AN ABSTRACT OF THESIS OF

LIPING ZHEN for the degree of Master of Science in Horticulture presented on December 14, 1988.

Title: Hydrogen Cyanamide on Triphenyltetrazolium Chloride Reduction, Sulfhydryl Group Binding, and Catalase Activity in Bromegrass (Bromus inermis Leyss) Cells

Abstract approved:

Leslie H. Fuchigami

Hydrogen cyanamide (CY) has been used to break rest in temperate crops, but levels used to overcome rest are sometimes toxic to plants. The rest breaking effect of CY is thought to involve peroxide metabolism. Inhibition of catalase by CY has been proposed as a mechanism for overcoming dormancy in plants.

The objectives of this study were to determine effects of CY on: (1) the viability of bromegrass cells; (2) the reduction of SH groups from SH-containing compounds, and the concentration of SH groups in

bromegrass cells; and (3) the relationship between TTC reduction and the amount of SH groups; and (4) the mode of inhibition of cyanamide on both bovine liver catalase from Sigma and on catalase extract from bromegrass cells.

The 2,3,5-triphenyltetrazolium chloride (TTC) method was used to determine cell viability. The TTC test indicates that the effect of cyanamide on cell viability is both concentration and time dependent. Relatively low concentrations of CY (1 and 5 mM) and short exposure times increased the reduction of TTC above the level of the controls, but reduction decreased with increasing exposure or higher concentrations.

Sulfhydryl (SH) groups are known to function as an antioxidant in cells and, therefore, have been proposed to protect cells against physical and chemical stresses. Hydrogen cyanamide reduced the titratable SH groups of SH-containing compounds in <u>in vitro</u> assays and the effect on SH groups depended on the ratio of CY concentration and the concentration of SH-containing compounds: the rate of reduction of titratable SH groups was fastest in dithiothreitol (DTT) followed by glutathione (GSH) and lastly by cysteine.

Hydrogen cyanamide also stimulates the production of nonprotein SH compounds in bromegrass cells <u>in vivo</u>. The

effect of CY on levels of SH compounds is concentration and time dependent. The concentration of SH compounds increased following relatively short exposure time (4 hours) to low concentrations of CY and then decreased with additional time. The level of TTC reduction was positively correlated with the concentration of SH groups in bromegrass cells.

Hydrogen cyanamide inhibited catalase activity and catalase activity was restored to near the level of the untreated control after the removal of CY by dialysis suggesting that hydrogen cyanamide is a reversible inhibitor of catalase. Enzyme kinetic studies indicate that the inhibition of catalase by cyanamide is of the mixed-type inhibition.

Hydrogen Cyanamide on Triphenyltetrazolium Chloride Reduction, Sulfhydryl Group Binding, and Catalase Activity in Bromegrass (Bromus inermis Leyss) Cells

by

Liping Zhen

A THESIS

submitted to

Oregon State University

in partial fulfillment of

the requirements for the

degree of Master of Science

Completed December 14, 1988

Commencement June, 1989

APPROVED:

Professor of Horticulture in charge of major

Head of Department of Horticulture

Dean of Graduate School

Date thesis is presented: December 14, 1988

ACKNOWLEDGMENTS

I would like to express my sincere gratitude to Dr. Leslie H. Fuchigami, my major professor, and Dr. Tony H.H. Chen, committee member, for their understanding, guidance, patience, encouragement and constant help throughout the course of my study at Oregon State University.

Appreciation is also extended to Dr. Joe B. Zaerr, my minor adviser, and Dr. Alvin R.Mosley, the graduate school representative, for their guidance and assistance in completing my thesis.

I also thank Karen Tanino, Renee Harber, Debbie Jones and the other fellow graduate students in the Department of Horticulture for their unconditional friendship and invaluable assistance.

My deepest and heartfelt appreciation to my wife Xiangqing Xu and daughter Yin Zhen for their love and unflinching support which made this work possible and worthwhile.

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CHAPTER 1

INTRODUCTION

Hydrogen cyanamide is an effective dormancy-breaking agent in both seeds and buds (Lin 1985; Nee 1986; Snir 1983; Shulman et al 1983). A major problem in using cyanamide to break rest is the variety of results obtained. Hydrogen cyanamide may either stimulate, delay or have no effect on bud break, or cause bud and stem injury. Also the hydrogen cyanamide concentration to overcome rest depended on the plant growth stage (Nee 1986). Because of the unpredictability of knowing when and how much hydrogen cyanamide to use to break rest, commercial usage has been limited.

Improper usage of hydrogen cyanamide may injure plant tissue. Amberger (1984) found that high

concentrations of cyanamide caused chlorosis and necrosis of plants. Bracho (1984) reported that vines treated with 6% cyanamide generally reduced budbreak compared to the control. Spiegel-Roy (1987) reported that applying cyanamide to Vitis vinifera seeds immediately after harvest and following 90 days of chilling reduced germination percentages although either application of cyanamide or chilling alone could increase germination percentages. Nee (1986) found that phytotoxicity of hydrogen cyanamide depended on the plant growth stage.

Kitson and Crow (1979) reported that under physiological conditions cyanamide reacts with amino and thiol groups, forming guanidine and isothiouronium compounds, respectively. Hydrogen cyanamide was reported to bind with thiol groups of GSH and one of the compounds from the reaction of cyanamide and glutathione was identified as a glutathione-cyanamide conjugate (Nee 1986).

Hydrogen cyanamide was reported to inhibit catalase activity (Amberger 1961; 1963; Nir 1986; Nee 1986). They found that the decrease in catalase activity was related to the effectiveness of the rest-breaking chemicals. The inhibition of catalase by cyanamide has been proposed as a mechanism for breaking rest. Amberger (1984) reported that the inhibition of catalase by cyanamide is a

specific and reversible process. Shirota (1984) showed that cyanamide irreversibly inhibits catalase by covalently binding to the apoprotein of the enzyme in a process requiring hydrogen peroxide. In contrast, Guthrie (1941) reported that rest breaking agents can stimulate catalase activity.

Amberger (1984) reported that a sharp drop of the catalase activity caused by cyanamide was followed by a marked but probably unspecific increase in peroxidase activity. It was thought that the resulting accumulation of H_2O_2 led to a higher content of peroxidases and also of glutathione. This was thought to control the metabolism of rest either directly or indirectly through an increased activity of peroxidase or other more specific oxidases (e.g. ascorbate oxidase) which were closely connected with the glutathione redox system.

Based on these studies it appears that hydrogen cyanamide affects the oxidant detoxification systems in plants that may play a role in overcoming rest. The objectives of this study are to characterize the effect of hydrogen cyanamide on TTC reduction for estimating plant cell viability, SH group binding, and the mode of action of cyanamide inhibition of catalase.

CHAPTER 2

REVIEW OF LITERATURE

Metabolism of Hydrogen Cyanamide

Hydrogen cyanamide was found to quickly penetrate all above ground parts of plants and to move in both directions in the vascular system (Amberger 1984). C^{14}-hydrogen cyanamide was rapidly absorbed by the root system. Within 30 minutes, one, and two hours of exposure, the C^{14} label from cyanamide was found in the vessels of leaf blades, tips, and mesophyll cells, respectively (Amberger 1984).

High concentration of cyanamide may cause plant chlorosis and necrosis. At sublethal doses cyanamide is rapidly metabolized in plants (Amberger 1984). At low concentrations, or if plants treated by cyanamide were transferred into a cyanamide-free nutrient solution, the plants always showed a deeper green color than those growing in nutrient solutions containing the same amount of nitrogen as calcium nitrate (Amberger 1984). Barley seedlings treated with cyanamide-nitrogen had high dry matter, total nitrogen, protein-N, and amid-N contents. Similar results were obtained with maize (Miller 1963).

These results suggests that hydrogen cyanamide may be directly involved in nitrogen metabolism and protein production.

The degradation of cyanamide in cotton was found to involve the formation of urea and other compounds which are utilized in production of amino acids (Miller 1963). Oats, barley, wheat and rye treated with cyanamide showed higher contents of alanine, tryptophane, lysine, leucine, phenylalanine and valine compared to those treated with calcium nitrate (Amberger 1984). Amberger (1984) further showed that arginine content of plants treated with CY increased dramatically.

Hydrogen Cyanamide on Rest Breaking

The regulation of bud break in temperate crops is of great economic importance. In warm regions of the world where a prolonged period of low temperature is not available to satisfy the chilling requirement of most temperate crops, researchers and commercial interests have been searching for artificial means of breaking dormancy. Numerous chemical and physical treatments have been developed for overcoming rest (Doorenbos 1953). So far hydrogen cyanamide is one of the most effective rest-breaking chemicals. Snir (1983) used cyanamide for breaking rest of the red raspberry. Shulman et al (1983)

and McColl (1986) found that cyanamide overcame rest in grapevine buds and resulted in earlier and more uniform budbreak and earlier fruit set and ripening. Hydrogen cyanamide was also found to affect bud break of vegetative buds in Kyoho grapevine cuttings (Lin 1985). Budbreak induced by hydrogen cyanamide depended on the concentration used and the physiological status of the plant (Nee 1986).

The effect of cyanamide on overcoming dormancy also depends on the plant's genetic makeup. Different grape varieties respond differently to cyanamide treatment. Generally, however, hydrogen cyanamide has been shown to improve the rate and uniformity of bud burst in different varieties of grapevines in warm areas. In cooler climates, cyanamide hastened budbreak in `Cabernet Sauvignon' and `Chardonnay' vines but had no effect on `Zinfandel' (Foott 1987).

Hydrogen cyanamide was effective in overcoming rest in red-osier dogwood (Cornus sericea L.) and `Radiant' crabapple (Malus floribunda Sieb.) (Nee 1986). The concentration of hydrogen cyanamide required to overcome rest depended on the growth stage. Before rest development (160'GS), when dormant buds were correlatively inhibited, hydrogen cyanamide did not promote bud break and was phytotoxic at concentrations

greater than 1 M. At the onset of rest, 180'GS, all concentrations of hydrogen cyanamide (0.5-2.0 M) were effective in overcoming rest. Higher concentrations of hydrogen cyanamide were required to break rest as the intensity of the rest increased (from 200 'GS to 270'GS). During post-rest (280 to 310'GS), the concentrations of chemical required to stimulate bud release decreased progressively with later growth stages. During quiescence, after 315'GS, hydrogen cyanamide either inhibited bud growth or severely injured the quiescent buds and stems (Nee 1986).

In `Sultana' and `Cabernet Sauvignon' grapevines, growing at separate locations, application of 0.83 M cyanamide about 30 days before natural bud burst did not hasten bud burst. With `Cabernet Sauvignon' the percentage of bud break was significantly decreased by cyanamide treatment. Cyanamide application two weeks after pruning had no appreciable effects on bud break. Cyanamide caused the death of the primary shoot in 10 and 23% of buds in `Sultana' and `Cabernet Sauvignon', respectively, probably due to excessive concentration and late application (Whiting 1984).

Bracho (1984) reported that cyanamide treatment of `Cabernet Sauvignon' grapevines at 1.25 % resulted in 99

% total bud break, significantly higher than the control. There were no significant differences in total bud break among the 1.25, 2.5 and 4.0 % cyanamide treatments. The rate of 6 % cyanamide reduced budbreak below the level of the controls.

Hydrogen cyanamide can also hasten budburst in resting seeds. Seeds of four Vitis vinifera. L. cultivars treated with either hydrogen cyanamide or with chilling showed a higher germination percentage than the controls. When seeds of `Queen of Vineyards' were treated for 5 minutes in a 2 % cyanamide solution, immediately after harvest, the percent of budbreak was equivalent to that of seeds exposed to a 90-day chill period. In the variety `Oz', treating the seeds with cyanamide concentrations between 0.5 or 2.0% immediately after harvest was proved significantly better in hastening budburst than chilling the seed for either 45 or 90 days. The combination of chilling the seeds for 45 days followed by cyanamide treatment resulted in a germination percentage significantly higher than any other treatment. For the medium-late cultivars, `Ribier' and `Dabouki', cyanamide application immediately after harvest resulted in germination percentages statistically similar to those obtained after 45 or 90 days of chilling (Spiegel-Roy 1987).

Hydrogen cyanamide stimulates ethylene production in dormant plants. Ethylene production and the stimulation of bud release during the rest period, 180 to 315'GS, were found to be highly correlated (Nee 1986). Cellular damage caused by hydrogen cyanamide failed to stimulate ethane production. Alfalfa cell cultures treated with hydrogen cyanamide could be induced to produce ethane by either removing the hydrogen cyanamide from the media or treating the cultures with glutathione (Nee 1986).

The increase in ethylene and prevention of ethane production in tissue exposed to sublethal dosages of hydrogen cyanamide may be due to an alteration in the cell membranes. This may account for the observed increase in electrolyte leakage (Nee 1986). Some researchers have also noted that an increase in membrane permeability may cause rest breaking (Doorenbos 1953). Nee's (1986) studies suggest that hydrogen cyanamide may increase membrane permeability without causing cellular death. Treatment of dormant `Radiant' crabapple plants with increasing concentrations of hydrogen cyanamide resulted in a progressive increase in the leakage of several readily mobile elements (K, Mg, Mn, Na, P, S, and Zn) (Nee 1986). Nee's (1986) study confirmed the findings of others (Doorenbos 1953) that a possible mechanism for

overcoming rest involves an increase in membrane permeability.

Hydrogen cyanamide was found to inhibit catalase activity (Amberger 1984; Nir 1986; Nee 1986). The decrease in catalase activity has been related to the effectiveness of the rest breaking chemicals such as hydrogen cyanamide, thiourea, hydroxylamine, nitrate and nitrite in seeds and plants (Hendricks 1975). Amberger (1984) found that the inhibition of catalase depends on the concentration of cyanamide (more than 10^{-3} M). Inhibition of beef liver catalase, after preincubation with 10^{-1} M cyanamide, was restored up to 90 % of its original activity by the addition of CaNO3 to the solution. This showed the inhibition of catalase by cyanamide to be reversible (Amberger 1984). In sugar beet seedlings grown in the same cyanamide concentration for 20 hours, catalase activity decreased to 30 % of the control; however, 8 days after they were transferred into a cyanamide free solution catalase activity increased to near normal level. Similar results were obtained with maize (Amberger 1984). In contrast, Shirota (1984) showed that cyanamide irreversibly inhibits catalase by covalently binding to the apoprotein of the enzyme in a process requiring hydrogen peroxide. Nee (1986) reported that in plants 3-amino-1,2,4-triazole (3-AT) was

considerably more effective than cyanamide in inhibiting catalase activity, suggesting that in plants the inhibition of catalase activity by cyanamide does not involve a complete blockage as with 3-AT.

Catalase activity in grapevine buds increased markedly in autumn, reaching a maximum at the end of October, then decreased, reaching the minimum in January (Nir 1986). Kaminski (1974) also reported catalase activity to be lowest at the end of rest. The change in catalase activity and the intensity of dormancy began to decrease parallel to the drop in temperature (Nir 1986).

Kitson and Crow (1979) reported that under physiological conditions cyanamide reacts with amino and thiol groups, forming guanidine and isothiouronium compounds, respectively. Hydrogen cyanamide was reported to bind with thiol groups of GSH in a cell free, in vitro, system and in germinating mung bean seeds (Nee 1986). With increasing concentrations of hydrogen cyanamide, the level of thiol groups decreased after 48 hours of incubation, indicating that cyanamide binds with free thiol groups (Nee 1986). The conjugation of hydrogen cyanamide with the thiol group in GSH was confirmed by HPLC, NMR and GC-MASS spectrometry. The conjugation appears to occur between the thiol group of

GSH and the carbon end of cyanamide (Nee 1986).

Occurrence of the SH Groups

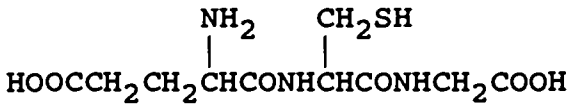
The sulfhydryl or thiol (SH) group participates in many biochemical processes in both plants and animals. In animals, free SH groups occur in plasma and other sites as so-called non-protein SH groups (NPSH), mainly in the form of reduced glutathione (GSH), and in many protein sulfhydryl groups (PSH) associated with structural proteins, enzymes, nucleoproteins, proteins associated with visual pigments, and others.

Water-soluble, non-protein sulfhydryl groups occur as glutathione and cysteine in plants. Glutathione was the predominant free sulfhydryl in water extracts of soybean leaves, constituting 95% of water-soluble, non-protein sulfhydryl. Cysteine was present in smaller amounts (Chiment 1986). Nieto-Sotelo (1986) reported one of the major thiols of maize roots to be GSH, which is present at least four times of amount greater than cysteine on either a fresh or dry weight basis. Grill (1979) reported that the predominant low molecular weight thiol in spruce needles is GSH which usually comprises more than 95% of the total nonprotein sulfhydryl compounds. Depending on the needle age, GSH concentration may range from 0.07 to 0.70 u mole/g fresh weight. Cysteine was found to be

present in small quantities with a concentration below 0.01 u mole/g fresh weight, except in April when cysteine rose up to 0.125 u mole for a short period (Esterbauer 1978).

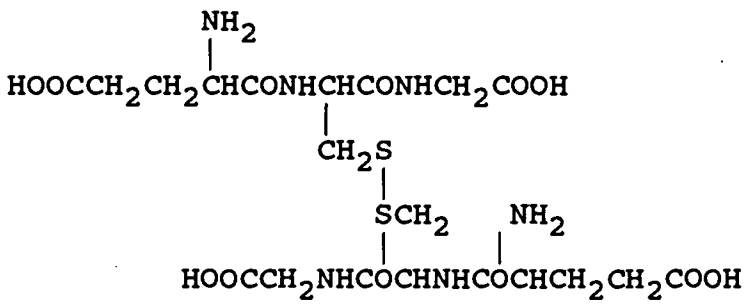
The Properties and Roles of Glutathione

Glutathione (GSH) is a tripeptide with the sequence glu-cys-gly, as shown in the following formula:



(GSH)

The disulfide derived from GSH by oxidation of the thiol group of cysteine residue is usually denoted as GSSG as shown by the following structure (Kosower 1976):



(GSSG)

GSH has been found in almost all eucaryotic cells studied (Meister 1983), except those of the apple fruit (Schroeder 1939) and the pathogen Entamoeba histolytica (Fahey 1984) which are reported to lack GSH entirely.

Synthetic pathways for GSH are well understood in mammals, but less in plants (Meister 1983). Synthesis occurs in the chloroplast of green plants as well as in the mitochondria and cytosol (Giovanelli 1980). Concentrations of 20 uM, 60 uM and 3.5 mM have been reported in the vacuole, cytosol, and chloroplast, respectively (Foyer 1976).

During sulfur transport and storage in plants the reduction of GSH increases with increasing levels of sulfate. Under these conditions the cysteine concentration in different compartments of the cell may still be maintained at a low level by the incorporation of excess cysteine into glutathione. Glutathione appears to be the storage form of reduced sulfur in higher plants (Rennenberg 1982).

An important finding is that cellular turnover of GSH is associated with its transport, in the form of GSH, out of cells. The functions of such GSH transport include the formation, by membrane-bound γ-glutamyl transpeptidase, of γ-glutamyl amino acids, which can be transported into certain cells, and thus serve as one mechanism of amino acid transport (Meister 1983). Transported GSH probably also functions in reductive reactions that may involve the cell membrane and the

immediate environment of the cell (Meister 1983).

Glutathione has long been known to protect plants against environmental stresses. Grill (1973) found that the total content of SH groups was fluctuated seasonally in healthy and damaged Pine needles. SO_2-damaged needles always contain more SH-groups than healthy needles except immediately after flushing. GSH and total protein SH and structural protein SH increased in needles of Picea abies growing in SO2-polluted areas (Grill 1980).

In the mung bean study GSH significantly reduced the electrolyte leakage caused by hydrogen cyanamide, thus suggesting that GSH reduced the injury caused by cyanamide (Nee 1986). In another study, when plants were pretreated with dithiothreitol (DTT) four hours before cyanamide treatment, the rate of budbreak was significantly reduced and similar to the DTT control treatment. In contrast, application of hydrogen cyanamide either without DTT or four hours before DTT treatment stimulated bud break. These studies suggest that DTT pretreatment "protected" the plant from cyanamide possibly by inactivating cyanamide via thiol-cyanamide binding. Treatment with DTT four hours after cyanamide treatment was ineffective indicating that the hydrogen cyanamide penetrates and translocates rapidly in dormant plant tissues, thus, binding with the natural thiol

groups before DTT can have an effect (Nee,1986).

Pretreatment with abscisic acid (ABA) caused a decrease in chilling injury of cotton cotyledonary tissue. ABA was found to maintain the level of GSH and of membranous phospholipids and reduce tissue leakiness and necrosis. After two days of chilling, pretreatment with 10^{-5} M ABA maintained GSH levels as before while GSH content of controls without ABA decreased by about 50% (Rikin 1979). Li (1986) found that pretreating illuminated rice seedlings with ABA prevents the decrease of GSH and increases the ratio of GSH/GSSG. The ABA pretreatment under light illumination also increased the survival of the rice seedlings following chilling stress.

Thiols may also play a significant role in the response of plants to heat-shock (Nieto-Sotelo 1986). Treatment of maize roots at 40'C resulted in a decrease in cysteine and an increase in GSH levels. Pulse labeling of maize with [^{35}S] cysteine showed that the rate of incorporation of ^{35}S into GSH-GSSG in heat-shocked tissue was twice that in non-heat-shocked tissue. During heat-shock GSH synthesizing capacity increases. The elevated synthesis of GSH may be related to the cell's capacity to cope with heat stress conditions (Nieto-Sotelo 1986).

Levels of GSH were found to vary depending on the

time of day. A maximum GSH level of about 400 n mole/g FW in tomato seedlings was found during mid day, fell to about 200 n mole/g FW at night and rose again after mid night. The level of GSSG also seems to vary. Maximum levels of GSSG was found when the levels of GSH were decreasing (Koike 1988).

Remarkable differences in the GSH content were found in needles of Picea abies collected at different times of the year (Esterbauer 1978). The needles had four to seven times higher GSH values in February than in August. GSH shows a characteristic seasonal rhythm with maximum concentration in winter and minimum in summer. Like GSH, the glutathione reductase (GR) activity showed an annual rhythm with a distinct maximum in winter and minimum in summer. GSH and GR begin to rise and fall at the same time each year (Esterbauer 1978).

In plants, Esterbauer (1978) and Guy (1982) reported that the GSH:GSSG ratio changes during the rest period. In Neurospora crassa, the glutathione thiol-disulfide status was found to be related to the dormancy status of the conidia (Fahey 1975). Oxidized (GSSG) and protein-bound glutathione (PSSG) content increased dramatically with dormancy. The GSH-GSSG ratio in freshly harvested dry conidia was about 150 but decreased to around 6 when dry conidia were aged (stored) for 10 days after harvest,

primarily through changes in GSSG levels, whereas GSH levels varied by a factor of only about two. The level of PSSG was found to generally parallel that of GSSG. When conidia were germinated, the ratio of GSH-GSSG increased to about 300 during the first 10 minutes of the 6 hour germination process. It was postulated that glutathione thiol-disulfide reaction is a mechanism for controlling dormancy and that the GSSG levels play a role in the regulation of protein synthesis (Fahey 1975). Dry embryos contained about 0.6 uM/g dw each of GSSG and protein-bound glutathione (PSSG) and these levels declined 5- to 10-fold within minutes after the onset of imbibition. After 90 minutes GSH declined from about 8 to 2 uM/g dw. Similar changes occurred when embryos were hydrated by storage at 100 % relative humidity (Fahey 1980).

Exposing red-osier dogwood (Cornus sericea L.) to low temperature (5'C) caused an increase of 550 % in GSH and 109 % in GSSG (Guy 1984). The ratio of GSH to GSSG was also influenced by low temperature treatment. Higher GR activity in cold-hardened tissue is largely responsible for the elevated GSH/GSSG ratio (Guy 1984). The accumulation of GSH at low temperature in dogwood cortical tissue appears to result from the reduced demand for sulfur-containing metabolites due to growth cessation

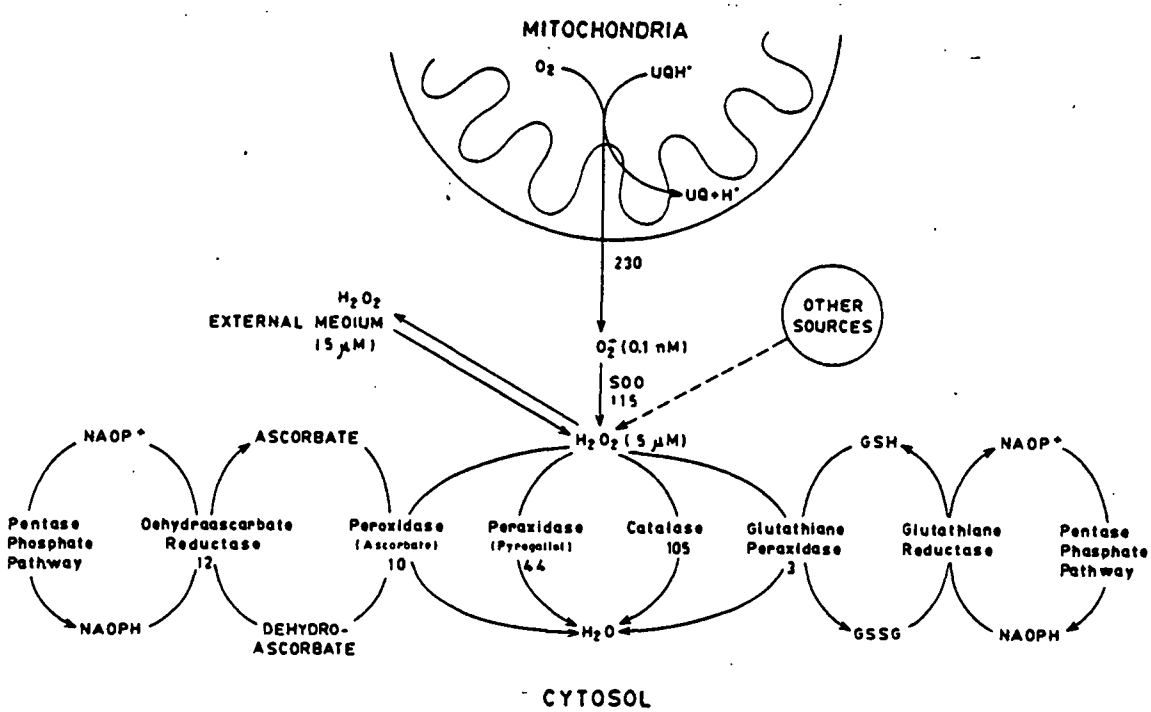
during cold acclimation. In this respect, GSH may not be associated with freezing tolerance in dogwood or citrus but may function as a storage form of reduced sulfur (Guy 1984). Glutathione content of spinach leaf discs increased up to four-fold when they were incubated with sulphate (10-100 mM) for 20 hours in light or darkness. This increased GSH content did not result in higher frost-tolerance of the spinach leaf discs (de Kok 1981). Increased levels of GSH and GR activity were found in spinach leaves upon low temperature hardening but GR activity was not involved in adaptation of spinach to saline conditions (de Kok 1983).

The Functions of Catalase and Peroxidase

Activated forms of O_2 produced by its incomplete reduction, such as the superoxide anion radical ($O_2^{\cdot-}$) and H_2O_2, are toxic to all living materials. Superoxide, $O_2^{\cdot-}$, is not directly reactive with most organic compounds, but it probably gives rise to more reactive oxygen species of higher potential toxicity. Hydrogen peroxide, like $O_2^{\cdot-}$, is not particularly reactive with most biologically important molecules; however, it is probably an intracellular precursor for more reactive oxidants such as HO' (Larson 1988).

One group of enzymes, the superoxide dismutases

(SOD), catalases and peroxidases, may act as an enzymic oxidant detoxification system to avoid excess formation of superoxide in living cells: SOD catalyzes the dismutation of O_2^- to H_2O_2 and O_2; catalase mediates the cleavage of H_2O_2 evolving O_2; and peroxidase reduces H_2O_2 to H_2O using several available reductants (Fridovich 1978). The following diagram integrates the various metabolic pathways related to the production and utilization of hydrogen peroxide (Puntarulo 1988).



Catalase activity has been shown to affect GSH content in plants (Smith 1984; 1985). Total GSH content of leaves of the catalase-deficient mutant barley increased 5- and 10-fold and similar results were obtained when wild-type barley leaves were treated with aminotriazole (3-AT), a catalase inhibitor (Smith 1984). Glutathione levels were increased in tobacco and soybean treated with 3-AT and thiosemicarbazide, catalase inhibitors (Smith 1985). Amberger (1984) also reported an increase in GSH levels due to the inhibition of catalase by hydrogen cyanamide, which resulted in an increase in hydrogen peroxide. Wendel (1979) reported that the level of GSH was important for lipid peroxidation and low levels of GSH promoted peroxidation.

The activity of catalase has been associated with stress resistance. Chilling injury, characteristic of tropical and subtropical plants, to cucumber plants exposed to 96 hours at 5'C and 85 % relative humidity caused a decline in catalase activity. When the plants were transferred to the control temperature (25'C), catalase activity was restored to its normal levels within 48 hours (Omran, 1980). An inhibitor of catalase activity was found in chilling-sensitive species (Patterson 1984). But peroxidase activity was not

affected by chilling (Omran 1980).

Catalase activity of 3-day-old cress seedlings decreased immediately after high oxygen pressure treatment. The decrease slowed to 6 hours and then rapidly decreased between 6 and 9 hours. No activity could be detected after 15 hours. In seedlings treated for 6 hours there was, after removal from oxygen, a time lag of about 24 hours before damage was apparent, and eventually most seedlings died. Seedlings treated for 9 hours collapsed soon after removal from the high pressure oxygen (Pritchard 1967).

Peroxidases are widely distributed among higher plants. Peroxidase has also been located histochemically in the plant cell wall (De Jong 1967). Peroxidases have been found in animals, fungi, bacteria and algae (Saunders 1964). The physiological function of plant peroxidases may be involved in indole-3-acetic acid (IAA) oxidation, malonate synthesis and lignin synthesis (Darusamin 1985).

Purified horse radish peroxidase activity increased slightly when the enzyme was reacted with cyanamide. In sugar beets a sharp drop in catalase activity caused by cyanamide was followed by a marked but probably unspecific increase in peroxidase activity in leaves and

roots combined with gradual wilting and protein degradation (Amberger 1984).

CHAPTER 3

HYDROGEN CYANAMIDE ON SULFHYDRYL GROUPS AND THE REDUCTION OF TRIPHENYL TETRAZOLIUM CHLORIDE

Abstract

The effect of cyanamide (CY) on 2,3,5-Triphenyl tetrazolium chloride (TTC) reduction and levels of sulfhydryl (SH) groups of bromegrass (Bromus inermis L.) cells was studied. Cyanamide increased TTC reduction greater than control levels in bromegrass cells for up to 10 hours of incubation, but decreased reduction with additional incubation. Maximum reduction of TTC occurred in cells incubated for 4 hours with 1.0 mM CY. The level of SH group also depended on exposure time and CY concentration. SH levels increased to a peak at 8 hours and decreased thereafter. The maximum level of SH group occurred at 8 hr. incubation with 1.0 mM CY. A positive correlation (r^2=.725) was found between SH group levels and TTC reduction. The relationship between SH group and TTC reduction is discussed.

To test the effect of CY on SH groups, three SH -

containing compounds [glutathione (GSH), cysteine (Cys), and dithiothreitol (DTT)] were treated with CY. The concentration of titratable SH groups of all three SH-containing compounds decreased in the presence of CY. Dithiothreitol was most reactive followed by glutathione and lastly by cysteine. This study confirms earlier reports that CY binds with SH group.

Additional index words: dormancy breaking, glutathione, cysteine, dithiothreitol, Bromus inermis L. cell cultures, rest.

Introduction

Hydrogen cyanamide has been used as a dormancy-breaking agent in many plant species (Spiegel-Roy 1987; Nee 1986). Depending on the growth stage of plants, however, the levels of CY used to overcome rest can either have no effect, enhance or delay bud break, or injure buds and stems. When and how much CY can be used to obtain optimum results is still unknown.

Sulfhydryl-containing compounds, such as glutathione (GSH), are proposed to function in sulfur transport and metabolism (Rennenberg 1982; 1984), amino acid transport

(Meister 1983), cellular antioxidant systems (Giovanelli 1980) and stabilization of enzyme systems (Higgins 1982). Glutathione has long been known to protect plants (Dass 1968; Dugger 1966) and animals (Fairchild 1959) against ozone and nitrogen dioxide injury. It has been suggested that GSH protects proteins by preventing the accumulation of H_2O_2 and free radicals within subcellular compartments (Meister 1981). Grill (1979) found one of the main functions of GSH in plants to be to protect the -SH groups in enzymes and structural proteins against oxidation either by acting as a scavenger for oxidizing substances or by repairing the -SH groups via the GSH-disulfide exchange reaction. Glutathione in conjunction with glutathione reductase is thought to play a major role in maintaining cellular redox potential (Rennenberg 1982; Halliwell 1981) and Smith (1984) hypothesized that GSH is involved in the reductive detoxification of hydrogen peroxide.

Koike (1988) reported that the levels of GSH varied depending on the time of day. Esterbauer (1978) reported that GSH showed a characteristic seasonal rhythm with maximum concentration in winter and minimum in summer. Guy (1984) showed that glutathione accumulates in plant tissues exposed to low temperatures but that GSH accumulation is not associated with freezing tolerance.

Hydrogen cyanamide was reported to bind with thiol groups of GSH in a cell-free, in vitro, system and in germinating mung bean seedlings (Nee 1986). With increasing concentrations of hydrogen cyanamide, the level of thiol groups decreased after 48 hours of incubation, indicating that cyanamide binds with free thiol groups (Nee 1986). The conjugation of hydrogen cyanamide with the thiol group in GSH was confirmed by HPLC, NMR and GC-MASS spectrometry. The conjugation appears to occur between the thiol group of GSH and the carbon end of cyanamide (Nee 1986).

The objectives of this study were to study the effect of cyanamide on the reduction of TTC (cell viability) and SH groups in bromegrass cells and the binding of SH-containing compounds.

Materials and Methods

Bromegrass cells were cultured in Ericksson's medium amended with B_5 micronutrients and vitamins, and supplemented with 0.5 mg/1 2,4-D as described by Chen and Gusta (1983). Three ml pack cell volume (PCV) of cells was transferred to 50 ml of medium in 250 ml erlenmeyer flasks and cultured for 4 days on a rotary shaker at 100 rpm at 25±2'C in the dark. Hydrogen cyanamide solutions

were filter-sterilized by passing through an 0.2 micro filter (Acrodisc 13, Gelman Sciences) and then added to the culture medium, so that the final concentrations of CY in the media were 1, 5, 10, 20, 30, and 100 mM, respectively. The pH values of the treated and control media were about 5.5. The cells were incubated in the control and CY-treated media for either 2, 4, 8, 24, 48 or 72 hours on a rotary shaker at 100 rpm in the dark at 25±2'C. The cell viability was determined by TTC reduction. The medium was discarded and cells were collected and incubated with 1 ml TTC solution (0.08% TTC in 0.05 M sodium phosphate buffer, pH=7.5) at 25'C in the dark for 24 hr. The TTC solution was removed; 3 ml of 95 % ethanol was added to the cells; incubated for 24 hours at 25'C in the dark; and the amount of reduced TTC was determined at 490 nm with a Bio-Tek Instrument Microplate Reader (EL309 Autoreader).

The method of Chiment (1986) was modified to determine concentrations of SH group. Three ml PCV of cells was collected and homogenized (3 times at 30 seconds each) in 3 ml of 0.02 M ethylenediaminetetraacetic acid-disodium (EDTA-Na2) solution (pH=4.8) with a Tissuemizer (Tekman, SDT-1810) set at 80 . The homogenate was added to 4 ml of 10 % trichloroacetic acid for 15 min. at 4'C and centrifuged

at 15,000 rpm (Beckman J-20 rotor) for 20 min. at 4°C. Four ml of 0.4 M Tris buffer containing 0.02 M EDTA-Na2 (pH=8.9) was added to 1 ml of the clear supernatant and absorbance was measured against a blank (reagents without the cell sample) at 412 nm on a Shimadzu 160 UV-vis spectrophotometer. Following the initial measurement, 0.1 ml of 10 mM 5,5'-dithiobis-(2-nitro-benzoic acid) (DTNB) in Tris, EDTA-Na2 buffer (pH=8.9), was then added and absorbance read again. The change in absorbance with the addition of DTNB was used to determine the amount of free sulfhydryl group present, expressed as n mole of water soluble, non-protein, free-SH per ml of PCV, when compared with a glutathione standard curve.

In order to determine the direct effect of CY on SH groups, three SH-containing compounds, GSH, Cys, and DTT (all from Sigma) were incubated with 1, 10, and 100 mM CY for 0, 4, 8, 24, 48, 72 and 96 hours, respectively. The amount of SH groups was determined by the method described above.

Regression analysis at the 1% level and standard deviation were used to analyze data.

Results

TTC reduction of the control cells was maintained at the same level throughout the incubation period (Fig.3.1). In cells treated with CY, the reduction of TTC depended on the CY concentration and incubation time (Fig.3.1). With 1 mM cyanamide, TTC reduction first increased by 2, 14, 10, and 4 % after 2, 4, 6, and 8 hour incubation, respectively, then decreased by 20, 53, and 67 % after 24, 48 and 72 hours of incubation, respectively. Similar results were obtained with the 5 mM CY treatment. At greater than 10 mM CY, however, there was no enhancement of TTC reduction throughout the incubation period.

Figure 3.2 illustrates the effect of cyanamide concentrations on TTC reduction at 3 incubation times. As above, TTC reduction depended on cyanamide concentration and incubation time. TTC reduction was enhanced at low cyanamide levels at 4 and 8 hours of incubation. As the concentration increased or at longer (24 hour) incubation TTC reduction decreased.

From these data the relationship of cyanamide concentration and incubation time on the LD_{50} of bromegrass cells was determined (curve B, Figure 3.3). This figure also illustrates the concentration of

cyanamide and exposure time which cause the enhancement of TTC reduction (below the area of curve A, Fig.3.3).

In bromegrass cells the effect of cyanamide on SH group also depended on CY concentration and incubation time (Fig.3.4). The SH group of the untreated sample did not change throughout the treatment period. At 1 and 5 mM cyanamide the level of SH group increased dramatically with up to 8 hours of incubation, then slowly decreased with longer incubation. In the 1 mM cyanamide treatment the SH level was greater than the control level throughout the treatment period. At 5 mM, the SH group level dipped below the control level only after 55 hours of incubation in cyanamide. Ten mM cyanamide caused a sudden decrease followed by a slight recovery of SH level, but levels remained lower than the control. These results were further verified by treating the cells with CY concentrations at two exposure times (Fig.3.5).
In cell free systems, cyanamide reduced the DTNB titratable SH groups and the effect on SH groups depended on the ratio of CY concentration and the concentration of SH-containing compounds (Fig.3.6-3.8). With the 100 mM cyanamide treatment, SH groups of the 1 mM (Fig.3.6), 10 mM (Fig.3.7), and 100 mM GSH (Fig.3.8) were reduced by 55, 27 and 0%, respectively.

The extent of binding of cyanamide to SH groups also

depended on the SH-containing compounds. DTT, with two SH groups per molecule, bound faster to cyanamide (Fig.3.9) than either GSH (Fig.3.7) or cysteine (Fig.3.10), both of which contain only one SH group per molecule.

Comparison of the TTC reduction with the SH levels following cyanamide treatment suggests a close relationship between the two (Fig.3.11 and 3.12). A positive correlation (r^2=0.725) was found between TTC reduction and SH groups (Fig.3.13). In every instance, the occurrence of the TTC reduction was slightly ahead but nearly parallel to the change in SH content.

Discussion

TTC reduction has been widely used to quantify the degree of freezing injury of plant cells (Stushnoff 1972; Chen 1983). The TTC viability assay is thought to be a good indicator of killing temperature in non-ABA- treated bromegrass cell suspension cultures (Harber 1988). However, the TTC method greatly overestimated the killing temperature of ABA-hardened cells because of the enhancement in TTC reduction when ABA-hardened cells were exposed to mild freezing temperatures. A linear regression model was therefore developed, based on a

comparison of cell viability by the regrowth test to the TTC test results, to predict the true killing temperature of ABA-hardened cells from TTC data (Harber 1988).

Similar to the above finding, bromegrass cells exposed to low concentrations of CY and short incubation periods also enhanced TTC reduction. The explanation for this is not known. However, since the reduction of TTC depends on NADPH, it is likely that CY at non-lethal concentrations may stimulate production of NADPH, possibly through the pentose phosphate pathway system. Nir (1986) hypothesized that CY overcame rest by inhibiting catalase activity, causing H_2O_2 to increase and activating the pentose phosphate pathway. The resulting accumulation of H_2O_2 led to a higher content of glutathione, which in turn may control the metabolism of dormancy either directly or indirectly through an increased activity of peroxidase or other more specific oxidases or ascorbate oxidase closely associated with the glutathione redox system (Amberger 1984). Although speculative, the increased NADPH could also explain the parallel increase in SH content observed, following non-injurious dosages of CY. The pool size of GSH is about 10-fold greater than that of NADPH and the GSH pool has a major influence upon the status of the NADPH pool (Reed

1986). The reduction of GSSG to yield GSH via glutathione reductase depends on NADPH. The slight delay in SH production as compared to TTC reduction observed may be due to transfer of NADPH from the pentose phosphate pathway in cytosol to other parts of the cell such as mitochondria and the additional reaction of glutathione reductase converting GSSG and NADPH to GSH and NADP.

Glutathione and cysteine are the major non-protein sulfhydryl groups in plants. Of these two compounds, glutathione constituted about 95 % of the water-soluble, non-protein sulfhydryl group in soybean and Picea (Chiment 1986; Grill 1979). The level and ratio of reduced (GSH) and oxidized (GSSG) glutathione change during the day (Koike 1988) and year (Esterbauer 1978) in response to environmental stresses. During heat shock GSH synthesizing capacity increases and the elevated GSH is thought to increase the cell capacity to cope with heat stress (Nieto-Sotelo 1986). Grill (1973) reported that SO_2-exposed spruce needles always contained higher SH content than healthy needles. Rikin (1979) found that ABA pretreatment (10^{-5} M ABA for 3-48 hr.) maintained GSH level, and greatly decreased the chilling injury in cotton cotyledonary tissue.

In plants, Esterbauer (1978) and Guy (1982) reported

that the GSH:GSSG ratio changes during the rest period. Fahey (1975) found that the glutathione thiol-disulfide status was related to the dormancy status of the conidia of Neurospora. Oxidized (GSSG) and protein-bound glutathione (PSSG) content increased dramatically with dormancy. The GSH:GSSG ratio in freshly harvested dry conidia was about 150 but decreased to around 6 when dry conidia were aged (stored) for 10 days after harvest, primarily through changes in GSSG levels whereas GSH levels varied by a factor of about two only. The level of PSSG was found to generally parallel that of GSSG. When conidia were germinated, the ratio of GSH-GSSG increased to about 300 during the first 10 minutes of the 6 hour germination process (Fahey 1975). Guthrie (1941) reported that GSH alone can overcome rest in many temperate crops. Fuchigami and Nee (1987) hypothesized that rest breaking agents overcome rest by altering the glutathione thiol-disulfide status in resting buds.

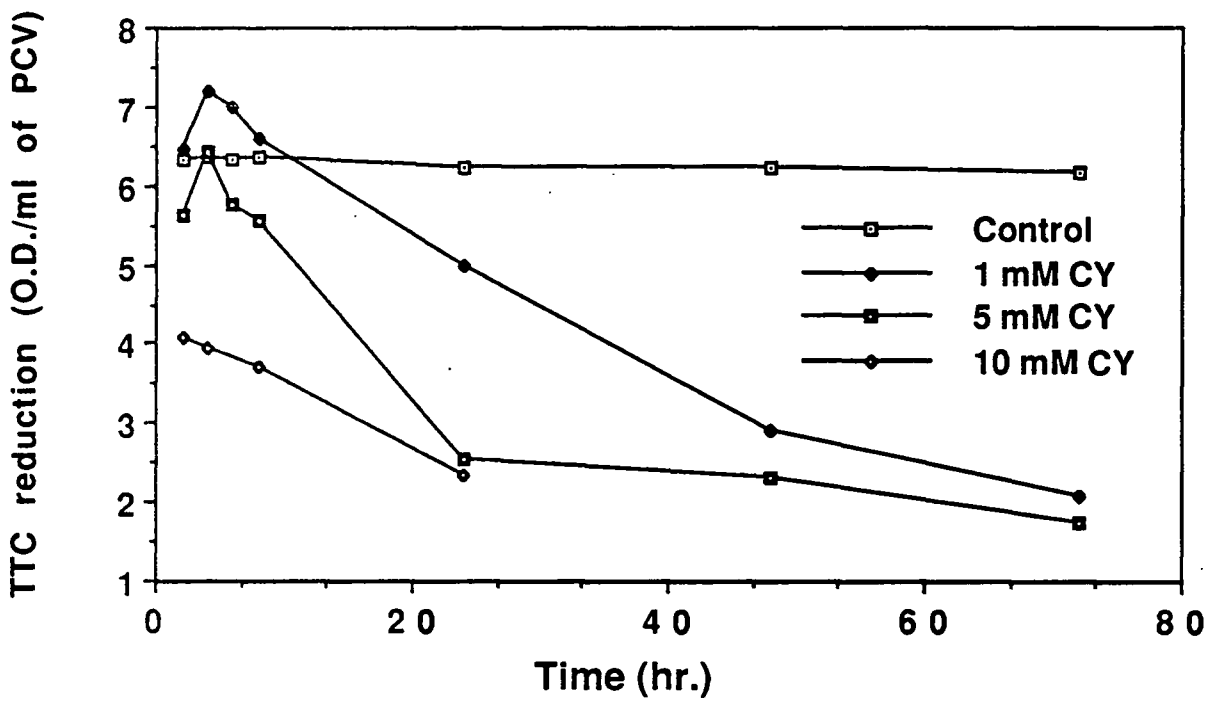


Fig.3.1. The effect of four cyanamide concentrations and incubation period on TTC reduction (TTC: triphenyltetrazolium chloride CY: hydrogen cyanamide)

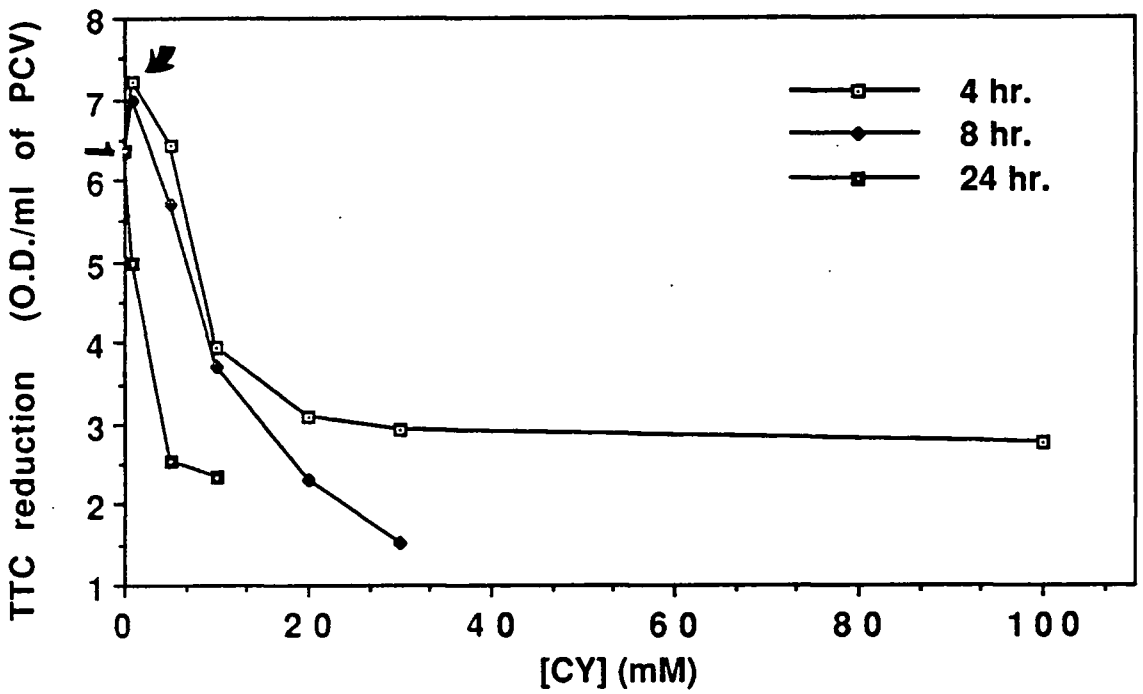


Fig.3.2. The effect of cyanamide on TTC reduction at three incubation periods. -➧ indicates TTC reduction of controls and => indicates TTC reduction at 1.0 mM cyanamide.
(TTC: triphenyltetrazolium chloride
CY: hydrogen cyanamide)

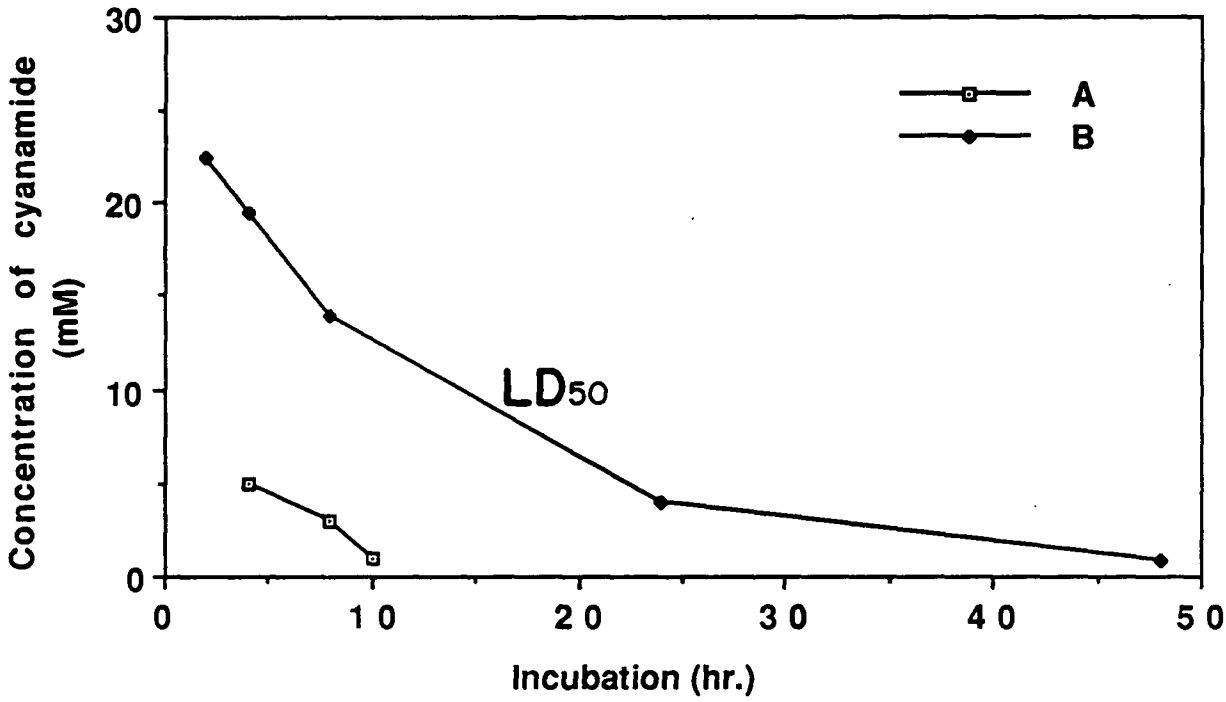


Fig.3.3. The effect of cyanamide concentration and incubation period on TTC reduction. Curve A indicates the same level of TTC reduction compared with control. Curve B indicates

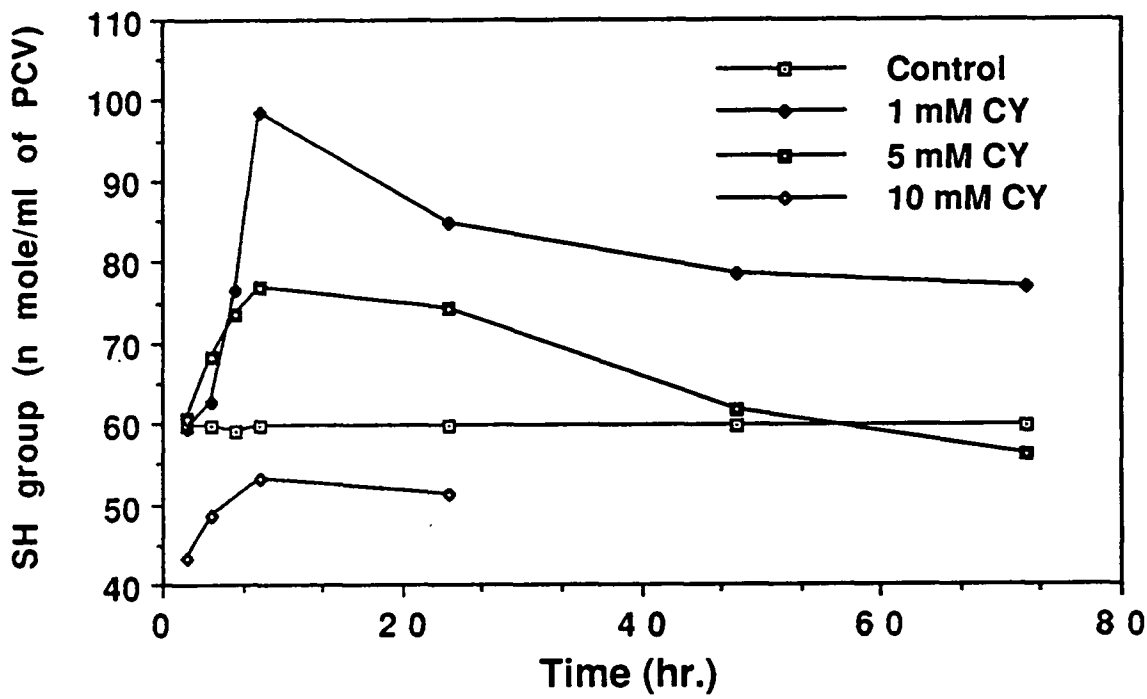


Fig.3.4. Incubation period of four cyanamide concentrations on SH groups in bromegrass cells. (SH: sulfhydryl CY: hydrogen cyanamide)

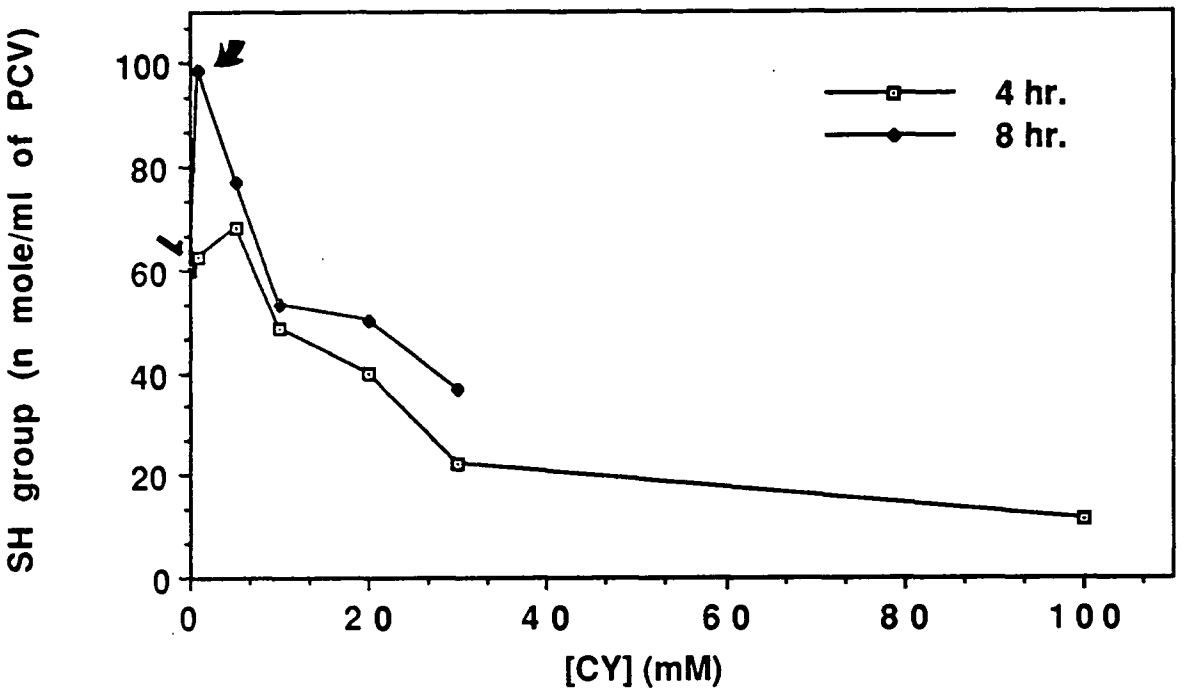


Fig.3.5. SH group level of bromegrass cells in cyanamide at two incubation periods. Control (-➸) and 1 mM cyanamide (=>). (SH: sulfhydryl CY: hydrogen cyanamide)

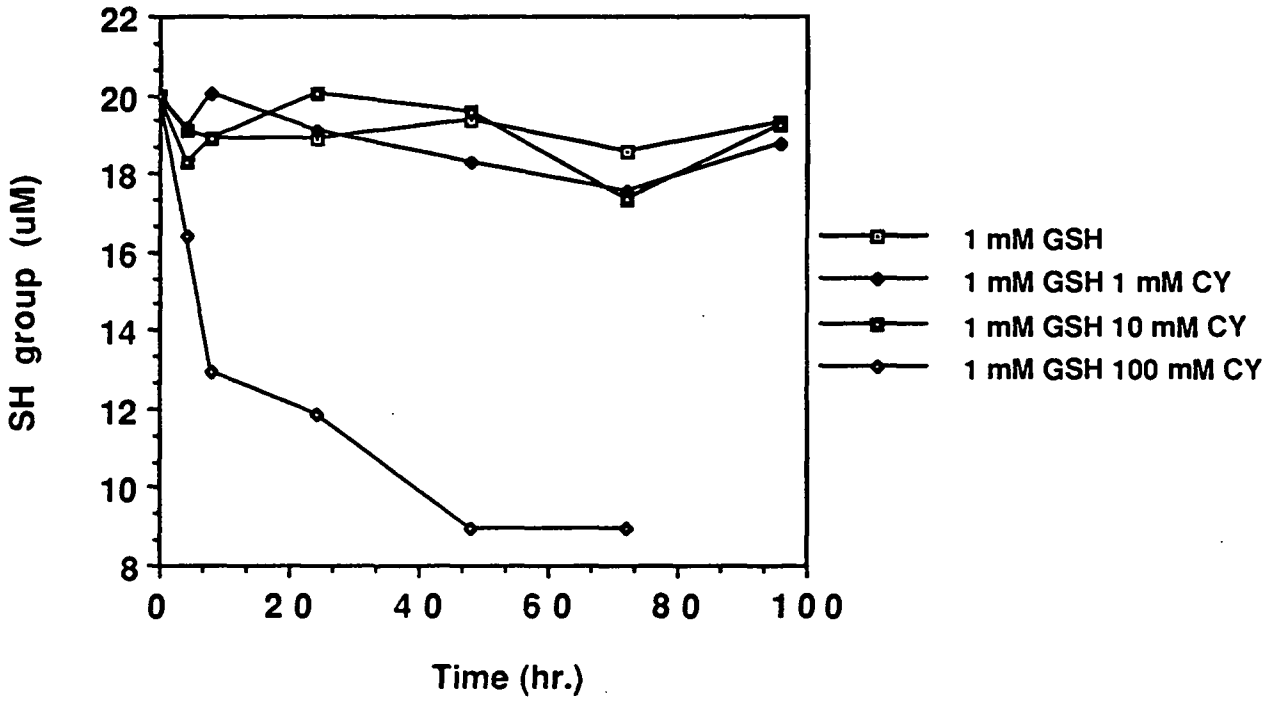


Fig.3.6. The effect of four cyanamide (CY) concentrations on 1 mM GSH. (GSH: glutathione SH: sulfhydryl)

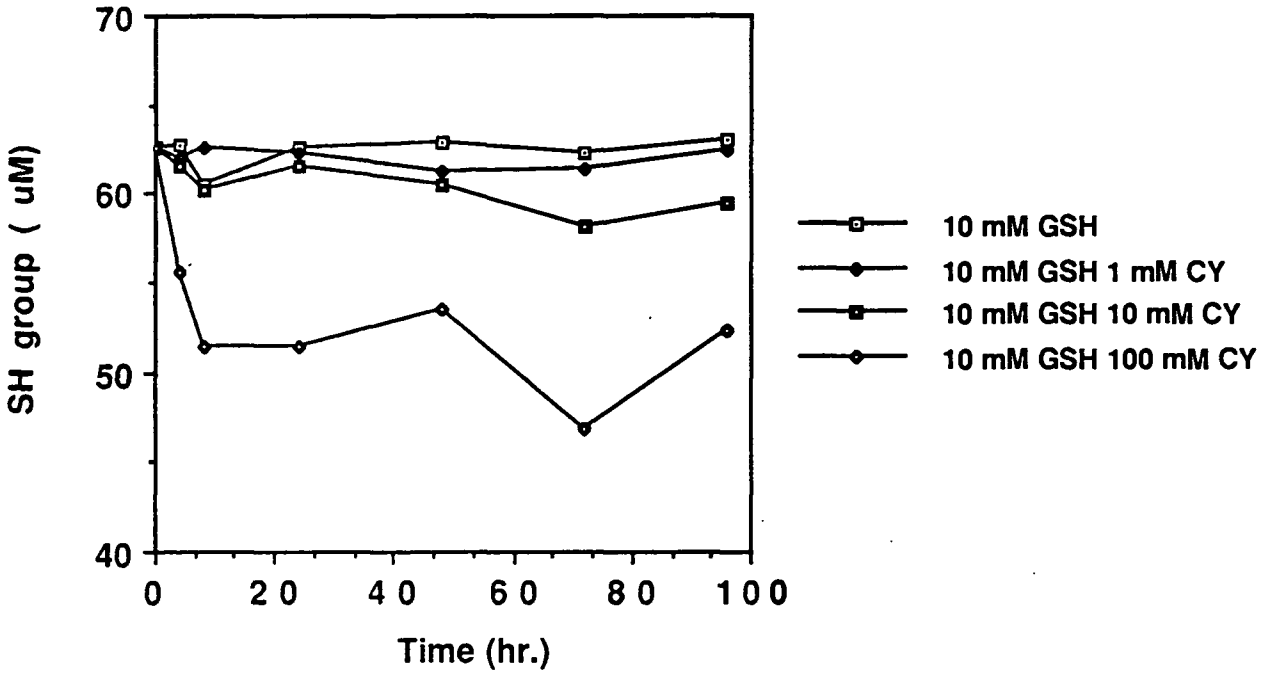


Fig.3.7. The effect of four cyanamide (CY) concentration on 10 mM GSH. (GSH: glutathione SH: sulfhydryl)

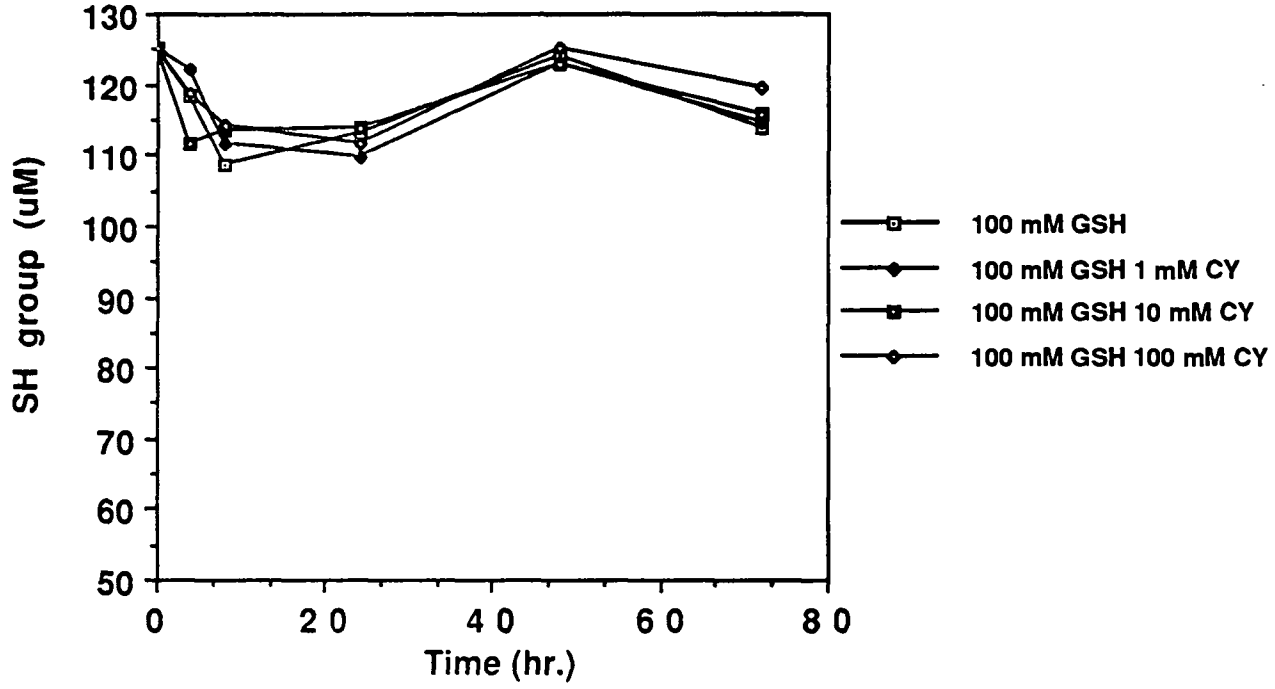


Fig.3.8. The effect of four cyanamide (CY) concentrations on 100 mM GSH. (GSH: glutathione SH: sulfhydryl)

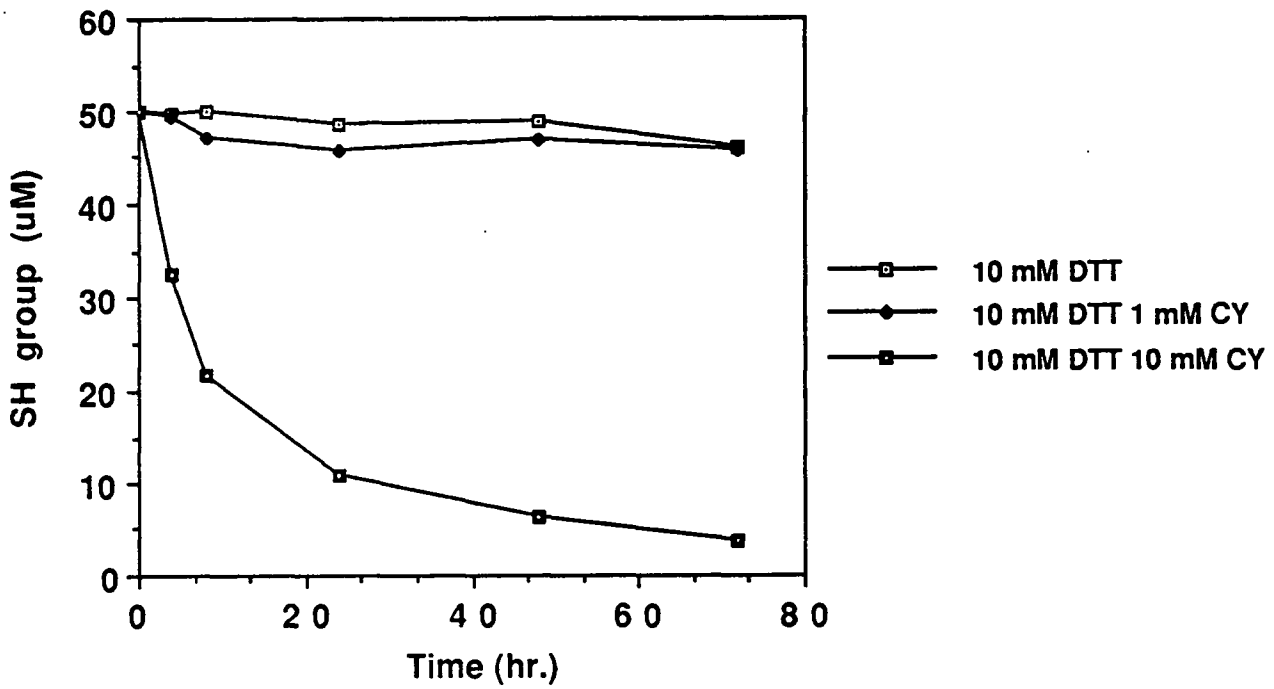


Fig.3.9. The effect of three cyanamide (CY) concentrations on 10 mM DTT. (DTT:dithiothreitol SH:sulfhydryl)

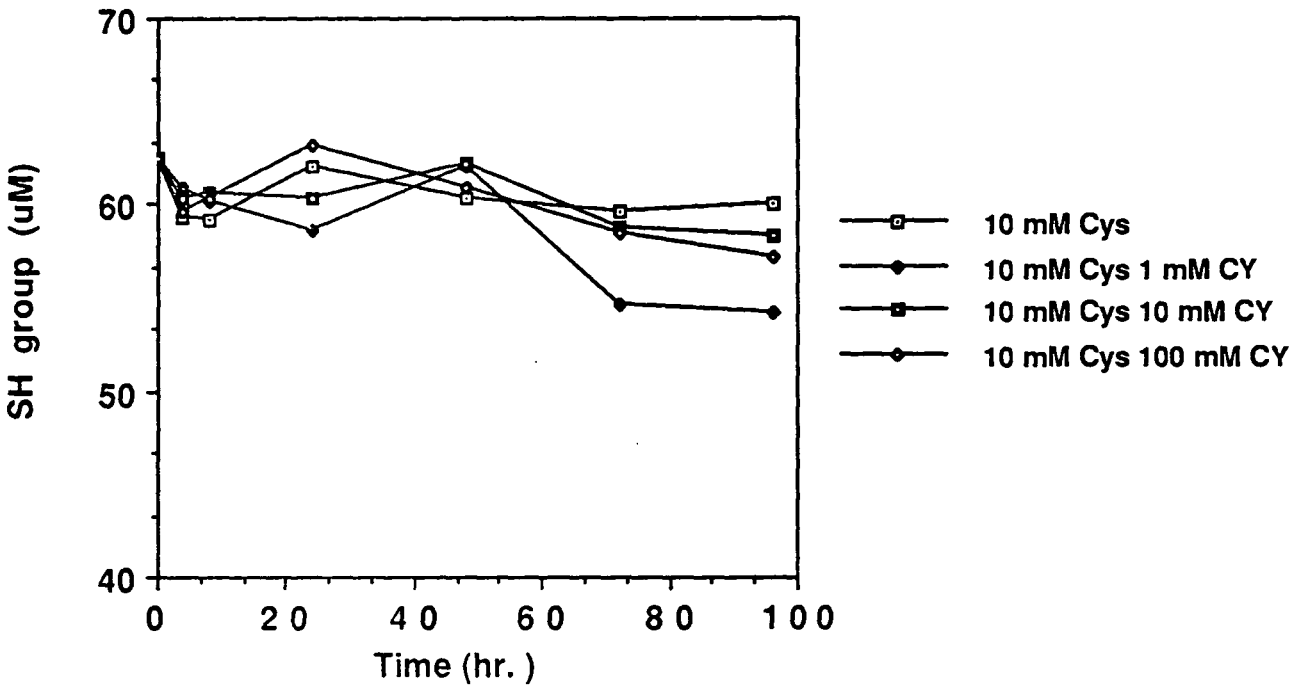


Fig.3.10. The effect of four cyanamide (CY) concentrations on 10 mM cysteine. (SH: sulfhydryl)

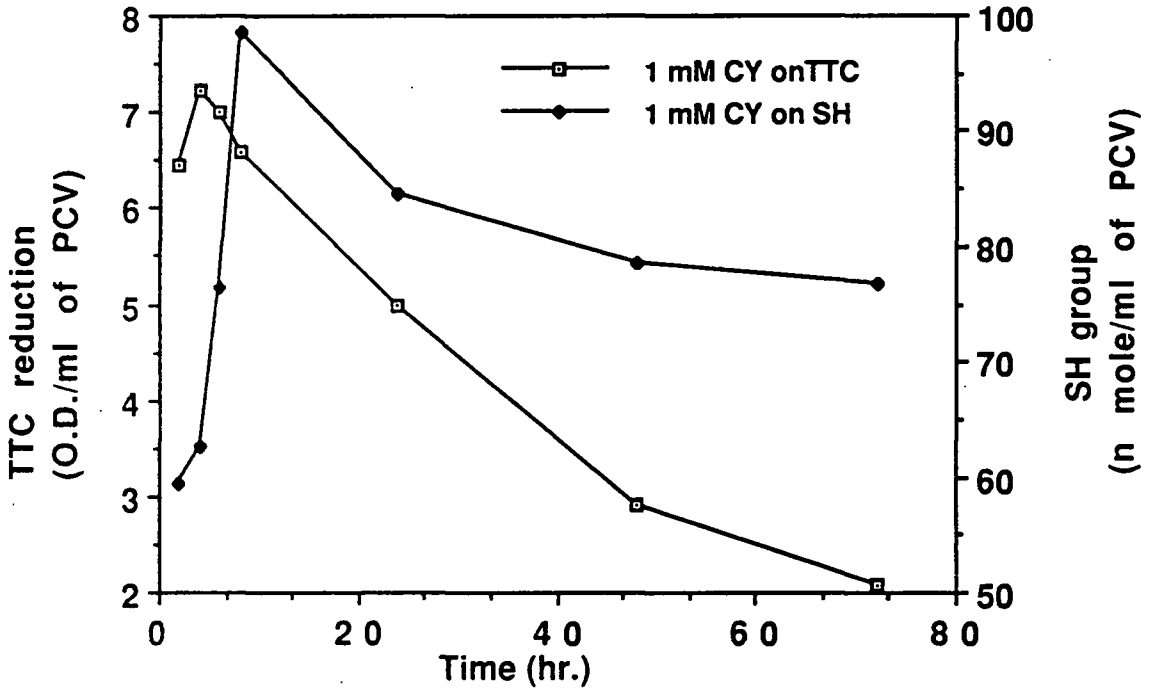


Fig.3.11. TTC reduction and SH group levels in bromegrass cells following incubation in 1 mM cyanamide (CY).
(TTC: triphenyltetrazolium chloride
SH: sulfhydryl)

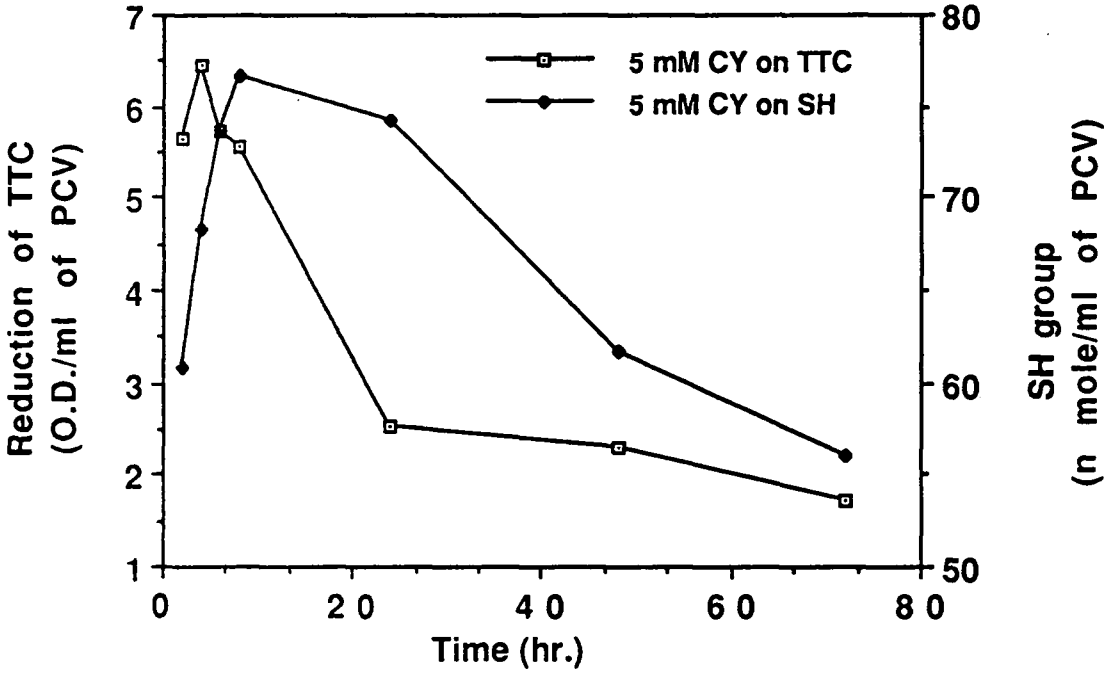
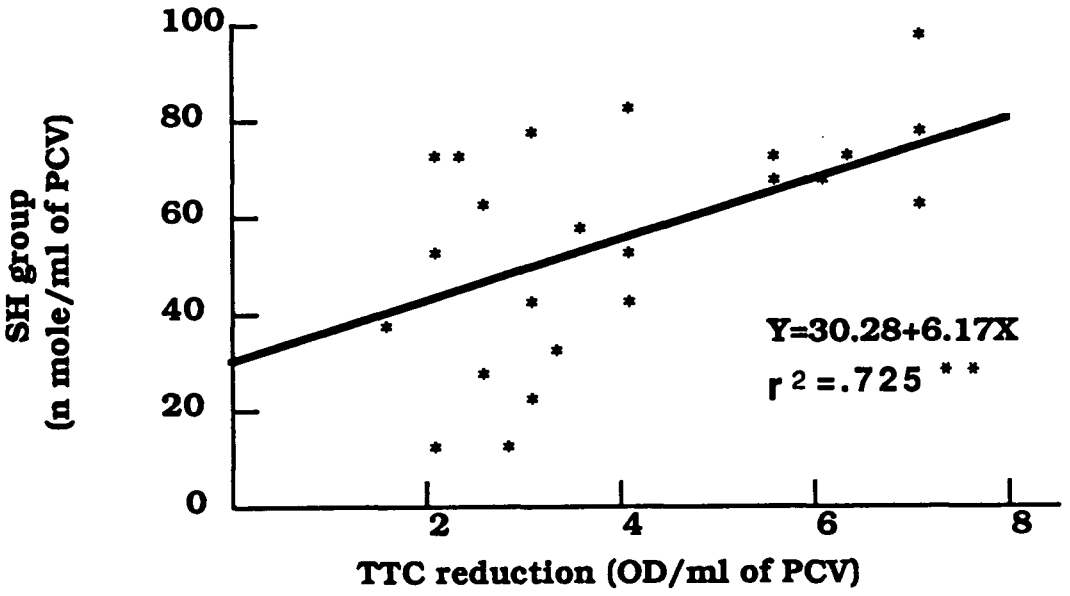


Fig.3.12. TTC reduction and SH group levels in bromegrass cells following incubation in 5 mM cyanamide (CY).
(TTC: triphenyltetrazolium chloride
SH: sulfhydryl)



** Significance at 1% level

Fig.3.13. The relationship between TTC reduction and SH group levels in bromegrass cells.
(TTC: triphenyltetrazolium chloride
SH: sulfhydryl)

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CHAPTER 4

INHIBITION OF CATALASE ACTIVITY BY HYDROGEN CYANAMIDE

Abstract

Inhibition of catalase activity by hydrogen cyanamide has been proposed as a mechanism for overcoming rest in plants. The objective of this study was to characterize the mode of inhibition of catalase by cyanamide. Bovine liver catalase in solution was treated with 0.1, 0.25, and 0.5 M hydrogen cyanamide for 24 hours, and catalase activity was measured before and after dialysis. Hydrogen cyanamide was found to inhibit catalase activity. However, catalase activity was restored to near the level of the untreated control after dialysis suggesting that hydrogen cyanamide is a reversible inhibitor of catalase. Enzyme kinetic studies indicate that the inhibition of catalase by cyanamide is of the mixed-type. Similar results were obtained by using catalase from bromegrass (Bromus inermis L.) cell suspensions.

Additional index words. enzyme kinetics, dormancy

breaking, rest, bromegrass (Bromus inermis L.) cells.

Introduction

Hydrogen cyanamide has been shown to be an effective dormancy-breaking agent in both seeds and buds (Lin 1984; Nee 1986). Cyanamide is thought to break rest via inhibition of catalase activity (Nir et al. 1986). Application of cyanamide resulted in lowered catalase activity and broke rest in grapevine (Nir et al. 1986). Catalase activity was also highly correlated to the natural dormancy status in plants. In grapevine, Nir et al. (1986) found that catalase activity increased to a maximum in October and, thereafter, decreased within 3 months to less than half its maximum rate. The decline in catalase activity coincided with the satisfaction of chilling requirement for dormancy release.

Although effective as a dormancy breaking agent, in certain instances, cyanamide can either have no effect, delay bud break, or injure buds. An understanding of the mechanism of action of cyanamide in overcoming rest may provide ways of either improving the efficacy of cyanamide in overcoming rest or assisting researchers in discovering more effective and safer chemicals to overcome rest. Results involving the mode of inhibition

of catalase by hydrogen cyanamide may provide clues for better understanding the mechanism of breaking rest in plants.

Currently, it is not clear how cyanamide inhibits catalase. Amberger et al (1961;1963;1984) reported that cyanamide inhibits catalase activity and the inhibition is a specific reversible process, while Shirota et al (1984) reported that cyanamide irreversibly inhibits catalase.

The objective of this study was to characterize the mode of inhibition of cyanamide on both bovine liver catalase and catalase of bromegrass cell cultures.

Materials and Methods

Bovine liver catalase (Sigma) was dissolved in 50 mM sodium phosphate buffer (pH=7.0). Cyanamide solution was added to the enzyme solution and incubated for 24 hr. at 4'C. The final enzyme concentration was 4.86 ug/ml with a cyanamide concentration of either 0.1, 0.25, or 0.5 M. After incubation, the enzyme solution was divided into two parts: one part was used for catalase assay at once and the other was dialysed in a dialysis membrane (molecular weight cut-off point 12,000) for 24 hr. at 4'C, against phosphate buffer (50 mM, pH=7.0). The buffer

was replaced several times during dialysis. The dialyzed enzyme solution was then used for catalase assay.

Bromegrass cells were cultured in Ericksson's medium amended with B_5 micronutrients and vitamins, and 0.5 mg/1,2,4-D as described by Chen and Gusta (1983). Three ml pack cell volume (PCV) of cells was transferred into 50 ml of medium in 250 ml erlenmeyer flasks and cultured for 4 days on a rotary shaker at 100 rpm in the dark at 25 ± 2'C. The hydrogen cyanamide solution was filter-sterilized by passing through an 0.2 micro filter (Acrodisc 13, Gelman Sciences) and then added to the culture medium. After 24 hours of incubation, 3 ml PCV of cells was collected and 2 ml phosphate buffer (100 mM, pH=7.0) and 0.02 g insoluble polyvinyl-polypyrrolidone (PVP) were added. The cells were homogenized (3 times at 30 seconds each) with a Tissuemizer (Tekman, SDT-1810) set at 80. The cells were then centrifuged at 15,000 xg for 20 min. at 4'C. The supernatant from either the dialysed or nondialysed solution was collected for enzyme assay. The conditions for dialysis were the same as that described above for Bovine liver catalase.

Two methods were used for catalase assay: 1. Titration method: phosphate buffer (100 mM and pH=7.0) containing 20, 40, 60, 80, and 100 mM sodium perborate

($NaBO_3$) was preincubated for 10 min. at 20'C. Bovine liver catalase solution was added to the buffered solution for 30 sec. while the crude enzyme extract from bromegrass cells was added to the buffered solution for 2 min. The final volume of the reaction mixture was 5 ml. The reaction was stopped by adding 3 ml of 2 N sulfuric acid. The remaining perborate concentration in the enzyme reaction solution was titrated with permanganate solution (0.01 N). Catalase activity was then calculated from the changes in $NaBO_3$ concentration (Aebi 1974). 2. Spectrophotometric method: catalase solution was added to the reaction mixture containing 5, 10, 15, and 20 mM H_2O_2, and absorbance was measured at 240 nm. The change in absorbance at 240 nm was recorded by a Shimadzu 160 UV-vis spectrophotometer. The catalase activity was determined by the method of Aebi (Aebi 1974).

Protein was determined by Lowry's method (Lowry 1951).

Results

Both U.V. and titration methods were acceptable for determining catalase activity for Bovine liver and bromegrass cell enzyme. Of the two methods tested, the U.V. method was simpler and faster. Therefore, the U.V.

method was used for determining catalase activity in the studies.

Inhibition of Bovine liver catalase by hydrogen cyanamide. Bovine liver catalase activity decreased as the concentration of cyanamide increased (Fig.4.1). Cyanamide inhibited catalase activity at all three substrate concentrations. Compared with the control level, catalase activity decreased 32, 54 and 80% when treated with 0.1, 0.25, and 0.5 M cyanamide, respectively in 600 uM concentration of subtrate. After dialysis, however, catalase activity was restored to the control level in all three substrate levels tested.

Enzyme kinetics from bovine liver catalase. Figure 4.2 shows the double reciprocal plot between enzyme activity and substrate concentrations. It was found that the interception of the three lines, when 1/V was plotted against 1/S, did not occur on either the X or Y axes. Similar results were obtained by using either the titration or U.V. method.

Catalase activity in both the dialyzed and non-dialyzed bromegrass call. In both the dialyzed and non-dialyzed bromegrass cell catalase, a linear relationship between the enzyme concentration and enzyme activity was found (Fig.4.3). The increase in enzyme activity as a

function of enzyme concentration was greater in the dialyzed samples.

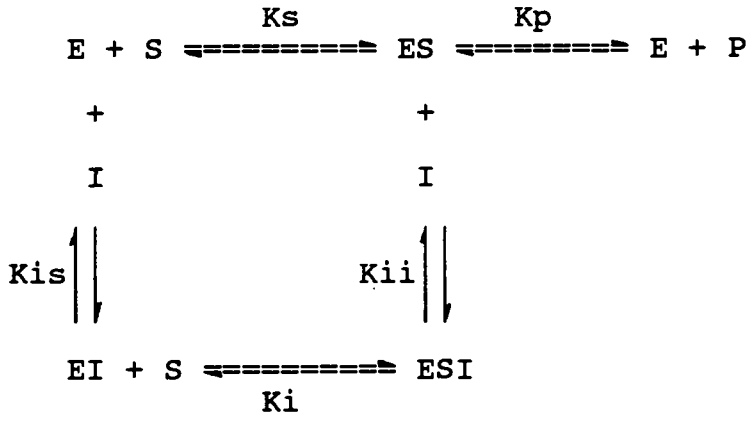
Enzyme kinetics from bromegrass cell. When bromegrass cells were treated with cyanamide at either 0.0, 0.1, or 5.0 mM for 24 hr. before extraction, the point of interception of the lines, when 1/V was plotted against 1/S (1/V vs 1/S), did not occur on either the X or Y axes (Fig.4.4), similar to that obtained from bovine liver catalase. In other experiments, catalase activity was similar to that shown in Figure 4.4 when cyanamide was added to the crude extract of catalase from bromegrass cells (Fig.4.5).

Discussion

Inhibition of catalase by cyanamide is specific, reacting with the Fe of catalase, and reversible (Amberger 1984). Cyanamide may act via its tautomeric carbodiimide form (HN=C=NH) (Smith 1965). In contrast, Shirota et al (1984) reported that cyanamide irreversibly inhibits catalase and the reaction requires hydrogen peroxide. Cyanamide inhibited erythrocyte catalase activity in vitro in the presence of hydrogen peroxide, and the apparent similarities between the inhibition of hepatic catalase by cyanamide and 3-amino-1,2,4-triazole

(3-AT) in vivo suggest that cyanamide belongs to the family of 3-AT-like catalase inhibitors (DeMaster 1986). Our results confirmed the reports of Amberger (1984) that cyanamide inhibits catalase activity and that the inhibition of catalase activity by hydrogen cyanamide is reversible. We observed that after dialysis, the activity of bovine liver catalase, treated with cyanamide, was restored to near the level of the control. In contrast to DeMaster's findings, we observed the inhibition of catalase by cyanamide in the absence of hydrogen peroxide.

From the double reciprocal plot, four kinds of reversible enzyme inhibition can be differentiated: 1) competitive, where the point of interception is on the Y axis; 2) non-competitive, where the point of interception is on X axis; 3) uncompetitive, where there is no point of interception; and 4) mixed-type, where the point of interception is neither on X nor on Y axes (Segel,1976). Our results (Figures 2, 4 and 5) suggest the inhibition of catalase activity by hydrogen cyanamide is of the mixed type inhibition because the interception point, when 1/V was plotted against 1/S, did not occur on either the X or Y axes. The equilibria shown below represent the simplest scheme for mixed-type inhibition.



where:

$$Ks = \frac{[E]\,[S]}{[ES]} \qquad Kis = \frac{[E]\,[I]}{[EI]}$$

$$Ki = \frac{[EI]\,[S]}{[ESI]} \qquad Kii = \frac{[ES]\,[I]}{[ESI]}$$

$$Kp = \frac{[ES]}{[E]\,[P]}$$

From table 4.1, if Kii < Kis, the apparent Km will decrease in the presence of cyanamide and the lines will intersect below the 1/S axis. If Kii > Kis, thus, the apparent Km will increase in the presence of cyanamide

and the lines will intersect above the 1/S axis. This indicates that the presence of cyanamide will affect the apparent Km of catalase. If Kii = Kis, the point of interception should be on the 1/S axis and the equation will be changed to that for the true non-competitive inhibition. Kalir and Polyjakoff-Mayber (1981) reported that different concentrations of NaCl can change the type of inhibition to catalase. The double reciprocal plots resemble the non-competitive effect for the 0.45 M NaCl treatment, but the 1 M treatment resembles the uncompetitive effect.

In conclusion, our results suggest that: (1) Cyanamide is a reversible inhibitor to catalase. The inhibition is observed in the absence of H2O2; (2) The inhibition of catalase by cyanamide belongs to the mixed-type inhibition; (3) Bromegrass cell culture treated with cyanamide showed inhibited catalase activity similar to that of bovine liver catalase. Further research is needed to study the physiological significance of inhibited catalase in plant cells with relation to dormancy status.

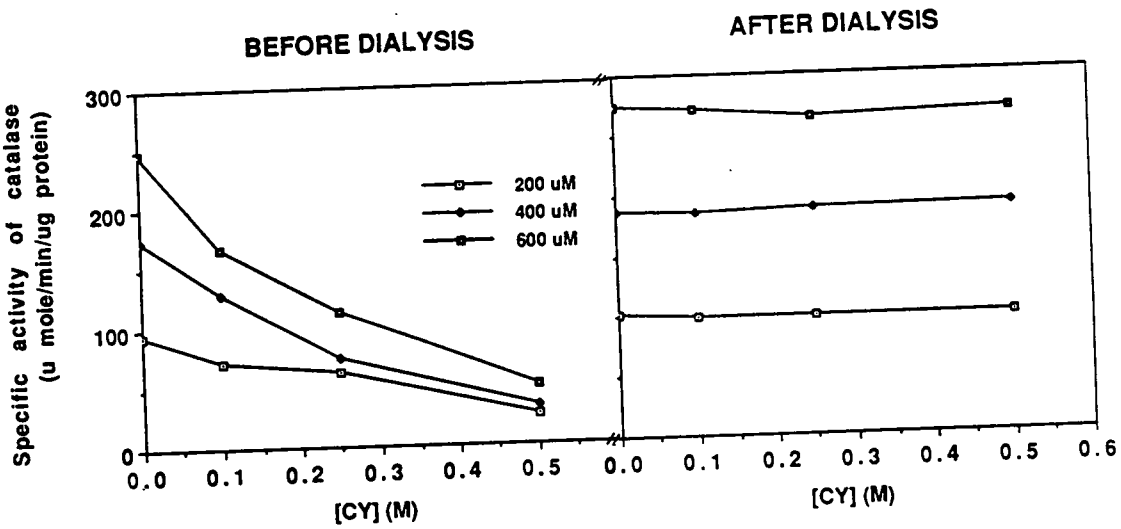


Fig.4.1. Inhibition of bovine liver catalase by cyanamide (CY) before and after dialysis at 3 substrate ($NaBO_3$) concentrations.

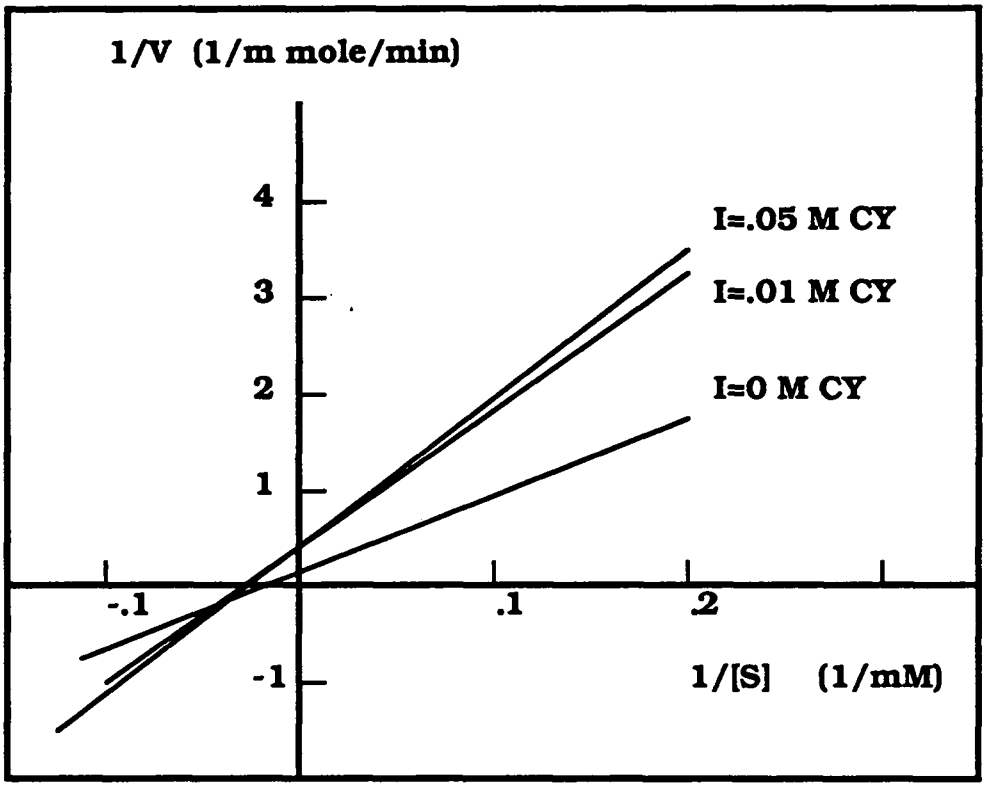


Fig.4.2. Double reciprocal plot of bovine liver catalase velocity dependence on substrate concentration in the presence of 3 inhibitor concentrations. (CY: hydrogen cyanamide)

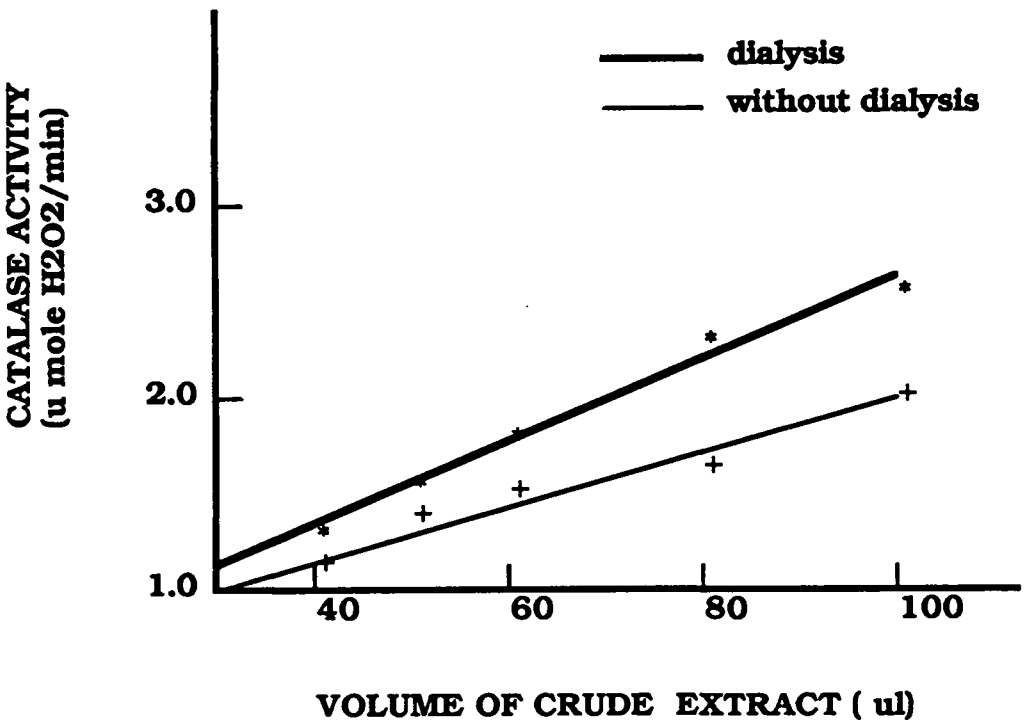


Fig.4.3. Relationship between catalase activity and enzyme concentrations in dialyzed and non-dialyzed bromegrass catalase. The protein concentration in the crude extract was 1.89 ug/ul.

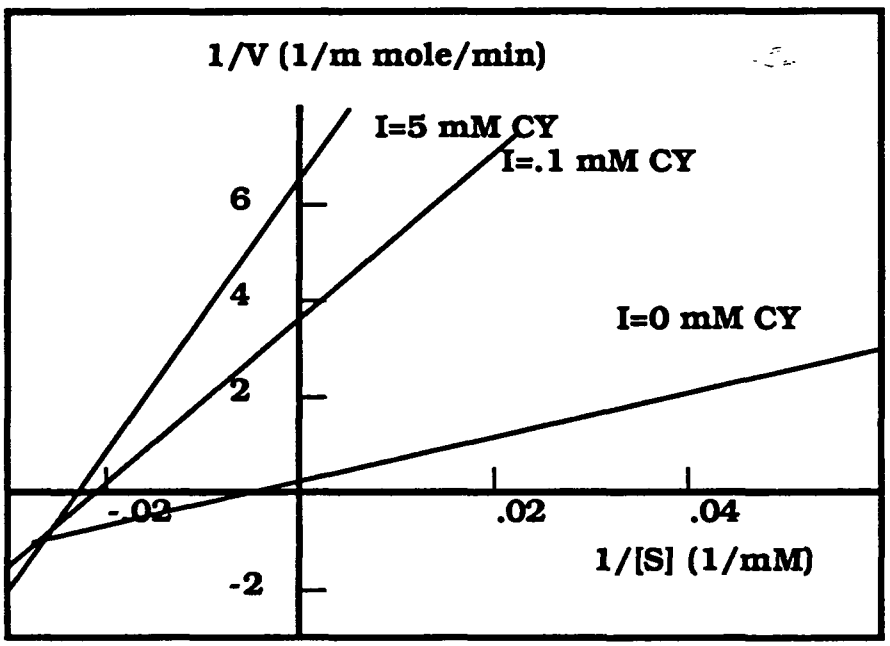


Fig.4.4. Double reciprocal plot of bromegrass catalase velocity at three inhibitor concentrations before dialysis. The bromegrass cells were treated with 3 concentrations of cyanamide (CY) for 24 hours before enzyme extraction.

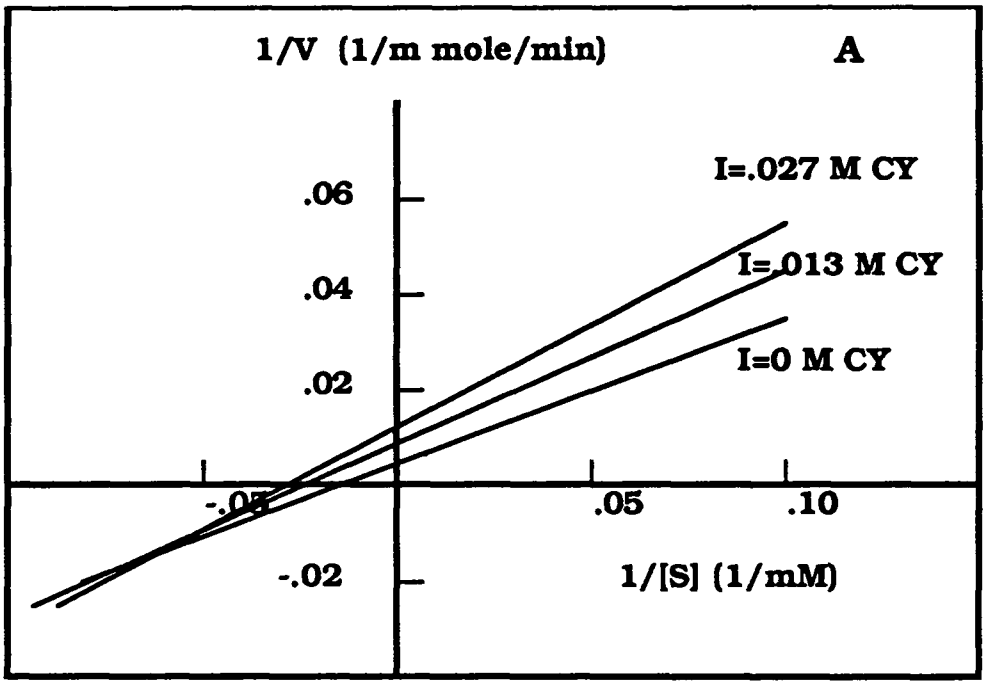


Fig.4.5. Double reciprocal plot of bromegrass catalase velocity at three inhibitor concentrations. The enzyme extract was added with the inhibitor and then assayed for catalase activity. (CY: hydrogen cyanamide)

Table 4.1. Dissociation constant of hydrogen cyanamide for catalase isolated from Bromegrass cells treated with cyanamide.

Treatment	Dissociation Constant (mM)	
	Kis	Kii
Medium + cyanamide	2.255	2.072
Extract + cyanamide	63.140	12.870

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