Inhibition of Fungal Cell Wall Synthesizing Enzymes by trans-Cinnamaldehyde

Kyu-Ho Bang, Dong-Won Lee, Hee-Moon Park, and Young-Ha Rhee

Department of Microbiology, College of Natural Sciences, Chungnam National University, Yusong, Taejon 305-764, Korea

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This study examined the inhibitory effects of trans-cinnamaldehyde (CA), an aromatic aldehyde derived from Cinnamomi Cortex, on Saccharomyces cerevisiae cell wall synthesizing enzymes in vitro. This compound was found to be a noncompetitive inhibitor of β-(1,3)-glucan synthase and a mixed inhibitor of chitin synthase 1 with 50% inhibitory concentrations (IC₅₀) of 0.84 and 1.44 mM, respectively. Chitin synthases 2 and 3 were less sensitive than chitin synthase 1 to CA. CA can be useful as a model compound of cell wall inhibitors for the development of effective antifungal agents.

Key words: β-(1,3)-glucan synthase inhibitor; chitin synthase inhibitor; noncompetitive and mixed inhibition; trans-cinnamaldehyde

Chitin and β-glucans are major structural components of fungal cell walls. Therefore, selective inhibitors of the synthesis of these polymers are considered as possible candidates for both pharmaceutical and agricultural applications. Our research to identify novel antifungal agents has focused on the discovery of cell wall active antifungal agents from natural resources. During the course of screening over 230 natural substances, trans-cinnamaldehyde (CA) from Cinnamomi Cortex was identified for its antifungal activity against 7 human pathogenic fungi at minimum inhibitory concentration (MIC) values ranging from 39 to 78 μg/ml. The antifungal activity of CA against many filamentous fungi has already been reported. However, its biochemical properties, particularly its mode of action in the antifungal effect, have not previously been investigated. In this paper, we describe the in vitro inhibitory effects of CA on the cell wall synthesizing enzymes, β-(1,3)-glucan synthase and chitin synthases (Chs) using Saccharomyces cerevisiae GS1-36 membrane preparations.

Membrane preparation and β-(1,3)-glucan synthase assay were done by the method reported previously. The assay measured the formation of radiolabeled trichloroacetic acid (TCA)-precipitable material formed from UDP-[14C]-Glu. The reaction mixture contained in a total volume of 40 μl, 5 mM UDP-[14C]-Glu (Du Pont, specific activity 250,000 cpm/μmol), 75 mM Tris chloride (pH 7.5), 25 mM potassium fluoride, 8% glycerol, 7.5 mg/ml bovine serum albumin (BSA, Sigma), an excess of membrane preparation (7.9 mg of protein), 20 μM guanosine-5′-O(3-thiotriphosphate), and 2 μl of CA solution diluted in DMSO at the appropriate concentration. Following incubation for 30 min at 30°C, the reaction was stopped with 1 ml of ice-cold 10% TCA, and the product was measured by filtration and a liquid scintillation counter (Packard). Each assay was run in duplicate. Protein was measured by the method of Lowry et al. with BSA as the standard. The activity of the enzyme was expressed as micromoles of UDP-Glu incorporated per minute per milligram of crude protein.

The preparation of the membrane fractions and the assay conditions for Chs 1, 2, and 3 activity were the same as described previously. To measure Chs 1 and 2 activity, 20 μl of membrane suspension (10 mg of protein) was first treated with 2 μl (experimentally measured for maximum activity) of trypsin (usually 1–3 mg/ml) by shaking at 30°C for 15 min. A 1.5-fold excess of soybean trypsin inhibitor was added, and the reaction was continued by the addition of CA dissolved in DMSO at the appropriate concentration to a standard 50 μl reaction mixture of 32 mM Tris-HCl (pH 7.5), 4.3 mM magnesium acetate, 1.1 mM UDP-[U-14C]-GlcNAc (400,000 cpm/μmol, Du Pont), and 32 mM GlcNAc. For the measurement of Chs 2 activity, the assay was done with 1.6 mM cobalt acetate as a substitute for 4.3 mM magnesium acetate. Incubations were done at 30°C for 90 min. The chitin formed was assayed by measurement of radioactivity after addition of 1 ml of cold TCA and filtration through a glass fiber filter (Whatman GF/C). For the measurement of Chs 3 activity, 20 μl of membranes were incubated with trypsin in the presence of CA in a standard 50 μl assay mixture: 32 mM Tris-HCl (pH 7.5), 4.3 mM magnesium acetate, 1.1 mM UDP-[U-14C]-GlcNAc (400,000 cpm/μmol), and 32 mM GlcNAc at 30°C. Each assay was run in duplicate. Activity was expressed as micromoles of GlcNAc incorporated per minute per mg of protein.

In view of the fact that the morphological effects suggested the inhibitory effect of CA on the biosyn-
thesis of the fungal cell wall, we assayed the activity of two enzymes that are the most likely targets of antifungal compounds. CA inhibited S. cerevisiae in vitro β-(1,3)-glucan synthesis with 50% inhibitory concentration (IC₅₀) of 0.84 mM. The inhibition activity against Chs 1 was observed at an IC₅₀ of 1.44 mM. The inhibitory action against Chs 2 and 3 was also assayed by measuring the formation of chitin with UDP-[U-¹⁴C]-GlcNAC. The IC₅₀ for Chs 2 and 3 were 3.94 mM and 2.71 mM, respectively, indicating that Chs 2 and 3 were less sensitive to CA than Chs 1. CA appears to be a selective inhibitor of β-(1,3)-glucan synthase and Chs 1 although it also has an inhibitory effect against Chs 2 and 3. The inhibitory effect of CA on cell wall synthesizing enzymes was compared with those of L688,786, nikkomycin Z, and poloxin D, which are well-known inhibitors of β-(1,3)-glucan synthase, Chs 1 and 3, and Chs 2, respectively. Under the same reaction conditions as used in this experiment, the IC₅₀ of L688,786, nikkomycin Z, and poloxin D for the corresponding enzymes were >48.25, 1.21, 2.02, and 42.19 μM, respectively. Although CA inhibits β-(1,3)-glucan synthase and Chs isoymes with a low potency, this is the first report that CA inhibits cell wall biosynthetic enzymes. The in vitro enzyme assay data and morphological effects in cell wall structures support the idea that CA inhibits the growth of fungal strains by inhibiting cell wall synthesizing enzymes.

We measured synthesis at different concentrations of CA and substrate to further evaluate the kinetics of the inhibition mechanism. A Lineweaver-Burk plot showed a noncompetitive inhibition for β-(1,3)-glucan synthase and a mixed inhibition for Chs 1, 2, and 3 using CA (Fig. 1A, B, C, and D). The inhibition constant, Kᵢ, was calculated from replots. An estimated Kᵢ was 0.39 mM for β-(1,3)-glucan synthase, 0.53 mM for Chs 1, 1.61 mM for Chs 2, and 0.97 mM for Chs 3 (Table 1).

Although many of the most useful antibacterial agents act via the inhibition of bacterial cell wall

### Table 1. Inhibition of β-(1,3)-Glucan Synthase, Chitin Synthases 1, 2, and 3 by trans-Cinnamaldehyde

<table>
<thead>
<tr>
<th>Compound</th>
<th>Kᵢ (mM)</th>
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<tbody>
<tr>
<td>β-(1,3)-GS⁺ Chs 1</td>
<td>0.39</td>
</tr>
<tr>
<td>β-(1,3)-GS⁺ Chs 2</td>
<td>0.53</td>
</tr>
<tr>
<td>β-(1,3)-GS⁺ Chs 3</td>
<td>1.61</td>
</tr>
<tr>
<td>trans-cinnamaldehyde</td>
<td>0.97</td>
</tr>
</tbody>
</table>

⁺ β-(1,3)-GS: β-(1,3)-glucan synthase, Chs 1: chitin synthase 1, Chs 2: chitin synthase 2, Chs 3: chitin synthase 3.

Fig. 1. Kinetics of trans-Cinnamaldehyde (CA) Inhibition.

(A), Inhibition of β-(1,3)-glucan synthase by CA. ○ 0.12 mM CA, ▽ 0.6 mM CA, ■ 1.5 mM CA; (B), Inhibition of chitin synthase 1 by CA. ○ 0.23 mM CA, ▽ 1.04 mM CA, ■ 2.55 mM CA; (C), Inhibition of chitin synthase 2 by CA. ○ 1.6 mM CA, ▽ 2.84 mM CA, ■ 6.97 mM CA; (D), Inhibition of chitin synthase 3. ○ 1.1 mM CA, ▽ 1.95 mM CA, ■ 4.5 mM CA.
biosynthesis, researchers have been less successful in developing commercially useful antifungal cell wall inhibitors. The results of this study clearly indicate that CA is an antifungal agent that inhibits the synthesis of β-(1,3)-glucan and chitin. Although CA did not show any detectable inhibitory effect \textit{in vitro} on the activity of DNA polymerase I and the synthesis of DNA (data not shown), further study of the effects of CA on other macromolecules is needed to prove that CA is a specific inhibitor of β-(1,3)-glucan synthase and Chs isoforms.

In conclusion, CA is a cell wall active antifungal agent that behaves kinetically as a noncompetitive inhibitor of β-(1,3)-glucan synthase, a cell wall enzyme in yeast. Additionally, it is a mixed inhibitor of Chs isoforms, enzymes for chitin synthesis in \textit{S. cerevisiae}.\textsuperscript{12-14} CA may be a useful lead compound for the development of antifungal agents through the control of β-(1,3)-glucan and chitin synthesis in yeasts and molds.

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\textbf{References}


