Analysis of the agglutinating activity from unicellular algae

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Abstract Agglutinating activity often varies both between and within the algal species assayed. However, it is difficult to interpret such variation without further analysis. We report a statistical analysis of agglutinating activities against human, cow, sheep, and pig erythrocytes, using cell extracts from 43 taxa (strains) of freshwater microalgae. Most of the extracts agglutinated erythrocytes from at least one of the sources, but pig erythrocytes appeared to be most suitable for the detection of agglutination reactions. *Chlorella* cell extracts preferentially agglutinated human erythrocytes, whereas extracts of other taxa were less active against mammalian erythrocytes. Cluster analysis generated four distinct subclusters of taxa, characterized by different specificities for antigens or carbohydrate receptors on the erythrocytes. Principal component analysis further separated the agglutination characteristics of *Chlamydomonas* from *Chlorella* on the first two components. Specificity for pig erythrocytes accounted for most of the clustering or grouping of algal taxa in multivariate analysis. However, clustering or grouping patterns of *Chlorella* species on haemagglutinating activity resembled that based on DNA sequences, revealing a possible genetic connection of agglutinins and their biochemical characteristics in algal cells. Variability of agglutination reactions among the algae investigated is simplified and interpreted most easily using multivariate analysis.

Key words agglutinating activity · microalgae · cluster analysis · principal component analysis

Introduction

Agglutinins are widely distributed in nature. They bind to specific carbohydrates and produce unique effects such as the aggregation of cells or the precipitation of glycoconjugates. Agglutinins can thus serve as probes of carbohydrates and are useful tools in cell biology, immunology, in the study and treatment of cancers, and in a range of biochemical and biomedical research areas.

Since the first report by Boyd et al. (1966), many researchers have investigated the agglutinating capacities of marine algal extracts (Blunden et al. 1978; Rogers et al. 1980; Hori et al. 1981; Fabregas et al. 1985; Freitas et al. 1997). For the microalgae, agglutinating activity has been reported for *Lyngbya majuscula* (Boyd et al. 1966) and *Chlamydomonas* spp. (Brown et al. 1968; Saito and Matsuda 1984). However, our knowledge of microalgal agglutinins and their agglutination characteristics is limited. Agglutinins from *Anabaena flosaquae* and *Microcystis aeruginosa* agglutinated mouse erythrocytes (Carmichael and Bent 1981), and Raphidophyceae, Bacillariophyceae species, and culture media conditioned by *Alexandrium cohorticula* and *Chattonella antiqua* caused agglutination (Hori et al. 1996). Some special characteristics of microalgal agglutinins have been reported, for example, that aerobic growth conditions could inhibit agglutinin production in *Microcystis viridis* (Yamaguchi et al. 1999), and that *M. aeruginosa* exhibited calcium-dependent agglutinating activity (Sakamoto et al. 1996). *Microcystis aeruginosa* and *Oscillatoria agardhii* agglutinins have unique properties

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that are not commonly found in higher plants (Yamaguchi et al. 1998; Sato et al. 2000).

In assaying for agglutination, sheep and rabbit erythrocytes are more sensitive than human erythrocytes in the detection of agglutinins from seaweeds (Hori et al. 1981; Fabregas et al. 1985; Freitas et al. 1997). However, monkey and human erythrocytes are more susceptible than sheep erythrocytes to the marine unicellular algal extracts in our previous studies (Liao and Huang 2000; Liao et al. 2001). Thus, microalgal agglutinins have diverse specificity for carbohydrate or antigens, as shown by seaweeds.

Freshwater microalgae have the advantage that they can be cultivated on a large scale at extremely high growth rates. These organisms thus form potentially important sources of novel agglutinins for biomedical research and commercial applications. Agglutinins have been identified in various types of cells and tissues of all taxonomies to higher vertebrates (Blunden et al. 1978; Carmichael and Bent 1981; Fabregas et al. 1985). Some reports show that agglutinin is more common in marine algae (Rogers et al. 1980), and agglutination assays with numerous marine algae indicated that agglutinins and their activities had taxonomic significance (Rogers and Fish 1991). Thus, genetic connection of haemagglutinating activity used on taxonomic significances is an interesting subject.

Because agglutinating activity varies greatly between and within the species assayed (Rogers and Fish 1991), it is often difficult to interpret such variations when large numbers of taxa are screened. In order to understand the relationship between the unicellular algal agglutinating activity and taxonomic significance, we analyzed the variability of agglutinating activity in freshwater microalgae using multivariate analysis. Additionally, the analysis results were used for comparison with an analysis based on DNA sequences.

Materials and methods

Forty-three strains of freshwater microalgae, mostly belonging to *Chlorella* and *Chlamydomonas*, were used in this study (Table 1). Most were obtained from Culture Collections such as ATCC (American Type Culture Collection), CCAP (Culture Collection of Algae and Protozoa, CCMP (The Provasoli-Guillard Center for Culture of Marine Phytoplankton) and IAM (IAM Culture Collection, Japan). *Chlorella* sp. I, *Chlorella* sp. 21, *Chlorella* sp. W, and *C. pyrenoidosa* were collected from rivers of Taiwan and isolated in our laboratory, while *Selenastrum capricornutum*, *Monoraphidium contorfum*, *Coslastrum astroideum*, *Eutetramorus fottii*, *Scenedesmus quadricauda*, and *S. ecornis* were kindly provided by Professor J.-T. Wu, Institute of Botany, Academia Sinica,

Taiwan, R.O.C. The algae were grown and maintained in proteose medium (Illman et al. 2000) at 25°C at an irradiance of 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 12:12 h light:dark cycle. Algal cells were harvested at the stationary growth phase and concentrated by centrifugation. The harvested cells were lyophilized and kept at -20°C until used for extraction.

Preparation of cell extracts and erythrocytes

The harvested cells were freeze-dried and ground into powder with liquid nitrogen. Cell powder was extracted at a 1:10 ratio (w:v) in 20% ethanol at 4°C overnight. Extracts were centrifuged and the supernatants collected for agglutination assays. Human, cow, sheep, and pig erythrocytes were used for the agglutination assay. Fresh human type A, B and O blood was provided by Union Clinical Laboratory, Taipei, Taiwan, and cow, sheep, and pig erythrocytes were from the Agricultural Experimental Farm, Department of Animal Science, National Taiwan University. Erythrocytes were concentrated and washed three times in phosphate buffer saline (PBS) by centrifugation, then diluted to a final concentration of 2% (v/v) in PBS.

Haemagglutination assay

Agglutinating activity was assayed according to Hori et al. (1981). An aliquot of algal extract (50 μL) was serially diluted two-fold with PBS in the wells of microtitre plates. An equal volume of erythrocyte suspension was then added to the well and mixed with the algal extract at room temperature for 2 h. The control used in this assay was aqueous ethanol without extract. Activity was expressed as a titre based on the reciprocal of the highest two-fold dilution that produced positive agglutination.

Data analysis

We used various statistical and multivariate techniques to interpret the variability in haemagglutination activity. Firstly, simple correlation coefficients were calculated, based on the haemagglutination reactions of each of the 43 cell extracts, paired with each erythrocyte type. This was followed by multivariate analysis. To reduce the bias caused by any absence of haemagglutination in the analyses, titres were $\log(x+1)$ transformed. Cluster analysis was carried out with complete linkage based on the similarity matrix of algal haemagglutination activities, using Primer 5 software (Primer-E, Plymouth, UK). Multivariate principal component analysis also using Primer 5 was used to interpret agglutination variability. In this analysis, all relationships among variables (sources of erythrocytes) were accounted for by the relatively independent

Table 1 Hemagglutinating activity of cell extracts from the study microalgae for different types of native erythrocytes; activity is expressed in titer. The numbers in parentheses are the serial numbers used in the Figs. 1 and 2

Algal species	Erythrocyte					
	Human			Cow	Sheep	Pig
	A	B	O			
Cyanophyceae						
(1) <i>Spirulina maxima</i> ATCC 53871	2 ³	2 ³	2 ³	0	2 ³	2 ³
(2) <i>S. platensis</i> ATCC 53844	2 ³	2 ³	2 ³	2 ³	2 ²	2 ³
(3) <i>S. subsalsa</i> IAM 223	0	0	0	0	2 ³	0
(4) <i>Synechococcus leopoliensis</i> IAM M6	0	0	0	0	2 ³	2 ⁵
Chlorophyceae						
(5) <i>Chlorella</i> sp.I	2 ⁶	2 ⁸	2 ⁶	0	0	0
(6) <i>Chlorella</i> sp. 21	2 ⁵	2 ⁶	2 ⁸	2 ⁷	2 ⁸	2 ⁷
(7) <i>Chlorella</i> sp.W	2 ³	2 ²	2 ⁴	0	0	2 ³
(8) <i>C. ellipsoidea</i> IAM87	2 ¹⁰	2 ¹¹	2 ¹⁰	2 ⁴	2 ⁴	2 ⁵
(9) <i>C. fusca</i> var. <i>fusca</i> IAM 101	2 ⁵	2 ⁶	2 ⁵	0	0	0
(10) <i>C. fusca</i> var. <i>vacuolata</i> IAM 28	2 ⁴	2 ⁶	2 ⁵	0	0	0
(11) <i>C. kessleri</i> IAM 208	2 ⁴	2 ⁵	2 ⁴	0	0	0
(12) <i>C. luteovirtidis</i> CCAP 213/9	2 ⁶	2 ⁸	2 ⁵	2 ⁴	2 ²	2 ⁷
(13) <i>C. protothecoides</i> IAM 150	2 ⁹	2 ¹¹	2 ⁹	0	0	0
(14) <i>C. protothecoides</i> var. <i>mannophila</i> IAM 206	2 ⁵	2 ⁶	2 ⁵	0	0	2 ⁶
(15) <i>C. pyrenoidosa</i>	2 ⁹	2 ¹¹	2 ¹⁰	2 ⁵	2 ⁴	2 ⁶
(16) <i>C. saccharophila</i> IAM 212	2 ⁵	2 ⁴	2 ³	0	0	2 ⁵
(17) <i>C. saccharophila</i> var. <i>ellipsidea</i> ATCC 30404	2 ³	2 ⁴	2 ⁶	0	0	0
(18) <i>C. sorokiniana</i> IAM 212	2 ⁷	2 ⁴	2 ³	0	2 ⁹	2 ⁶
(19) <i>C. vulgaris</i> CCAP 211/13	2 ⁷	2 ⁷	2 ⁷	2 ⁸	2 ⁶	2 ⁸
(20) <i>C. vulgaris</i> NIES 227	2 ⁵	2 ³	2 ³	2 ⁴	2 ⁴	2 ⁵
(21) <i>C. xanthella</i> ATCC 30411	2 ⁵	2 ⁷	2 ⁵	0	0	2 ⁸
(22) <i>C. zofingiensis</i> IAM 111	2 ⁸	2 ⁹	2 ⁸	0	2 ³	2 ⁹
(23) <i>Chlamydomonas applanata</i> IAM 237	0	0	0	0	0	0
(24) <i>C. asymmetrica</i> IAM 234	2 ⁵	2 ⁴	2 ⁵	0	2 ²	2 ⁵
(25) <i>C. debaryana</i> IAM 215	0	0	0	0	0	0
(26) <i>C. fimbriata</i> IAM 230	0	0	2 ²	0	0	0
(27) <i>C. leiostraca</i> IAM 284	0	0	0	0	0	0
(28) <i>C. noctigama</i> IAM 557	0	0	0	2 ²	2 ⁴	2 ⁵
(29) <i>C. oblonga</i> IAM 265	0	0	0	0	0	0
(30) <i>C. proteus</i> IAM 560	0	0	0	0	0	0
(31) <i>C. pulvinata</i> IAM 267	2 ³	2 ³	2 ³	0	2 ²	2 ⁵
(32) <i>C. reinhardtii</i> IAM 238	0	0	0	0	0	0
(33) <i>C. segnis</i> IAM 254	0	0	0	0	0	0
(34) <i>C. simplex</i> IAM 281	0	0	0	0	0	0
(35) <i>C. subangulosa</i> IAM 563	0	0	0	0	0	0
(36) <i>C. ulvaensis</i> IAM 566	2 ³	2 ³	2 ³	2 ³	2 ⁴	2 ⁹
(37) <i>C. zebra</i> IAM 221	0	0	0	0	0	0
(38) <i>Coelastrum astroideum</i>	0	2 ⁵	0	0	0	2 ³
(39) <i>Eutetramorus fottii</i>	0	2 ²	0	0	0	0
(40) <i>Monoraphidium contorfum</i>	0	0	0	0	0	0
(41) <i>Scenedesmus quadricauda</i>	2 ⁶	2 ⁷	2 ⁷	2 ⁴	2 ⁵	2 ⁸
(42) <i>S. ecorni</i>	0	0	0	0	0	2 ²
(43) <i>Selenastrum capricornutum</i>	2 ⁶	2 ⁸	2 ⁷	2 ²	2 ³	2 ⁴

and interpretable, but non-observable, components. Eigenvectors forming the principal components were obtained from the similarity coefficients in linear combinations of variables.

Comparison of the phylogenetic trees

The phylogenetic relationship between species of *Chlorella* species based on 18 SSU DNA sequence (Wu et al. 2001)

was modified for comparison with that on haemagglutinating activity of this study. A slight modification was made by deleting the species which were not used in this study. The remaining species were maintained in their original positions on the phylogenetic tree. The encoded SSU DNA sequence data in *Chlorella* species 21, *Chlorella* sp. I and *Chlorella* sp. V were obtained from PCR amplifications using genomic DNA in the laboratory and the identified species GenBank/EMBL databases. In addition, the NS12 region of SSU sequence from the GenBank/EMBL data base was employed. The phylogenetic tree was produced by parsimony method using of a heuristic search in PAUP 4.0 from cladistically informative characters. Values above branches are confidence levels estimated by 100 bootstrap replicates.

Results

Agglutination of human erythrocytes by cell extracts

Thirty-two of the 43 strains tested exhibited agglutinating activity against at least one of the erythrocyte types assayed (Table 1). All *Chlorella* cell extracts agglutinated human A, B and O erythrocytes. Of these, *C. ellipsoidea*, *C. protothecoides*, and *C. pyrenoidosa* extracts exhibited the strongest activity, reaching titres of 2^9 – 2^{11} . Haemagglutination by *Chlamydomonas* was limited to 4 of 15 taxa, with an activity titre of less than 2^5 . *Scenedesmus quadricauda* and *Selenastrum capricornutum* extracts preferentially agglutinated human erythrocytes, while *S. ecornis* and *Monoraphidium contorfum* extracts failed to react with any of the erythrocyte species used. The prokaryotes *Spirulina maximum* and *Sp. platensis* also contained agglutinins that strongly agglutinated human ABO erythrocytes, while the other two species *Sp. subsalsa* and *Synechococcus leopoliensis* did not agglutinate any. However, all cell extracts giving positive reactions with human erythrocytes were non-specific, with the exception of *Coelastrum astroideum*, which showed anti-(B+H) activity for erythrocytes from type B blood.

Agglutination of ungulate erythrocytes by cell extracts

Fewer cell extracts were reactive against ungulate than against human erythrocytes. Twenty-four taxa exhibited agglutinating activity against cow, sheep, and pig erythrocytes; 12 of these belonged to *Chlorella* and 4 to *Chlamydomonas* species. In general, agglutinating activity was lower than for human erythrocytes. More taxa contained agglutinins that reacted with pig erythrocytes than with sheep or cow erythrocytes. The strains *Chlorella* sp. 21 and *C. vulgaris* CCAP 211/13 exhibited the strongest

activity, with titres of 2^6 – 2^8 , against cow, sheep, and pig erythrocytes. *C. sorokiniana* exhibited stronger activity against sheep erythrocytes, and *Chlorella xanthella*, *C. protothecoides*, and *C. sorokiniana* exhibited stronger activity against pig erythrocytes.

Statistical and multivariate analysis

The correlations (r values) for agglutination reactions with human type A, B and O erythrocytes were higher than for cow, sheep, and pig erythrocytes (Table 1). However, all coefficients were significant ($p < 0.05$), indicating that the agglutination activity of cell extracts against the six erythrocyte types used varied together, but independently. The agglutinating characteristics of cell extracts against human erythrocytes differed from those against cow and sheep, but not pig erythrocytes (Tables 2 and 3). However, there were no significant differences in agglutination reactions between sheep and cow erythrocytes.

The result of the cluster analysis for 39 green algae is shown in Fig. 1, with two large clusters represented mainly by *Chlamydomonas* (C1) and *Chlorella* taxa (C2). Each was further divided into two subclusters with unequal numbers of taxa and distances. Subcluster 1 (Sc1) contained taxa whose extracts did not cause agglutination or that agglutinated one of ungulate or human erythrocyte species tested. Subcluster 2 (Sc2) contained taxa having agglutination activity towards two or more ungulate erythrocytes and all human erythrocytes at low titres if any. Subcluster 3 (Sc3) contained taxa with specific activity for all human erythrocytes at high titres and one or two ungulate erythrocytes. Subcluster 4 (Sc4) contained taxa that agglutinated all human erythrocytes tested and pig erythrocytes. Taxa in subclusters 3 and 4 contained agglutinins that preferentially agglutinated human erythrocytes, whereas those in subclusters 1 and 2 preferentially agglutinated ungulate erythrocytes. In general, the cluster-

Table 2 Correlation coefficients (r) of the paired agglutination reactions of cell extracts with different erythrocytes

Erythrocyte		r^*				
Human	B					
	O	0.930				
	A	0.932	0.941			
Pig		0.502	0.516	0.579		
Cow		0.442	0.522	0.478	0.592	
Sheep		0.324	0.415	0.478	0.690	0.693

* Significance level at least $p < 0.05$.

Analysis was performed based on $\log(x+1)$, where x = activity in titer, and $n=43$

Table 3 *t* values of the paired agglutination reaction of *Chlorella* cell extracts with different erythrocytes

Erythrocyte		Mean	<i>t</i>				
Human	B	1.106					
	O	0.987	1.143				
	A	0.956	1.298	0.619			
Pig		0.899	1.670	1.310	1.275		
Sheep		0.515	3.055**	2.896**	2.843**	2.585*	
Cow		0.331	3.705***	3.617***	3.585***	3.371**	2.313

* $p < 0.05$, $t_{0.05(2)76} = 1.992$ ** $p < 0.01$, $t_{0.01(2)76} = 2.642$ *** $p < 0.001$, $t_{0.001(2)76} = 3.423$ Analysis was performed based on $x = \log(X+1)$, where X = activity in titer, and $n = 43$

ing of taxa was more dependent on specificity for ungulate than for human erythrocytes. Distance between taxa in the dendrogram is inverse to similarity, thus the greater the disparity between two taxa or clusters, the greater their distance.

Principal component analysis (PCA) yielded two major components, PC1 and PC2, with eigenvalues explaining 87.7% of the total variability of reactions (Table 4). These were used to interpret the variability of haemagglutination assays. The positions of taxa in Fig. 2 reveal eigenvectors or weights on taxa on these two components. The PC1 axis expresses the activity component from taxa with higher positive eigenvectors that did not agglutinate any of the erythrocytes used. Thus, the higher value on this axis, the lower the activity found. The PC2 axis expresses the component of specificity for erythrocytes: taxa whose extracts strongly agglutinated human erythrocytes have higher positive values, and those

with activity against ungulate erythrocytes have negative values.

PCA analysis separated *Chlorella* and *Chlamydomonas* taxa into two regions designated R-Clo (5–22) and R-Cla (23–37). Most *Chlorella* taxa lie above the PC1 axis, but *Chlamydomonas* lies below this axis on the positive side of the PC2 axis. This reflects the different binding specificities for human and non-human erythrocytes. The remaining algae, *Coelastrum*, *Monoraphidium*, *Scenedesmus*, and *Selenastrum* (38–43), are closely distributed on or near the PC1 axis on the positive side, in association with most of the *Chlamydomonas* taxa. The blue-green algae *Spirulina* and *Synechococcus* (1–4) are also located inside the *Chlamydomonas* region and form a small group (not shown in the figure). The arrow represents a general trend in haemagglutination characteristics from *Chlorella* to *Chlamydomonas*, with decreasing activity against human erythrocytes, but with increasing activity against ungulate erythrocytes.

Fig. 1 Dendrogram showing the clusters of 39 taxa of green microalgae. Algal species numbers refer to Table 1

Table 4 Eigenvectors of the variables in the first five principal components

Variable		Principal component				
		PC1	PC2	PC3	PC4	PC5
Human	A	−0.451	0.321	0.079	0.239	0.006
	B	−0.425	0.434	−0.030	−0.129	−0.690
	O	−0.443	0.352	−0.162	0.030	0.706
Cow		−0.365	−0.451	−0.665	−0.436	−0.042
Sheep		−0.369	−0.521	0.030	0.727	−0.119
Pig		−0.387	−0.332	0.724	−0.455	0.096
Cum. % var.		68.8	87.7	94.3	98.2	99.3

Comparison on phylogenetic analysis

After a slight modification, the phylogenetic tree (by the parsimony analysis) based on 18SSU DNA sequence of the 11 species produced two main clusters. The first cluster was further divided into two subclusters, corresponding to subcluster SC3 and SC4 in our cluster analysis based on haemagglutinating activity (Fig. 3). Species in the two subclusters were *C. zofingiensis*, *C. pyrenoidosa*, *C. luteoviridis*, *C. saccharophila*, *C. ellipsodea* and *C. kessien*, *Chlorella* species V. The second cluster, represented by *C. sorokiniana*, *Chlorella* species 21, *Chlorella* species I and *C. vulgaris*, corresponds to SC2 in the cluster analysis. Therefore, phylogenetic grouping of *Chlorella* species based on agglutinin activity agrees to a considerable extent with that based on nuclear sequence.

Fig. 2 Thirty-nine taxa of green microalgae distributed along two principal components based on haemagglutination characters against six types of erythrocytes. The arrow indicates the change in haemagglutination characters with decreasing activity against human erythrocytes but with increasing activity against ungulate erythrocytes. Numbers indicate species numbers referred in Table 1

Discussion

Most of the microalgae studied here contained agglutinins capable of agglutinating at least one type of mammalian erythrocyte. All *Chlorella* extracts preferentially agglutinated human erythrocytes, whereas haemagglutination by *Chlamydomonas* extracts was limited to fewer species and to lesser degrees. Nevertheless, all the algae showing positive reaction towards human erythrocytes contained non-specific agglutinins for ABH antigens, with the exception of *Coelastrum astroideum*, which had anti-(B+H) specificity. Such activity has been reported previously for the marine algae *Ptilota plumosa* (Rogers et al. 1977) and *Bryopsis hypnoides* (Hori et al. 1981).

Sheep and rabbit erythrocytes have been commonly used for the detection of haemagglutinating activity from seaweeds (Hori et al. 1981; Fabregas et al. 1985; Freitas et al. 1997). However, human erythrocytes are superior to sheep erythrocytes for this purpose in evaluating extracts of marine microalgae (Chu et al. 2005; Liao and Huang 2000; Liao et al. 2001). Our assays confirmed this. In addition, pig erythrocytes appeared to be more sensitive than cow and sheep erythrocytes in the agglutination assay.

Haemagglutinating activity has some taxonomic significance (Rogers and Fish 1991), as it exhibits great variability between species, as demonstrated in seaweeds from various ecological habitats (Blunden et al. 1978; Fabregas et al. 1985; Freitas et al. 1997). However, the assumption was never given with statistical analysis of the variability. Thus, we are interested in what the variability is between species in relation to specificity for antigens or carbohydrate receptors on erythrocytes, and whether the statistical analysis of haemagglutinating activity would show some taxonomic pattern. Multivariate analysis tools, such as principal component analysis or cluster analysis, have been widely used to interpret and group the variability of morphometric measurements of various organisms as well as in genetics and immunology (Blackith and Reymont 1971). In the present study, the erythrocyte types assayed could be used as the determining variables in clustering or grouping of the algae, provided the within-assay variations were small in comparison with those between taxa or erythrocyte types. Statistical analysis showed that the agglutination reactions of algal extracts with all the erythrocytes tested were related. Furthermore, agglutination reactions with pig erythrocytes did not differ significantly from those with human erythrocytes, but did differ from cow and sheep erythrocytes ($p < 0.05$). This suggests that pig erythrocytes share more common antigens with human erythrocytes than those of other two ungulates, by approximately 7–10% (estimated from t values in the two-tailed distribution table; Zar 1974). Thus, the variability of agglutinating activity and

Fig. 3 Phylogenetic relationships inferred by *Chlorella* nuclear SSU NS12 nucleotide sequence data and modified from Wu et al. (2001). SC2, SC3, and SC4 are corresponding subclusters based on haemagglutinating activities

the subsequent clustering or grouping of taxa by multivariate analyses may have been due mainly to the presence of anti-pig agglutinins in algal extracts.

Cluster analysis revealed four subclusters of taxa with significant differences in agglutination patterns, which were not observable when considering individual species. Principal component analysis produced a two-dimensional interpretation. This proved satisfactory in distinguishing the haemagglutination characteristics of *Chlamydomonas* extracts from those of *Chlorella* extracts. Similarities and dissimilarities among and between taxa are evident from their relative positions on the two components in this analysis. For example, *C. fusca* and its variety *C. fusca* var. *vacuolata*, closely placed in Fig. 2, exhibited identical haemagglutination reactions. By contrast, the two strains *C. vulgaris*-CCAP and *C. vulgaris*-NIES appear in the same region as *Chlorella* although they were widely separated in two subclusters in the dendrogram (Fig. 1). Thus, principal component analysis can provide complementary information on agglutinin characteristics that is not shown by cluster analysis. It is not surprising that *Scenedesmus quadricauda* and *S. eiconis* are seen as separate, as they have extremely different haemagglutination characteristics. Whether this separation is of taxonomic relevance is not known. By contrast, the four prokaryotes in *Synechococcus* and *Spirulina* species lie within the *Chlamydomonas* region, suggesting that their binding specificities overlap with those of *Chlamydomonas* cells.

It appeared that the clustering or grouping of *Chlorella* species based on haemagglutinating activities was similar to results with grouping based on 18S rDNA sequences (Wu et al. 2001). Thus, we propose that agglutinating characteristics can be used in taxonomy (see also Blunden et al. 1978).

In this study, we used native or untreated mammalian erythrocytes. Treatment of erythrocytes to improve the agglutination assay could cause a bias in analysis and thus distort the true haemagglutination variability in the assayed algae. Proteases such as pronase, trypsin, and papain have been used to improve the detection of agglutinins in some marine algae (Rogers and Fish 1991; Freitas et al. 1997; Liao and Huang 2000; Liao et al. 2001). Additionally, the presence of haemolysin, which masks haemagglutination reactions in the extracts, can also distort analysis (Rogers et al. 1980; Hori et al. 1981). Nevertheless, the well-defined haemagglutination characteristics of *Chlorella* and *Chlamydomonas* extracts seen in this study suggest that agglutinins in microalgae have evolved through changes in binding specificity, and that these have genetic significance.

Recently, we have purified algal agglutinins, CPH (*Chlorella pyrenoidosa* hemagglutinin; 58.0 kDa) and CLH (*Chlorella luteoviridis* hemagglutinin; 58.3 kDa), from *C. pyrenoidosa* and *C. luteoviridis*, respectively, and found those algal lectins possess similar properties (Chu et al. 2006). We have also developed specific monoclonal antibodies for CPH and CPL. Immunogold labeling and electron microscopy revealed that both CPH and CPL are localized beneath the surface layer of the cell, between the cytoplasm and cell membrane. Furthermore, western blotting revealed that above antibodies recognized each microalga of sc3 clearly (unpublished data). However, microalgae in both sc2 and sc3 were not easily recognized by those antibodies. Those results also support our hypothesis that algal agglutinating activity could be an important taxonomic character.

The multivariate analysis used here gives useful information and is useful in the interpretation of haemagglutination variability between algal extracts, particularly when many data are available from screening studies. Several reports indicate that microalgae contain agglutinins that agglutinate human and animal erythrocytes to varying degrees (Hori et al. 1996; Liao and Huang 2000; Sato et al. 2000). Some agglutinins have shown pronounced antitumor effects, such as a glycoprotein from *Chlorella vulgaris* (Tanaka et al. 1998). They also exhibit unique structure and characteristics, as shown in extracts from *Microcystis aeruginosa*, *M. viridis*, *Oscillatoria agardhii* and related species (Sakamoto et al. 1996; Yamaguchi et al. 1998, 1999; Sato et al. 2000). Thus, microalgae may prove to be a good source of diverse agglutinins for biochemical studies or commercial applications.

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