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Influence of Metal Ions on Dehydrohalogenase Activity

[1]R. Latha, [2]I.M. Mandappa, [1]M.S.Thakur and [1]H.K. Manonmani

[1]Fermentation Technology and Bioengineering Department
[2]Food Safety and Analytical and Quality Control Department Central Food Technological Research Institute (Council of Scientific and Industrial Research)
Mysore-570 020, Karnataka, India

Abstract: The effect of few metal ions on DDT- and HCH-dehydrohalogenase activity was studied. Both the enzymes were inactivated in the presence of these metals. The apparent rate constants of inactivation at different metal concentrations and conformational changes in the presence of 100mM metals were obtained. The conformational changes of both the enzymes were followed by fluorescence spectra and circular dichoism spectra. Comparison of the results of both the enzymes indicated that both the enzymes were inactivated by the addition of metal ions. There was a progressive loss in catalytic activity of dehydrohalogenase enzymes with increasing concentrations of metals. Both dehydrohalogenases studied had almost the same effect. The fluorescence spectrum of both the enzymes exposed to metal ions showed a shift towards the red region. The CD spectrum of these metal treated enzymes showed significant changes such as reduction in α-helices and β-sheets. Alkali metals, alkaline earth metals, heavy metals and transition metals studied had almost the same changes in the secondary structure of both dehydrohalogenases.

Key words: Conformational change · DDT- dehydrohalogenase · HCH dehydrohalogenase, Inactivation, Metal ions

INTRODUCTION

Biodegradation is the conversion of chemical substances by the action of living organisms. The biodegradation of organic compounds by microorganisms is an intrinsically complex process. A large number of sub-processes, like penetration of compounds to the cells, biochemical catalysis and the release of products will take place during biodegradation. For nearly half a century active research on biodegradation has been focussed on cells, enzymes or nucleic acids. It has become possible to view the biodegradative process as the biochemical reactions taking place in the active site of proteins as a result of technical development of X-ray diffraction and spectroscopic techniques. Dehalogenases are the key enzymes that catalyze dehalogenation of aromatic hydrocarbons by cleaving carbon-hydrogen bond. Based on substrate range, reaction type and gene sequences, the dehalogenating enzymes are classified in different groups, including hydrolytic dehalogenases, glutathione transferases, monooxygenases and hydratases- [1]. Hydrolytic dehalogenation is one of the most important mechanisms used by microorganisms to initiate the dehalogenation process. Dichlorodiphenyltrichloroethane (DDT) and hexachlorocyclohexane (HCH), the most widely used organochlorine pesticides have been shown to undergo the initial dehydrohalogenation to form dichlorodiphenyldichloroethane (DDD) [2] and TCB [3]. In our laboratory we have isolated, purified and characterized DDT-dehydrohalogenase from *Pseudomonas putida,* T5 [4] and HCH-dehydrohalogenase [5].

The intrinsic fluorescence of proteins, which is mainly due to tryptophan and tyrosine residues, has recently been used to obtain structural information about these macromolecules [6, 7]. Upon excitations at 290 - 295 nm, where tyrosine absorption is negligible, the fluorescence of a protein is extensively due to the tryptophan residues. Since it has been shown that the fluorescence properties of these residues, such as fluorescence emission maximum (λ_{max}), band width and quantum yields are strongly influenced by their particular location in the protein molecules [8]. One can use tryptophan residues as "built-in" probes of the

Corresponding Author: H.K. Manonmani, Fermentation Technology and Bio-engineering Central Food Technological Research Institute Mysore- 5700 20, India. Tel: +91-821 2515792, Fax: +91-821 2517233.
E-mail: manonmani_99@yahoo.com / manonmani@cftri.res.in.

confirmation of the protein molecule. Sometimes fluorescence spectroscopy can show structural changes in the protein that are too subtle to be observed by other techniques due to high sensitivity of protein fluorescence to the microenvironment surrounding the emitting tryptophan(s) [9]. CD spectroscopy is a very powerful technique to study metal–protein interactions. CD gives specific structural information and is a quick method that does not require large amounts of proteins or extensive data processing. Circular dichorism (CD) is a spectroscopic technique that can be used to determine the secondary structural content of proteins.

We have reported the purification and characterization of two pesticide degrading dehydrohalogenases from bacteria [4, 5]. The objective of this study was to elucidate the role of metal ions on the activity and stability of DDT- and HCH-dehydrohalogenases by measurement of activity, fluorescence emission properties and CD-spectral studies.

MATERIALS AND METHODS

Metals: Na^+, K^+, Co^{2+}, Cu^{2+}, Mg^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, Zn^{2+}, Fe^{2+} and DDT (98% pure), γ-Hexachlorocyclohexane (HCH) were purchased from Sigma-Aldrich chemical company, MO, USA. All other chemicals were of analytical grade. DDT-dehydrohalogenase and HCH-dehydrohalogenase were purified according to the methods described in our earlier paper [4, 5].

Dehydrohalogenase Assay: Dehydrohalogenase activity was carried out with DDT and HCH as the substrates. Known amount of DDT-dehydrohalogenase enzyme (suitably diluted) in 2 ml of phosphate buffer (50 mM, pH 7.5) containing dimethyl formamide (DMF) and substrate (DDT) were incubated at ambient temperature (26- 28°C) for 1 h. Reaction was stopped by adding nitric acid to pH 2.0 and halide production was determined spectrophotometrically at 460 nm with mercuric thiocyanate and ferric ammonium sulphate [10]. One unit of enzyme activity is defined as the amount of enzyme that catalyzed the formation of 1 μmol halide min^{-1}. Specific activity was expressed as units per mg protein. Similarly HCH-dehydrohalogenase enzyme was assayed using γ-HCH as substrate. Other reaction conditions were same as above.

Effect of Metals on Enzyme Activity: Aliquots of the enzyme (specific activity of 90 U) were incubated separately with metals Na^+, K^+, Co^{2+}, Cu^{2+}, Mg^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, Zn^{2+} and Fe^{2+} for 5 to 60 min. prior to measuring the enzyme activity. The concentrations of these metals ranged from 1 μM to 2 M. Enzyme in phosphate buffer (50 mM, pH 7.5) was incubated with different concentrations of metals for 1 h, dialyzed extensively before adding the substrate (DDT and / or HCH). The assay was carried out as described above. Activity measurements were done calorimetrically as described above. Fluorimetric measurements were carried out using Shimadzu model No.RF 5301PC fluorescence spectrophotometer in the ratio mode using split widths of 5mm for excitation and emission. The fluorimetric assay of the enzyme was conducted by following increase in the intensity of emission fluorescence 330 to 340 nm (excitation at 280 nm). The spectra were recorded with a 5nm band width for both excitation and emission monochromators.

CD Measurements: CD measurements were made using Jasco J-810 automatic spectropolarimeter fitted with a xenon lamp. The instrument was calibrated with dextro-10-camphor-sulphonic acid. Slits were programmed to yield 1 nm bandwidth at each wave length from 300 to 200 nm. The chart speed, wavelength expansion and time constant of the instrument were set to obtain the best signal- to-noise ratio. The lamp was purged continuously with nitrogen before and during experiments. The scans were recorded twice. The far-UV CD spectra were recorded between 200 and 260 nm using a quartz cell of 1 mm path length. The near UV CD spectra were recorded between 260 and 340 nm using a quartz cell of 1 mm path length. The enzyme protein concentration used was in the range of 0.4 to 0.44 mg ml^{-1}. The base line for the spectrum was obtained using buffer blank (50 mM phosphate buffer, pH 7.5). The measurements were made at room temperature. Mean residue ellipticity values $[\theta]_{mrw}^2$ were calculated using IIS for mean residue weight. CD analyses were done using the programs CONTIN and K2D recommended for proteins with predominant β-structure.

Measurement of Inactivation Time: The kinetic course of inactivation of HCH- and DDT-dehydrohalogenase enzymes was monitored through measurements of dehydrohalogenase activities at different time intervals with 1 μM to 2 mM metal concentrations at 30°C.

ESULTS AND DISCUSSION

Effect of Metals on Enzyme Activity: In general there was a progressive loss in catalytic activity of dehydrohalogenase enzymes with increasing concentrations of metals. Both dehydrohalogenases

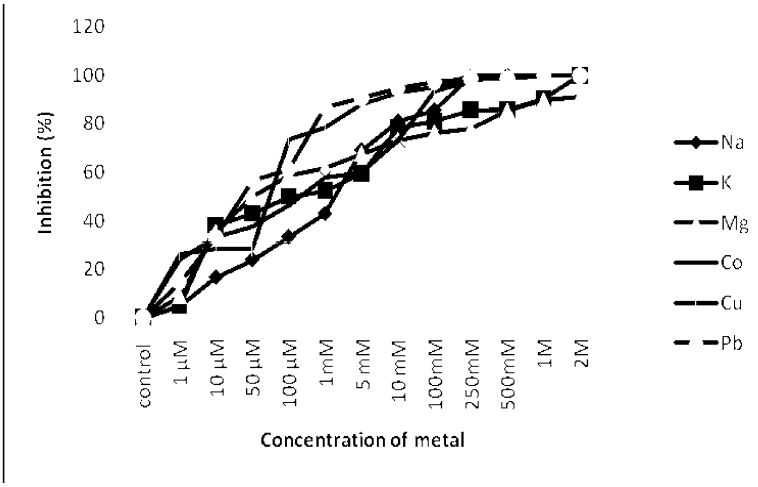

Fig. 1: Effect of metal ions on HCH-dehydrohalogenase activity.

HCH-dehydrohalogenase enzyme was incubated separately with metals Na^+, K^+, Co^{2+}, Cu^{2+}, Mg^{2+} and Pb^{2+} prior to measuring the enzyme activity The concentrations of these metals ranged from 1µM to 2M. Enzyme in phosphate buffer (50mM, pH 7.5) was incubated with different concentrations of metals for 1 h, dialyzed extensively before adding the substrate (DDT). The assay was carried out as described above. The results are an average of triplicate values.

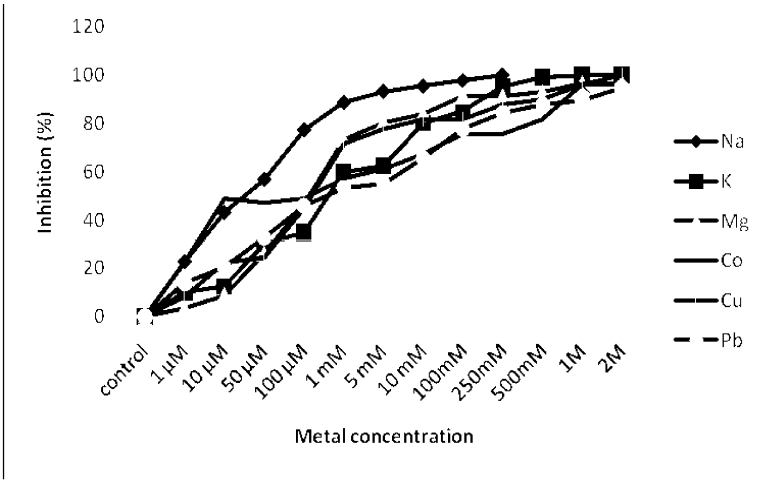

Fig. 2: Effect of metals on DDT-dehydrohalogenase activity.

DDT-dehydrohalogenase enzyme was incubated separately with metals Na^+, K^+, Co^{2+}, Cu^{2+}, Mg^{2+}, Pb^{2+} prior to measuring the enzyme activity The concentrations of these metals ranged from 1µM to 2M. Enzyme in phosphate buffer (50mM, pH 7.5) was incubated with different concentrations of metals for 1 h, dialyzed extensively before adding the substrate (DDT). The assay was carried out as described above. The results are an average of triplicate values

studied had almost the same effect. With alkali metals, 50% of enzyme activity was lost at 0.01 , 0.83 mM and 1.17, 100 mM of Na^+ and K^+ levels respectively for DDT and HCH-dehydrohalogenases (Fig. 1 and 2). At 250 mM Na^+, the activity of both-dehydrohalogenase was completely lost. But the enzyme activities were lost completely at 1 M and 2 M K^+ respectively for DDT- and HCH- dehydrohalogenases. The loss in enzyme activity was progressive with increase in concentrations of Na^+ and K^+ (Fig. 1 and 2). There was no complete loss in enzyme activity when alkaline earth metal (Mg^{2+}) was tested (Figure 1 and 2). 50% loss in DDT- and HCH-dehydrohalogenase enzymes was observed respectively at 186.56 and 50 µM concentrations of Mg^{2+}. Still 4 and 10% enzyme activity was still remaining respectively for DDT- and HCH-dehydrohalogenases after treatment with

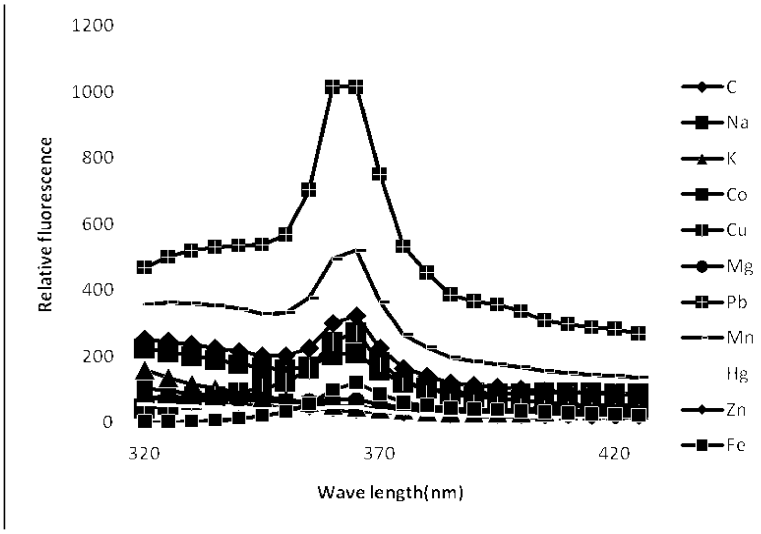

Fig. 3: Effect of metal ions on fluorescence spectral properties of HCH-dehydrohalogenase enzyme.
HCH-dehydrohalogenase enzyme was incubated separately with metals: Control (C), Na^{+}, K^{+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, Zn^{2+} and Fe^{2+} prior to measuring the enzyme activity The concentrations of these metals ranged from 1µM to 2M. Enzyme in phosphate buffer (50mM, pH 7.5) was incubated with different concentrations of metals for 1 h, dialyzed extensively . Fluorimetric measurements were carried out using Shimadzu model No.RF 5301PC fluorescence spectrophotometer in the ratio mode using split widths of 5mm for excitation and emission. The fluorimetric assay of the enzyme was conducted by following increase in the intensity of emission fluorescence 330 to 340 nm (excitation at 280 nm). The spectra were recorded with a 5nm band width for both excitation and emission monochromators.

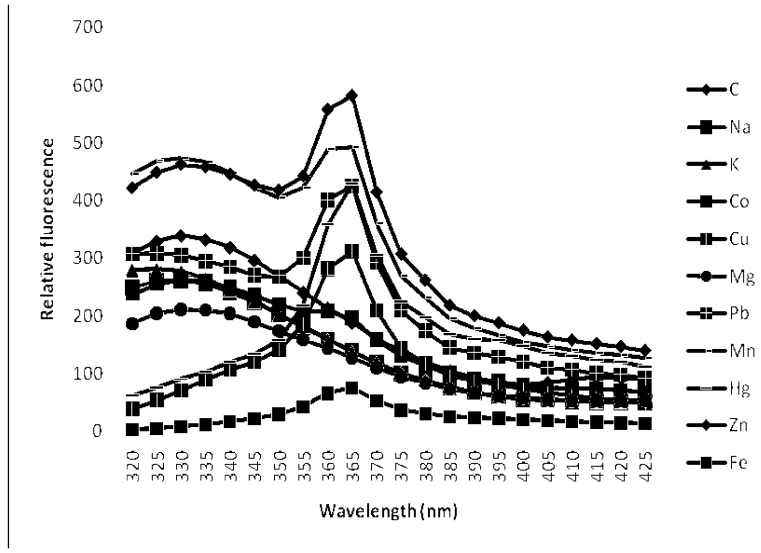

Fig. 4: Effect of metal ions on fluorescence spectral properties of DDT-dehydrohalogenase enzyme.
DDT-dehydrohalogenase enzyme was incubated separately with metals: Control (C), Na^{+}, K^{+}, Co^{2+}, Cu^{2+}, Mg^{2+}, Pb^{2+}, Mn^{2+}, Hg^{2+}, Zn^{2+} and Fe^{2+} prior to measuring the enzyme activity The concentrations of these metals ranged from 1µM to 2M. Enzyme in phosphate buffer (50mM, pH 7.5) was incubated with different concentrations of metals for 1 h, dialyzed extensively . Fluorimetric measurements were carried out using Shimadzu model No.RF 5301PC fluorescence spectrophotometer in the ratio mode using split widths of 5mm for excitation and emission. The fluorimetric assay of the enzyme was conducted by following increase in the intensity of emission fluorescence 330 to 340 nm (excitation at 280 nm). The spectra were recorded with a 5nm band width for both excitation and emission monochromators.

Table 1: Effect of metal ions on the secondary structure content of Dehydrohalogenase

	Secondary structure (%)					
	α-helix	β-sheets		α-helix	β-sheets	
Metal ions	HCH-dehydrohalogenase		Random	DDT-dehydrohalogenase		Random
(Control)	3.5	41	55.5	3.5	82.1	14.6
Na^+ treated	2.7	22	75.3	3.1	62.4	34.5
K^+ treated	3.7	15	81.3	0.0	3.0	97.0
Co^{2+}treated	2.4	40	57.6	1.5	22.7	75.8
Cu^{2+}treated	1.8	55	43.2	1.2	39.4	59.4
Mg^{2+}treated	1.6	34	64.4	3.6	17.0	79.4
Pb^{2+} treated	2.9	23	74.1	2.9	13	84.1
Mn^{2+}treated	2.6	56	41.4	3.0	68.3	28.7
Hg^{2+}treated	1.1	55.3	43.6	0.9	54.3	44.8
Zn^{2+} treated	2.3	34.8	62.9	1.4	38.5	60.1
Fe^{2+}treated	0.9	44.7	54.4	0.8	23.0	76.2

2 M Mg^{2+}. Co^{2+}, a transition metal inhibited the activity of HCH-dehydrohalogenase completely at 500 mM level while the activity of DDT-dehydrohalogenase was not lost even at 2 M levels (Figure 1 and 2). 102.06 and 107.13 μM Co^{2+} was required to cause 50% loss in activity of DDT- and HCH-dehydrohalogenase enzymes respectively. Cu^{2+}, a heavy metal also inhibited the enzyme activity in a similar way. There was a progressive loss in enzyme activity with increase in concentration of Cu^{2+} with 50% loss shown at 11.33 and 67.73 μM levels respectively for DDT- and HCH-dehydrohalogenases. . The DDT-dehydrohalogenase activity was lost completely at 2M of Cu^{2+} while HCH-dehydrohalogenase enzyme lost its activity completely at 250 mM levels.

Conformational Changes

Effect of Metals on the Fluorescence Property of the Enzyme: The control (untreated DDT- and HCH-dehydrohalogenase) enzymes had an excitation maximum of 280 nm and the emission maximum of 333 nm. Effect of Na^+, K^+ on the fluorescence spectrum of DDT- and HCH dehydrohalogenase is given in the Figures 3 & 4. The emission spectrum of Na^+ and K^+ treated HCH-dehydrohalogenase enzymes showed no shift in the fluorescence spectrum. There was an increase in the intensity of emission fluorescence of Na^+ treated enzyme by 25 RFU, (as compared to that of native enzyme). With the addition of K^+, there was an increase in the intensity by 54 RFU. No other changes were observed on the spectral pattern of Na^+ and K^+ treated HCH-dehydrohalogenase enzyme. When these metals were added to DDT-dehydrohalogenase enzyme, there was shift in the emission peak towards blue region. There was a decrease in the intensity of both Na^+ and K^+ treated enzymes by 80 and 69 RFU respectively. Mg^{2+} addition showed no shift in the spectrum, but there was a reduction of 126 RFU with DDT-dehydrohalogenase enzyme at 333 nm. The intensity of emission spectrum of Mg^{2+} treated HCH-dehydrohalogenase at 333 nm increased by 10 RFU to that of untreated enzyme. Co^{2+} treated enzyme showed a large increase in the emission intensity by 130 RFU for HCH-dehydrohalogenase enzyme. DDT dehydrohalogenase enzyme exhibited a decrease in the emission intensity by 86 RFU. There was a slight shift in the fluorescence spectrum towards blue region (Figure 3 and 4). Heavy metals Hg^{2+} and Cu^{2+} showed very high reduction in the emission intensity with DDT-dehydrohalogenase enzyme. There was a decrease of 242 and 258 RFU respectively. However there was a reverse response with Zn^{2+} treated DDT-dehydrohalogenase enzyme. It showed an increased intensity of 62 RFU. The emission intensity of HCH - dehydrohalogenase enzyme showed a reduction of 15 and 9 RFU by exposure to Hg^{2+} and Cu^{2+}. However, addition of Zn^{2+} showed an enhanced emission intensity of 164 RFU. The Cu^{2+} treated enzyme showed a shift in the peak towards the red region. Mn^{2+} treated enzyme showed a shift in the peak to the red region with both the enzymes. There was an increase of 133 and 293 RFU with DDT and HCH- dehydrohalogenase enzymes respectively. Pb^{2+} treated enzyme also behaved in the same way showing a shift towards the red region.

Effect of Metals on Secondary Structural Properties: The near-UV CD spectrum exhibited a minimum at 205 nm. The secondary structure analysis of the enzyme suggested a predominance of β-structure in the enzyme. The enzyme DDT-dehydrochlorinase had 82.1 % β-structure, 14.6% random. HCH-dehydrohalogenase had 41 % β-structure and 51.5 % random.

Effect of Alkali Metals: The UV CD-spectrum of Na^+ and K^+ treated DDT-dehydrohalogenase enzyme showed significant changes as compared to the native enzyme. There was complete loss of α-helix with K^+ treated enzyme .and there was substantial reduction in β sheets. i.e., there was 96.35% reduction in the β sheets. There was substantial increase in the random (Table 1). Na^+ treated enzyme showed decrease in both α-helix and β sheets .But there was increase in turns. HCH-dehydrohalogenase enzyme treated with Na^+, an alkali metal showed a slight decrease in α-helix and ~50% reduction in reduction in β sheet. Enzyme treated with K^+ showed a slight increase in α-helix and 63.4% decrease in β sheet. Thus the enzyme appeared to undergo detectable conformational changes in the backbone structure.

Effect of Alkaline Earth Metals: Effect of Mg^{2+} was different with both these enzymes. There was 54% reduction in α-helix and 17% reduction in β sheets with HCH-dehydrohalogenase enzyme. DDT-dehydrohalogenase enzyme treated with Mg^{2+} showed substantial reduction in β sheets. α-helix was not much affected. There was a substantial increase in random coil.

Effect of Transition Metals: Mn^{2+} and Co^{2+} effected 25.72% and 31.43% reduction in α-helix. There was 36.5% increase and 25.41% decrease in β sheets and random coils with Mn^{2+} treated HCH-dehydrohalogenase enzyme. Both α-helix and β sheets decreased by 14.29%, 57.15% and 16.81%, 72.36% in Mn^{2+} and Co^{2+} treated DDT-dehydrohalogenase enzyme. The random coils increased substantially in both the enzymes with addition of both the metals.

Effect of Heavy Metals: There was a substantial reduction in α-helix in Hg^{2+}, Cu^{2+} and Zn^{2+} treated dehalogenases. Both the enzymes behaved in the same way. There was reduction in β sheets too. The effect was more pronounced with DDT-dehydrohalogenase enzyme. There was an increase in random coils in both the cases.

In general, the aromatic amino acids exhibit fluorescence in any protein and proteinaceous enzymes in the range 310 nm to 340 nm. The hydrophobicity of these aromatic amino acids determines the intensity of fluorescence. Moreover, an exposure of aromatic amino acids such as tyrosine, tryptophan and phenylalanine those are buried in the core of the protein to hydrophilic or aqueous environment results in the reduction of protein fluorescence. Therefore, the reduction in the fluorescence intensity of dehydrohalogenase enzyme in presence of metals was an indication of loss of 3D conformation. Metals could have made the aromatic amino acids of dehydrohalogenase enzyme to get exposed to an aqueous environment that has lead to the decrease in the protein fluorescence. Also, reduction in the intensity of emission fluorescence may be related to the reduced hydrophobicity of metal exposed protein in comparison to the native enzyme. The near-UV CD spectrum exhibited a minimum at 205 nm. It is indicative of parallel β-structure.

The effect of metals upon the activity and optical spectroscopic properties of dehalogenases is an important aspect to study, since it is well known that activity of these inducible enzymes is strictly related to their conformational state, mostly controlled by the presence or the absence of aromatic substrate undergoing dehalogenation. In our studies decrease in enzyme activity was a concentration dependent phenomenon. All the metals studied exhibited inhibition of enzyme activity. The recognition of a metal ion by the enzyme binding site depends upon the ion size, charge and the chemical nature [11, 12]. In addition, simple electrostatic effects are also important for metal binding. [13]. In addition to bringing about alterations in the activity there were changes in the overall binding capacity of the enzymes. The metal ions could have brought about changes in over all structure of the domains of these dehalogenases.

Metals have been shown to inhibit urease and glutamate dehydrogenase activities $Cu^{2+,}$ Cr^{2+} and Hg^{2+} were most inhibitory, while Co^{2+}, Mn^{2+} and Zn^{2+} stimulated the enzyme activities [14, 15].

The activity inhibition of dehydrohalogenases by metals has been supported by spectral studies. λ_{max} of the tryptophan fluorescence of dehydrohalogenase enzymes in aqueous buffer was 333 nm which was blue- shifted about 26 nm from that of free tryptophan. This fact indicated that tryptophan residues as a whole are located in the relatively hydrophobic interior part of the enzyme [16]. Exposure of enzyme to metal ions brought changes in the fluorescence emission properties. There was shift in the emission peak compared to control. Increase in the fluorescence intensity implies binding of the metal ion thus causing conformational change. The rate of this change was found to be independent of the concentration of the metal ion implying that it may be a structural change in the enzyme-metal-complex. Also, the interaction with metal ions at a high affinity site might be leading to structural change in dehydrohalogenase enzymes.

The metal-treated dehydrohalogenase enzymes underwent basic structural changes such as decrease in the number of α-helix and β sheets. Proteins which contained a markedly high amount of primary sequence ordered with respect to helical amphiphilicity underwent

the same basic structural changes upon addition of metals. This change includes decrease in α-helix and β-sheets. There could be conversion of α-helices in to β-sheets. This conformational transition seems likely to be the fundamental molecular event that causes the activity loss. Removal of metal by dialysis does not restore back the activity.

CONCLUSIONS

Dehalogenases are the key enzymes that catalyze dehalogenation of aromatic hydrocarbons by cleaving carbon-hydrogen bond. Few dehalogenases have been cloned and isolated. But their catalytic nature has not been worked in detail. We studied the effect of few metal ions on DDT- and HCH-dehydrohalogenase activity. Both the enzymes were inactivated in the presence of these metals. The apparent rate constants of inactivation at different metal concentrations and conformational changes in the presence of 100mM metals were followed by fluorescence spectra and circular dichoism spectra. These metals exhibited significant influence on the secondary structure of the enzyme. These kinds of studies will help in carrying out bioremediation of contaminated environment successfully by taking care of all these factors that influence the degradative enzyme activity.

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