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MANNAN GLYCOPEPTIDE ELICITS *p*-COUMAROYLAMINO ACIDS IN *EPHEDRA DISTACHYA* CULTURES

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Key Word Index—*Ephedra distachya*; Ephedraceae; cell suspension culture; yeast extract; mannan glycopeptide; elicitor; *p*-coumaroylglycine; *p*-coumaroyl-D-alanine.

Abstract—A mannan glycopeptide (Con A-II) which elicits the accumulation of *p*-coumaroylamino acids in *Ephedra distachya* cells was purified *ca* 20-fold from commercial yeast extract by ethanol precipitation, DEAE-cellulose, CM cellulose, gel filtration and Con A–Sepharose chromatography, consecutively. There was no significant elicitor activity in the glucan fraction. The elicitor active fraction (Con A-II) was partially hydrolysed with 2 M TFA and the hydrolysate chromatographed on Sephadex G-25 followed by ethanol precipitation. The activity was found in the 60% ethanol soluble but 80% ethanol insoluble fraction (MGP) whose M_r on gel filtration HPLC was nearly homogeneous (*ca* 4600). The GC-mass spectral analysis of the partially *O*-methylated alditol acetate and ^{13}C NMR spectroscopy revealed that MGP contained terminal-(25.4%), α-1,2-(33.6%), α-1,6-(20.6%) and α-1,2,6- (20.4%) mannan glycosyl linkages. MGP elicitor also contained 17.4% (by weight) of peptide which was composed of 10 kinds of amino acid. Ser and Thr comprised 45 molar% of the total amino acid content. Hydrazinolysis of MGP revealed that the mannan moiety was possibly linked to Ser and Thr of the peptide through *O*-glycosidic bonds. Enzymatic degradation of MGP using α-mannosidase and proteinase K suggested that both mannan and peptide moieties were essential for elicitor activity.

INTRODUCTION

It has been known that plants induce a series of biosynthetic enzymes triggered by external factors and the signalling substances which stimulate the induction of these enzymes are termed elicitors [1]. The production of phytoalexins, low M_r antibiotic materials which are induced as a result of plant–pathogen interaction, is one of the most important and well studied plant defence mechanisms against fungal infections [2–4]. However, the molecular basis of the interaction mechanisms between elicitors and plants is still obscure. The main reason is the general lack of homogeneous elicitor preparations of known structure and of appropriate bioassay methods for evaluating elicitor activities. The heterogeneity of plant materials to be tested has also been a serious problem as long as intact plants are employed for bioassay. Plant cell cultures may offer reduced complexity and can be controlled to obtain the best conditioned cells which are free from any phytopathogens. In fact, it has been shown that the plant–pathogen interaction in intact plant could be mimicked by the use of cultures as model systems [5].

The accumulation of phytoalexins has been demonstrated in at least 17 plant families, although most of the studies so far have been focused on Angiospermae belonging to crop plants [2]. Only a few reports are available for gymnospermous plants. Suspension cultures of *Pinus banksiana* treated with an elicitor prepared from an ectomycorrhizal fungus, *Thelephora terrestris*, rapidly accumulated thioglycolic acid-extractable cell wall-bound phenolics with the increased level of phenylalanine-ammonia lyase (PAL) activity [6]. However, the chemical structure of the elicitor has not been determined.

Cell suspension cultures of *Ephedra distachya*, a gymnospermous medicinal plant which produces clinically important alkaloids represented by *l*-ephedrine, were found to accumulate *p*-coumaroylglycine and *p*-coumaroyl-D-alanine by addition of yeast extract to the culture. The amount of accumulated *p*-coumaroyl conjugates was linear against the added amount of yeast extract up to 0.5 g per 100 ml medium with good reproducibility [8]. Some synthetic *N*-acylamino acids were reported to inhibit the growth of several phytopathogens [8]. From the underground part of *Ephedra*, *N*-feruloyl histamine

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had been isolated [9]. These facts and the induction of *p*-coumaroylamino acids by yeast extract addition to the culture under appropriate conditions strongly suggested that they might be produced as phytoalexins in intact *Ephedra*. In this context, we set out to trace and characterize the yeast-derived elicitor active materials which stimulate the accumulation of *p*-coumaroylamino acids in *E. distachya* cultures.

RESULTS AND DISCUSSION

Ephedra distachya callus was induced from the aerial part of the plant and the callus was transferred to MS liquid medium to propagate suspension cultures as described previously [7]. Two to three flasks of 27- to 28-days old stock culture of *Ephedra* cells were combined in a flask just before elicitation and subdivided into small volumes. Aliquots of sterilized elicitor solutions were added to the subdivided cells and further incubated under appropriate conditions. Elicited cells were extracted with methanol and the extract analysed by HPLC. Produced amounts of *p*-coumaroylglycine and *p*-coumaroyl-D-alanine were quantitated from the calibration curves prepared by synthetic standard samples [7]. Three to six different concentrations of elicitors were used for one set of bioassays and at least three sets of bioassays were made against each elicitor fraction. The activity of each fraction was expressed as the sum (nmol) of the produced *p*-coumaroylamino acids (*p*-CAA) g^{-1} fr. wt of cells. To compare the specific elicitor activity, the amount of each elicitor frs required to give an arbitrary amount of products (30 nmol g^{-1} fr. wt) was calculated from the range that exhibit a linear correlation between added amount of elicitors and produced *p*-CAA, and each elicitor activity was normalized to that of yeast extract as 1. This can be summarized by the equation: Specific activity = the amount (mg) of yeast ext. required to produce 30 nmol *p*-CAA (=4.4 mg)/the amount (mg) of elicitor required to produce 30 nmol *p*-CAA.

In order to decide the optimal elicitation timing, 15-, 27- and 40-day-old cells were treated with yeast extract (30 mg 10 ml^{-1} medium). The 15- and 40-day-old cells (log phase and late stationary phase, respectively) responded reluctantly to yeast extract addition, while the 27-day-old cells (early stationary phase) accumulated significant of *p*-CAA (*ca* 210 nmol g^{-1} fr. wt). The optimum growth phase of *Ephedra* culture for elicitor addition was found to be around the end of the exponential growth phase (Fig. 1). Yeast-elicited (30 mg 10 ml^{-1}) 27-day-old cells were harvested after 2, 4, 6, 9, 12, 24 and 48 hr of incubation in order to examine optimal duration for elicitation. The accumulation of *p*-CAA was increased with incubation time up of 12 hr and reached its maximum level about 24 hr after elicitation. Further incubation up to 48 hr did not increase the accumulation of *p*-CAA (Fig. 2). From these results, 26- to 28-day-old cells and 48 hr of duration for elicitor contact were chosen for optimum bioassay conditions.

Fractionation of yeast extract was initiated by 80% ethanol precipitation according to the reported method

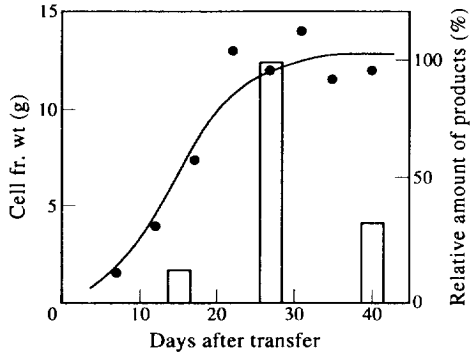

Fig. 1. Optimum growth phase of *E. distachya* cultures for elicitation. Logarithmic graph indicates the growth curve and bar graphs show the relative amount of elicitor-induced *p*-CAA. 100% represents *ca* 210 nmol of *p*-CAA per g fr. wt cells.

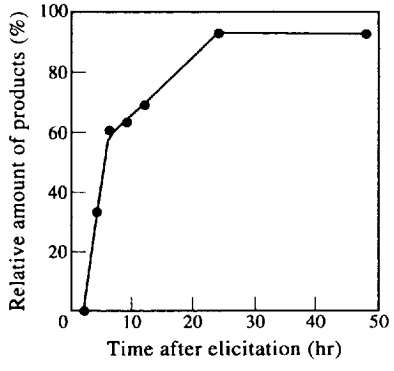

Fig. 2. Optimum duration for elicitor contact. 100% represents *ca* 210 nmol of products per g fr. wt cells.

[10] with some modifications. Yeast extract was precipitated with 80% ethanol and separated into soluble (80 S) and insoluble fractions (80 I). 80 I was dialysed against water and the inner and outer parts of the dialysis bag were concentrated and lyophilized (designated as 80 I-DI and 80 I-DO, respectively). The autoclaved solutions containing 2.5, 5 and 10 mg of each 80 I-DI, 80 I-DO, 80 S and yeast extract were added to the culture to compare their elicitor activities. The critical separation of elicitor activity of 80 I-DI and 80 I-DO was not achieved while the activity of 80 S was slightly lower than that of other two frs. 80 I-DI was chosen for further purification since this fraction has been known as a source of β-glucan elicitor of glyceollin accumulation in *Glycine max* [10]. The purification of 80 I-DO will be published elsewhere. The sugar and protein contents as well as specific activities of the fractions are presented in Table 1. Neutral sugar composition analysis using anion exchange HPLC revealed that mannose was the major neutral sugar component of the fraction (Table 2).

DEAE-cellulose chromatography of 80 I-DI afforded a non-binding fraction (DE-I) and four binding fractions

Table 1. Specific activity and the contents of carbohydrate and protein of elicitor active fraction

Fraction	Specific acitivity*	Protein ($\mu g\ mg^{-1}$)†	Carbohydrate ($\mu g\ m^{-1}$)†
Yeast extract	1.0	64	175
80 I-DI	3.0	283	521
DE-I	14.2	87	866
CM-I	13.0	70	881
GP-I	13.1	37	692
Con A-II	19.8	393	546
MGP	22.0	174	705
SG-60 I	—	118	845

*See text.
†Assayed by Lowry's method and anthrone–sulphuric acid, respectively.

Table 2. Neutral sugar composition* of elicitor active fractions

Fr.	Ara	Glc	Man	Rib	Gal
80 I-DI	0.5	0.9	93.4	4.6	0.6
DE-I	0.5	2.0	97.2	—	0.3
CM-I	—	9.7	90.3	—	—
GP-I	—	1.6	98.3	—	—
Con A-I	—	100.0	—	—	—
Con A-II	—	—	100.0	—	—
MGP	—	—	100.0	—	—
SG-60 I	—	—	100.0	—	—

*Analysed by anion exchange HPLC.
†Not determined.

(DE-II to DE-V). About 35% (be weight) of 80–I-DI was not retained on the DEAE-cellulose column (Fig. 3). The main elicitor activity was found in the void fraction, DE-I, which contained 90 $\mu g\ mg^{-1}$ of Lowry-positive materials and 870 $\mu g\ mg^{-1}$ carbohydrate. Neutral sugar composition analysis of DE-I revealed the presence of mannose, glucose, and small amounts of galactose and arabinose (Table 2). Then DE-I was applied to CM cellulose column to yield void (CM-I) and retained (CM-II) fr. Only a small amount of DE-I (3.8% by weight) was retained on CM cellulose and more than 95% of it was collected from the void fr. CM-I showed slightly higher activity than CM-II. Neutral sugar composition and the carbohydrate and protein contents of CM-I are presented in Tables 1 and 2.

Further purification of CM-I was attempted by gel filtration with Toyopearl HW 55S column. Three frs [GP-1($M_r \geq 200\,000$), GP-II (*ca* 35 000) and GP-III (*ca* 10 000)] were roughly separated according to their M_r. This chromatographic separation did not afford the critical separation of elicitor activity although GP-I showed slightly higher elicitor activity than others. This fact suggests that the M_r of elicitor is not essentially important for activity. Considering the yield of gel filtration frs, the most abundant GP-I was employed for further purification. Neutral sugar composition analysis revealed that GP-I consisted of a high percentage of mannose and contained minute amount of glucose (Table 2). Lowry positive materials were still found in GP-I in an amount of 37.2 $\mu g\ mg^{-1}$.

Since mannose occupied a great portion of GP-I, it was applied on a Concanavalin A (Con A)–Sepharose column. The anthrone positive material which has no affinity for Con A–Sepharose (Con A-I) was eluted by equilibration buffer. The retained material (Con A-II) was washed out with 4% α-methylmannopyranoside prepared in the same buffer. Con A-I, which contains glucose and Lowry-positive material, had no significant elicitor activity, whereas Con A-II, which is composed of mannose and peptide, showed significant activity (Table 2, Fig. 5). This fact suggests that the mannan moiety is essential for *p*-CAA-inducing elicitor, although the β-glucan elicitor purified from the cell wall of *Phytophthora megasperma* (Pmg) or yeast extract has been asserted to be the most effective on the accumulation of phytoalexin glyceollin in hypocotyls or cotyledons of *Glycine max* [11, 12].

To investigate which moiety is necessary for the elicitor activity, Con A-II was digested with exo-α-mannosidase

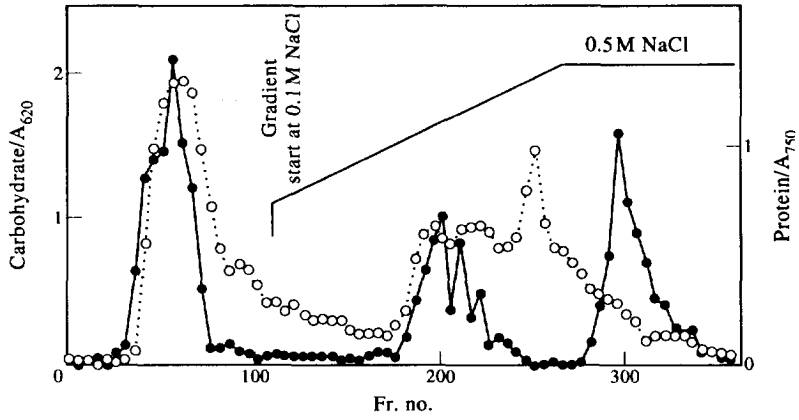

Fig. 3. Elution profile of 80 I-DI on DEAE-cellulose. Frs 25–75, frs 76–175, frs 176–225, frs 226–275 and frs 276–359 were collected, dialysed, lyophilized and designated as DE-I, DE-II, DE-III, DE-IV and DE-V, respectively. Carbohydrate (—●—) was monitored by anthrone–sulphuric acid and protein (---○---) was by Lowry's method.

or proteinase K. Time dependent decrease of *p*-CAA-inducing activity of Con A-II by these enzymatic digestions indicated that both sugar and peptide moieties were essential for elicitor activity (Table 3).

To identify the minimum structure required for elicitor activity, Con A-II was partially hydrolysed with 2 M trifluoroacetic acid (TFA). The elicitor activity remained intact after 2 or 4 hr of hydrolysis, even though the gel filtration HPLC analysis of these hydrloysate showed the shift of M_r distribution to the lower side. Hydrolysate (2 M TFA, 4 hr) of Con A-II was applied on Sephadex G-25. The activity was found in void fraction (SG-25) whose M_r distribution was still heterogeneous when analysed by gel filtration HPLC using TOSOH G-3000 PWXL (Fig. 4).

SG-25 was fractionated into 80% ethanol soluble fraction (SG-80 S), 60% ethanol insoluble fraction (SG-60 I) and 60% ethanol insoluble but 80% ethanol soluble active (MGP) fraction. MGP and SG-60 I whose M_rs were estimated to be 4700 and 21 700, respectively, were found to be relatively homogeneous (Fig. 4). M_rs was calculated from the retention times on HPLC by comparison with those of standard pullulan M_r markers. MGP exhibited almost the same elicitor activity as Con A-II, while SG-60 I did not show any significant activity.

Table 3. Effect of enzymatic digestion on elicitor activity

Incubation time (hr)	Relative elicitor activity (%)*	
	α-Mannosidase	Proteinase K
24	90.0	88.0
48	77.9	82.5

*Remaining elicitor activity after enzyme treatment (presented as relative % against boiled enzyme-treated control)

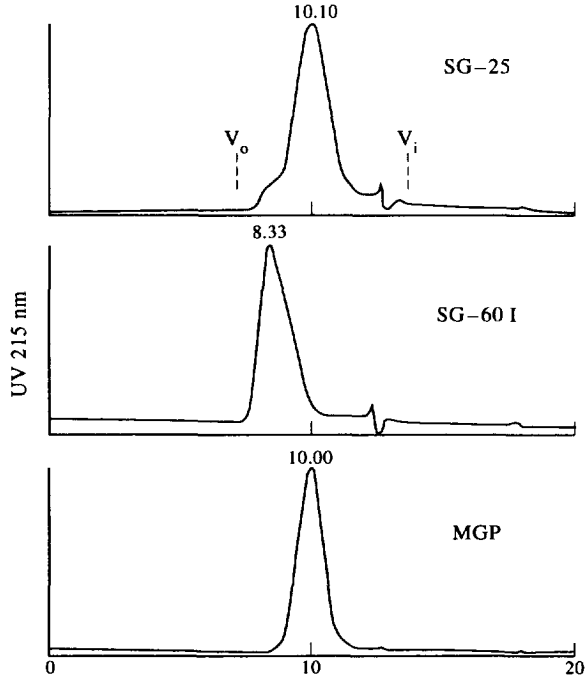

Fig. 4. Gel filtration HPLC chromatogram of SG-25, SG-601 and MGP. Detection was carried out by both UV 215 nm and refractive index. Chromatograms under UV detection are presented only, since no significant differences between them were recognized.

When calculated with their average M_r, half maximal concentration of Con A-II and MGP was *ca* 17 and 15 nmol, respectively.

Although Con A-II and MGP were purified *ca* 20–22 fold from yeast extract, they failed to accumulate the maximum amount (*ca* 210 nmol) of *p*-CAA shown in yeast extract elicitation (fig. 5). Two possibilities were

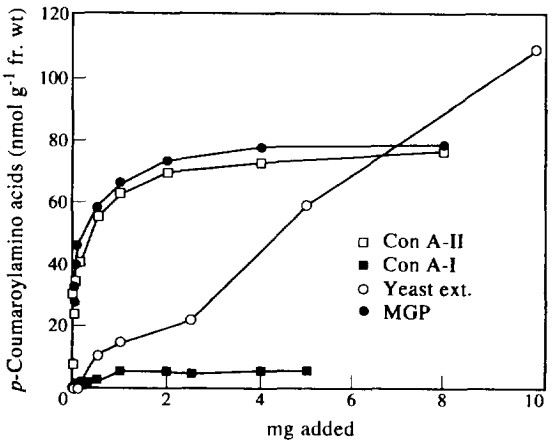

Fig. 5. Elicitor activity of Con A-I, Con A-II, MGP and yeast extract.

assumed by this fact: one is the inactivation of elicitor during the purification procedure, and the other was the existence of another elicitor active material or the presence of an enhancer of the elicitor. The former was less likely since MGP elicitor was relatively stable to heat and pH. On the contrary, the latter possibility was more reasonable because the co-addition of 80% ethanol soluble fraction (80 S) with MGP greatly enhanced the elicitor activity of MGP (data not shown). 80 S contained ninhydrin positive materials, and TLC analysis of this fraction revealed the presence of free amino acids.

Elicitor active MGP and elicitor inactive SG-60 I were permethylated with solid NaOH and methyliodide in DMSO. The permethylated MGP and SG-60 I were hydrolysed, reduced to their alditols and acetylated [13, 28]. The molar % of each partially *O*-methylated alditol acetate was determined by the peak areas in GC. The total ion chromatogram showed four peaks and each peak was analysed by GC-EI-mass spectrometry. The fragmentation pattern and relative retention times of partially-*O*-methylated alditol acetates in GC or GC-EI-mass spectrometry revealed the presence of terminal-, 1,2-, 1,6-, and 1,2,6-mannosyl linkages in its structure [14]. Compared with the elicitor active MGP, no significant differences in its glycosyl linkage composition was recognized in the inactive SG-60 I, however, they showed a notable difference in their peptide contents (Tables 1 and 4). The inactive SG-60 I contained only negligible amounts of peptide, whereas active MGP contained 174 $\mu g\,mg^{-1}$ of peptide. This fact, in combination with the result of the proteinase digestion of Con A-II, clearly indicated that the peptide moiety was essential for the elicitor activity.

Four kinds of anomeric carbon resonances were detected in the ^{13}C NMR spectrum of MGP at δ103.6, 102.1, 100.8, and 99.6 ppm. They were assigned to anomeric carbons of terminal-, 1,2-, 1,6-, and 1,2,6-mannosyl moiety, respectively by comparison with the reported data [15–17]. The $^1J_{C\text{-}H}$ coupling constants of anomeric carbons (171.4–173.4 Hz) indicated that the anomeric

Table 4. Glycosyl linkage analysis* of SG-60 I and MGP

Glycosyl linkage	Mol% glycosyl residues		
	T value	MGP	SG-60 I
t-Man	1.00	25.4	25.3
2-Man	1.33	3.6	21.4
6-Man	1.45	20.6	14.9
2,6-Man	2.13	20.5	38.4

*Analysed by GC-EI-MS as their alditol acetates.

protons of MGP should have an α-configuration (Fig. 6) [18].

MGP was hydrolysed by HCl and analysed for its amino acid composition and contents. Ten kinds of amino acids were detected and Ser and Thr comprised 45 molar% of the total detected amino acids (Table 5). To investigate the type of glycopeptidal linkage, MGP was treated with hydrazine [19]. The UV spectrum of the hydrazinolysed MGP showed an increased *A* at 241 nm, indicating the formation of a conjugated double bond in the β-position. On the contrary, elicitor inactive SG-60 I

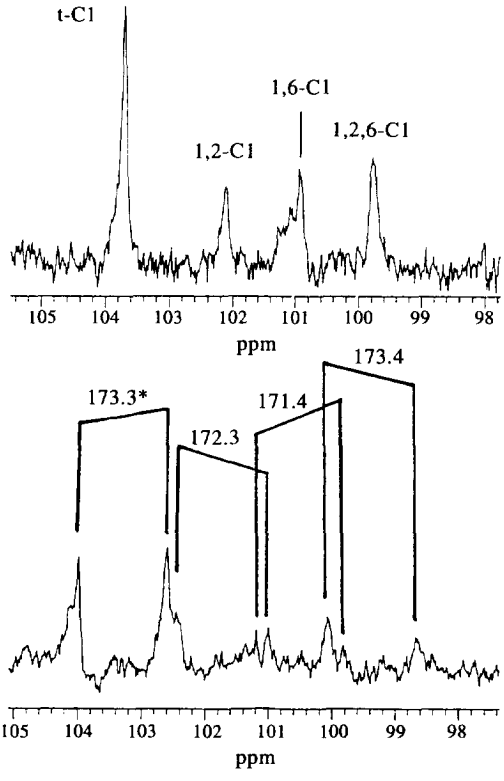

Fig. 6. ^{13}C NMR spectrum of anomeric carbons of MPG. The lower spectrum is a non-proton decoupled and the upper one is a complete decoupled spectrum. NMR spectra were recorded on 125 MHz in D_2O at 35°. Chemical shifts are given in δ (ppm) relative from TMS. *$^1J_{C-H}$, Hz.

Table 5. Amino acid contents of MGP and its hydrazinolysate

Amino acids	mol/mol aspartic acid	
	Non-hydrazinolysed	Hydrazinolysed
Asp	1.00	1.00
Thr	2.43	0.39
Ser	2.66	0.59
Glu	1.12	1.06
Gly	0.57	0.55
Ala	1.51	1.68
Val	0.67	0.60
Ile	0.38	0.24
Leu	0.39	0.32
Lys	0.59	0.56

did not show any increase in *A* at 241 nm after hydrazine treatment. The hydrazinolysed MGP was analysed for its amino acid content. Compared with the non-hydrazinolysed control, the content of Ser and Thr was decreased significantly (14% of total detected amino acid), suggesting that the peptide moiety of MGP was *O*-glycosidically linked through Ser and Thr to its mannan moiety.

It has been known that the carbohydrate component of yeast cell wall mannan was attached covalently to protein or peptide-like material. One type has been identified as a branched polymannose of high M_r that appeared to be attached to the peptide by the bond between *N*-acetylglucosamine and Arg [20]. The other type which has been found by the β-elimination reaction was small oligosaccharides linked by *O*-glycosidic bonds to the hydroxyaminoacids Ser and Thr in the peptide [21]. Therefore, MGP was supposed to be yeast cell wall fragment which could be involved in the latter type of mannan glycopeptide.

Several glycoproteins have been reported as effective elicitors of phytoalexin production. Glycoproteins from various races of *Cladosporium fulvum* were found to be elicitors or rishitin accumulation in tomato. As in the case of MGP elicitor, the activity was dependent on both glycosyl and peptide portions of the molecule [22, 23]. A similar type of glycoprotein, obtained by autoclaving the germ tubes of *Uromyces phaseoli*, was shown to elicit the accumulation of phytoalexins in *Phaseolus vulgaris* [24]. The glycoprotein that could be released from the cell walls of Pmg by the action of pronase was a glucomannan conjugate which stimulated phytoalexin accumulation in soybean. Glycoproteins from different races of the fungus were found to exhibit race-specific elicitation [12, 25]. However, none of the sugar moieties of reported glycoprotein elicitors was composed of mannose alone, but they contained glucosyl or galactosyl moiety together with the mannosyl group. MGP was the first example of elicitor active glycopeptide whose glycosyl residue consisted of homomannan.

EXPERIMENTAL

Bioassay of elicitor activity. Two flasks (10 ml per 100 ml flask) of elicited cells were combined in a flask and the combined cells were filtered through filter paper. Cells (1 g fr. wt, almost equivalent to 10 ml culture) was extracted with 10 ml MeOH by ultrasonics for 30 min. Supernatant (1 ml) was taken out and filtered through Toyo-Pak ODS (Tosoh). 40 μl filtrate was injected to HPLC for *p*-CAA quantitation. HPLC conditions were the same as those described in the previous paper [8] except that the column temp. was maintained at 35°.

Elicitation. Two to three flasks of 27- to 28-day-old stock culture (100 ml medium in 500 ml conical flask) of *E. distachya* cultures were combined in a flask just before elicitation and they were subdivided into 10 ml in a 100 ml flasks with an autoclaved pipette. Samples to be tested were dissolved in 0.5 ml H_2O and autoclaved at 121° for 20 min. Elicited cells were further incubated for 48 hr or an appropriate time.

Colorimetric assays. The sugar contents were determined by the method described in ref. [26] using mannose as a standard. The protein contents were determined by the method of ref. [27] using BSA as a standard.

Neutral sugar composition. One to two milligrams of each fr. was dissolved in 1–2 ml of 2 M TFA in a sealed tube and hydrolysed at 121° for 1 hr [28]. The hydrolysate was cooled to room temp. and filtered through a Whatman GF/D filter. The filtrate was evapd to dryness and 5 ml *iso*-PrOH was added to the residue the soln was evapd again in order to remove any remaining TFA. This process was repeated ×3 and the final dark brown residue was dried *in vacuo*. After 0.5–1 ml elution buffer (described below) was added, aliquots of soln were injected for HPLC analysis. HPLC conditions were as follows: column; TOSOH Sugar AXI (4.6 × 150 mm), column temp.; 60°, detection; refractive index and/or UV 215 nm, flow rate; 0.4 ml min^{-1}, eluent; 0.5 M Na borate buffer (pH 8.7).

Fractionation of yeast extract. (1) 80% EtOH pptn: yeast ext. (Bacto Difcolab, 100 g) was suspended in 1 l H_2O and 4 l EtOH was added to make 80% aq. EtOH soln. The turbid soln was allowed to settle at 4° for 3 days to ppt. insoluble materials. The clear yellow supernatant was decanted off from the sticky dark brown residue. The residue was dissolved in H_2O and EtOH pptn was repeated ×3 in the same manner. The soluble fr. was combined and concd and stored at 4°. The final 80% EtOH insoluble fr. (80 l) was dissolved in 400 ml deionized H_2O and dialysed at 4° against H_2O for 3 days, changing H_2O ×3. The insoluble material which formed inside the dialysis bag was filtered in a Whatman GF/D filter and the filtrate was lyophilized (80 I-DI, 5.8 g). The outer fr. of the dialysis bag was concd *in vacuo* and lyophilized (80 I-DO, 13.5 g). All frs were stored in a desiccator until they were used.

(2) DEAE-cellulose: 4.2 g 80 I-DI was applied on a DEAE-cellulose (Whatman DE 52) column (3.5 × 47 cm) which has been equilibrated with 5 mM Na-Pi buffer and 5 ml eluates collected (total *ca* 500 ml). The non-binding fr. which was positive to both anthrone–H_2SO_4 and Lowry tests was combined and dialysed (DE-I). The bound material was eluted from the column by a linear gradient elution of 0.1–0.5 M NaCl prepared in the same

buffer (total *ca* 800 ml), and fractionated into DE-II to DE-V (total 2.8 g).

(3) CM cellulose: 1.2 g DE-I was dissolved in 30 ml equilibration buffer and applied on a CM cellulose column [Whatman CM 52, 3.5 × 27 cm equilibrated with 20 mM NaOAc buffer (pH 3.5)]. The column was eluted with 200 ml equilibration buffer (CM-I) and subsequently washed with a linear gradient of NaCl (0.1–0.5 M) prepared in the same buffer (total gradient vol. was 400 ml). Finally, the column was washed with 200 ml equilibration buffer containing 1 M NaCl (CM-II).

(4) HW55S gel filtration: 75 mg CM-I was applied on a gel filtration (Tosoh HW55S) column (2.2 × 28 cm) and eluted with H_2O. The eluate was monitored by both UV (215 nm) and refractive index. Three frs [GP-I (48 mg), GP-II (15 mg) and GP-III (5 mg)] were obtained. This chromatography was repeated × 4.

(5) Concanavalin A–Sepharose: About 0.1 g of GP-I was applied to Concanavalin A–Sepharose (Pharmacia, Con A–Sepharose) column (2.5 × 21 cm) which had been equilibrated with Con A buffer (0.1 M NaOAc buffer (pH 5.2) containing 150 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$ and 1 mM $CaCl_2$). The column was washed with 400 ml of equilibration buffer. Anthrone positive material which has no affinity for Con A–Sepharose came out in the first 100 ml (Con A-I, 6 mg). The retained material was eluted from the column by 400 ml of 4% α-methyl-mannopyranoside in Con A buffer (Con A-II, 56 mg). Because of the high content of mannose in GP-I, 100 mg was the maximum amount to be applied to 100 ml Con A–Sepharose, therefore the same procedure was performed twice for 190 mg of GP-1.

Partial hydrolysis of Con A-II. Con A-II (2 mg) was dissolved in 2 ml of 2 M TFA in a sealed tube. Three tubes were prepared and hydrolysed at 85° for 2, 4 and 8 hr. The reaction mixture was allowed to cool at room temp., followed by filtration through Whatman GF/D filter and evapd to dryness. Water (1 ml) was added to the residue and the soln filtered through cotton. Aliquots (500 μl) were tested for their elicitor activity and the rest was submitted to gel permeation HPLC. A large-scale hydrolysis of Con A-II was carried out by the same method described above.

Sephadex G-25. Hydrolyzed (4 hr) Con A-II (67 mg) was applied on Sephadex G-25 (5 × 30 cm, eluted with H_2O). Three milliliter eluates was collected and monitored by anthrone assay. Aliquots (200 μl) of eluate were assayed for their elicitor activities.

Ethanol precipitation of SG-25. SG-25 (35 mg) was dissolved in 8 ml H_2O and 12 ml EtOH was added to make 60% aq. EtOH soln. After being centrifuged (10 000 *g* for 20 min at 4°), the supernatant was taken out carefully from the tube and evapd under red. pres. The remaining pellet was lyophilized (SG 60 l, 7 mg). The 60% EtOH soluble fr. was dissolved in 4 ml H_2O and 16 ml EtOH added to make 80% aq. EtOH soln. The centrifugation of the soln afforded supernatant (SG 80-S) and pellet (MGP, 14 mg).

Gel filtration HPLC. HPLC conditions were as follows: column; Tosoh G3000 PWXL (7.8 × 300 mm), column temp.; 40°, detection UV 215 nm and refractive index, eluant; 0.1 M NaCl, flow rate; 0.8 ml min^{-1}.

^{13}C NMR, GC and GC-EI mass spectrometry. ^{13}C NMR spectra were recorded in D_2O, 35° on 125 MHz (GSX 500). Chemical shifts are given in δ (ppm) from TMS. GC was carried out by Shimadzu GC-14A equipped with fused silica capillary column (Supelco SP-2330, 0.32 mm i.d. × 30 m). GC conditions were as follows: detection; FID, detector temp.; 250°. injection temp.; 250°, column temp.; programmed 170° to 220° at 4° min^{-1}, carrier gas; He at 30 ml min^{-1}. GC-EI-MS was measured by Jeol JMS-DX303.

Enzymatic degradation. Proteinase K (from *Tritirachium album*) and exo-α-mannosidase (from almond) were purchased from Sigma. Con A-II (2 mg) was suspended in 900 μl 0.25 M K-Pi buffer (pH 7.5) and 1 mg (*ca* 20 unit) proteinase (dissolved in 100 μl buffer) was added. The enzyme reaction mixture was incubated at 37° for 24 or 48 hr. Another 2 mg Con A-II was suspended in 90 μl 50 mM KOAc buffer (pH 4.5) and 1 mg (*ca* 30 unit) α-mannosidase was added. The enzyme mixture was incubated at 27° for 24 hr or 48 hr and the reaction was stopped by boiling the mixture. Boiled enzymes served as controls.

Hydrazinolysis. MGP elicitor (1 mg) or SG-60 I was dissolved in 0.5 ml of 25% hydrazine monohydrate. β-Elimination was carried out in a sealed tube at 110° for 18 hr. After cooling at room temp., the soln was evaporated under red. pres. at 40°. The residual material was dried over $CaCO_3$ *in vacuo* for 18 hr.

Amino acid analysis. Typical hydrolysis was performed as follows; 1 mg sample was dissolved in 200 μl 5.7 mM HCl and 20 μl soln was transferred to a minivial, then evapd to dryness. The residues located in Waters Pico-Tag hydrolysis kit were hydrolysed with 5.7 M HCl for 2 hr. The hydrloysate was dried again and dissolved in 40 μl 5.7 mM HCl, followed by centrifuging to ppt. insoluble materials. Supernatant (2 μl) was injected for HPLC analysis. HPLC conditions were as follows: column; Tosoh IEX-215 (4.6 × 50 mm), elution buffer; Na-citrate buffer (pH 3.2-10.5), reaction buffer; NaOCl or OPA in 0.7 M Na_2CO_3 buffer (pH 10), flow rate; 0.3 ml min^{-1} for elution buffer and 0.2 ml min^{-1} for reaction buffer, column temp.; 60°, detection; fluorescence (Ex. 235, Em. 389 nm). Amino acid contents were determined by comparison of their retention times and peak heights with those of standard materials.

Methylation analysis. Methylation analysis was carried out by the reported method using solid NaOH and MeI [13] with some modification. Briefly, 4 mg MGP in centrifuge tube was dried *in vacuo* in a desiccator prior to methylation. After 0.5 ml of freshly distilled DMSO was poured into the dried MGP, 20 mg powdered solid NaOH and 120 μl MeI were added to the soln simultaneously. The reaction mixture was stirred for 1 hr at room temp. under N_2. The mixture was extracted with $CHCl_3$ and the organic layer evpd to dryness (3.3 mg). The residue was applied to the Sephadex LH-20 column (1 × 20 cm, eluted with $CHCl_3$–MeOH, 2:1). One millilitre fractions were collected and frs 5–7 combined and evapd

(3.0 mg). The residue was hydrloysed by 660 μl 0.25 M H_2SO_4 in 95% HOAc at 80° for 16 hr in a sealed tube. After cooling at room temp., 660 μl H_2SO_4 was added, then re-sealed for further hydrolysis at 100° for 3 hr. The partially *O*-methylated monosaccharides were reduced with $NaBH_4$ overnight. The resultant partially *O*-methylated alditols were acetylated with pyridine and Ac_2O. Partially *O*-methylated alditol acetates were dissolved in $CHCl_3$ just before the GC or GC-EI-MS analysis.

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