SHORT COMMUNICATION

Relative Inhibition of Insect Phenoloxidase by Cyclic Fungal Metabolites from Insect and Plant Pathogens

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ABSTRACT The fungal metabolite kojic acid, which is produced by Aspergillus and Penicillium species fungi that may be pathogens of both insects and plants, was a significant inhibitor of phenoloxidase of different representative beetle and caterpillar insect species. Fusaric acid and picolinic acid, produced by Fusarium spp., were also significant inhibitors of phenoloxidase, while dipicolinic acid and beauvericin were ineffective at concentrations tested. Previous reports of the ability of kojic and fusaric acid to inhibit defensive enzymes of plants suggest that these compounds may be important in allowing the producing fungi to be pathogens of both insects and plants. Published in 1999 by John Wiley & Sons, Ltd.

Key words: kojic acid; fusaric acid; picolinic acid; Aspergillus; Fusarium

INTRODUCTION

Cyclic fungal metabolites that can act as chelators or ionophores are produced by many species of fungi that can potentially attack both plants and arthropods. Kojic acid produced by several species of Aspergillus and Penicillium (Cole and Cox, 1981), which can be pathogenic on plants (Frisvad and Samson, 1991) and insects (Brooks and Raun, 1965; Madelin, 1963; Steinhaus, 1949). Fusaric, picolinic and dipicolinic acids are produced by several species of Fusarium (Turner, 1971; Turner and Aldridge, 1983; Bacon et al., 1996), which can also be pathogenic on insects (Claydon and Grove, 1982) or plants (Turner, 1971; Turner and Aldridge, 1983; Bacon et al., 1996; Desjardins, 1992). Beauvericin is a cyclic peptide produced by different species of Beauveria and Fusarium (Turner, 1971; Turner and Aldridge, 1983; Kraska et al., 1996). Although generally low toxicity to vertebrates, invertebrates or plants, compared to other secondary metabolites produced by the same fungal species (Cole and Cox, 1981; Bacon et al., 1996; Desjardins, 1992; Dowd, 1992; Khachatourians, 1991), some of these fungal cyclic metabolites can be effective enzyme inhibitors. Kojic acid inhibits oxidative enzymes from both plants and arthropods (Saruno et al., 1979; Chen et al., 1991a,b; Dowd, 1988, 1994; Dowd et al., 1994; Lee and Anstee, 1995). Fusaric acid also inhibits oxidative enzymes from plants (Bossi, 1960) and arthropods (Dowd, 1988), while picolinic acid and dipicolinic acids inhibits enzymes from other sources (Hachisuka et al., 1965; Fortnagel and Freese, 1968; Mann and Byerrum, 1974). The ability of some of these fungal aromatic acids to inhibit defensive enzymes in insects and plants suggests an adaptation that allows the same species of fungi that produce them to be potential pathogens of diverse organisms such as insects and fungi (Dowd, 1994). This multihost adaptation has also been suggested for proteolytic enzymes produced by insect and plant pathogens (Clarkson and Charley, 1996). The present report indicates that these cyclic metabolites differentially inhibit phenoloxidase (monophenol oxidase, catechol oxidase E.C. 1.10.3.1), the enzyme responsible for wound healing and pathogen...

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encapsulation in insects, further supporting the role of some of these compounds in the insect pathogenic process.

### MATERIALS AND METHODS

#### Insects

Fall armyworms (*Spodoptera frugiperda* (J. E. Smith)), corn earworms (*Helicoverpa zea* (Boddie)) and Freeman sap beetles (*Carpophilus freemani* Dobson) were reared at 27 ± 1°C, 40 ± 10 % r.h. and a L14:D10 photoperiod on pinto bean-based diet (Dowd, 1988; Dowd and Weber, 1991). Cigarette beetles (*Lasioderma serricorne* (F.)) were reared at 27 ± 1°C, 60 ± 10 % r.h. and a L14:D10 light:dark photoperiod on a flour, maize meal, brewer’s yeast mix (Dowd, 1989a). Greenhouse whitefly adults (*Trialeurodes vaporariorum* (Westwood)) were obtained from infested tobacco in the greenhouse c. 1 week after emergence.

#### Enzyme Extraction

Last instar caterpillar larvae were used as an enzyme source of both hemolymph and cuticle phenoloxidase, which can differ in types of phenoloxidase properties and activity (Marmaras et al., 1996). To collect hemolymph, prolegs were clipped off and hemolymph was allowed to drip into test tubes held at 0°C. Approximately 50 μl of hemolymph could be obtained from one individual. The hemolymph was diluted 1:9 with pH 7.4, 0.1 M phosphate buffer, and frozen solid at −20°C in order to rupture hemocytes and free the phenoloxidase (Brookman et al., 1989; Dunphy, 1991). The material was then thawed to a liquid and centrifuged at 10 000 x g for 5 min at 4°C. The supernatant was further diluted 1:10 and used in assays. Protein was from c. 0.125–0.250 mg ml⁻¹ in the different assay series using the Bio-Rad protein reagent and instructions (Bio-Rad, Richmond, CA, USA). The cylinder of cuticle behind the last pair of true legs and in front of the last pair of prolegs was split lengthwise, and gut, fat body and Malpighian tubules were removed. The cuticle strip was rinsed in the phosphate buffer, and then two strips were homogenized in 2 ml of the buffer with a ground glass homogenizer. The homogenate was centrifuged at 10 000 x g for 5 min, and the supernatant was removed. The supernatant was diluted 1:10 for assays; protein content in assays was c. 0.175–0.320 mg ml⁻¹ using the Bio-Rad kit.

Due to the small size of the other insects, whole body preparations were used. One hundred last instar *L. serricorne* and *C. freemani* larvae and *T. vaporariorum* adults were chilled and homogenized in 250–500 μl of the phosphate buffer. Homogenates were otherwise treated as for the caterpillar preparations. Final protein concentration in assays was c. 0.9–1.1 mg ml⁻¹ for *L. serricorne* and *C. freemani*, and 0.03–0.045 mg ml⁻¹ for *T. vaporariorum*.

#### Enzyme Assays

The reaction mixture for phenoloxidase activity consisted of 700 μl of room temperature buffer: 100 μl of 10⁻³ M kojic acid (in buffer), fusaric acid (in ethanol), picolinic acid (in ethanol), beauvericin (in ethanol) or dipicolinic acid (in 50 % ethanol) with appropriate solvent controls: 100 μl of 0.2 % L-DOPA (L-3, 4-dihydroxyphenyl alanine) in water; and 50–100 μl of enzyme source. All chemicals were obtained from Aldrich Chemical Co., Minneapolis, MN, USA or Sigma Chemical Co., St Louis, MO, USA. The oxidation of DOPA was monitored at 470 nm for 10 min using a Perkin-Elmer Lambda 4B spectrophotometer equilibrated to 30°C. Assays were run at least in duplicate on at least two separate occasions. Assays were initially run with *S. frugiperda* to determine the most active compound (kojic acid). Kojic acid was then tested as an inhibitor at 10⁻³ M against the other insect enzyme sources.

### RESULTS AND DISCUSSION

At 10⁻³ M, kojic acid inhibited phenoloxidase activity by fall armyworm (*Spodoptera frugiperda*) cuticle and hemolymph by c. 80 %. At 10⁻⁴ M, significant inhibition of *S. frugiperda* cuticular and hemolymph phenoloxidase activity by kojic acid (35–40 %) was again noted (Table 1). Both sources showed about the same susceptibility to inhibition by kojic acid. Kojic acid was also a highly effective inhibitor of phenoloxidase activity in other species of Lepidoptera and Coleoptera tested, but was much less effective against adult whiteflies (Table 2). Levels of inhibition of *S. frugiperda* hemolymph phenoloxidase by kojic acid (Dowd et al., 1994; present study) are similar to those reported for *S. littoralis* hemolymph (Lee and Anstee, 1995). The levels of inhibition for insects were similar to those reported for

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Hemolymph</th>
<th>Cuticle</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusaric acid 10⁻³ M</td>
<td>38.7 ± 2.2 a</td>
<td>34.6 ± 5.2 a</td>
</tr>
<tr>
<td>Picolinic acid 10⁻³ M</td>
<td>16.2 ± 3.5 b</td>
<td>41.2 ± 6.5 a</td>
</tr>
<tr>
<td>Dipicolinic acid 10⁻³ M</td>
<td>−23.8 ± 14.4 bc</td>
<td>−8.6 ± 2.6 c</td>
</tr>
<tr>
<td>Beauvericin 10⁻³ M</td>
<td>−51.5 ± 5.5 c</td>
<td>−9.8 ± 6.5 c</td>
</tr>
<tr>
<td>Kojic acid 10⁻³ M</td>
<td>88.2 ± 1.2 d</td>
<td>80.7 ± 2.1 d</td>
</tr>
<tr>
<td>Kojic acid 10⁻⁴ M</td>
<td>40.8 ± 1.8 a</td>
<td>36.6 ± 1.9 a</td>
</tr>
</tbody>
</table>

Values are means ± SE. Values in columns followed by the same letter are not significantly different at p < 0.05 by analysis of variance.

### Table 1. Inhibition of *S. frugiperda* hemolymph and cuticular phenoloxidase by fungal cyclic metabolites

L. serricorne and *C. freemani*, and 0.03–0.045 mg ml⁻¹ for *T. vaporariorum*.
lobster polyphenoloxidase when similar concentrations of kojic acid were used and DL-DOPA was used as a substrate (Chen et al., 1991a). A mixed type of inhibition was noted with kojic acid and polyphenoloxidase from white shrimp, spiny lobster and grass prawn (Chen et al., 1991b). Kojic acid also inhibits monophenol monoxygenase (tyrosinase: E.C. 1.14.18.1) from mushroom (Saruno et al., 1979), kidney D-amino acid oxidase (E.C. 1.4.3.3; Klein, 1953), different plant catechol oxidases (polyphenoloxidases: (E.C. 1.10.3.1; Chen et al., 1991a,b), plant peroxidase (E.C. 1.11.1.7; Dowd, 1994), insect unspecific monoxygenase (E.C. 1.14.14.1; Dowd, 1988) and insect phenoloxidase (Dowd et al., 1994; Lee and Anstee, 1995).

Fusaric and picolinic acids were significantly less active than kojic acid at the same concentration against S. frugiperda phenoloxidase (Table 1). Dipicolinic acid was inactive to slightly stimulatory for hemolymph phenoloxidase, while beauvericin was strongly stimulatory for hemolymph phenoloxidase (Table 1). Picolinic acid was less active towards hemolymph phenoloxidase compared to cuticle phenoloxidase. Fusaric acid inhibits oxidative enzymes such as mammalian dopamine B-hydroxylase (monoxygenase: E.C. 1.14.17.1; Hidaka, 1971), mammalian tyrosinase (Nagatsu et al., 1972), plant catechol oxidase (Bosi, 1960) and insect unspecific monoxygenase (Dowd, 1998). Picolinic acid inhibits oxidative enzymes such as bacterial aldolase (aldose 1-dehydrogenase: E.C. 1.1.1.121; Fortnagel and Freese, 1968) and plant quinolidic acid phosphoribosyl transferase (E.C. 2.4.2.19; Mann and Byerrum, 1974). Fusaric acid also synergizes the toxicity of fungal metabolites, insecticides and plant allelochemicals (Dowd, 1988, 1989b). Dipicolinic acid inhibits bacterial glucose dehydrogenase (E.C. 1.1.99.10; Hachisuka et al., 1965) but stimulates maize NADPH oxidase (dehydrogenase: E.C. 1.6.99.1; Tyagi et al., 1987). Presumably inhibition of metalloenzymes, such as unspecific monoxygenase, peroxidase and phenoloxidase are due to the ability of the fungal aromatic acids to function as chelators. This causes it to complex with metal ions of these enzymes (Dowd, 1988, 1994). At the molecular level this complexing appears to result in interference of oxygen uptake and reduction of o-quinones that may be produced (Chen et al., 1991b).

The main defense reaction of insects against fungi is the encapsulation response, which involves hemocyte phenoloxidase (Hajek and St Leger, 1994), an enzyme that is critical to the process (Marmaras et al., 1996). The present study indicates that kojic, fusaric, and picolinic acids are all capable of inhibiting the enzyme involved in the hemocyte encapsulation response, as well as wound healing by the cuticle (a site of penetration for the fungi). In addition to direct enzyme inhibition, the insect phenoloxidase also requires Ca$^{2+}$ ions for activation and activity, although this varies among insect species (Brookman et al., 1989; Dunphy, 1991). The aforementioned chelating/ionophore activity of these compounds suggests that this is an additional way that they can inhibit insect phenoloxidase, as well as plant peroxidase, which also requires Ca$^{2+}$ ions (Gaspar et al., 1982). Although dipicolinic acid and beauvericin were sometimes stimulatory to the phenoloxidase, ability to interact with Ca$^{2+}$ ions may ultimately result in enzyme inhibition. However, their ability to enhance phenoloxidase activity suggests the insects have partially adapted to pathogens that produce these compounds.

Kojic acid is produced relatively early overall in solid culture and in much higher quantities compared to aflatoxin, a more toxic secondary metabolite (Lee et al., 1986). This early production suggests that kojic acid is important in the infection process, compared to highly toxic metabolites such as aflatoxin, which may ultimately kill the insect. Quantities of kojic acid produced in liquid culture can be in the 100 g kg$^{-1}$ range (Wilson, 1971), suggesting high quantity production could also occur in insects at a level at least comparable to that tested in the present study. Although there are no reports for kojic acid concentration in infected insects to the author's knowledge, aflatoxin has been reported at 0.05 μmole per A. flavus infected Bombyx mori larva (Ohtomo et al., 1975). Assuming a larva weighs 0.5 g and has the density of water (which is conservative), the concentration of aflatoxin B$_1$ would be 1.0 × 10$^{-4}$ M. Even assuming kojic acid was only produced at the same rate as aflatoxin, this would still put its concentration in the insect at a level biologically relevant to concentrations tested in the present study.

The relatively short, three-step biosynthetic pathway for production of kojic acid from glucose (Wilson, 1971) suggests isolation and cloning of biosynthetic genes is a possibility for increasing the virulence of insect pathogens that do not produce this compound. Determining the metabolic pathway difference for picolinic and dipico-

### Table 2. Inhibition of insect whole body, hemolymph or cuticular phenoloxidase by 10$^{-5}$ M kojic acid

<table>
<thead>
<tr>
<th>Insect</th>
<th>Hemolymph % Inhibition</th>
<th>Cuticle % Inhibition</th>
<th>Whole Body % Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. frugiperda</td>
<td>88.2 ± 1.2 a</td>
<td>80.2 ± 2.1 a</td>
<td>ND</td>
</tr>
<tr>
<td>H. zea</td>
<td>83.8 ± 0.7 a</td>
<td>89.0 ± 0.4 a</td>
<td>ND</td>
</tr>
<tr>
<td>L. serraticum</td>
<td>ND</td>
<td>96.7 ± 3.0 a</td>
<td>ND</td>
</tr>
<tr>
<td>C. treemani</td>
<td>ND</td>
<td>88.4 ± 0.9 a</td>
<td>ND</td>
</tr>
<tr>
<td>T. vaporariorum</td>
<td>ND</td>
<td>19.0 ± 4.2 b</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE. Values in the same column followed by different letters are significantly different at p < 0.05 by analysis of variance.

ND = not determined.

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linic acid production, and altering it to favor picolinic acid by mutation or genetic engineering, may enhance the virulence of insect pathogens that produce dipicolinic acid (which includes Beauveria bassiana and Paecilomyces fumoso-roseus (ICI Ltd. 1983). The ability of the fungal aromatic acids to inhibit defensive enzymes in both plants and insects helps explain the ability of different strains of the producing organisms to be both plant and insect pathogens.

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INHIBITION OF INSECT PHENOLOXIDASE BY FUNGAL METABOLITES


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