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STIMULATORY EFFECT OF DIMBOA ON NADH OXIDATION CATALYSED BY HORSERADISH PEROXIDASE

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Key Word Index—Hydroxamic acids; 1,4-benzoxazin-3-ones; DIMBOA; peroxidase; NADH oxidase.

Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) effectively enhanced the rate of NADH oxidation catalysed by horseradish peroxidase isoenzyme C (HRPC), a reaction that generates H_2O_2. The effect showed saturation kinetics with increasing DIMBOA concentrations and the extrapolated maximum rate gave an activating factor of 246 for this hydroxamic acid. After 80% of the NADH had been oxidized, DIMBOA had converted into a species that showed absorption bands centered at 430 and 345 nm. A product with the same absorption properties was formed upon oxidation of DIMBOA with H_2O_2. The V_{max} for the latter reaction was 3.3 times lower than the maximum rate of NADH oxidation in the presence of DIMBOA. Ferulic and *p*-coumaric acids were also stimulators of NADH oxidase activity of HRPC, giving saturating kinetics. Maximum rates obtained for these effectors give activating factors of 35 and 1170, respectively. The DIMBOA analogue 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA) that lacks the methoxy substitution in the aromatic ring was only 16% efficient in stimulating NADH oxidase as compared with DIMBOA at the 0.1 mM level. On the other hand, the benzoxazolinones 6-methoxy-benzoxazolin-2-one (MBOA) and benxoxazolin-2-one (BOA) had negligible effect on the rate of NADH oxidation and did not alter the activating effect of DIMBOA over this reaction.

INTRODUCTION

Peroxidases (EC 1.11.1.7) participate in the modulation of cell growth in plant tissues catalysing the oxidative cross linking of cell wall polysaccharides and proteins [1]. Moreover, peroxidases can generate the H_2O_2 required by these reactions through the oxidation of NADH with molecular oxygen [2–4]. The latter reaction has been postulated as the possible source of hydrogen peroxide at the cell wall level [1, 3] where this oxidant is needed in the last phase of plant growth for the cross linking of ferulic and *p*-coumaric acid residues bound to polysaccharides and of tyrosine residues bound to proteins [5, 6]. The finding of a malate dehydrogenase in the cell wall that could generate the NADH required as substrate supports this proposition [7].

We have been interested in characterizing the cell wall peroxidases present in oat coleoptiles in relation to their role in cell growth modulation and we have found that NADH oxidase activity present in oat cell wall preparations is increased by a factor of about 400 upon addition of the natural hydroxamic acid DIMBOA (unpublished). This compound is native to maize and wheat and we have found that it markedly inhibits the growth of oat coleoptiles (unpublished). The enhancement of NADH oxidation by DIMBOA could account for the growth inhibitory effect shown by this compound by increasing the concentration of H_2O_2 at the cell wall level.

Because the oat cell wall preparation contains many proteins and the study of the effect of DIMBOA and NADH oxidation by peroxidases may be influenced by several interferences, we decided to further study this effect with a commercial preparation of horseradish peroxidase (HRP) as a model system. It has already been shown that hydroxamic acids alter several reactions catalysed by HRP [8, 9]: NADH oxidation is enhanced by benzhydroxamic and salicylhydroxamic acids, while phenol oxidation with H_2O_2 is inhibited by these compounds. Other peroxidase reactions such as ferrocyanide oxidation are stimulated by hydroxamic acids [9]. The enhancement by hydroxamic acids of NADH oxidase activity of HRP is probably analogous to the stimulatory effect already described for phenols. Activating phenols are also oxidized by this enzyme in the presence of H_2O_2,

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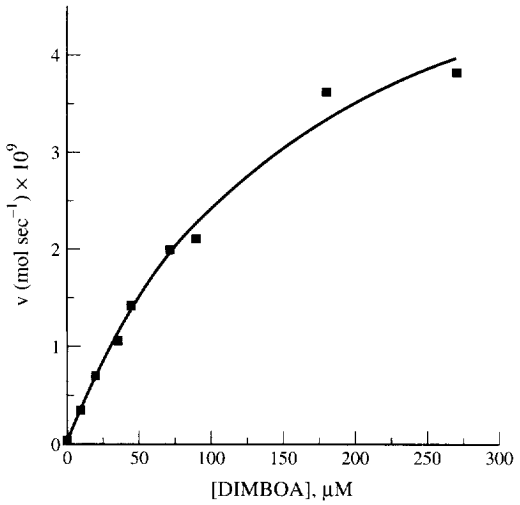

Fig. 1. Effect of DIMBOA on the rate of NADH oxidation by horseradish peroxidase isoenzyme C. Reaction conditions as described in Experimental with HRPC 0.017 μM.

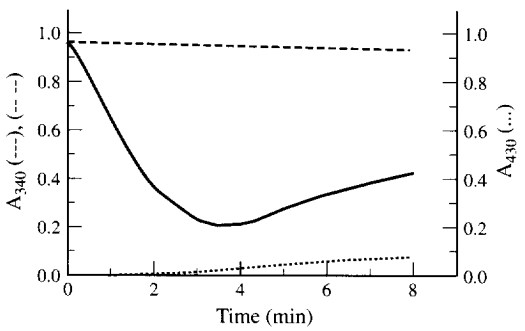

Fig. 2. Time course of NADH and DIMBOA oxidation during NADH oxidase activity determination. (——) Absorbance at 340 nm with DIMBOA 180 μM; (– – –) absorbance at 340 nm in the absence of DIMBOA; (....) absorbance at 430 nm with DIMBOA 180 μM. Reaction conditions as described in Experimental with HRPC 0.005 μM.

and an inverse correlation has been found between the rate of oxidation of these compounds and their effectiveness in activating NADH oxidase [10]. The results reported here support this hypothesis as we found that the natural hydroxamic acid DIMBOA is a very efficient activator of NADH oxidation catalysed by HRPC, while this compound is poorly utilized by the enzyme in the presence of H_2O_2.

RESULTS AND DISCUSSION

Enhancement of NADH oxidation by DIMBOA

The rate of NADH oxidation in the presence of 30 μM $MnCl_2$ and 0.017 μM HRPC (0.026×10^{-9} mol sec^{-1}) increased when DIMBOA, the major hydroxamic acid found in maize, was added to the reaction media. The rate enhancement levelled off at effector concentrations higher than 200 μM (Fig. 1). The data gave a good fit to a hyperbolic function with an extrapolated maximum velocity of 6.4×10^{-9} mol sec^{-1}. The half saturating concentration, that gives a measure of the affinity of DIMBOA for the enzyme was 166 μM. Thus, this hydroxamic acid effectively enhances the NADH oxidase activity of HRP.

When the time course of NADH oxidation in the presence of DIMBOA was monitored at 340 nm (Fig. 2), a biphasic curve was obtained: the *A* decreased from an initial value of 0.96 to a value of 0.21 and then it increased again to give a value of 0.4 after 8 min of reaction. Concomitant with the latter phase, a brown-yellow compound was formed in the reaction media, that showed absorption bands in the visible region at 430 nm and at 345 nm. When the reaction was monitored at the former wavelength (Fig. 2), an increase in *A* was observed after a lag of about 3 min, parallel with the increase in *A* at 340 nm. This result suggested that DIMBOA was oxidized in the reaction medium and that the oxidizing agent was not molecular oxygen but hydrogen peroxide that accumulated as a result of NADH oxidation. One confirmation for this suggestion came from the fact that DIMBOA was not oxidized in the absence of NADH under the above reaction conditions. Data in Fig. 2 also showed that DIMBOA was not transformed until NADH oxidation had almost been completed and thus it can be deduced that the activating effect of DIMBOA does not involve its oxidation.

Stimulation of NADH oxidation by salicyl and benzhydroxamic acids has already been described with HRP [9] and it has been suggested that hydroxamate free radicals would mediate the effect, acting as cyclic intermediates by generating substrate radicals that would react with oxygen. On the other hand, phenols, that are well known activators of NADH oxidation catalysed by HRP [2, 11], have been demonstrated to enhance the rate of decomposition of compound III, an inactive peroxidase intermediate generated during NADH oxidation [2]. Decomposition of compound III could also be increased by DIMBOA but we have not yet explored this possibility.

Several spectroscopic studies have revealed that aromatic hydroxamic acids form complexes with HRP [8, 11–14]. The binding of these compounds is competitive with respect to hydrogen donor substrates. Iron chelation is not involved in hydroxamate binding to the enzyme and it has been suggested that the hydrophobic site that binds phenols would be implicated in this interaction [8, 12, 15]. However, binding of hydroxamic acids to free peroxidase is probably not related to their activating effect on NADH oxidation but instead this effect would be related to the binding to some of the enzymatic intermediates that occur during the catalytic cycle.

Oxidation of DIMBOA by H_2O_2

In order to investigate the origin of the product formed from DIMBOA during NADH oxidation, the transformation of this hydroxamic acid by added

Table 1. Effect of DIMBOA and *p*-hydroxycinnamic acids on NADH oxidation by horseradish peroxidase isoenzyme C

ACTIVATOR	$[A]_{0.5}$ (μM)	$V_{max} \times 10^9$ (mol sec^{-1})	Activating factor*
Ferulic acid	197	0.9	35
p-Coumaric acid	39	30.4	1170
DIMBOA	166	6.4	246

Reaction conditions as described in Experimental with HRPC 0.017 μM.

* Ratio between V_{max} and the rate of NADH oxidation in the absence of effectors (0.026×10^{-9} mol sec^{-1}).

$[A]_{0.5}$, effector concentration that gives half of the maximum rate.

H_2O_2 was monitored at 430 nm. DIMBOA was a substrate of HRP under these conditions, giving a product that showed absorption bands centred at 345 nm and at 430 nm, the same that were observed at the end of NADH oxidation in the presence of DIMBOA. From the A obtained when DIMBOA oxidation was complete, a molar extinction coefficient of 1260 cm^{-1} M^{-1} was calculated for its oxidation product at 430 nm. With this value, DIMBOA oxidation rates were calculated at different substrate concentrations (Fig. 3). These data revealed that DIMBOA is utilized in the presence of 0.017 μM HRPC and 5 mM H_2O_2 with a maximum rate of 1.93×10^{-9} mol sec^{-1}, 3.3 times lower than that obtained for NADH oxidation in the presence of DIMBOA. The apparent K_m value for DIMBOA was 135 μM. The effectiveness of substrate oxidation with H_2O_2 by HRP has been correlated with the rate of reaction of H donors with compound II, that appears to be the limiting step in peroxidase catalysed reactions [16]. Pedreño *et al.* [10] found an inverse correlation between the efficiency of phenol peroxidation by lupin peroxidase and the effectiveness of these compounds in activating NADH oxidation. The activating effect of DIMBOA found for HRPC in the present work, points in the same direction, for this hydroxamic acid is an efficient activator of NADH oxidation and a less efficient substrate in the peroxidation reaction. The low utilization efficiency of DIMBOA in the presence of H_2O_2 would also account for the fact that this hydroxamic acid is not oxidized during NADH utilization (Fig. 2) even when inhibition of DIMBOA peroxidation by NADH could also contribute to this effect. Other hydroxamic acids such as salicyl and benzhydroxamic acids have been shown to suffer oxidative destruction by HRP in the presence of H_2O_2 giving nitrite as a product [8]. This would account for their strong inhibitory effect in the peroxidation of other substrates.

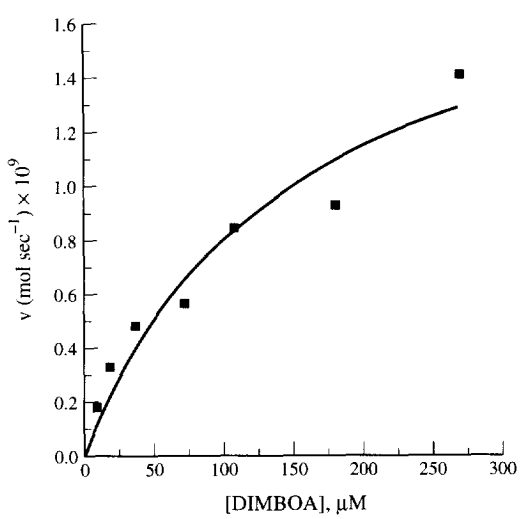

Fig. 3. Oxidation of DIMBOA by H_2O_2 and horseradish peroxidase isoenzyme C. Reaction conditions as described in Experimental with H_2O_2 5 mM and HRPC 0.017 μM.

Stimulatory effect of p-*hydroxycinnamic acids on NADH oxidation catalysed by HRPC*

The natural plant phenolic compounds ferulic and *p*-coumaric acids also enhanced NADH oxidase activity of HRPC with a hyperbolic dependence on the effector concentration. The values obtained from these data for the maximum velocities and for the half saturating concentrations are shown in Table 1 and compared with those found for DIMBOA.

The hydroxycinnamic derivative, *p*-coumaric acid, enhanced the reaction rate by a factor of 1170, that is 4.7 times higher than the activating factor found for DIMBOA. Ferulic acid on the other hand, was a very poor activator, giving a rate enhancement of only 35 times. These findings are relevant because ferulic and *p*-coumaric acids are present in plant cell walls and have been postulated as possible natural effectors of peroxidases [17]. Our results suggest that only *p*-coumaric acid would have a significant role in H_2O_2 generation through the oxidation of NADH. Stimulation of NADH oxidase activity of HRP by salicyl, benzhydroxamic and phenylacetohydroxamic acids has been shown to be similar to that obtained with *p*-cresol or 2,4-dichlorophenol. The activating factors obtained for the above hydroxamic acids were slightly higher than that found for phenols, in the range of 200–600 [9], similar to the value reported here for DIMBOA.

Table 2. Effect of maize hydroxamic acids and their decomposition products on NADH oxidase activity of horseradish peroxidase isoenzyme C

EFFECTOR*	NADH oxidation rate $\times 10^9$ (mol sec^{-1})	Relative rate
DIMBOA	2.02	1
DIMBOA	0.32	0.16
BOA	0.1	0.05
MBOA	0.08	0.04
DIMBOA + DIBOA	1.22	0.6
DIMBOA + MBOA	2.02	1
DIMBOA + BOA	2.06	1.02

* All effector concentrations were 0.1 mM. Reaction conditions as described in the Experimental with HRPC 0.017 μM.

Effect of DIMBOA analogues on the rate of NADH oxidation

Even when the effect that has been described for salicyl and benzhydroxamic acids is similar or higher than that reported here for DIMBOA, we found that the hydroxamate function is not enough to give an activating effect. The methoxy substituent in the aromatic moiety is also necessary, since DIBOA, a hydroxamic acid present in rye and wheat that does not contain this substitution, is a very poor activator of NADH oxidation catalysed by HRPC (Table 2). On the other hand, the decomposition products of DIMBOA and DIBOA (MBOA and BOA), that lack the hydroxamate function were not activators of NADH oxidase. Experiments done with DIMBOA in the presence of both MBOA or BOA at the 0.1 mM level, showed that these compounds did not alter the activating effect of DIMBOA (Table 2), suggesting that they do not bind or bind with very low affinity to HRP. On the other hand, DIBOA decreased the rate of NADH oxidation by 40% in the presence of DIMBOA, as expected for a competing effector ligand.

The results here reported indicate that DIMBOA is an efficient activator of NADH oxidation catalysed by HRPC and thus support our preliminary results obtained with DIMBOA and an oat cell wall protein fraction. Activation by DIMBOA appears not to involve the oxidation of this hydroxamic acid. We also report here that DIMBOA is less effectively utilized by HRPC in the presence of H_2O_2 than NADH in the presence of O_2 and this hydroxamic acid. Thus, DIMBOA can effectively enhance the peroxidase-catalysed generation of H_2O_2 from NADH.

EXPERIMENTAL

Reagents. All reagents used were of analytical grade. Horseradish peroxidase (Type IX, isoenzyme C; R/Z = 3.2) was purchased from Sigma. DIMBOA and DIBOA were obtained from maize seedlings according to the procedure of ref. [18]. BOA was purchased from Aldrich. MBOA was synthesized as described [19].

Enzymatic assays. NADH oxidation by horseradish peroxidase was recorded at 340 nm and 22° in a Shimadzu UV-120 spectrophotometer. The reaction medium contained: 0.1 M K-Pi buffer pH 7, 30 μM $MnCl_2$ and 160 μM NADH, HRPC (0.005 μM or 0.017 μM) and different concns of the effectors. The molar concn of HRPC was determined using the Soret *A* at 403 nm and an extinction coefficient of 102 000 cm^{-1} M^{-1}. A molar extinction coefficient of 6220 cm^{-1} M^{-1} was utilized for NADH at 340 nm. DIMBOA oxidation was monitored at 430 nm, utilizing the value determined in this work for the extinction coefficient of its oxidation product (1260 cm^{-1} M^{-1}).

Curve fittings. Rates were determined from the initial slopes of the progress curves and the rate values obtained at different effector or substrate concns were fitted to hyperbolic functions using the program Sigma Plot. V_{max} values as well as semisaturation effector concns or K_m values, were obtained as the constant parameters of the respective hyperbolic functions.

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