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Purification and some properties of hemagglutinin from the Myxomycete, *Physarum polycephalum*

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Abstract. A new hemagglutinin was isolated from the plasmodium of *Physarum polycephalum* by salting out with ammonium sulphate followed by chromatography on DE-32, DEAE-Toyopearl and hydroxyapatite. This hemagglutinin, named physarumin, was purified 1000-fold over crude extracts. The molecular weight of physarumin was determined to be 22,000 by size exclusion chromatography on Bio-Gel P-60 and 8,700 by SDS-polyacrylamide gel electrophoresis. Physarumin agglutinated rabbit, guinea pig, horse and human erythrocytes. Physarumin-induced hemagglutination was inhibited by fetuin and α_1-acid glycoprotein, but not by commercially available mono- and disaccharides. Hemagglutinating activity was blocked by EDTA, and was restored by adding Ca^{2+} but not by Mg^{2+}.
Key words. Myxomycete; Physarum; hemagglutinin; Ca^{2+}-dependent lectin.

A number of carbohydrate-binding proteins (lectins) have been isolated and purified from various microorganisms, plants and animals[1–7]. Since these proteins can recognize complex carbohydrates on the cell surface, and are involved in cell functions, they have been widely used as reagents to detect complex carbohydrates on cell surfaces and for other cell biology studies.

Slime molds are the most primitive eukaryotic cells and can be divided into true slime molds, such as *Physarum polycephalum*, and cellular slime molds, such as *Dictyostelium discoideum* and *Polysphondylium pallidum*. The slime molds have the simplest form of differentiation processes among eukaryocytes. The life cycle is apparently similar for both kinds, but true slime molds can form a multinucleate plasmodium stage, whereas the cellular slime molds retain their individual cell identity at all stages. For these reasons, slime molds have been used to study fundamental biological problems such as cell differentiation, cell movement, and the control of cell growth and nuclear division[8,9]. Discoidin isolated from *D. discoideum* and pallidin from *P. pallidum* are lectins that bind glycoconjugates containing N-acetyl-D-galactosamine or D-galactose[10,11]. Both lectins seem to play an important role in the life of slime molds during morphogenesis. A similar lectin was also found in the fruiting body formation of a gram-negative myxobacterium (slime bacterium), *Myxococcus xanthus*[12]. In this paper, we describe the purification of a new hemagglutinin (termed physarumin) from the multinucleate plasmodium stage of a true slime mold, and its properties.

Materials and methods

Culture of plasmodia. A plasmodium of *P. polycephalum* was supplied by the National Institute for Basic Biology (Okazaki, Japan) and cultured according to the method of S. Hatano et al.[13]. After harvest, the microorganism was stored at −80 °C until use.

Preparation of crude fraction. The plasmodia (200 g) were extracted with 5 volumes of 10 mM Tris-HCl buffer (pH 7.4) under gentle stirring at 0 °C overnight. The extract was centrifuged at 13000 × *g* for 70 min. The supernatant (crude fraction) was heated at 50 °C for 30 min, and the denatured pellets were removed by centrifugation as described above. Ammonium sulphate was added to the supernatant (heated fraction) to a concentration of 80% at 0 °C. The solution was left standing for 2 h and then centrifuged at 6000 × *g* for 20 min to yield an $(NH_4)_2SO_4$-precipitated fraction (1125 mg protein, see table 1).

Purification of physarumin. The $(NH_4)_2SO_4$-precipitated fraction was dissolved in 10 mM Tris-HCl buffer (pH 8.0) (350 ml), followed by dialysis against the same buffer for 48 h. The solution was loaded onto a column (5 × 20 cm) of DE-32 (Whatman) equilibrated with the same buffer, and then eluted with a linear gradient elution of 0 to 450 mM NaCl in the same buffer. The active fraction eluted with 80–190 mM NaCl was pooled

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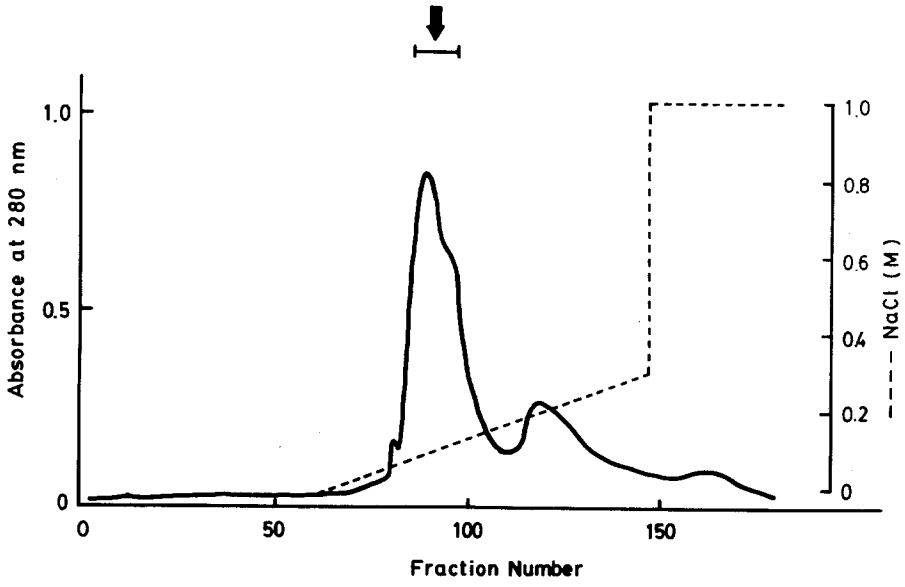

Figure 1. DEAE-Toyopearl chromatography of physarumin. The active fraction eluted from a DE-32 column (200 ml) was applied to a DEAE-Toyopearl column (5.0 × 10 cm). Fractions of 10 ml each were collected and the flow rate was kept under 20 ml/h or less. Fractions indicated with bars were pooled for further studies.

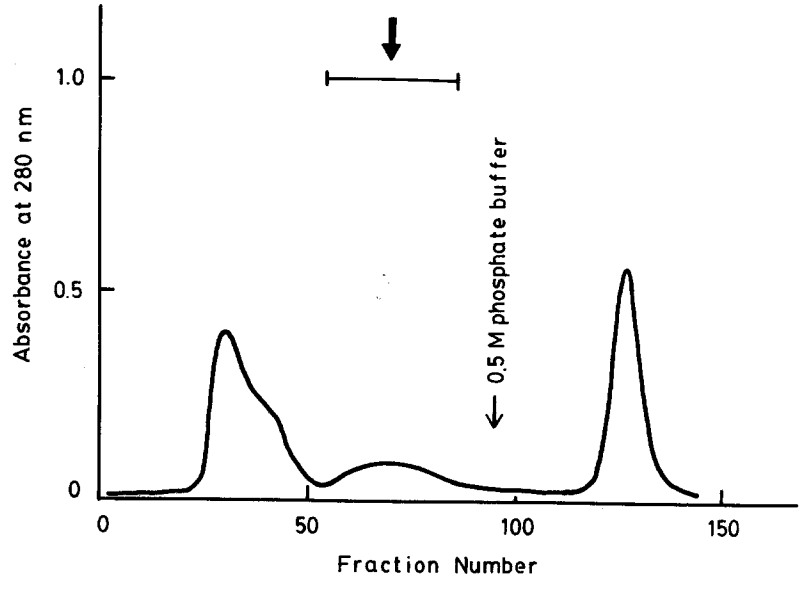

Figure 2. Hydroxyapatite chromatography of physarumin. The active fraction eluted from the DEAE-Toyopearl column (70–100 ml) was concentrated to 7–10 ml by absorption on a small DE-32 column (5.0 × 1.0 cm). The concentrated sample was applied to a hydroxyapatite column (2.5 × 12 cm). Fractions of 2 ml each were collected and the flow rate was kept under 8 ml/h. Fractions indicated with bars were pooled, dialyzed against distilled water and lyophilized.

and dialyzed against 10 mM Tris-HCl buffer (pH 8.0). The dialyzed sample was applied to a column of DEAE-Toyopearl (Tosoh, Japan), and eluted with a linear gradient elution of 0 to 300 mM NaCl in the same buffer (see fig. 1). The active fractions were pooled, dialyzed against 5 mM dipotassium hydrogenphosphate-potassium dihydrogenphosphate buffer (pH 6.6), and then applied to a column of hydroxyapatite (Wako Pure Chemical Ind., Japan) equilibrated with the same buffer. After elution of the major unadsorbed fraction, the retarded unadsorbed fraction was pooled, dialyzed against distilled water and lyophilized (see fig. 2 and table 1). The purified hemagglutinin (physarumin) was stored at −80 °C for further studies.

Table 1. Purification of physarumin.

Fraction	Volume (ml)	Protein (mg/ml)	Total protein (mg)	$Titer^{-1}$	Specific activity ($titer^{-1}$/mg protein)	Purification (fold)	Total activity	Recovery (%)
Crude	1000	2.76	2760	16	5.8	1.0	16008	100
Heated	990	1.36	1346	16	11.8	2.0	15883	99
$(NH_4)_2SO_4$-precipitated	450	2.50	1125	32	12.8	2.2	14400	90
DEAE-Toyopearl	17	0.28	4.8	512	1828.6	315.3	8777	55
Hydroxyapatite	6.4	0.17	1.1	1024	6023.5	1038.5	6626	41

Determination of molecular weight and chemical analysis. The purity and molecular weight of physarumin were determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) and by size exclusion chromatography using Bio-Gel P-60 (100–200 mesh, 1.6 × 66 cm). SDS-PAGE was performed according to the method of Fling and Gregerson using a 20% acrylamide slab gel[14]. Physarumin (10–15 µg of protein) was dissolved in the sample buffer and treated at 100 °C for 3 min. Gels were stained for 30 min in 0.025% Coomassie Brilliant Blue R-250 solution. Size exclusion chromatography was performed with medium A (0.2 mM $CaCl_2$, 150 mM NaCl, 10 mM Tris HCl, pH 7.4) and medium B (0.1 mM EDTA, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4). Isoelectric focusing was performed in IsoGel™ plates (pH range 3–7) on the Multiphor II apparatus. The gel was stained with Coomassie Brilliant Blue and an isoelectric focusing calibration kit (Pharmacia Biotech, LKB 1860-202 pI markers, pI range 2.4–5.65) was used as pI marker. Protein concentrations were determined by the method of Lowry et al. using bovine serum albumin as a standard[15]. The amino acid composition of physarumin was analyzed with a Hitachi model L-8500 amino acid analyzer. Physarumin was hydrolyzed in 6 N HCl under N_2 gas at 110 °C for 24 h[16]. The values of Thr, Ser and Tyr were extrapolated to zero time after 72 h hydrolysis. Val and Ile had a 72 h hydrolysis value.

Hemagglutination and inhibition assay. Fresh erythrocytes (human, rabbit, guinea pig, horse, sheep, calf, chicken, goose and rat) were washed five times with medium A. A sample (25 µl) was double-diluted in series with medium A in microtiter V-plates (Cook Engineering) and the dilutions mixed with 25 µl of 2% erythrocyte suspension (25 µl). The hemagglutinating activity was expressed as a titer defined as the reciprocal of the highest dilution causing hemagglutination after 60 min at room temperature. The specific activity was defined as a $titer^{-1}$/mg protein[5,6].

Neuraminadase digestion of erythrocytes was performed as follows. A rabbit erythrocyte suspension (10%, 1 ml) was treated with 0.07 U neuraminidase from *Vibrio cholerae* (Sigma, type III) in 50 mM acetate buffer (pH 5.5) containing 0.15 M NaCl and 4 mM $CaCl_2$ at 37 °C for 1 h. After enzyme treatment, erythrocytes were washed 3 times with medium A and tested[17].

Various saccharide solutions at an initial concentration of 150 mM, dissolved in medium A (25 µl) were serially diluted in microtiter V-plates. Physarumin (25 µ) of titer 4 was added to each well, and incubated at room temperature with occasional shaking for 60 min, followed by addition of 2% rabbit or human erythrocyte suspension (25 µl)[5,6].

Effects of EDTA, Ca^{2+}, and Mg^{2+} on the hemagglutinating activity. Medium B (25 µl) was added to each well in microtiter V-plates, and mixed with physarumin (25 µl) of titer 4 dissolved in medium C (150 mM NaCl, 10 mM Tris-HCl, pH 7.4) and incubated at room temperature for 60 min. Then 0.2 mM $CaCl_2$ or 0.2 mM $MgCl_2$ solution (25 µl) in medium C was added to each well and incubated at room temperature for 60 min, followed by addition of 2% rabbit or human erythrocyte suspension (25 µl).

Results

Purification and chemical properties of physarumin. Figures 1 and 2 show the elution patterns of fractions separated in the purifying process of physarumin. The hemagglutinating activities were adsorbed on DE-32 and DEAE-Toyopearl columns and eluted with 80–190 mM NaCl (data not shown) and 70–126 mM NaCl (fig. 1), respectively. The activity after hydroxyapatite column chromatography was present in fractions with low protein absorption (fig. 2). The purification factor for each fraction is shown in table 1. The hemagglutinin was purified 1000-fold with a yield of 41%.

The purity of physarumin as analyzed by SDS-PAGE is shown in fig. 3. Physarumin migrated as a single band with a molecular weight of 8,700 in the presence or absence of 2-mercaptoethanol. The size exclusion chromatography on Bio-Gel P-60 showed that the approximate molecular weight of physarumin was 22,000 in the presence or absence of Ca^{2+} (data not shown). Thus, physarumin under non-denaturing conditions behaves as a dimer. On isoelectric focusing, physarumin has an acidic isoelectric point (pI 4.45, data not shown). The

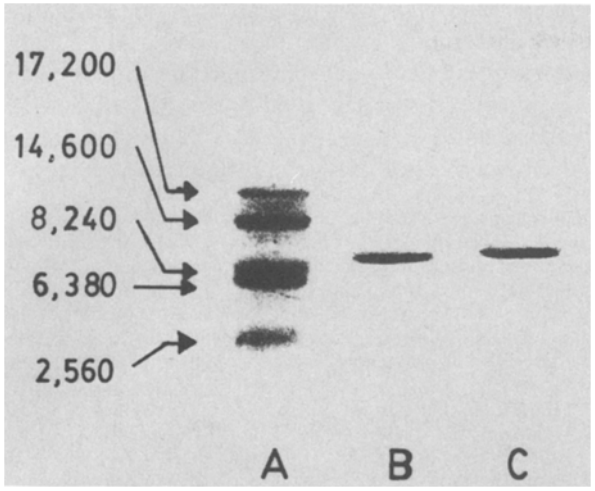

Figure 3. SDS-PAGE of physarumin. Electrophoresis was performed as described in the text on a 20% polyacrylamide gel under reducing (lane B) and nonreducing (lane C) conditions. Migration was from top to bottom. Lane A, marker proteins; lane B, physarumin under reducing conditions; lane C, physarumin under nonreducing conditions. Markers were: myoglobin (intact), 17,200; myoglobin I + II, 14,600; myoglobin I, 8,240; myoglobin II, 6,380; myoglobin III, 2,560.

amino acid composition of physarumin is shown in table 2. Physarumin is rich in Asp plus Asn, Val and Ser but poor in Met, Tyr and His.

Biological activity of physarumin. Table 3 shows physarumin-induced hemagglutination of erythrocytes from various species. Physarumin was highly active against rabbit, guinea pig and horse erythrocytes, and weakly active against human erythrocytes. No change of agglutinability was observed when rabbit erythrocytes were digested with neuraminidase (table 3). Table 4 shows the results of a hemagglutination inhibition assay using intact rabbit and human erythrocytes. The hemagglutination was inhibited at low concentrations of fetuin and α_1-acid glycoprotein but not by a range of mono- or disaccharides. The inhibitory capacity of asialofetuin was similar to that of fetuin (table 4). To examine the influence of chelating agents and divalent cations on physarumin-induced hemagglutination (rabbit and human erythrocytes), EDTA (0.1 mM), $CaCl_2$ and $MgCl_2$ (0.2 mM) were added to the assay system (see 'Materials and methods'). The hemagglutinating activity was completely blocked by EDTA. The blocked hemagglutinating activity was restored by the addition of $CaCl_2$, but not by adding $MgCl_2$ (data not shown). These results indicate that physarumin requires Ca^{2+} to promote hemagglutinating acitivy. Although hemagglutinating activity was not reduced at temperatures lower than 63 °C for 20 min, the activity was reduced to one-sixteenth when physarumin solution was heated at 100 °C for 10 min (data not shown).

Table 2. Amino acid compositions of physarumin.

Amino acid	Residues/100 residues
Asp + Asn	16.9
Thr	6.7
Ser	10.4
Glu + Gln	7.5
Gly	7.9
Ala	6.0
Half-Cys	0.5
Val	11.5
Met	0.3
Ile	4.5
Leu	4.9
Tyr	1.0
Phe	8.7
Lys	4.9
His	1.4
Arg	4.7
Pro	2.2
Trp	ND[a]

[a]ND = not determined.

Table 3. Hemagglutinating activity of physarumin with erythrocytes from various species[a].

Source of erythrocytes	Specific activity (titer^{-1}/mg protein)
Human type A	94
B	94
O	94
Rabbit	
intact	6024
+ neuraminidase	6024
Guinea pig	3012
Horse	1506

[a]Physarumin did not agglutinate calf, chicken, goose, rat or sheep erythrocytes.

Table 4. Inhibition of hemagglutinating activity of physarumin by glycoproteins and polysaccharides[a].

Glycoproteins/polysaccharides	Inhibition
Fetuin	Complete inhibition
Asialofetuin	at 0.8–1.6 mg/ml
α_1-Acid glycoprotein	
Heparin	None at 50 mg/ml
Mannan	

[a]The following saccharides did not inhibit the physarumin-induced hemagglutination, even at 150 mM concentration: L-arabinose, D-fucose, D-ribose, D-xylose, L-rhamnose, D-glucose, D-galactose, D-mannose, D-fructose, D-glucosamine, N-acetyl-D-glucosamine, D-galactosamine, N-acetyl-D-galactosamine, sialic acid, and lactose.

Discussion

When amoebae of cellular slime molds differentiate from a vegetative noncohesive to a cohesive stage, carbohydrate-binding proteins (lectins) appear on their cell surface[18]. Three lectins have been isolated from cellular

slime molds: tetrameric proteins (M_r: approximately 100,000), discoidin I (M_r: 26,000) and discoidin II (M_r: 23,000), from *D. discoideum*; and pallidin (Mr: 25,000) from *P. pallidum*[10,19–21]. Discoidin I and II bind well to rabbit erythrocytes. The hemagglutinating activities of both lectins are inhibited by N-acetyl-D-galactosamine, D-fucose, D-galactose and lactose. Since discoidin I binds well to formalinized sheep erythrocytes whereas discoidin II does not, the carbohydrate-binding sites of these lectins are distinct[20]. Pallidin-induced hemagglutination is inhibited by lactose[21]. Discoidin I and II agglutinate fixed cohesive *D. discoideum* cells in preference to fixed vegetative *D. discoideum* cells. The increased agglutinability of *D. discoideum* cells by discoidin I or II with differentiation is correlated with the appearance of increased quantities of discoidin and development of cohesiveness[22]. Thus, these lectins may contribute to the recognition of species specificity and intercellular adhesion of slime mold cells.

Generally, lectins are purified using specific sugars as affinity adsorbents. Discoidin I and II have a high affinity for D-galactose, and are capable of binding to Sepharose 4B[10]. Native discoidin is a tetramer and tends to aggregate in the absence of galactose. The isoelectric point of discoidin is 6.1 in the presence of galactose[10]. Since physarumin is an acidic protein (pI 4.45), and failed to bind mono- or disaccharides (table 4), we attempted to use anion-exchange and adsorption chromatography for its isolation (see figs 1 and 2). The molecular weight of physarumin was 22,000 by size exclusion chromatography, and 8,700 by SDS-PAGE. Since a single band was observed in SDS-PAGE under the reducing and nonreducing conditions (fig. 3), the lectin was considered a homodimer.

Although physarumin has hemagglutinating activity like discoidin and pallidin, its activity was inhibited by fetuin and α_1-acid glycoprotein, but not by N-acetyl-D-galactosamine, D-galactose or lactose which inhibited discoidin- or pallidin-induced hemagglutination (tables 3 and 4). Animal lectins are classed in three categories, C-type (Ca^{2+}-dependent), S-type (thiol-dependent) and P-type lectins[23,24]. Considering that physarumin requires Ca^{2+} for its hemagglutinating activity, we may assume that this hemagglutinin belongs to C-type lectins.

While the amoebae of cellular slime molds never fuse to form a multinucleate plasmodium, the life cycle of *P. polycephalum* comprises a unicellular amoeboid stage, a multinucleate plasmodium stage and a sporulation stage[8]. Physarumin, isolated from plasmodia of *P. polycephalum*, may have a different function from discoidin or pallidin. To test the validity of this assumption, further detailed studies of its sugar binding, biological and physicochemical properties will be necessary.

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