Vol. 199, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1994 Pages 199-206

PURIFICATION AND CHARACTERIZATION OF THIOL-SPECIFIC ANTIOXIDANT PROTEIN FROM HUMAN RED BLOOD CELL: A NEW TYPE OF ANTIOXIDANT PROTEIN

Yoo-Shick Lim, Mee-Kyung Cha, Chul-Ho Yun, Ha-Kun Kim, Kangwha Kim+, and Il-Han Kim*

Department of Biochemistry, Pai-Chai University, Taejon 302-735, Republic of Korea
+ Department of Food and Nutrition, Chonnam National University, Kwangju, Republic of Korea

Received January 11, 1994

A thiol-specific antioxidant protein (Protector Protein, PRP) was purified from human red blood cells (RBC). The PRP exists as a predominant protein in human RBC, which showed distinct thiol-specific antioxidant activities in the presence of dithiothreitol (DTT) as a reducing equivalent. The human RBC PRP (HRPRP) completely inhibited visible absorption spectral changes of oxyhemoglobin, DNA cleavage, and the peroxidation of RBC membrane by a nonenzymatic Fe^{3+}/O_2/thiol mixed-function oxidation system capable of generating hydroxyl radical. These observations suggest that HRPRP could act as a new type of antioxidant protein to maintain the RBC integrity by scavenging reactive oxygen species.

In biological systems, $\cdot O_2^-$ and H_2O_2 react together to generate the highly reactive ·OH which can attack and destroy almost all known biomolecules (1,2). Free radical damage in biological system is also attributed to transition metal-dependent generation of ·OH radical, called 'the metal-catalyzed Haber-Weiss reaction' (3). To survive, all aerobes are equipped with several antioxidant enzymes which prevent O_2 cytotoxicity. Preventive antioxidant enzymes including catalase and glutathione peroxidase catalyze the conversion of hydrogen peroxide and hydroperoxides, respectively, to stable nonradical products, resulting in reducing the rate of formation of ·OH. Another type of antioxidant enzymes, called 'chain-breaking antioxidant enzyme', acts by trapping oxygen radicals (4). Superoxide dismutase (SOD) catalyzes the disproportionation of $\cdot O_2^-$ to H_2O_2. However, it has not been clearly understood how the other oxygen radicals such as ·OH and peroxy radicals are trapped *in vivo*. Some chemical antioxidants such as ascorbate and urate have been recognized as a chain-breaking antioxidant *in vivo*. However, these chemical substances may act to potentiate oxidizing damage rather than to inhibit it (5).

A thiol-specific antioxidant protein, PRP, first found in *Saccharomyces cerevisiae*, (YPRP) does not show any known antioxidant enzyme activities (6). PRP is the antioxidant protein which specifically protects the inactivation of various enzymes (7)

*To whom correspondence should be addressed: Fax 82-042-520-5379.

0006-291X/94 $5.00

and the cleavage of DNA (8) by a nonenzymatic mixed-function oxidation (MFO) system containing a sulfhydryl compound (Fe^{3+}, O_2, and DTT: thiol-MFO system). These antioxidant properties were completely abolished by reacting with N-ethylmaleimide only in the presence of DTT, suggesting sulfhydryl group(s) on PRP make a very important contribution to the thiol-specific antioxidant activities (8). The YPRP synthesis was significantly enhanced by oxidative pressure (9). Furthermore, the *prp* mutant of *Saccharomyces cerevisiae* was viable under air, but the growth rates was significantly retarded only under aerobic condition (10). These observations clearly indicate its physiological significance as a new type of antioxidant protein.

In this comunication, we first report the existence of PRP in human RBC which contains well known antioxidant enzymes such as SOD, catalase, and glutathione peroxidase. The discovery of PRP existing as a predominant antioxidant protein in human RBC implicates distinct antioxidant property of HRPRP from those of other antioxidant proteins. The experiments with HRPRP have suggested that HRPRP may be a new type of antioxidant protein which directly scavenges reactive oxygen species, presumably hydroxyl radical-like species.

Materials and Methods

Purification of HRPRP from Human RBC: Human RBCs were obtained from freshly drawn heparinized blood. Crude extract of Human RBCs was prepared from RBCs which were three times washed with phosphated-buffered saline (PBS) solution and lysed in 50 mM Tris-HCl buffer, pH 7.0. After centrifugation at 18,000 x g for 30 min. the clear lysates were precipitated with 70% ammonium sulfate. The dissolved precipitate was extensively dialyzed against DEAE column equilibrium buffer (50 mM Tris-HCl buffer, pH 7.6). After centrifugation, the clear supernatant was loaded into a DEAE column. The column was washed with equilibrium buffer and eluted with a linear KCl gradient (0-400 mM). The presence of the PRP was assayed throughout the purification by monitoring its ability to inhibit the inactivation of *E. coli* glutamine synthetase (GS) by thiol MFO system as previously described (6). Broad peak fractions of PRP activity eluted at a KCl concentration of 150-250 mM were pooled and precipitated with 70% ammonium sulfate. The dissolved ammonium sulfate-precipitates were applied to a G-75 column previously equilibrated with 100 mM Hepes buffer, pH 7.4, containing 100 mM KCl. Like the case of YPRP (6), dimeric form of HRPRP was eluted. The fractions from each peak were pooled separately, washed with 50 mM Hepes buffer, pH 7.0 and concentrated. Homogeneous fractions showing one PRP band on 12% SDS-PAGE were used for this experiment.

DNA Cleavage by Nonenzymatic Thiol-MFO System: After reaction buffer, 100 mM Hepes pH 7.0, containing the thiol-MFO system (3 μM $FeCl_3$, 10 mM DTT) was incubated with or without HRPRP for 40 min at 37 °C, 2 μg of pUC19 plasmid was added for additional 4 hr incubation. The resulting reaction mixtures were subjected to phenol/chloroform extraction to obtain DNA which was applied to 1% agarose gel to examine its cleavage.

Oxidation of HbO_2 by Nonenzymatic Thiol-MFO System: Oxyhemoglobin (HbO_2) purified by the method reported (11) was incubated at a concentration of 8 mg/ml in 50 μl containing 100 mM Hepes, pH 7.0, and the thiol-MFO components for 20 min at 37 °C. After dilution to 1 ml of chilled deionized water at appropriate time, the

increase in absorbance at 635 nm due to the oxidation of HbO_2 by the thiol-MFO system was measured.

Measurement of Lipid Peroxidation: The peroxidation of RBC membrane lipids by a thiol-MFO system was determined by measurement of TBAR (thiobarbituric acid-reactive products). In the presence of the thiol-MFO system, one ml of reaction mixture containing 0.6 mmol of membrane lipid was incubated for one hour at 37 ^{o}C. Whole reaction mixture was boiled for 20 min with thiobarbituric acid and trichloroacetic acid, in the presence of 0.01% butylated hydroxyltoluene, as described by Buege and Aust (12). After centrifugation at 2000 x g, the malonyldialdehyde content of supernatant was then determined by their absorbance at 535 nm. The concentration of phospholipid was determined by assay of elemental phosphorous (13).

Assays for catalase, SOD, and Glutathione Peroxidase: Catalase activity was determined by direct measurement of the decrease of absorbance at 250 nm caused by the decomposition of H_2O_2 by catalase (14). SOD activity to scavenge $\cdot O_2^-$ was measured according the method by Crapo et al. (15). The rate of production of GSSG by glutathione peroxidase was measured in the presence of excess glutathione reductase by following the rate of NADPH oxidation (16).

Results and Discussions

Purification and Characterization of HRPRP from Human RBC: We first found PRP in human RBC. Westhern blot shown in Fig. 1-A indicates that PRP exist in the stroma of RBC, not in the blood plasma. The only one purification step, DEAE chromatography, yielded a major PRP protein band (lane 3 of Fig. 1-C), showing that the PRP except for Hb is the most abundant protein in the stroma of the RBC. This PRP possessing M_r of 25 kDa was shown in the crude extract (lane 2 of Fig. 1-B,C). Another band showing M_r of 32 kDa was known to be a dimer form of the globin originated from Hb. The second purification step as the last step, G-75

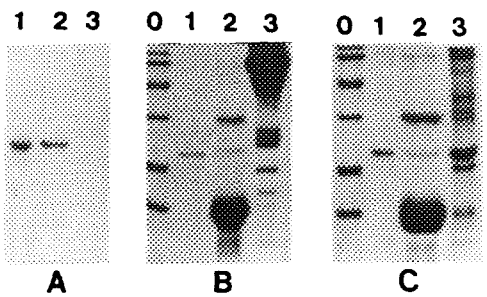

Fig. 1. Analysis of protector protein (PRP) from human red blood cell (RBC). A and B: SDS-PAGE and its Westhern blot, respectively, for crude proteins from the stroma of RBC (lane 2) and the plasma (lane 3), and for purified 1 μg of yeast PRP (lane 1). C: 12 % SDS-PAGE for crude proteins from the stroma (lane 2), DEAE fraction before loaded to G75 column (lane 3), and the RBC PRP eluted from G75 chromatography. 0 lane of B and C: low molecular weight of protein size markers (from top, 94, 67, 45, 31, 21.5, 14.4 kDa). The polyclonal antibodies against yeast PRP bound to nitrocellulose blots were detected by immunoblot using peroxidase-conjugated goat anti-rabbit IgG.

chromatography, gives a homogeneous PRP protein band on SDS-PAGE (lane 1 of Fig. 1-C). The M_r of HRPRP was observed to be sligtly larger than that of YPRP (lane 1 of Fig. 1-A,B)

Inactivation of yeast GS by the thiol-MFO system is completely prevented by HRPRP. Fig. 2-A shows time-coursed inactivation of GS in the presence of various concentrations of HRPRP. The HRPRP did not protect the inactivation of GS by a non-thiol MFO system which contains 10 mM of ascorbate instead of 10 mM DTT (Fig. 2-B), showing a distinct thiol-specific antioxidant activity of YPRP. The plot of % protection as a function of HRPRP concentration (Fig. 2-C) shows that the concentration of HRPRP required to preserve completely GS activity was 0.2 mg/ml, which is comparable to that of YPRP (0.3 mg/ml). Under the assay conditions (see Materials and Methods). 10 μg of HRPRP did not show any significant activities for SOD, catalase, and glutathione peroxidase.

It is believed that the hydroxyl radical is a strong oxidant capable of destroying DNA (17,18). Claycamp (19) showed that the oxidation of thiol in neutral buffer can significantly damage DNA and its constituents. A number of laboratories verified the production of ·OH from a thiol-MFO system (19-23). Recently, we also reported that the

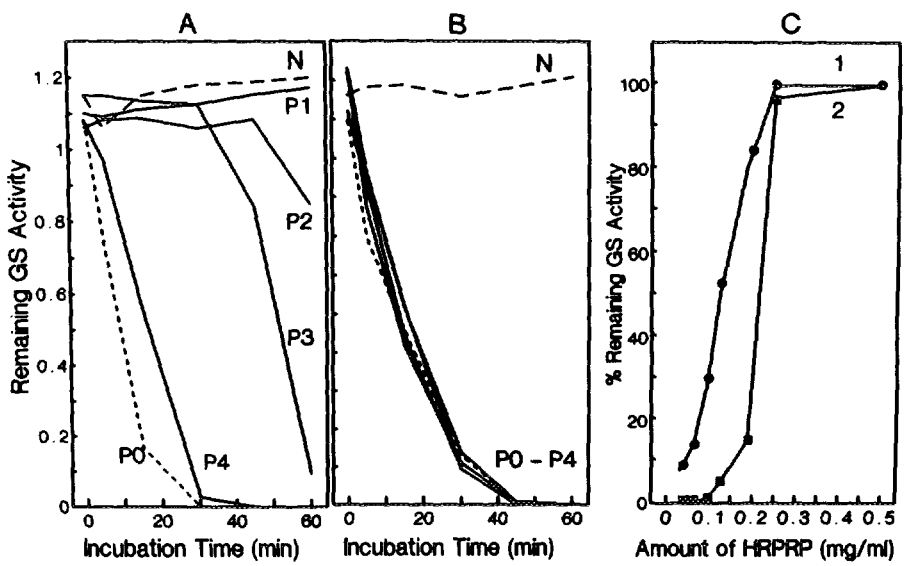

Fig. 2. Characterization of RBC protector protein (HRPRP): A and B: protection of glutamine synthetase (GS) by HRPRP against DTT/Fe^{3+} (thiol-MFO) system, and by HRPRP against ascorbate/Fe^{3+} (nonthiol-MFO) system. At various times, aliquots were removed and assayed for remaining GS activities. Abbreviations are as follows: N, GS only as a control; P1,P2,P3,P4,P0 are 500, 250, 125, 62.5, 0 μg/ml of HRPRP added, respectively. C: Potency of HRPRP against the thiol-MFO system. Various amounts of HRPRP were added into the inactivation mixture. After 30 min (1 of C) or 60 min (2 of C) inactivation, remaining GS activities were measured and normalized against the activity without the thiol-MFO system.

supercoiled form of pUC19 plasmid was mostly converted to the nicked form by the thiol-MFO system capable of producing ·OH, and its cleavage was completely inhibited by YPRP (8). Therefore, the preventive effect of HRPRP on the DNA cleavage was examined and compared with that of YPRP (data not shown). The result indicates that the concentration of HRPRP required to protect perfectly the DNA cleavage was about 50 μg per ml of reaction mixture, which is nearly same as that of YPRP (ca., 62.5 μg/ml) repoprted (8).

Protection of the Hemoglobin Oxidation: Human Hb has been observed to promote the formation of reactive species equivalent to ·OH *in vivo* (24). Reactive oxygen radicals such as ·OH have been known to cause to produce oxidized forms of Hb (25,26). Fig. 3 shows spectral changes indicative of the differences in the rates of

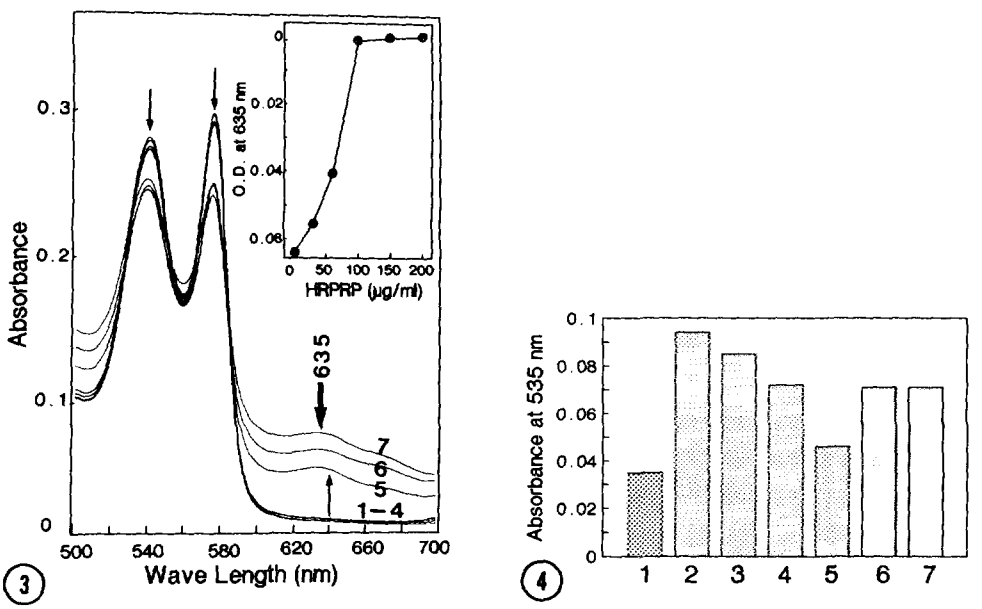

Fig. 3. Spectral changes of hemoglobin (Hb) associated with the oxidation of Hb caused by the thiol-MFO system and protection of HRPRP against the oxidation of Hb. The spectra of oxyhemoglobin after 20 min incubation without the MFO system,1; with the MFO system ,7; with the MFO system containing 200 μg/ml, 2; 150 μg/ml, 3; 100 μg/ml, 4; 60 μg/ml, 5; 40 μg/ml of HRPRP, 6. (*Inset*): the protection against the oxidation of Hb as functions of concentration of HRPRP. Arrows indicate the direction of change caused by the MFO system.

Fig. 4. Effect of HRPRP on the lipid peroxidation by a MFO system. After one hour incubation of one ml reaction mixture at 37 °C, malonyldialdehyde was determined by measuring the absorbance at 535 nm. The standard reaction mixture contains 0.6 M of RBC membrane in term of phospholipid, 20 mM DTT, 2 mM $FeCl_3$, and 100 mM Hepes, pH 7. Each experimetal reaction conditions as follow: (*lane 1*), without DTT and $FeCl_3$; (*lane 2*), the standard reaction mixture; (*lane 3,4, and 5*), the standard reaction mixture *plus* 0.05 mg/ml, 0.25 mg/ml, and 0.50 mg/ml of added HRPRP, respectively. (*lane 6 and 7*), included 20 mM ascorbate instead of DTT without HRPRP and with HRPRP (0.5 mg/ml), respectively.

Hb oxidation caused by during 20 min exposure to the thiol-MFO system capable of producing ·OH. The addition of the thiol-MFO system to the solution of 100 mM Hepes, pH 7.0 containing human Hb resulted in decreasing the *Soret* band, and increasing the absorbance at 635 nm of Hb. Spectral examination at 635 nm of Hb treated with the thiol-MFO system reveals a slow increase in absorbance with time as a consequence of the oxidation of human Hb (data not shown). The *inset* of Fig. 3 shows that HRPRP retards the increases as a function of the concentration of HRPRP, indicating that HRPRP prevent the oxidation of human Hb. The concentration of HRPRP required to preserve completely the Hb against the oxidation was observed to be about 100 μg/ml. However, an excess amount of SOD, 2300 units (2 mg/ml), did not retard the oxidation of human Hb (data not shown). Catalase also prevents the oxidation of human Hb as function of the activity (data not shown), indicating that 140 units of catalase (7.5 units per μg of catalase) was required to inhibit completely the oxidation. HRPRP has a capability to destroy H_2O_2 to the almost same extent (0.006 μmole of H_2O_2/min/μg, or 0.006 unit/μg) as YPRP previously reported (8). From the plots for H_2O_2 destroying activities of HRPRP or catalase *versus* the extent of the protection (data not shown), it was known that 0.03 unit of HRPRP (i.e., 100 μg/ml) in term of H_2O_2-destroying activity or 140 units of catalase (i.e.,18.7 μg/ml) were required for the complete protection, respectively. These results indicate that the preventive effect of HRPRP is not due to H_2O_2-removing capability of HRPRP.

Protection of the Membrane Lipid Peroxidation: Membrane lipid peroxidation induced by the thiol-MFO system was assayed in term of increased absorbance 535 nm (12), which is shown in Fig. 4. In the presence of the thiol-MFO system, the peroxidation of RBC membrane was accelerated (lane 1 and 2 of Fig. 4-A), but the peroxidation decreased as the concentration of HRPRP was increased. The peroxidation induced by the thiol-MFO system was nearly completely prevented in the presense of 0.5 mg/ml of HRPRP (lane 5 on Fig. 4). However, the peroxidation induced by a non-thiol MFO system containing ascorbate instead of DTT was not inhibited by HRPRP (0.5 mg/ml) (lane 6 and 7 of Fig. 4). These results indicate that HRPRP can prevent the membrane lipid peroxidation resulting in the membrane damage.

It is widely believed that the ·OH is the primary cause of the adverse oxidative reaction that causes tissue damage (27). Hb as a major iron source in RBC (i.e., greater than 95% of the soluble protein) may promote the generation of reactive oxygen radicals *via* iron-dependent oxidation reaction. RBC is believed to have a very effective defence mechanism against the cell damages by highly reactive oxygen species such as ·OH. A new type of antioxidant protein called 'HRPRP' was first found to exist as the most predomonant protein except for Hb and purified in the stroma of human RBC. HRPRP did not show any significant activities for SOD, catalase, and glutathione peroxidase, but HRPRP has a distinct activity to prevent the inactivation of enzyme such as *E. coli* GS, the cleavage of DNA, the oxidation of Hb, and the

membrane damage by the thiol-MFO system, wherein the production of ·OH should occur. Although there is no direct evidence supporting this hypothesis, these results suggest that HRPRP could act as an *in vivo* radical scavenger (or 'chain-breaking antioxidant') to remove reactive oxygen radicals capable of damaging cells. The synergistic interactions and/or the integrations of HRPRP acting as a 'chain-breaking antioxidant' to other preventive antioxidants such as catalase and glutathione peroxidase could be a very effective defence mechanism against damaging cells by reactive oxygen species including hydroxyl radical-like species. The antioxidant activities of HRPRP (data not shown) and YPRP (8) were completely inhibited by reacting with N-ethylmaleimide only in the presence of DTT, indicating that the reduced form of the cysteine involves in its activity. Physiological thiol-reductants such as glutathione and the reduced form of lipoic acid have replaced DTT (6). This thiol-dependent activity of PRP may indicate that a physiological thiol-reductant or an enzyme-mediated process (i.e., NADPH-dependent thioredoxin system) may contribute to the recovery of PRP inactivated by oxidation during the process of scavenging reactive oxygen species. However, it remains to be much more studies to understand the mechanism of the antioxidant property of HRPRP.

Acknowledgment: This work was supported by KOSEF Grant 91-07-00-13 awarded to Il-Han Kim.

References

1. Fridovich, I. (1975) *Annu. Rev. Biochem.* **44**, 147-159
2. Tauber, A. I., Gabia, T. G., and Babior, B. M. (1979) *Blood* **53**, 666-676
3. McCord, J. M. (1974) *Science* **185**, 529-531
4. Burton, G. W., and Ingold, K. U. (1989) *Handbook of Free Radicals and Antioxidant in Biomedicine* Vol. II, pp 29-43, CRC press
5. Borg, D.C. and Schaich, K. M. (1989) *Handbook of Free Radicals and Antioxidant in Biomedicine* Vol. I, pp 63-80, CRC press
6. Kim, K., Kim, I. H., Lee, K. Y., Rhee, S.G., and Stadtman, E. R. (1988) *J. Biol. Chem.* **263**, 4704-4711
7. Kim, K., Rhee, S. G., and Stadtman, E. R. (1985) *J. Biol. Chem.* **260**, 13394-13397
8. Lim, Y.S., Cha, M. K., Kim, H. K., Uhm, T. B., Park, J. W., Kim, K., and Kim, I.H. (1993) *Biochem. Biophys. Res. Commun.* **192**, 273-280
9. Kim, I. H., Kim, K., and Rhee, S. G. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6018-6022
10. Chae, H.Z., Kim, I.H., Kim, K., and Rhee, S.G. (1993) *J. Biol. Chem.* **268**, 16815-16821
11. Fagan, J. M., Waxman, L., and Goldberg, A. L. (1986) *J. Biol. Chem.* **261**,5705-5713
12. Buege, J. A., and Aust, A. D. (1978) *Methods Enzymol.* **52**, 302-310
13. Vaskovsky, V. E., Kostetsky, E. Y., and Vasendin, I. M. (1975) *J. Chromatogr.* **114**, 129-141
14. Chance, B. and Maehly, A. C. (1955) *Methods Enzymol.* **2**, 764-775
15. Crapo, J. D., McCord, J. M., and Fridovich, I. (1978) *Methods Enzymol.* **53**, 382-393
16. Tappel, A. L. (1978) *Methods Enzymol.* **52**, 506-513
17. Chevio, M (1988) *Free Radical Biol. and Med.* **260**, 757-764
18. Sakurai, H., Nakai, M., Miki, T., Tsuchiya, K., Takada, J., and Matsushita, R. (1992) *Biochem. Biophys. Res. Commun.* **189**, 1090-1095
19. Claycamp, H. G. (1987) *Biochem. Biophys. Res. Commun.* **144**, 432-437
20. Rowley, D. A. and Halliwell, B. (1982) *FEBS Lett.* **138**, 33-36

21.McClune, G. J. (1972) *Biochemistry of the SH Group*, pp 98-99, Academic Press
22.Halliwell, B. and Gutteridge, J. M. C. (1985) *Free Radical in Biology and Medicine* pp 119- 125, Clarenden Press, Oxford
23.Misra, H. P. (1974) *J. Biol. Chem.* **249**, 2151-2155
24.Sadrzadehm, S. M., Panter, C. E., Hallaway, P. E., and Eaton, J. W. (1984) *J. Biol. Chem.* **259,** 14354-14356
25.Caughey, W. S., and Watkins, J. A. (1985) *CRC Handbook of Methods for Oxygen Radical Research*, pp 95-104, CRC Press
26.Alayash, A., Fratantoni, J. C., Bonaventura, C., Bonaventura, J., and Bucci, E. (1992) *Arch. Biochem. Biophys.* **298**, 114-120
27.Halliwell, B. and Gutteridge, J. M. C. (1986) *Arch. Biochem. Biophys.* **246**, 501-514