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ORIGINAL ARTICLE

Effects of alginate oligosaccharides with different molecular weights and guluronic to mannuronic acid ratios on glyceollin induction and accumulation in soybeans

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Abstract Alginate oligosaccharides (AOs) are linear oligosaccharides with alternating sequences of mannuronic acid (M) and guluronic acid (G) residues. AOs can be used as a safe elicitor to induce glyceollins, which have many human health benefits, in soybean seeds. In this research, four AO fractions with different chemical structures and molecular weights were separated, purified, and then characterized by NMR spectroscopy and ESI–MS. With a 4,5-unsaturated hexuronic acid residue (△) at the non-reducing terminus, the structures of these four AO fractions were △G, △MG, △GMG and △MGGG, which exhibited glyceollin-inducing activities of 1.2339, 0.3472, 0.6494 and 1.0611 (mg/g dry weight) in soybean seeds, respectively. The results demonstrated that a larger molecular weight or a higher G/M ratio might correlate with a higher glyceollin-inducing activity. Moreover, the alginate disaccharide △G could be introduced as relatively safe and efficient elicitor of high glyceollin content in soybeans.

Keywords Alginate oligosaccharides · Glyceollin · Soybean · Molecular weight · Ratio of guluronic to mannuronic acid (G/M)

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Abbreviations

AO	Alginate oligosaccharide
G	Guluronic acid
M	Mannuronic acid
HPLC	High-performance liquid chromatography
NMR	Nuclear magnetic resonance
ESI–MS	Electrospray ionization–mass spectrometry
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
△	*O*-(4-deoxy-α-L-erythro-hex-4-enopyranosyluronic acid)-(1→
-G-	→4)-*O*-(α-L-gulopyranosyluronic acid)-(1→
-G	→4)-*O*-β-L-gulopyranuronic acid
-M-	→4)-*O*-(β-D-mannopyranuronic acid)-(1→

Introduction

Soybeans are a major part of the Chinese diet and have become popular in other parts of the world as well. The popularity of soybeans is associated with their health-promoting properties, which include reduced risks of cancer and cardiovascular disease (Jenkins et al. 2010). These health benefits result from the presence of bioactive compounds such as isoflavones in soybeans (Boue et al. 2009). Glyceollins, a major family of phytoalexins, are important isoflavones in soybeans (Bhattacharyya and Ward 1986). They have many human health benefits, such as preventing breast and ovarian carcinoma (Salvo et al. 2006), preventing hyperglycemia and insulin resistance (Yoon et al. 2013), and regulating lipid and carbohydrate metabolism (Wood et al. 2012). Glyceollins accumulate in soybeans in response to various elicitors, including substances of pathogen origin (biotic elicitors), such as cell walls and

spores (Fett and Zacharius 1982; Moesta and Grisebach 1980; Yoshikawa and Yoshikawa 1978), and abiotic elicitors, such as heavy metal salts and detergents (Pitta Alvarez et al. 2000; Stintzi et al. 2001; Stössel 1984). However, those elicitors are either complex microorganic mixtures or poisonous compounds, which may impact the consumption of induced soybean products because of food biosafety considerations and restrict their application in the food industry (Angelova et al. 2006).

Alginate oligosaccharides (AOs) are marine oligosaccharides generated from alginate. They contain guluronic acid (α-L-guluronate, G) and mannuronic acid (β-D-mannuronate, M) units linked by 1 → 4 *O*-glycoside bonds; these units are arranged in homopolymeric G blocks, M blocks, and random heteropolymeric G and M blocks (Nagasawa et al. 2000; Zhang et al. 2013). To date, the structures of AOs have been elucidated using ESI–MS, NMR spectroscopy and ESI–MS techniques (Holtan et al. 2006; Jochum et al. 2002; Scherz et al. 2005). AOs have multiple roles and can be used as growth factors to stimulate the VEGF-mediated growth and migration of human endothelial cells (Kawada et al. 1997, 1999) and to regulate plant developmental and defensive processes (Akimoto et al. 2000; Chandía et al. 2004; Iwasaki and Matsubara 2000; Ma et al. 2010; Natsume et al. 1994). However, there still have been very few studies on the induction capacity of AOs for glyceollin accumulation in plants, especially soybeans (Zhang et al. 2015).

In our previous research, we reported the glyceollin-inducing activity of AOs in soybeans; however, the AOs used as elicitors were a mixture of sodium alginate oligomers (Jia et al. 2012). The identification of AOs derived from the M-, G-, or MG-blocks and their sequence determination are known to be important for better understanding the structure–function relationships of alginates at the molecular level (Schürks et al. 2002). Therefore, in this study, the structures of four AO fractions were completely determined, and then each fraction was used to induce glyceollin accumulation in soybeans in order to investigate the relationship between the structure and glyceollin-inducing activity of AOs. The final aim of this research is to find a suitable structure for use as an efficient elicitor that is safe for humans to increase the amount of glyceollins in soybeans in order to improve their health benefits.

Materials and methods

Materials and chemicals

The mixture of alginate oligosaccharides (AOs, enzymatically hydrolyzed) used was donated by the Dalian Institute of Chemical Physics (Dalian, China). The glyceollin standard was prepared in our lab by preparative HPLC (Eromosele et al. 2013). Soybean was purchased from Kefeng Group Co. (Haerbin, China). Deuterium oxide (99.9 atom %D) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). HPLC-grade methanol and acetonitrile were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA). Only HPLC-grade water was used to prepare reagent solutions. All other analytical-grade reagents were purchased from Beijing Chemicals Reagent Company (Beijing, China).

Methods

Separation of alginate oligosaccharides

The AO powder (1 g) was mixed with 100 ml of water for 2 h in room temperature. The mixture was centrifuged at 15,000 g for 5 min, and the supernatant was filtered through a 0.22 μm sterile syringe filter. The filtered extract was applied as a crude AO solution. A 1 ml sample was fractionated and collected by a Sykam S1125 preparative HPLC system equipped with an S3245 UV detector operating at a wavelength of 230 nm, Clarity ver. 7.1 software and a TSKgel DEAE-2SW preparation column (20.0 mm I.D. × 25 cm, Tosoh Corp., Tokyo, Japan) using a 180 min linear gradient of 0–0.25 M NaCl and a flow rate of 7.0 ml/min.

Alginate oligosaccharides desalination

The separated fraction samples were desalinated by a solid phase extraction (SPE) cartridge with a porous graphitic carbon column (Varian Bond Elut Carbon, 500 mg, 6 ml). Each sample was totally evaporated and then redissolved in 10 ml of 0.1% (v/v) trifluoroacetic acid (TFA). The small column was previously conditioned with 5 ml of acetonitrile and 5 ml of 0.1% (v/v) TFA. In each procedure, a 500 μl sample was loaded into column, the column was washed with 6 ml 0.1% (v/v) TFA, and then the oligosaccharide was eluted with 5 ml of 0.1% (v/v) TFA containing 25% (v/v) acetonitrile. The desalination procedures described above were repeated several times until NaCl was completely removed, which was determined by NaCl detection with 1% (w/v) $AgNO_3$. Finally, the desalinated oligosaccharide samples were freeze-dried and stored at − 20 °C.

Purity test on AO fractions

The collected AO fractions were checked on an analytical HPLC Waters 2695 system equipped with a 2996 UV detector and Empower Pro software under previously described conditions (Chaki et al. 2006): Column, TSKgel

DEAE-2SW 4.6 mm I.D. × 25 cm (Tosoh Corp., Tokyo, Japan); column temperature, 30 °C; monitor wavelength, 230 nm; mobile phase, water-0.25 M NaCl with a linear gradient (0–60 min); flow rate, 1.0 ml/min; injection amount, 20 µl; analytical time, 60 min. The purity of each fraction was calculated via the HPLC area normalization method.

NMR spectroscopy for structural analysis

Each AO fraction sample (20 mg) was evaporated by lyophilization twice with 0.5 ml of deuterium oxide to remove exchangeable protons before final dissolution in 0.5 ml of deuterium oxide for 1D and 2D NMR analysis on a Bruker Avance 500 spectrometer. ^{1}H-1D, ^{1}H-^{1}H COSY-2D, ^{1}H-^{13}C HSQC-2D and ^{1}H-^{13}C HMBC-2D NMR spectra were recorded at 500 MHz at 25 °C. ^{13}C-1D NMR spectra were obtained at 125 MHz at 25 °C. The water peak in ^{1}H NMR spectra was 4.70. DMSO was used as an internal standard; the DMSO peak in ^{13}C NMR spectra was 37.83.

ESI–MS analysis

ESI–MS was conducted in negative-ion mode with a Waters Xevo TQ-s instrument (Waters Corp.). Each sample was dissolved in water and diluted in 50% aqueous methanol. The ESI–MS conditions were as follows: injection volume, 2 µl; ESI voltage, 4 kV; capillary temperature, 275 °C; capillary voltage, 350 V; tube lens, 250 V; scan range, 400–2000 m/z. Nitrogen was used as the sheath gas and auxiliary gas at a flow rate of 30 and 5 arb, respectively. The mobile phase (methanol/water = 1:1, v/v) was delivered at a flow rate of 200 µl/min.

Glyceollin induction

Glyceollin induction in soybean cotyledons was performed according to the method described by Boué with some modifications (Boué et al. 2000). The AO mixture and the AO single compound samples were dissolved in water to provide 1% (w/v) solutions. Each solution (60 µl) was applied to the cut surface of a soybean cotyledon. In the control group, water (60 µl) was applied. All chambers were sealed with parafilm and incubated for 4 days at 25 °C in the dark.

Glyceollin determination

Soybean cotyledon extraction and sample preparation were conducted according the methods described by Eromosele in our previous research (Eromosele et al. 2013). The accumulation of glyceollins was calculated by the following formula: $y = 1.0 \times 10^{7}x + 754.32$ ($R^2 = 0.9999$).

Results

Separation of alginate oligosaccharides

AOs are a kind of polyanionic oligosaccharides and thus were separated on an anion-exchange column by preparative HPLC (Fig. 1a). This method yielded a series of distinct and well-resolved peaks, which were defined as F1 to F12. All the fractions were collected and analyzed further via analytical HPLC (Fig. 1b–n). The retention time and purity of each faction are shown in Table 1. The relative purities of these fractions mainly reached a high level of ≥ 97%; however, F6 and F10 were observed to be divided into 2 peaks upon further analytical HPLC analysis (Fig. 1h, l), which indicated that one-step separation was not sufficient to distinguish every oligomer of the AO mixture, as oligomers with similar structures could have had the same retention time.

Structural confirmation of the separated alginate oligosaccharide fractions

High-resolution NMR spectroscopy was performed to confirm the structure of the AO fractions. Finally, the structures of 4 fractions (F1, F3, F5 and F7) were completely determined, as they displayed clear signals in their NMR spectra. In our study, because the AOs were obtained by lyase cleavage, a 4,5-unsaturated hexuronic acid residue (△) was located at the non-reducing terminus (Zhang et al. 2006). The chemical shift values of ^{1}H and ^{13}C peaks are given in Table 2. Peaks in the 1D NMR spectra were assigned based on 1D ^{1}H and ^{13}C NMR experiments and 2D NMR experiments, including ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC and ^{1}H-^{13}C HMBC (see Supplemental Fig. 1-6). According to the previous data of NMR spectroscopy of alginate oligomers (Holtan et al. 2006; Zhang et al. 2004), the structures of F1, F3, F5 and F7 in our research were established as follows: F1: △G, *O*-(4-deoxy-α-L-erythro-hex-4-enopyranosyluronic acid)-(1 → 4)-*O*-β-L-gulopyranuronic acid; F3: △MG, *O*-(4-deoxy-α-L-erythro-hex-4-enopyranosyluronic acid)-(1 → 4)-*O*-(β-D-mannopyranuronic acid)-(1 → 4)-*O*-β-L-gulopyranuronic acid; F5: △GMG, *O*-(4-deoxy-α-L-erythro-hex-4-enopyranosyluronic acid)-(1 → 4)-*O*-(α-L-gulopyranosyluronic acid)-(1 → 4)-*O*-(β-D-mannopyranuronic acid)-(1 → 4)-*O*-β-L-gulopyranuronic acid; and F7: △MGGG, *O*-(4-deoxy-α-L-erythro-hex-4-enopyranosyluronic acid)-(1 → 4)-*O*-(β-D-mannopyranuronic acid)-(1 → 4)-*O*-(α-L-gulopyranosyluronic acid)-(1 → 4)-*O*-(α-

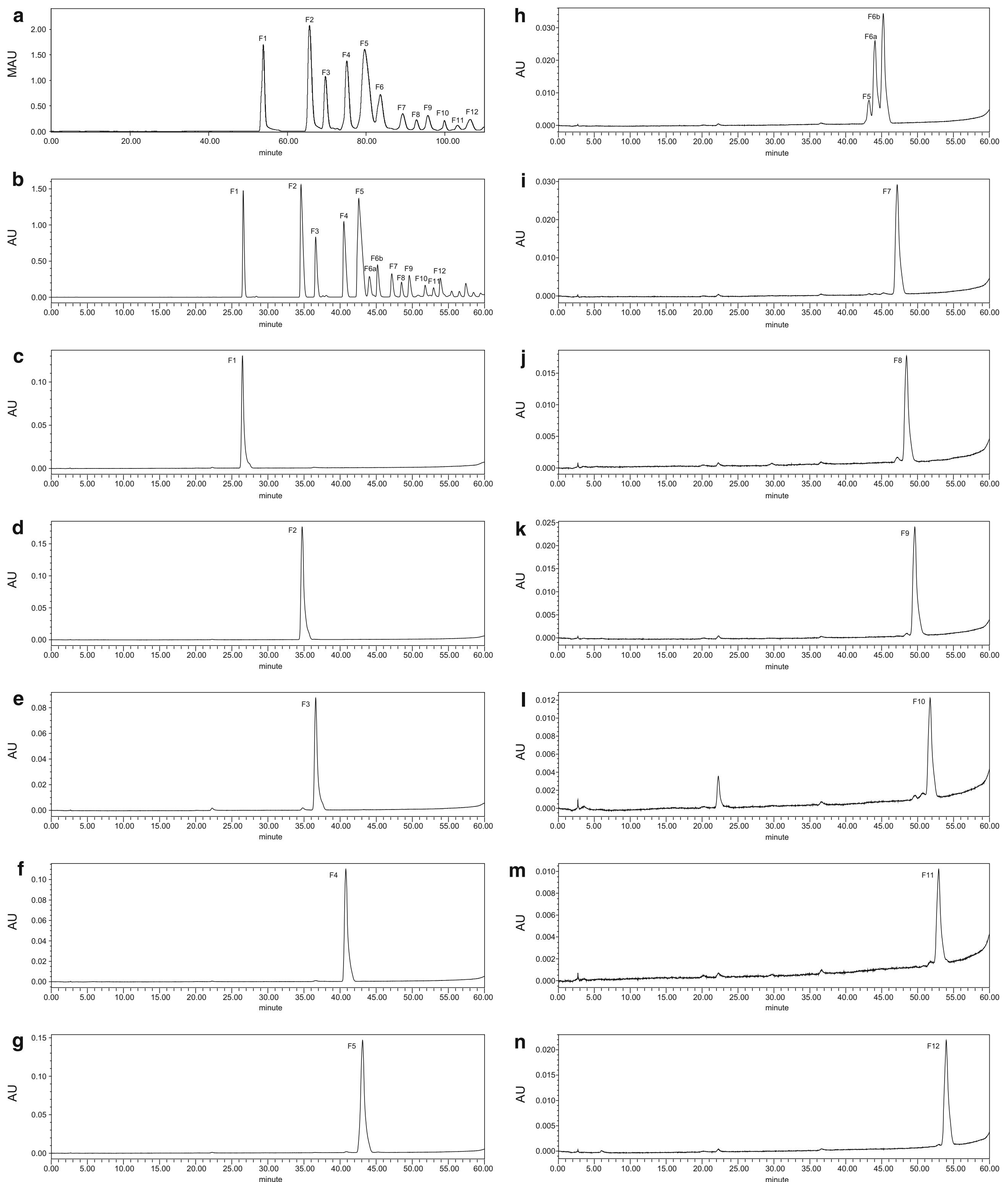

Fig. 1 HPLC profiles of AO separation. **a** Preparative HPLC profile of the AO mixture. A total of 12 fractions collected in 110 min are shown. **b** Analytical HPLC profile of the AO mixture. Fraction F6, which was defined based on the preparative HPLC chromatogram, was divided into 2 fractions for analytical HPLC. **c**–**n** Analytical HPLC profiles of each oligosaccharide fraction collected by preparative HPLC. The purity of each fraction was calculated via the HPLC area normalization method

Table 1 The retention time and the relative purity of AO fraction F1–F12

Fraction	Preparative HPLC (min)	Analytical HPLC (min)	Relative purity (%)	Fraction	Preparative HPLC (min)	Analytical HPLC (min)	Relative purity (%)
F1	53.9	26.476	98.08	F7	89.4	47.158	99.86
F2	65.6	34.740	99.67	F8	92.9	48.471	99.34
F3	69.7	36.618	97.66	F9	95.8	49.623	99.78
F4	75.1	40.801	98.19	F10	100.0	51.741	–
F5	79.6	43.120	99.39	F11	103.3	52.929	99.16
F6a	83.7	44.076	–	F12	106.5	53.983	99.76
F6b	83.7	45.231	–				

Table 2 ^{1}H and ^{13}C NMR chemical shifts of oligomers of F1, F3, F5 and F7

	Proton chemical shifts (ppm)					Carbon chemical shifts (ppm)				
	H-1	H-2	H-3	H-4	H-5	C-1	C-2	C-3	C-4	C-5
F1										
△G dimer										
$\triangle G_{red\alpha}$	5.20	3.88	4.31	6.08	–	100.73	66.51	62.24	111.97	141.16
$\triangle \underline{G}_{red\beta}$	4.86	3.50	4.21	4.16	4.63	93.47	68.56	69.59	79.54	72.12
F3										
△MG trimer										
$\triangle MG_{red\alpha}$	5.17	3.92	4.39	6.02	–	99.99	66.40	63.07	112.17	140.92
$\triangle \underline{M}G_{red\beta}$	4.72	3.89	3.73	3.95	3.95	101.65	70.17	70.68	77.27	73.63
$\triangle M\underline{G}_{red\beta}$	4.86	3.62	4.25	4.15	4.63	93.45	68.49	69.90	79.61	72.25
F5										
△GMG tetramer										
$\triangle GMG_{red\alpha}$	5.17	3.84	4.32	6.07	–	100.78	66.31	62.33	112.90	140.34
$\triangle \underline{G}MG_{red\alpha}$	4.88	3.76	4.08	4.20	4.97	99.44	64.29	68.58	79.73	66.16
$\triangle G\underline{M}G_{red\beta}$	4.69	3.80	3.70	3.79	3.97	101.72	70.54	70.74	76.50	73.64
$\triangle GM\underline{G}_{red\beta}$	4.83	3.57	4.20	4.12	4.61	93.43	68.45	69.86	79.50	72.11
F7										
△MGGG pentamer										
$\triangle MGGG_{red\alpha}$	5.17	3.91	4.40	6.02	–	99.99	66.40	63.06	112.17	140.93
$\triangle \underline{M}GGG_{red\beta}$	4.74	3.89	3.73	3.94	3.96	101.54	70.14	70.71	77.24	73.60
$\triangle M\underline{G}GG_{red\alpha}$	5.05	3.89	4.14	4.24	4.65	100.97	64.41	68.66	79.47	66.53
$\triangle MG\underline{G}G_{red\alpha}$	5.04	3.83	3.98	4.15	4.69	100.92	64.86	68.66	79.59	66.40
$\triangle MGG\underline{G}_{red\beta}$	4.88	3.56	4.09	4.07	4.65	93.46	68.76	69.72	79.81	71.96

L-gulopyranosyluronic acid)-(1 → 4)-*O*-β-L-gulopyranuronic acid.

As shown in Fig. 2, the negative-ion mode mass spectra of F1, F3, F5 and F7 displayed signals corresponding to the main deprotonated oligomers $[M–H]^{-}$ (M is the molecular weight of the main solute) and a series of ions with Na, i.e., $[M + Na–2H]^{-}$, $[M + 2Na–3H]^{-}$, $[M + 4Na–5H]^{-}$, etc. The most abundant oligomer in the fractions was consistent with the most abundant deprotonated ion, which indicated that the molecular weights of F1, F3, F5 and F7 were 351.00 (DP2), 527.00 (DP3), 703.00 (DP4) and 878.92 (DP5), respectively (Chaki et al. 2006).

Glyceollin induction and determination

To determine the glyceollin-inducing activities of AOs with different molecular weights and G/M ratios, the glyceollin concentration in soybean cotyledons was calculated by HPLC analysis. As shown in Fig. 3a, b, glyceollins were synthesized after induction by the AO mixture. Glyceollins showed peaks with retention times of 23–24 min, which corresponded to glyceollin I, II and III (Boué et al. 2000). The concentration of glyceollins was calculated according to the method described by Eromosele (Eromosele et al. 2013). Figure 3c shows the glyceollin-

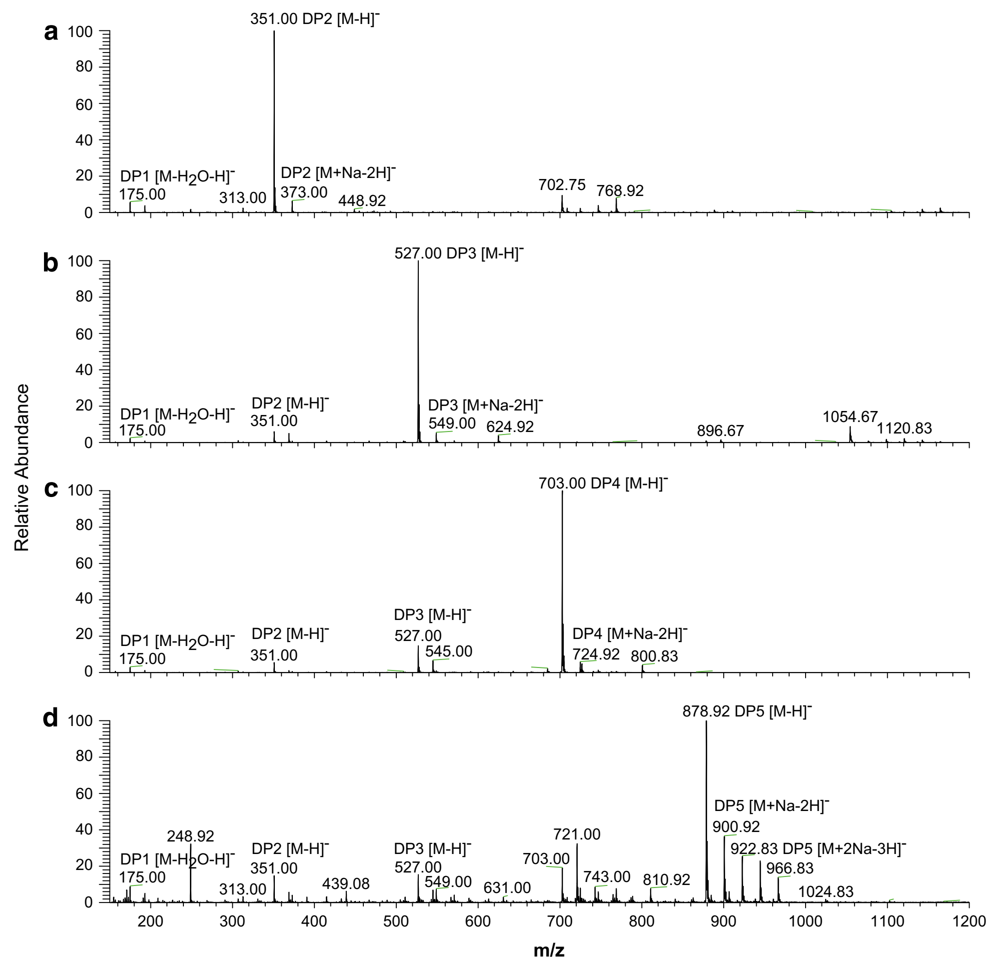

Fig. 2 Negative-ion ESI mass spectra of oligouronic acid fractions F1, F3, F5 and F7, which were collected by preparative HPLC and then subjected to desalination. DP1-5 refers to the degree of oligomer polymerization. The ESI–MS conditions were as follows: injection volume, 2 F7; ESI voltage, 4 kV; capillary temperature, 275 °C; capillary voltage, 350 V; tube lens, 250 V; scan range, 400–2000 m/z. Nitrogen was used as the sheath gas and auxiliary gas at a flow rate of 30 and 5 arb, respectively. The mobile phase (methanol/ water = 1:1, v/v) was delivered at a flow rate of 200 µl/min

inducing activities of the AO mixture and purified AO fractions with different molecular weights and G/M ratios. The glyceollin accumulation levels (mg/g dry weight) induced using the AO mixture, F1 (△G), F3 (△MG), F5 (△GMG) and F7 (△MGGG) were 0.6373, 1.2339, 0.3472, 0.6494 and 1.0611, respectively. Moreover, in our research, the disaccharide △G had outstanding glyceollin-inducing activity.

Discussion

In this research, we characterized 4 AO fractions with degrees of polymerization of 2–5: △G, △MG and △GMG, and △MGGG. These purified fractions were used for the first time as elicitors for glyceollin induction to determine the structure–activity relationship of AOs at the molecular level.

We found that as the molecular weight increased, the tri-, tetra-, and pentasaccharides separated from the AOs mixture had increasing glyceollin-inducing activities in soybeans (Fig. 4a). Such a molecular weight-activity relationship was also observed for the antimicrobial activity of chitosan (Zheng and Zhu 2003) and the growth promotion activity in lettuce seedling roots of pectate oligosaccharide mixtures (Iwasaki and Matsubara 2000).

Moreover, as shown in Fig. 4b, we found that AOs with a higher G/M ratio induced a higher glyceollin concentration in soybeans, indicating that the glyceollin-inducing activities are related not only to their molecular weight but also to their chemical structures, such as the G/M ratio. This finding is not unique: Küpper found that alginate oligomers with a higher G/M ratio could elicit a higher reactive oxygen species level in the sporophytes of the kelp *Laminaria digitata* (Küpper et al. 2002). In plants, reactive oxygen species are important in signaling cascades and are continuously produced as byproducts of various metabolic pathways involved in the plant immune system (Apel and Hirt 2004). Glyceollins are the main type of secondary metabolites in soybeans and play crucial roles in plant–microbe interactions (Hai et al. 2010; Landini et al. 2003). Therefore, we propose that the AOs with a higher G/M ratio induce a higher reactive oxygen species level in soybean cotyledons, which may ultimately correspond to a stronger signal for the glyceollin synthesis pathway. To

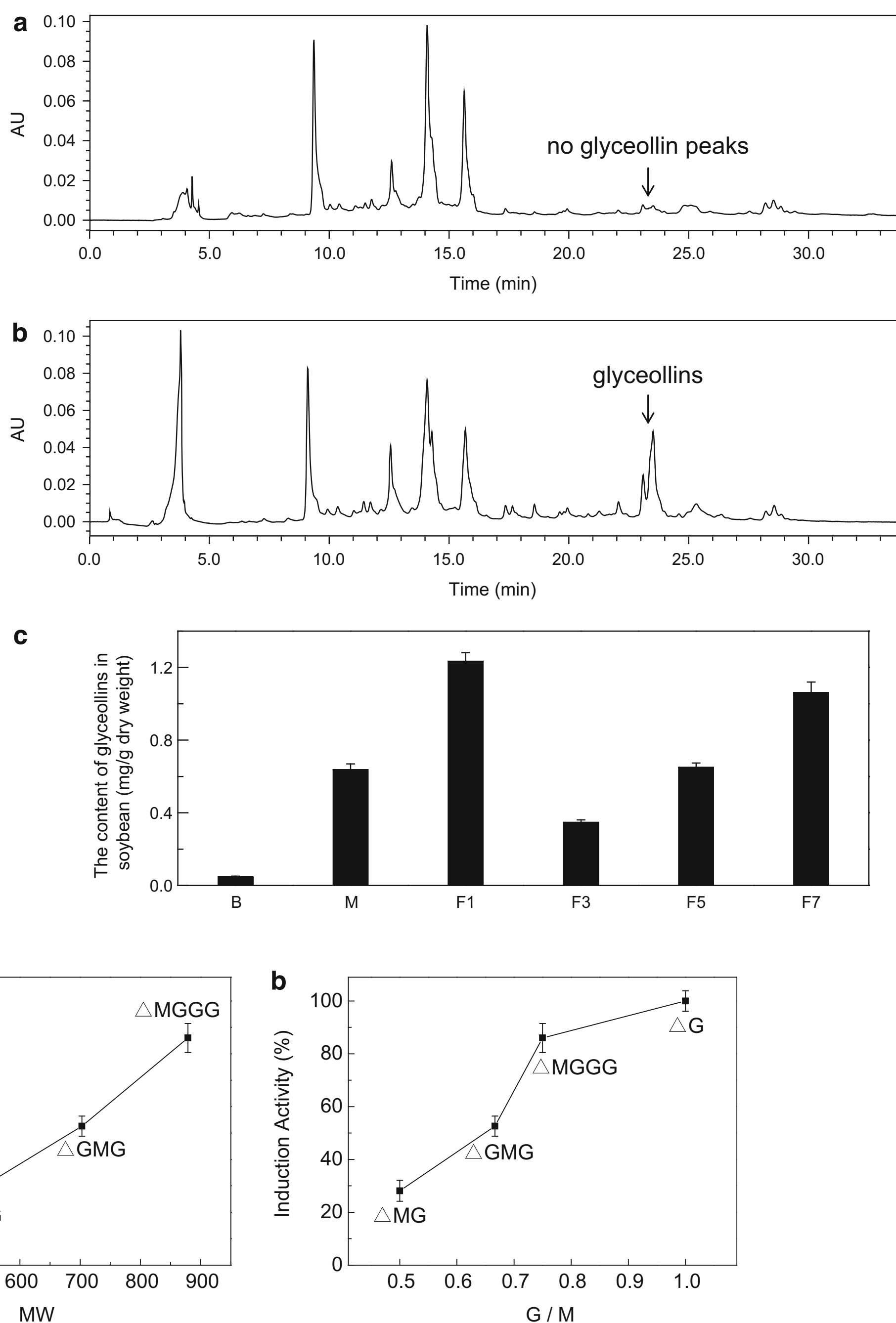

Fig. 3 Glyceollin induction by the AO mixtures and purified AO fractions with different molecular weights and G/M ratios. **a** HPLC chromatogram of 4-day-old uninduced cotyledons showing no glyceollin peaks. **b** HPLC chromatogram of 4-day-old cotyledons induced by the AO mixture showing glyceollin isomer peaks with retention times of 23–24 min. The chromatograms were obtained by recording the absorbance at 285 nm. **c** The glyceollin-inducing activities of the AO mixture and purified AO fractions with different molecular weights and G/M ratios. B represents the blank, and M represents the AO mixture. The glyceollin accumulation levels (mg/g dry weight) induced using the AO mixture, F1 (△G), F3 (△MG), F5 (△GMG) and F7 (△MGGG) were 0.6373, 1.2339, 0.3472, 0.6494 and 1.0611, respectively

Fig. 4 Glyceollin-inducing activities of F1 (△G), F3 (△MG), F5 (△GMG) and F7 (△MGGG). **a** Glyceollin-inducing activities arranged by molecular weight. **b** Glyceollin-inducing activities arranged by G/M ratio

confirm this hypothesis, further studies, as well as large amounts of AOs with different structures, are necessary.

Conclusion

To the best of our knowledge, this is the first report of the different induction capacities of alginate oligosaccharides with different molecular weights and G/M ratios for glyceollin accumulation. Among the 4 AO fractions tested in this research, the alginate disaccharide △G was found to exert the best induction effect. AOs are better elicitors to induce glyceollin biosynthesis in soybeans than metal ions, microorganisms and chemicals because they do not affect the edibility of soybeans and have many human health benefits. Moreover, soybean products with high glyceollin contents can be consumed to confer these health benefits. It is therefore suggested that AOs, especially the alginate

disaccharide △G, could be introduced as relatively safe and efficient elicitors to produce soybeans with high glyceollin contents.

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Compliance with ethical standards

Conflict of interest There are no conflicts of interest. The author and co-authors alone are responsible for the content and writing of this paper.

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