/min; the detection was at 280 nm. The amount of studied material placed onto the column was 0.02 mg (sample volume 20 μl).

The activity of purified SOD was determined by the quercetin method [25].

The concentration of H_2O_2 in Fenton's system was determined at 230 nm with molar absorption coefficient 71 $M^{-1}{\cdot}cm^{-1}$ [26]. The tables and figures present average results of three or four experiments.

RESULTS

At the initial stage of the work, we studied the dependence of rates of tryptophan oxidation and of dity-

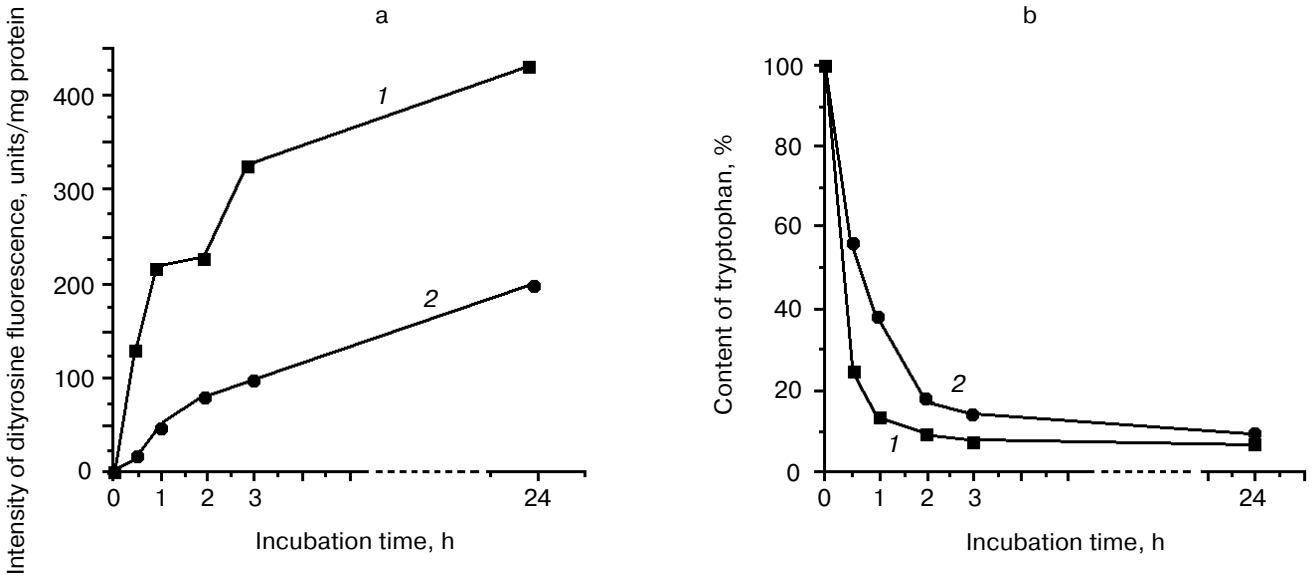


Fig. 1. Time dependence of oxidation of tryptophan and tyrosine of HSA (*2*) and SOD (*1*) in Fenton's medium (Fe^{2+}/H_2O_2): a) oxidation of tyrosine; b) oxidation of tryptophan. The experimental conditions are described in the text.

rosine cross-link production in HSA and in human erythrocyte SOD on the time of incubation in Fenton's medium. The tryptophan oxidation of the proteins was studied in the incubation mixture as follows: 0.065 M phosphate buffer (pH 7.4), 0.2 mM Fe^{2+}, 0.086 mM EDTA, and 0.1 M H_2O_2 [27, 28]. Dityrosine is known to have a blue fluorescence in alkaline medium [29, 30]. Therefore, tyrosine residues of HSA and SOD were oxidized under the same concentrations of the Fenton's medium components but using 0.1 M carbonate-bicarbonate buffer (CBC, pH 9.5) (Fig. 1). The Fig. 1 shows that 2 h of the incubation resulted in the oxidation of virtually all tryptophan in SOD and HSA. The oxidation of tyrosine residues in HSA and SOD under the same conditions but in the presence of CBC was slower, and the level of dityrosine was gradually increasing during 24 h.

We studied the free radical sensitivity of some purified proteins in Fenton's system with contents of its components decreased 16-, 8-, 4-, and 2-fold compared to their following initial values: 0.1 mM Fe^{2+}, 0.043 mM EDTA, and 0.05 M H_2O_2. The incubation was for 2 h at 37°C. The level of oxidation of tryptophan residues of SOD, trypsin, thrombin, and HSA (0.5 mg/ml) increased with an increase in contents of the Fenton's system components (Fig. 2). SOD was the most sensitive to radicals, and a dramatic decrease in the content of tryptophan was recorded even at the 16-fold decreased contents of the Fenton's system components.

The production of dityrosine cross-links also increased in all proteins studied with an increase in the contents of the Fenton's system components. The highest level of dityrosine was recorded for SOD and trypsin at the lowest contents of the diluted Fenton's medium (16-fold dilution).

Thus, the oxidation of tyrosine and tryptophan in the studied proteins depends on the concentration of the Fenton's system components and on the incubation time. SOD was the most sensitive protein. Its high sensitivity is suggested to be due to the presence of Cu^{2+} in the active center of the enzyme. In the presence of H_2O_2, this results in an additional generation of $OH^{\bullet}$. This hypothesis is confirmed by our finding of the increased intensity of dityrosine fluorescence up to 90 units/mg protein after the 1-h incubation of SOD in the medium containing CBC and 0.05 M H_2O_2 and of the decrease in the tryptophan content to 21% in the medium containing phosphate buffer and 0.05 M H_2O_2.

The effect of the Fenton's system components is believed to be mainly associated with the generation of $OH^{\bullet}$. However, the detection of dityrosine cross-links after 24 h makes it doubtful. During such a prolonged incubation the degradation of H_2O_2 is possible. We determined the content of H_2O_2 in the medium containing carbonate buffer (pH 9.5), HSA, and the Fenton's system components by the absorption at 230 nm [26]. Even after 2 h, only trace amounts of H_2O_2 were found in the incubation medium. This dramatic decrease in the level of hydrogen peroxide in the incubation medium could be caused by the influence of carbonate ions or of pH. To elucidate the cause of the decrease in the H_2O_2 content, we compared amounts of H_2O_2 in borate and phosphate buffers (table) in incubation medium of the following composition: HSA (0.5 mg/ml), Fe^{2+} (0.2 mM), and EDTA (0.086 mM). A pronounced degradation of H_2O_2

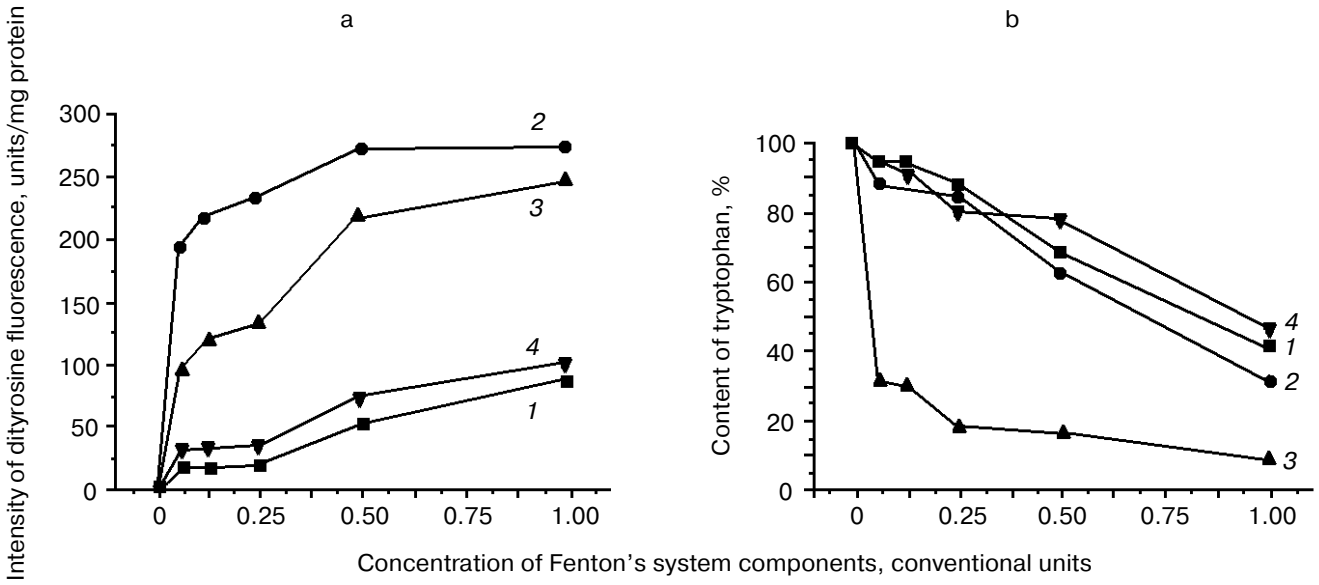


Fig. 2. Dependence of oxidation of tryptophan and tyrosine residues of proteins on contents of Fenton's system (Fe^{2+}–EDTA/H_2O_2) components on incubation for 2 h: a) oxidation of tyrosine; b) oxidation of tryptophan. *1*) HSA; *2*) trypsin; *3*) SOD; *4*) thrombin. Concentrations of the Fenton's system components are given in conventional units (1 unit corresponds to $1.0 \cdot 10^{-4}$ M Fe^{2+}, $0.43 \cdot 10^{-4}$ M EDTA, and 0.05 M H_2O_2).

occurred in phosphate buffer already after 2 h. And under these conditions, the level of dityrosine cross-links in HSA was the highest compared to other buffer systems. A decrease in the content of H_2O_2 was recorded after 24 h of incubation (independently of the buffer used). However, the extent of this decrease was different in different buffers.

Thus, in carbonate and borate buffers virtually no H_2O_2 remains, in phosphate buffer (pH 9.5) its content decreases twofold and, on average, by 30% at pH 7.4. However, independently of the hydrogen peroxide content, the level of dityrosine in the protein increased. In alkaline medium and in the presence of phosphate and borate buffers the levels of dityrosine cross-links were nearly the same, whereas their concentration increased twofold when bicarbonate buffer was used, and this suggests an involvement of other reactive compounds in the oxidation of tyrosine residues in proteins.

To determine the time interval of decreasing in the H_2O_2 content in carbonate buffer, we determined the

Changes in the content of hydrogen peroxide in Fenton's medium depending on the buffer system used and on pH

Buffer system	Content of hydrogen peroxide in sample, M			Intensity of fluorescence, units/mg protein	
	initial	after 2 h of incubation	after 24 h of incubation	after 2 h of incubation	after 24 h of incubation
0.065 M phosphate buffer (pH 7.4)	0.107 ± 0.006	0.092 ± 0.014	0.067 ± 0.007	36 ± 3	110 ± 18
0.065 M phosphate buffer (pH 9.5)	0.108 ± 0.008	0.081 ± 0.012	0.048 ± 0.005	53 ± 13	135 ± 9
0.1 M carbonate-bicarbonate buffer (pH 9.5)	0.099 ± 0.001	trace amount	not determined	83 ± 17	218 ± 17
0.0125 M borate buffer (pH 9.5)	0.109 ± 0.026	0.069 ± 0.032	not determined	39 ± 6	86 ± 4

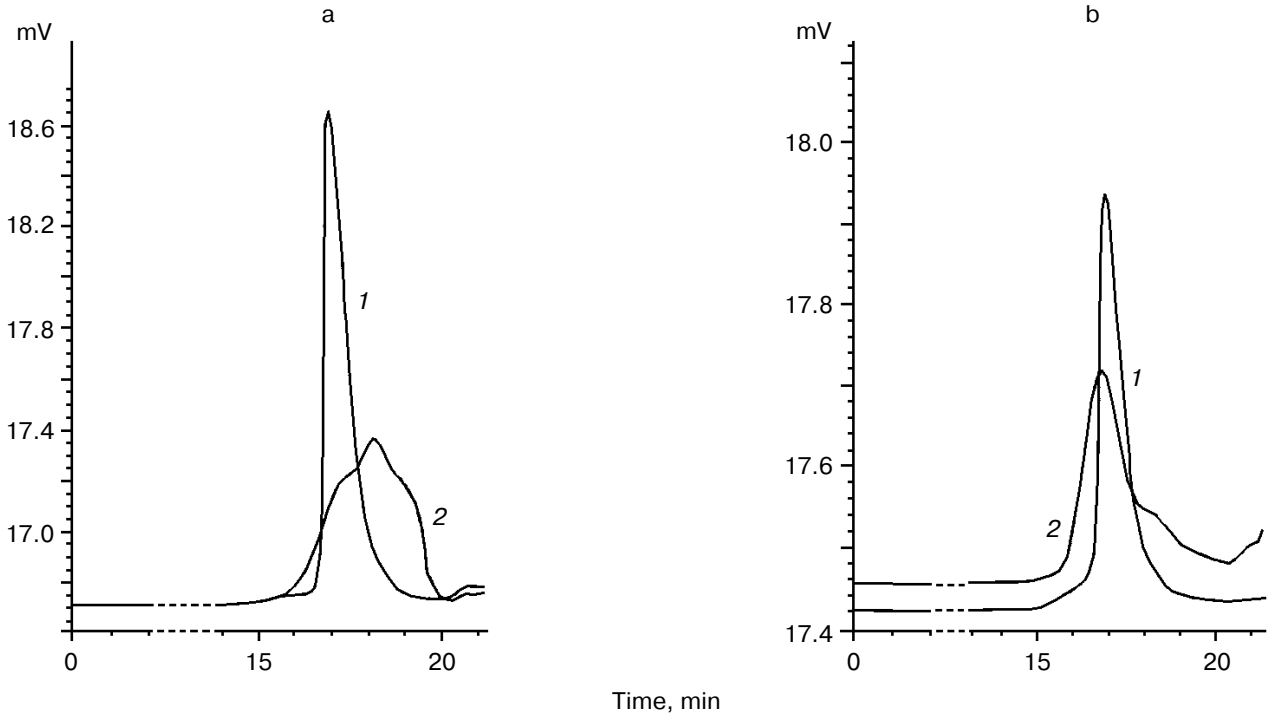


Fig. 3. Chromatographic separation of the native (*1*) and oxidized (*2*) SOD. Fenton's medium: Fe^{2+} ($1.0 \cdot 10^{-4}$ M), EDTA ($0.43 \cdot 10^{-4}$ M), H_2O_2 (0.05 M) in phosphate buffer (pH 7.4) (a) and in 0.1 M carbonate-bicarbonate buffer (pH 9.55) (b).

content of hydrogen peroxide in the incubation mixture every 5 min within the first 20 min of the incubation. Even in 10 min the content of H_2O_2 was decreased to 0.03 M, and 20 min later only a trace amount of H_2O_2 was recorded. No pronounced decrease in the content of H_2O_2 was recorded when 0.065 M phosphate buffer (pH 7.4 and 9.5) and 0.0125 M borate buffer (pH 9.5) were used. Thus, the degradation of H_2O_2 in Fenton's system is mainly associated with carbonate buffer.

Changes in the structure of the oxidized proteins were studied by separation of SOD by GPC. The incubation was performed for 2 h at 37°C in medium containing 0.1 mM Fe^{2+}, 0.043 mM EDTA, and 0.05 M H_2O_2 in phosphate buffer (pH 7.4) and in CBC (pH 9.55). The resulting chromatograms show that changes in the structure of SOD depended on pH of the medium and on the buffer used (Fig. 3). When 0.065 M phosphate buffer (pH 7.4) was used, the shift towards low-molecular-weight fragments of SOD was more pronounced.

Electrophoresis of oxidized proteins in the presence of SDS revealed the most pronounced changes in the structure of SOD (Fig. 4). Thus, the oxidative modification of SOD recorded by the oxidation level of tyrosine and tryptophan residues was associated with large structural changes resulting in a decrease in the enzyme activity. Even after the incubation of SOD for 1 h in the presence of Fe^{2+}-EDTA and H_2O_2, the activity of SOD completely disappeared, independently of the buffer used (carbonate, phosphate) and of pH (7.4 and 9.5).

To elucidate the role of hydroxyl radical generated during Fenton's reaction in the oxidation of amino acid

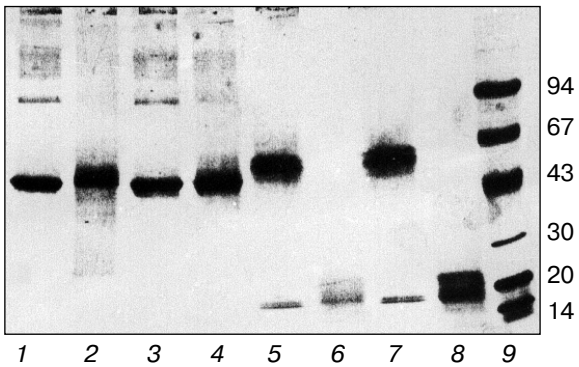


Fig. 4. Electrophoretic separation of SOD in the gradient polyacrylamide gel in the presence of SDS: *1*) HSA (phosphate buffer (PB)); *2*) oxidized HSA (PB); *3*) HSA (carbonate-bicarbonate buffer (CBC)); *4*) oxidized HSA (CBC); *5*) SOD (PB); *6*) oxidized SOD (PB); *7*) SOD (CBC); *8*) oxidized SOD (CBC); *9*) marker proteins. Molecular weights of the marker proteins (kD) are shown to the right.

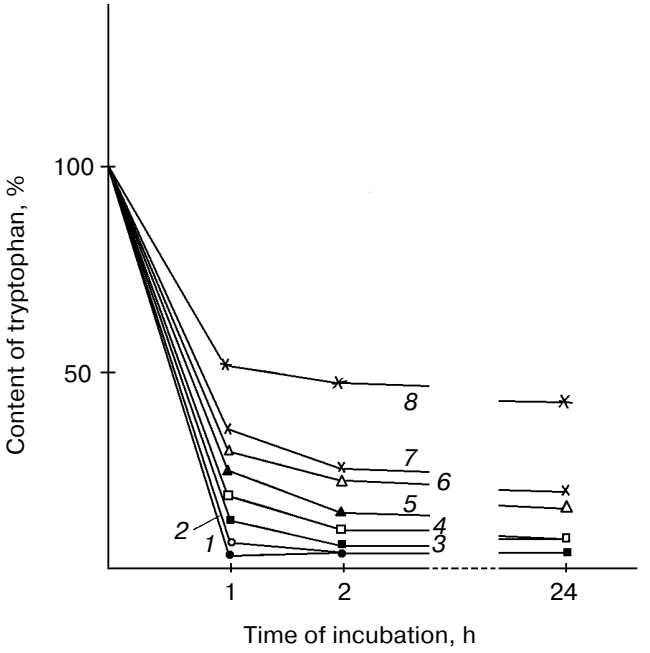


Fig. 5. Effect of ethanol on oxidation of tryptophan residues of HSA. The concentration of ethanol (mM): *1*) 0 (control); *2*) 17; *3*) 34; *4*) 60; *5*) 120; *6*) 240; *7*) 480; *8*) 1000.

residues in proteins, we performed experiments using mannitol and ethanol as radical scavengers. Mannitol at the final concentration of 25 mM did not prevent the oxidation of tryptophan in HSA in the presence of 0.2 mM Fe^{2+}, 0.086 mM EDTA, 0.1 M H_2O_2, 0.065 M phosphate buffer (pH 7.4) on incubation for 1, 2, and 24 h. At the content increased to 123 mM, mannitol displayed a weak antioxidant effect during the 2-h incubation with HSA in Fenton's medium. The oxidation level of tryptophan was decreased by 20-25% compared to the samples without mannitol.

Mannitol completely inhibited the production of dityrosine cross-links in HSA on incubation for 1 and 2 h. On incubation for 24 h, mannitol virtually had no protective effect: the fluorescence intensities of dityrosine cross-links of the experimental and control samples (without mannitol) were similar (150.0 and 160.0 units/mg protein, respectively).

Ethanol prevented the oxidation of tyrosine and tryptophan residues of HSA. The antiradical effect of ethanol on the oxidation of tryptophan of the protein increased with an increase in the alcohol content from 17 mM to 1 M in 0.065 M phosphate buffer (pH 7.4) (Fig. 5). At the ethanol content from 1 to 8 M, the fluorescence intensity of tryptophan residues was decreased by 51-58%, and the same level was recorded 2 and 24 h later.

The maximal antiradical activity of ethanol in the oxidation of tyrosine and production of dityrosine cross-links in HSA was recorded at its content of 240-480 mM regardless of the time of incubation (Fig. 6). An increase in the ethanol content (to 1 and 2 M) resulted in a decrease in its antiradical activity.

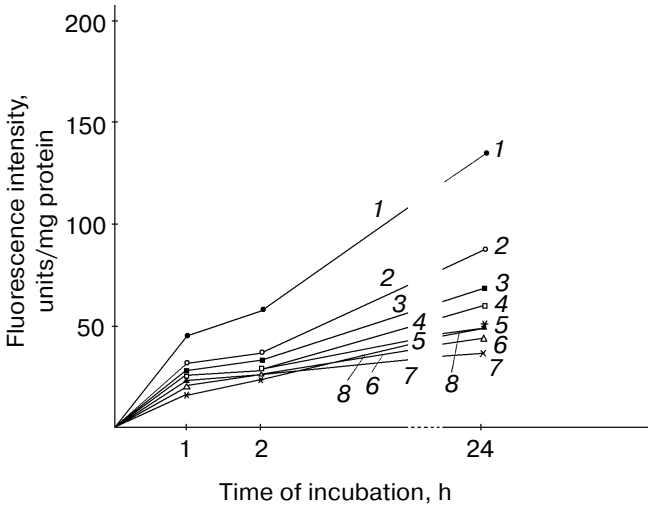


Fig. 6. Effect of ethanol on generation of dityrosine cross-links. The concentration of ethanol (mM): *1*) 0 (control); *2*) 17; *3*) 34; *4*) 60; *5*) 120; *6*) 240; *7*) 480; *8*) 1000.

DISCUSSION

The findings suggest that oxidation of tyrosine and tryptophan residues of purified proteins is associated not only with $OH^{\bullet}$ generated in the presence of Fe^{2+}-EDTA and H_2O_2 but also with other reactive compounds: ferryl and perferryl ions and $CO_3^{\bullet-}$. The involvement of some or other reactive compounds in oxidation of proteins depends on the reaction conditions: content of hydrogen peroxide, buffer mixtures used, pH of the medium, time of incubation, and on the presence of radical scavengers. It is likely that within 1 h of incubation the effect of $OH^{\bullet}$ is predominant, and it is responsible for oxidation of the adjacent amino acid residues of proteins.

The failure of mannitol as an $OH^{\bullet}$ scavenger during oxidation of tryptophan of HSA contradicts our hypotheses. But, taking into account that $OH^{\bullet}$ is a short-lived radical and is effective only in the place of its generation, it is likely that mannitol has no time for scavenging the $OH^{\bullet}$ generated. On the other hand, at pH 7.0 the rate constant of the HSA reaction with $OH^{\bullet}$ is $7.8 \cdot 10^{10}$ $M^{-1} \cdot sec^{-1}$ [31] and the rate constant of the mannitol reaction under the same conditions is $1.7 \cdot 10^{9}$ $M^{-1} \cdot sec^{-1}$. Gutteridge and Wilkins [32] suggested that mannitol as an $OH^{\bullet}$ scavenger cannot affect the site-specific reaction of protein oxidation.

Tyrosine residues in proteins are thought not mainly oxidized by the short-lived hydroxyl radical. This hypothesis is based on certain reasons. First, the oxidation of tyrosine depends on the time of incubation. Unlike the case of tryptophan, the maximal increase in the fluorescence intensity of dityrosine cross-links was recorded after 24 h of incubation. The oxidation of amino acid residues for such a long time can be caused only by stable reactive compounds, not by $OH^{\bullet}$. Second, we have found that the level of H_2O_2 is sharply decreasing during 15-20 min of incubation in carbonate buffer (pH 9.5).

Under aerobic conditions, the generation of $OH^{\bullet}$ and ferryl (perferryl) ions in Fenton's medium is thought to depend on the ratio of $[O_2]$ to $[H_2O_2]$ [21]. If the ratio $[O_2]/[H_2O_2]$ is lower than 10, the generation of hydroxyl radical prevails, whereas at ratio higher than 100, the generation of this is insignificant. On the assumption that the content of O_2 dissolved in water is 0.25 mM at 23°C [29] and at the H_2O_2 concentration used of 0.1 M, the $[O_2]/[H_2O_2]$ ratio should be <10. According to the literature, under these conditions Fenton's reaction resulting in $OH^{\bullet}$ generation should dominate. And within the first 5-10 min of the incubation in carbonate buffer the protein is mainly oxidized by $OH^{\bullet}$ with production of dityrosine cross-links. An increase in the incubation time results in a decrease in the content of H_2O_2 and provides the generation of ferryl and perferryl radicals which promote the further oxidation of tyrosine with production of tyrosyl radical and of dityrosine cross-links. The antiradical effects of mannitol and ethanol under these conditions seem to be caused by their interaction with tyrosyl radicals of HSA.

When phosphate and borate buffers are used and a high content of H_2O_2 is maintained, the amino acid residues can be also oxidized by $OH^{\bullet}$ radical on incubation for 2 h. However, after 24 h of incubation the relative levels of dityrosine were similar in both phosphate and borate buffers, and this suggests the protein oxidation by ferryl and perferryl ions.

The oxidation rate of tyrosine residues in HSA is higher on the background of a sharply decreased level of hydrogen peroxide in carbonate buffer compared to phosphate and borate buffers on incubation for 2 and 24 h. We proposed the explanation of the findings as follows. The tyrosine oxidation in HSA in carbonate buffer is associated not only with $OH^{\bullet}$ and ferryl (perferryl) ions, but also with radical $CO_3^{\bullet-}$. The $CO_3^{\bullet-}$ radical is generated in carbonate buffer during the interaction of $OH^{\bullet}$ with CO_3^{2-} and HCO_3^- [31]:

$$OH^{\bullet} + CO_3^{2-} \xrightarrow{k = 4.2 \cdot 10^8\ M^{-1}\cdot sec^{-1}} CO_3^{\bullet-} + OH^-,$$

$$OH^{\bullet} + HCO_3^- \xrightarrow{k = 8.5 \cdot 10^6\ M^{-1}\cdot sec^{-1}} CO_3^{\bullet-} + H_2O.$$

Although $CO_3^{\bullet-}$ is less reactive than hydroxyl radical, it can diffuse for greater distances and cause oxidative damages to biomolecules [33]. This radical can rapidly react with tyrosine, tryptophan, and other aromatic compounds. The rate constant of this reaction is 10^7-$10^8\ M^{-1}\cdot sec^{-1}$ [34]:

$$\text{tyrosine}{-}OH + CO_3^{\bullet-} \rightarrow \text{tyrosine}{-}O^{\bullet} + HCO_3^-.$$
$$\downarrow$$
$$\text{dityrosine}$$

We have recorded a very high sensitivity of SOD to the metal-dependent oxidation. SOD can be inactivated in Fenton's medium in two ways. First, the $OH^{\bullet}$ generated can produce a complex of $SOD{-}Cu^{2+}{-}OH^{\bullet}$ that results in oxidation of His61 in the active site and in the inactivation of SOD [35]. Second, the enzyme can also be inactivated with the involvement of H_2O_2 itself as a substrate of the "peroxidase" activity of SOD [35-39]:

$$SOD{-}Cu^{2+} + H_2O_2 \rightarrow SOD{-}Cu^{+} + O_2^{\bullet-} + 2H^+,$$

$$SOD{-}Cu^{+} + H_2O_2 \rightarrow SOD{-}Cu^{2+}{-}OH^{\bullet} + OH^-,$$

$$SOD{-}Cu^{2+}{-}OH^{\bullet} \xrightarrow{\text{His61} \rightarrow \text{oxidized His61}} Cu^{2+} +$$

$$+ \text{inactive SOD}.$$

Consequently, in parallel with the oxidation of tryptophan of SOD, at physiological values of pH His61 in the active site of the enzyme is oxidized with its subsequent fragmentation. In alkaline medium $OH^{\bullet}$ oxidized His61 in the active site of SOD within the first 10-15 min of the incubation.

$SOD{-}Cu^{2+}{-}OH^{\bullet}$ can be a source of $CO_3^{\bullet-}$ in the presence of carbonate buffer [36, 37, 40]:

$$SOD{-}Cu^{2+}{-}OH^{\bullet} + HCO_3^- \rightarrow$$

$$\rightarrow SOD{-}Cu^{2+} + H_2O + CO_3^{\bullet-}.$$

Thus, it is concluded that, depending on the reaction conditions, in the presence of Fenton's system components not only $OH^{\bullet}$ can be produced, but also other reactive compounds (ferryl and perferryl ions, $CO_3^{\bullet-}$) which are involved in protein oxidation under conditions of oxidative stress.

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