J Ind Microbiol Biotechnol (2006) 33:967–973
DOI 10.1007/s10295-006-0145-9

ORIGINAL PAPER

Purification and characterization of a novel haemagglutinin from *Chlorella pyrenoidosa*

Chun-Yao Chu · Rang Huang · Liang-Ping Ling

Received: 7 June 2005 / Accepted: 3 May 2006 / Published online: 15 June 2006
© Society for Industrial Microbiology 2006

Abstract We previously identified a strong haemagglutination activity in the freshwater unicellular green alga, *Chlorella pyrenoidosa*. Here, we sought to purify and characterize the haemagglutinin associated with this activity. Ammonium sulfate precipitation, gel filtration on sephacryl S-200 and DEAE-Sepharose ion-exchange chromatography were used to purify the haemagglutinin, which was designated CPH (*Chlorella pyrenoidosa* haemagglutinin). The molecular weight of CPH was estimated as 58 kDa by SDS-PAGE and 60 kDa by gel filtration of the native protein, indicating that this haemagglutinin exists as a monomer. The haemagglutinin activity of CPH was inhibited by glycoproteins, especially yeast mannan, but not by monosaccharides or disaccharides, indicating that CPH is carbohydrate-specific. In addition to the composition of CPH shown to be rich in glycine and acidic amino acids, heamagglutinating activity of CPH was insensitive to variations in pH or the presence of divalent cations, and atomic force microscopy revealed that the protein is rod-shaped. These results indicate that the characteristics of CPH are consistent with its identification as a haemagglutinin, and suggest that CPH may be a viable candidate for applications in a variety of biomedical fields.

C. -Y. Chu · L. -P. Ling (✉)
Institute of Microbiology and Biochemistry,
National Taiwan University,
Taipei 106, Taiwan
e-mail: Sping.bbs@yahoo.com.tw

C. -Y. Chu · R. Huang
Institute of Oceanography, National Taiwan University,
Taipei 106, Taiwan

Keywords Haemagglutinin · *Chlorella pyrenoidosa* · Purified · Rod-shaped

Introduction

The haemagglutinins, which are widely distributed in nature, are carbohydrate-binding proteins associated with important effects, such as cell aggregation and glycoconjugate precipitation. As such, haemagglutinins can be used as carbohydrate probes, making them useful tools in a variety of biochemical and biomedical research areas. Although the physiological function of agglutinins in algae is not clear, biochemical studies indicate that algal haemagglutinins have potentially useful applications in biochemical, drug, and clinical studies. For example, several studies have reported that algal haemagglutinins exhibit immunomodulatory and antitumour activities in vitro and in vivo [16, 19, 22].

Since Boyd et al. [3] first reported identification of marine algae haemagglutinin, many researchers have investigated the haemagglutinating capacities of marine algae extracts [1, 7, 8, 10, 24], and several algal haemagglutinins have been isolated to date [2, 16, 26]. The majority of previous studies have focused on haemagglutinins from marine macroalgae. Unfortunately, the practical use of marine macroalgal haemagglutinins are limited by some critical problems, including few scientists study this subject, low concentrations of molecules in algal extracts, and some high haemagglutinating activity algae are difficult to collect [16, 25].

Recent works have suggested that these limitations may be overcome by the use of microalgae, which are more easily cultured and manipulated [4, 18, 27, 30]. To date, haemagglutinins have been isolated from the

freshwater microalgae *Microcystis aeruginosa* (M228), *M. viride* [29, 30], and *Oscillatoria agardihii* [27]. However, only a few studies have examined the possible applications of haemagglutinins from freshwater microalgae.

Although only few attempts have so far been made at freshwater microalgal haemagglutinin, we still believe that microalgae haemagglutinins could complement faults of macroalgal haemagglutinins and have wide application in many fields. In a recent screening for haemagglutinating activity of freshwater microalgae, we found that *Chlorella pyrenoidosa* displayed a strong and stable activity [4]. The aim of this study was to purify and characterize this novel haemagglutinin from *C. pyrenoidosa* as one of a series of studies of agglutinins and their activities focusing on the microalgae.

Materials and methods

Cells and culture conditions

The *C. pyrenoidosa* cells used in this study were isolated from the river in Taiwan, and were grown and maintained on proteose medium [14] composed of KNO_3 (0.25 g L^{-1}), $MgSO_4 \bullet 7H_2O$ (75 mg L^{-1}), K_2PO_4 (0.175 g L^{-1}), NaCl (25 mg L^{-1}), $CaCl_2$ (10 mg L^{-1}), Fe solution (1 ml L^{-1}), A5 solution (1 ml L^{-1}), and proteose peptone (1 g L^{-1}). The pH was adjusted to 6.0 before the medium was autoclaved at 121°C for 20 min. Algae were maintained at 25°C in 1.2 L containing 1.0 L culture under 80 μmol m^{-2} S^{-1} illumination with a 12 h light:12 h dark cycle. Cells were cultured for 10–14 days, and then harvested at the stationary phase by centrifugation at 10,000×*g* for 20 min. Harvested cells were lyophilized and stored at −20°C until use.

Preparation of extracts from harvested cells

Lyophilized *Chlorella* cells (10 g) were resuspended in 200 ml of Tris–HCl buffered saline (TBS; pH 7.4, containing 25 mM NaCl) at 4°C, and sonicated (Virsonic, USA) in an ice bath for ten 3 min cycles Each mixture was then centrifuged at 15,000×*g* for 20 min, and the supernatant was used as the extract for haemagglutinin purification.

Haemagglutination assay

Human B erythrocytes were used to purify haemagglutinin as previously reported [9]. The erythrocytes were concentrated and washed three times with phosphate-buffered saline (PBS; pH 7.2, containing 0.1 M NaCl), collected by centrifugation, and diluted 1:50 PBS. This erythrocyte suspension was used directly in the agglutination activity assay, using serial two-fold dilutions as previously reported [7]. Algal extracts were placed in wells (50 μL to which equal volumes of human B erythrocyte suspension were added. The plates were gently shaken, and left for 2 h at room temperature. The reciprocal of the highest dilution exhibiting positive haemagglutination was expressed as the extract titer.

Purification of haemagglutinin from C. pyrenoidosa

The *C. pyrenoidosa* cellular extracts were precipitated with increasing percentages of ammonium sulfate. Precipitates showing positive haemagglutination reactions (primarily from the 40–60% ammonium sulfate fractions) were pooled, dissolved in distilled ion-exchanged water, and then dialyzed against TBS (containing 50 mM NaCl) at 4°C for 24 h. During dialysis, the buffer was refreshed once every 4 h. The dialyzed samples were centrifuged, and the supernatants were subjected to gel filtration chromatography on a sephacryl S-200 column (26×60 cm; Pharmarcia) previously equilibrated with TBS and eluted at a flow rate of 1.0 ml min^{-1}. The eluted protein fractions were detected their haemagglutining activity by human B erythrocyte. Samples showing positive reactions were collected and dialyzed against TBS (containing 50 mM NaCl) at 4°C for 24 h. Each dialyzed fraction was centrifuged and the supernatant was subjected to ion-exchange chromatography using a DEAE-Sepharose column equilibrated with the same buffer and eluted by stepwise increases in NaCl concentration up to 0.5 M at flow rate of 1.0 ml min^{-1}. The active fractions were collected and dialyzed against TBS (containing 50 mM NaCl). Finally active elutes were dialyzed against distilled ion-exchanged water, lyophilized, stored in buffer at −20 C, and considered to be purified haemagglutinin (designated as CPH). SDS-PAGE and gel filtration methods were used for molecular weight determination using thyoglobulin (Mw 670 kDa), bovine gamma globulin (Mw 158 kDa), ovalbumin (Mw 44 kDa), and Myoglobin (Mw 17 kDa) as markers (Pharmacia; Uppsala, Sweden).

Protein and sugar contents

Protein contents were quantitated by bovine serum albumin as the standard according to the Lowry's [20] method. Sugar contents were quantitated by phenol-sulfuric methods [6] with D-galatose as the standard.

Amino acid composition

Amino acid composition of the purified protein was analyzed by an Amino Acid Analyzer (Schimazu, SCL−10 A). The protein sample was hydrolyzed with 6N HCl in an evacuated tube at 110°C for 24 h. The hydrolyzed sample was then applied to the analyzer for amino acid composition.

Haemagglutination-inhibition test

Purified CPH with an activity titer of eight was tested for vulnerability to inhibition by various carbohydrates and glycoproteins according to the methods of Hori et al. [9]. The utilized carbohydrates and glycoproteins were obtained from Sigma Co (USA), and included the monosaccharides L(−)arabinose, L(-)fucose, D(+)glucose, D(+)galactose, D(+)mannose, D(+)xylose and *N*-acetyl-D-galactosamine, the disaccharides lactose, D(+)maltose and D(+)sucrose, and the glycoproteins γ-globulin, asialofetuin, fetuin, mucin, and yeast mannan.

Testing the effect of pH and divalent cations on CPH activity

The effects were determined following the methods used by Hori et al. [9], using B erythrocytes for activity assay. For examination of the effect of pH on haemagglutination activity, each 1.0 mL of purified CPH was dialyzed against 0.1 M buffer solutions with pH values ranging from 3.0 to 11.0 at 4°C for 24 h, followed by thoroughly dialysis against PBS to rule out the effect of pH on the activity assay. The buffers used were: citrate buffer (pH 3.0–4.0), acetate buffer (pH 4.0–5.0), phosphate buffer (pH 6.0–8.0), Tris-HCl buffer (pH 8.0–9.0) and carbonate buffer (pH 10.0–11.0). For divalent cations, 1.0 ml of protein solution was mixed with or without 10 mM EDTA plus an equal volume of 5 mM $CaCl_2$, $ZnCl_2$, $MgCl_2$ or $MnCl_2$. Each mixture incubated at room temperature for 2 h, centrifuged, and then assayed.

Morphology of CPH

For observing the image of CPH, the purified protein was attached through electrostatic interactions by placing it in contact with freshly cleaved mica that had been coated with poly L-lysine, a positively charged compound. After cleaning the mica with methanol and Milli-Q water, 5 μL of 10^{-2} M poly L-lysine solution was applied and incubated for 30 min. The mica surface was then washed with Milli-Q water before introduction of the purified CPH specimens. Specimens of 5 μL were applied onto the poly L-lysine-treated mica for 5 min at room temperature, followed by washing with distilled water and drying in air prior to the Atomic force microscope (AFM) experiments.

Atomic force microscope (AFM) experiments in tapping mode of operation were carried out using scanning probe microscope (NT-MDT, Russia). Commercial silicon cantilevers nano-probe with a spring constant 0.03 and 0.1 N m^{-1} were used for tapping modes in this study. Tapping mode images were collected in a broad range of frequencies, 10–20 kHz. The tip of the silicon nitrite probe being 10 nm in diameter, the default gain value 10 nA, and data acquisition and image processing software an integral part of each scanning probe microscope from NT-MDT.

Electrophoresis

The purified protein was fractionated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [13] on 12.5% gels. Samples and standards were prepared in 10% SDS with 1% 2-mercaptoethanol at 100°C for 5 min prior to application to electrophoresis. The separated proteins were stained with Coomassie brilliant blue R−250 for 60 min. Proteins (Mark 12™, Novex) of known molecular weights (14; 20; 30; 43; 67; 94 kDa) were used as reference proteins.

Results

Isolation of a haemagglutinin from C. pyrenoidosa

Crude protein extracts from *C. pyrenoidosa* were precipitated with increasing quantities of ammonium sulfate and purified with a two-step chromatographic purification procedure. During ammonium sulfate precipitation, the highest haemagglutinating activity was present in fractions precipitated with 40–60% ammonium sulfate (maximum haemagglutination titer = 2^{12}), with the 0–20% and 60–80% fractions showing moderate activity and the 20–40, and 80–100% fractions showing negligible activity (Fig. 1). Notably, most of the algal proteins precipitated in the 40–60% ammonium sulfate range.

The 40–60% ammonium sulfate fraction was subjected to gel filtration on a Sephacryl S-200 column, yielding peaks in both activity and protein absorbance at fractions 46–55. The active fractions were collected and further purified by ion-exchange chromatography on a DEAE-Sepharose column eluted by increasing concentrations of NaCl. Haemagglutination activity was detected in the fractions eluted with 0.1 M NaCl,

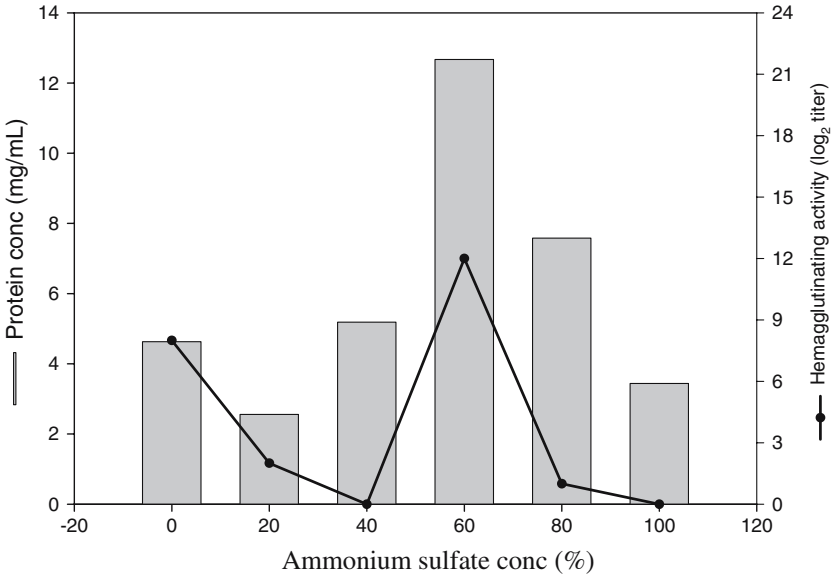

Fig. 1 Ammonium sulfate-precipitation steps of haemagglutinin from *C. pyrenoidosa*. Protein concentration (*bars*) and haemagglutinating activity (*circles*) were detected from various steps. Haemagglutination activity was expressed as a titer, the reciprocal of the highest two-fold dilution exhibiting positive haemagglutination

and the eluted haemagglutinin was designated CPH (*C. pyrenoidosa* haemagglutinin). The results of purification are summarized in Table 1, where approximately 926 mg protein was extracted from 10 g of *C. pyrenoidosa* cells (dry weight). The total protein and specific activity of CPH was 1.45 mg and 1544.83 (titer mg^{-1}) from the final DEAE ion-exchange step, respectively. The purified haemagglutinin was approximately 27.7-fold more active than that from the extracts.

Chemical properties of CPH

SDS-PAGE and gel filtration were used to determine the molecular weight of purified CPH. A single band at 58 kDa was detected on SDS-PAGE (Fig. 2), while a single symmetrical peak at 60 kDa was obtained by molecular exclusion chromatography of the native protein on a Sephacryl S-200 column (Fig. 3), indicating that CPH exists as a monomer. Furthermore, the neutral sugar content of CPH with the phenol-sulfate method was not determined.

The amino acid composition of CPH (Table 2) predominantly consists of glycine (18.3%), aspartic acid/asparagine (14.8%), and glutamic acid/glutamine (16.4%). The content of acidic amino acids was fairly larger than that of basic amino acids, i.e., histidine, lysine and arginine. The purified protein also contained a relatively small quantity of sulfur-containing amino acids, such as methionine and cystine shown in Table 2.

Carbohydrate binding specificity

The binding specificity of CPH for carbohydrates was detected using a haemagglutination inhibition test, which revealed that the haemagglutination activity of CPH was not inhibited by any of the tested monosaccharides or disaccharides (Table 3), whereas all five tested glycoproteins showed inhibitory effects. Yeast

Table 1 Purification of CPH from C. *pyrenoidosa*

Purification step	THA[a]	Total protein (mg)	Specific activity (titer mg^{-1})	Purification (fold)	Yield (%)
Crude extract	51,600	926	55.72	1.00	100
40–60% of $(NH_4)_2SO_4$ ppt	61,440	190	323.37	5.80	119
Gel filtration chromatography	3,840	10.6	362.26	6.50	7.44
DEAE chromatography	2,240	1.45	1,544.83	27.72	4.34

Haemagglutinating active (HA) was expressed as titer, and the titer is reported as the inverse of the last dilution with positive agglutination against human B-type erythrocytes

[a] Total haemagglutination titer (HA × vol)

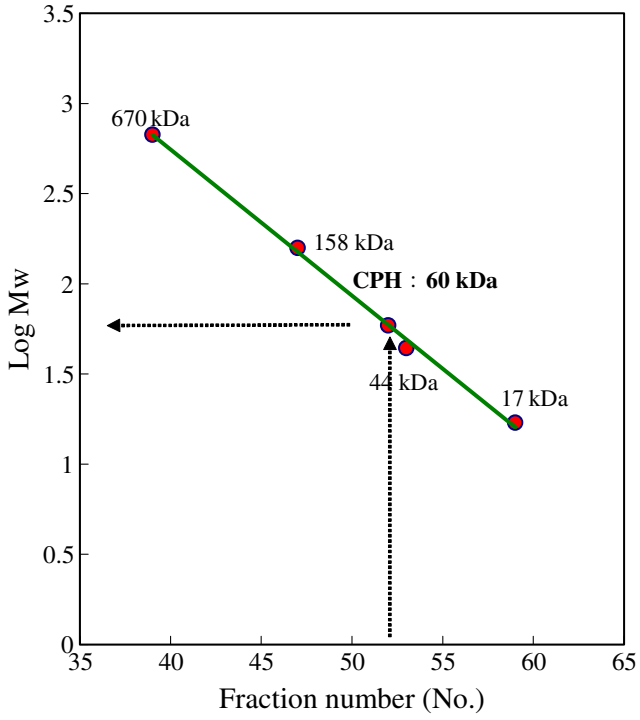

Fig. 2 Determination of the molecular weight of CPH from *C. pyrenoidosa* by gel filtration. The Mw standards (Bio-Rad) were used Thyroglobulin (670 kDa); Bovine gamma globulin (158 kDa); Chicken Ovalbumin (44 kDa), and Myoglobin (17 kDa). The molecular weights on the vertical axes are logarithmic function of the original value, and the predicted CPH molecular weights are indicated on the plot

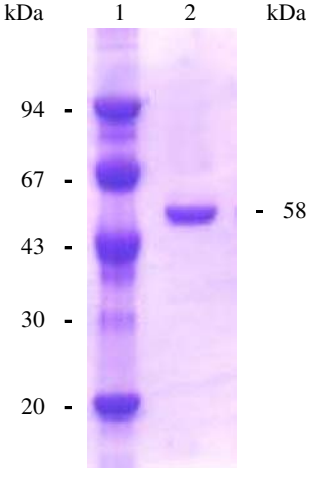

Fig. 3 SDS-PAGE of hemagglutinin from *C. pyrenoidosa* and molecular weight standards. *Lane 1* shows molecular weight standards, which are composed of phosphorylase b (94 kDa), Bovine serum albumin (67 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (30 kDa), and Trypsin inhibitor (20 kDa)

mannan showed the strongest inhibitory effect, followed by mucin, asialofetuin and fetuin, with globulin showing the weakest inhibitory effect.

Effects of pH and divalent cations on CPH activity

The agglutinating activity of CPH was fairly stable over pH 4–11, but was slightly reduced at a pH of 3. CPH activity was not significantly affected by the presence of divalent cations such as Ca^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+}, with or without EDTA. These results indicate that the CPH-directed haemagglutination reaction is independent of divalent cations. In addition, the Fig. 4 shows the two dimension atomic force micrographs of CPH from *C. pyrenoidosa.* The result of atomic force micrographs shows the morphology of CPH as rod-shaped.

Discussion

The *Chlorella* species are widespread in the air and soil, as well as in fresh and salt water. These simple unicellular algae reproduce asexually via non-motile autospores, grow quickly and have highly effective photosynthesis. These characteristics make *Chlorella* a good choice for use in the lab [31]. In addition, *Chlorella* cells are rich in protein and contain special functional compounds, making them an ideal health food source [17, 23, 28]. Thus, scientists have sought to investigate *Chlorella* cellular biology for both laboratory and commercial applications.

We previously examined the haemagglutination activity of extracts from 44 species of freshwater microalgae and identified *C. pyrenoidosa* as having a high haemagglutination activity [4]. In previous works, marine algal haemagglutinins/lectins have been isolated by salt precipitation followed by gel filtration and ion-exchange chromatography [11, 16]. Here, we sought to purify and characterize the relevant protein using chromatography of ammonium sulfate-precipitated cell extracts. We found that the fraction precipitated with 40–60% ammonium sulfate had increased agglutination activity than the raw extract, suggesting that salt precipitation is useful for removal of inhibitory compounds from the algal extracts. However, we also found that the haemagglutination activity decreased following the gel filtration step, suggesting that this portion of the purification process could be improved for better yield of active CPH.

Previous reports have revealed that the freshwater microalgal haemagglutinins/lectins are monomeric proteins with high affinities for glycoproteins but not monosaccharides [27, 29, 30]. These molecules contain high percentages of acidic amino acids, and show no dependence on metal ions for their haemagglutination activities. Specifically, *Oscillatoria agadihii* agglutinin (OAA) was shown to be a monomer protein with a

Table 2 Amino acid composition of CPH

Amino acid	Mol. %	Amino acid	Mol. %
Asx[b]	14.8	Val	4.5
Thr	4.9	Met	1.0
Ser	3.1	Ile	3.2
Glx[c]	16.4	Leu	5.0
Pro	4.8	Tyr	3.8
His	6.1	Phe	2.0
Ala	5.8	Gly	18.3
Cys (half)	ND [a]	Lys	3.1
Arg	2.7		

[a] ND means not detected

[b] Asx means Asp and Asn

[c] Glx means Glu and Gln

Table 3 Haemagglutination-inhibition with carbohydrates and glycoproteins

Carbohydrate and glycoprotein[a]	Minimum inhibitory concentration ($\mu g\ mL^{-1}$)
Monosaccharide	
L-arabinose	–[b]
L-fucose	–
D-glucose	–
D-galactose	–
D-mannose	–
D-xylose	–
N-acetyl-D-glucosamine	–
Disaccharide	–
Maltose	–
Sucrose	–
Lactose	–
Glycoprotein	
γ-Globulin (bovine)	125
Asialofetuin	62.5
Fetuin	62.5
Mucin	31.3
Yeast mannan	15.6

[a] The minimum inhibitory concentration that is required to inhibit completely the haemagglutinating activity of a titer, 8

[b] "–" indicates no inhibitory activity at the concentration of 100 mM carbohydrates

strong affinity for yeast mannan and no cation dependency for its haemagglutination activity [27], while a lectin from the cyanobacterium, *Microcystis aeruginosa* (M228), was found to be a monomer protein with a molecular weight of 57–72 kDa and a particular affinity for galactose and *N*-acetyl-galactosamine [29]. In this work, the molecular weight of CPH was estimated to be 58 kDa by SDS-PAGE and 60 kDa by gel-filtration, suggesting that CPH exists as a monomeric protein with no disulfide bonds. Furthermore, CPH had affinities for glycoproteins but not monosaccharides or disaccharides, and its haemagglutination activity was

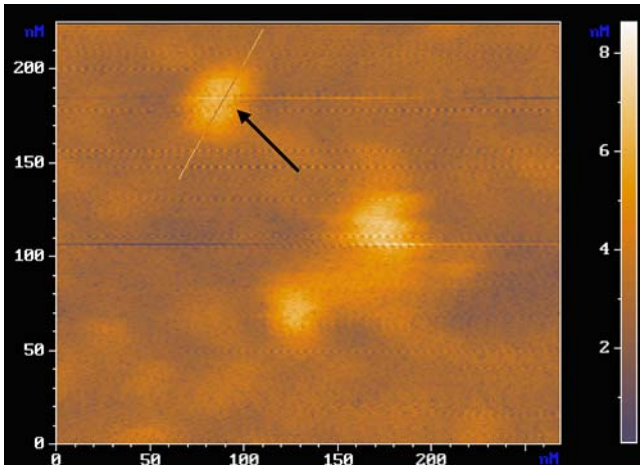

Fig. 4 AFM image of *C. pyrenoidosa* heamagglutinin (CPH) on a mica surface and the *arrow* indicated the molecules of CPH

cation independent. Collectively, these characteristics indicate that CPH should be classified as a freshwater microalgal lectin.

The interaction of proteins with biomaterial surfaces plays a key role in the host response to implanted devices [15, 21]. Algal haemagglutinin/lectin molecules are considered potentially useful in biochemical and clinical applications, due to their carbohydrate-binding specificities [5, 12, 25]. However, the precise physiological role(s) of these proteins are not yet known. A number of recent reports have suggested that algal haemagglutinin/lectin receptors present on the surfaces of marine microalgae may function in cell recognition, cell surface adhesion, symbiosis with marine invertebrates or other algae, and phagocytosis of virus and bacteria [12]. To provide additional insight into our novel haemagglutinin molecule, we recently examined CPH using atomic force microscopy (AFM), which has been used to observe the morphologies of biological macromolecules such as proteins, antibodies, and DNA fragments [15].

AFM images revealed that CPH is a rod-shaped protein. In the future, it will be interesting to use AFM to examine conformational changes in CPH during glycoprotein adsorption or desorption; these studies may provide new insights into microalgal physiology, protein structure, and the function of the CPH/carbohydrate interaction. Finally, we recently developed a CPH-specific monoclonal antibody. Immunogold labeling and electron microscopy revealed that CPH is localized beneath the surface layer of the cell, between the cytoplasm and cell membrane (unpublished data). Thus, we hypothesize that CPH is widely distributed on the *Chlorella* cell surface and may function in cell recognition, adherence, symbiosis, and/or may play additional yet-unknown physiological roles.

In sum, we herein purified and characterized a novel haemagglutinin from *C. pyrenoidosa* with pH stability, good carbohydrate specificity and high-level activity, making it a strong candidate for future applications in lectin research and biomedical applications.

Acknowledgments We wish to acknowledge the financial support for this study received from the National Science Council (Taiwan) (NSC: 92–2317-B-002–030).

References

1. Ainouz IL, Simpaio H, Benevides NMB, Freitas ALP, Costa FHF, Carvalho MR. Joventino FP (1992) Agglutination of enzyme treated erythrocytes by Brazilian marine algal extracts. Bot Mar 35:475–479
2. Benevides NM, Holanda ML, Melo FR, Freitas ALP, Simpaio H (1998) Purification and partial characterization of the lectin from marine red alga *Enantiocladia duperreyi* (C. Agardh) Falkenberg. Bot Mar 41:521–525
3. Boyd WC, Almodovar LR, Boyd JG (1966) Agglutinins from marine algae for human erythrocytes. Transfusion (Philadelphia) 6:82–83
4. Chu CY, Laio WR, Huang R, Lin LP (2004) Hemagglutinating and antibiotic activities of freshwater microalgae. World J Microbiol Biotechnol 20:817–825
5. Chiles TC, Bird KT (1990) *Gracilaria tikvahiae* agglutinin: partial purification and purification and preliminary characterization of its carbohydrate specificity. Carbohydr Res 207:319–326
6. Dudois M, Gilles KA, Hamilton JK, Rogers PA, Smith F (1956) Colorimetric method for determination of sugar and related substances. Anal Chem 28:350
7. Fabregas J, Llovo J, Munoz A (1985) Hemagglutinins in red seaweeds. Bot Mar 28:517–520
8. Freitas ALP, Teixeira DIA, Costa FHF, Farias WRL, Lobato ASC, Sampaio AH, Benevides NMB (1997) A new survey of Brazilian marine algae for agglutinins. J Appl Phycol 9:495–501
9. Hori K, Miyazawa K, lto K (1986) Preliminary characterization of agglutinins from seven marine algal species. Bull Jpn Soc Fish 52:323–331
10. Hoir K, Oiwa C, Miyazawa K, Ito K (1988) Evidence for wide distribution of agglutinins. Bot Mar 31:133–138
11. Hori K, Miyazawa K, Ito K (1990) Some common properties of lectins from marine algae. Hydrobiol 204/205:561–566
12. Hori K, Ogata T, Kamiya H, Mimuro M (1996) Lectin-like and lectin receptors in marine microalgae hemagglutination and reactivity with purified lectins. J Phycol 32:783–790
13. Hu CY, Lin. LP (2003) Purification and characterization of hydrolytic enzymes in *Sinorhizobium fredii* CCRC15769. World J Microbiol Biotechnol 19:515–522
14. Illman AM, Scragg AH, Shales SW (2000) Increase in *Chlorella* stains calorific values when grown in low nitrogen medium. Enz Microbiol Tech 27:631–635
15. Kapila WM, Nabil AA, Jayne CG, Xu S, Liu GY (2001) Fabrication of nanometer-sized protein patterns using atomic force microscopy and selective immobilization. Biophys J 80:1891–1899
16. Kawakubo A, Makino H, Ohnishi JI, Hirohara H, Hori K (1997) The marine red algae *Eucheuma serra* (J. Agardh), a high yielding source of two isolectins. J Appl Phycol 9:331–338
17. Konishi F, Mitsuyama M, Okuda M, Tanaka K, Hasegawa T, Nomoto K (1996) Protective effect of an acidic glycoprotein obtained from culture of *Chlorella vulgaris* against myelosuppression by 5-fluorouracil. Cancer Immunol Immunotherapy 42:268–274
18. Liao WR, Huang R (2000) Agglutination of human and animal erythrocytes in marine unicellular algae. J Ind Microbiol Biotechnol 24:262–266
19. Lima HC, Costa FHF, Simpaio AH, Neves SA, Benevides NMB, Teixeira DIA, Rogers DJ, Freitas ALP (1998) Induction and inhibition of human lymphocyte transformation by the lectin from the red marine algae *Amansia multifida.* J Appl Phycol 10:153–162
20. Lowry OM, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265
21. Marchin KL, Berrie CL (2003) Conformational changes in the plasma protein fibrinogen upon adsorption to graphite and mica investigated by atomic force microscopy. Langmuir 19:9883–9888
22. Neves SA, Baruffi MD, Freitas ALP, Barreira MCR (2001) Neutrophil migration induced in vivo and vitro by marine algae lectins. Inflam Res 50:486–490
23. Noda K, Tanaka K, Yamada A, Ogata J, Tanaka H, Shoyama Y (2002) Simple assay for antitumour immunoactive glycoprotein derived from *Chlorella vulgaris* strain CK22 using ELISA. Phytother Res 16:581–585
24. Rogers DJ, Blunden G, Topliss JA, Guiry MD (1980) A survey of some marine organisms for hemagglutinins. Bot Mar 23:569–577
25. Rogers DJ, Hori K (1993) Marine algal lectins: new developments. Hydrobiol 260/261:589–593
26. Sampaio AH, Rogers DJ, Barwell CJ, Sampaio SS, Costa FHF, Ramos MV (1999) A new isolation procedure and further characterization of the lectin from the red alga *Ptilota serrata*. J Appl Phycol 10:539–546
27. Sato Y, Murakami M, Miyazawa K, Hori K (2000) Purification and characterization of a novel lectin from a freshwater cyanobacterium, *Oscillatoria agardhii*. Comp Biochem Phys Part B 125:169–177
28. Tanaka K, Yamada A, Noda K, Hasegawa T, Okuda M, Shoyama Y, Nomoto K (1998) A novel glycoprotein obtained from *Chlorella vulgaris* strain CK22 shows antimetastatic immunopotentiation. Cancer Immunol Immunother 45:313–320
29. Yamaguchi M, Mituru J, Ryuichi S, Koji M, Kamiya H (1998) Purification and characterization of *Microcystis aeruginosa* (freshwater cyanobacterium) lectin. Comp Biochem Phys Part B. 119:593–597
30. Yamaguchi M, Ogawa T, Muramoto K, Kamio Y, Jimbo M Kamiya H (1999) Isolation and characterization of mannan-binding lectin from the freshwater cyanobacterium (blue-green algae) *Microcystis viridis*. Biochem Biophys Res Comm 265:703–708
31. Wu HL, Hseu RS, Lin LP (2000) Utilization of ribosomal DNA sequences to identify *Chlorella* spp. isolates. Bot Bull Acad Sin 42:115–121