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REACTION OF DIMBOA, A RESISTANCE FACTOR FROM CEREALS, WITH α-CHYMOTRYPSIN

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Key Word Index— Gramineae; benzoxazinones; hydroxamic acids; DIMBOA; α-chymotrypsin plant defence.

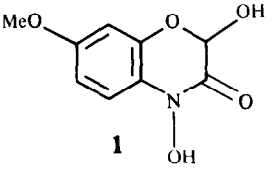
Abstract—2,4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA), a hydroxamic acid involved in pest resistance of cereals, inactivated α-chymotrypsin (EC 4.21.2). Semilog plots of the residual activity of α-chymotrypsin in the presence of an excess of DIMBOA as a function of time were not linear. A kinetic model which considers the spontaneous decomposition of DIMBOA provided a quantitative account for these results. *N*-Acetyl-L-phenylalanine ethyl ester protected the enzyme against DIMBOA inactivation. Amino acid analysis showed a significant decrease in lysine residues in the modified enzyme as compared with the native one. The loss of enzyme activity by reaction with DIMBOA was simultaneous with the decrease in titre of active site serine. These results suggested reaction of DIMBOA with the active site serine residue. The DIMBOA analogue 4-hydroxy-2,7-dimethoxy-1,4-benzoxazin-3-one showed no effect on the enzyme. This result suggested that the reaction of DIMBOA with α-chymotrypsin occurred with the participation of the aldol tautomer of DIMBOA.

INTRODUCTION

2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA, **1**), the main hydroxamic acid (Hx) isolated from extracts of maize and other cereals [1], has been implicated as a resistance factor toward the European corn borer, *Ostrinia nubilalis*, a major pest of corn [2]. When larvae of *O. nubilalis* were fed with diets containing DIMBOA at concentrations higher than 0.4 mg DIMBOA/g diet, consumption increased, a behaviour interpreted as an attempt of the larvae to compensate for the antinutritional effects of DIMBOA [2].

Gut extracts of *O. nubilalis* showed proteinase activities toward different synthetic substrates. Highest specific activities were obtained with *N*-benzoyl arginine ethyl ester (BAEE) and with *N*-benzoyl tyrosine ethyl ester (BTEE). DIMBOA inhibited these activities both *in vivo* and *in vitro*. The major inhibitions were obtained when BAEE and BTEE were used as substrates [F. Campos, J. Houseman, J. Atkinson and J. T. Arnason, personal communication], suggesting that the main proteinase activities in the gut extracts affected by DIMBOA are tryptic and chymotryptic in nature.

Because enzymes from insects are, in general, comparable to the corresponding enzymes from vertebrates [3], we studied the reaction of DIMBOA with well-known bovine α-chymotrypsin, as a model for DIMBOA inhibition of *O. nubilalis* proteinases.



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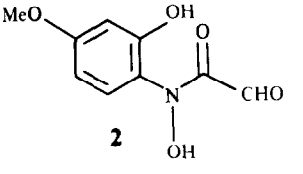

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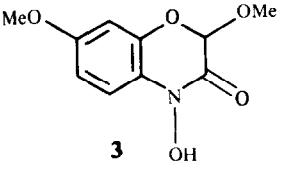

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RESULTS AND DISCUSSION

Kinetic analysis

Semilog plots of the decrease with time of α-chymotrypsin activity in the presence of an excess of DIMBOA at pH 7.5 were not linear. After 8 hr, complete inactivation was not obtained at any DIMBOA concentration studied and the curves describing the inactivation process were asymptotic with time (Fig. 1). Several models in which the inactivator partially modifies the catalytic activity of the enzyme may account for the results [4]. However, DIMBOA decomposes in aqueous solution with first order kinetics, the decomposition rate at pH 7.5 and 30° being 8×10^{-2} min^{-1} [5]. Hence, after 8 hr only

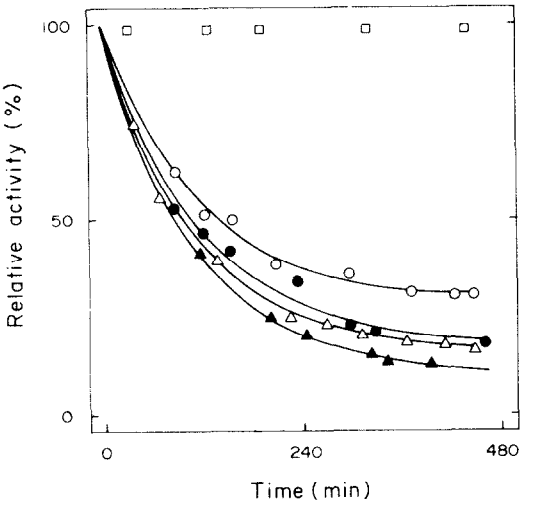

Fig. 1. Kinetics of α-chymotrypsin inactivation by DIMBOA at different concentrations: 0 (□); 10 (○); 15 (●); 20 (△) and 40 (▲) mM. The curves represent the least-squares fit of the experimental points to equation 1. Initial specific activity was 4.9 μmol/min mg protein.

2% of the initial DIMBOA concentration remains in solution. A kinetic model for the inactivation of an enzyme by an unstable inactivator that decomposes spontaneously with first order kinetics has been described [6–9]. The model may be represented by the scheme below, in which k_d is the unimolecular decomposition rate of the inhibitor and E* is the inactive enzyme.

$$E + I \underset{}{\overset{1/K_1}{\rightleftharpoons}} EI \xrightarrow{k_2} E^*$$

$$\downarrow k_d$$

$$P$$

$$\ln \frac{E}{E_o} = \frac{k_2}{k_d} \ln \frac{K_1 + (I)_o e^{-k_d t}}{K_1 + (I)_o} \quad (1)$$

Two approaches were employed for the determination of the kinetic constants in this model: analysis of the time dependence of the inactivation process, and analysis of the dependence of the end point of the enzyme inactivation on the initial inactivator concentration. For this latter procedure eqn 1 was transformed to eqn 3 by setting $t = \infty$ and establishing the condition $(I)_o \gg K_1$.

$$\log \frac{E}{E_o} = \frac{k_2}{k_d} \log \frac{K_1}{K_1 + (I)_o} \quad (2)$$

$$\log \frac{E}{E_o} = -\frac{k_2}{k_d} \log (I)_o + \frac{k_2}{k_d} \log K_1 \quad (3)$$

The experimental points for the time dependence inactivation of α-chymotrypsin at different DIMBOA concentrations were least-squares fitted to eqn 1. The theoretical lines generated are shown in Fig. 1 and the values obtained for k_2 and K_1 are shown in Table 1. The values for the constants determined through the end point method (eqn 3) did not differ significantly from those calculated above (Table 1).

Table 1. k_2 and K_1 values from inactivation kinetics of α-chymotrypsin by DIMBOA in 0.1 M MOPS buffer with 5 mM $CaCl_2$, pH 7.5, at 30° ($k_d = 8 \times 10^{-2}$ min^{-1}, [4])

DIMBOA (mM)	k_2	$K_1 \times 10^{-3}$
10	0.106 ± 0.024	6.4 ± 2.5
15	0.102 ± 0.024	5.3 ± 3.1
20	0.116 ± 0.016	7.8 ± 2.0
40	0.093 ± 0.03	6.0 ± 2.0

k_2 and K_1 values determined through the end point method (equation 3) were 0.076 ± 0.0063 min^{-1} and 5.2 ± 1.3 × 10^{-2} M, respectively.

Chemical modification of α-chymotrypsin by DIMBOA

Inactivation of α-chymotrypsin by DIMBOA was carried out in the presence of different concentrations of *N*-acetyl-L-phenylalanine ethyl ester (AFEE), a synthetic substrate of the enzyme. The results (Table 2) showed that AFEE protected the enzyme against DIMBOA inactivation, suggesting that the reaction occurred at or near the active site. Amino acid analysis of α-chymotrypsin inactivated by DIMBOA, as compared with native α-chymotrypsin, indicated that only the number of lysine residues was significantly reduced by reaction with DIMBOA (Table 3).

Tryptophan and methionine were not detected by this method. It is, however, unlikely that these amino acids suffer changes since they do not react with DIMBOA in aqueous solutions [10, 11]. Lysine, on the other hand, reacts with DIMBOA in aqueous solution with the intermediacy of the open chain aldol **2** [12] and lysine residues in α-chymotrypsin are located in the outer surface of the enzyme [13]. Although normal serine residues in enzymes are not expected to react with DIMBOA [11], the enhanced nucleophilicity of the active site serin in α-chymotrypsin [14] warranted the parallel determination of the active site serine titre [15] and enzyme activity in the presence of excess DIMBOA. The kinetic patterns for both processes were similar (Fig. 2), suggesting that the inactivation of α-chymotrypsin by DIMBOA was due to its reaction with the active site serine. The precision of amino acid analysis did not allow the detection of the modification of this single serine residue.

DIMBOA reacts with nucleophiles either through its hydroxamic nitrogen or through the reactive aldehyde group of its aldol (**2**) with which it forms an equilibrium mixture in solution [16]. Hence, inactivation experiments were carried out with 4-hydroxy-2,7-dimethoxy-1,4-benzoxazinone (**3**), a compound that cannot form aldol **2**, and hence does not generate a reactive aldehyde group. Compound **3** did not inactive α-chymotrypsin in the range of concentrations studied (5 to 20 mM), suggesting that the reaction of DIMBOA with α-chymotrypsin occurred through the aldol tautomer **2**. In support of this conclusion, α-chymotrypsin was inactivated by phenylglyoxal (data not shown), a compound containing an α,β-dicarbonyl function similar to that of aldol **2**.

Insect digestive enzymes have been suggested as possible target sites for the action of plant defence chemicals [17]. It appears likely that the toxicity of DIMBOA towards phytophagous insects such as *O. nubilalis* is

Table 2. Relative activities of α-chymotrypsin inactivated by 30 mM DIMBOA after incubation (pH 7.5, 30°) for 3 hr in the presence of different concentrations of *N*-acetyl-L-phenylalanine ethyl ester (AFEE)

AFEE (mM)	Relative activity (%)*
0	18
10	37
20	51.5
40	63

*Initial specific activity was 4.9 μmol/min mg protein.

Table 3. Amino acid analysis of native α-chymotrypsin and α-chymotrypsin modified by DIMBOA

Amino acid	Native* α-chymotrypsin	Modified* α-chymotrypsin
Trp†	—	—
Lys	12.9 ± 0.1	4.9 ± 0.6
Arg	3.0	3.0
Met†	—	—
Asp	21.4 ± 0.2	22.3 ± 0.2
Thr	22.0	22.0
Ser	27.5 ± 2.1	25.2 ± 1.1
Glu	18.4 ± 0.6	17.8 ± 0.2
Pro	9.6 ± 0.1	9.2 ± 0.1
Gly	29.0 ± 0.9	27.7 ± 0.6
Ala	24.8 ± 0.1	25.9 ± 0.5
Val	20.6 ± 0.1	21.1 ± 0.2
Ile	6.6 ± 0.1	7.2 ± 0.1
Leu	17.0 ± 0.1	16.7 ± 0.1
Tyr	2.8 ± 0.1	2.8 ± 0.2
Phe	4.9 ± 0.1	5.0 ± 0.1

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

†Not detected by this method.

related to its capacity to inhibit serine or thiol [18] proteinases in the larval digestive tract.

EXPERIMENTAL

Enzymes and reagents, α-Chymotrypsin (3 × crystallized), *N*-acetyl-L-phenylalanine-ethyl ester (AFEE), *N*-carbobenzyl-L-tyrosine-*p*-nitrophenyl ester, *N-trans*-cinnamoylimidazol, MOPS and $CaCl_2$ were purchased from Sigma. 4-Hydroxy-2,7-dimethoxy-1,4-benzoxazin-3-one was a gift from Dr J. Atkinson (University of Ottawa, Canada).

2,4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA). This compound was isolated from ethereal extracts of *Zea mays* L. cv T 129s, as described in ref. [19].

Assay of α-chymotrypsin activity. The hydrolytic activity of α-chymotrypsin was measured at 30° with *N*-carbobenzyl-L-tyrosine-*p*-nitrophenyl ester as substrate. The increase in A_{400} due to the release of *p*-nitrophenol was followed [20]. The

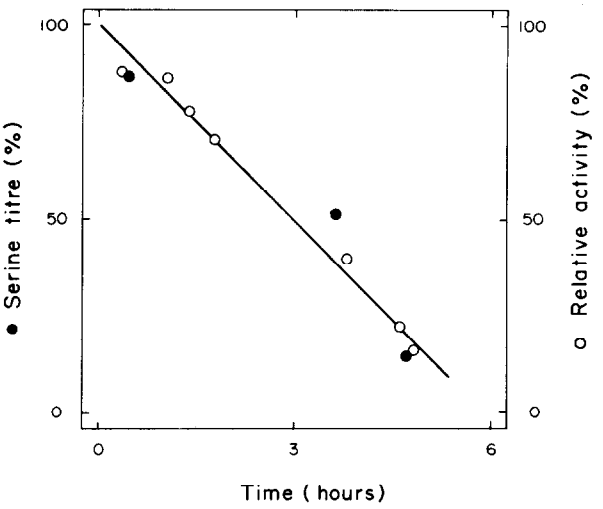

Fig. 2. Proportionality of α-chymotrypsin activity and serine titre as percentage of initial values, during the inactivation of the enzyme by DIMBOA. Initial specific activity was 4.9 μmol/min mg protein and initial serine titre was 0.97 mol serine/mol enzyme.

reaction mixture consisted of 14 μl 3.2×10^{-4} M substrate, 2.6 ml 0.1 M MOPS buffer with 5 mM $CaCl_2$, pH 7.5 (buffer A), 350 μl MeCN and 5 μl 3.2×10^{-6} M α-chymotrypsin. The non-enzymatic hydrolysis of the substrate was taken into account by adding the substrate to the reference cuvette.

Inactivation of α-chymotrypsin by DIMBOA. The reactions were followed under pseudo-first order conditions with an excess of DIMBOA, by taking 5 μl from the reaction mixture (1 ml) after appropriate time intervals and measuring the decrease in the enzymatic activity by the procedure previously described.

Substrate protection. A soln (14 μl) of 3.2×10^{-6} M α-chymotrypsin in 1 ml buffer A was incubated at 30° in the presence of 30 mM DIMBOA and concentrations of AFEE ranging from 0 to 40 mM. Aliquots (5 μl) were withdrawn from the reaction mixture and activity was measured according to the assay previously described.

Amino acid analysis. A mixture consisting of 1.6×10^{-4} M α-chymotrypsin and 40 mM DIMBOA was incubated in buffer A at 30° for 12 hr. After this time, α-chymotrypsin had lost its activity. The reaction mixture was filtered through a Sephadex G-25 column equilibrated with the same buffer, in order to eliminate excess DIMBOA. The filtrate was concd by ultrafiltration and then lyophilized. The protein was hydrolysed with 6 M HCl at 110° for 24 hr and analysed in a Beckman amino acid analyzer model 120. A sample of native α-chymotrypsin was submitted to the same procedure.

Titration of active site serine of α-chymotrypsin. The titre of active site serine of α-chymotrypsin was determined as described in ref. [15]. To a mixture containing 2.9 ml 0.1 M acetate buffer with 3.2% of MeCN, pH 5, and 24 μl 10 mM *N-trans*-cinnamoylimidazol (ε 9115 M^{-1} cm^{-1}), different vols of 3.2×10^{-4} M α-chymotrypsin were added. Decrease in A_{325} was measured with respect to a control without enzyme. The enzyme concentration was measured at 280 nm (ε 50000 M^{-1} cm^{-1}) [21]. A relation of 0.97 mol of serine per mol α-chymotrypsin was obtained for native α-chymotrypsin.

Kinetics of the loss of active site serine titre of α-chymotrypsin in the presence of DIMBOA. After different time intervals 200 μl

aliquots were withdrawn from a reaction mixture consisting of 0.5 ml 3.2×10^{-4} M α-chymotrypsin, 1.9 ml buffer A and 100 μl 0.5 M DIMBOA in DMSO. The aliquots were filtered through a Sephadex G-25 column as described [22] in order to eliminate excess DIMBOA. Active site serine titre and enzyme activity were measured in the filtrates after different time intervals according to the assays previously described.

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