Inhibitory effect of sakuranetin on (1,3)-β-glucan synthase

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SUMMARY

An examination of the kinetic properties of UDP-glucose, (1,3)-β-glucans (callose) synthase, from mung bean seedlings (Sorbus commixta cortex) shows that these enzymes have a complex relationship with UDP-glucose and various effectors. Fluorescence assay showed that deoxynojirimycin increased the inhibitory effect of (1,3)-β-glucan synthase in a concentration-dependent manner. The inhibitory effect of sakuranetin (34.34%) was higher than that of deoxynojirimycin (80.63%). Disk diffusion method revealed that sakuranetin inhibited the growth of Candida albicans to a 1.5 mm inhibition zone. These results suggest that sakuranetin, isolated from Sorbus commixta cortex extract, can be used as stable antifungal material.

Key words: (1,3)-β-glucan synthase; Sorbus commixta cortex; Fluorescence assay; Inhibitory effect; Sakuranetin; Candida albicans

INTRODUCTION

(1,3)-β-Glucans play important roles in the morphogenesis of fungi and higher plants. In higher plants, this polymer has a common name, callose. Callose is only found as a wall polymer in specialized cell types such as pollen mother cells and pollen tubes. In addition, callose is deposited rapidly in plants in environmental conditions, and in response to pathogen attack (Stone et al., 1992). Essentially all higher plants contain a UDP-glucose [(1→3)-β-glucan (callose) synthase]. This enzyme is largely found on the plasma membrane, and in most cases is latent, only becoming activated by perturbed conditions which lead to some loss of membrane permeability (Delmer et al., 1977; Delmer, 1983). In 1979, Ray (1979) named this enzyme glucan synthetase II and assayed it at high concentrations of UDP-Glc in the absence of divalent cations. However, others have reported that its activity can be enhanced by Mg²⁺ (Delmer et al., 1977), and variable stimulation by β-glucosides has frequently been observed (Henry et al., 1982). In fungi, (1,3)-β-glucans containing some (1,6)-β branches is a major wall constituent (Cabib et al., 1982) and due to the increasing problems associated with pathogenicity of yeasts and fungi, drugs that might interfere with its synthesis are widely sought after. In plants, callose synthase is a high-molecular-weight complex situated in the plasma membrane. The enzyme is activated by micromolar levels of Ca²⁺ and a β-glucoside. By contrast, regulation of fungi (1,3)-β-
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MATERIALS AND METHODS

General experimental procedures
All solvents were distilled before use. Rotary evaporation was used to remove solvent at temperatures up to 40°C. Column chromatography was performed with davisil silica 60 (35-70 μm silica gel, Alltech). Optical rotations were measured on a Perkin-Elmer 241 Polarimeter. Mass, UV, and IR spectra were recorded on Kratos MS-80, Shimadzu UV 240, and Perkin-Elmer 1600 FT-IR Instruments, respectively. NMR spectra, at 25°C, were recorded at 300 MHz for 1H and 75 MHz for 13C on a Varian VXR-300 spectrometer.

Plant material
S. commixta cortex bark was purchased from a herbal market in Iksan city, Korea, in June 2006. The identification of the specimen was confirmed by Prof. K.Y. Chai, and a voucher specimen, 060609-63, has been kept in the Division of Nanobiochemistry, Wonkwang University in Korea.

Extraction and isolation
Air-dried S. commixta cortex bark (1.2 kg) were ground before extracted thrice with methanol (5 l) at room temperature. The combined filtrates were evaporated under reduced pressure below 40°C which produced a dark green gum (18.84 g, 1.57%). The methanolic extract was partitioned into n-hexane, ethyl acetate, n-BuOH, and water layers. This ethyl acetate fraction (4 g) was subjected to flash chromatography on silica gel (150 g) with a ethyl acetate : methylene chloride : methanol gradient. The most active fraction 3 was eluted with 1 : 9 ethyl acetate : methylene chloride (183.6 mg). This was subjected to flash chromatography on silica gel (150 g) with a ethyl acetate: methylene chloride: methanol gradient. The most active fraction 3 was eluted with 1 : 9 ethyl acetate : methylene chloride (183.6 mg). This was subjected to reversed-phase HPLC with an acetonitrile : H2O (20 ~ 100%) gradient. A fraction eluted with 40 : 60 acetonitrile : H2O was sakuranetin (1, 24.0 mg); 1H and 13C-NMR spectra as published (Rakwal et al., 1996; Lee et al., 1999).
Growth, harvesting and preparation of mung bean (1,3)-β-glucan (callose) synthase
Mung bean (Vigna radiata) were purchased locally, soaked overnight in water, and grown for 5 days in the dark at 25°C in water-saturated vermiculite. All subsequent isolation procedures were carried out at 47°C. Hypocotyls were harvested into cold 10 mM hepes/KOH, pH 7.3, containing 1 mM DTT and weighed. The hypocotyls were then homogenized with Polytron homogenizer in 50 mM hepes/KOH, pH 7.3, containing 5 mM EDTA, 1 mM DTT, 5 μM leupeptin, and 20 mM pefabloc. The homogenate was filtered through three layers of miracloth and centrifuged for 5 min at 12,000 g, after which the pellet was discarded. The supernatant was centrifuged for 1 h at 100,000 g, and the resulting membrane pellet was resuspended in 50 mM hepes/KOH, pH 7.3. Membranes were stored in aliquots at -80°C until use. Protein was determined by using the Bio-Rad protein assay kit (Thelen et al., 1986; Hayashi et al., 1987).

Protocols for assay of mung bean (1,3)-β-glucan synthases
All standard curves and enzyme assays were carried out in flat-bottomed 96-well microtiter plates in a total volume of 50 μl. For the mung bean enzyme, reactions were modified from those used by Hayashi et al. (1987) and contained 50 mM hepes/KOH, pH 7.3, 0.01% digitonin, 1 mM CaCl₂, 10 mM cellobiose, 0.4 mM UDP-Glc, and 2.5 μg of membrane protein. For the yeast enzyme, reactions were modified from those used by Frost et al. (1994) and contained 50 mM tris/HCl, pH 7.5, 20 μM GTP, 4 mM EDTA, 0.5% Brij 35, 6.6% glycerol, 2 mM UDP-Glc, and 100 μg of yeast membrane protein. All reactions were incubated for 30 min at 25°C and terminated by addition of 10 μl of 6 N NaOH. Glucan produced was solubilized by floating the microtiter plate in a water bath at 80°C for 30 min, followed by addition of 210 μl of aniline blue mix. This mix was prepared by combining 40 volume of 0.1% aniline blue in water, 21 volume of 1 N HCl, and 59 volume of 1 M glycine/NaOH buffer, pH 9.5, and is stable and can be stored at room temperature indefinitely. The plate was vortexed briefly and then incubated at 50°C for 30 min, and an additional 30 min at room temperature to allow reaction with the fluorochrome and decolorization of the aniline blue. Fluorescence was quantified with a fluorescence plate reader [either the Cytefluor 2350 Fluorescence Measurement System (Milipore, Vienna, Austria) or the FL500 Microplate Fluorescence Reader (Bio-Tek Instruments)]. Excitation wavelength was 400 nm/slit width 30 and emission wavelength was 460 nm/slit width 40 (Thelen et al., 1986; Shedletzky et al., 1997).

Antimicrobial activity
The yeast C. albicans was maintained in Potato Dextrose broth (Difco, USA) after incubation for 24 h at 37°C. Testing was performed in Potato Dextrose broth and the serial dilution technique was applied with ELISA reader at 620 nm. The final inoculum concentration was 105 CFU/ml. A set of tubes containing only inoculated broth was kept as control. After incubation for 24 h at 37°C, the last tube with no growth of microorganisms was recorded to represent MIC expressed in µg/ml. Every experiment in the antibacterial assay was triplicated in order to defines the MIC values (Chun et al., 2003; Lee et al., 2003). Sample was used in various concentrations such as 50, 100, 200, 300, 500 mg/ml.

RESULTS AND DISCUSSION
Methanol extract of S. commixta cortex bark showed antifungal activity. Antifungal activity-directed isolation using HPLC and silica column chromatography gave sakuranetin (1). Structure (1) was
Inhibitory effect of sakuranetin on (1,3)-β-glucan synthase

derived from a combination of NMR experiments and confirmed by comparison with published NMR data (Fig. 1) (Rakwal et al., 1996; Lee et al., 1999, 2003).

Suitability of assay for screening of glucan synthases inhibitors
Table 1 is as an example of mung bean callose synthase inhibition by deoxynojirimycin, a compound that was previously shown to inhibit spinach sucrose-phosphate synthase. Inhibition is easily detectable with fluorescence assay, as it is with the standard radioactive assay (Delmer et al., 1977; Hayashi et al., 1987; Stone et al., 1992).

Advantages of fluorescence assay
In radioactive assays, sensitivity can be adjusted by varying the specific activity of the substrate, but increased sensitivity is limited by the high cost of UDP-[14C]Glc. Comparing the sensitivity of previously reported high-throughput glucan synthases radioactive assay with the assay presented here,

Table 1. Inhibition effect of deoxynojirimycin on (1,3)-β-glucan synthase

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.00</td>
</tr>
<tr>
<td>100</td>
<td>93.41</td>
</tr>
<tr>
<td>150</td>
<td>88.63</td>
</tr>
<tr>
<td>250</td>
<td>83.76</td>
</tr>
<tr>
<td>500</td>
<td>81.23</td>
</tr>
<tr>
<td>1,000</td>
<td>68.34</td>
</tr>
<tr>
<td>1,500</td>
<td>47.23</td>
</tr>
<tr>
<td>2,000</td>
<td>33.54</td>
</tr>
</tbody>
</table>

the inspection of deoxynojirimycin suggest that the radioactive assay was capable of detection at a IC50 1.47 mg/ml on 1,3-β-glucan synthase (Table 1). Deoxynojirimycin, which exhibited inhibition effect of 80.63%, inhibited (1,3)-β-glucan synthase in a concentration-dependent manner. Sakuranetin and the methanol extract of S. commixta cortex were shown 34.34 and 69.70 inhibition percentages against (1,3)-β-glucan synthase, respectively (Table 2) (Delmer et al., 1977; Hayashi et al., 1987; Stone et al., 1992).

The antimicrobial activity of sakuranetin and S. commixta cortex methanol extract were determined against C. albicans using the serial dilution and fluorescence methods. Among these, sakuranetin showed higher antimicrobial activity than that of S. commixta cortex methanol extract against C. albicans. These antimicrobial activities increased in a concentration-dependent manner (Tables 3 and 4) (Jang et al., 2003; Chun et al., 2004; Kim et al., 2006). The significant antimicrobial activity is due to a characteristic feature of two aromatic hydroxyl compounds. Sakuranetin contains both phenolic hydroxyl groups and a system of delocalized electrons, which were found to possess antimicrobial activity against C. albicans (Ultee et al., 2002). Ultee et al. (2002) also reported that the hydroxyl group of carvacrol is important for its effect on membrane

Table 2. Inhibition effects of sakuranetin and methanol extract of S. commixta cortex (1 mg/ml) on (1,3)-β-glucan synthase

<table>
<thead>
<tr>
<th>Sample</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sakuranetin</td>
<td>34.34</td>
</tr>
<tr>
<td>Deoxynojirimycin</td>
<td>80.63</td>
</tr>
<tr>
<td>MeOH Extract</td>
<td>69.70</td>
</tr>
</tbody>
</table>

Table 3. Absorbance of C. albicans in various concentrations of sakuranetin and S. commixta cortex methanol extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Sakuranetin</td>
<td>0.857</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>0.603</td>
</tr>
<tr>
<td>MeOH Extract</td>
<td>0.987</td>
</tr>
</tbody>
</table>

Fig. 1. Molecular structure of sakuranetin (1).
Table 4. Fluorescence of C. albicans in various concentrations of sakuranetin and S. commixta cortex methanol extract

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/ml)</th>
<th>Fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sakuranetin</td>
<td>1.0</td>
<td>++</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>1.0</td>
<td>+++</td>
</tr>
<tr>
<td>MeOH Extract</td>
<td>1.0</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 5. Antifungal activity of sakuranetin and S. commixta cortex methanol extract against C. albicans

<table>
<thead>
<tr>
<th>Sample</th>
<th>Concentration (mg/ml)</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sakuranetin</td>
<td>1.0</td>
<td>++</td>
</tr>
<tr>
<td>MeOH Extract</td>
<td>1.0</td>
<td>++</td>
</tr>
</tbody>
</table>

Antimicrobial activity

Table 5 shows the antimicrobial activity of sakuranetin obtained from S. commixta cortex. As clearly evident, with an inhibition zone of 1.5 mm, sakuranetin obtained from S. commixta exhibited significant activity against C. albicans. However, the antimicrobial activity of S. commixta cortex methanol extract was established with an inhibition zone of 0.5 mm (Jang et al., 2003; Kang et al., 2003; Chun et al., 2004; Kim et al., 2006).

The assay was performed in microtiter plates and is extremely inexpensive compared to other standard assays for these enzymes. The reduction in price is achieved by replacing the conventional substrate UDP-[14C]Glc with its non-radioactive counterpart, and the non-radioactive glucan produced is quantified as a fluorescent complex following specific interaction with the fluorochrome present in commercial aniline blue. As such, the assay is highly suitable for high-throughput screening for inhibitors of this enzyme.

CONCLUSION

(1,3)-β-Glucan (callose) synthase from mung bean seedlings (S. commixta cortex) showed that this enzyme has a complex relationship with UDP-glucose and various effectors. Fluorescence assay showed that deoxynojirimycin increased the inhibitory effect of (1,3)-β-glucan synthase in a concentration-dependent manner. The inhibitory effect of sakuranetin (34.34%) was higher than that of deoxynojirimycin (80.63%). Sakuranetin inhibited the growth of C. albicans to a 1.5 mm inhibition zone. These results suggest that sakuranetin, isolated from S. commixta cortex, extract can be used as stable antifungal material.

ACKNOWLEDGEMENTS

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Inhibitory effect of sakuranetin on (1,3)-β-glucan synthase

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