IJCRT.ORG ISSN : 2320-2882

INTERNATIONAL JOURNAL OF CREATIVE RESEARCH THOUGHTS (IJCRT)

An International Open Access, Peer-reviewed, Refereed Journal

Reversal Of Fungicidal Action By Suitable Activators

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Abstract **– The mechanism of fungicidal action was studied by reversing the activities of fungicides by adding suitable activators to the enzyme - fungicide mixture. Various activators containing - NH2, - SH, - OH and - COOH groups were tested by incubating their different concentrations with the inhibited enzyme solution at 55 ± 2 °C for 60 minutes. For this purpose nine activators were selected - histidine, sodium propionate, alanine, n - propyl alcohol, cysteine, glycerol, tryptophan, glutathione and sodium thio glycollate. The enzyme activity was measured by Nelson - Somogyi method. Out of the various activators tested, sodium thio glycollate, glutathione and tryptophan were found to reverse appreciably the inhibited activity of purified enzymes, followed by glycerol and cysteine. Various concentrations of these activators were tried and it was found that 1.5 × 10^{-2} M concentration of sodium thio glycollate reactivated the enzyme activity inhibited by pentachloro phenol (PCP) to the extent of 100% and that inhibited by m - dinitrobenzene (MDB) only to the extent of 73%. A similar concentration of tryptophan reactivated the enzyme activity inhibited by PCP to the extent of 77.5% of the original activity and that inhibited by MDB to the extent of 65% of the original activity. Inhibition of enzyme activity by using conventional thiol - Inhibitors, like iodoacetamide and pentachloro mercuric benzoate of 1.5 × 10^{-3} M concentration was recorded as 92% and 87%, respectively. The inhibition was reversed by using thiol compounds, such as, sodium thio glycollate and glutathione, each of 1.5 × 10^{-2} M concentration. It was observed that sodium thio glycollate reactivated the inhibited activity caused by iodoacetamide and p- chloro mercuric benzoate to the extent of 85% and 79.8% , respectively. However, glutathione caused the reversal of enzyme activity inhibited by iodoacetamide and p-chloro mercuric benzoate to the extent of 82 % and 86.5 % respectively. These reactivation studies with inhibited enzymes indicated that MDB binds at the - SH as well as - NH_2 groups nearly to the same extent. However, PCP mainly blocks the - SH groups and to some extent inhibits the - NH_2 dependent systems. It was assumed that the toxic action of both the fungicides on the activity of enzymes may be due to excessive withdrawal of electrons at the active sites, thus oxidising active groups of protein moiety.**

Index Terms **– Activators, Sodium thio glycollate, Glutathione, p-chloro mercuric benzoate.**

I. INTRODUCTION

In order to establish the mechanism of action of fungicides, the reversal of toxicity by various activators was studied. The site of action of fungicides in inhibiting the cellulase activity of fungus *Aspergillus niger* was studied. Suitable fungicides were used for the inhibition of growth and enzyme activity of the fungus. Two potent fungicides m-dinitrobenzene and pentachloro phenol were used for this purpose. The inhibition of growth of a wild strain of *Neurospora crassa* by Cu^{++} is counteracted by histidine, histidine methyl ester, histidinol and Mn^{++} (Subramanyam and Venkateswerlu, 1979). The data suggested that copper toxicity in the mould is due to the suppression of histidine biosynthesis. Soni and Bhatia have reported the inactivation of cellulase enzymes of *Fusarium oxysporum* by phenols and related compounds and the reversal of inactivation by cysteine HCl, sodium thio glycollate and hydroxyl amine hydrochloride indicated the involvement of - NH_2, - OH and - SH groups in fungicide - enzyme interaction. In 1980, Widholm and Slife have found that the growth inhibition of carrot cell suspension cultures by 4-amino benzene sulfonyl carbamate was reversed by adding p- amino benzoic acid or folic acid, thereby suggesting the structural relationship between the fungicide and the reactivator.

The inhibition - reactivation studies made by Singh et al. (1990) exhibited that L- cysteine- HCl or reduced glutathione could to a great extent reverse the inhibition of purified cellobiase of *Aspergillus niger* AS - 101 and thus indicated the involvement of- SH group (s) at the active site of the enzyme. All the five ions, viz. Suramin, Streptomycin, neomycin, viomycin and kanamycin were found to be negative modulators of purified cellobiase, whereas viomycin was the most potent Inhibitor.

The structure - fungicidal activity relationship of fungicides was studied in detail by Weidenborner et al (1990). They tested flavones and flavanones for fungicidal activity against the growth of five storage

fungi of genus *Aspergillus*. It was observed that unsubstituted flavone and flavanone were highly active and caused mycelial inhibition up to 90% in case of *A. glaucus*, while *A. flavus* and *A. petrakii* were inhibited only upto 70%. It has been further reported that the antifungal activity of flavonol was more effective than 7 - hydroxy flavone. However, introduction of a hydroxyl group at position 3 gave no guarantee for antifungal activity, if other hydroxyl groups are present in the molecule. The growth of *Aspergillus chevalieri* was reported to be stimulated by (+) - catechin. Earlier, O' Neill et al. (1982) found that 5,7 - dihydroxy flavone (chrysin) was inactive against *Botrytis cinerea*. This shows that even a further hydroxylation at position 5 caused no increase in antifungal activity. High polarity due to several hydroxyl groups seems to reduce the activity.

II. EXPERIMENTAL

I. Materials & Method:

Chemicals – All the inorganic chemicals used were of analytical grade obtained from BDH Laboratories Bombay, India. Carboxymethyl cellulose was obtained from Loba Chemie Indo Austranal.

Microorganisms – The cellulolytic fungus Aspergillus niger was obtained from National Sugar Institute, Kanpur. The culture was grown on potato dextrose agar slants containing filter paper strips as cellulosic substrate at a temperature of 30 ± 2 °C for a period of ten days and maintained at 4 °C by subculturing every month.

Elaboration of enzymes – On 7th day of growth of the fungus the mycelia were harvested by filtration through four layers of cheese cloth. The culture filtrates were directly centrifuged at 5000 rpm for 20 minutes at 4°C. Simultaneously the mycelial mat was collected on preweighed Whatman filter paper (No. 1), washed with distilled water and dried at 70°C until constant weight was obtained. The supernatant obtained was used as a source of crude Extracellular Enzyme preparation.
After removing the supernatant, the mycelial mat was dried between the folds of filter paper, crushed in a grinder with small amount of distilled water which was diluted to obtain 2.0% solution of mycelia. This suspension was used for the study of Intracellular Enzymes.

Study of inhibitory effects of fungicides – Chemical protection of cellulose from fungal attack was best accomplished by impregnation methods which includes insolubilizing biocidal toxicants onto the surface. Different concentrations of fungicides were added to the broth and potato dextrose agar media to observe the qualitative and quantitative growth of the organism and the enzyme activity was determined.

Reversal of fungicidal activity – To measure the reversal of fungicides by suitable activators, 24 h old mycelia were aseptically transferred, after proper washing with distilled water, to 10 ml medium containing suitable activator having structural relationship with the fungicides. These mycelia were allowed to grow for a further period of 24, 48 and 72 h. At the end of the experiment, aliquots were withdrawn and the content of the activator was determined by Tabor (1957). Restoration of 90% growth was used as criteria for reversal of toxicity. Similarly, reversal of enzyme activity was assessed by examining the inhibited enzyme activity in presence of suitable reactivating compounds having - SH, - NH_2, - COOH etc. groups.

II. Result and Discussion:

The activity of the purified enzymes, inhibited by fungicides, was reversed by the addition of suitable activators having structural relationship with the fungicides. For this purpose, several compounds having - NH_2, - SH, - OH and - COOH groups were tested to examine the possibilities of recovering the inhibited enzymes. Different concentrations of activators were mixed with the inhibited enzyme solution and incubated at 55°C. After 60 minutes of incubation, the enzyme activity was assessed for the reducing sugars formed at the end of enzymic hydrolysis of carboxymethyl cellulose. Assessment of enzyme activity in absence of fungicides and activators served as control. The results obtained are recorded in Table (1) and Table (2).

Table (1) shows that the activity of purified enzyme inhibited by m-dinitrobenzene (MDB) and pentachloro phenol (PCP) is restored appreciably by the activators – sodium thio glycollate, glutathione and tryptophan. Glycerol and cysteine are also effective to some extent in restoring the activity. The activity of glutathione is approximately the same as that of sodium thio glycollate. Table (2) presents the inhibition of carboxymethyl cellulase by a definite concentration of MDB and PCP and its reactivation by different concentrations of sodium thio glycollate and tryptophan. While 1.5×10^{-2}M concentration of sodium thio glycollate reactivates the enzyme activity inhibited by PCP to the extent of 100%, it

Table 1

Reactivation of inhibited carboxymethyl cellulase by different activators

Inhibitor (1.5×10^{-3}M)	Activators (1.5×10^{-2}M)	Enzyme activity (% of initial)
m-Dinitrobenzene	Nil	5.0
	Histidine	6.0
	Sodium propionate	7.5
	Alanine	10.0
	n-Propyl alcohol	18.0
	Cysteine	21.2
	Glycerol	37.0
	Tryptophan	63.0
	Glutathione	72.0
	Sodium thioglycollate	72.5
Pentachlorophenol	Nil	8.0
	Histidine	10.5
	Sodium propionate	15.0
	Alanine	20.0
	n-Propyl alcohol	22.5
	Cysteine	29.0
	Glycerol	44.7
	Tryptophan	77.0
	Glutathione	100.0
	Sodium thioglycollate	100.0
Control	Nil	100.0

Number of determinations were three in each case.

Table 2

Inhibition and reactivation of carboxymethyl cellulase activity

Inhibitor (1.5×10^{-3} M)	Activator	Concentration of activator (M)	Enzyme activity (% of initial)
m-dinitrobenzene	Nil	Nil	5.0
	Sodium thioglycollate	1.5×10^{-2}	73.0
		1.5×10^{-3}	26.7
		1.5×10^{-4}	18.0
		1.5×10^{-5}	7.0
	Tryptophan	1.5×10^{-2}	65.0
		1.5×10^{-3}	33.0
		1.5×10^{-4}	8.2
		1.5×10^{-5}	5.5
Pentachlorophenol	Nil	Nil	8.0
	Sodium thioglycollate	1.5×10^{-2}	100.0
		1.5×10^{-3}	46.0
		1.5×10^{-4}	23.0
		1.5×10^{-5}	15.6
	Tryptophan	1.5×10^{-2}	77.5
		1.5×10^{-3}	52.0
		1.5×10^{-4}	19.9
		1.5×10^{-5}	12.7
Control	Nil	Nil	100.0

Number of determinations were three in each case.

Table 3

Inhibition of carboxymethyl cellulase by conventional thiol inhibitors and activation by thiol compounds

Inhibitor (1.5×10^{-3} M)	Activator	Concentration of activator (M)	Enzyme activity (% of initial)
Iodoacetamide	Nil	Nil	8.0
	Sodium thioglycollate	1.5×10^{-2}	85.0
		1.5×10^{-3}	80.0
		1.5×10^{-4}	32.5
		1.5×10^{-5}	21.0
	Glutathione	1.5×10^{-2}	82.0
		1.5×10^{-3}	56.6
		1.5×10^{-4}	20.0
		1.5×10^{-5}	7.0
p-Chloromercuric benzoate.	Nil	Nil	13.3
	Sodium thioglycollate	1.5×10^{-2}	79.8
		1.5×10^{-3}	54.0
		1.5×10^{-4}	23.4
		1.5×10^{-5}	14.0
	Glutathione	1.5×10^{-2}	86.5
		1.5×10^{-3}	43.0
		1.5×10^{-4}	13.9
		1.5×10^{-5}	9.5
Control	Nil	Nil	100.0

Number of determinations were three in each case.

reactivated the enzyme activity inhibited by MDB only to the extent of 73%. A similar concentration of tryptophan reactivates the enzyme activity inhibited by PCP to the extent of 77.5% of the original activity and that inhibited by MDB to the extent of 65% of the original activity.

Table (3) describes the inhibition of cellulase activity by using conventional thiol - inhibitors and its reversal by thiol compounds. It has been observed that a definite concentration (1.5×10^{-3} M) of iodoacetamide and p-chloro mercuric benzoate inhibited the carboxymethyl cellulase activity to the extent of 92% and 87% respectively. Different concentrations of sodium thio glycollate and glutathione were tried to reactivate the inhibited enzyme and it was found that 1.5×10^{-2} M concentration of sodium thio glycollate reactivated the inhibited activity caused by iodoacetamide and p-chloro mercuric benzoate to the extent of 85% and 79.8% respectively. A similar concentration of glutathione caused the reversal of enzyme activity inhibited by iodoacetamide and p-chloro mercuric benzoate to the extent of 82% and 86.5% respectively.

The fungicides inactivate the enzymes by forming enzyme - fungicide complex at the active sites. PCP mainly blocks the -SH group and to some extent inhibits the $-NH_2$ dependent systems, while MDB binds at both - SH as well as $-NH_2$ groups of enzyme protein nearly to the same extent. Inactivation of carboxymethyl cellulase enzymes of *Fusarium oxysporum* by various phenols and related compounds and its reversal by cysteine HCl, sodium thio glycollate and hydroxyl amine hydrochloride has been reported (Soni and Bhatia, 1979). Since phenolics and related compounds react readily with such groups in proteins as are capable of undergoing electron interactions, e.g. $-NH_2$, -OH or -SH groups, they inactivate the enzyme by acting as non - specific reactants. Reversal of Inhibition by cysteine HCl, sodium thio glycollate and hydroxyl amine hydrochloride confirms this view. The effects of various metal ions, chelating agents and sodium dodecyl - sulphate (SDS) on B - glucosidase was observed (Sanyal et al, 1988). Among the metal ions tested, Hg^{++} had a pronounced Inhibitory effect on the enzyme. The addition of EDTA was found to reverse the inhibitory effect of Hg^{++}. There was a slight stimulation of enzyme activity (10%) in the presence of a Mn^{++} or Co^{++} ion. EDTA alone had no inhibitory effect. Sodium azide similarly had no inhibitory effect. The activity of enzyme was found to be slightly affected by SDS at a lower concentration, but at a higher concentration (1%), there was a 40% reduction in enzyme activity. While N - ethyl maleimide (NEM) did not show any Inhibitory effect, p - chloro mercuric benzoate (PCMB), a well-known - SH blocking agent exhibited marked Inhibitory effect.

Addition of bovine serum albumin (BSA), glycerol or sodium azide protected the purified cellobiase of *Aspergillus niger* from inactivation on storage (Singh et al, 1990). Among divalent metal ions Mn^{++}, Co^{++}, Ca^{++} and Mg^{++}, were found to be good positive modulators while Pb^{++} and Hg^{++} were strong Inhibitors. Dome sulphydryl group reagents were tested for their possible effects on cellobiase activity. Reduced glutathione was a mild activator and L- cysteine HCl showed no effect on cellobiase activity. Sodium sulphite and sodium arsenite slightly inhibited the cellobiase activity. PCMB inhibited the cellobiase activity of *A. niger* by 68% and has similarly been reported to be a strong inhibitor of ß-glucosidases from *Sporotrichum thermophile* (Meyer and Canevascini, 1981) and *Myceliopthora thermophila* (Sen et al, 1986). The inhibition - reactivation studies exhibited that L-cysteine HCl or reduced glutathione could to a great extent reverse the inhibition of purified cellobiase caused by PCMB and thus indicated the involvement of - SH groups at the active site of the enzyme (Singh et al., 1990). Kojima et al. (2004) reported an example of fungicidal effects through hyperactivation of a fungal signal transduction pathway. They showed that fludioxonil exhibits fungicidal activity through phosphorylation and subsequent activation of a Hog - 1 - related MAPK in the fungal pathogen *Colletotrichum langenarium*. Gracini et al. (2012) studied that the inhibitory effect of PGE2 on the killing of Paracoccidioides brasiliensis by human monocytes can be reversed by cellular activation with cytokines.

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