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HEMAGGLUTININS FROM THE GREEN ALGAE, CHLOROPHYTA

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SUMMARY

The extracts from 30 species of the green algae were examined for hemagglutination activity with a variety of different animal and human erythrocytes which were untreated and treated with enzymes. Almost all extracts showed the activity at least toward one type of erythrocytes tested. Strong activity was detected in the extracts from 5 Chlorophyta species (*Anadyomene plicata*, *Avrainvillea erecta*, *Boodlea struveoides*, *Halimeda velasquezii* and *Halimeda discoidea*) with enzyme-treated rabbit and sheep erythrocytes. The hemagglutinins of some active species were examined for carbohydrate binding specificity, pH and temperature stability, and effect of divalent cation on their hemagglutination activities. All of the hemagglutinins had no affinity for monosaccharides, except hemagglutinin of *Codium arabicum* whose activities were inhibited by N-acetyl-D-galactosamine and N-acetyl-D-glucosamine. On the other hand, hemagglutination activities of the hemagglutinins were inhibited by some glycoproteins. The inhibition profiles with glycoproteins were different, depending on hemagglutininproducing species, and suggest the presence of lectins specific for high mannose N-glycans, complex N-glycans, or O-glycans. The hemagglutination activities of the green algal hemagglutinins were stable in a wide range of pH and temperature, and independent on divalent cations. Therefore, Vietnamese green algae may promise as a valuable source of new lectins for application.

Keywords: Chlorophyta, carbohydrate-binding specificity, hemagglutinins, lectins, stability

INTRODUCTION

Lectins, hemagglutinins or agglutinins are carbohydrate binding proteins isolated from a wide variety of organisms and play important roles as recognition molecules in cell–cell or cell–matrix interactions. Owing to the capability of discriminating carbohydrate structures, not only are lectins used as valuable biochemical reagents in many research fields, including glycomics, but they are promising candidates for medicinal and clinical application (Sharon, Lis, 2003).

Marine algal hemagglutinin was first demonstrated by Boyd *et al*. (1966). Until now, more than 800 algal species have so far been reported to contain hemagglutinins, but the number of these lectins purified and characterized is still small in comparison to lectins from higher plants (Singh *et al*., 2015; Singh, Walia, 2018). Algal lectins possess unique molecular structures and carbohydrate-binding specificities distinct from known lectins from other sources, which make them useful for applications (Cheung *et al*., 2015; Barre *et al*., 2019). Recently, some the high-mannose

specific lectins from the eukaryotic marine algae attracted some attention as potential sources of new lectins with antibacterial, antiviral (HIV, SARS-CoV and influenza viruses) and anticancer activities, such as ESA-2 from *Eucheuma serra* (Hori *et al*., 2007; Sato *et al*., 2015), Griffithsin from *Griffithsia* sp. (O'Keefe *et al*., 2010), BCA from *Boodlea coacta* (Sato *et al.,* 2011a), KAAs from *Kappaphycus alvarezii*(Sato *et al*., 2011b; Hirayama *et al*., 2016), KSA-2 from *K. striatum* (Le Dinh Hung *et al*., 2011, 2015a), EDA-2 from *E. denticulatum* (Le Dinh Hung *et al*., 2015b), HRL40 from *Halimeda renschii* (Mu *et al.,* 2017), SfLs from *Solieria filiformis* (Chaves *et al*., 2018a) and MEL from *Meristiella echinocarpa* (Chaves *et al*., 2018b) Although all of them share both carbohydrate binding specificities and repeated domain structures, they differ from each another in amino acid sequences and recognizing branched oligosaccharide structures, which may lead to subtle differences in the degree of their inhibiting activities. Thus, marine algal lectins may become a novel source of antiviral and anticancer compounds for applications.

Vietnam is located in the tropical and subtropical zone with a long coastline of about 3,260 km, where there is a rich algal flora, including green, red and brown algae (Nguyen Van Tu *et al.,* 2013; Le Nhu Hau *et al*., 2015; Phang *et al.,* 2016). These algal species may be potential sources of lectins. Thus, the objective of research was to report on presence and diversity of carbohydrate binding specificities of hemagglutinins from green algae, which may provide valuable information for application of lectins in biochemistry and biomedicines.

MATERIALS AND METHODS

Materials

Green algal specimens were collected at Ninh Thuan and Khanh Hoa provinces in Vietnam in March and August from 2013 to 2015. After collection, they were immediately transferred to the laboratory on ice, washed with distilled water, and kept at – 20^{o}C until used. The green algal species were identified by Dr. Le Nhu Hau (Nha Trang Institute of Technology Research and Application). Bloods from rabbit, sheep, and chicken were obtained from the Institute of Vaccine - Nha Trang, Vietnam. Human A, B, and O bloods were obtained from Blood transfusion and Hematology Center of Khanh Hoa General Hospital, Vietnam. L-fucose, D-glucose, D-mannose, D-galactose, N-acetyl-D-glucosamine, N-acetyl-D-mannosamine, N-acetyl-D-galactosamine, N-acetyl neuraminic acid, transferrin, fetuin, asialo-fetuin, porcine stomach thyroglobulin and bovine submaxillary mucin were purchased from Sigma Chemical Co. Yeast mannan was from Nakarai Chemical Co. All other chemicals used in this study were of the highest purity available.

Preparations of algal extracts and ammonium sulfate-precipitates

Two hundred gram of each fresh alga were cut into small pieces, homogenized for 1 min in a blender with 2 volumes of 0.02M phosphate buffer, pH 7.0 containing 0.85% NaCl (PBS), and kept at 4^{o}C for 12 h with occasionally stirring. After filtration through a cheese cloth, the filtrate was centrifuged at 3000 rpm for 5 min. To the supernatant (extract), solid ammonium sulfate was slowly added to attain a 70% saturation. The mixture was gently stirred and then kept at 4^{o}C for 18 h. The precipitates were recovered by centrifugation at 6000 rpm for 20 min, dissolved in a small volume of PBS, and thoroughly dialyzed against the same buffer. The non-dialyzable fraction was recovered as ammonium sulfate-precipitates (Le Dinh Hung *et al*., 2009).

Preparation of a 2% suspension of native or enzyme-treated erythrocytes

Each blood sample was washed three to five times with 50 volumes of 0.85 % saline. After washing, a 2% erythrocyte suspension (v/v) was prepared in saline and used as native erythrocytes. Trypsin or papain-treated erythrocytes were prepared as follows. One tenth volume of 0.5% (w/v) trypsin or papain solution

was added to a 2% native erythrocyte suspension, and the mixture was incubated at 37ºC for 60 min. After incubation, theerythrocytes were washed three to five times with saline and a 2% suspension (v/v) of trypsin or papain-treated erythrocyte was prepared in saline (Le Dinh Hung *et al*., 2009)

Hemagglutination assay

Hemagglutination assay was carried out by a microtiter method using a 96-well microtiter V-plate (Le Dinh Hung *et al*., 2009). First, 25 µL each of the serially two-fold dilutions of a test solution were prepared in saline on a microtiter V-plate. To each well, 25 µL of a 2% erythrocyte suspension were added and the mixtures were gently shaken and incubated at room temperature for 2 hrs. Hemagglutination was observed macroscopically and judged as positive in the case that more than 50 % of erythrocytes in wells are agglutinated. Hemagglutination activity was expressed as a titer, the reciprocal of the highest dilution showing positive hemagglutination.

Hemagglutination-inhibition test

Hemagglutination-inhibition test was carried out using ammonium sulfate-precipitates according to the method previously described by (Le Dinh Hung *et al*., 2009). Briefly, first, 25 µL each of the serially two-fold dilutions of sugar or glycoprotein were prepared in saline. To each well, an equal volume of extract solution with a hemagglutination titer of four was added, and the plate was mixed gently and allowed to stand at room temperature for 1 h. Finally, 25 µL of a 2 % suspension of trypsin-treated rabbit erythrocytes was added to each well, and the plate gently shaken and incubated for a further 1 h. Inhibition was observed macroscopically and inhibition activity was expressed as the lowest concentration of sugar or glycoprotein at which complete inhibition of hemagglutination was achieved. The assay was performed in duplicate per a sugar compound.

Effects of divalent cations, pH, and temperature on hemagglutination activity

Briefly, a 1 mL aliquot of a hemagglutinin solution was dialyzed at 4ºC overnight against 100 mL of 50 mM EDTA in PBS. The non-dialyzable fraction was recovered and determined in the presence or absence of divalent cations (10 mM $CaCl_2$ or $MgCl_2$) for hemagglutination activity. The effect of temperature, a 1 mL aliquot of a hemagglutinin solution was heated at various temperatures (30~100°C) for 30 min, then immediately cooled on ice, and determined for hemagglutination activity. The effect of pH, a 1 mL aliquot of a hemagglutinin solution was dialyzed at 4ºC overnight against 0.05M buffers of various pH (3 ~ 10) and then dialyzed against saline to eliminate the pH effect. The non-dialyzable fractions were determined for hemagglutination activity (Le Dinh Hung *et al*., 2009).

RESULTS AND DISCUSSION

Screening of hemagglutinins from green algae

The results of the screening of hemagglutinins from green algae are summarized in Table 1. Rabbit erythrocytes were the most suitable to detect the hemagglutination activity followed by sheep, chicken and human A, B, O erythrocytes (Figure 1).

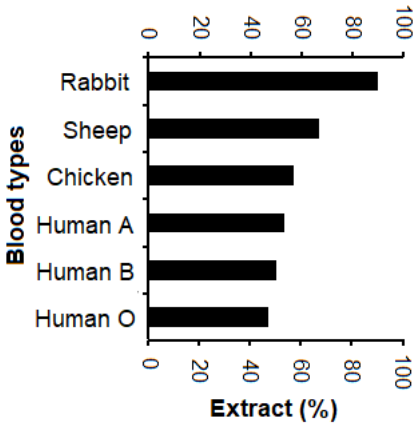

Figure 1. The algal extract amount agglutinated the different types of erythrocytes expressed as % for Rabbit (90 %), Sheep (66.7 %), Chicken (56.7 %), Human A (53.3 %), Human B (50 %) and Human O (46.7 %).

Table 1. Hemagglutination activity of extracts from green algae, Chlorophyta.

Chlorophyta Species	Hemagglutination titer of algal extracts[a]																	
	Rabbit			Sheep			Chicken			Human A			Human B			Human O		
	N[b]	T[c]	P[d]	N	T	P	N	T	P	N	T	P	N	T	P	N	T	P
Cladophorales																		
Anadyomenaceae																		
Anadyomene plicata	32	4096	4096	16	512	1024	8	1024	2048	8	64	128	16	64	128	8	32	64
Cladophoraceae																		
Chaetomorpha linum	-[e]	-	2	-	4	4	-	8	8	-	-	-	-	-	-	-	-	-
Chaetomorpha crassa	-	32	128	4	8	4	8	8	16	8	4	8	8	6	8	4	8	16
Caulerpales																		
Caulerpaceae																		
Caulerpa cupressoides	-	16	256	2	4	4	-	2	2	8	16	16	-	4	8	8	16	8
C.racemosa	-	256	256	-	64	128	-	16	16	-	2	2	-	-	4	-	-	-
C.racemosa var. occidentalis	-	32	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. racemosa var. clavifera	-	-	1024	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. sertularioides	-	64	128	-	16	32	-	16	16	-	4	8	-	4	8	-	4	8
C. sertularioides f. longipes																		
C. serrulata	-	128	256	-	32	64	-	32	32	-	-	4	-	4	8	-	4	8
C. serulata var. boryana	-	16	1024	2	8	4	-	4	-	8	16	16	-	-	-	4	4	8
Udoteaceae																		
Avrainvillea erecta	16	2048	2048	8	1024	1024	-	512	1024	-	-	-	-	-	-	-	-	-
A. obscura	-	32	128	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Halimeda opuntia	-	2	8	-	8	8	-	8	8	-	-	4	-	4	8	-	2	8
H. opuntia f. triloba	-	2	2	-	-	2	-	2	2	-	-	-	-	-	-	-	-	-
H. velasquezii	64	2048	2048	16	64	32	8	64	128	16	64	32	16	32	32	16	32	32
H. discoidea	64	2048	2048	16	64	32	8	64	128	16	64	32	16	32	32	16	32	32
Codiales																		
Codiaceae																		
Codium geppiorum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
C. arabicum	-	32	32	-	4	8	-	-	-	-	4	4	-	4	4	-	-	-
Siphonocladales																		
Boodleaceae																		
Boodlea struveoides	64	128	256	-	1024	1024	-	64	128	-	-	32	-	-	16	-	-	64
B. coacta	-	4	64	-	2	-	-	-	-	-	-	-	-	-	-	-	-	-
B. composita	-	16	128	2	8	2	-	-	-	-	-	-	-	-	-	-	-	-
Valoniopsis pachynema	-	64	512	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Siphonocladaceae																		
Boergesenia forbesii	-	8	32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Valoniaceae																		
Dictyosphaeria cavernosa	-	32	128	-	-	-	-	2	2	-	-	-	-	-	-	-	-	-
D. versluysii	-	32	64	4	4	8	4	2	4	-	-	8	-	-	16	4	4	32
Valonia fastigiata	-	16	256	8	8	16	8	4	4	-	8	64	4	16	64	16	16	32
Ulvales																		
Ulvaceae																		

Enteromorpha kylinii	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
E. intestinalis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ulva papenfussii	-	-	8	-	-	-	-	-	-	-	8	16	-	8	2	-	8	64	
U. reticulata	-	8	16	-	2	8	-	-	-	-	16	64	-	16	16	-	16	512	

[a]Hemagglutination activity was expressed as a titer, the reciprocal of the highest dilution showing positive hemagglutination.
[b]Native erythrocytes; [c]Trypsin-treated erythrocytes; [d]Papain-treated erythrocytes. [e]Negative agglutination.

Of the 30 Chlorophyta species surveyed, extracts from 27 species agglutinated at least one type of erythrocytes tested (Table 1). Extracts from *Anadyomene plicata* and *Avrainvillea erecta* showed strong activities with trypsin- or papain-treated rabbit, sheep, and chicken erythrocytes. The extract from *Boodlea struveoides* also strongly agglutinated trypsin- or papain-treated sheep erythrocytes. Extracts from eight species of the genus Caulerpa showed relatively strong activities with enzyme-treated rabbit erythrocytes, but weak or no hemagglutination with the other types of erythrocytes irrespective of treatment with enzymes. The extract from *Valoniopsis pachynema* did not agglutinate any type of human, sheep, horse, and chicken erythrocytes, although they agglutinated enzyme-treated rabbit erythrocytes. The extracts from *Halimeda velasquezii* and *H. discoidea* showed the strongest activity toward rabbit erythrocytes following with enzyme-treated chicken, sheep and human A, B and O erythrocytes. The extracts from *Dictyosphaeria cavernosa*, *D. versluysii* and *Valonia fastigiata* were more active with animal erythrocytes than with human erythrocytes. On the other hand, the extracts from *Ulva reticulata* and *U. papenfussii* were more active with papain-treated human A, B, and O erythrocytes than animal erythrocytes. The extracts from *Codium geppirosum*, *Enteromorpha kylinii* and *E. intestinalis* were inactive with any type of erythrocytes used. Thus, the hemagglutination titers of the green algal extracts were higher with animal erythrocytes than with human erythrocytes.

A total of 90 % of algal species surveyed were active. Of the algal species surveyed, 13 Chlorophyta were newly found to contain hemagglutinins. In this survey, hemagglutinins from green algae preferentially agglutinated animal erythrocytes than human ones. This study is consistent with other reports on the occurrence of marine algal hemagglutinins, using animal erythrocytes were more suitable for lectin detection in marine algae than human cells (Le Dinh Hung *et al*., 2009, 2012; Singh *et al*., 2015; Singh, Walia, 2018).

Carbohydrate-binding specificities

For the 12 active species, sugar-binding specificity of eachhemagglutinin was examined using ammonium sulfateprecipitates prepared from each extractwith a variety of monosaccharides and glycoproteins (Table 2). The hemagglutination activities of these algal species were not inhibited by any of the monosaccharides examined, except that hemagglutinin of *C. arabicum* was inhibited by amino sugars. On the other hand, hemagglutination activities of hemagglutinins were inhibited by glycoproteins bearing high mannose N-glycan, complex N-glycan, or O-glycan. The hemagglutination-inhibition profiles with a variety of glycoproteins differed depending on algal species. With respect to the hemagglutinins of *A. plicata*, *B. struveoides* and *B. composite*, yeast mannan bearing high mannose N-glycans was mostinhibitory, suggesting that these algal species each contain, at least, a lectin specific for high-mannose N-glycans. The activities of the *A. erecta*, *A. obscura*, *H. velasquezii*, *H. discoidea* and *D. versluysii* hemagglutinins were strongly inhibited by asialo-transferrin bearing biantennary complexN-glycan,

suggesting that it can discriminate differences inthe branched structures of complex type-N-glycans. The activities of hemagglutinins from *H. opuntia, H. opuntia f. triloba* and *H. discoidea* were strongly inhibited by asialo-fetuin bearing both complex N-glycans and O-glycans, suggesting that these algal species contain, at least, a lectin specific for O-glycans. Similarly, it is suggested from the inhibition profiles that *C. arabicum* and *C. sertularioides*, each contain a lectin specific for O-glycans. Hemagglutinin of *C. arabicum* appears to recognizethe non-reducing terminal N-acetyl-D-galactosaminyl residue(s) of O-glycans, because its activity was stronglyinhibited by bovine submaxillary mucin and its asialo-derivative as well as by N-acetyl-D-galactosamine. The hemagglutinins of *D. cavernosa* and *D. versluysii* were strongly inhibited by asialo-fetuin bearing both complex N-glycansand O-glycans as well as by porcine thyroglobulin bearing both complex type and high mannose type N-glycans, suggesting that these two algal species contain, at least, a lectin specific for complex type N-glycans.

Of the 12 green algal hemagglutinins examined, only *C. arabicum* hemagglutinin was inhibited by amino sugars. Activities of almost all the hemagglutinins were inhibited by glycoproteins bearing high mannose N-glycans, complex N-glycans, or O-glycans. Among the inhibitory glycoproteins tested, asialo-transferrin, asialo-fetuin were the best inhibitors against almost all hemagglutinins, indicating that elimination of sialic acid residues from parental glycoproteins enhanced greatly inhibitory potential of parental glycoproteins. The lack of specificity for simple sugars from these algal preparations in this survey is in agreement with several works already reported that many algal lectins have affinity for glycoproteins, but not for monosaccharides and appears to be a common feature of many algal lectins (Singh *et al*., 2015; Singh, Walia, 2018).

Table 2. Hemagglutination-inhibition test of the green algal hemagglutinins with sugars and glycoproteins.

Chlorophyta samples	Sugars and glycoproteins									
	GlcNAc	GalNAc	T	A-T	F	A-F	YM	PTG	BSM	A-BSM
Anadyomene plicata	-[a]	-	500.0	125.0	125.0	62.5	31.2	500	250.0	250.0
Avrainvillea erecta	-	-	500.0	7.8	250.0	31.2	-	62.5	62.5	125.0
Boodlea struveoides	-	-	-	500.0	500.0	62.5	15.6	125.0	62.5	62.5
B. composita	-	-	-	250.0	500.0	15.6	15.6	250.0	250.0	250.0
Caulerpa sertularioides	-	-	125.0	15.6	62.5	31.2	125.0	250.0	31.2	31.2
Codium arabicum	100.0	6.2	31.2	31.2	1.9	1.0	31.2	500.0	0.5	0.5
Dictyospheria cavernosa	-	-	250.0	15.6	500.0	7.8	-	31.2	250.0	250.0
D. versluysii	-	-	125.0	1.9	250.0	7.8	-	31.2	250.0	125.0
Halimeda opuntia	-	-	500.0	31.2	31.2	7.8	31.2	62.5	15.6	15.6
H. opuntia f. triloba	-	-	-	125.0	125.0	7,8	62.5	500.0	125.0	125.0
H. discoidea	-	-	250.0	3.9	1000.0	15.6	62.5	15.6	500.0	500.0
Valonia fastigiata		-	-	62.5	500.0	250.0	-	250.0	500.0	500.0

T: transferrin, A-T: asialo-transferrin, F: fetuin, A-F: asialo-fetuin, YM: yeast mannan, PTG: porcine thyroglobulin, BSM Bovinestomach mucin, A-BSM asialo-bovine stomach mucin.
[a] Indicates no inhibition at the concentration of 100 mM for monosaccharide and 2,000 µg mL-1 for glycoprotein.

Effects of divalent cations, pH, and temperature on hemagglutination activity

The effects of divalent cations, pH, and temperature on hemagglutination activity of the heamaglutinins of the 12 active species listed in Table 2 were examined. Hemagglutination activities of these agglutinins were unchanged

after dialyses against 50 mM EDTA and in the presence of 10 mM $CaCl_2$ or $MgCl_2$ after dialyses, indicating that these algal agglutinins do notrequire the presence of divalent cations for hemagglutination.

The hemagglutination activities of *A. plicata, A. erecta*, *D. versluysii* and *H. discoidea* hemagglutinins were unchanged even when heated at 100°C for 30 min (Figure 2a and 2b). The hemagglutination activities of *D. cavernosa* hemagglutinin were also thermostable because their activities were unchanged by heating at 90 °C for 30 min. On the other hand, activities of *B. struveoides, C. arabicum, C. sertularioides, H. opuntia* and *H. opuntia f. triloba* hemagglutiniins were also thermostable because their activities were unchanged by heating at 70-80°C for 30 min. The other algal hemagglutinins were moderately stable over a relatively wide range of temperature.

Most of the algal hemagglutinins examined maintained their activities over a wide range of pH values between 5 and 9 with a slight decrease in activity in more acidic and alkaline media (Figure 3a and 3b). The activities of *A. erecta* and *A. plicata* were unchanged at a pH range between 3 and 10. On the other hand, the activities of *B. composita*, *D. versluysii* and *V. fastigiata* hemagglutinins were unchanged at a pH range between 3 and 9.

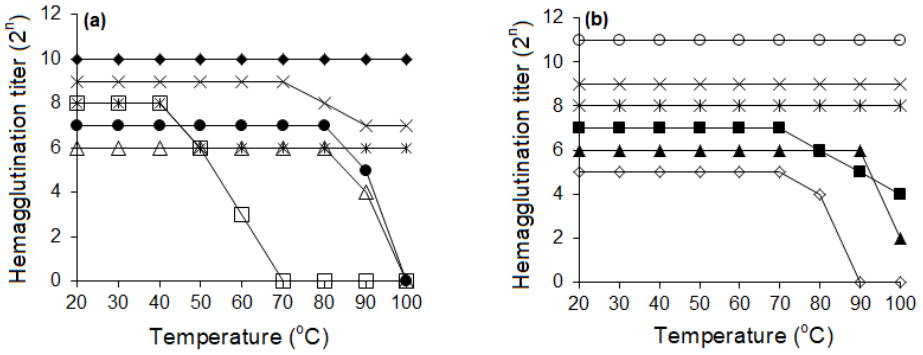

Figure 2. The effects of temperature on hemagglutinating activities of hemagglutinins from the green algae (Chlorophyta). **(a)** *Anadyomene plicata* (-◆-); *Codium arabicum*(-⊞-); *Halimeda opuntia* (-△-); *Caulerpa sertularioides* (-✳-); *Boodlea struveoides* (-✳-);*Valonia fastigiata*(-●-). (**b**) *Avrainvillea erecta* (-○-);*Boodlea composite* (-■-); *Dictyospheria cavernosa* (-▲-); *Dictyospheria versluysii* (-✳-); *Halimeda opuntia f. triloba* (-◇-); *Halimeda discoidea* (-✳-).

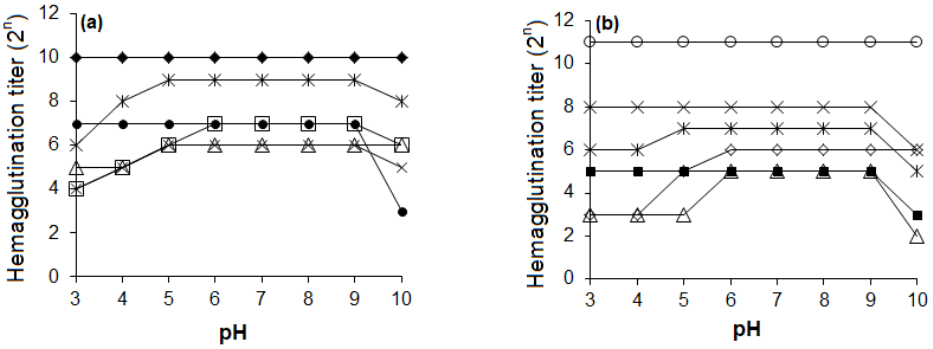

Figure 3. The effects of pH on hemagglutinatin activities of hemagglutinins from the green algae (Chlorophyta). **(a)** *Anadyomene plicata*(-◆-); *Codium arabicum*(-⊞-); *Halimeda opuntia* (-△-); *Caulerpa sertularioides* (-✳-); *Boodlea struveoides*(-✳-); *Valonia fastigiata* (-●-). (**b**) *Avrainvillea erecta* (-○-); *Boodlea composite* (-■-); *Dictyospheria cavernosa* (-▲-); *Dictyospheria versluysii* (-✳-); *Halimeda opuntia f. triloba* (-◇-);*Halimeda discoidea* (-✳-).

CONCLUSION

Thirty Chlorophyta species were surveyed for hemagglutination activity with rabbit, sheep, chicken and human A, B, O erythrocytes, among them the extracts from 27 species agglutinated at least one type of erythrocytes tested. The hemagglutination activities of hemagglutinins were stable over a wide range of pH, temperature and showed independence for divalent cations. The hemagglutination-inhibition profiles of hemegglutinins are diverse, depending on the algal species extracted, suggesting that the green algal species contain lectins specific for high mannose type N-glycan, complex type N-glycan, or O-glycan. Therefore, Vietnamese green algae may be promising as a valuable source of novel lectins for applications. The wealth of information obtained in the present study would be helpful for identification of new lectins from marine algae.

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