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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 morphogensis 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 e*t 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 Mg^{2+} (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 Ca^{2+} and a β-glucoside. By contrast, regulation of fungi (1,3)-β-

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glucans synthases differs considerably from that of the plant enzymes, since the fungal enzymes do not require divalent cations and are activated by GTP. The latter effect has been clarified in the yeast Saccharomyces cerevisiae, where activation has been shown to occur via interaction of the enzyme with the GTP-bound form of the small GTPase Rho1p (Drgonova *et al*., 1996; Qadota *et al*., 1996). Re-examination of the kinetic properties of UDP-glucose, (1→3)-β-glucan (callose) synthase, from mung bean seedlings (*Vigna radiata*) and cotton fibers (Gossypium hirsutum) shows that these enzymes have a complex relationship with UDP -glucose and various effectors. Stimulation of activity by micromolar concentrations of Ca^{2+} and millimolar concentrations of β-glucosides or other polyosis is highest at low (< 100 micromolar) UDP-β-glucose concentrations. These effectors act both by raising the Vmax of the enzyme, and by lowering the apparent Km for UDP-β-glucose from > 1 mM, to 0.2 mM. Mg^{2+} markedly enhances the affinity of the mung bean enzyme for Ca^{2+}, but not for β-glucoside, where with saturating Ca^{2+}, Mg^{2+} only slightly stimulates further production of glucan (Hayashi *et al*., 1987).

Sorbus commixta (S. commixta) cortex has long been used in the field of traditional Oriental medicine as a tonic to treat coughing, asthma, and other bronchial disorders (Bae *et al*., 2000). From the cortex of *S. commixta cortex*, triterpenoids such as lupenone and lupeol have been isolated (Lee *et al*., 2006; Zhang *et al*., 2006). Recently, the methanol extract of *S. commixta cortex* (MSC) was shown to have a potent radical scavenging activity (Na *et al*., 2002). MSC has also been observed to dilate vascular smooth muscle via the up-regulation of an endothelium-dependent NO-cyclic GMP pathway (Kang *et al*., 2005). This pharmacological effect on vascular tissue may be useful for the treatment of cardiovascular diseases such as atherosclerosis (Sohn *et al*., 2005). In this study, the antimicrobial activity of sakuranetin from *S. commixta cortex* on *Candida albicans (C. albicans)* was investigated.

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 ^{1}H and 75 MHz for ^{13}C 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 reversed-phase HPLC with an acetonitrile : H_2O (20 ~ 100%) gradient. A fraction eluted with 40 : 60 acetonitrile : H_2O was sakuranetin (1, 24.0 mg); ^{1}H and ^{13}C-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 $CaCl_2$, 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-Glic, 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). Solution for assay was dried onto 6.25 mm filter paper discs, which were then placed onto seeded agar Petri dishes and incubated. Activity was observed as a zone of inhibition around the disc, with its width recorded from the edge of the disc in mm (Chun *et al*., 2004; Kim *et al*., 2006). 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

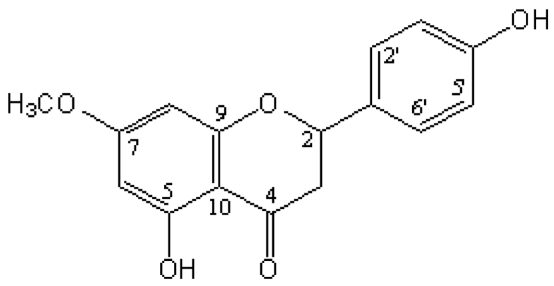

Fig. 1. Molecular structure of sakuranetin (1).

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

Concentration (μg/ml)	Inhibition (%)
0	100.00
100	93.41
150	88.63
250	83.76
500	81.23
1,000	68.34
1,500	47.23
2,000	33.54

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

Sample	Inhibition (%)
Sakuranetin	34.34
Deoxynojirimycine	80.63
MeOH Extract	69.70

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

Sample	Concentration (mg/ml)			
	100	200	300	500
Sakuranetin	0.857	0.754	0.719	0.658
Ampicillin	0.603	0.340	0.246	0.130
MeOH Extract	0.987	0.895	0.842	0.823

Table 4. Fluorescence of *C. albicans* in various concentrations of sakuranetin and *S. commixta cortex* methanol extract

Sample	Concentration (mg/ml)			
	100	200	300	500
Sakuranetin	156.25	148.58	135.26	128.56
Ampicillin	63.53	55.17	54.12	52.11
MeOH Extract	195.68	188.73	182.36	178.89

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

Sample	Concentration (mg/ml)	Inhibition (%)
Sakuranetin	1.0	++
Ampicillin	1.0	+++
	0.5	++
	0.25	++
MeOH Extract	1.0	++

Positive control (+) : 0 - 0.5 mm +, 0.5 - 2 mm ++, 2 - 4 mm +++.

properties, and possibly for its antimicrobial activity. Although carvacrol causes destabilization of the membrane and a decrease in the membrane potential, its antimicrobial activity is most probably caused by an additional decrease in pH as a result of a hydroxyl group presence and a system of delocalized electrons. These characteristics lead to the conclusion that sakuranetin possess antimicrobial activity.

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.

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