Food Sci. Technol. Res., **6** (2), 99–101, 2000

Note

Isolation and Structure of Antimicrobial Substances from Paprika Seeds

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Received September 28, 1999; Accepted January 18, 2000

Antimicrobial substances were isolated from the 50% ethanol extract of paprika seeds by ODS open column chromatography and reverse phase HPLC. Ten compounds (1–10) which demonstrated antimicrobial activity against *Saccharomyces cerevisiae* (MIC=1.3–2.6 mg/*l*) were isolated. The structure of the major compound, 4 (MIC=2.6 mg/*l*), was determined to be 3β-*O*-{β-D-glucopyranosyl-(1→3)-β-D-glucopyransyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyransyl-(1→4)-β-D-galactopyranosyl}-2α-hydroxyl-(25R)-5α-spirostane by MS, NMR and sugar analyses.

Keywords: paprika seeds, antimicrobial substance, saponin, gitogenin

Paprika is a sweet or mildly pungent substance that belongs to the genus *Capsicum*. The brilliant red powder obtained from dried paprika fruits is used as a flavoring and garnish. We previously found that a water extract from paprika seeds exhibited strong antimicrobial activity towards yeasts (Yajima *et al.*, 1996). The antimicrobial substance partially purified from paprika seeds was very stable at high temperatures, over a wide pH range. Furthermore, several application studies showed that this antimicrobial substance was a good food preservative to prevent yeast growth (Yajima *et al.*, 1997; Yajima *et al.*, 1998a; 1998b). However, this antimicrobial substance has not yet been completely purified, nor has its structure been elucidated. We report here the purification and structure of antimicrobial substances from paprika seeds.

To extract the antimicrobial substances, paprika seeds (100 g) were ground into a powder using a mechanical coffee grinder. The powder was mixed with 50% ethanol (700 ml) and stirred at room temperature for 3 h. The stirred solution was filtered through a cotton cloth and then centrifuged (10,000×*g*, 20 min). The filtrate was concentrated to 1/2 of its original volume by rotary evaporation at 40°C and then applied to a reverse phase open column (16×300 mm, Cosmosil 140 C_{18}-OPN, Nacalai Tesque) that had been equilibrated with 5% ethanol solution. After washing the column with 300 ml of 5% ethanol solution, antimicrobial substances were eluted using a stepwise gradient of 20% to 80% ethanol. The fraction that eluted at 60% ethanol showed the highest antimicrobial activity. The antimicrobial activity against *Saccharomyces cerevisiae* W-3 was measured using a 96-well microplate (Yajima *et al.*, 1996). The pre-culture of *S. cerevisiae* W-3 (50 μl), a 50-μl sample, and 150 μl of YM medium were placed in each well of a 96-well microplate (U-bottom) and incubated at 25°C for 48 h. The pre-culture was prepared by diluting (20-fold) the culture which had been incubated at 25°C for 24 h. The minimum inhibitory concentration (MIC) was determined by visually monitoring the growth of W-3.

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The active fraction that eluted with 60% ethanol was concentrated by rotary evaporation at 40°C and then separated by reverse phase HPLC on a μBondasphere C_{18} column (19×150 mm, Waters) using 50% ethanol as a eluent. Many peaks were detected by a RI detector (Hitachi L-3300). The compounds (**1–10**) obtained from ten peaks demonstrated the antimicrobial activity (MIC=1.3–2.6 mg/*l*) (Fig. 1).

Structure of the major compound, **4** [MIC=2.6 mg/*l*, yield = 0.1% (based on weight of the starting seed sample)], was elucidated by MS, NMR and sugar analyses. The monosaccharide composition of compound **4** was analyzed by acid hydrolysis and HPLC analysis (Takayanagi *et al.*, 1994). Compound **4** (1 mg) was dissolved in 1 ml of 2.5 M trifluoroacetic acid and incubated for 5 h at 100°C. Then the cooled trifluoroacetic acid solutions were evaporated by a centrifugal concentrator at 50°C just until the samples dried. The dry samples were injected onto an HPLC equipped with a CarboPac PA-1 column (4×250 mm, Dionex) and eluted with 15 mM sodium hydroxide. The eluted oligosaccharides were monitored using a pulsed amperometric detector (Hardy *et al.*, 1988). Compound **4** had a sugar chain consisting of D-glucose (Glc) and D-galactose (Gal) in a proportion of 4.3 : 1 (Glc : Gal).

An aglycon of compound **4** was obtained according to the method of Lavaud *et al.* (1998). Compound **4** (10 mg) was dissolved in 1 ml of a mixture containing (1 : 1) 6.5% aq. $HClO_4$ and 0.02 N H_2SO_4, and heated at 140°C for 2 h. After cooling, the obtained aglycon precipitate was rinsed with H_2O and dried *in vacuo*.

^1H- and ^{13}C- NMR spectra were recorded by a Varian Unity Inova 500 spectrometer at 500 MHz (^1H) and 125 MHz (^{13}C), and tetramethylsilane was used as an internal standard. Mass spectra, EI- and FAB-MS, were measured by a benchtop quadrupole mass spectrometer (JOEL) and a HITACHI M-80B mass spectrometer, respectively. The ^{13}C-NMR data of the aglycon of compound **4** ($[M]^+$ m/z 432) agreed with those of (25R)-5α-spirostane-2α, 3β-diol (VanAntwerp *et al.*, 1977; Jain *et al.*, 1987; Uniyal *et al.*, 1991) (Table 1, Fig. 2).

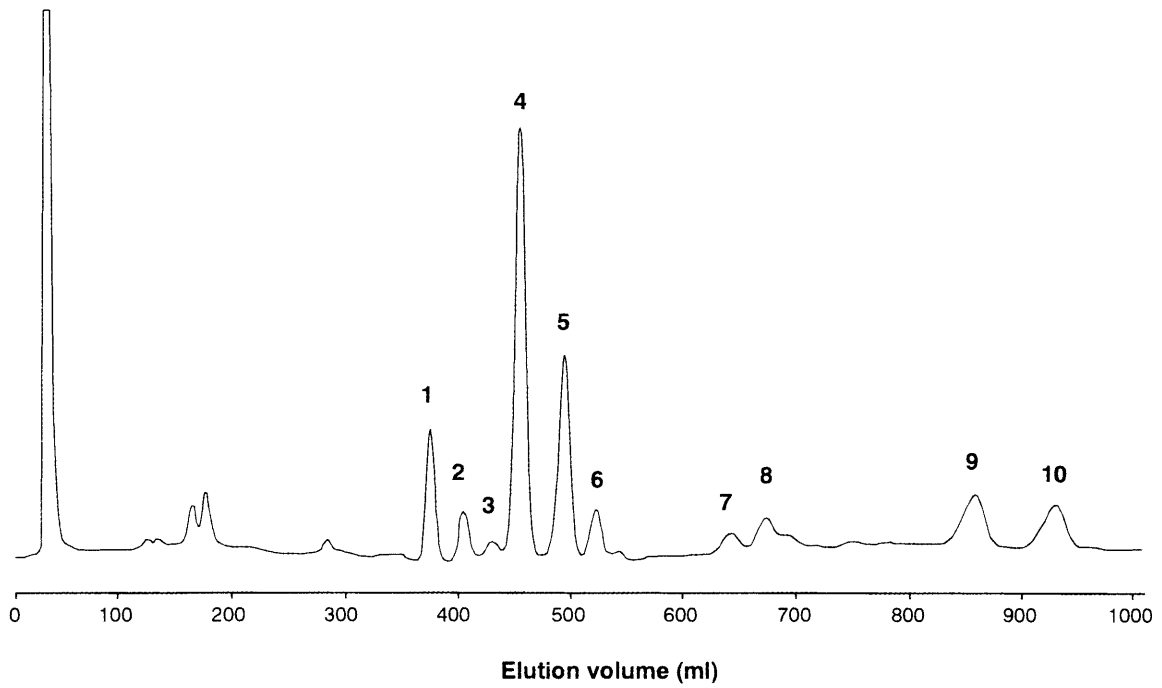
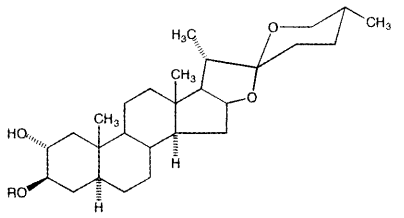


Fig. 1. HPLC chromatogram of the active fraction obtained from ODS open column chromatography. Active fraction eluted at 60% ethanol was separated by reverse phase HPLC on a μBondasphere C_{18} column (19×150 mm, Waters) using 50% ethanol as the eluent. Eluted substances were detected by an RI detector (Hitachi L-3300).

Table 1. ^{13}C NMR chemical shifts for aglycon of compound **4** in $CDCl_3$ (δ values; 125 MHz).

Position	δ (ppm)	Position	δ (ppm)
1	45.0	15	31.7
2	73.1	16	80.8
3	76.4	17	62.2
4	35.6	18	16.5
5	44.8	19	13.6
6	27.8	20	41.6
7	32.1	21	14.5
8	34.4	22	109.2
9	54.3	23	31.4
10	37.6	24	28.8
11	21.2	25	30.3
12	40.0	26	66.8
13	40.6	27	17.1
14	56.1		

Aglycone of Compound **4**: (25R)-5α-spirostane-2α, 3β-diol (R = H)



Compound **4** R = sugar moiety: ß-D-glucopyranosyl-(1→3)-ß-D-glucopyranosyl-(1→3)-[ß-D-glucopyranosyl-(1→2)]-ß-D-glucopyransyl-(1→4)-ß-D-galactopyranoside

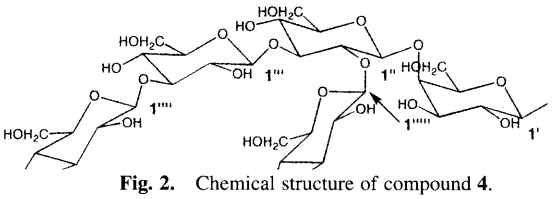


Fig. 2. Chemical structure of compound **4**.

Compound **4** showed an $[M-H]^-$ ion at m/z 1241 in the negative-ion FAB-MS data. The ^1H- NMR spectrum of this compound showed five sugar anomeric protons [δ 4.46 (d, J=7.8 Hz); 4.72 (d, J=7.7 Hz); 4.87 (d, J=7.9 Hz); 4.66 (d, J=7.9 Hz); 5.05 (d, J=7.9 Hz)]. All the proton and carbon signals due to the sugar moiety of compound **4** were assigned according to ^1H-^1H COSY, TOCSY, and GHSQC experiments (Table 2). The HMBC spectra of the compound showed long-range correlation between 1′- H of galactose and C-3 of the aglycon, indicating a glycosidic linkage of galactose to C3-OH of the aglycon (Fig 2). Other long-range correlations between 1″-H of glucose and C-4′ of galactose, 1‴-H of glucose and C-3″ of glucose, 1⁗-H of glucose and C-3‴ of glucose, and 1⁗′-H of glucose and C-2″ of glucose were also observed. The anomeric con-figurations of galactose and glucose were concluded to be all β based on the large coupling constants (J=7.7–7.9 Hz). Thus, the structure of the sugar moiety was determined to be β-D-glucopyranosyl-(1→3)-β-D-glucopyransyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-glucopyransyl-(1→4)-β-D-galactopyranoside (Fig. 2).

The structure of compound **4** was similar to the gitogenin oligoglycosides isolated from red peppers (Yahara *et al.*, 1994). The biological activities of gitogenin, including inhibition of tumour promoters (Mimaki *et al.*, 1996) and the hypocholesterol effect (Sauvaire *et al.*, 1991) have previously been investigated, but their antimicrobial activity toward yeasts has not been reported. Thus, to the best of our knowledge, this is the first report of antimicrobial activity of a gitogenin glycoside isolated from paprika seeds.

Table 2. ^{1}H and ^{13}C NMR chemical shifts for sugar moiety of compound **4** in CD_3OD (δ values; 125 MHz).

	^{1}H	^{13}C
Gal		
1′	4.46 (d, *J*=7.8)	103.1
2′	3.79	73.2
3′	3.64	75.8
4′	4.14	80.2
Glc		
1″	4.72 (d, *J*=7.7)	104.7
2″	3.85	81.3
3″	3.89	87.9
4″	3.45	71.4
Glc		
1‴	4.87 (d, *J*=7.9)	103.9
2‴	3.58	75.0
3‴	3.70	88.2
4‴	3.51	70.4
Glc		
1⁗	4.66 (d, *J*=7.9)	105.4
2⁗	3.38	75.7
3⁗	3.47	71.9
4⁗	3.45	70.9
Glc		
1⁗′	5.05 (d, *J*=7.9)	104.4
2⁗′	3.30	76.0
3⁗′	3.44	78.0
4⁗′	3.45	78.2

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