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# CHEMICAL MODIFICATION STUDIES ON ISOENZYMES OF SUPEROXIDE DISMUTASE FROM BAJRA SEEDLINGS

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**Key Word Index**—*Pennisetum typhoideum*; Gramineae; bajra; superoxide dismutase; chemical modification.

**Abstract**—Chemical modification of bajra superoxide dismutases (SODs) with phenyl glyoxal and hydrogen peroxide reduces the catalytic activity of SOD-I and SOD-III (Cu–Zn enzymes) but not that of SOD-II (Mn-SOD). The loss in activity of both the Cu–Zn enzymes is accompanied by modification of arginine and histidine residues with a stoichiometry of 1:1. The second order rate constants for the inactivation of SOD-I and SOD-II were 0.007 and 0.0086 $M^{-1}$ $min^{-1}$ for phenyl glyoxal and 0.004 and 0.006 $M^{-1}$ $min^{-1}$ for hydrogen peroxide respectively. Acetylation of the lysine residues of each isoenzyme has no effect on its catalytic activity. However, the modified SOD-III bears a more negative charge than the native enzyme.

## INTRODUCTION

Superoxide dismutases are a group of metalloenzymes (Cu–Zn, Fe and Mn) which protect aerobic cells against the deleterious effects of superoxide radicals by catalysing their dismutation to hydrogen peroxide and molecular oxygen [1]. The catalytic mechanism involves the alternate oxidation and reduction of the metal ion present at the active site of the enzyme [2]. The amino acid residues present around the metal ion provide electrostatic channelling of superoxide radicals to the active site [3]. The identification of such functionally important amino acid residues in multiple forms of an enzyme like SOD will be of immense value in the elucidation of the structural differences responsible for the catalytic process. This can be achieved through application of reagents which preferentially attack specific chemical groups [4]. Earlier, we have reported on the purification and characterization of two Cu–Zn SODs (SOD-I and III) and one Mn–SOD (SOD-II) in bajra seedlings [5]. The present report describes the effect of chemical modification of amino acid residues such as histidine, arginine and lysine present at the active site of bajra SODs.

## RESULTS

### *Effect of phenyl glyoxal*

The two Cu–Zn enzymes were found to be sensitive to phenyl glyoxal while the Mn–SOD was unaffected even at 20 mM. The time course of inactivation of SOD-I and III by different concentrations (0–2.5 mM) of phenyl glyoxal followed pseudofirst order kinetics as shown by plots of $\log(V/V_c)$ vs time. The slope of the replot of log $K_{app}$ against log [phenyl glyoxal] gave an '*n*' value of 0.9 and 1.2 for SOD-I and III indicating that a single molecule of phenyl glyoxal was reacting with one arginine residue at the active site of the Cu–Zn enzymes. The second-order rate constant for the reactivity of phenyl glyoxal with both the Cu–Zn enzymes (SOD-I and III) were 0.007 and 0.008 $M^{-1}$ $min^{-1}$ respectively.

### *Inactivation by hydrogen peroxide*

Both the Cu–Zn enzymes were susceptible to inactivation by hydrogen peroxide and the time course of inactivation followed pseudo-first-order kinetics with different concentrations (0–2.0 mM) of hydrogen peroxide. From the results obtained it was clear that of the two Cu–Zn enzymes, SOD-I appears to be more sensitive to hydrogen peroxide than SOD-III. An '*n*' value of 1.0 and 1.3 was obtained from the replot of log $K_{app}$ against log $[H_2O_2]$ indicating that one molecule of hydrogen peroxide was interacting with one histidine residue at the active site of both the Cu–Zn enzymes. The second-order rate constant for hydrogen peroxide was calculated to be 0.004 and 0.006 $M^{-1}$ $min^{-1}$ for SOD-I and III (Cu–Zn enzymes) respectively. In contrast, the Mn-SOD was found to be insensitive to hydrogen peroxide and was not inactivated even at 5 mM.

### *Acetylation of lysine residues*

Acylated bajra SODs were found to be similar to the native enzymes with respect to catalytic activity. But when analysed by PAGE and activity staining, it was found that the acylated SOD-III had increased mobility towards the anode when compared to the native enzyme. Whereas the electrophoretic mobility of the SOD-I and II was not altered significantly.

## DISCUSSION

The inactivation of the Cu–Zn enzymes on modification of the arginine residue at the active site by phenyl glyoxal clearly indicates that arginine is needed for catalytic activity. This may be due to the interaction of the negatively charged substrate ($O_2^-$) with a positively charged arginine residue in the vicinity of the $Cu^{2+}$ at the active site. Arginine has been identified as the positively charged residue providing for the binding of anions to the active site of mung bean Cu–Zn SODs [6] and bovine erythrocyte SOD [7].

Recently it has been shown that hydrogen peroxide has a high affinity for histidine residues [8]. The time course of inactivation of SOD-I and III by hydrogen peroxide suggests that a histidine residue plays an important role in the catalytic processes of each of these enzymes. The stoichiometric inactivation ratio of 1:1 observed is, by analogy with yeast [9] and mung bean SOD [6], indicative that the inactivation is accompanied by concomitant modification of a single histidine residue per sub unit. It has been reported that the affinity of hydrogen peroxide towards SOD is increased when the pH is raised from 9 to 11.5 due to generation of the reactive species $HO_2^-$, which then forms a reactive complex with the Cu present in the enzyme [10]. However, in the present study the reactivity of hydrogen peroxide observed at pH 8.0 supports the requirement of a histidine residue for the catalytic activity of both the Cu–Zn enzymes.

The activity and altered electrophoretic mobility exhibited by the acylated enzymes indicates that lysine may not be critical for the catalytic activity of bajra SODs, although it does impose a net negative charge on the native enzyme. This is in contrast to the observation made with bovine and *E. coli* SODs [11, 12]. The marked anodal mobility exhibited by acylated SOD-III indicates that it may possess a greater number of lysine residues than the other two (SOD-I and II) enzymes. However, the insensitivity of Mn-SOD towards phenyl glyoxal and hydrogen peroxide suggests that its active site environment may differ from the Cu–Zn enzymes.

## EXPERIMENTAL

Bajra SODs were purified as described earlier [5] and the activity was assayed by the ability of the protein to inhibit adrenalin autoxidation at pH 9.8 as described in ref. [13]. One unit of enzyme activity is defined as the amount of enzyme which inhibits the rate of autoxidation by 50% under standard conditions. Protein concentration was determined by the method of ref. [14].

*Arginine modification.* The isoenzyme (30 $\mu$M) in 20 mM $Na_2CO_3/HCO_3^-$ buffer, pH 9.5, was incubated with different concentrations (0–2.0 mM) of phenyl glyoxal at 30° [15]. At specific time intervals aliquots were removed and assayed for residual activity. Arginine was not added to quench the unreacted phenyl glyoxal, as it interfered with the assay. However, the amount of phenyl glyoxal carried over did not effect the assay.

*Histidine modification.* The isoenzyme (30 $\mu$M) was incubated in 25 mM K-Pi buffer, pH 8.0, containing 0.1 mM EDTA with different concentrations of (0–2.5 mM) $H_2O_2$ at 30°[6]. The time course of inactivation was followed by assaying the activity in aliquots removed from the incubation mixture at specified time intervals. The amount of $H_2O_2$ carried over into the assay mixture did not interfere with the assay.

*Acetylation of lysine residues.* Modification of lysine residues was carried out by the method of ref. [16].

The enzyme was mixed with an equal vol. of saturated NaOAc and the mixture kept in ice. $Ac_2O$ was added to 20 mM and aliquots taken after 10 and 15 min and dialysed against 10 mM Na-Pi buffer at 4° for 24 hr. The dialysed modified proteins were subjected to PAGE and activity staining was performed as described in ref. [17].

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