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REACTION OF DIMBOA, A RESISTANCE FACTOR FROM CEREALS, WITH PAPAIN

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Key Word Index—Gramineae; resistance factor; hydroxamic acids; 1,4-benzoxazin-3-ones; thiols; papain inactivation; DIMBOA.

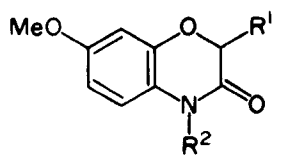
Abstract—2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a hydroxamic acid from cereals involved in host plant resistance to insects, inactivated papain (EC 3.4.2.2). Semilog plots of the residual activity of papain in the presence of an excess of DIMBOA as a function of time were concave downwards. Cysteine and serine were significantly modified by DIMBOA inactivation. Loss of enzyme activity by reaction with DIMBOA parallelled loss of thiol titre of the enzyme. DTT partially restored the activity of papain inactivated by DIMBOA. A kinetic model involving the reaction of a non-essential serine residue of papain followed by the reaction of essential Cys-25 with DIMBOA quantitatively accounted for these results. 4-Hydroxy-7-methoxy-1,4-benzoxazin-3-one inactivated papain in a manner similar to DIMBOA, whereas 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one showed no effect on papain. These results suggest that the inactivation of papain by DIMBOA occurs by interaction of the sulphur atom of Cys-25 with the hydroxamic nitrogen of DIMBOA. The results are discussed in relation to the toxicity of DIMBOA towards insects.

INTRODUCTION

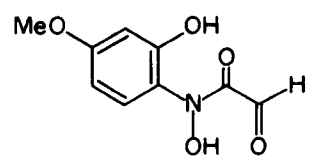
Hydroxamic acids (Hx) such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, **1**), play a defensive role in wheat and maize plants against insects [1–3]. The toxicity of these acids is associated with their interference with key metabolic processes, such as energy transduction in mitochondria [4].

The chemical properties of DIMBOA in solution [5, 6] suggested that enzymic inhibitions may be due to the reaction of DIMBOA with nucleophilic residues in the enzymes [7]. The reaction of DIMBOA with thiols in aqueous solutions was described and a mechanism for it proposed [8, 9]. It remains to be demonstrated whether enzymic sulphydryl groups are able to react with DIMBOA.

An ideal model system for the study of the reaction of DIMBOA with a sulphydryl group in an enzyme is papain (EC 3.4.2.2), since it has a single free cysteine residue, that of the active site (Cys-25), and its structure and mechanism of catalysis are well known [10, 11]. In this work we show that DIMBOA inactivates papain through reaction of its nitrogen atom with Cys-25.



	R^1	R^2
1	OH	OH
3	H	OH
4	OH	H



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RESULTS AND DISCUSSION

Inactivation of papain by DIMBOA

The semilog plots of the decrease with time of papain activity in the presence of an excess of DIMBOA, gave curves whose slopes increased in a negative sense with time (Fig. 1). This pattern may be interpreted by several kinetic models involving one or more amino acid residues in the inactivation process [12]. Amino acid analysis showed that only cysteine and serine decreased significantly in the modified enzyme as compared with the

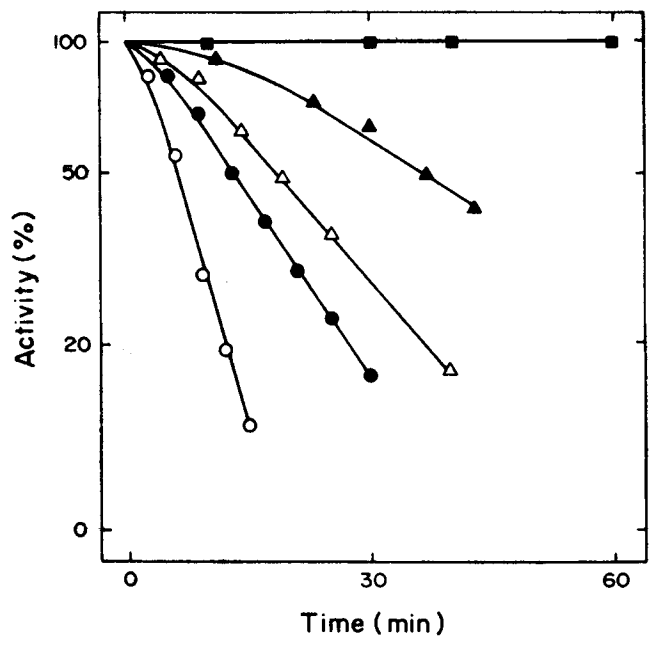

Fig. 1. Kinetics of papain inactivation at different DIMBOA concentrations: 0 (■), 3 (▲), 5 (△), 8 (●), and 15 mM (○), in 50 mM phosphate buffer, pH 6.8, at 28°. The concentration of papain was 1.1×10^{-5} M. The curves represents the fit of the experimental points to eqn (1). Initial specific activity was 8.1 μmol/min/mg protein.

Table 1. Amino acid analysis of papain and papain modified by DIMBOA

Amino acid	Native* papain (ref. [13])	Modified* papain	% Change
Try	5	nd†	—
Lys	10	10.4	3.8
Arg	12	12	0
Cystine (half)	6	nd†	—
CM-Cys	1	0.5	50
Asp	19	19.6	3.2
Thr	8	7.7	3.8
Ser	13	9.1	30
Glu	20	20	0
Pro	10	9.6	4
Gly	28	27.8	1.3
Ala	14	14.8	5.4
Val	18	16.8	6.6
Ile	12	12	0
Leu	12	11.6	3.3
Tyr	19	19.3	1.6
Phe	4	4.1	2.5

*Residues per mol protein. Data was normalized with respect to arginine and glutamic acid.

†Not detected by this method.

native one (Table 1) [13]. Although tryptophan and cysteine were not detected by this method, these amino acids do not react with DIMBOA in aqueous solution [14]. On the other hand, the ε-amino group of lysine does react with DIMBOA. However, this reaction is negligible at the pH used in the inactivation of papain by DIMBOA [15].

Two models may account for the results. In the first model, the modification of either residue separately will not affect enzyme activity, but modification of both will inactivate the enzyme. In the second model, the modification of one amino acid residue does not affect enzyme activity, but does affect the protein molecule in such a way that a second amino acid residue, essential for enzyme activity, becomes susceptible to inactivator attack. In this latter model, the modification rate of the second amino acid residue must correlate with the loss of enzyme activity.

Papain was incubated with DIMBOA and the thiol titre and activity were determined in parallel. A linear correlation with a slope close to one was obtained between the loss of activity and the loss of thiol titre (Fig. 2), indicating that the second model is consistent with our kinetic data. The equations describing this model are as follows:

$$E+I \xrightarrow{k_1} E_1+I \xrightarrow{k_2} E_{1,2}$$

$$\frac{E}{E_0}=\frac{k_2}{k_2-k_1}e^{-k_1t}-\frac{k_1}{k_2-k_1}e^{-k_2t} \qquad (1)$$

The experimental points for the inactivation kinetics were least-squares-fitted to this equation. The theoretical lines generated are shown in Fig. 1. Pseudo-first order rate constants were obtained from the fit. Second order rate

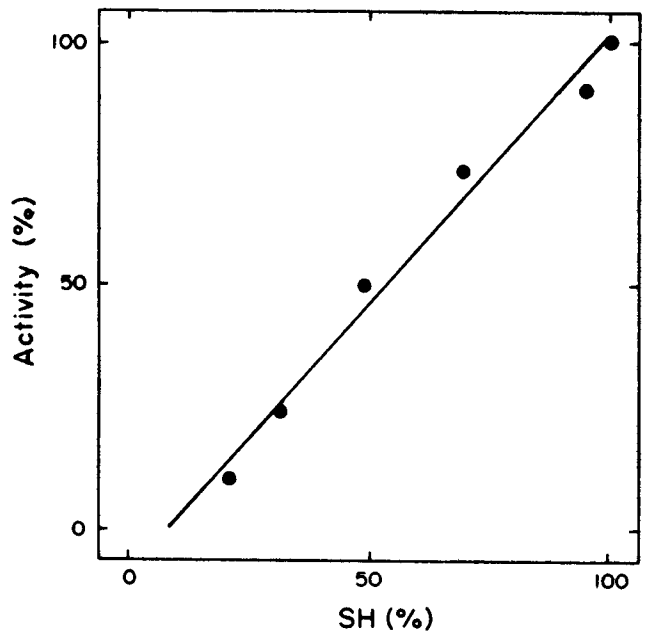

Fig. 2. Proportionality of activity and thiol content of papain as percentage of initial values, during inactivation of the enzyme by DIMBOA. Initial specific activity was 8.9 μmol/min/mg protein, and initial thiol titre was 0.57 mol SH/mol enzyme.

constants determined from the slope of a plot of pseudo-first order rate constants versus DIMBOA concentration were $k_1=50\pm5\ \text{min}^{-1}\ \text{M}^{-1}$ and $k_2=10\pm2\ \text{min}^{-1}\ \text{M}^{-1}$.

Inactivation of papain by DIMBOA analogues

DIMBOA is known to react with thiols through the reactive aldehyde group of aldol **2**, in equilibrium with DIMBOA in solution, or through the hydroxamic nitrogen atom [9]. The reaction of DIMBOA with Cys-25 of papain was determined from studies with DIMBOA analogues **3** and **4**. Compound **3** cannot form an open chain tautomer, and hence does not generate a reactive aldehyde group. Compound **4** forms an open chain tautomer with a reactive aldehyde group, but does not have a hydroxamic nitrogen. Inactivation studies with these compounds (50 mM phosphate buffer, pH 6.8) showed that while compound **3** inactivated papain with a kinetic pattern (results not shown) and rate constants ($k_1=47\pm4\ \text{min}^{-1}\ \text{M}^{-1}$; $k_2=9\pm1\ \text{min}^{-1}\ \text{M}^{-1}$) similar to that of DIMBOA, compound **4** did not alter papain activity in the same time period.

These results indicate that the reaction of papain with DIMBOA occurs by interaction of the sulphur atom of Cys-25 with the hydroxamic nitrogen of DIMBOA, in a way presumably analogous to the reduction of DIMBOA by thiols [9]. In the non-enzymatic reaction, an intermediate with the sulphur atom bonded to nitrogen reacts with a second thiol molecule to generate a disulphide and the corresponding lactam of DIMBOA [9]. In the reaction of DIMBOA with papain this is unlikely due to steric and concentration effects. Consistent with this proposition, activity was recovered by addition of dithiothreitol (DTT) to inactivated papain (Fig. 3). The DTT added presumably reacted at the sulphur atom of Cys-25 bonded covalently to the hydroxamic nitrogen of DIMBOA, causing the formation of DIMBOA-lactam and a mixed disulphide (enzyme-DTT), which upon reaction with further DTT liberated the active enzyme.

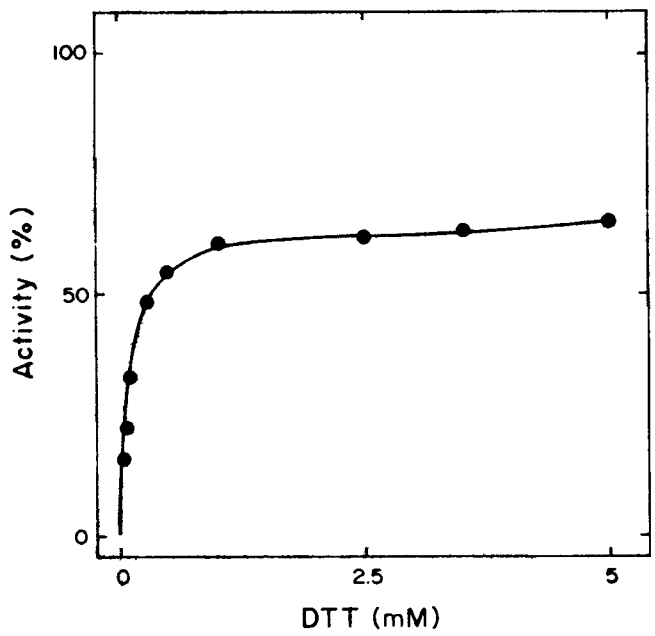

Fig. 3. Recovery of activity by different DTT concentrations of papain inactivated by DIMBOA.

Toxicity of DIMBOA towards insects

DIMBOA is toxic towards several cereal pest insects such as the larvae of the European corn borer, *Ostrinia nubilalis*, and the cereal aphids [1]. The mechanism of toxicity is unknown.

Insect digestive enzymes have been suggested as possible targets for the action of plant defence chemicals [16]. Thiol proteinases have been isolated from the gut of *Callosobruchus maculatus F.* a phytophagous insect [17]. Recently, it was demonstrated that DIMBOA inhibits proteinases isolated from the gut of *Ostrinia nubilalis* [F. Campos, J. Houseman, J. Atkinson and J. T. Arnason, personal communication]. DIMBOA also inactivated α-chymotrypsin [L. Cuevas, H. M. Niemeyer and F. J. Pérez, unpublished work]. Thus, it appears likely that the toxicity of DIMBOA towards phytophagous insects is related to its capacity to inhibit thiol or serine proteinases acting as digestive enzymes.

This proposition is unlikely to explain the toxicity of DIMBOA towards aphids, since aphids are sucking insects which utilize mainly protein-poor plant sap as food source [18]. We were unable to detect serine and thiol proteinase activity in extracts of the cereal aphid *Rhopalosiphum padi*. Acetylcholinesterases in aphids possess a cysteine residue that reacts covalently with thiol reagents leading to enzyme inactivation [19]. DIMBOA inactivated cholinesterase activity from an aqueous extract of the aphid *Rhopalosiphum padi* (results not shown). This may be related to the toxicity of DIMBOA towards aphids. Further studies are underway to test this hypothesis.

EXPERIMENTAL

Enzyme and reagents. Papain (twice recrystallized), *N*-carbobenzyloxyglycine *p*-nitrophenyl ester, 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) and dithiothreitol (DTT) were purchased from Sigma.

2,4-*Dihydroxy*-7-*methoxy*-1,4-*benzoxazin*-3-*one* (**1**). This compound was isolated from ethereal extracts of *Zea mays* L. cv. T129s, as described [8].

7-*Methoxy*-4-*hydroxy*-1,4-*benzoxazin*-3-*one* (**3**). Nitration of 3-methoxy-phenol followed by reaction with methylbromoacetate and further reductive cyclization, according to the method of ref. [20], afforded this compound. ^{1}H NMR (60 MHz, acetone-d_6, TMS): δ: 3.3 (*s*, 3H), 4.3 (*d*, 2H), 6.7–7 (*m*, 3H), 10.2 (*s*, 1H); EIMS (probe) 70 eV, *m/z* (rel. int.): 195 $[M]^+$ (8), 179 (93), 167 (23), 165 (23); 149 (100), 136 (48).

7-*Methoxy*-2-*hydroxy*-1,4-*benzoxazin*-3-*one* (**4**) was synthesized from 2-amino-5-methoxy-phenol and dichloroacetylchloride essentially by the method described in ref. [21]. ^{1}H NMR (60 MHz acetone-d_6, TMS): δ 3.7 (*s*, 3H), 5.5 (*s*, 1H), 6.6–6.9 (*m*, 3H), 8.0 (*s*, H), 10.7 (*s*, 1H); EIMS (probe) 70 eV, *m/z* (rel. int.): 195 $[M]^+$ (42), 166 (100), 150 (13), 138 (25), 124 (40), 110 (46).

Activation of papain. Papain was activated for 30 min at 30° in a soln containing 20 mM cysteine, 50 mM acetate buffer, pH 5.2, and 1 mM EDTA. It was separated from excess activator by filtration through a Sephadex G-25 column equilibrated with the same buffer without cysteine, using a rapid centrifugation-filtration technique [22]. Papain concentration was estimated from the absorption coefficient at 278 nm ($\varepsilon = 52\,000$ l/mol cm), and a M_r of 23 700 [13]. Activated papain showed 0.6 mol of SH groups/mol protein, measured according to Ellman's procedure [23]. This figure is in agreement with earlier reports [24].

Assay of papain activity. The hydrolytic activity of papain was measured at 28° with 0.2 mM *N*-carbobenzyloxyglycine *p*-nitrophenyl ester as substrate in 50 mM acetate buffer, pH 5.2. The increase in absorbance at 340 nm due to the release of *p*-nitrophenol was followed [25]. The stock substrate soln (6.1 mM) was prepared in spectroscopic quality MeCN just before use. The MeCN concentration in the assay mixture was 3%. The non-enzymatic hydrolysis of the substrate was taken into account by using substrate in the reference cuvette.

Inactivation of papain by DIMBOA. The reactions were followed under pseudo-first order conditions with an excess of DIMBOA, by taking 0.1 ml aliquots from the reaction mixture (1 ml) after appropriate time intervals and measuring the decrease in the enzymatic activity by the procedure previously described. The dilution of aliquots for the enzymatic assay was at least 30-fold.

Amino acid analysis. A soln (0.5 ml) of 0.5 mM activated papain was filtered through a Sephadex G-25 column equilibrated with 50 mM Pi buffer, pH 6.8. To this soln DIMBOA (0.5 M in 5 μl DMSO) was added, and the mixture incubated for 2 hr at 28°. After this time, papain had lost its activity and was filtered through a Sephadex G-25 column to eliminate the excess of DIMBOA. To this fraction, iodoacetic acid (107 mM, 10 μl) was added and the mixture incubated for 15 min. After this time, the soln was filtered to eliminate the excess of iodoacetic acid, and lyophilysed. The protein was hydrolysed with 6 M HCl at 110° for 20 hr and analysed in a Beckman aminoacid analyzer model 120C.

Titration of the sulphydryl groups in the enzyme. From a reaction mixture (1 ml) containing 0.1 mM gel-filtered activated papain and 20 mM DIMBOA in 50 mM acetate buffer, pH 5, aliquots (0.1 ml) were withdrawn after different time intervals and diluted with 0.7 ml Tris–HCl buffer, pH 8. The resulting aliquot was then filtered through a Sephadex G-25 column equilibrated with 0.1 M Tris–HCl, pH 8, to eliminate excess DIMBOA before adding the thiol reagent DTNB, since it was found that DIMBOA reacted with it. Protein was determined in each filtrate by its absorption coefficient at 278 nm. A soln of DTNB (0.1 ml, 1 mM) was added, and 5 min later the absorbance was measured at 412 nm ($\varepsilon = 13\,600$ l/mol cm). After the same time intervals, 10 μl aliquots were withdrawn and enzymatic activity was assayed according to the standard procedure.

Reversion by dithiothreitol of the inactivation of papain by DIMBOA. A papain soln (1 ml) of specific activity 8.9 μmol/min/mg of protein in 50 mM Pi buffer, pH 6.8, was inactivated with 20 μl of 0.5 M DIMBOA in DMSO. Once the enzyme had lost all its activity, it was filtered through a Sephadex G-25 column to eliminate the excess of DIMBOA. To different aliquots of this enzyme soln, DTT was added in concns ranging from 0.025 to 5 mM, and the kinetics of recovery of activity were followed. Enzyme activity was measured as described previously. Controls were run without the enzyme soln at different DTT concentrations: no release of nitrophenol was observed under the experimental conditions employed.

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