STUDIES OF THE STATUS OF ANTIOXIDANT ENZYMES AND METABOLITES FOLLOWING BURN INJURY, AND THE PRESENCE OF ANTIOXIDANT ENZYMES IN THE *ALOE VERA* PLANT

DISSERTATION

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Part I: The effects of skin burn injury on the levels of oxidized and reduced glutathione, malondialdehyde, and on the activities of glutathione peroxidase, glutathione S-transferase, and glutathione reductase were determined in liver and lung of rabbit models, 24-h post-burn. The data obtained are indicative of a major oxidative stress in liver and lung tissues due to burn injury at a remote site. Tumor necrosis factor (TNF), a mediator in the pathogenesis of endotoxic shock and burn injury, is associated with decreased glutathione levels. Depletion of cellular glutathione by chemical agents enhanced the release of TNF from lipopolysaccharide (LPS)-stimulated rabbit lung macrophages. Glutathione repletion of macrophages, using glutathione diesters, inhibited LPS-stimulated TNF secretion. Thus, glutathione diesters may have therapeutic value in treating endotoxic shock and burn injury.

Part II: Two antioxidative enzymes, glutathione peroxidase (GSHPx) and superoxide dismutase (SOD), which are involved in scavenging reduced oxygen species, have been purified and characterized from the *Aloe vera* plant. GSHPx activity was purified

to homogeneity by ion exchange and gel filtration chromatography. The enzyme is apparently a tetramer with a subunit molecular mass of 16 kD, with one atom of selenium per subunit. The Km values are 3.2 mM for glutathione and 0.26 mM for cumene hydroperoxide. The enzyme is competitively inhibited by N, S, bis-fluorenylmethoxy-carbonyl glutathione. Superoxide dismutases from both the gel and the rind of *Aloe vera* were purified by ion exchange chromatography. Seven SOD activities were detected, with identifiable differences in their relative distribution in rind and gel. Two of these contain manganese with native molecular masses of 42 and 43 kD and five others are copper/zinc SODs with molecular masses of 31-33 kD. *Aloe vera* SODs have high specific activities; these high activities may relate to the plant's healing properties of inflammatory disorders.

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CHAPTER TWO

CHAPTER III

Scheme

Figure

LIST OF ABBREVIATIONS

β-Mercaptoethanol ..... BME

Buthionine Sulfoximine ..... BSO

Cumene Hydroperoxide ..... CHP

Copper-Zinc Superoxide Dismutase ..... Cu/ZnSOD

Glutathione ..... GSH

Glutathione diethyl ester ..... GDEE

Glutathione disulfide ..... GSSG

Glutathione Peroxidase ..... GSHPx

Glutathione Reductase ..... GSSGRd(GRd)

Glutathione S-Transferase ..... GST

Hydrogen Peroxide ..... H_2O_2

Iron Superoxide Dismutase ..... FeSOD

Lipopolysaccharide ..... LPS

Macrophage ..... MΦ

Malondialdehyde ..... MDA

Manganese Superoxide Dismutase ..... MnSOD

Nitro-Blue-Tetrazolium ..... NBT

Phospholipid Hydroperoxide Glutathione Peroxidase ..... PLGSHPx

Selenium ..... Se

Superoxide anion (radical) ..... $O_2^{-\cdot}$

Tumor Necrosis Factor ..... TNF

PART I

THE STUDY OF OXIDATIVE STRESS DUE TO BURN INJURY AND IN RELATION TO GLUTATHIONE

INTRODUCTION

All living cells are prone to oxygen toxicity. This toxicity arises from reduced oxygen species formed as the reduction of molecular oxygen occurs, initially resulting in the formation of superoxide anion ($O_2^{-\bullet}$), hydroperoxyl radical ($HO_2\cdot$), and then hydrogen peroxide H_2O_2. Although neither $O_2^{-\bullet}$ nor H_2O_2 at physiological concentrations is particularly harmful, they are converted into the most reactive oxygen species, the hydroxyl radicals ($\cdot OH$). These reduced oxygen species are considered to be mediators involved in inflammation and cell injury. Their formation is greatly increased during stress conditions such as a burn injury, irradiation and drug metabolism [1-4]. Oxidative stress during a burn injury causes an accelerated oxidant release that can promote peroxidation of cell membrane lipids. These lipid peroxides can be carried from the site of injury to other organs through the plasma lipids to produce membrane damage in various organs or tissues. Other documented consequences of reduced oxygen species, in animals, are in ischemia/reperfusion injury, in the aging process, in the development of inflammatory diseases such as rheumatoid

arthritis, in the cytotoxicity of tumor necrosis factor (TNF), and in DNA mutation [5-7].

Survival of organisms in an oxygen rich environment depends on a wide variety of defense metabolites and/ or enzymes, antioxidant defenses, for the constant repair of oxidative damage. These metabolites and enzymes are designed to ensure that superoxide radical and hydrogen peroxide are disposed of before coming in contact with each other, which may lead to formation of the most reactive radical, ·OH. Cells have a number of mechanisms for dealing with these reduced oxygen species. The primary lines of antioxidant defenses are intracellular enzymes and metabolites, such as superoxide dismutases (EC 1.15.1.1), catalase (EC 1.11.1.6), glutathione peroxidases (EC 1.11.1.9), reduced glutathione (GSH), ascorbic acid, α-tocopherol, β-carotene, and uric acid. There are other glutathione-dependent enzymes involved in detoxification, such as glutathione S-transferases (EC 2.5.1.18); the GSH-regenerating enzyme, glutathione reductase (EC 1.6.4.2); and probably the glyoxalase system, glyoxalases I and II (EC 4.4.1.5 and EC 3.1.2.6, respectively). GSH and GSH-dependent enzymes are also involved in protection against lipid peroxidation [8].

A major goal of the work described herein is the determination of the role of GSH and its dependent enzymes in alleviating oxidative stress, induced as a result of deep burn injury to the skin leading to distal organ damage. The effect of short-term burn injury, after 24 h, on the major antioxidation system, GSH-system, has been

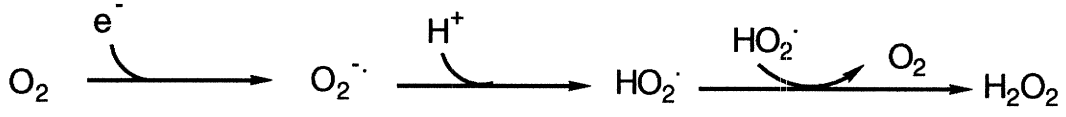
evaluated in rabbit model. The long-term effect of burn injury has been associated with a number of metabolic and immunological abnormalities. These may in part be due to trans-location of gut exotoxin and endotoxin [9], which can induce tumor necrosis factor (TNF-α) and prostaglandin E (PGE) production by various types of macrophages. A second goal of this study is the investigation of possible therapeutic measures that could ameliorate the damage to organs and tissues following massive oxidative insult and resultant TNF-α release.

Reactive Oxygen Species in *Vivo*

A free radical is defined as any species that has one or more unpaired electrons. Free radicals, in living systems, are generated in two ways : 1) enzymatically catalyzed, one-electron reduction of O_2, and 2) reactions initiated by xenobiotics. In this study the emphasis is on oxygen radicals and their effects on biological systems. While oxygen is essential for the survival of living organisms, the formation of its reactive reduction products seems to be commonplace in aerobically metabolizing cells, when the concentration of O_2 is elevated. Cytochrome oxidase and other proteins that reduce O_2 have been designed not to release $O_2^{-\bullet}$; however, in some reactions such as oxidation of the ferroheme (Fe^{2+}) group of hemoglobin to ferriheme (Fe^{3+}) some $O_2^{-\bullet}$ is formed unavoidably. Another source of this radical, $O_2^{-\bullet}$, is leakage of electrons onto O2 from biological oxidations such as the electron transport chain of mitochondria [10], chloroplasts, and the

endoplasmic reticulum. Heart mitochondria have been recently discovered to have a more active superoxide anion generator that is localized at the cytosolic face of the inner membrane [11]. It is also well known that phagocytes such as neutrophils, monocytes, and macrophages produce $O_2^{-\bullet}$ during respiratory burst. Activation of these phagocytes after burn injury increases the level of reactive intermediates $O_2^{-\bullet}$ and H_2O_2, designed for the killing of possible invading bacteria; however, tissue damage with resultant organ failure may result [12-14]. $O_2^{-\bullet}$ has been reported to inactivate *Escherichia coli* dihydroxy-acid dehydratase [15] and cardiac creatine kinase [16].

Protonation of the superoxide anion gives rise to the formation of hydroperoxyl radical, leading to the production of organic hydroperoxides. Hydroperoxyl radical ($HO_2\cdot$) can react with another superoxide anion to form hydrogen peroxide (as seen below).



The pKa of $HO_2\cdot$ is 4.7-4.8 [17], and so more $HO_2\cdot$ will form in proximity to membranes where the pH is considerably lower than physiological pH. Since this reactive oxygen intermediate is less polar than $O_2^{-\bullet}$, it can cross the biological membranes as effectively as can H_2O_2. $HO_2\cdot$ is more reactive than $O_2^{-\bullet}$ and can attack fatty acids directly, converting them to their peroxide forms [17]. H_2O_2 is produced when oxygen accepts two electrons by enzymatic or

nonenzymatic reactions. Superoxide dismutase and several oxidases, i.e., urate oxidase and glucose oxidase produce H_2O_2 directly. Studies in models of ischemia reperfusion injury have suggested that xanthine oxidase (XO), an enzyme of purine catabolism, uses increased levels of molecular oxygen, leading to the production of superoxide radical $O_2^{-}\cdot$ and a hydrogen peroxide H_2O_2 (see reactions; 1 and 2). These two oxygen metabolites cause post-ischemic tissue damage [19, 20]. Xanthine oxidase also exists as an NAD+-reducing form, xanthine dehydrogenase (XDH), which catalyzes reaction (3). Xanthine dehydrogenase, in the presence of oxidizing agents such as oxidized glutathione or sulfhydryl oxidase, can be converted to xanthine oxidase *in vitro* as shown below.

$$\text{Xanthine} + 2O_2 + H_2O \longrightarrow \text{Uric acid} + 2O_2^{-}\cdot + 2H^+ \quad (1)$$

$$\text{Xanthine} + O_2 + H_2O \longrightarrow \text{Uric acid} + H_2O_2 \quad (2)$$

$$\text{Xanthine} + NAD^+ + H_2O \longrightarrow \text{Uric acid} + NADH + H^+ \quad (3)$$

H_2O_2 production has also been observed from phagocytic cells, bacteria of several species, mitochondria, and chloroplasts, mainly via $O_2^{-\bullet}$ [21]. The human eye lens has been reported to contain micromolar concentrations of H_2O_2 [22]. H_2O_2 is not a radical and has limited reactivity, but it can cross biological membranes. Toxicity of H_2O_2 to animal cells and bacteria depends on a relationship involving the activity of H_2O_2-removing enzymes and

the rate of conversion of H_2O_2 into more highly reactive radicals, such as the hydroxyl radical.

The hydroxyl radical (·OH) is highly reactive, perhaps one of the most reactive species known in chemistry [7]. It reacts at or close to the site of its formation in *vivo*. This radical can damage DNA by modification of purines and pyrimidines or by breaking DNA strands [23]. Reaction of ·OH with biological molecules will produce less reactive radicals in *vivo*, the so called "secondary" radicals that are able to initiate lipid peroxidation by abstracting hydrogen atoms to form peroxyl radicals.

In addition to its generation by excessive radiation, ·OH is generated in *vivo* by metal-ion dependent breakdown of H_2O_2. According to the Haber-Weiss reaction superoxide radical serves as a reducing agent for oxidized metal ions, and so promote the breakdown of hydrogen peroxide H_2O_2, leading to the formation of hydroxyl radicals (·OH) [24-26], as shown below.

$$Fe^{3+} + O_2^{-\cdot} \longrightarrow Fe^{2+} + O_2 \qquad (1)$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + HO^- + HO^\cdot \qquad (2)$$

$$O_2^{-\cdot} + H_2O_2 \longrightarrow O_2 + HO^- + HO^\cdot \qquad (3)$$

(Haber-Weiss reaction)

The major determinant of the cytotoxicity of H_2O_2 and $O_2^{-\cdot}$ is the location and availability of the metal ion involved in the catalysis of the Haber-Weiss reaction (Fenton chemistry) leading to .OH formation. During the 1970's and early 1980's, biochemists spent considerable time looking for "iron promoters" in human and animal body fluids [27]. Organisms have evolved elaborate systems for safely sequestering transition metal ions in storage or transport proteins such as ferritin and transferrin, respectively. In addition, cells contain a small iron pool, largely compartmentalized into a vacuole [27], which is used for the synthesis of ferroproteins. However, oxidative stress can provide elevated available iron levels for the Haber-Weiss reaction, though the degradation of heme proteins by H_2O_2 formation and by the mobilization of iron from ferritin by $O_2^{-\cdot}$.

In recent years, the growing interests in the field of free radicals and oxygen toxicity have been very great because of the harmful effects associated with these intermediates to all aerobic organisms. These species interact with the intracellular environment and lead to the pathophysiology of several diseases, including the development of lung injury such as pulmonary O_2 toxicity and the adult respiratory distress syndrome (ARDS) [28, 29]. Accelerated oxidative metabolism of pulmonary parenchymal cells and phagocytes might be the sources of O_2 metabolites in lung injury from hyperoxia [28-30]. There is evidence that during thermal trauma the increased vascular permeability is related to the

generation of toxic oxygen metabolites, as a result of xanthine oxidase activation, leading to damage of microvascular endothelial cells [31].

Lipid peroxidation

Knowledge of the physical, biochemical and cellular events in burned skin is limited. Occurrence of a "toxic factor" after burn injury has gathered much attention since Cannon and Bayliss reported in 1919 [32] that fluid loss was not the only factor responsible for the circulatory failure often seen in battle casualties. In 1968, this toxic factor was identified as a lipid-protein polymer by Allgower [33]. In 1973, Helmkamp [34] reported on the decrease in the levels of polyunsaturated fatty acids such as linoleate, arachidonate, and docosahexaenoate in the red cell membrane phospholipids of severely burned humans. Loebl and Baxter [35], in 1973, reported on the existence of a substance circulating in thermally injured patients that causes erythrocyte destruction in early post burn period. Rai and Courtemanche [36] found significant reduction in the vitamin A level as burn index increase. Sugiyama [37] reported on the extensive release of prostaglandins into the blister a few hours after burn injury. There is strong evidence that the toxic products of burn oxidative stress are systemically circulating lipid peroxides. The formation of these peroxides is caused by reduced oxygen species formed during thermal injury. Appearance of lipid peroxidation products, extractable from skin, plasma, and from distant organs such as

liver, lung, and heart after burn injury have been reported by several investigators [38-43]. Lipid peroxides have numerous deleterious effects on biological systems, including alterations in membrane fluidity, decrease in membrane potential, increased permeability to H^+ and other ions, and rupturing of membranes leading to the release of organelles and their contents from the cell [21], such as lysosomal hydrolytic enzymes [44]. These peroxides ultimately give rise to malon-dialdehyde (MDA) and other toxic aldehydes including 4,5-dihydroxydecenal [45] and 4-hydroxynonenal [46]. Malondialdehyde has been shown to react with proteins [47, 48] and amino acids [49]. Lipid peroxides and/ or cytotoxic aldehydes can block macrophage action, inhibit protein synthesis, inactivate enzymes, cross-link proteins, generate thrombin, and act as chemotaxins for phagocytes.

Chemistry of Lipid Peroxidation

Hydroxyl radicals or any species that has sufficient reactivity to abstract a hydrogen atom from a methylene ($-CH_2-$) group can initiate lipid peroxidation in a membrane or polyunsaturated fatty acid, as shown.

$$-CH_2- + \cdot OH \longrightarrow -C\cdot H- + H_2O$$

Other reactive oxygen intermediates such as $O_2^{-\cdot}$ are insufficiently reactive to abstract H from lipids, but its protenated form $HO_2\cdot$, is more reactive and is capable of such abstractions from some fatty

acids. However, there is no evidence of the capability of $HO_2\cdot$ to initiate lipid peroxidation in cell membranes. Various iron-oxygen complexes can abstract H and initiate lipid peroxidation. Abstraction of H from methylene ($-CH_2-$) groups lead to the formation of carbon radicals which are stabilized by a molecular rearrangement to form a conjugated diene. Under aerobic conditions, these conjugated dienes react with O_2 and lead to the formation of peroxyl radical, $ROO\cdot$ [21]. These peroxyl radicals can abstract H from another lipid molecule and give rise to lipid hydroperoxide and another carbon radical. The later product can react with O_2 to form another peroxyl radical that can start the propagation stage of lipid peroxidation, as seen in the equation below.

$$RO_2^{\cdot} \quad + \quad -CH_2- \longrightarrow \quad ROOH \quad + \quad -CH^{\cdot}-$$

Iron ions can also take part in accelerating lipid peroxidation. Addition of Fe^{2+} salts to a peroxide-free unsaturated fatty acid can initiate lipid peroxidation by the formation of $\cdot OH$ radical [50]. Iron (III) salts (Fe^{3+}) can accelerate decomposition of lipid hydroperoxides, leading to the formation of peroxyl and alkoxyl radicals.

Lipid peroxides are also formed enzymatically by the actions of cyclooxygenase and lipoxygenase. Thus, membrane fractions isolated from disrupted cells should contain some lipid peroxides, since lipid peroxidation is favored in injured cells. Hydroperoxides and endoperoxides produced by these enzymes are stereospecific and have important functions in biological systems. Many investigators

report that depletion of tissue GSH levels' results in lipid peroxidation of membranes. Since GSH and GSH-dependent enzymes are believed to be involved in protection against lipid peroxidation, it has been generally accepted that the initiation of peroxidative processes must take place after depletion of GSH. In general, chemicals that initiate lipid peroxidation by depleting the GSH levels, demonstrate a lag time before occurrence of lipid peroxides. There are some apparent exceptions to the correlation between depletion of glutathione and initiation of lipid peroxidation, however. Compounds such as Phorone cause depletion of GSH in tissue homogenate but do not result in lipid peroxidation, while an opposite observation applies for compounds such as diethylfumarate [51].

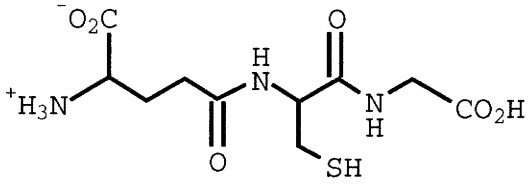
Glutathione

Glutathione (GSH) is an important antioxidant in the reduction of reactive oxygen species and metabolism of many drugs and endogenous substances. Glutathione protects cellular molecules from alteration by xenobiotics, irradiation, lipid peroxidation, oxygen free radicals, and hydrogen peroxide. Glutathione is a widely distributed tripeptide thiol (L-γ-glutamyl-L-cysteinyl-glycine) which is synthesized in virtually all animal cells, many microorganisms and plants by the sequential actions of two enzymes: γ-glutamylcysteine synthetase and glutathione synthetase. In 1888, deRey-Pailhade extracted a substance from a living organism that he named philothion [52, 53]. He suggested that "philothion plays a role analogous to that of hemoglobin toward oxygen and is endowed

with the property of hydrogenated sulfur." The structure of this substance was not known until, Hopkins isolated and hydrolyzed the compound 33 years later [54]. He obtained L-glutamic acid and L-cysteine, with the empirical formula $C_8H_{14}O_5N_2S$, and named it *glutathione*. Hopkins stated: " there can be little doubt that the chief significance of the occurrence of cysteine in the dipeptide, rather than free, lies in the fact that it is thereby protected from metabolic breakdown." Harris determined the molecular weight of glutathione to be 307± 0.8 [55]. Pirie and Pinhey [56] determined the pK value of glutathione, from the titration curves in water and in dilute formaldehyde, to be as follow: SH, 9.62; NH_2, 8.66; COOH, 3.53; and COOH, 2.12. On the basis of these values, they favored the structure γ-glutamyl-cysteinyl-glycine. During the period 1921-1936 the interest in this compound was not only in its molecular structure but also with its promise as an important and crucial component in cellular function.

In 1951, Barron stated that glutathione provides the cell with reducing power and it can protect protein sulfhydryl groups by interacting with heavy-metal ions, xenobiotics, and other toxic substances (including oxygen) [57]. Indeed, there is a strong evolutionary link between GSH and eukaryotic aerobic metabolism, involving protection by GSH, against oxygen toxicity [58]. Most of the total cellular glutathione is reduced glutathione (99.5%) rather than oxidized glutathione. The export of the reduced glutathione serves to protect the cell membrane against oxidative and other

types of damage by maintaining essential thiol groups, or other components of the cell membrane such as α-tocopherol. There is evidence that glutathione reduces the free-radical oxy form of α-tocopherol present in cell membranes to α-tocopherol [59].



Structure of Glutathione

Glutathione is also involved in the protection of other anti-oxidants such as ascorbate that can be oxidized to dehydroascorbate. Dehydroascorbate is irreversibly degraded if it is not reduced, this reduction is GSH-dependent [60, 61].

The export of glutathione from the cell may facilitate the transport of certain compounds, such as disulfides. Erythrocytes transport GSH, though the membrane, into the plasma as either GSSG or thioether conjugates [62]. In 1879, Baumann and Preusse [63] and Jaffe [64] showed that administration of certain organic compounds

to dogs was followed by urinary excretion of compounds which they called mercapturic acids. The source of the cysteine moiety of mercapturate was determined, many years later, to be glutathione [65, 66]. A soluble enzyme activity, glutathione S-transferase (GST), present in rat liver homogenates was found that catalyzes this conjugation of GSH with other foreign compounds (xenobiotics) [67]. Interaction of foreign compounds with GSH occurs spontaneously or may be catalyzed by the enzyme, GST. S-Conjugation of GSH is of importance in the metabolism and function of prostaglandins and leukotrienes formed from arachidonic acid metabolism [68-71].

Moreover, glutathione serves as a substrate or cofactor for many other enzymes, beside GST, which are involved in detoxification of toxic compounds. Among these enzymes, the glyoxalase system catalyses the detoxification of toxic α-ketoaldehydes, i.e., methylglyoxal, which forms an intermediate thiolester with GSH by the action of glyoxalase I [72]. This intermediate is subsequently hydrolyzed by glyoxalase II to glutathione and α-D-lactate [73].

The glutathione peroxidases are involved, in the reduction of reactive oxygen species and their products (hydrogen peroxides, organic hydroperoxides, and phospholipid hydroperoxides); these enzymes along with the GSH regenerating enzyme, glutathione reductase (GSSGRd) provide an efficient protection against oxidative stress and damaging free radicals. Indeed, glutathione seems to be

connected with metabolism, catalysis, transport, cellular protection against reactive oxygen species, xenobiotics, and free radicals.

Glutathione Peroxidases

The discovery of glutathione peroxidase in erythrocytes by Mills in 1957 [74] elucidated a major function of glutathione, that of reducing hydrogen peroxide by serving as a substrate for this enzyme. Glutathione peroxidase (GSHPx), the selenium-dependent, is the major scavenger of hydrogen peroxide in the cell [75]. Both H_2O_2 and $O_2^{-\bullet}$ are formed extensively in the cells and can produce other reactive oxygen species that may lead to the formation of organic peroxides [76]. Beside the classic selenium-dependent GSHPx, there are other GSHPxs; a non-selenium-containing glutathione S-transferase, which reduces organic hydroperoxides but not H_2O_2 [77]; phospholipid hydroperoxide glutathione peroxidase (selenium-dependent), which can protect biomembranes from peroxidative damage by reducing hydroperoxides derived from phospholipids, cholesterol and cholesterol esters [78]. Cellular peroxidase activities are diminished by selenium deficient diet; this increases the susceptibility of tissues to the damaging effect of reactive oxygen species [79]. The cellular defenses against oxidant cell damage are heavily dependent on GSH and the glutathione peroxidase-glutathione reductase system [80, 81]. The formation of oxidized glutathione (GSSG) in response to an oxidant challenge reflects the activity of glutathione peroxidase. Increases in the

efflux of GSSG often are used as a quantitative index for the measurement of the magnitude of oxidant stress [82].

Glutathione Reductase

Glutathione disulfide reductase is a flavin enzyme, which reduces the GSSG formed by glutathione peroxidase, back to GSH, at the expense of NADPH oxidation. There is also an active transport for the elimination of intracellular GSSG from the cell. This kind of transport processes has been reported for rat liver and heart [83, 84]. Erythrocytes transport GSH, though the membrane, into the plasma as either GSSG or thioether conjugates [62]. The main metabolic pathway involved in coping with oxidative stress is the glutathione redox cycle, which prevents the harmful buildup of H_2O_2 and reduces the peroxidized cellular structures. Glutathione reductase is part of this redox cycle and thus is involved in the protection of nucleic acids and membrane lipids. Cells and tissues can be rendered deficient of this enzyme by 1,3-bis(chloroethyl)-1-nitrosourea that is the specific inhibitor of glutathione reductase activity. According to some investigations, cells containing approximately 10% glutathione reductase activity compared to controls cannot cope with physiologically occurring challenges by oxidants [85]. The normal activity of glutathione reductase can effectively recycle low level of GSSG that is formed by the action of glutathione peroxidase on H_2O_2 and organic peroxides. Therefore, it protects against oxidative damage by turning over GSSG rather than by maintaining a high [GSH]/[GSSG] ratio [85].

Glutathione S-Transferase

GST play a central role in detoxification though their multiple catalytic activities towards a wide range of xenobiotics [86]. The proteins also serve an equally important role as an intracellular binding protein for several organic anions [86, 87]. GSTs catalytic and binding functions have been considered to facilitate the detoxification of various substances in the liver. Oxidative damage mediates lipid peroxide formation possibly leading to cell death. A certain lipid peroxide product, most toxic of these being 4-hydroxy-2-3-trans-alkenals of different chain length [88], exhibit toxic effects on the tissues where they are formed. These 4-hydroxyalkenals occur in the cell as a result of oxidative metabolism of endogenous as well as foreign compounds. A major product of peroxidative degradation of arachidonic acid is 4-hydroxynon-2-enals [89, 90]. Certain isoenzymes of the glutathione S-transferase families catalyze the conjugation of these toxic metabolites with glutathione [91]. These activities had been detected in various tissues.

Tumor Necrosis Factor

Postburn metabolic and immunological alterations may be due to bacterial infection and translocation of gut exotoxin and endotoxin [9], which can result in tumor necrosis factor (TNF). The constellation of clinical changes secondary to infection are directed by the toxins and the cytokines released (e.g., TNF) from the host in response to injury. Tumor necrosis factor is a 17-kD cytokine that

is released from macrophages upon an endotoxin-stimulus [92]. This peptide has an antitumor action [93] and participates in homeostatic tissue repair. However, its clinical utility is limited because it plays a role in liver toxicity and modulates immune and inflammatory reactions. Circulating TNF has been detected in patients afflicted with malaria [94] and infections associated with thermal injury [95]. Elevated serum TNF levels were also detected in one-third of adult burn victims who had no evidence of concomitant infection [96].

Exaggerated or prolonged secretion of TNF has been implicated in the pathogenesis of the clinical syndromes of septic shock. TNF is also involved in microvascular coagulation [97], endothelial injury (leading to morphological changes), actin filament redistribution, reduction of the intracellular glutathione (GSH), and elevation of oxidized GSH. Hepatocytes exposed to recombinant human TNF-α (1-10 μg/ml), in *vitro* exhibited intracellular GSH depletion and GSSG increase. This level of TNF-α was toxic to the animal treated with 1,3-bis(chloroethyl)-1-nitrosourea, inhibitor of glutathione reductase activity [98]. GSH deficiency in animals causes focal necrosis in liver, proximal tubular damage in kidney, and disruption of lamellar bodies in the lung. In this study, we determine the role of GSH depletion and augmentation on the release of TNF from macrophages in response to an endotoxic shock.

EXPERIMENTAL SECTION

Reagents and Chemicals

The following reagents and chemicals, glutathione (reduced and oxidized forms), 1-chloro-2,4-dinitrobenzene (CDNB), β-nicotinamide adenine dinucleotide phosphate (reduced form, NADPH), glutathione reductase (type III, Bakers yeast), cumene hydroperoxide, hydrogen peroxide (30% sol.), o-phthalaldehyde (OPA), phenylmethylsulfonyl fluoride (PMSF), N-ethylmaleimide (NEM), tumor necrosis factor, lipopolysaccharide, thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (malonaldehyde- bis-dimethylacetal, MDA), xanthine, xanthine oxidase (from butter milk), superoxide dismutase (bovine erythrocyte), cytochrome C (horse heart), phosphoric acid (85.8%), and n-butanol were all obtained from Sigma (St. Louis MO). Acetic acid glacial and methanol were purchased from Mallinckrodt. Pyridine was purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ). Ethanol (100%) was purchased from McCormick Distilling Co, Inc.

Cell Culture

All tissue culture media and components were purchased from GIBCO, Grand Island, NY. Murine fibroblast cell line L929 was obtained from American Type Culture Collection (ATCC), Rockville, MD. L-Buthionine-SR-sulfoximine was purchased from Sigma, and the glutathione diethyl ester was synthesized in our laboratory according to the method of Levy et al. [99].

Animals

Liver and lung tissues from sham burn and burn models rabbits, were donated by Dr. J. W. Horton, Professor of Surgery, University of Texas Southwestern Medical Center, Dallas, Texas. New Zealand white rabbits (2 to 3 Kg) were housed and used in compliance with the regulations of the Animal Care Facility of The University of Texas Southwestern Medical Center. The animals were divided into 3 experimental groups: control (n=4), sham (n=7), and burn (n=7). The control animals were given a lethal dose of sodium pentabarbital (4 ml of 50 mg/ml), and the organs were harvested immediately after death and freeze-clamped. The other two groups were anesthetized with isoflurane; central venous and thermistor tipped aortic catheters (Edwards, 94-011) were placed in the jugular vein and carotid artery (arterial blood pressure, cardiac output, heart rate). After stabilization, baseline parameters were measured for 20 minutes and arterial blood sample collected (pH, pO_2, pCO_2, HCO_3, and WBC). After baseline measurements animals were deeply anesthetized and placed in a template device, and the surface area of the shaved skin exposed though the device (on the animal's back and on each side) was immersed in 100^{o} water for 12 seconds. With this technique [100], full thickness burns comprising 30% of the total body surface area (TBSA) were obtained. Control (sham-burned) rabbits were subjected to identical anesthesia and treatment, except they were immersed in room temperature water. The animals were then dried, allowed to recover from anesthesia, monitored, and

immediately after burn injury, were given fluid resuscitation (4 ml/Kg/% burn; Parkland/Baxter formula) to maintain urine output. The animals were also given an analgesic (buprenorphine, 0.05 mg/Kg) immediately postburn (or sham burn) and at each succeeding 8 h interval throughout a 24 h period. The animals were then sacrificed (seven burn and seven sham-burn), and organs (lung, liver) were harvested, freeze clamped, and stored at -80° until used for analysis.

Tissue Preparation for Enzyme Assays

Tissues were homogenized (100 g/l) in cold, 10 mM sodium phosphate buffer, pH 7.4, containing 100 μM phenylmethylsulfonyl fluoride. Homogenization was carried out using a Polytron homogenizer at a setting of 12.5 to 13.0 for 30 seconds. Homogenates were centrifuged at 20,000 xg for 30 minutes, the supernatant solutions were then collected and stored in 1 ml aliquots, at -80° for the enzyme assays.

Determination of Tissue Lipid Peroxides

Lipid peroxides, in terms of malondialdehyde produced, were determined by a method similar to that of Ohkawa, et al. [101] with some modification. In the standard assay, 1.0 ml of (100 g/l) tissue homogenate (Polytron homogenization in, 1.15% w/v, KCl) was mixed with 0.4 ml of (80 g/l) aqueous sodium dodecylsulfate. Then 1.0 ml of thiobarbituric acid solution, prepared from 0.8% TBA aqueous solution and acetic acid glacial (1:1, v/v) as previously described

[102], was added, and the reaction mixture was diluted to a total volume of 5.0 ml with physiological saline. This mixture was incubated at 95° in a water bath, with gentle shaking, for one hour. After cooling with tap water, 5.0 ml of n-butanol-pyridine solution (15:1, v/v) was added, and the mixture was vortexed vigorously. The mixture was then centrifuged for 15 minutes (4,000 x g) to extract the pink reaction product. The absorbance of this reaction product was measured at 532 nm, and readings were compared to a standard curve prepared from solutions of known concentrations of malondialdehyde treated as described above.

Glutathione and Glutathione Disulfide Determinations

Glutathione (GSH) and glutathione disulfide (GSSG) levels in lung and liver tissues were determined by the method of Cohn and Lyle [103] as modified by Hissin and Hilf [104]. This method measures the fluorescence emmision of the o-phthalaldehyde(OPA) reaction product with these metabolites. At pH 8, GSH reacts with OPA to yield an activation (absorption) peak at 350 nm, and a fluorescence emmision peak at 420 nm. The reaction of GSSG with OPA occur at pH 12; while GSH can be complexed to N-ethyl-maleimide (NEM) to prevent its interference with GSSG measurement. Tissue extracts, for these measurements, were made by homogenizing 1.0 g of liver or lung tissue in 19 ml of a cold solution prepared from 15 ml of 100 mM phosphate-5 mM EDTA buffer, pH 8.0, and 4.0 ml of 25% phosphoric acid. The precipitated proteins (specially albumin which can react with OPA) were then

removed by centrifugation at 4° at 30,000 x g for 30 min, and the supernatant solutions were used for the glutathione and glutathione disulfide assays. A small amounts of these supernatants were mixed with phosphate-EDTA buffer (as previously described), and 100 µl of OPA (1mg/ml Solution) in a final assay volume of 2 ml. The reaction mixture were incubated at room temperature for 15 minutes and immediatly read. For GSSG assay, 200 µl portion of the original supernatant was incubated with 160 ml of 0.02 M NEM for 30 minutes, at room temperature in order to interact with GSH present in the tissue. This mixture was diluted to 5.0 ml with 0.1 N NaOH. A 100 µl portion of this alkaline mixture was diluted to 1.9 ml with 0.1 N NaOH, then incubated for 15 min with 100 µl of OPA (1mg/ml Solution) and read immediatly.

Fluorescence measurements were compared to a standard curve constructed from fluorescence values obtained for known concentrations of glutathione or glutathione disulfide (6 different concentrations 0 - 2 µg). Recovery studies were conducted on several tissue samples by spiking the tissue extracts with known quantities of glutathione or glutathione disulfide. In all cases recoveries of both glutathione and glutathione disulfide ranged from 90 to 95%. A Hitachi F-2000 fluorescence spectrophotometer was used for all determinations of fluorescence intensity.

Glutathione Peroxidase Activity Assay

Glutathione peroxidases catalyze the reduction of hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH) and in some cases phospholipid hydroperoxides by glutathione (GSH):

$$2GSH + ROOH \longrightarrow GSSG + H_2O + ROH$$
$$(H_2O_2) \qquad\qquad (2H_2O)$$

R can be an aliphatic or aromatic organic group or a phospholipid.

Glutathione peroxidase activity toward cumene hydroperoxide, hydrogen peroxide, and phospholipid hydroperoxide was determined by the coupled enzyme method of Paglia and Valentine [105] and rate of oxidation of NADPH at 340 nm in the presence of GSH reductase that catalyzes the reduction of GSSG produced by GSHPx activity. The reaction mixture (1.0 ml final volume), contained 50 mM Tris buffer (pH 7.4), 0.12 mM NADPH, 1.0 mM GSH, 1.0 IU GSH reductase, 0.5 mM cumene hydroperoxide or hydrogen peroxide or oxidized liposomes, and some tissue homogenate. The nonenzymatic oxidation rates of GSH with all peroxides were determined in the absence of tissue homogenate; the non-enzymatic rate was then subtracted from the rate determined in the presence of enzyme. A unit of GSHPx activity is expressed as amount of enzyme required to oxidize 1.0 μmole of NADPH/min under the described assay conditions.

Glutathione S-transferase Activity Assay

Activity of glutathione S-transferase was measured, similar to the procedure of Habig et al. [106]. This assay measures the formation of the conjugate of glutathione (GSH) with 1-chloro-2,4-dinitro-benzene (CDNB), spectrophotometrically at 340 nm. The assay mixture contained a suitable amount of 100 mM sodium phosphate buffer, pH 6.5, containing 1.0 mM EDTA, 1.0 mM GSH and 1.0 mM CDNB (dissolved in ethanol), and a small amount of diluted tissue homogenate in a final volume of 1.0 ml. The increase in absorbance at 340 nm is monitored (ε = 9.6 $mM^{-1}cm^{-1}$). The spontaneous reaction rate, between GSH and CDNB in the absence of enzyme, was measured and subtracted from the rate determined in the presence of enzyme. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1.0 μmole/min of thioether by nucleophilic attack of GSH on CDNB with displacement of chloride.

Glutathione Reductase Activity Assay

Glutathione reductase (EC 1.6.4.2), a flavoprotein, catalyzes the reduction of GSSG to GSH by utilizing NADPH as a reducing agent:

$$GSSG + NADPH + H^+ \longrightarrow 2GSH + NADP^+$$

Activity of glutathione reductase was determined according to the method of Carlberg et al. [107], at 25 ^oC, in a 0.5 ml of 0.2 M potassium phosphate buffer containing 2.0 mM EDTA, 0.1 mM NADPH, 1.0 mM GSSG, an adequate volume of tissue homogenate, and a volume of deionized water (final assay volume is 1.0 ml). The reaction was

initiated by addition of tissue homogenate, and the disappearance of NADPH was measured at 340 nm. A unit of activity is determined as the amount of glutathione reductase that catalyzes the oxidation of 1 μmole of NADPH/min under this assay condition.

Protein Determinations

Proteins content of all tissues were determined by using dye-binding method of Bradford [108]. Protein concentration can determined in the presence of sulfhydryl reagents. The procedure was standardized with bovine serum albumin.

All enzyme assays and spectrophotometric readings were made on a Shimadzu UV-1201 or a Beckman DU-70 spectrophotometer. All specific activities are expressed in term of milliunits per milligram of protein.

TNF Bioassay

Alveolar macrophages (MΦ) were lavaged from healthy New Zealand white rabbits. The cells were suspended (0.5×10^6 cells/ml) in either RPMI media (plus 10% FCS) alone or with buthionine sulfoximine (BSO; 1 mM final concentration), a specific inhibitor of γ-glutamylcysteine synthetase [109]. After 24 h, the supernatants were removed, and replaced with media containing either glutathione (GSH; 5 mM) or glutathione diethylester (GDEE; 5 mM). At the end of the incubation period (3 h), the cells were washed and then stimulated with lipopolysaccharide (LPS) at one of the 3 concentrations (0, 2.5 and 5 μg/ml). After 18 h, the

supernatants were collected, and stored at -80° until assayed for TNF. TNF functional activity was quantitated by using the L929 cell killing assay of Aggarwal with some modification [110].

Under anaerobic conditions, L929 murine fibroblasts are relatively resistant to lysis, which suggests that oxygen-dependent metabolic processes or free radicals facilitate killing. Oxygen consumption and ATP synthesis drop shortly after exposure of cells to TNF, and precede cell death. Cells metabolically crippled and sensitized to the cytotoxic action of TNF, by inhibition of protein synthesis with cycloheximide or inhibition of transcription with actinomycin D. Cells are plated in 96-well culture plates ($70X10^3$ cell per well) in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 50 μg streptomycin and 50 U penicillin per ml. After incubation for 2 h at 37°C, the samples were applied (5 μl) by serial dilution and 50 μl of cycloheximide (3 mg/ 10 ml) was added. Viability of the cells was determined by vital dye uptake analysis after 18 h incubation time. The later was accomplished by washing the plates with saline using a plate washer and staining with a 0.5% solution of crystal violet for 4 minutes. Plates were then rinsed 4 times in saline and dye uptake assessed at 540 nm with a Titertek Multiscan MC ELISA Reader (Flow Laboratories, McLean, VA). A preparation of recombinant human TNF-α was used as a standard.

The statistical analyses of the data (mean, standard error of the mean, and p value [Student t-test]) were calculated using the Macintosh software program, Microsoft Excell, Version 4.0.

RESULTS AND DISCUSSION

Lipid peroxide levels were measured, in terms of malondialdehyde produced therefrom (88,111), in liver and lung tissues of burn, sham burn and control rabbit models, 24 h post-burn or post-sham burn. Liver and lung tissues from burn models have an average increase of 17% ($p < 0.001$) and 29% ($p < 0.001$) in malondialdehyde levels, respectively, when compared to sham burn models. The malondialdehyde level of the burn models increased by 26% and 55% in the liver and lung respectively, when compared to control models ($p<0.001$). The effects of anesthetic/analgesic on the liver and lung malondialdehyde levels were increases of 8% ($p<0.03$) and 20% ($p<0.001$), respectively, (compared with control model values). These data are summarized in Table I (see also Fig. 1).

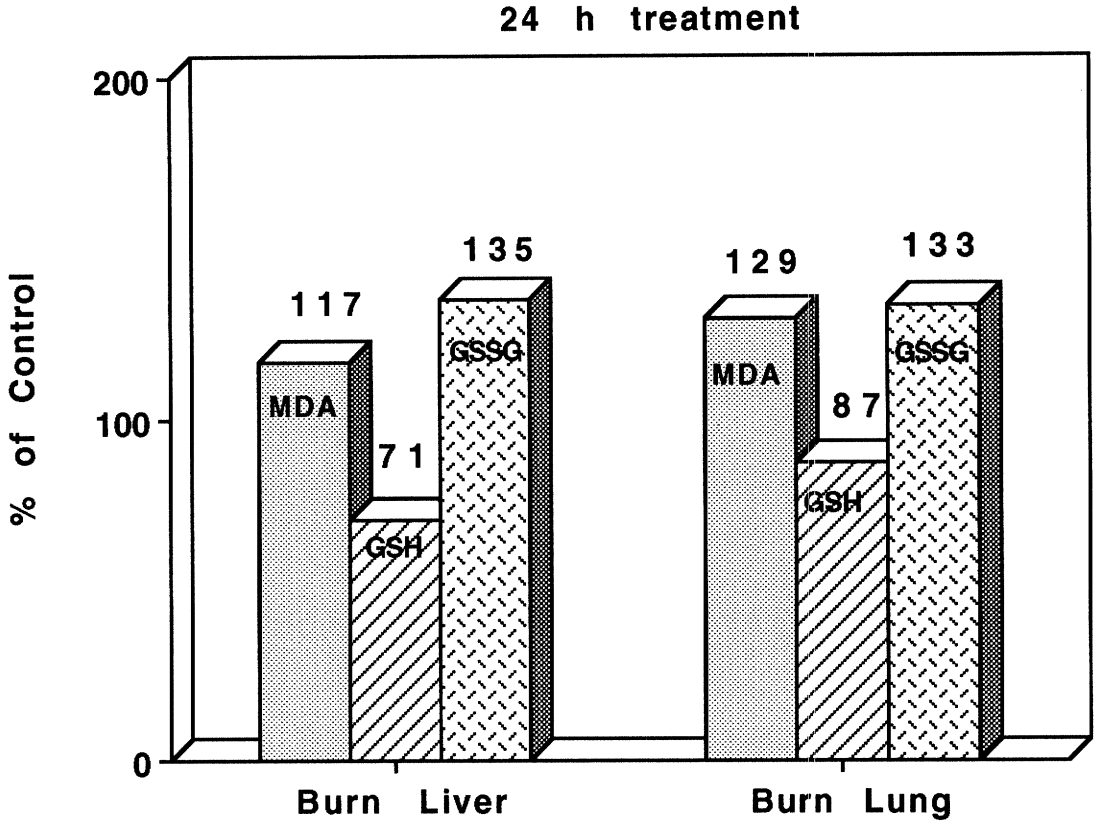
Glutathione and glutathione disulfide determinations of liver and lung tissues from 24 h burn, sham burn, and control models are also summarized in Table I and Fig. 1. In liver tissues from burn models there is an average 29% ($p<0.001$) decrease in glutathione concentration accompanied by an average 35% ($p<0.01$) increase in glutathione disulfide concentration when compared to sham burn models. In the liver tissues of control models, compared to burn models, there is an average 2-fold decrease ($p<0.001$) in glutathione concentration and a 2-fold increase ($p<0.001$) in glutathione disulfide concentration. Sham burn also caused some alteration in the levels of these metabolites in the liver; a 30% ($p<0.001$)

Table I. MDA, GSH and GSSG Levels in Sham Burn and Burn, Liver and Lung Tissues.

Tissue	Treatment	MDA (nmol/g)	GSH (μmol/g)	GSSG (μmol/g)
	Control	71.5 ± 1.32	8.00 ± 0.09	1.15 ± 0.06
Liver	Sham	77.3 ± 1.70	5.60 ± 0.20	1.70 ± 0.10
	Burn	90.2 ± 1.80	4.00 ± 0.30	2.30 ± 0.20
	Control	81.5 ± 1.03	3.54 ± 0.07	0.88 ± 0.03
Lung	Sham	97.4 ± 1.15	2.76 ± 0.15	0.99 ± 0.05
	Burn	126.0 ± 2.03	2.40 ± 0.09	1.32 ± 0.03

See Experimental Section for details. Data are expressed as mean ± SEM, (n=4 for control, 7 for sham and 7 for burn). Statistical significances, p values, are given in the Results and Discussion section. The units for MDA, GSH, and GSSG are expressed in nmol or μmol per gram of tissue.

Figure 1. MDA, GSH, and GSSG levels in burn liver and lung tissues as percentages of control values (sham burn liver and lung). See the Experimental Section and Table I respectively, for experimental details and actual values obtained.


24 h treatment
% of Control
200
100
0
117
71
135
129
87
133
MDA
GSH
GSSG
MDA
GSH
GSSG
Burn Liver
Burn Lung

decrease in glutathione and a 48% ($p<0.01$) increase in glutathione disulfide levels, compared to control values. The corresponding values for glutathione in burn lung compared to sham burn and control lungs give a 13% ($p<0.07$) and 32% ($p<0.01$) decrease, respectively. The concentration of glutathione disulfide in burn lung tissue increased by 33% ($p<0.001$) and 50% ($p<0.001$) over sham burn and control values, respectively. The glutathione and glutathione disulfide levels of sham burn lung versus control lung show a 22% ($p<0.001$) decrease and a 13% ($p<0.2$) increase, respectively.

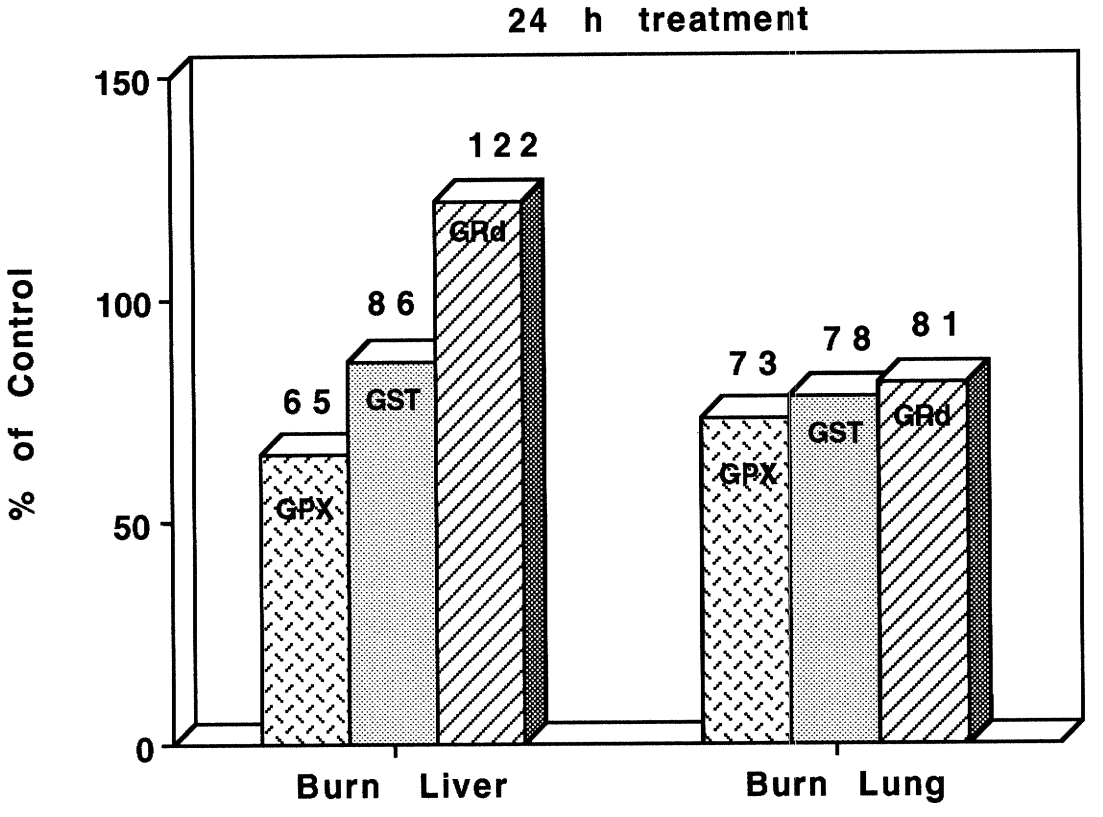
The glutathione-dependent enzymes, glutathione peroxidase, glutathione S-transferase, and glutathione reductase, were also studied in 24 h tissues from liver and lung in burn, sham burn, and control rabbit models. The results are summarized in Table II and Fig.2. The values given are statistically significant. The glutathione peroxidase specific activity (toward cumene hydroperoxide) is decreased by an average of 35% ($p<0.001$) in burn liver, and by 27% ($p<0.001$) in burn lung in comparison to sham burn. When this activity was measured using hydrogen peroxide, there were 27% and 23% decrease in burn liver and lung, respectively, compared to sham burn (data not shown). The specific activity of glutathione S-transferase is decreased by an average of 14% ($p<0.01$) and 23% ($p<0.001$) in the liver and lung of burn animal models, respectively, compared to sham burn. On the other hand, the specific activities of glutathione reductase are increased by an average of 22% ($p<0.001$) in the livers of models subjected to thermal injury, when compared

Table II. GSH-Dependent Enzyme Specific Activities in Sham Burn and Burn, Liver and Lung Tissues.

Tissue	Treatment	Glutathione Peroxidase	Glutathione S-Transferase	Glutathione Reductase
		(units per mg of protein)		
Liver	Control	911 ± 81	5228 ± 40	69.3 ± 1.4
	Sham	904 ± 40	5200 ± 160	72.1 ± 2.6
	Burn	591 ± 47	4470 ± 120	88.2 ± 2.0
Lung	Control	166 ± 7.3	819 ± 13	69.1 ± 1.5
	Sham	162 ± 5.2	830 ± 30	69.4 ± 1.7
	Burn	118 ± 3.4	640 ± 20	56.1 ± 2.0

See Experimental Section for details. Data are expressed as mean ± SEM, (n=7 for burn and sham burn, n=4 for control). Statistical significances, p values, are given in the Results and Discussion section.

Figure 2. Glutathione peroxidase (GPx), glutathione S-transferase (GST), and glutathione reductase (GRd) specific activities in burn liver and lung tissues as percentages of control values (sham burn liver and lung). See the Experimental Section and Table II respectively, for experimental details and actual values obtained.


24 h treatment
% of Control
150
100
50
0
6 5
8 6
1 2 2
7 3
7 8
8 1
GPX
GST
GRd
GPX
GST
GRd
Burn Liver
Burn Lung

to sham burn. Although the actual differences in glutathione reductase specific activities of burn and sham burn models are rather small, statistical analyses indicate that these differences are significant. In contrast, in lung tissues of burn and sham burn models, the glutathione reductase specific activities reflect the trends of the other two glutathione-dependent enzymes studied; there is an average 19% ($p<0.001$) decrease in specific activity in burn models as compared to sham burn models. No differences were found in these enzyme activities that could relate to effects of anesthetic/analgesic (control versus sham). Others have reported differences in glutathione metabolism and in glutathione-dependent enzyme activity responses to various chemical challenge in lung and liver tissues (112). In a separate study, we measured the activities of two glutathione-independent, anti-oxidative enzymes (superoxide dismutase and catalase) in liver and lung homogenates of control, sham burn and burn models. The activities of these enzymes within the three groups were not statistically different.

In the present study, the malondialdehyde levels, derived from the breakdown of fatty acid peroxide moieties, are increased in both liver and lung of burn models, when compared with malondialdehyde levels in sham burn animals (Table I). This increase could result from deleterious oxidative effects on membrane lipids [38-42] at sites remote from the site of injury. Thus, hydrogen peroxide (or other organic peroxides), hydroxyl radicals or superoxide radicals, produced at the site of injury, could oxidize fatty acid moieties of

membrane lipids at distal sites. Another explanation could be that lipid peroxides, produced from damaged cell membranes at the injury site, enter the circulation and become incorporated into membrane lipids in other tissues, such as liver and lung [113]. Yet another explanation could relate to the influx into liver and lung tissues of phagocytes, such as neutrophils, in response to the burn injury, which could generate reactive oxygen species [12, 13]. A previous study by others (Mileski et al., unpublished observations), involving skin burns in rabbit models, showed that circulating neutrophils increase approximately 30% within 4 h following burn injury, and remain at that level at least 24 h. As also seen in Table I, malondialdehyde levels in both liver and lung of control rabbits (no sham burn, no anesthetic/analgesic) are the lowest of the test groupings. It is evident that there are increases in peroxide formation due to oxidative processes involving the anesthetics/ analgesics (sham burn). However, these findings do not negate the evidence of even greater oxidative stress (highest malondialdehyde levels) found in the burn animal group.

Since glutathione-dependent enzymes (i.e., glutathione peroxidase) play a major role in decreasing the levels of various peroxides, it would not be surprising that the oxidative insult evidenced by the increases in malondialdehyde in burn liver and lung would have an effect on the relative amounts of the glutathione and glutathione disulfide. The results in this study indicate this to be the case. The glutathione disulfide/glutathione ratio in sham burn

liver is 0.30; the ratio increases almost 2-fold, to 0.58 in burn liver. Similarly, the glutathione disulfide/glutathione ratio in sham burn lung is 0.36; in burn lung the ratio is 0.55, an increase of over 1.5-fold (data derived from Table I).

It is noteworthy that the glutathione disulfide levels in both liver and lung of sham burn models are extraordinarily high and give testimony to an oxidative stress situation [114]. Thus, the glutathione disulfide/glutathione ratio in the control groups for liver and lung were found to be 0.14 and 0.25, respectively (data derived from Table I). The value for this ratio in normal rat liver has been reported to be 0.05 [115]. The low glutathione and high glutathione disulfide levels in both burn and sham burn animals, when compared to control rabbit tissues, could well reflect a depletion of glutathione (and increase in glutathione disulfide) in liver and lung tissues via detoxification processes of xenobiotic anesthetic and analgesic supplements (isoflurane and buprenorphine, respectively) during the 24 h period (see Experimental Section). It has been established that a variety of drugs cause oxidative stress [116]; relief of such stress is addressed, in part, through the action of glutathione-dependent enzymes. Glutathione peroxidase produces glutathione disulfide, which competes with glutathione conjugates (from glutathione S-transferase activity) for a common carrier to be transported from cells [117]. Both of these processes could lead to the accumulation of intracellular glutathione disulfide.

In the studies of glutathione-dependent enzymes from liver and lung of burn and sham burn models, (Table II, and Fig. 2) the results are consistent with an assumption that the burn models have experienced oxidative damage of tissue protein and/or membranes at sites remote from the thermal injury site. There is one exception in these results, however. In the case of liver tissue, there is a small, but statistically significant increase in glutathione reductase activity in burn versus sham burn models. There is evidence that some enzyme activities are increased when the glutathione disulfide/glutathione ratio increases (oxidative stress situation). This has been suggested as an explanation for the increased blood glutathione reductase activity in the uremic rat where the glutathione disulfide/glutathione ratio is increased [115]. On the other hand, one of the causes of a decrease in the activity of an enzyme under oxidative stress is the formation of protein disulfide bridges, with consequent diminution of catalytic capability [118]. The difference in the responses to oxidative stress of glutathione reductase in liver and lung tissue in this study is presently unclear. We found that the in vitro incubation of liver homogenates, obtained from control models, with hydrogen peroxide at two different concentrations (0.01 and 0.10 mM) did not significantly alter the activities of the three enzymes studied herein.

This study gives strong evidence that full thickness skin burns cause significant effects on the oxidation-reduction status of glutathione in liver and lung tissues of animal models. It is

reasonable to propose that the increased glutathione disulfide/ glutathione ratios reflect the organ's response to ameliorate the deleterious effects of reactive oxygen species (as reflected by increased malondialdehyde levels) produced at the site of the burn injury. It is also apparent that when the burn injury is massive (30%, total body surface area, in this study), organ response to oxidative damage in a 24 h period may be inadequate to fully compensate for the insult to the organism. The residual metabolite and antioxidant enzyme levels determined in this study generally support this conclusion. Therapeutic amelioration of the consequences of burn injury could well invoke methodologies that correct for the related increase in glutathione disulfide/ glutathione ratios, since the oxidation-protective glutathione-dependent enzymes, glutathione peroxidase and glutathione S-transferase, demand high levels of glutathione for optimal activity.

Decreases in the levels of GSH in tissues after burn injury may indirectly contribute to tissue damage, since the loss of this essential thiol renders cells and organs unprotected against reactive oxygen species, leading to lipid peroxidation of bio-membranes. The cell membrane proteins contain cysteine residues in the thiol form that are accessible mainly from the cytoplasmic side of the membrane [119]; some of the depletion of GSH might be due to its involvement in protecting these proteins from oxidation. GSH is also known to be involved in protection against membrane lipid peroxidation, removal of hydrogen peroxide and organic peroxides

[120, 121]; therefore, increasing the intracellular levels of GSH may be therapeutically important in amelioration of some of the consequences of burn injury and other oxidative stress related conditions.

The second goal of this study was to find a possible therapeutic role for GSH in preventing the release of TNF or if TNF is released to relieve its cytotoxicity. This cytokine is known to be secreted by macrophages after oxidative insult, such as burn injury [96,122]. TNF mediated cytotoxicity is also enhanced under conditions of oxidative stress [123]. Previous studies (see Introduction) have reported the role of GSH in cellular protection against free radicals and oxidative damage mediated by increased production of reactive oxygen species.

This study was conducted with rabbit alveolar macrophages in culture (see Experimental Section). The design of experiments involved the determination of the effects of cellular GSH depletion (buthionine sulfoximine; BSO treatment) and of GSH replenishment (GSH or GSH diethylester; GDEE) on the release of TNF from these cells. GSH does not readily enter most cells; on the other hand, certain hydrophobic derivatives of GSH (e.g., GDEE) have been shown to readily traverse cell membranes, and after subsequent hydrolysis by intracellular esterases, to increase intracellular GSH levels [99]. The releases of TNF from macrophages (MΦ) in response to LPS stimulation, for untreated and BSO-treated MΦ, are presented in Fig. 3 and 4. The level of TNF released from stimulated MΦ, treated with

BSO, is higher than that from cells not treated with BSO. However, when the cells are treated with GDEE (but not with GSH), the release of TNF is greatly reduced in comparison to both BSO-treated and untreated MΦ. The differences in TNF production among GDEE-treated groups, and both control- and GSH-treated groups are statistically significant ($p < 0.01$).

These data indicate that GSH may act as an indirect, endogenous factor in TNF production. Thus, it is possible that GSH acts by removing activated oxygen species that may be more directly involved in the release of TNF. In a separate experiment, 0.7 mM GDEE protected target cells such as L929 murine fibroblasts by 50% against the cytotoxicity of TNF ($2.5\text{-}2 \times 10^4$ U), while the same concentration of GSH did not influence cell survival (Data not shown.).

The significantly greater effects shown by GDEE over that of GSH, in decreasing LPS-induced TNF release and in protection against the cytotoxicity of TNF, probably reflect the important role of adequate levels of intracellular GSH in countering certain effects of oxidative stress. Because glutathione depletion may occur in trauma, shock and burn, treatments that help maintain intracellular GSH levels (e.g., GDEE), may be of therapeutical value in the treatment of endotoxic shock and burn injury.

Figure 3: Alveolar Macrophage TNF response to LPS stimulation in the presence and absence of a glutathione source. See Experimental Section for details.
Abbreviations used: GSH (Glutathione) and GDEE (Glutathione diethyl ester).

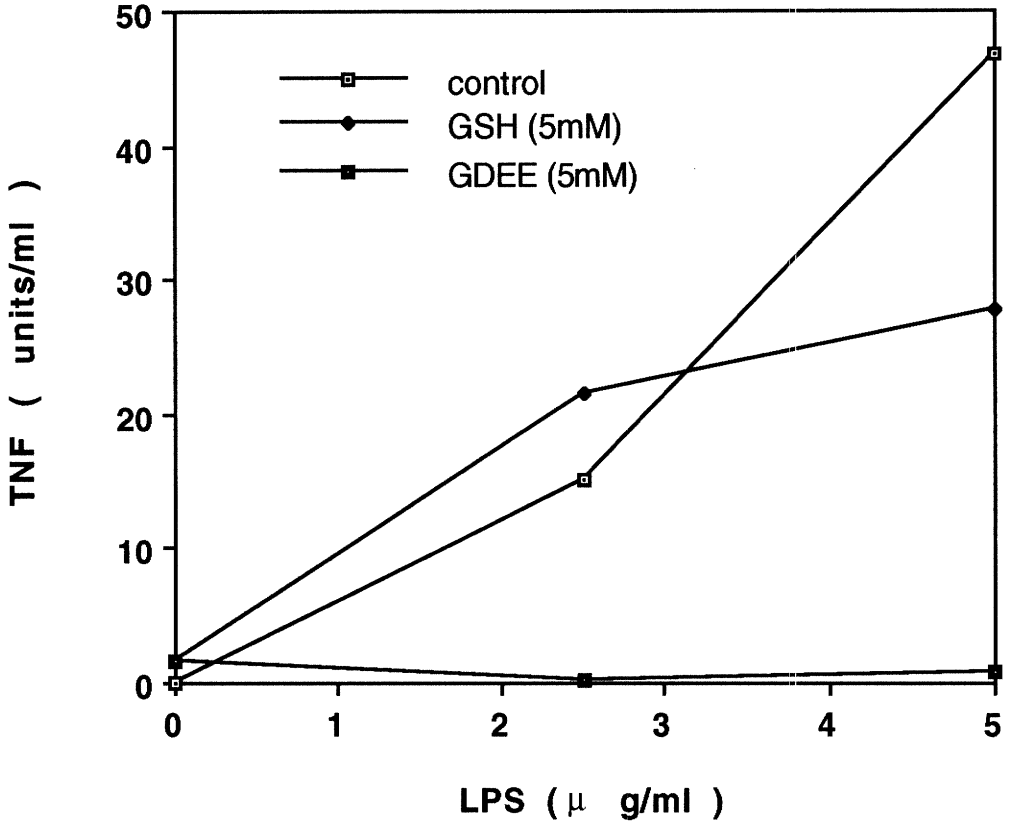
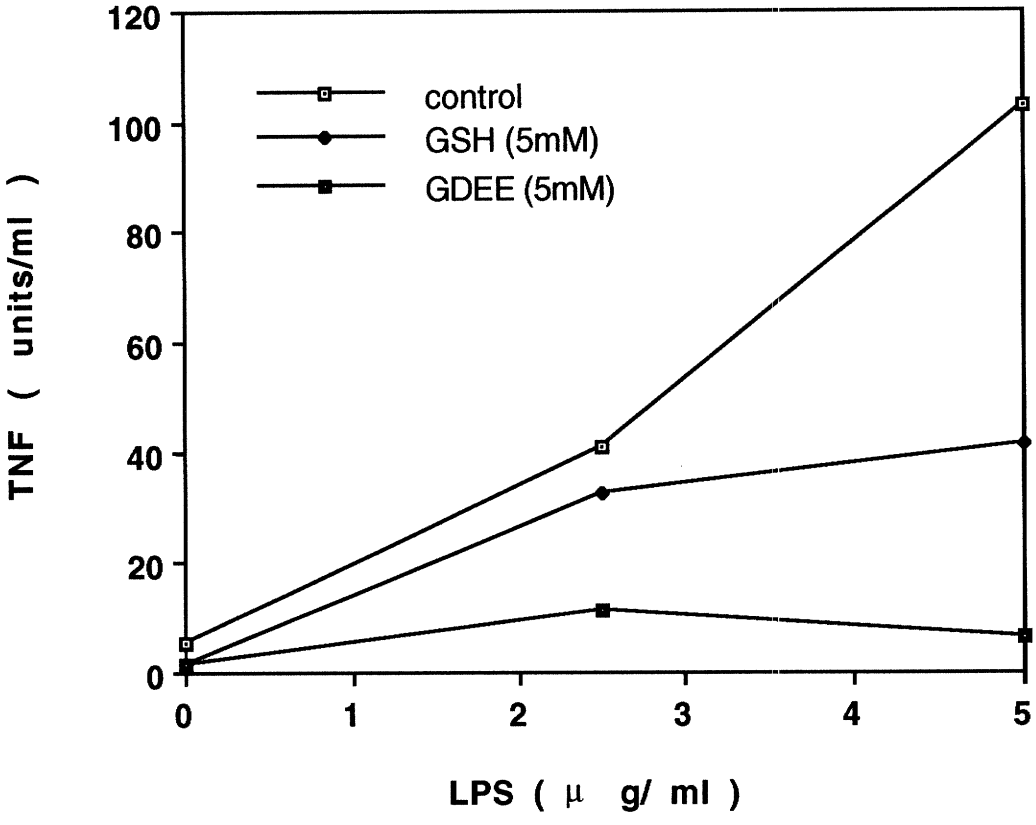

50
40
30
20
10
0
TNF (units/ml)
control
GSH (5mM)
GDEE (5mM)
0
1
2
3
4
5
LPS (μ g/ml)

Figure 4: GSH-depleted alveolar Macrophage TNF response to LPS stimulation, in the presence and absence of GSH and/ or GDEE. See Experimental Section for details.
Abbreviations used: GSH (Glutathione) and
GDEE (Glutathione diethyl ester)


120
100
80
60
40
20
0
TNF (units/ml)
control
GSH (5mM)
GDEE (5mM)
0
1
2
3
4
5
LPS (μ g/ ml)

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PART II

STUDIES OF ANTI-OXIDATIVE/ ANTI-INFLAMMATORY ENZYMES IN *ALOE VERA*

CHAPTER ONE

ALOE VERA

GENERAL INTRODUCTION

For centuries, plants and herbs have been commonly used as folk remedies for basic health care. A fairly well documented plant in traditional medicine is *Aloe vera*. There are numerous optimistic, and in some cases very promising, claims for its medicinal usage. Modern clinical use of the gel (parenchyma) of *Aloe vera* began in the 1930s, with reports of successful treatment of X-ray and radium burns, which led to further experimental studies using laboratory animals in the 1940s. Numerous experiments of this nature and favorable case histories did not give conclusive evidence, because much of the work suffered from poor experimental design [1]. In recent years, new experimental work has indicated that *Aloe vera* gel does contain substances having interesting physiological effects. *Aloe vera* has been used to treat different forms of inflammatory disorders, ranging from infected wounds to rheumatoid arthritis,

skin injury, X-radiation dermatitis and burns [2-4]. The mechanisms of these therapeutic effects are not well elucidated.

Aloe vera (*Aloe barbadensis* Miller) is a desert succulent, a member of the lily family (*Liliaceae*). It entered the New World initially by transport from northern Africa to the island of Barbados. The yellow sap (or latex), the mucilaginous material between the parenchymal tissue (gel) and the outer green rind, has been used as a laxative source for centuries. The gel of *Aloe vera* that constitutes the bulk of the leaf substance, serves as a water storage organ for the plant; it contains more than 200 identified substances. Chief among these are polysaccharides, glycoproteins, vitamins, minerals and enzymes. The gel is more than 95% water, so these solutes are found in very limited amounts. The gel, a mucilaginous jelly, is mainly polysaccharide, and its composition is shown to vary widely with the species [5-9]. Polysaccharide materials from plants, bacteria and yeast are shown to stimulate the host cell defense systems. There is evidence that several polysaccharides, e.g., the mannans from *Saccharomyces* and *Candida albicans*, induce lymphocytes and macrophages to produce a wide range of immunologically active substances [10-12]. Lebbar et al. [13] reported on the chemical portion of lipopolysaccharide (LPS), that provides the signal for macrophage activation, to be 2-keto-3-deoxy-D-manno-octulosonic acid. These immunologically active substances manifest specific antiviral activity though alteration of viral glycoprotein synthesis. A number of pharmacological studies

have been conducted on extracts of different *Aloe vera* species; e.g., *Aloe arborescens* extracts have been shown to have a protective effect on mouse skin X-irradiation-induced by its ability to scavenge hydroxyl radicals and to suppress the changes of activity in glutathione peroxidase and superoxide dismutase [14]. Hart et al. have isolated low molecular weight constituents from the gel of *Aloe vera*, that inhibit the release of reactive oxygen species from stimulated human neutrophils [15]. Comparative evaluation of *Aloe vera*-treated animal burn wounds with non-treated and silver sulfadiazine-treated wounds, showed a significant improvement in the *Aloe vera*-treated animals over the others [16].

Aloe extract has also been shown to contain a lectin-like substance that reacts with serum proteins such as α_2-macroglobulin and α_1- antitrypsin [17]. The dried sap of the *Aloe* plant (aloes) has the ability to lower blood glucose, and has been used as an anti-diabetic remedy [18-20]. A case study was conducted with five thousand patients having atheromatous heart disease, over a period of five years. Those who were given juice of the *Aloe vera* as a dietary supplement, had a marked reduction in total serum cholesterol, serum triglycerides, total lipids, and an increase in HDL. Simultaneously the clinical profile of these patients showed reduction in the frequency of anginal attacks; those mostly benefited were diabetics [20]. Other components isolated from *Aloe* (*Aloe arborescens Mill var. natalensis Berger* and *Aloe saponaria*), such as the enzyme serine carboxypeptidase that hydrolyzes

bradykinin, might explain reported anti-inflammatory effects [21,22]. Aloe has also been reported to be useful in treating leg ulcers and dermatoses [23]. Topical application of *Aloe barbadensis* gel extract to the skin of uv-irradiated mice ameliorates uv-induced immune suppression [24]. *Aloe vera* has other active components such as salicylates, substances that inhibit thromboxane formation *in vivo*, and components that exhibit anti-bacterial and antifungal effects [25]. The enzymes, glyoxalases I and II have also been purified to homogeneity in this laboratory, from the rind of *Aloe vera* [26]; these enzymes catalyze the detoxification of reactive α-ketoaldehydes that may be formed in cellular oxidative processes. The anti-inflammatory properties of the *Aloe vera* plant against burns and wounds have long been reported and studied to some extent. This activity of *Aloe vera* has been tested against various irritants, such as gelatin-induced and kaolin-induced edema; in both cases *Aloe vera* was active and the response was time-dependent [27]. The purpose of the studies reported herein is to investigate and characterize certain additional enzymes known to be involved in the scavenging of reactive oxygen species; these enzymes may therefore also be anti-inflammatory and antioxidative components of *Aloe barbadensis.* Two of the most important enzymes in the destruction of reactive oxygen species are glutathione peroxidase, which has recently been purified and characterized in this laboratory from *Aloe vera* gel [28], and superoxide dismutases [29]. These enzymes, along with catalase (peroxisomal enzyme) are the

major de-toxification enzymes known to prevent oxidative damage in animals and plant cells. Superoxide dismutases (SODs) convert superoxide anions to hydrogen peroxide and O_2, while glutathione peroxidase (GSHPx) destroys hydrogen peroxide in the cytosol. These enzymatic processes ensure that superoxide anion and hydrogen peroxide do not accumulate (especially, the latter species) to produce particularly reactive and harmful, hydroxyl radicals. These reactive oxygen species have been proposed to be involved in the aging process in general, and of the brain, in particular; also they are implicated in the development of inflammatory disorders such as rheumatoid arthritis [30].

An enhancement of cellular defenses by augmentation of antioxidative enzymes, such as GSHPx and SOD, could influence the efficiency of cells to combat oxidative stress situations. The substantial presence of these enzymes in *Aloe vera* juices could be a major reason for the reported anti-inflammatory and wound healing properties associated with this plant.

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CHAPTER TWO

THE STUDY OF A GLUTATHIONE PEROXIDASE FROM THE *ALOE VERA* PLANT

INTRODUCTION

Glutathione peroxidase (GSHPx, EC 1.11.1.9), is one of the major detoxification and hydrogen peroxide-scavenging enzymes [1]. The GSHPx enzymes reduce the products of aerobic respiration and exposure to oxygen, hydrogen peroxide and organic hydroperoxides, with a preferred thiol, glutathione (GSH). This enzyme activity was first discovered in erythrocytes [2, 3] by Mills and was subsequently shown to require selenium (selenocysteine) as an integral component [4]. The enzyme from mammalian sources is generally tetrameric, with four identical subunits; molecular weights range from 76-105 KD [5]. Recently, a monomeric form of GSHPx was purified from rat liver. It is different from the typical GSHPx activities and is more active in the presence of detergent [6].

A second type of Se-containing GSHPx, phospholipid hydroperoxide glutathione peroxidase (PLGSHPx), was isolated in 1982 from porcine liver and was reported to be different in its physical properties, cellular localization, and substrate specificity from the typical cytosolic GSHPx. The PLGSHPx enzyme is believed to be

important in protecting cellular components and biomembranes from peroxidative damage and lipid hydroperoxides formation [7]. GSHPx is widespread in many mammalians and nonmammalian species, including fish [8], the marine diatom, *Thalassiosira pseudonana* [9], and a green alga, *Chlamydomonas reinhardtii* [10].

Plants like other aerobic organisms, are also prone to oxygen toxicity. High oxygen concentrations can lead to the formation of reactive oxygen species including hydrogen peroxide, by the action of urate oxidase, amino acid oxidases, and especially glycollate oxidase, an enzyme of the photorespiratory pathway. Almost all plant tissues contain high activities of catalase, in peroxisomes, which breaks down high concentrations of H_2O_2 [11]. Plant tissues can also dispose of H_2O_2 by using peroxidase enzymes, which catalyze H_2O_2-dependent oxidation of substrates (SH_2). There is evidence for the existence of an unstable enzyme activity, in leaf tissue homogenates, which brings about removal of H_2O_2 in the presence of GSH [12]. This enzyme has been partially purified from cultured cells of higher plants such as spinach, maize and sycamore [13]. Two types of GSHPx activity have been found in *Euglena*; first, a form that was co-purified with glutathione transferase and reduces ROOH, but not HOOH, and a second form that reduces both ROOH and HOOH. The latter activity was reported to be a selenium-independent enzyme form [14].

It has been shown that glutathione peroxidases from *Euglena* [15] and mammalian sources [1] can also remove lipid peroxides from membranes, where the oxidative stress leads to membrane lipid peroxidation. The presence of this enzyme in plants may play a role as a protective agent in photosynthetic tissues. Traditional medicine in many countries has long held a therapeutic value for the parenchymous leaf-gel of *Aloe vera* in the treatment of inflammatory-based diseases [16]. Glutathione peroxidase activity, which may be capable of destroying the toxic products of oxidative stress that are produced in burns and other wounds [17,18], has not previously been purified to homogeneity from higher plant sources. In this study we report on the purification and characterization of GSHPx from the parenchymous tissue of *Aloe vera*.

EXPERIMENTAL SECTION

Materials

Aloe vera (*Aloe barbadensis* Miller) leaves were provided by Active Organics, Inc., Dallas, Texas. The following chemicals were purchased from Sigma Chemical Co.: reduced glutathione (GSH), cumene hydroperoxide, glutathione reductase (Type III, Baker's yeast), β-NADPH, egg albumin, bovine serum albumin, carbonic anhydrase, β-amylase, blue dextran, IgM, apoferritin, phenylmethylsulfonyl fluoride (PMSF), and all inorganic salts and organic reagents. DEAE-Sephadex A-50 and the Suprose-12 column for FPLC use were obtained from Pharmacia. S-Octyl glutathione [19], and N, S-bis-FMOC glutathione (DiFMOC-G) were synthesized in this laboratory [20]. Enzyme assays were conducted using a Varian DMS 70 UV-Vis spectrophotometer, and the final purification step was conducted using a biocompatible Pharmacia Fast Protein Liquid Chromatography (FPLC) system at an operating pressure of 1.5 MPa.

Enzyme Assays

Glutathione peroxidase activity during the purification steps and for kinetics studies was determined by the coupled enzyme method of Paglia and Valentine, with some modifications [21]. This procedure measures the rate of oxidation of NADPH at 340 nm in the presence of GSH reductase, which catalyzes the reduction of GSSG produced by GSHPx activity. The typical reaction mixture (1.0 ml final volume) contained 50mM Tris buffer (pH 7.5), 0.12 mM NADPH,

1.0 mM GSH, 1.0 IU GSH reductase, 1.0 mM cumene hydroperoxide, and a rate-limiting amount of *Aloe vera* GSHPx. The reaction was initiated by the addition of cumene hydroperoxide, and the disappearance of NADPH was followed spectrophotometrically at 25°C. The nonenzymatic oxidation rate of GSH was determined in the absence of GSHPx; the nonenzymatic rate was then subtracted from the rate determined in the presence of enzyme. A unit of GSHPx activity is expressed as the amount of enzyme required to oxidize one umole of NADPH per min. under the described assay conditions. Protein concentrations were determined using the dye binding assay of Bradford [22].

GSHPx Purification Procedure

All purification steps were conducted at 4°C. The inner mucilagenous, parenchymal tissue (250 g) was removed from two *Aloe vera* leaves and homogenized using a Waring blender (one min.) in the presence of PMSF and GSH (50 μM and 500 μM final concentrations, respectively). The homogenate was placed in a 500 ml Erlenmeyer flask and the pH of the mixture was adjusted to 7.0 by the addition of 10 mM Tris buffer, pH 8.8. The flask was stoppered and placed in the cold room overnight. During this period considerable carbohydrate-rich material precipitated, and the viscosity of the very viscous solution decreased substantially. The homogenate mixture was then filtered though several layers of cheesecloth, and the filtrate was utilized for further purification steps.

Wet DEAE-Sephadex (100 ml total volume), previously equilibrated with 10 mM Tris buffer, pH 8.8 (Buffer A) was added to the homogenate filtrate with occasional stirring with a glass rod. After approximately one hour, virtually all of the GSHPx activity had disappeared from the supernatant portion of the mixture. The column material was separated by filtration though a Buchner funnel and washed exhaustively with Buffer A to remove unbound (or weakly bound) proteins. The DEAE-Sephadex was then added to a column and washed again with Buffer A. The column was developed in a linear gradient of NaCl (0.0-0.5 M) in Buffer A. Fractions containing GSHPx activity were combined and concentrated by use of polyethylene glycol.

The GSHPx concentrate from the DEAE-Sephadex column was applied, in 500 μL aliquots, to a FPLC system equipped with a Suprose-12 gel filtration column (23 ml). The column had been previously equilibrated with 50 mM Tris buffer, pH 8.8, and elution was effected employing the same buffer. Protein profiles in the eluate were monitored at 280 nm. Fractions of 3 ml were collected and assayed for GSHPx activity.

Native Enzyme Molecular Mass Estimation

Utilizing FPLC, the Suprose-12 column (23 ml) was calibrated by determining the elution volumes of protein standards. The elution volume of the GSHPx was then compared with those of the protein standards (see Experimental Section) and the molecular weight estimated.

Subunit Molecular Mass Estimation

SDS-PAGE of *Aloe vera* GSHPx was conducted according to the method of Weber and Osborn [23]. Protein detection was determined by the silver staining method of Merill, et al. [24]. The subunit molecular weight was estimated by comparison with the electrophoretic migrations of standard molecular weight proteins.

Selenium Determination

The Se content of *Aloe vera* GSHPx was kindly determined by Dr. Farida Saleh, Environmental Sciences, the University of North Texas. The determinations were made by atomic absorption spectrometry using a Perkin Elmer Model 2380 Spectrometer equipped with a graphite furnace, PE-HGA Model 2100, with a temperature programmer, PE-HGA Model 400. The enzyme sample was 100 μl (X μg/ ml); four determinations were made, averaged, and compared to the readings obtained from standard selenium solutions.

RESULTS AND DISCUSSION

As shown in Fig. 1, FPLC-gel filtration chromatography gave a single large peak of GSHPx activity that was subsequently shown to contain a single protein. Table I show the purification steps employed; a 600-fold purification of the enzyme, with an overall yield of 77%, was obtained. The step involving DEAE-Sephadex A-50 was conducted by a batch procedure, since the viscous gel extract precluded the initial use of a column. After binding of the GSHPx to the DEAE, followed by filtration and washing (effectively removing the bulk of the mucilagenous material), a gradient elution of GSHPx from a column was possible. The mucilagenous *Aloe vera* gel contains very little protein (only 12.5 mg in the 250 ml extract). Calculations, assuming a 100% recovery, indicate that GSHPx is less than 0.2% of the total protein in the original extract, with an activity of about 0.03 enzyme units per ml of *Aloe vera* gel.

Early attempts to purify *Aloe vera* GSHPx were unsuccessful due to a rapid loss of activity in the original homogenate; this activity loss occurred even when the homogenates were stored at -80°C. The instability problem was corrected by the addition of GSH (final concentration, 0.5 mM) to the gel during the homogenization process.

Dithiothreitol (DTT) at 0.5 mM also effectively stabilized the enzyme. When either GSH or DTT is present in all buffers, GSHPx activity is stable for several months when stored at -30°C.

Figure 1. FPLC gel filtration elution profiles in the final step of the purification of glutathione peroxidase from *Aloe vera*. See the Experimental Section for details.

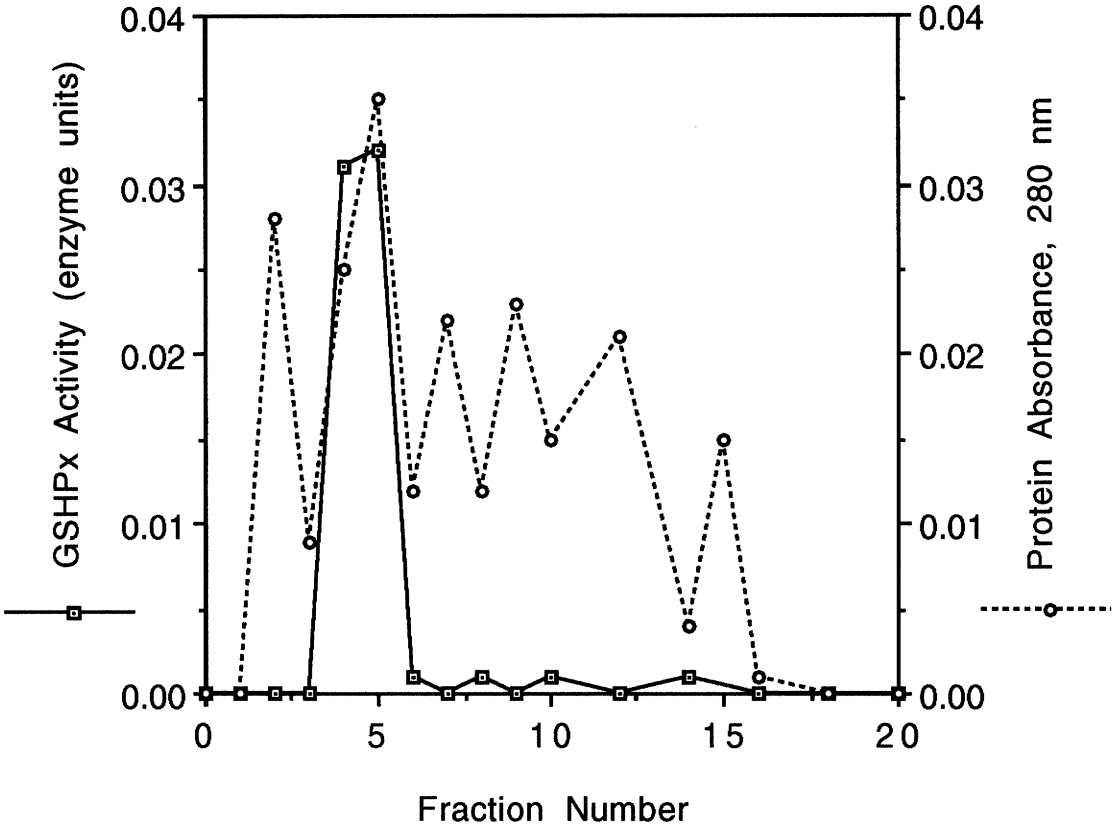

0.04
0.03
0.02
0.01
0.00
GSHPx Activity (enzyme units)
Protein Absorbance, 280 nm
0
5
10
15
20
Fraction Number

TABLE I. Summary of the Purification of Glutathione Peroxidase from *Aloe vera*.

Purification Steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Overall yield (%)	Purification fold
Crude Homogenate	12.5	7.3	0.58	100	1
DEAE-Sephadex	0.61	6.0	9.8	82	17
Gel-filtration Suprose-12	0.016	5.6	350	77	603

Details of the purification procedures are given in the Experimental Section.

The elution volume of *Aloe vera* GSHPx from a Suprose-12 gel filtration column was compared with those of standard proteins (IgM, apoferritin, β-amylase, IgG, bovine serum albumin, and carbonic anhydrase). A plot of log molecular mass vs. elution volumes gave a straight line and indicated that the native GSHPX has a molecular mass of about 62 kD. SDS-PAGE of the GSHPx from *Aloe vera* was also conducted (see Fig. 2). A single, dense protein band, detected by silver staining, was observed. This band corresponds to a molecular mass of about 16 KD; apparently the GSHPx from *Aloe vera* is a tetramer composed of identical subunits. This plant enzyme is thus similar in subunit constitution to most of the GSHPxs that have been studied from animal sources [3-5], although the overall molecular weight is somewhat less than typically found from those sources.

The Km values for the substrates, cumene hydroperoxide and GSH, were determined from double reciprocal plots according to the method of Cleland [25]. Velocity responses for each substrate were determined at increasing concentrations of that substrate in the presence of a high concentration of the other substrate. The Km values were found to be 0.26 mM and 3.2 mM for cumene hydroperoxide and GSH, respectively (see Fig. 3 and 4). This values are similar to those reported for GSHPx isolated from other sources [26].

Figure 2. SDS-PAGE of GSHPx activity peaks from the DEAE Sephadex and Suprose 12 gel filtration (FPLC) purification steps. Lane 1: Standard molecular weight proteins[bovine serum albumin (66 kD), fumarase (48 kD), α- lactalbumin (14.2 kD)]; Lane 2: Activity peak from the gel filtration step; Lane 3: Activity peak from the DEAE Sephadex step. See the Experimental Section and references 23 and 24 for further details.

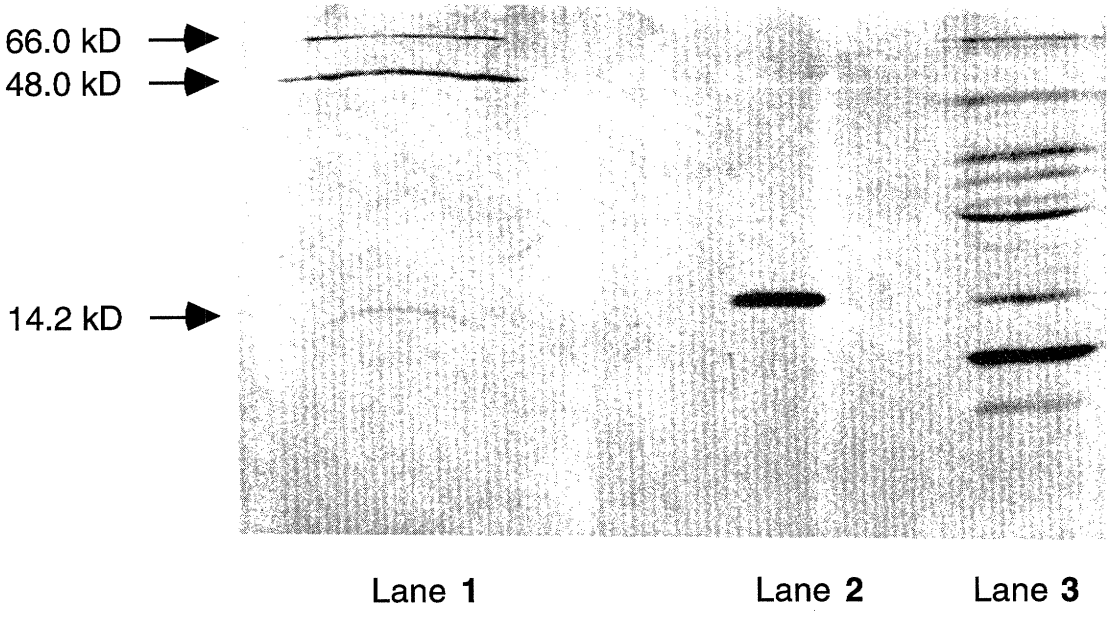

66.0 kD
48.0 kD
14.2 kD
Lane 1
Lane 2
Lane 3

Figure 3. Double-reciprocal plots of *Aloe vera* glutathione peroxidase for K_m determination with respect to GSH. Velocity responses were determined at increasing concentrations of GSH and a high concentration of CHP. K_m for GSH was found to be 3.2 mM. See the Experimental Section for details.

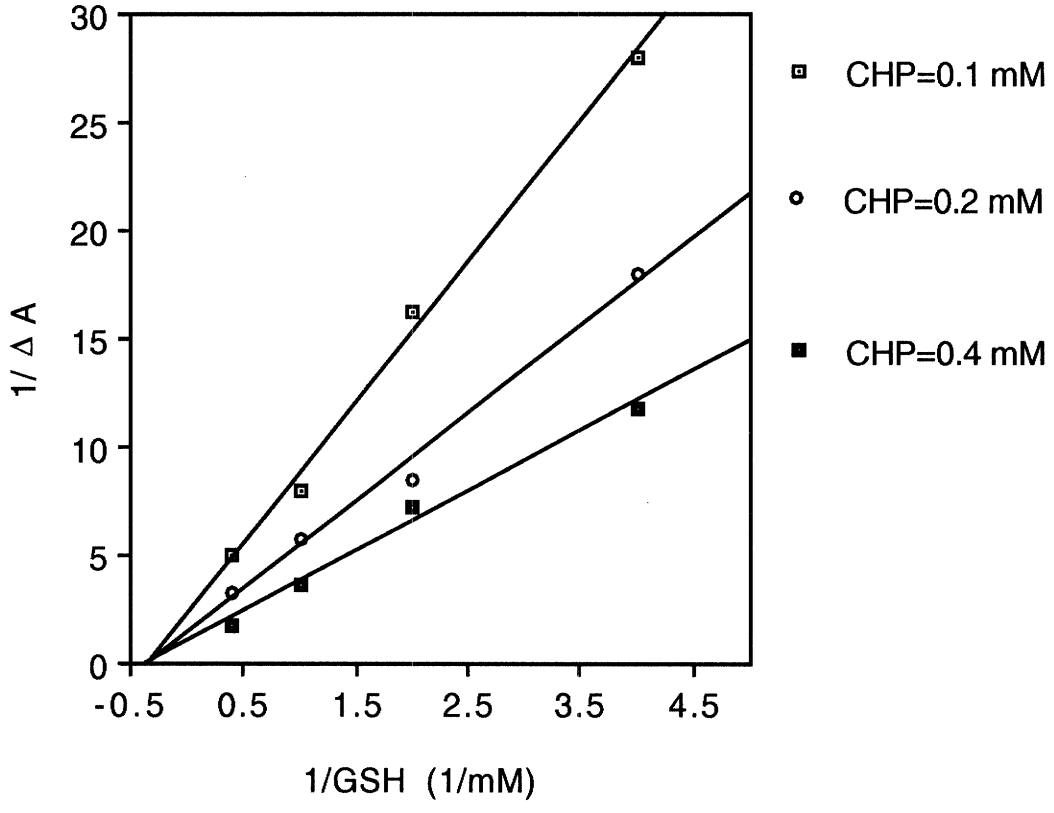
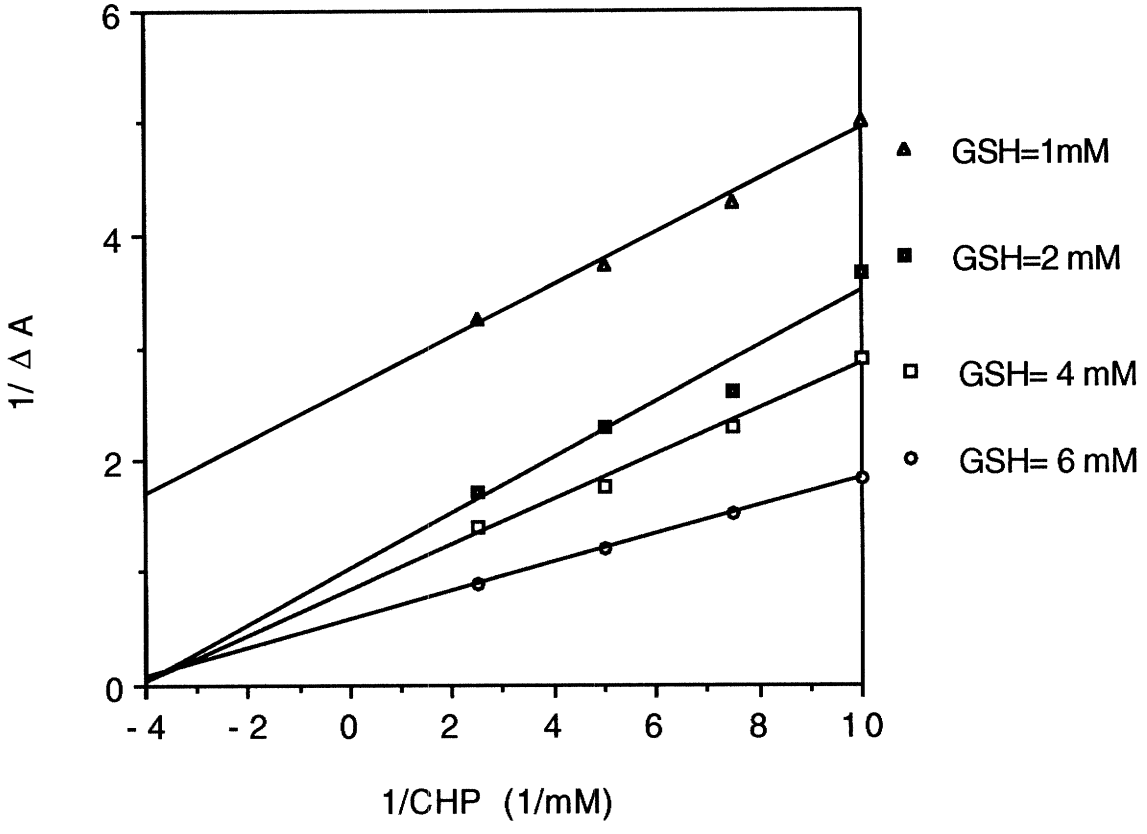

30
25
20
15
10
5
0
1/ Δ A
-0.5
0.5
1.5
2.5
3.5
4.5
1/GSH (1/mM)
CHP=0.1 mM
CHP=0.2 mM
CHP=0.4 mM

Figure 4. Double-reciprocal plots of *Aloe vera* glutathione peroxidase for K_m determination with respect to CHP. Velocity responses were determined at increasing concentrations of CHP and a high concentration of GSH. K_m for CHP was found to be 0.26 mM. See the Experimental Section for details.

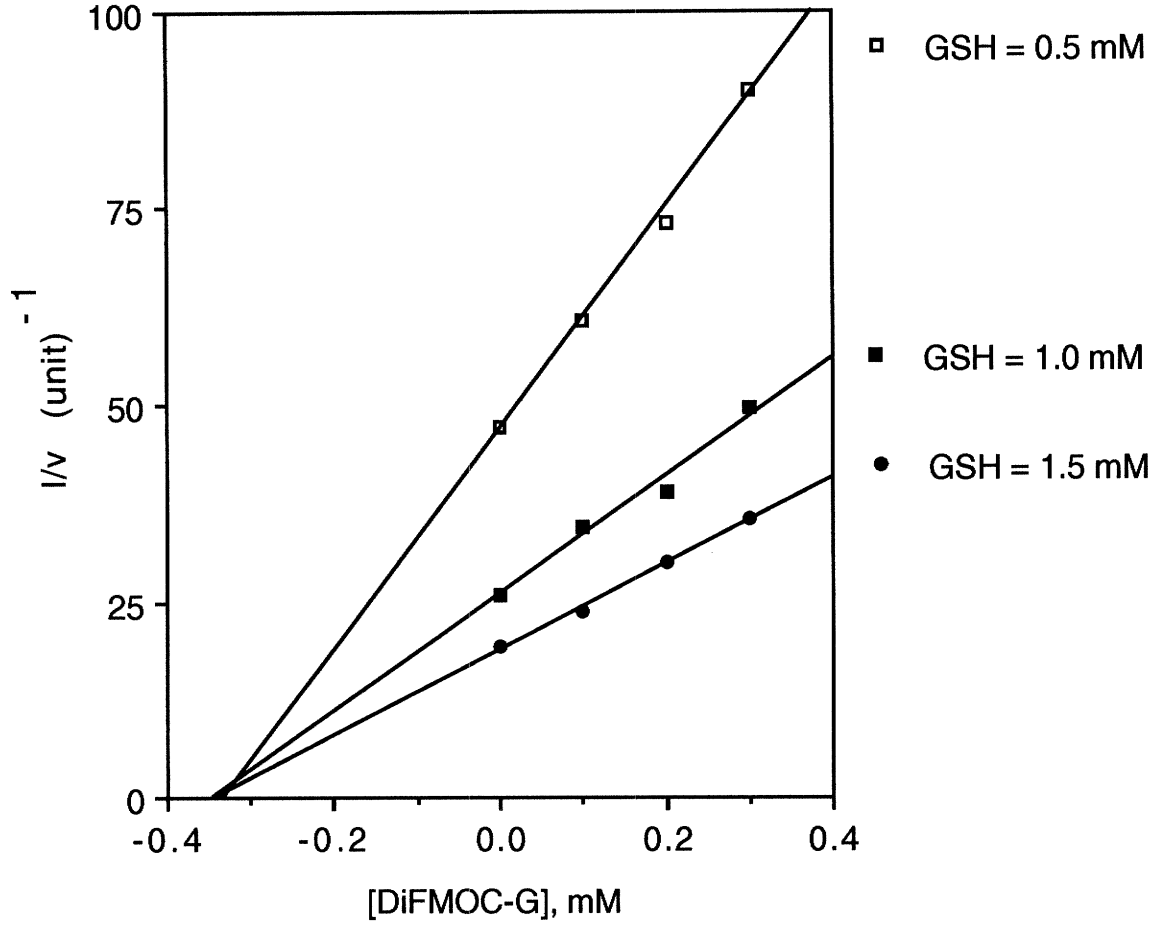

6
4
2
0
1/ Δ A
- 4
- 2
0
2
4
6
8
10
1/CHP (1/mM)
GSH=1mM
GSH=2 mM
GSH= 4 mM
GSH= 6 mM

For *Aloe vera* GSHPx, the calculated value of V_{max} per μg enzyme is about 4.8 μmole/min. Assuming a molecular mass of 62 KD, the turnover number (kcat) for the enzyme activity is approximately 5,000 moles of product (GSSG) mole-1 enzyme sec^{-1}.

Relative apparent affinities for the active site of GSHPx for GSH and GSH-containing compounds were studied using S-octyl-glutathione and DiFMOC-G, dead-end inhibitors of glyoxalase I [19] and glyoxalase II [20], respectively. While S-octylglutathione exhibited no inhibition at all levels tested, DiFMOC-G gave significant, apparently competitive inhibition of *Aloe vera* GSHPx, with a Ki value of 0.32 mM (see Dixon plot, Fig. 5). This Ki value is significantly lower than the Km value for GSH; however, the binding of DiFMOC-G to GSHPx is of much lower affinity than is found with mammalian glyoxalase II (Ki = 0.75 μM for the calf liver enzyme) [20].

Is the GSHPx from *Aloe vera* a seleno-enzyme? Four determinations by atomic absorption spectrometry (see Experimental Section) were made on a pure GSHPx solution containing approximately 1.0 μg protein/ml. The selenium concentration of this solution was found to be 4.76 + 0.26 X10-5 mM. Assuming the subunit molecular mass of the GSHPx to be 16 kD, the enzyme concentration of the solution would be about 6.25 mM. Considering these values, which suggest one selenium atom/ enzyme subunit, it is apparent that the *Aloe vera* GSHPx is also a seleno-enzyme in which there is a selenocysteine residue in each active

Figure 5. Dixon plot [27] of *Aloe vera* GSHPx kinetic data in the presence of DiFMOC-G. Enzyme velocity is given as the μmoles NADPH oxidized min-1 (see Enzyme Assays in the Experimental Section). The Ki value for DiFMOC-G is 0.32 mM.


100
75
50
25
0
-0.4
-0.2
0.0
0.2
0.4
I/v (unit)$^{-1}$
[DiFMOC-G], mM
GSH = 0.5 mM
GSH = 1.0 mM
GSH = 1.5 mM

site [2,5].

The GSHPx from the parenchymous leaf-gel of the *Aloe vera* plant, while having a lower tetrameric molecular weight than those found in GSHPxs purified and studied from mammalian sources, is similar to the mammalian enzymes in subunit composition, selenium content, and GSH Km values. The types of organic hydroperoxide substrates preferred and the functional role(s) of higher plant GSHPxs remain to be elucidated.

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CHAPTER THREE

STUDY OF SUPEROXIDE DISMUTASES FROM *ALOE VERA*

INTRODUCTION

It has been well established that all aerobic organisms contain enzymes, known as superoxide dismutases (EC 1.15.1.1)(SOD) which catalyze the conversion of superoxide anion to oxygen and hydrogen peroxide [1]. Superoxide is thus rapidly removed, preventing its conversion (via hydrogen peroxide) to the most reactive of oxygen radicals, the ·OH radical. The production of free radicals has been implicated in the aging process, in animals [2]. In plants, air pollutants such as ozone [3], sulfur dioxide [4] and certain herbicides [5, 6] often lead to the formation of highly reactive oxygen radicals and increased oxygen toxicity. High oxygen concentrations in plants inhibit chloroplast development [7], decrease seed viability and root growth [8], damage the membranes of leaves and roots, increase growth abnormalities [9] and leaf senescence in higher plants [10]. The concept of free reactive oxygen radicals did not capture much interest until the discovery of SOD in 1969 by McCord and Fridovich. These researchers isolated the enzyme from bovine erythrocytes and found it to be a blue-green copper-containing protein [1]. Superoxide

dismutases are a family of metalloproteins. Three forms of this enzyme have been found, as classified by their metal cofactor: copper-zinc, manganese, and iron forms. The copper-zinc SOD (Cu/ZnSOD) is localized mainly in the cytosol of all eukaryotic cells [11]; however it has also been found in mitochondria [12] and in the chloroplasts of higher plants [13-15], with the exception of some green algae, such as *Euglena* which contains only the iron and manganese enzymes [16-18]. SODs from prokaryotic cells [19] and from chloroplasts of some plants (tomato leaves and lemon leaves), contain iron at their active sites [20,21]. The manganese SOD (MnSOD) is widely distributed among prokaryotic and eukaryotic organisms, and in eukaryotes it is most often found in the mitochondrial matrix. In the plant kingdom, MnSOD has been purified from green peas [22], maize [23], and spinach [14]. This activity has also been detected in extracts from wheat germ [24], kidney bean leaves [25], corn [26], tea [27] and jerusalem artichoke [12].

Most of the SOD (Cu/ZnSOD) activity of green leaves is located in the chloroplasts; some activity is bound to the thylakoids and found free in the stroma. Two types of Cu/ZnSOD isozymes were found from cytosol and chloroplasts of angiosperms (spinach and rice), fern (horsetail) and green alga. Amino acid sequence differences among the cytosol Cu/ZnSODs are greater than those among the chloroplast Cu/ZnSODs [28]. Chloroplasts are especially affected by superoxide anion, hydrogen peroxide, the hydroxyl radical, and lipid peroxides due to a high internal oxygen

concentration. To allow their continued functioning, all plant tissues, especially chloroplasts, have multiple protective mechanisms against these species. The main ones are the enzyme, SOD, and the compounds, ascorbic acid [29], glutathione [30], α-tocopherol [31] and carotenoids [32].

The purpose of this investigation was to evaluate the abundance and characteristics of SODs in the leaves of *Aloe vera* (*Aloe barbadensis* Miller). The presence of SODs in the highly aqueous interior of this succulent plant was considered to be possible factors in reported healing and anti-inflammatory properties against skin burn injury and rheumatoid arthritis which is the clinical model of chronic phagocyte-mediated inflammation [33].

EXPERIMENTAL SECTION

Materials

Aloe vera (Aloe barbadensis Miller) leaves were provided by Active Organics, Inc., Dallas, Texas. The following chemicals were purchased from Sigma Chemical Co.: xanthine, xanthine oxidase (from butter milk), superoxide dismutase (bovine erythrocyte), cytochrome C (horse heart), riboflavin, 4-nitro blue tetrazolium chloride, N, N, N', N'-tetramethylethylenediamine (TEMED), bovine serum albumin (66 kD), β-amylase (200 kD), apoferritin (443 kD), thyroglobulin (669 kD), fumarase (48.5 kD), carbonic anhydrase (29 kD), β-lactoglobulin (18.4 kD), a-lactalbumin (14.2 kD), phenylmethyl- sulfonyl fluoride (PMSF), and all inorganic salts and organic reagents. Q-Sepharose, Suprose-12 and the mono-Q column for FPLC use were obtained from Pharmacia. Enzyme assays were conducted using a Shimadzu UV-1201 or a Beckman DU-70 spectrophotometer. A Biocompatible Pharmacia Fast Protein Liquid Chromatography (FPLC) system was used in second purification step and for relative molecular mass determinations.

Enzyme and Protein Assays

SOD activity, during the purification steps, was determined by the indirect method of McCord and Fridovich involving ferricytochrome c reduction, with some modifications [10]. All SODs, regardless of their metallo component, catalyze the following

reaction.

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

The main problem in the development of an assay for SOD has been the instability of the substrate, the superoxide radical. In the assay employed, the radical is generated enzymatically from the xanthine/ xanthine oxidase reaction. Measurement of the rate of reduction of cytochrome c by superoxide radicals is monitored at 550 nm. The presence of SOD activity in these reduction process results in an inhibition; the extent of inhibition is a measure of the activity of the SOD. The typical reaction mixture (1.0 ml final volume) contained 50 mM phosphate buffer (pH 7.8), 0.1 mM EDTA; 17 μM cytochrome c; 45 μM xanthine; 0.01 U of xanthine oxidase; and a sufficient amount of *Aloe vera* extract (containing SOD activity) to give measurable inhibition. As the activity of xanthine oxidase may vary upon exposure to light and air, one should use a sufficient amount of enzyme to produce a rate of cytochrome c reduction of 0.025 absorbance units/min in the assay without SOD. A calibration curve was prepared using various concentrations of purified bovine SOD (used as a standard: 0.0-1.2 U) and plotted as $1/\Delta A\ min^{-1}$ versus standard, for the measurement of the relative activities of *Aloe vera* SOD preparations during the purification procedure. One unit of SOD activity is expressed as the amount of enzyme required to inhibit the rate of reduction of cytochrome c by 50%. Protein concentrations were determined using the dye binding assay of

Bradford [34]. All of the specific activities are expressed in term of units per mg of protein.

SOD Purification Procedure

Freshly cut *Aloe vera* leaves were washed and the inner mucilagenous, parenchymal tissue (gel) and the green outer rind were separated. The rind (65 g) was homogenized using a Waring blender (one min.) in the presence of 100 ml of 20 mM Tris-acetate buffer, pH 6.7, containing 1 mM phenylmethyl-sulfonyl fluoride (PMSF). To the homogenate 3 volumes of cold ethanol (-20 ^{o}C) were slowly added with stirring. The mixture was stirred for 30 min, allowed to stand for a further 1.5 h, and then centrifuged at 10,000 xg for 15 min. No SOD activity was detected in the ethanol supernatant fraction. The sediment (19.5 g) was resuspended in 120 ml of 20 mM Tris-acetate buffer, pH 6.7, containing 1 mM PMSF and stirred overnight. After centrifugation at 14,000 xg for 30 min, the clear viscous supernatant solution was added to 50 ml of Q-Sepharose, previously equilibrated with 20 mM Tris-acetate buffer, pH 7.8 (Buffer A). The mixture was periodically stirred with a glass rod for 24 hours. Under these conditions all SOD activity was bound to the Q-Sepharose. The column material was separated by filtration through a Buchner funnel and washed exhaustively with Buffer A to remove unbound (or weakly bound) proteins. The Q-Sepharose was then added to a column (Column 1) and washed again with Buffer A and then with Buffer B (20 mM Tris-acetate buffer, pH 7.0). After washing with 5 volumes of Buffer B, the activity was

eluted with a linear gradient of NaCl (0.0-0.5 M) in 50 mM Tris-acetate buffer, pH 6.8. All active fractions were collected, concentrated by use of polyethylene glycol, and dialyzed in several changes of 20 mM Tris-acetate buffer, pH 7.6, to remove most of NaCl. After desalting, the SOD concentrates from the Q-Sepharose column (Column I) was applied, in 500 µl aliquots, to a FPLC system equipped with a Mono-Q anion exchange column (Column II) (5 ml). The column had been previously equilibrated with 20 mM Tris-acetate buffer, pH 7.6, and elution was effected employing a stepwise gradient elution using eluent A: 50 mM Tris-acetate buffer, pH 7.6 and eluent B: 50 mM Tris-acetate buffer, pH 5.2, 0.2 M NaCl.

The gel part of the *Aloe vera* leaves (180 g), mostly water, was homogenized in the presence of 1 mM PMSF, using a Waring blender (one min.). The homogenate was treated with 3 volumes of cold ethanol as described above. After centrifugation at 14,000 xg for 30 min, the ethanol supernatant was assayed for SOD activity. The rate of reduction of cytochrome c with this supernatant was higher than control (ethanol alone). The pellet (10 g) was stirred, in 100 ml of 20 mM Tris-acetate buffer, pH 6.7, containing 1 mM PMSF, overnight at 4^0C. After centrifugation at 14000 xg for 30 min, the clear viscous supernatant was collected and assayed. The total number of units of SOD activity in this supernatant was higher than the total number of units in the crude homogenate. The supernatant recovered from the pellet was mixed with 50 ml of Q-Sepharose (Column I), previously equilibrated with 20 mM Tris-acetate buffer,

pH 7.8 (Buffer A), and was eluted in the same fashion as described for the rind preparation. The active fractions from Column I were then concentrated, desalted, and applied, in 500 µl aliquots, to Column II. The elution procedure was, again, the same as that described for the rind preparation.

Activity Stain of Native Enzyme

Characterization of SOD is most conveniently done by demonstrating coincidence of protein staining and enzyme activity after polyacrylamide gel electrophoresis [35]. Enzyme activity staining was accomplished by monitoring SOD in nondenaturing polyacrylamide gels. Proteins were loaded on 12% w/v gel in the presence and absence of specific inhibitors for each isoform of SOD. Immediately after termination of the electrophoresis, the gel was placed in a solution of nitro-blue-tetrazolium (5 mg/20 ml) and riboflavin (2 mg/20 ml). After 20 min the gel was then placed in a solution of TEMED (1% w/v). With this procedure, generated superoxide radicals reduce the colorless nitro blue tetrazolium to a blue formazan [36]. SOD, by scavenging the radicals, inhibits the blue color formation and the active SOD bands appear colorless against a blue background.

Native Enzyme Molecular Mass Estimation

The relative molecular weights of the SODs were determined using an FPLC, Suprose-12, gel filtration column. The molecular mass standards used to calibrate the column were: bovine serum

albumin (66 KD, fumarase (48.5 kD), β-amylase (200 kD), apoferritin (443 KD), and thyroglobulin (669 KD). The elution volumes of these proteins were recorded and the elution volumes of the SOD isozymes were then compared with those of the protein standards.

Subunit Molecular Mass Estimation

SDS Polyacrylamide gel electrophoresis of *Aloe vera* SOD was performed according to the method of Weber and Osborn [37], using the following molecular mass standards (Mr): bovine serum albumin (66000), fumarase from porcine heart (48500), carbonic anhydrase (29000), α-lactalbumin from bovine milk (14200). Protein detection was determined by the silver staining method of Merill, *et al.* [38]. The subunit molecular weight was estimated by comparison with the electrophoretic migrations of standard molecular weight proteins.

Enzyme Sensitivity to Inhibitors

To distinguish the different forms of metallo-SODs from each other, several inhibitors, specific for each family were used. Complete inhibition of CU/ZnSOD by 2 mM cyanide is utilized to differentiate this family from the mangano- and iron-containing enzymes [39]. Inhibition of Fe-containing SODs with 5 mM H_2O_2 or 5 mM sodium azide is a good test to distinguish MnSOD from FeSOD [40,41].

RESULTS AND DISCUSSION

The rind of *Aloe vera* contains about 23 mg of extractable proteins per 100 g of rind. Calculations, assuming a 100% recovery, indicate that rind SODs represent about 2.5% of the total protein in the crude extract. Similar calculations with the gel of *Aloe vera* show it to contain about 3.5 mg of protein per 100 g of gel; the gel SODs represent about 2% of the total gel protein. The *Aloe vera* SOD activities were purified by the process summarized in Scheme I. Tables I and II show the details of the purification process for the rind and gel, respectively. Average purifications of all the SOD activities of 28- and 31-fold, with overall yields of 69% and 40%, were obtained for the rind and gel, respectively. The overall yields and purification factors were calculated relative to the ethanol fractions; the presence of certain factor(s) in *Aloe vera* crude homogenates, especially in the rind, cause an enhancement in the rate of cytochrome c reduction. This enhancement is not enzymatic, because upon use of the boiled (five min) crude homogenate in the enzyme assay, the rate of reduction of cytochrome c is increased to an even greater extent. It was found in other experiments that *Aloe vera* SOD activity in partially purified preparations are completely destroyed by boiling for five minutes. This factor(s) is soluble in ethanol, since when the ethanol supernatant is assayed, the rate of reduction of cytochrome c is increased relative to the control (ethanol alone).

Scheme I: Flow Chart of Purification of SODs from *Aloe vera*

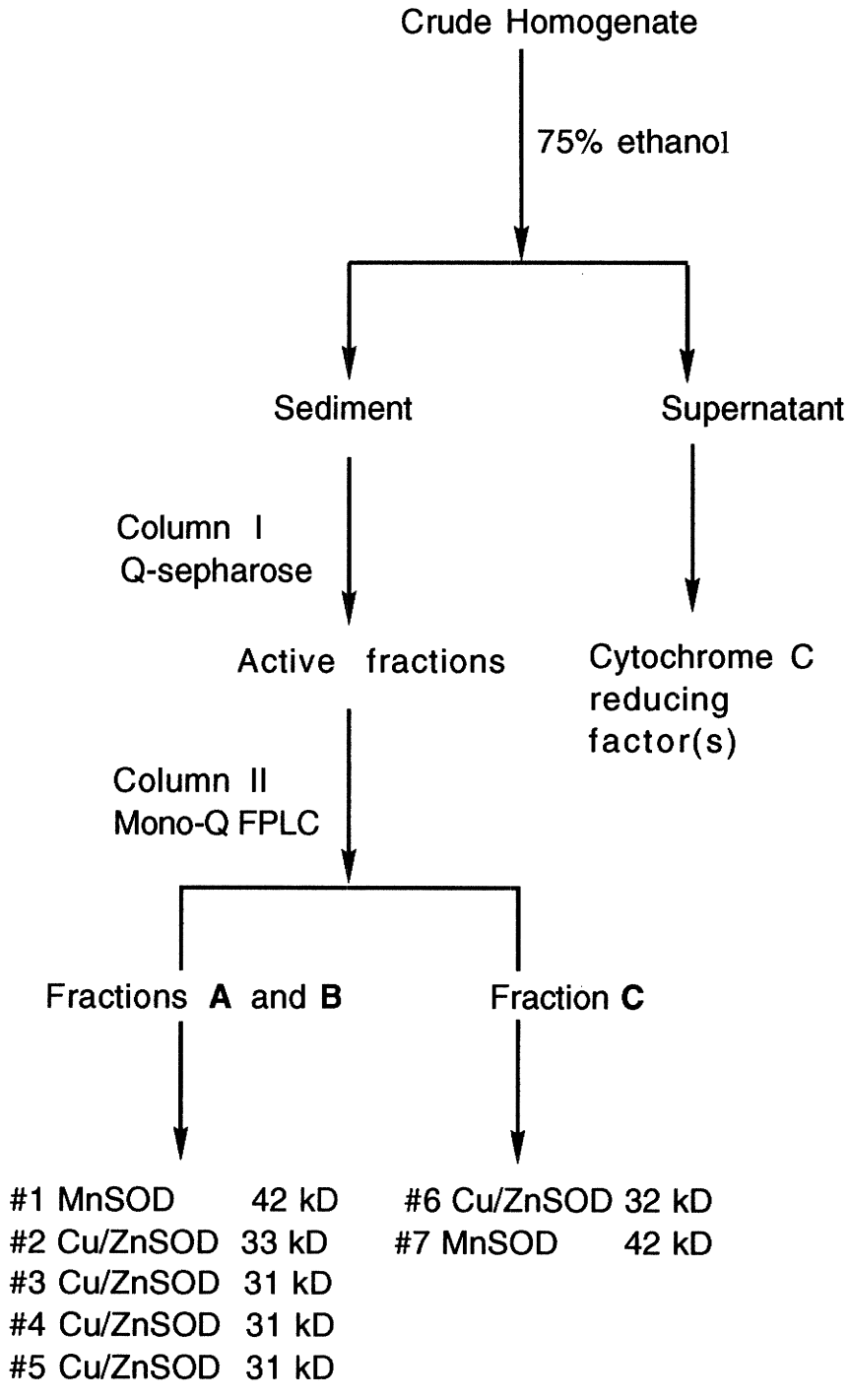


Table I. Summary of Purification of Superoxide Dismutase from *A. vera* Rind.

Purification Steps	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Overall yield (%)	Purification fold
Crude Extract	15	ND[a]	ND	ND	ND
Ethanol Fraction	12	1430	120	100	1.0
Col. 1: Q-Sepharose	3.0	1230	410	86	3.4
Col. 2: Mono Q					
Frac. **A**	0.11	200	1700	14	14
Frac. **B**	0.10	160	1600	11	13
Frac. **C**	0.09	620	6900	43	58
Total (Avg.)	0.30	980	3300[b]	69[b]	28[b]

ND[a] = not determined; because of the existence of some factors in the crude homogenates interfering with the rate of cytochrome c reduction; see the Experimental and Results and Discussion sections for further details.

[b] = data are calculated based on the total number of units of activities in fractions **A**, **B** and **C** and the total mg of protein in those fractions.

Table II. Summary of Purification of Superoxide Dismutase from *A. vera* gel.

Purification Step	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Overal yield (%)	Purification fold
Crude Extract	6.3	280	44	100	1.0
Ethanol Fraction	3.8	420[a]	111	100[b]	- -
Col. 1: Q-Sepharose	0.3	200	670	48	6.0
Col. 2: Mono Q					
Frac. **A**	0.02	70	3500	17	32
Frac. **B**	0.02	50	2500	12	23
Frac. **C**	0.01	50	5000	12	45
Total (Avg.)	0.05	170	3400[c]	40[c]	31[c]

[a] The number of units of activities recovered in ethanol fraction are higher than those in the crude homogenates. Some factor(s) causing interferences in the assay were disposed in the ethanol supernatant. See Experimental Section and Results and Discussion section for further details.

b The recovery (% yield) in the ethanol fraction was assumed to be 100%; the true number of activity units in the crude homogenates can not be determined.

c Data are calculated based on the total number of units of activities in the fractions **A**, **B** and **C**; and the total mg of protein in those fractions.

The step involving Q-Sepharose (Column I) was conducted by a batch procedure, since the viscous *Aloe vera* extracts (rind or gel) precluded the initial use of a column. After binding of the SODs to the Q-Sepharose, followed by filtration and washing, a gradient elution of SOD from a column was possible. The native gel electrophoresis of the combined active fractions from Column I showed seven identifiable SOD activity bands for both the rind and gel (See Fig. 1 for the designation of the bands).

The combined active fractions from Column I for both rind and gel were passed (separately) through a Mono Q ion exchange column (FPLC); the activity elution profiles are shown in Fig. 2. The elution profiles for both the rind and gel are quite similar with respect to the fraction numbers giving activity maxima; however, the maximal activity values appear reversed. That is, for the rind, the activity shown for elution region **C** is much higher than that given for elution regions **A** and **B**; for the gel, the **A** and **B** regions are relatively higher than that for region **C**. This finding appears to indicate that while similar (if not identical) SOD activities occur in rind and gel, the relative activity levels differ significantly.

The SOD activities in the rind and gel do indeed appear to be identical. As shown in Figures 3 and 4 SOD activity staining of native PAGE of Column II elution regions **A**, **B** and of **C** for the gel, and the rind, gave virtually identical migration patterns. Thus, the gel elution region **A** gave two discrete activity bands, while elution region **B** gave four, perhaps five additional activity bands, the faster

moving band (possibly two bands) being very faint. The elution region **C** gave two activity bands corresponding to the weak, fast moving activity band(s) of elution region **B**. The rind elution region **A** gave two strong discernable activity bands and the **C** region gave two intense activity bands having similar migration rates. The elution region **B** gave three new activity bands, the others that are observed on the gel are apparently contaminants from elution regions **A** and **C**.

In a separate experiment, the elution volumes of the active fractions (Column II) of rind *Aloe vera* SOD from a Suprose-12 gel filtration column were compared with those of standard proteins (see Experimental Section). A plot of log molecular weights vs. elution volumes gave a straight line; the native molecular weights of SODs (two activity peaks) were estimated to be centered at 32 KD, and 42 KD (See Fig. 5).

SDS-PAGE of the SODs from the rind elution region **C** (Column II) gave only two bands, after a prolonged silver staining period, with molecular weights of 32 and 42 KD, when no β-mercaptoethanol (BME) was employed. Two additional bands appeared (16 and 2I KD) when BME was used. When one half the amount of the protein solution used for the silver staining of the SDS-gel (above) was loaded onto a native gel, electrophoresed, and protein bands having SOD activity identified by activity staining, two very dense bands appeared (Fig. 4). In a parallel study, the region corresponding to

Figure 1. Nitro blue tetrazolium staining of *Aloe vera* SODs (active fraction from column I) after electrophoresis in polyacrylamide gel.

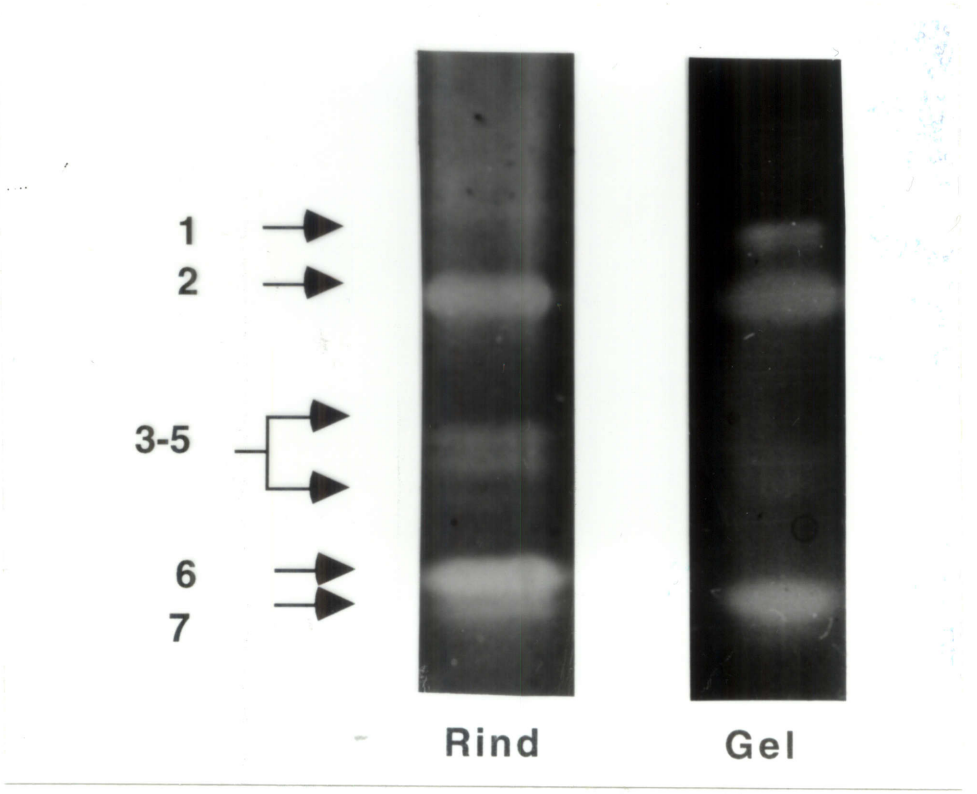

1
2
3-5
6
7
Rind
Gel

Figure 2. FPLC ion-exchange (Mono-Q) elution profiles in the final step of the purification of superoxide dismutases from *Aloe vera* rind and gel. **A**, **B** and **C** are the active SOD fractions.

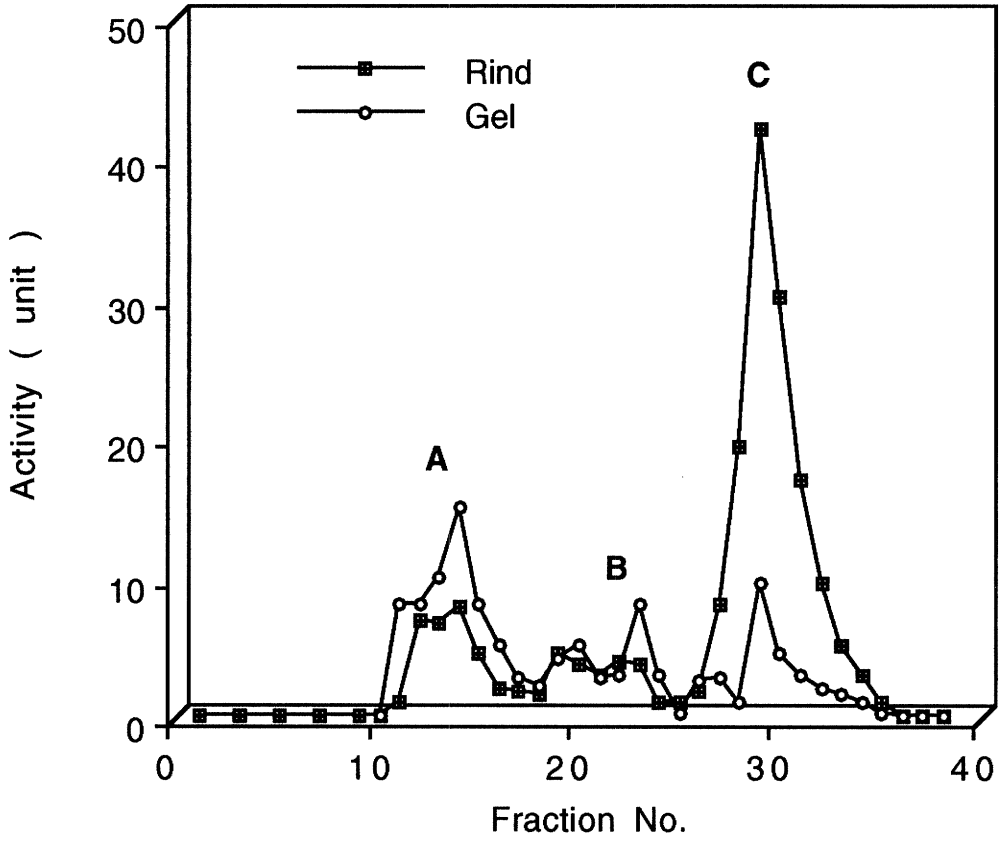
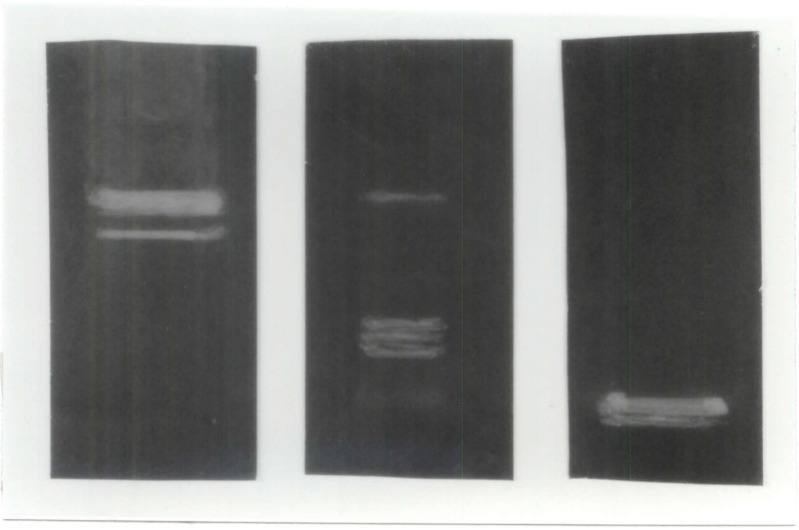
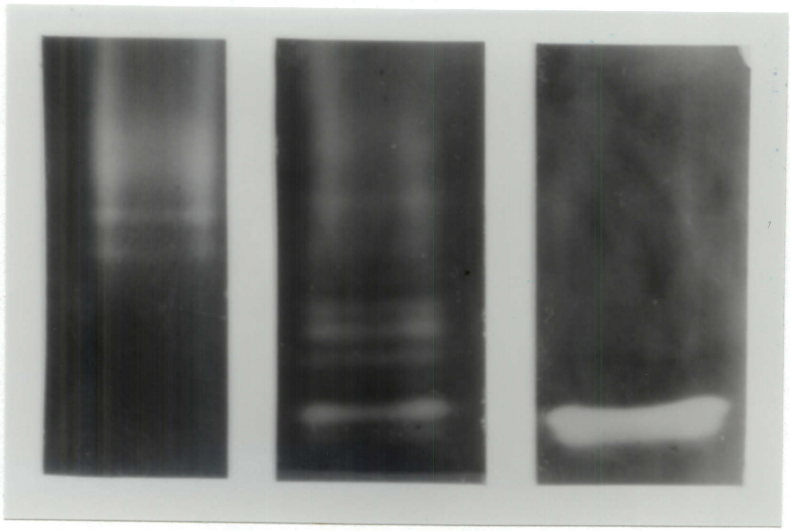

Rind
Gel
A
B
C
Activity (unit)
Fraction No.
0
10
20
30
40
50

Figure 3. Nitro blue tetrazolium staining of *Aloe vera* gel SOD Active fractions from Column II) after electrophoresis in polyacrylamide gel. A, B, and C correspond to active fractions **A**, **B**, and **C** respectively.



A B C

Figure 4. Nitro blue tetrazolium staining of *Aloe vera* rind SOD Active fractions from Column II) after electrophoresis in polyacrylamide gel. A, B, and C correspond to active fractions **A**, **B**, and **C** respectively.



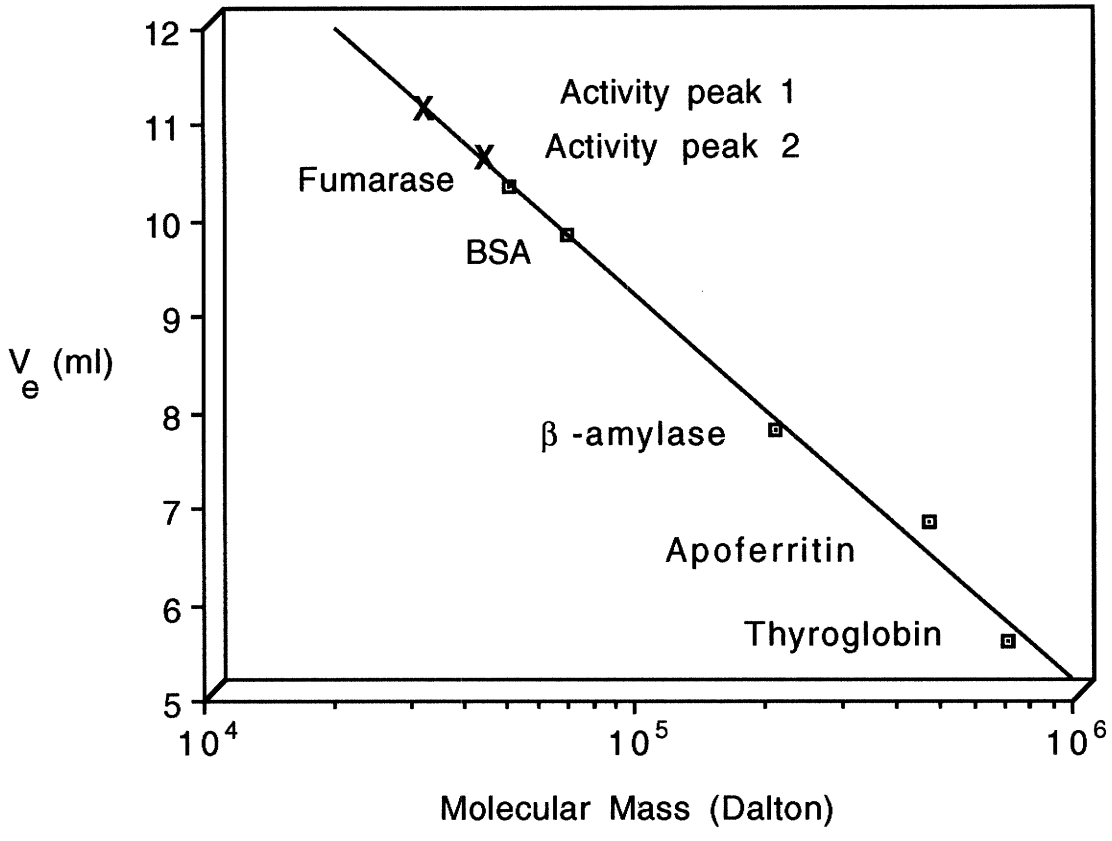
A B C

these two activities was cut from the native gel, the proteins removed by electro-elution and loaded onto SDS-PAGE. Upon electrophoresis (no BME), followed by silver staining, two bands appeared, having molecular weights of 32 and 42 KD.

SDS-PAGE (without BME) of the SODs from the active fractions **A** and **B** (rind and gel) gave three bands, after a prolonged period of silver staining, with approximate molecular weights of 42, 33 and 31 KD. SDS-PAGE (with BME) of the above fractions gave six bands with approximate molecular weights of 42, 33, 31, 21, 16 and 15 KD. In a parallel study, when the region corresponding to activity bands # 3, 4, and 5 (see Fig. 1) was cut from the native gel and the proteins removed by electro-elution, followed SDS-PAGE (no BME), and silver stained, one band appeared with a corresponding molecular weight of 31 kD. These results indicate that the three activity bands (3, 4 and 5) are isozymes having approximately the same molecular weights. The molecular weights of the proteins in the region corresponding to the activity bands # 1 and 2, when eluted and examined by SDS-PAGE (no BME) were found to have molecular weights of 42 kD (band 1) and 33 kD (band 2).

The seven SOD activities identified in *Aloe vera* gel and rind were examined with respect to the metal component of each, employing published methodologies (24, 39-40; see also the Experimental Section). These metalloenzymes, depending on the specific associated metal ion, are subject to inhibition by certain reagents. Cu/ZnSODs are cyanide-sensitive; MnSODs are cyanide-

Figure 5. Determination of the relative native molecular masses of *Aloe vera* SODs. Molecular exclusion chromatography on a column of Superose12 (Pharmacia).


12
11
10
9
8
7
6
5
V_e (ml)
Activity peak 1
Activity peak 2
Fumarase
BSA
β -amylase
Apoferritin
Thyroglobin
10^4
10^5
10^6
Molecular Mass (Dalton)

insensitive; while FeSODs are sensitive to hydrogen peroxide and sodium azide. This study was conducted on the active fractions from the Q-Sepharose column (Column I), for both the rind and the gel, and the results are summarized in Table III.

The same families of Cu/ZnSODs and MnSODs, but not FeSODs, were found in both the rind and the gel with apparently the same molecular weights and relative mobilities in nondenaturing PAGE (see Fig. 1 and also Table III). The native molecular weights of the *Aloe vera* Cu/ZnSOD isozymes, regardless of their localization (rind or gel), were found to be 31-33 kD (see Table III). As has been reported, all Cu/ZnSODs from different species are a remarkably conserved family, with respect to their gross structural properties. Without exception, the purified enzymes have been shown to be dimers with native molecular weights usually among 31 and 33 kD [41], The *Aloe vera* Cu/ZnSODs are thus similar in native molecular weight to all Cu/ZnSODs reported to date . The Mn/Fe SODs are generally dimeric; tetramers have also been identified in all Mn-containing enzymes from eukaryotes and in some bacteria [42]. The native molecular weights in all of the reported MnSODs vary from 37 to 90 kD. *Aloe vera* has two identifiable bands (in both the rind and the gel) that are cyanide- and H_2O_2 insensitive; their molecular weights are approximately 42 kD, which is comparable to the 40 kD MnSOD from red alga [43]. It has been reported that the plant MnSOD proteins are highly homologous to MnSODs from other organisms [44]. MnSODs are reported to be induced dramatically during stress

conditions. This induction is always accompanied by an increase in cytochrome oxidase activity, which suggests a specific protective role for MnSOD during conditions of increased mitochondrial respiration [44].

There are identifiable differences in the relative distribution of *Aloe vera* SOD isozymes in the rind and the gel. There is one MnSOD (band #1, Fig. 1) that is more abundant in the gel while the other MnSOD (band #7, Fig. 1) is more abundant in the rind. This could be due to the differential expression of this multigene family that is both spatially and developmentally regulated in other plants, such as maize [45]. The Cu/ZnSOD isozymes, overall, are more abundant in the rind; this might be due to the presence of chloroplasts and stroma in the green part of the leaves, where the SODs are present largely as the Cu/Zn enzyme forms [13-15]. Chloroplasts are especially affected by superoxide anion and other reactive oxygen species due to a high internal oxygen concentration, therefore they have multiple protective mechanisms against these species; SODs play a major role in the protective mechanism. The expression of specific SOD genes in plant chloroplasts has been shown to be dependent upon the location of the oxidative stress, and the type of stress conditions, such as light, photoinhibitory processes, and in the response to certain xenobiotics (e.g., herbicides) [46].

We have also found, in the present study, that there are differences in the relative abundances and tissue distributions of the five identifiable Cu/ZnSOD activities in *Aloe vera*. This might be due to the origin and organelle localization of these isozymes (from cytosol or from chloroplast). The expressions of the Cu/ZnSOD genes in other plants, such as tomato and Scots Pine, have been found to be dependent on their organ localization and the plant developmental stages. In non-photosynthetic organs, most of the Cu/ZnSOD activity is cytosolic, while in the expanded leaf the chloroplast contains most of the Cu/ZnSOD activity [47,14]. The relative abundances of the SODs of the rind and those of the gel are summarized in Table III.

The specific activities of SODs in the ethanol fractions from the *Aloe vera* rind and gel were compared to the specific activities of the ethanol fractions from spinach leaves, rabbit liver, rabbit heart, and lung. It was found that the specific activities of SODs from *Aloe vera* are comparable to those of spinach leaves and rabbit liver while *Aloe vera* specific activities are higher than the those found in rabbit heart and lung (see Table IV). The abundance of this enzyme (SOD) in *Aloe vera* , as in other plants, is important in combating the damaging effects of elevated oxygen concentrations, and to the increase of tolerance to the toxic effects of many xenobiotics that promote formation of active oxygen species [48]. This presence of significant activities of SODs in the *Aloe vera* plant, at least in part, might be operative in its reported therapeutic efficacy in the amelioration of a variety of inflammatory disorders.

Table III: SOD Specific Activity in *Aloe vera* Compared with Other Species (Ethanol Fractions):

Species	Specific Activity (U/mg of Protein)
Aloe vera Rind	118
Aloe vera Gel	112
Spinach	118
Rabbit Liver	145
Rabbit Lung	45
Rabbit Heart	54

Specific activities for all are calculated based on the total units of activities and total mg of proteins found in the ethanol fractions.

Table IV: Summary of the SOD Activities in the Rind and the Gel of *Aloe vera*.

Band Number	*Aloe* SOD (Metallo Family)	Mr.(Kd) (approx)	Relative Abundance	
			Rind	Gel
# 1	Mn	42	++	++++
# 2	Cu/Zn	33	++++	+++++
# 3	Cu/Zn	31	+++	+
# 4	Cu/Zn	31	+++	+
# 5	Cu/Zn	31	++	+
# 6	Cu/Zn	32	+++++	+++
# 7	Mn	42	+++	+

The relative abundance of each band was judged based on its appearance (intensity) on the native gel (Fig. 1) and on the elution profile (Fig. 2).

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