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Peroxidase Activity Boosting by Various Nitrogenous Compounds

Dong Joo Lee, Soung Soo Kim and Mi Young Lee†,*

Department of Biochemistry, College of Science and Bioproducts Research Center, Yonsei University, Seoul 120-749,
†Department of Life Science, College of Science, Soonchunhyang University, Shinchang, Asan, Choongnam 336-745, Korea

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Effects of various nitrogenous compounds on the peroxidative activity of Korean radish (*Raphanus sativus L.*) isoperoxidase A_1 were examined by using anilino substrates, such as dianisidine and phenylenediamine. We also used phenolic substrates such as guaiacol, chlorogenic acid, caffeic acid, ferulic acid and esculetin. The peroxidation of dianisidine was stimulated by adenine and imidazole as much as 5 fold and 11 fold, respectively at pH 8. Moreover, about 4.8 fold and 8 fold stimulation of phenylenediamine peroxidation occurred by adenine and imidazole, respectively at pH 8. The stimulation by adenine and imidazole did not occur at the acidic pH range. The peroxidations of phenolic substrates, such as guaiacol, chlorogenic acid, caffeic acid, ferulic acid and esculetin, were not boosted greatly by any of the nitrogenous compounds tested. Notably, ammonium salt, which has been known for the excellent booster of horseradish peroxidase, did not affect the peroxidation of the Korean radish isoperoxidase A_1. The kinetic studies of dianisidine peroxidation with imidazole, as a model of boosting reaction, showed that neither the affinity of imidazole against dianisidine, nor the activation energy of dianisidine peroxidation changed during the activity boosting of isoperoxidase A_1.

Keywords: Anilino substrate, Isoperoxidase, Nitrogenous compound, Phenolic substrate, *Raphanus sativus L.*

Introduction

Peroxidases are ubiquitous enzymes widely distributed in various living organisms (Lee *et al.*, 1998; Cha and Kim, 1999; Dunford, 1999; Lee and Kim, 1999). Plant peroxidases have been extensively investigated; however, the physiological role of peroxidase isozyme in the cell has not yet been properly elucidated. Since the finding of the 160 fold stimulating effect of ammonium salt on the peroxidation of dianisidine by horseradish peroxidase (HRP) (Fridovich, 1963), several investigations containing various boosters for HRP have been reported (Sitter *et al.*, 1985; Kuo and Fridovich, 1988; Bakardjieva *et al.*, 1999). The peroxidation of anilino substrate dianisidine by HRP was markedly stimulated in the presence of imidazole; whereas, the peroxidation of phenolic substrate guaiacol was not affected. In terms of the affinity of the substrate toward HRP, phenolic substrates had exhibited a greater affinity for the enzyme, suggesting the existence of hydrogen bonding and hydrophobic interactions in the binding of phenols to HRP (Schejter *et al.*, 1976). Moreover, Ca^{2+} binding by HRP C and the heme environmental structures of HRP have been reported (Ogawas *et al.*, 1979; Barberk *et al.*, 1995). Direct interaction of HRP with Ca^{2+} and the amino acids Pro, Trp, Ala and Val brought about a great thermostability, as well as activity boosting of HRP, as reported by Bakardjieva *et al.* (1999).

In Korean radish (*Raphanus sativus L.*) root there are at least eight distinguishable peroxidase isoenzymes, among them are two cationic isoperoxidases, such as C_1 and C_3, and four anionic isoperoxidases, such as A_1, A_2, A_{3n} and A_3, that were purified to near homogeneity (Lee and Kim, 1994). Their substrate utilities and physicochemical properties were examined in detail (Yoo and Kim, 1987; Lee and Kim, 1990; Lee and Kim, 1993; Lee *et al.*, 1994; Kim and Kim, 1996; Lee and Kim, 1998), and peroxidase promoter regulations by various phytohormones were also investigated (Lee *et al.*, 1999; Lee and Kim, 1999). Moreover, the entire cDNA sequence of one cationic isoperoxidase prxK1 has been determined (Park and Kim, 1996a). The deduced amino acid sequence of prxK1 cDNA showed only about 46% homology to horseradish peroxidase (Park and Kim, 1996b). In particular, prxK1 cDNA had Phe40 and His42 in the acid/base catalysis region instead of a conserved acid/base catalysis region of His40 and His42 that is found in many plant peroxidase. Chemical modification studies of Korean radish isoperoxidase A_1 also suggested that there might be different heme environmental conformations between Korean radish peroxidase and HRP (Lee and Kim, 1993). However, an investigation of peroxidase activity in Korean radish has not yet been undertaken.

*To whom correspondence should be addressed.
Tel: 82-41-530-1355; Fax: 82-41-530-1350
E-mail: miyoung@asan.sch.ac.kr

In this report, the effects of various nitrogenous compounds on the peroxidations of anilino substrate and phenolic substrate by Korean radish isoperoxidase A_1 are described. The biochemical basis of peroxidase activity boosting was also investigated by using kinetic analysis.

Materials and Methods

Preparations of isoperoxidase A_1 Korean radish isoperoxidase A_1 was purified by using DEAE-Sephacel chromatography and Sephadex G-150 gel filtration as reported earlier (Lee and Kim, 1994).

Effect of nitrogenous compounds on the peroxidative activities of isoperoxidase A_1 The stimulatory effects of nitrogenous compounds on the peroxidations of several substrates were examined. The reaction mixtures contained 0.18 mM nitrogenous compounds, 0.36 mM H_2O_2, and 42 nM isoperoxidase A_1 in a total volume of 1 ml. The buffer systems were 50 mM sodium acetate buffer, 50 mM sodium phosphate buffer and 50 mM Tris-HCl buffer, depending upon the pH ranges studied. Typical assay conditions and wavelengths for dianisidine, guaiacol, phenylenediamine, chlorogenic acid, caffeic acid, ferulic acid and esculetin were determined according to the method of Kim *et al.* (1980). The nitrogenous compounds tested here included: adenine, guanine, cytosine, ammonia, histidine, imidazole, nicotinic acid, tryptophan and uracil.

Effect of imidazole on the kinetics of dianisidine peroxidation The effects of various concentrations of imidazole on the kinetics of dianisidine peroxidation were examined in order to know whether or not imidazole might influence the binding affinity of dianisidine. Reaction mixtures contained 50 mM Tris-HCl buffer (pH 8.0), 0.36 mM H_2O_2, the indicated concentrations of dianisidine, 42 nM isoperoxidase A_1 and various concentrations of imidazole (0-0.18 mM). Initial velocities of enzyme reactions in the absence and presence of imidazole were determined on reciprocal coordinates.

Effect of imidazole on the activation energy of dianisidine peroxidation The activation energy for the dianisidine peroxidation by isoperoxidase A_1 was measured at 283 K (10°C), 288 K (15°C), 293 K (20°C) and 303 K (30°C) in the absence and presence of imidazole. The slope was measured according to the Arrhenius equation (Kuo and Fridovich, 1988).

Results and Discussion

Effect of various nitrogenous compounds on the peroxidative activity of isoperoxidase A_1 Effects of various nitrogenous compounds on the peroxidations of anilino substrates as well as phenolic substrates by Korean radish isoperoxidase A_1 were examined. Isoperoxidase A_1 was reported to be the most abundant isoperoxidase in Korean radish root (Lee and Kim, 1994). As shown in Table 1, the peroxidations of anilino substrate dianisidine and phenylenediamine were stimulated by adenine, as much as 5 fold and 4.8 fold respectively at pH 8. Moreover, about 11 fold and 8 fold stimulations of dianisidine and phenylenediamine peroxidation occurred by imidazole, respectively. Ammonium ion, guanine, histidine and nicotinate showed only a slight activity increase, and tryptophan and uracil inhibited peroxidative activity to some extent when dianisidine or phenylenediamine was used as a substrate. Therefore, imidazole seemed to be the most powerful booster of anilino substrates, such as dianisidine and phenylenediamine, in the Korean radish peroxidase system. On the contrary, the peroxidations of phenolic substrates, such as guaiacol, caffeic acid, ferulic acid and esculetin, were not boosted by various nitrogenous compounds such as ammonium ion, cytosine, histidine, imidazole, nicotinic acid, tryptophan, uracil and so on. Only a slight increase in peroxidation of phenolic substrate chlorogenic acid was found with adenine and ammonium ion.

Interestingly, ammonium ion, which had been reported to be about a 160 fold stimulator of dianisidine peroxidation in the HRP boosting system (Fridovich, 1963), had no effect on the Korean radish peroxidase boosting, although the boosting effects of imidazole were indiscriminately found in isoperoxidase A_1 as well as HRP. These results might reflect some structural differences of Korean radish peroxidase from HRP. Different heme environmental conformations between the Korean radish peroxidase and HRP had been suggested by chemical

Table 1. Effects of various nitrogenous compounds on the peroxidative activity of isoperoxidase A_1* from Korean radish

Substrate	Booster	Adenine	Ammonium ion	Cytosine	Guanine	Histidine	Imidazole	Nicotinate	Tryptophan	Uracil
Anilino Substrate	Dianisidine	500	120	90	104	104	1100	110	68	65
	Phenylenediamine	480	114	105	122	118	800	118	80	86
Phenolic Substrate	Guaiacol	113	95	90	105	98	120	105	98	79
	Chlorogenic acid	125	125	103	69	92	89	103	70	80
	Caffeic acid	96	96	88	87	92	80	80	80	74
	Ferulic acid	101	83	98	88	80	90	95	95	78
	Esculetin	106	85	96	95	95	97	101	74	70

*The values presented here have been shown relative to the peroxidative activity without booster which was given a value of 100. All experiments were repeated four times.

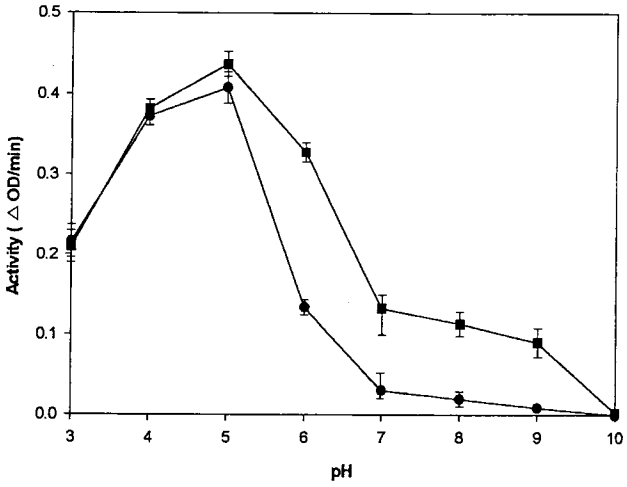

Fig. 1. Effect of pH on the peroxidation of dianisidine by the Korean radish isoperoxidase A_1 in the presence of imidazole. Reaction mixtures contained 50 mM buffer (pH 3.0-pH 5.0: sodium acetate buffer, pH 6.0-pH 7.0: sodium phosphate buffer, pH 8.0-pH 10.0: Tri-HCl buffer), 0.36 mM H_2O_2, 0.18 mM dianisidine, 40 unit peroxidase. ●-●, no imidazole; ■-■, 0.18 mM imidazole.

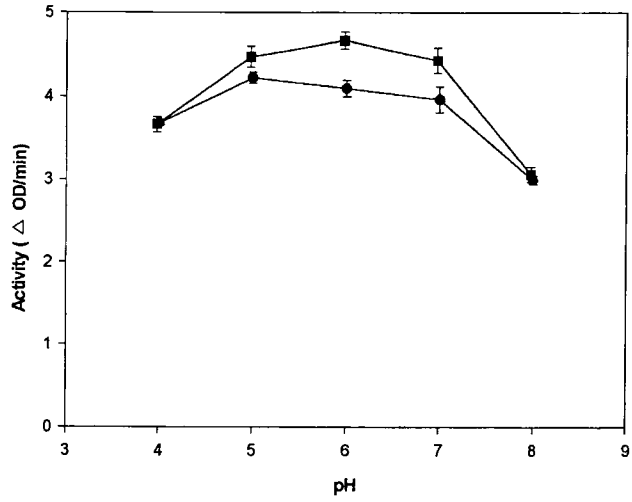

Fig. 2. Effect of pH on the peroxidation of guaiacol by the Korean radish isoperoxidase A_1 in the presence of imidazole. Reaction mixtures contained 50 mM buffer (pH 4.0-pH 5.0: sodium acetate buffer, pH 6.0-pH 7.0: sodium phosphate buffer, pH 8.0-pH 10.0: Tris-HCl buffer), 0.36 mM H_2O_2, 15 mM guaiacol, 40 unit peroxidase. ■-■, no imidazole; ●-●, 0.18 mM imidazole.

modification (Lee and Kim, 1993b) as well as R.Z. (Reinheitzal) value (Lee and Kim, 1994). The R.Z. values of the Korean radish isoperoxidases, such as A_1, A_2, A_{3n} and A_3, ranged in 1.5-1.8; whereas those of the horseradish peroxidase were 3.04. A computer analysis of the deduced amino acid sequence of Korean radish cationic isoperoxidase prxK1 cDNA showed only about 46% homology to horseradish peroxidase (Park and Kim, 1996a). Instead, the Korean radish prxK1 had 92% homologous sequences with turnip peroxidase (Park and Kim, 1996b). In particular, prxK1 cDNA had Phe40 and His42 in the acid/base catalysis region (Park and Kim, 1996b). However, all known plant peroxidases, except turnip TP7, were reported to have His40 and His42 in the acid/base catalysis region (Lobarzewski *et al.*, 1991). Therefore, a unique activity stimulatory reaction seems to occur at or near the heme environmental structure of the Korean radish isoperoxidase A_1.

Effect of pH on the stimulating effect of imidazole As a model of boosting reaction, the stimulating effect of imidazole on dianisidine peroxidation was investigated at various pH values. Figure 1 shows that the boosting effect of imidazole on dianisidine peroxidation occurred in a pH dependent manner. The notable stimulating effect of imidazole could be seen only in the alkaline pH range. The peroxidative activity with imidazole at pH 6.0 was 0.32, and the control activity without imidazole was 0.13. This indicates that about 2.5 fold activity boosting occurred with imidazole at pH 6. At pH 8, the stimulated peroxidative activities with imidazole and without imidazole, were 0.11 and 0.01, respectively. This shows approximately an 11 fold stimulation of dianisidine peroxidation. On the other hand, the stimulation by imidazole did not occur

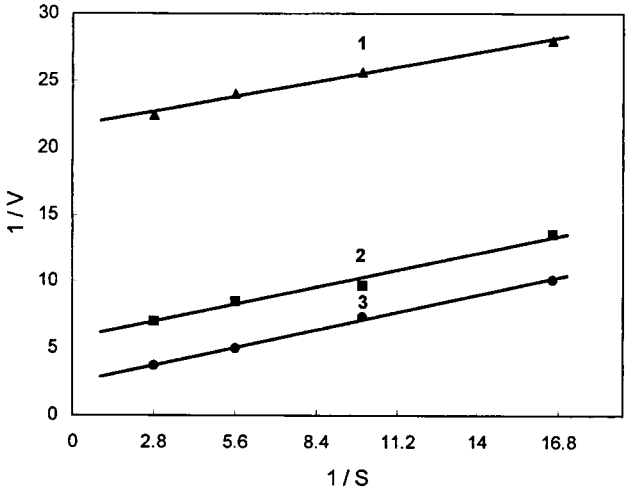

Fig. 3. Effect of imidazole on the kinetics of peroxidation of dianisidine by the Korean radish isoperoxidase A_1. Reaction mixtures contained 50 mM Tris-HCl buffer (pH 8.0), 0.36 mM H_2O_2, 40 unit peroxidase, the indicated concentrations of dianisidine, and the following concentrations of imidazole: line 1, no imidazole; line 2, 0.09 mM imidazole; line 3, 0.18 mM imidazole. Initial rates are presented here as a function of dianisidine concentration on reciprocal coordinates.

at lower pH values. The optimum pH for dianisidine peroxidation by A_1 was reported to be pH 5.0 (Lee and Kim, 1994), thus activity boosting versus pH might shift into the alkaline pH region with imidazole. These results suggested that the rise of pKa value of some essential amino acids at or near the active site of isoperoxidase A_1 might be involved in the boosting effect of imidazole on dianisidine peroxidation.

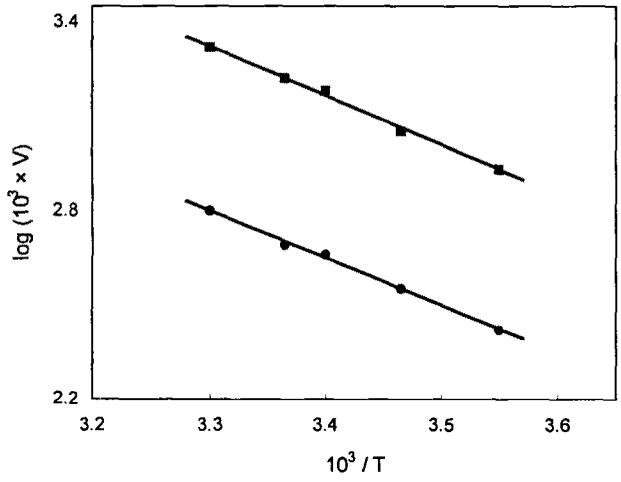

Fig. 4. Effect of imidazole on the activation energy for dianisidine peroxidation by the Korean radish isoperoxidase A_1. Reaction mixtures contained 50 mM Tris-HCl buffer (pH 8.0), 0.36 mM H_2O_2, 0.18 mM dianisidine, 40 unit peroxidase and 0.18 mM imidazole at various temperatures. The log of the initial rate of peroxidation of dianisidine is presented here as a function of reciprocal absolute temperature ●-●, no imidazole; ■-■, 0.18 mM imidazole.

However, the boosting effect of imidazole on the dianisidine peroxidation did not occur in myeloperoxidase, chloroperoxidase, *E. coli* hydroperoxidase and methemoglobin peroxidase as reported earlier (Kuo and Fridovich, 1988).

As shown in Fig. 2 the stimulating effects of imidazole on anilino substrate did not occur when phenolic substrate guaiacol was used. Highly conserved hydrogen bond networks in the distal site were reported to be required for peroxidase catalysis. The disruption of hydrogen bonds with distal His exhibited very low V_{max} in HRP (Nagano *et al.*, 1996). Furthermore, phenolic substrates were reported to hydrogen-bond directly to ferryl oxygen of heme moiety. Therefore, the reason for no response of phenolic substrate toward imidazole in isoperoxidase A_1 might be explained by the direct H-bonding of guaiacol to the heme moiety of isoperoxidase A_1. This direct H-bonding might eliminate the possibility of being stimulated by nitrogenous boosters as suggested by Kuo and Fridovich (1988).

The kinetics of the dianisidine peroxidation by Korean radish isoperoxidase A_1 The kinetics of dianisidine peroxidation by isoperoxidase A_1 with various concentrations of imidazole were shown in Fig. 3. Parallel increases of the K_m values and V_{max} for dianisidine without the change of the slope of the Lineweaver-Burk plot in the presence of imidazole were found. These results showed that imidazole did not increase its apparent affinity for dianisidine, but it did greatly stimulate the peroxidative activity of isoperoxidase A_1 in terms of K_m values and V_{max}. In order to know the possibility of the reaction mechanism change during activity boosting by imidazole, the activation energy was measured in the absence and in the presence of imidazole (Fig. 4). The results in Fig. 4, plotted according to the Arrhenius equation, showed a parallel slope in the absence and in the presence of imidazole. These data suggest that the boosting effect of imidazole is not accompanied by a change of activation energy; therefore, it seems that imidazole does not use a new boosting reaction pathway with a lower activation energy. Therefore, a more detailed clarification of the reaction mechanism and active site structure of the Korean radish isoperoxidase A_1 will be needed in order to understand the boosting effect completely.

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