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STIMULATION OF PRENYLATED FLAVANONE PRODUCTION BY MANNANS AND ACIDIC POLYSACCHARIDES IN CALLUS CULTURE OF *SOPHORA FLAVESCENS*

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Key Word Index—*Sophora flavescens*; Leguminosae; callus culture; prenylated flavanone; sophoraflavanone G; lehmannin; yeast extract; mannan; pectin; pectinic acid; elicitor.

Abstract—The effect of polysaccharides on the production of prenylated flavanones sophoraflavanone G and lehmannin in *Sophora flavescens* callus culture was investigated. The production of these flavanones was stimulated up to 5 times by the addition of 2 mg/ml yeast extract. The active component was partially purified as a mannan (*c* 92% of mannose and *c* 5% glucose) by DEAE–cellulose and Concanavalin A–Sepharose column chromatographies. Acidic polysaccharides also stimulated the production of prenylated flavanones. The effect of pectin was higher than that of pectinic acid from the same source. Other polysaccharides such as cellulose, β-1,3-glucan and chitosan did not influence the productivity.

INTRODUCTION

In the previous paper, we reported that callus cultures of *Sophora flavescens* produced the prenylated flavanones, sophoraflavanone G and lehmannin, and the pterocarpans, maackiain and its glycosides [1]. At the same time, it was shown that the production of prenylated flavanones increased in cells transferred to a low-nutrient medium such as White's [2] or M-9 [3] medium. In order to investigate the regulatory mechanism of the prenylation of flavonoids, we sought to establish another system in which it is possible to stimulate prenylated flavanone production. In this paper, we report on the effects of polysaccharides partially purified from yeast extract and acidic polysaccharides on prenylated flavanone production in *Sophora flavescens* callus culture.

RESULTS

Effect of yeast extract on the production of prenylated flavanone

Prenylated flavanone production by *S. flavescens* callus was stimulated by the addition of yeast extract, and at concentrations of 2–8 mg ml^{-1} the flavanone content of the callus was some five times higher than that of the control. At these concentrations, both cell growth and pterocarpan production were unaffected. When the yeast extract concentration was higher than 16 mg ml^{-1}, both cell growth and the flavonoid production were inhibited (Fig. 1).

Partial purification of yeast extract

200 g of yeast extract were partially purified by EtOH precipitation and DEAE–Sephadex column chromatography. On DEAE–Sephadex separation, the activity was present mainly in the 0 M NaCl fraction (Fig. 2). The specific activity of this fraction was about 100 times higher than that of the yeast extract. This fraction consisted of 79% mannose, 15% glucose, 2% galactose and small amounts of rhamnose and arabinose GC analysis of the alditol acetate derivatives prepared from the acidic hydrolysates of polysaccharides (Table 1).

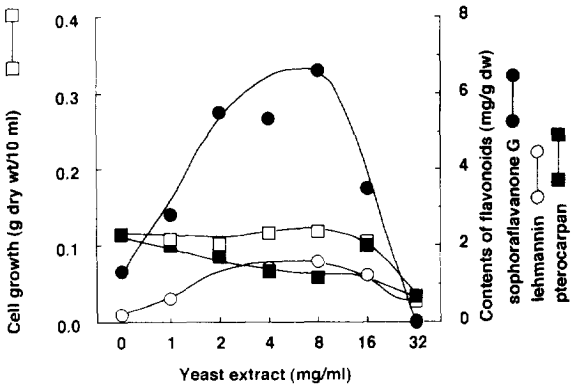

Fig. 1. Effect of yeast extract on the production of prenylated flavanones by *S. flavescens* callus culture.

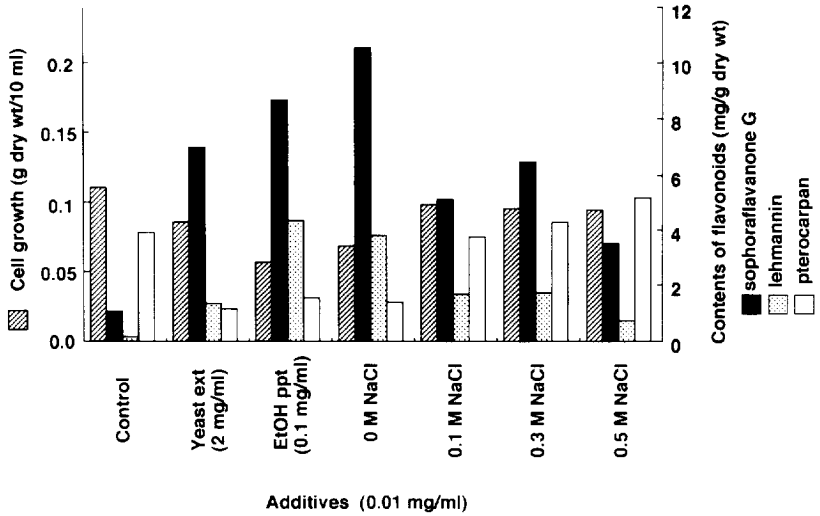

Fig. 2. Effect of polysaccharides separated by DEAE-Sephadex A-50 on the production of prenylated flavanones by *S. flavescens* callus culture.

Table 1. Sugar composition of fractions separated by DEAE-Sephadex column chromatography

Fraction	Glc	Man	Gal	Ara	Rha
0 M NaCl	15.4	79.4	2.0	0.9	2.3
0.1 M NaCl	—*	100.0	—	—	—
0.3 M NaCl	3.1	94.9	—	—	1.9
0.5 M NaCl	—	62.3	—	36.7	—

*Not detected.

Harn and Albersheim reported that a glucan in yeast extract purified by Concanavalin A-Sepharose chromatography was the elicitor of glyceollin accumulation in soybeans [4]. By means of their method, we separated the 0 M NaCl fraction by Concanavalin A-Sepharose column chromatography. The column was eluted with buffer containing 0% (Fr. I) and 4% (Fr. II) α-methyl-D-mannopyranoside (6.9% and 93.1% recoveries, respectively). The stimulatory activity was observed only in Fr. II (Fig. 3), and the relative activity of Fr. II did

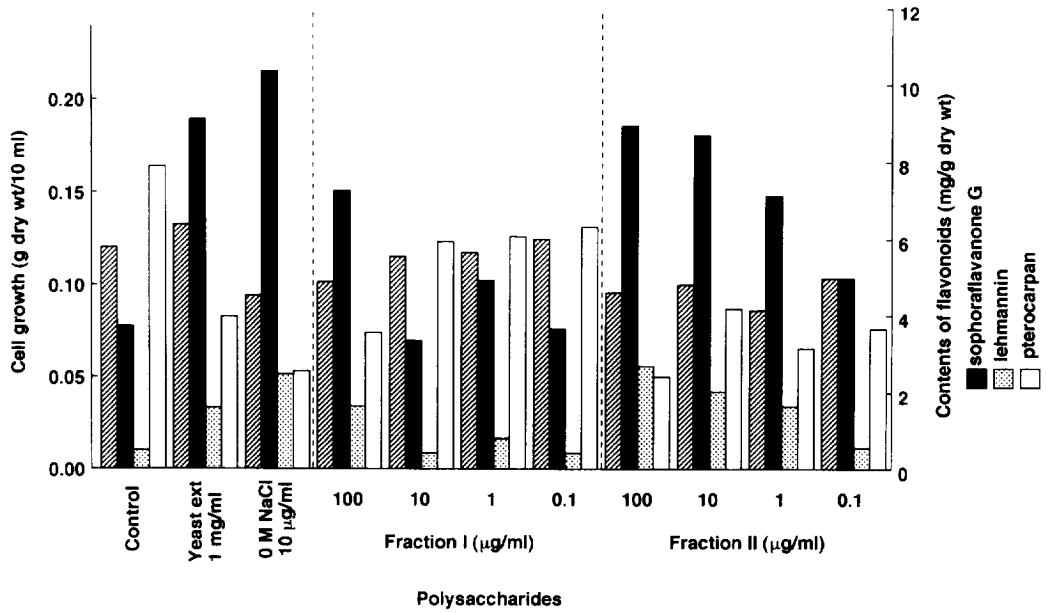

Fig. 3. Effect of polysaccharides separated by Concanavalin A-Sepharose on the production of prenylated flavanones by *S. flavescens* callus culture.

not change compared with that of 0 M NaCl fraction. The sugar composition of Frs I and II are shown in Table 2. Glucose was the main constituent of Fr. I whereas Fr. II consisted of 92% mannose and a small amount of glucose (5%). From these results, it was concluded that a mannan in yeast extract was the elicitor which stimulated prenylated flavanone production in *S. flavescens*.

Table 2. Sugar composition of fractions separated by Concanavalin-A-Sepharose column chromatography

Fraction	Glc	Man	Gal	Ara	Rha
Fr. I	94.0	3.5	*	2.0	0.5
Fr. II	5.0	92.0	1.0	0.7	1.3

*Not detected.

Effect of other polysaccharides on prenylated flavanone production

Some polysaccharides such as polygalacturonic acid and chitosan are known to act as elicitors [5]. We next investigated the effects of these polysaccharides on prenylated flavanone production. Both citrus pectin and commercial pectinic acid stimulated flavanone production (Fig. 4), but other polysaccharides, β-1,3-glucan, chitosan and cellulose, were without effect (data not shown). The effective concentration of pectin was almost 10 times lower than that of pectinic acid. It is possible that the different origins of these acidic polysaccharides was the cause of the diverse activities observed. To investigate this possibility pectinic acids were prepared from the citrus pectin by enzymatic or alkaline hydrolysis and were examined for their effect on prenylated flavanone production. As shown in Fig. 5, the pectinic acids preparations did not stimulate the production of prenylated flavanone at 1 mg ml^{-1}, whereas citrus pectin and denatured enzyme-treated pectin increased the productivity.

DISCUSSION

Harn and Albersheim were the first to isolate a glucan elicitor from yeast extract [4]. Sumaryono *et al.* also purified glucomannan elicitors from yeast extract [6]. Schumacher *et al.* reported that the mannan obtained from yeast extract stimulated benzophenanthridine alkaloid production in *Eschscholtzia californica* cell suspension cultures [7], but the full experimental details were not given. Recently, Song *et al.* isolated a mannan as the elicitor that induces the accumulation of *p*-coumaroylamino acids in *Ephedra distachya* cell cultures [8, 9]. In the present study, we have provided another example in which a mannan (Fr. II) in yeast extract is able to act as an elicitor. A glucan (Fr. I) separated from the 0 M NaCl fraction by Concanavalin A–Sepharose chromatography also stimulated prenylated flavanone production at 0.1 mg ml^{-1}. However, the 0 M NaCl fraction stimulated flavanone production at 0.01 mg ml^{-1}, an effective concentration which is almost 10 times lower than that of Fr. I. Moreover, the percentage of Fr. I in the 0 M NaCl fraction was only 7%. From these results, it was obvious that the glucan was a very poor elicitor in *S. flavescens* cells. In yeast extract, many kinds of elicitors must be present to account for the isolation of different elicitors from yeast extracts of different origins and/or purification methods.

Song *et al.* reported that commercial yeast mannan (Sigma) had no elicitation activity [10]. We also investigated the effect of commercial yeast mannan but it did not stimulate prenylated flavanone production (data not shown). These results might be due to the different purifi-

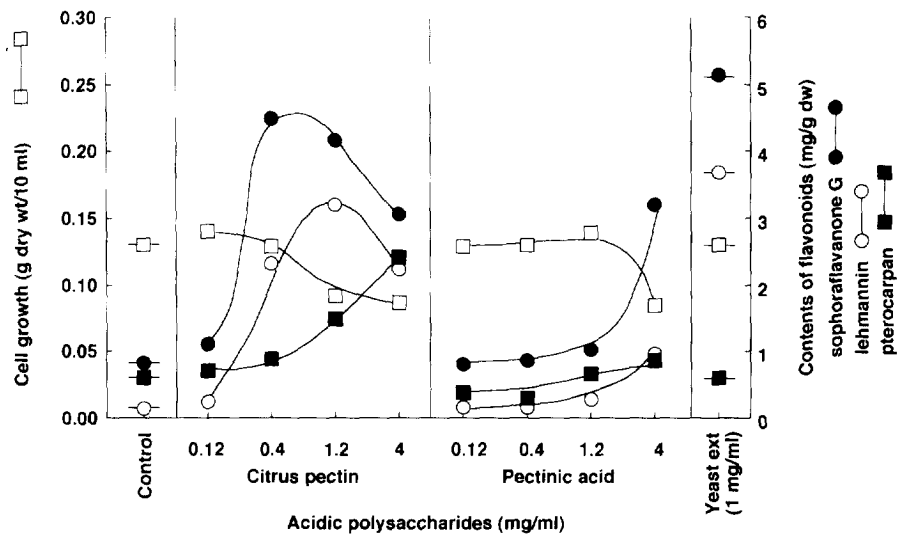

Fig. 4. Effect of commercial acidic polysaccharides on the production of prenylated flavanones by *S. flavescens* callus culture.

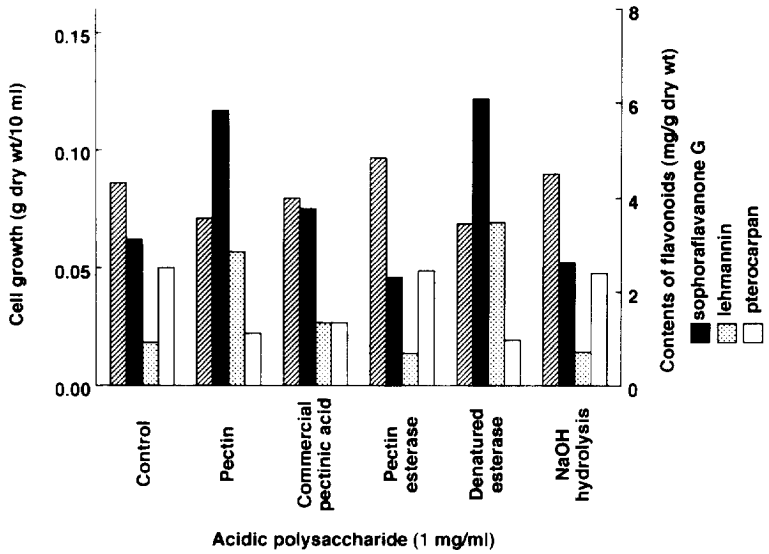

Fig. 5. Effect of pectinic acids prepared from citrus pectin on the production of prenylated flavanones by *S. flavescens* callus culture.

cation methods of mannan which cause a change in its conformation.

We also found that the production of prenylated flavanone in *S. flavescens* cells was stimulated by acidic polysaccharides such as pectin and pectinic acid, and the effect of pectin was higher than that of pectinic acid. These acidic polysaccharides are constituents of plant cells and their fragments which possess elicitation activities are termed "endogenous elicitors" [10]. Some workers have purified those fragments and shown them to be oligomers of galacturonic acid [5, 10, 11]. In these studies, the acidic residues of galacturonic acid were not methylated. Oligogalacturonides easily form an 'egg box' conformation with divalent cations, [12], and these unique conformations are regarded as the signal that induces the plant's responses in plant–microbe interactions [13]. In *Sophora* cells, in contrast to the above-mentioned results, pectin had the strongest effect on prenylated flavanone production compared with pectinic acid. To our knowledge, this is the first report that partially methylated polygalacturonides rather than unmethylated ones have higher elicitation activity. In *S. flavescens* cells, the receptor conformation may be different from that of other plants.

EXPERIMENTAL

Plant material and culture method. The origin and subculturing of the callus of *Sophora flavescens* were described previously [1]. For the experiments reported here, 0.3 g of cells subcultured on MS medium [14] containing 1 μM 2,4-D and 1 μM kinetin, solidified with 0.3% gellan gum were inoculated on the same medium containing polysaccharide supplements, and cultured for 1 month at 25° in the dark. Each polysaccharide was dissolved in the medium, and the pH was adjusted to 6.0–6.2 before autoclaving. In each experiment, 3 replicates were used and the experiment was repeated at least twice.

Quantitative analysis of flavonoids. Extraction and quantitative analysis of flavonoids produced by cultured cells were carried out according to the method of Yamamoto *et al.* [15].

Partial purification of elicitor from yeast extract. 200 g of yeast extract (Kyokuto Seiyaku, Japan) was dissolved in 750 ml of H_2O without heating and then mixed with 3 l EtOH and kept at 4° for 2 days to precipitate the polysaccharides. The supernatants were removed by decantation, and the gummy precipitate dissolved in 300 ml H_2O, then dialyzed against 15 l H_2O at 4° for 3 days, twice. The precipitate formed inside the dialysis bag was removed by centrifugation, and the supernatant was precipitated by 80% EtOH again. The precipitate suspended in H_2O was dialyzed against H_2O, and 10 g of crude polysaccharide fraction was obtained. This fraction was dissolved in 10 mM Tris–HCl buffer (pH 7.5) and applied to a DEAE–Sephadex A–50 (Pharmacia) column (5 × 25 cm) which had been equilibrated with the same buffer. The column was eluted with 4 l of the equilibration buffer followed by 0.1 M, 0.3 M and 0.5 M NaCl solutions prepared in the same buffer, respectively (3 l each). Each litre was fractionated, concentrated, dialyzed against H_2O, lyophilized and checked for elicitation activity. About 100 mg of elicitor-active fraction dissolved in 10 ml of 100 mM NaOAc buffer (pH 5.2) containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$ was applied to a Concanavalin A–Sepharose (Pharmacia) column (2 × 30 cm) equilibrated with the same buffer. The column was eluted with 500 ml of the equilibration buffer, then by 4% α-methyl-D-mannopyranoside containing buffer (1000 ml). The 0% and

4% α-methyl-D-mannopyranoside fractions were concentrated separately, dialyzed against H_2O to remove the α-methyl-D-mannopyranoside and inorganic salts, and lyophilized (7.1 mg of 0% fraction and 96.2 mg of 4% fraction, respectively).

Determination of sugar composition. The sugar composition was determined by GC after the preparation of their alditol acetate derivatives according to the method of Selvendran *et al.* [16]. FID GC analysis was carried out on a silicone OV-225 (2% Unioport, 60/80 mesh, GL Science) glass column (3 mm × 2.1 m) at 185° with N_2 at 60 ml min^{-1}.

Demethylation of pectin by pectin esterase. Demethylation of pectin obtained from citrus (Wako Pure Chemicals) by pectin methylesterase was carried out according to Kertesz [17]. After the incubation, both enzyme and pectinic acid were precipitated by 90% EtOH. The precipitate was dialyzed against H_2O, then lyophilized. In the elicitor assay, pectin treated with heat-denatured enzyme was used as a control.

Demethylation of pectin by NaOH. 100 mg of pectin was dissolved in 1 M NaOH (100 ml) under vacuum without heating, and stirred at 25° overnight under N_2 gas. The resulting sol was neutralized by 2 M HCl, dialyzed against H_2O, then lyophilized.

NMR analysis. The degree of demethylation of pectin was determined by NMR: 270 MHz, D_2O, 25°, monitoring of loss of 3.7 ppm signal ($-OMe$).

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