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Galactose-binding lectin from mulberry (*Morus alba* L.) seeds with growth hormone-like activity

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ABSTRACT

Plant lectins are well documented to participate in multiple physiological activities based on selective binding to the carbohydrate structures. They have been reported to play significant roles in various processes such as growth and development, differentiation and plant protection. Nevertheless, the intrinsic roles of plant lectins still remain undefined. We purified a galactose-binding lectin, named MAL, from mulberry (*M. alba* L.) seeds and analyzed its properties. The lectin is composed of one polypeptide of 17 kDa, which is abundant in the seed protein fraction. MAL interacted with GalNAc and galactose residues of saccharides with high binding ability. Western blotting analysis suggested that MAL is deposited in the mulberry leaves and inflorescence. MAL was examined for growth stimulatory activity on mulberry hypocotyls and internodal sections of *in vitro* grown *P. euphratica* cultures. Elongation of mulberry hypocotyls was detected in the apical parts of the hypocotyl, where the growth increment was 58%. MAL had no significant effect on the stem elongation and induction of new leaves. Our results suggest that MAL may be involved in the growth and cell elongation at initial stages of tissue development.

1. Introduction

Plant growth and development is coordinated by signaling molecules including phytohormones and hormone peptides. Apart of small lipophilic compounds referred to as traditional plant hormones there is a growing interest in biologically active peptides in plants, currently regarded as a new class of plant hormones involved in the regulation of plant growth [1,2]. Over the past decade many investigators reported polypeptides of plant origin that act as signaling molecules. Peptide hormone, RALF (rapid alkalinization factor), regulates plant cell expansion by direct peptide-receptor interaction [3]. The family of peptide hormone-transporters was identified that function as transporters of signaling auxin and other plant hormones [4]; Secretory peptides control plant gravitropic responses by regulation of auxin carrier turnover [5]. Plant phytohaemagglutinins or lectins have been reported to play significant role in various processes such as growth and development, differentiation, plant protection [6]. In our previous report galactose-specific mulberry lectin affected the growth of wheat coleoptiles by enhancing the cell elongation [7]. The results revealed that endogenous lectins might be involved in the plant growth processes. The objective of present study was to investigate the effects of galactose-binding lectin (MAL) from *M. alba* seeds on cell elongation of mulberry hypocotyls and *in vitro* cultures of *P. euphratica*.

2. Materials and methods

2.1. Materials

The seeds of mulberry (*M. alba* var. Gruzia L.) were collected from Kartli region in May-Juneand stored at 25 °C until use. For isolation of seed proteins whole mature fruits were used. *Populus euphratica* twigs were collected from field-grown adult trees in Vashlovani Game Reserve at Dedoplistskaro municipality in March–April.

2.2. MAL purification

M. alba lectin (MAL) was prepared as described with some modifications [8]. Mulberry seeds (5 g) were homogenized with 0.9% NaCl, 0.04 M potassium phosphate saline (pH 7.4) at the ratio

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of 1:10 (w/v), and filtered using Whatman CF/C filter. The filtrate was centrifuged at 5000 × g for 15 min. Partial purification of the proteins was performed by successive precipitation in ammonium sulfate under 0–90% saturation. Suspension was centrifuged at 20,000 × g for 20 min at 40 °C. Precipitate was dissolved in 0.04 M potassium phosphate saline (pH 7.4) at the ratio of 1:10 (w/v) and centrifuged at 5000 × g for 15 min. The supernatant was filtered through Whatman CF/C paper and 0.45μ filter (Millipore). Dialysis was performed by chromatography on a Sephadex G-10 column (50 cm × 2.7 cm) equilibrated with 0.9% NaCl, 0.02 M potassium phosphate saline (pH 7.4). Gel-filtration was performed on Toyopearl HW-55 column (45 cm × 1.5 cm) equilibrated with the same buffer. Affinity purification of lectins was performed using chromatography in agarose-GalNAc column (60 mm × 8 mm). Molecular mass of MAL was determined by SDS-PAGE as described by Laemmli [9]. Haemagglutination activity was detected by using of trypsin-treated 2% rabbit erythrocytes [10]. Specific agglutinating activity (SA) of MAL was calculated as the maximum dilution of 1 mg of protein, causing agglutination of rabbit erythrocytes. Protein concentration was estimated according to Lowry [11].

2.3. Preparation of anti-MAL antiserum

Anti-MAL antiserum was raised in rabbits by repeated injection of *M. alba* agglutinin emulsified with Freund's complete adjuvant. The titer and specificity of antibodies were estimated by double immunodiffusion analysis on agar gels [12] and enzyme-linked immunosorbent assay (ELISA). After three booster injections, serum was collected and stored at −20 °C. Purified *M. alba* agglutinin was transferred electrophoretically to *Trans*-Blot PVDF membrane (BIO-RAD, CA) by the method of Laurriere [13]. Membrane was incubated in the blocking buffer containing 50 mM Tris-HCl, 0.15 M NaCl, 5% skim milk (pH 7.5) for 40 min at 37 °C. The membrane was then incubated with anti-MAL antiserum at the 1:10 000 dilution for 90 min at 37 °C, washed three times with TBS containing 0.05% Tween 20 and were subsequently incubated with goat peroxidase-conjugated anti-rabbit IgG (JacsonImmunoresearch Laboratories, West Baltimore, PA) at the dilution 1:1000 for 90 min at 37 °C. Blots were developed with 3,3'-diaminobenzidine and H_2O_2 as substrate in 50 mM Tris-HCl buffer (pH 7.5).

2.4. Establishment of in vitro cultures

Seeds were isolated from mature fruits of mulberry. Refined seeds were washed in tap water and soaked in 1% H_2O_2 for 48 h at 4 °C, followed by disinfestation with 30% H_2O_2 for 15 min. After being rinsed in sterile distilled water, they were germinated aseptically on agar (6 g l^{-1}, plantMedia™, Dublin, Ohio) in growth chamber at 24 ± 0.5 °C under 16 h light/8 h dark photoperiod with an irradiance 40–42 μmol m^{-2} s^{-1} from cool-white fluorescent tubes. The hypocotyls 10–12 mm in length were aseptically removed from 10 day old seedlings and used for MAL assays.

In vitro cultures of *P. euphratica* were established using axillary buds as explants source. Sprouting shoots were washed and surface disinfested in sodium hypochlorite (2% available chlorine) for 10 min. The explants were then rinsed several times in distilled water, the bud scales were aseptically removed and buds were cultured in glass tubes (20 mm × 170 mm) containing 10 ml of culture medium. All cultures were incubated at 24 ± 0.5 °C day/night with 16 h photoperiod with an irradiance of 40–42 μmol m^{-2} s^{-1}. After three weeks elongated shoots were subcultured in 200 ml glass jars (60 mm × 95 mm) containing 50 ml nutrient medium with four-five explants per jar. Further shoot production was accomplished every 4–5 weeks by sequential subculture of an individual shoots with significant acceleration in the proliferative rate. The internodal sections 14 mm in length was used in all experiments. WPM [14] basal medium were used for all cultures supplemented with the 1.1 μM of N^6-6-Benzylaminopurine (BAP, Sigma). Sucrose was supplied at 30 g l^{-1}. The media were then solidified with plantMedia agar (8 g l^{-1}) and adjusted to pH 5.7 before autoclaving. Mulberry hypocotyls were obtained from *in vitro* seedlings of *M. alba*. Seeds were washed in tap water and soaked in 1% H_2O_2 for 48 h at 4 °C, followed by disinfestation with 30% H_2O_2 for 15 min. After being rinsed in sterile distilled water, they were germinated aseptically on agar (6 g l^{-1}, plantMedia™, Dublin, Ohio) in growth chamber at 24 ± 0.5 °C under 16 h light/8 h dark photoperiod with an irradiance 40–42 μmol m^{-2} s^{-1} from cool-white fluorescent tubes. The hypocotyls were cut from the 10-day-old seedlings and used for MAL bioassay.

2.5. MAL assays

Endogenous auxins were separated by the solvent mixture of butyl/wine acid/water (40:12:28) and activity of auxins was studied according to the method of Kefeli [15]. Growth stimulation of *M. alba* hypocotyls by MAL was tested by using hypocotyl clippings and i internodal segments of *P. euphratica.* Internodal sections of *P. euphratica* 14 mm in length were cultured in vessels (9 mm × 150 mm) containing WPM culture medium containing 1.1 μM BAP alone or in combination with MAL at different concentrations (10 μg/ml, 30 μg/ml and 50 μg/ml). In other experiments, growth stimulatory activity of the endogenous auxins and MAL were tested using mulberry hypocotyl clippings. Mulberry hypocotyls 10–12 mm in length were cut from 10 days old *in vitro* seedlings. Hypocotils were cropped in 3–4 mm segments and transferred in the solution containing endogenous auxinsat 30 μg/ml or MAL at 50 μg/ml concentration. Elongation of the hypocotyls in the absence of growth regulators was valued as 100% of growth.

3. Results

3.1. Purification and subunit structure of MAL

Extraction of mulberry seeds followed by Toyopearl HW-55 chromatography yielded 17.0 mg of crude protein from 1 g of mature seeds (49% yield) (Table 1). The protein fraction, subjected to agarose-GalNAc chromatography gave one major peak designated MAL. The yield of the fraction was 1 mg. MAL, which was not retarded on the latter column, gave a single band corresponding to 17 kDa on SDS-PAGE under non-reducing and with ME treatment (Fig. 1). No sugars were detected in MAL either by Schiff staining or the 4-N,N-dimethylamino-4'-azobenzenesulfonyl-hydrazide method [16], indicating that MAL was not a glycoprotein.

Table 1
The purification and yield of Galactose-specific mulberry seed lectin.

Purification step	Protein	Recovery	Heamagglutination activity
	mg[a]	%	μg/ml[b]
Homogenate	35	100	0.72
$(NH_4)_2SO_4$	28	83	0.068
Toyopearl HW-55	17	49	0.029
Agarose-GalNAc	1	3	0.00098

[a] Values were calculated per 1 g of mature seeds.
[b] Minimum concentration required to agglutinate 2% rabbit erythrocytes.

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