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Short Communication

Method for detecting the lectin activity of *Momordica charantia* transferred from micro two-dimensional electrophoretic gel on to nitrocellulose

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ABSTRACT

A method was devised for detecting both the molecular mass and the isoelectric point (p*I*) of the lectin in the seed extract of *Momordica charantia* on a nitrocellulose membrane. It was associated with the electrophoretic blotting technique that produced replicas of proteins separated on micro two-dimensional polyacrylamide gels. The red blood cell adherence procedure on the blotted membrane exhibited only one red spot with molecular mass $107 \cdot 10^3$ and p*I* 5.3, which indicated the lectin activity. Additionally, the lectin appeared to be a glycoprotein with mannose and/or glucose, because it was stained by concanavalin A-peroxidase staining.

INTRODUCTION

Lectins are sugar-binding and cell-agglutinin proteins, which have been utilized extensively as cell-surface probes to investigate the distribution and function of carbohydrates on the cell [1,2].

Isolation of the D-galactose-binding agglutinin from *Momordica charantia* seeds has been reported by Tomita and co-workers [3,4] and its lectin has been shown to agglutinate human type O red blood cells, but not Yoshida sarcoma

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cells. Its physiochemical characteristics have been studied by affinity column and gel permeation methods [3–8].

Recently, we have reported the identification of lectin activity in the extract of plant seeds without these methods [9]. The new method was based on the adherence of red blood cells to a nitrocellulose membrane, to which the polypeptides were transferred from sodium dodecyl sulphate (SDS) polyacrylamide. It showed the molecular mass of the polypeptide with lectin activity.

This paper describes a development of the technique using micro two-dimensional polyacrylamide gel electrophoresis (micro 2D-PAGE), which allows the molecular mass and isoelectric point (p*I*) of a lectin from *M. charantia* seeds to be determined.

EXPERIMENTAL

Chemicals

Tris(hydroxymethyl)aminomethane (Tris), SDS, acrylamide, N,N′-methylenebisacrylamide (Bis), bromophenol blue, glycine, N,N,N′,N′-tetramethylenediamine and ammonium persulphate were purchased from Wako (Osaka, Japan). Phosphoric acid, sodium hydroxide, bovine serum albumin and sucrose were purchased from Kanto (Tokyo, Japan). Coomassie Brilliant Blue R-250 was purchased from Nakarai (Kyoto, Japan). Ampholine (pH 3.5 9.5) and the low-molecular-mass calibration kit were purchased from LKB (Uppsala, Sweden).

Extract of seeds.

M. charantia seeds were obtained from Tochigi prefecture in Japan, and 1 g of the seeds was decorticated and homogenized in 10 ml of 0.01 *M* phosphate-buffered saline (pH 7.3). The homogenate was kept overnight at 4°C and then centrifuged at 1000 *g* for 10 min. After centrifugation, the supernatant was filtered through a membrane filter with pore size of 0.45 μm (Millipore, Tokyo, Japan).

The protein concentration of the lectin solution was determined by the method of Lowry *et al.* [10].

Hemagglutination tests

Hemagglutination procedures were performed by the method of American Association of Blood Banks [11]. Human and animal red blood cells obtained from the heparinized peripheral blood of healthy donors were washed three times in physiological saline. Each 50-μl portion of serial two-fold dilutions of the *M. charantia* lectin was added to an equal volume of a 3% suspension of the red blood cells, and then was incubated for 30 min at 37°C. After incubation the mixture was centrifuged at 1000 *g* for 15 s.

Preparation of sample

The solution of *M. charantia* lectin was added to an equal volume of the sample buffer containing 2% SDS in 0.5 *M* Tris–HCl (pH 6.8) overnight at room temperature.

Solid-phase red blood cell adherence assay

The dot blot technique was performed according to the method described by Plapp *et al.* [12]. A 5-μl volume of the sample was applied to the centre of the nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), which was cut into 1-cm squares. Each square was covered with a 3% solution of bovine serum albumin for 2 h at room temperature, and then washed three times in physiological saline. One drop of packed red blood cells was added to the centre of each membrane square and then incubated for 5 min at room temperature. The membrane was washed gently with physiological saline.

SDS-PAGE

SDS-PAGE was performed according to the method of Laemmli [13]. A 40-μl volume of the sample was placed in each well of the slab gel. The electrophoretic separation was carried out in a 5–10% gradient gel that was covered with a stacking gel of 3% acrylamide. Electrophoresis was performed at 12 mA constant current in the stacking gel and 16 mA constant current.

Micro 2D-PAGE

Micro 2D-PAGE was performed by the method of Manabe *et al.* [14]. The first dimension was isoelectric focusing of the protein mixture, and the second dimension was electrophoresis on a slab gel containing SDS. A 6-μl volume of the sample was applied to the isoelectric focusing gel. Isoelectric focusing was performed at a constant current of 0.1 mA per tube for 40 min. After the first-dimension electrophoresis, the gel was treated with a sample buffer for 15 min at room temperature and placed on the top of the slag gel. The slab gel was made of a 4–17% continuous gradient gel containing 1% SDS. Electrophoresis was performed at a constant current of 8 mA per gel.

Electrophoretic blotting and red blood cell adherence

After micro 2D-PAGE, the electrotransfer of polypeptides to a nitrocellulose membrane was performed in a bufffer containing 0.025 *M* Tris, 0.192 *M* glycine and 0.1% SDS at a constant current of 200 mA for 15 min, according to the method of Manabe *et al.* [14]. The membrane was stained with Coomassic Brilliant Blue R-250 according to the method of Fairbanks *et al.* [15] or the method of red blood cell adherence [9]. Briefly, the membrane was covered with a 3% solution of bovine serum albumin to block polypeptide-unattached sites for 2 h at room temperature and then washed three times in physiological saline. Then 1 ml of a 0.25% red blood cell suspension was mounted on each 1 cm^2 of membrane

for 2 h at room temperature, and the membrane was washed gently with physiological saline.

Computer image analysis

The micro 2D electrophoresis pattern of the polypeptide with lectin activity was analysed with an image analyser (Immuno Medica, Shizuoka, Japan). This computer program processed and compared the micro 2D-PAGE images.

RESULTS AND DISCUSSION

The extract of *M. charantia* was light green and its pH was 6.2. The protein concentration was 500 μg/ml. The extract showed a lectin activity that agglutinated all of human group A, B, O and AB red blood cells. Its agglutination titre was 8192 in physiological saline at room temperature. On the other hand, the lectin did not agglutinate red blood cells from chimpanzees, marmosets, tamarines, cows, pigs, goats, sheeps, dogs, cats or rabbits.

The solid-phase red blood adherence assay was a quick test for the detection of lectin acitivity against red blood cells, which was very easy to interpret visually. The positive reaction produced a red dot on the nitrocellulose membrane, because of the adherence of red blood cells to the solid-phase lectin (Fig. 1). All of the group A, B, O and AB red bloods cells formed red dots on the membranes according to this procedure.

The SDS-PAGE analysis was performed on the *M. charantia* extract followed by blotting on to two sheets in nitrocellulose membrane. The Coomassie-stained membrane revealed fourteen polypeptide bands in the 20 000 to 115 000 molecular mass range (data not shown). On the other hand, a red band corresponding to one of these polypeptides was observed in the red blood cell-adhered membrane, and it was located in the region of *ca.* 107 000 molecular mass, as previously reported [9]. The peptide is henceforth called the 107 000 polypeptide.

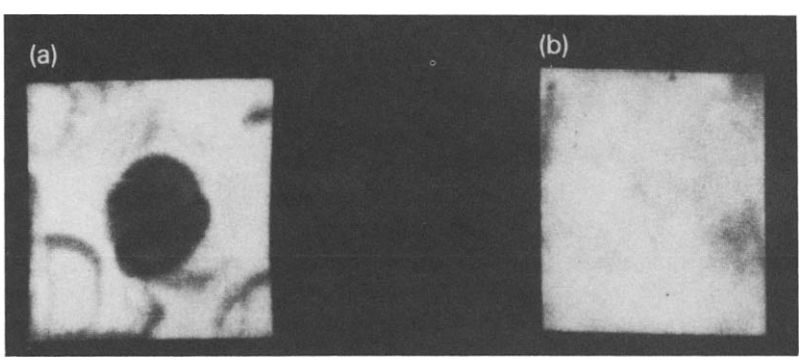

Fig. 1. Photograph of solid-phase red blood cell adherence assays, showing (A) positive (dot in centre) and (B) negative (blank) reactions.

To examine the lectin in more detail, the *M. charantia* extract was subjected to micro 2D-PAGE, followed by electrophoretic blotting on to a nitrocellulose membrane. The blotted membrane was treated with the same procedure as SDS-PAGE. Twenty-five polypeptide spots were visualized on the Coomassie-stained 2D-PAGE gel (Fig. 2A). Among these spots, the three large ones that were deeply stained were termed "major spots". Most polypeptides were located within an effective focusing range of p*I* of 4.8 7.2. The inhibition of the entrance of high-molecular-mass polypeptides into the gel was not observed. All the polypeptides were seen in the 12 000 to 107 000 molecular mass range. The procedure of red blood cell adherence to a blotted membrane showed only one red spot, with molecular mass 107 000 and p*I* 5.3 (Fig. 2B). The molecular mass and p*I* values are compatible with previous values [4–8]. This spot was a major one with the highest molecular mass, and corresponded to the 107 000 polypeptide band observed in the SDS-PAGE system.

The three-dimensional map of Fig. 2 using the image analyser is shown in Fig. 3. The 107 000 polypeptide occupied *ca.* 1.76% of the total volume of protein. The 107 000 polypeptide was stained with concanavalin A-peroxidase (Fig. 4). These results suggest that the lectin of *M. charantia* is a glycopeptide with molecular mass 107 000 and p*I* 5.3, with the sugar chain of mannose and/or glucose.

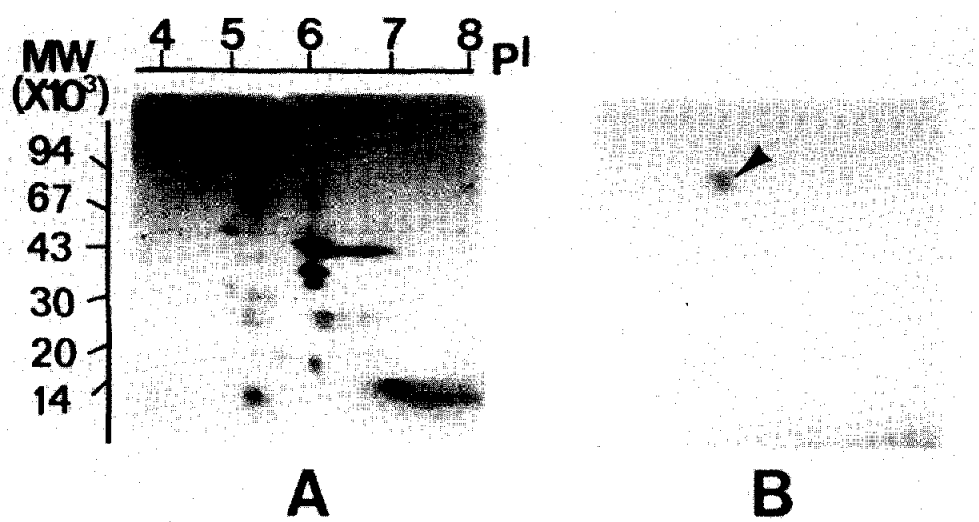

Fig. 2. Extract components of *M. charantia* separated with micro 2D-PAGE and electrophoretically transferred to a nitrocellulose membrane. (A) A Coomassie-stained gel; (B) a red blood cell adhered membrane. The arrowhead shows the positive band with a molecular mass of 107 000 and an isoelectric point (p*I*) of 5.3, which reacted against the red blood cells.

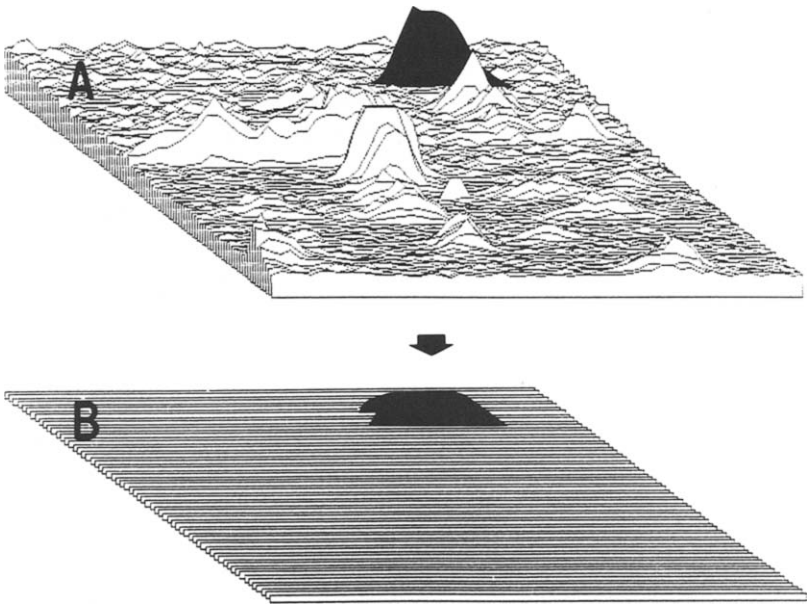

Fig. 3. Computer image analysis of the Coomassie-stained micro 2D-PAGE gel of *M. charantia* (A) and its transferred nitrocellulose membrane adhered by red blood cells (B). The shaded area shows the component of *M. charantia* with lectin activity detected by the adherence of red blood cells.

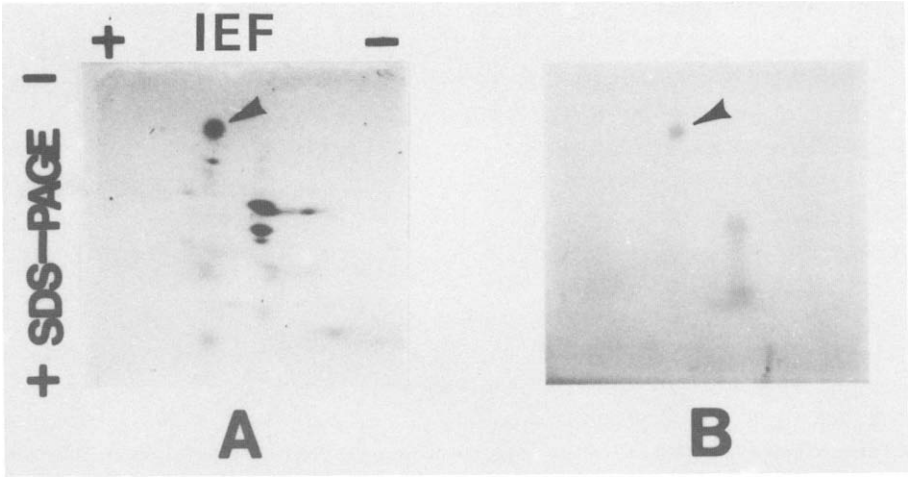

Fig. 4. (A) Coomassie-stained micro 2D-PAGE gel of *M. charantia*, and (B) its immunoblotting pattern with concanavalin A-peroxidase.

CONCLUSION

The procedure described here allows the molecular mass and p*I* of a lectin from *M. charantia* to be determined. However, the general application of the method for the detection of lectins after SDS electrophoretic separation will be limited to those cases of lectins that are not dissociated under the conditions of SDS electrophoresis.

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