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Purification and Characterization of Anti-H Lectin from the Seed of *Momordica charantia* and the Inter-Specific Differences of Hemagglutinating Activity in *Cucurbitaceae*

[1]J. Toyama, [2]H. Tanaka, [3]A. Horie, [4]T. Uchiyama and [2]R. Akashi
[1]JST Innovation Satellite Miyazaki, 1-7-4 Tachibana-Dori Higashi, Miyazaki 880-0805, Japan
[2]Frontier Science Research Center, University of Miyazaki, 1-1 Gakuen Kibanadai Nishi, Miyazaki 889-2192, Japan
[3]Fuji Chemical Co. Ltd., 1683-1880 Nasubigawa, Nakatsugawa, Gifu 509-9132, Japan
[4]Forensic Science Laboratory, Miyazaki Prefectural Police Headquarters, 1-8-28 Asahi, Miyazaki 880-8509, Japan

Abstract: Thirteen *Cucurbitaceae* species have been investigated for anti-H activity of seed lectins. The lectin was extracted from seed powder and concentrated by ethanol precipitation method. *Momordica charantia*, *Trichosanthes kirilowii*, *T. cucumeroides* and *T. bracteata* had potent hemagglutinating (HA) activity toward human type-H(O) erythrocytes, in which *M. charantia* exhibited considerably lower activity toward human type-O_m^h (para-Bombay, H-deficient). Hence, it was characterized as anti-H activity. Eight Japanese cultivars exhibited almost same degrees of anti-H activity. A lectin from seeds of *M. charantia* has been purified by affinity chromatography and gel-permeation. The lectin was shown to be a glycoprotein containing approximately 10% neutral sugar, which gave a single band on native polyacrylamide gel electrophoresis (PAGE) and four bands of 31.5, 30.5, 30.0 and 28.5 kDa on SDS-PAGE under reducing conditions suggesting that the lectin is a tetramer. The HA activity was stable at 50°C for 1 h, but sharply decreased beyond 55°C. The lectin agglutinated human type-O erythrocytes and the agglutination was inhibited by D-galactose and its derivatives, particularly human blood type-H (O) antigen trisaccharide (Fucα1→2Galβ1→4GlcNAc). These results suggest that *M. charantia* seed lectin has anti-H (O) activity and D-galactose specificity. Inter-specific differences in anti-H activity of the seed among *Cucurbitaceae* may exist.

Key words: *Momordica charantia*, anti-H(O) lectin, *Cucurbitaceae*, inter-specific difference

INTRODUCTION

Cucurbitaceae is widely distributed in the tropics and warm temperate regions of the world. It is widely cultivated for food (as fruit) throughout the world. In most cases, the seeds are unused, but some of them have been traditionally used as medicine. Recently, various functional components in the seed are reported by EI-Fiky *et al*. (1996), Telang *et al*. (2003), Grover and Yadav (2004), Tsoi *et al*. (2005) and Sathishsekar and Subramanian (2005) and the lectin is found as one of such components.

Lectins are carbohydrate-binding and cell agglutinating proteins of non-immune origin (Goldstein *et al*., 1980). They have diverse biological functions, such as, antifungal (Melo *et al*., 2005), anti-insect (Shukla *et al*., 2005; Majumder *et al*., 2005), mitogenic (Lis and Sharon, 1986) and hemagglutinating (HA) activity. Some lectins, as an antigenic determinant of blood group, have come to be an important tool in the identification of different blood groups (Khan *et al*., 2002).

Anti-H lectin has the specificity to human blood group H(O) antigen. Several anti-H lectins have previously been isolated and characterized, which include the lectins from *Anguilla anguilla* (Watkins and Morgan, 1952), *Streptomyces* sp. (Kameyama *et al*., 1979) and some leguminous seeds (Matsumoto and Osawa, 1969, 1974; Datta and Basu, 1981; Konami *et al*., 1983; Khan *et al*., 2002). Among these lectins, the *Ulex europaeus* lectin is commonly used as blood type checking reagent (Khan *et al*., 2002). However, *U. europaeus* is originally from Europe and is difficult to obtain in Asian countries. Moreover, commercially available *Ulex* anti-H lectin is expensive.

Corresponding Author: Ryo Akashi, Frontier Science Research Center, University of Miyazaki, 1-1 Gakuen Kibanadai Nishi, Miyazaki 889-2192, Japan Tel/Fax: +81 985 58 7257

In *Cucurbitaceae*, some lectins with HA activity have been purified from the seeds and roots and then have been characterized (Van Damme *et al*., 1998; Padma *et al*., 1999; Wu *et al*., 2000; Sultan and Swamy, 2005). Among these lectins, the *Trichosanthes japonica* root lectin (TJA-II; Yamashita *et al*., 1992) and the *Momordica dioica* seed lectin (Joshi *et al*., 2005) are reported to have anti-H activity and are valuable in forensic practices (Takahashi *et al*., 2001).

Momordica charantia (bitter gourd), belonging to the *Cucurbitaceae*, has been widely used in Asia as a foodstuff. Some lectins from *M. charantia* seeds have been isolated and characterized (Lin *et al*., 1978; Barbieri *et al*., 1980; Wang and Ng, 1998; Sultan and Swamy, 2005). However, these lectins have not been evaluated for H antigen specificity and also have not been used as a practical reagent yet. In this study, we investigate 13 *Cucurbitaceae* species for anti-H activity of seed lectins. The *M. charantia* seed lectin was purified and some properties were investigated.

MATERIALS AND METHODS

The studies were conducted at University of Miyazaki during the period of 2004-2008.

Plant materials: Seeds of *M. charantia* Hyaku-nari were collected from industrial waste of a food-processing company (Ishihara foods Co. Ltd., Miyazaki, Japan). Seeds of *Trichosanthes cucumeroides*, *T. kirilowii* and *T. bracteata* were collected at Kyushu region, Japan. Other *Cucurbitaceae* seeds were purchased from local seed companies of Miyazaki, Japan. Seeds of *U. europaeus* were purchased from Sanko Junyaku Co. Ltd. of Osaka, Japan.

To investigate the distribution of lectin in different tissues of *M. charantia* plants (Hyaku-nari or Sadowara No. 3), the seeds were sown in the peat moss in a phytotron and then transferred to a planter in the greenhouse. The planter size was 22 L and two plants were cultivated in each planter. Ten grams of compound fertilizer (N-P_2O_5-K_2O = 6-6-6) were applied to each pot at every 2 weeks interval. The leaves, stems, cotyledons and roots were sampled from one-week and one-month old plants. In addition, pulp and aril of fruits were also harvested from two-month old plant. Furthermore, in order to investigate the lectin with seed maturity, seeds were also collected from the fruits associated with 1-2, 2-3 weeks and more than 3 weeks post-pollination. Three fruits were used for analysis from each stage.

Extraction and concentration of lectin: Dried seeds or fresh plant tissues were ground using a mill (IFM-700G, Iwatani, Tokyo, Japan). Compared to sample amount, two (for fresh plant) or five (for dried seeds) volumes of phosphate-buffered saline (PBS, pH 7.4) was added to sample and the suspension was magnetically stirred at 4°C for 3 h. Subsequently, the mixture was then centrifuged at 10,000 x g for 30 min at 4°C. One and a half volumes of absolute ethanol were then added to the supernatant and allowed to settle down overnight at 4°C for precipitation. The precipitate was collected by centrifugation at 10,000 x g for 15 min at 4°C and then dried (25°C) to obtain crude lectin powder. The crude lectin powder was re-suspended using PBS at a concentration of 0.1 g mL^{-1} and then centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was used for the analysis.

Purification of *M. charantia* seed lectin: *M. charantia* seed extract obtained by ethanol precipitation method was loaded onto a galactose-coupled Sepharose 6B (Epoxy-activated Sepharose 6B; GE Healthcare Bio-Science, Piscataway, NJ, USA) affinity column, preequilibrated with PBS. The column was washed with PBS and the lectin was eluted with 0.2 M galactose in PBS. The effluent stream was monitored for absorbance at 280 nm and fractions were dialyzed against distilled water, lyophilized and suspended in PBS. It was then loaded onto a TSK-gel HW55S (Tosoh Corporation, Tokyo, Japan), preequilibrated with PBS. A single peak of HA activities coincided with the absorbance at 280 nm was obtained. This fraction was dialyzed against distilled water and lyophilized, to get the purified lectin. All purification procedures were carried out at 4°C using the BioAssist-eZ system (Tosoh).

Hemagglutination assay: The HA activity was assessed using 2-3% erythrocyte suspensions of human type -A, -B, -O (Ortho-Clinical Diagnostics K. K., Tokyo, Japan) or human type-O_m^h (H-deficient phenotype; *Ulex europaeus* anti-H lectin negative) or various animals (Nippon Biotest Laboratories Inc., Tokyo, Japan) in a 96-well microtiter plates. Crude or purified lectin (25-50 μL) was placed in the first well and serially diluted two-fold into the successive wells with PBS, then an equal volume of erythrocyte suspension was added to all the wells. The plates were gently shaken for 30 min at room temperature. The HA activity was expressed as titer, i.e. the highest dilution of the lectin that caused agglutination.

Hemagglutination inhibition assay: For the inhibition assay, saccharides or human blood group antigens (Dextra Laboratories, Reading, UK) were serially diluted and mixed with an equal volume of the purified lectin (1.4 μg mL^{-1}) for a total of 25 μL. After incubation at room

temperature for 1 h, 25 μL of 2-3% human type-O erythrocyte suspension was added and the reaction examined after 30 min. The inhibitory activity was determined as the concentration required for complete inhibition of the hemagglutination.

Protein and neutral sugar contents determination: Protein content was determined following the method of Bradford (1976) with bovine serum albumin as a standard. Total neutral sugar contents were measured by the phenol-sulfuric acid method (Dubois *et al*., 1956) with reference to glucose as standard.

Polyacrylamide gel electrophoresis: The molecular mass of lectin was estimated by native-polyacrylamide gel electrophoresis (Native-PAGE) with a 10% separating gel under the non-denaturing condition using the method of Davis (1964). SDS-PAGE was carried out with a 10% separating gel as described by Laemmili (1970) and the samples were denatured and reduced by heating in the presence of SDS and 2-mercaptoethanol. Electrophoresis was run at 20 mA of constant current. Gels were stained for protein with Coomassie Brilliant Blue R-250.

Thermal stability: Thermal stability of the lectin was determined by incubating at various temperatures for 0.125, 0.25, 0.5, 1.0 and 2.0 h. The samples were then chilled on ice and assayed for HA activity as described before. The residual activity was expressed as percentage of the initial activity.

Effect of chelating agent and metal ions: For the test of HA activity in the presence of EDTA, the purified lectin was dialyzed against 5 mM EDTA at 4°C for 16 h and then submitted to HA assay. Effect of metal ion on HA activity was studied by submitting the EDTA-treated lectin to two-fold serial dilutions in PBS with or without 5 mM $CaCl_2$ and 5 mM $MgCl_2$.

RESULTS AND DISCUSSION

Hemagglutinating (HA) activities of seed extracts of various *Cucurbitaceae* species: Out of the 13 *Cucurbitaceae* species seed extracts examined, seed extracts of only 4 species had potent for HA activities toward human type-H(O) erythrocytes. These were *M. charantia*, *T. bracteata*, *T. cucumeroides* and *T. kirilowii*. Among of them, *M. charantia* exhibited stronger activity (titer 2^{12}) compared with the other species (Table 1).

The above stated high potent 4 species seed extracts were also tested for HA activities toward human type-O_m^h erythrocytes (Table 2). It was observed that all of *Trichosanthes* species exhibited almost same levels of HA activities toward both type-O_m^h and -O erythrocytes and it appeared that they were not anti-H lectin. However, *M. charantia* had considerably weaker activity toward human type-O_m^h erythrocytes (titer 2^5) than type-O erythrocytes (titer 2^{12}); hence it was characterized as anti-H activity. In other *Cucurbitaceae* seeds, the *M. dioica* seed lectin was only characterized as anti-H lectin (Joshi *et al*., 2005). Therefore, inter-specific differences in anti-H activity of the seed among *Cucurbitaceae* may exist. The *M. charantia* seed lectin was further characterized.

Table 1: Hemagglutinating (HA) activities of the seed extracts of various *Cucurbitaceae* species

Species	HA activity (titer)
Benincasa hispida, Nagatohgan	[1]ND
Benincasa hispida, Lion tohgan	2^3
Citrullus lanatus, Kabuki	2^1
Cucumis melo, Earls seinu	ND
Cucumis sativus, Furesco 100	ND
Cucurbita maxima, Kuriyutaka	ND
Cucurbita moschata, Miyazaki-wase No. 2	ND
Cucurbita pepo, Dainer	ND
Lagenaria siceraria, Sennari hyoutan	ND
Lagenaria siceraria, Dantotsu yugao	2^3
Luffa cylindrical, Daicho rokushaku hechima	ND
Luffa cylindrical, Hutohechima	ND
Momordica charantia, Hyaku-nari	2^{12}
Trichosanthes bracteata	2^7
Trichosanthes cucumeroides	2^8
Trichosanthes kirilowii	2^{11}

[1]ND: Not detected

Table 2: Hemagglutinating (HA) activities of the seed extracts of 4 *Cucurbitaceae* species toward human type-O and -O_m^h erythrocytes

	HA activity (titer)	
Species	O	O_m^h
M. charantia, Hyaku-nari	2^{12}	2^5
T. cucumeroides	2^8	2^7
T. bracteata	2^7	2^7
T. kirilowii	2^{11}	2^{11}

The distribution of HA activities in *M. charantia* plants and cultivar differences: The HA activities toward human type-O erythrocytes of different tissues of *M. charantia* were studied. The HA activity could not be detected in the extracts of leaves, stems, roots, fruit pulp, aril and immature seeds (1-2 weeks post-pollination). The high levels of HA activities (titer 2^{12}) were detected in the seeds at more than 2 weeks post-pollination, in which an embryo was formed (Table 3). In seedlings, cotyledons exhibited the HA activities (titer 2^9) and leaves, stems and roots had no activities.

The seed extracts of Japanese 8 cultivars exhibited almost same degrees of HA activities (titer 2^{11}-2^{12}) (Table 4). These results indicated that *M. charantia* seed from Japanese cultivars could be utilized effectively as a source of materials for extracting anti-H lectin.

Table 3: Hemagglutinating (HA) activities of the seed extracts of *M. charantia* (Sadowara No. 3) at different weeks post-pollination

Weeks post-pollination	HA activity (titer)
1-2	[1]ND
2-3	2^{12}
3-	2^{12}

[1]ND: Not detected

Table 4: Hemagglutinating (HA) activities of the seed extracts of *M. charantia* cultivars

Cultivar	Protein (mg mL^{-1})	HA activity (titer)
Fushinari	12.0	2^{12}
Hyaku-nari	12.0	2^{12}
Miyazaki koi midori	12.9	2^{11}
Miyazaki midori	10.8	2^{12}
Shimasango	12.8	2^{12}
Satsuma daichou	12.3	2^{12}
Powerful	12.1	2^{11}
Sadowara No. 3	13.6	2^{12}

Table 5: Hemagglutinating (HA) activities of *M. charantia* seed extract

Type of erythrocyte	HA activity (titer)
Human A_1	2^9
Human A_2	2^{11}
Human B	2^9
Human O	2^{12}
Human O_m^h (para Bombay)	2^6
Rabbit	2^4
Horse	[1]ND
Bovine	ND

[1]ND: Not detected

Table 6: Purification steps of lectin in *M. charantia* [1]seeds

Step	[2]Total protein (mg)	Total activity (titer×mL)	Specific activity (titer×mL mg^{-1})	Yield (%)
Crude extraction	522.00	73216	140	100
Ethanol precipitation	41.80	45056	1078	62
Affinity chromatography	0.26	17981	68267	25
Gel-permeation	0.15	10363	68267	14

[1]Forty gram of seeds were used as starting material, [2]Protein determined by Bradford method using BSA as standard

Hemagglutinating (HA) activities of *M. charantia* seed extract toward human and animal erythrocytes: *M. charantia* seed extract showed lower HA activity toward human type-O_m^h as compared to other human erythrocytes and also weaker activity toward rabbit erythrocytes as compared to human erythrocytes, but exhibited the strongest HA activity toward human type-O erythrocytes (Table 5). The suitable diluted extract could be prepared for H antigen assays due to large differences of it's HA titers between type-O and type-O_m^h erythrocytes, which showed agglutination with type-O erythrocytes and no agglutination with type-O_m^h erythrocytes. Using the same amount of seeds and same extraction methods, the *U. europaeus* seed extract showed lower HA activities (titer 2^6) toward human type-O erythrocytes compared to HA activity (titer 2^{12}) of the *M. charantia* seed extracts.

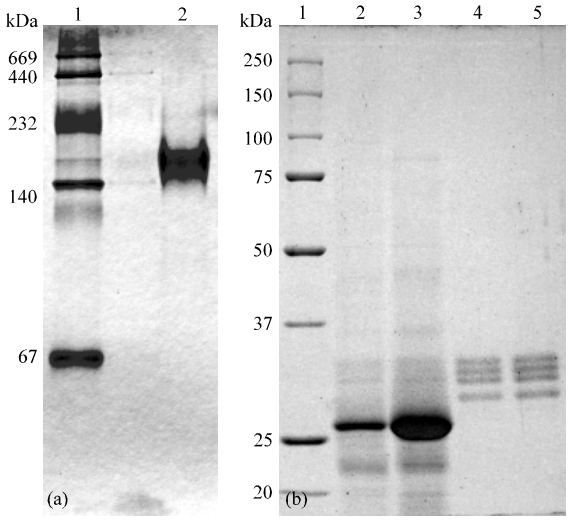

Fig. 1: Electrophoresis of *M. charantia* seed lectin. (a) Native PAGE of the *M. charantia* seed lectin. Lane 1, standard proteins; lane 2, purified lectin. (b) SDS-PAGE (10%) of the purification steps. Lane 1, standard proteins; lane 2, crude extract; lane 3, ethanol precipitated fraction; lane 4, affinity-purified fraction; lane 5, gel permeation purified lectin.

Extraction procedure and concentration of lectin of *M. charantia* seed: The ethanol precipitation step resulted in 62% yield with a 7.7-fold increase in the specific activity of the lectin compared to crude extraction step (Table 6). Routinely, about 2 g of crude lectin powder was obtained from 100 g of dry seeds. The HA activity of the crude lectin powder solution (0.1 g mL^{-1}) was high enough to evaluate the activity of the lectin.

Purification of *M. charantia* seed lectin and its properties: A *M. charantia* seed extract obtained by ethanol precipitation step was subjected to the affinity column (galactose-coupled Sepharose 6B). A retained protein with HA activity was eluted with PBS containing 0.2 M galactose as a single protein peak. Subsequently, gel-permeation step resulted in one major protein peak which coincided with the peak of HA activity. In addition, the gel-permeation step gave the lectin in 14% yield with a 487-fold purification as compared to crude extraction step (Table 6). The purified lectin was homogeneous as judged by native PAGE and SDS-PAGE, showed a molecular mass of approximately 150 kDa (Fig. 1a) and dissociated into four subunits: 31.5, 30.5, 30.0 and 28.5 kDa under reducing conditions (Fig. 1b). The molecular weight of purified lectin in the present study was similar with the previous isolated lectin of Barbieri *et al.* (1980).

Table 7: Inhibitory effect on the hemagglutination by various saccharides

Saccharide	Minimum concentration for inhibition (mM)	Relative inhibition (galactose = 1.0)
D-Galactose	12.50	1.0
N-acetyl-D-galactosamine	12.50	1.0
Methyl-α-D-galactopyranoside	12.50	1.0
Galβ1→4Glc (D-Lactose)	3.13	4.0
Galβ1→4GlcNAc (*N*-Acetyl-D-lactosamine)	3.13	4.0
Galβ1→3GlcNAc (Lacto-*N*-biose I)	6.25	2.0
Galβ1→3GlcNAc 4 ↑ Fucα1 (Lewis[a] trisaccharide)	25.00	0.5
Fucα1→2Galβ1→3GlcNAc 4 ↑ Fucα1 (Lewis[b] tetrasaccharide)	12.50	1.0
GalNAcα1→3Gal 2 ↑ Fucα1 (Blood type-A antigen trisaccharide)	25.00	0.5
Galα1→3Gal 2 ↑ Fucα1 (Blood type-B antigen trisaccharide)	100.00	0.1
Fucα1→2Gal (Blood type-H antigen disaccharide)	3.13	4.0
Fucα1→2Galβ1→4GlcNAc (Blood type-H antigen trisaccharide)	0.10	125.0

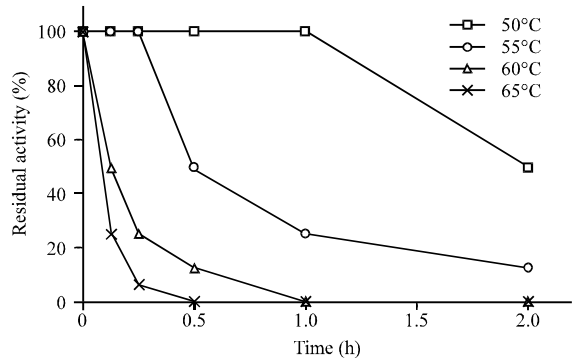

Fig. 2: Effect of temperature on the HA activity of a purified lectin from *M. charantia* seeds. The residual activity was expressed as percentage of the initial HA activity

The carbohydrate analysis following the method of phenol-sulfuric acid with glucose as standard showed that the lectin contained approximately 10% of neutral sugars. The hemagglutination was not influenced by the addition of 5 mM Ca^{2+}, Mg^{2+}, or the chelating agent EDTA, suggesting that any divalent cations are not essential for the HA activity. The thermal stability of lectin was determined by incubating in a temperature range of 50 to 65°C. The lectin remained stable at 50°C for 1 h, but lost its activity rapidly at higher temperatures (Fig. 2).

The carbohydrate-binding specificity of the lectin was studied by carrying out saccharide inhibition assays (Table 7). The following saccharides did not inhibit at concentrations of up to 100 mM: L-fucose, D-mannose, D-fructose, sucrose, *N*-acetyl-D-glucosamine, *N*, *N*'-diacetylchitobiose and *N*-acetylneuraminic acid. Galactose and its derivatives *N*-acetyl-D-galactosamine and Methyl-α-D-galactopyranoside were found to inhibit the agglutination at a moderate rate. Lactose, *N*-acetyl-D-lactosamine and lacto-*N*-biose, all containing one galactose moiety, showed stronger inhibition than galactose and its two derivatives. Human blood type-H antigen trisaccharide, Fucα1→2Galβ1→4GlcNAc, showed the strongest inhibition of all tested reagents. The other blood antigens, types A, B, Le^a and Le^b were found as weak inhibitors. These results indicate that fucose and *N*-acetyl-D-glucosamine might not be directly involved in binding to the lectin. However, these saccharides may play a crucial role in decreasing the binding energy due to interaction with amino acid side chains around the recognition site. The minimum hemagglutinating concentrations of the lectin were 0.03, 0.14, 0.03, and 0.14 μg mL^{-1} against human type -O, $-A_1$, $-A_2$, -B erythrocytes, respectively. Furthermore, human type-O_m^h erythrocytes exhibited more resistant to hemagglutination activity than the other erythrocytes (above 0.55 μg mL^{-1}). These observations suggested that the lectin from *M. charantia* seeds had an anti-H activity. Although it

could not be classified this lectin into the two major classes of anti-H lectin, eel-serum or *Cytisus* type (Matsumoto and Osawa, 1969), but appears to be similar to galactose-specific lectins from *Erythrina variegata* (Datta and Basu, 1981) and *Laburnum alpinum* (LAA-II) (Konami *et al*., 1983), which was shown an anti-H activity.

In conclusion, we have extracted and purified an anti-H lectin from the seeds of *M. charantia* using affinity and gel-permeation chromatography. Its anti-H properties were not observed in the lectins of other examined *Cucurbitaceae* seed. Because of its low cost, thermal stability and blood-type specificity, this lectin can be used quickly and economically to determine a certain blood type from extremely small bloodstains. This method, patent 3849945 registered with the Japan Patent Office, has superior sensitivity compared to conventional H antigen assays.

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