

Variability of Allergenicity in Eight Strains of the Green Algal Genus *Chlorella*¹

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Abstract. Biochemical and immunological properties of allergen extracts from eight strains within four species of the unicellular green alga *Chlorella* were compared. The total protein and carbohydrate contents ranged from 13 to 29 and 18 to 47% of the dry weight, respectively. It was shown, both with RAST inhibition and immunoblotting, that *Chlorella* contain components which specifically bind to patient IgE antibodies. The relative allergen potency as assayed by RAST inhibition varied fivefold. Between seven and twenty IgE-binding components were identified in the various strains with molecular weights from 13 to 80 kD. Thus, the use of thoroughly characterized *Chlorella* strains is mandatory for preparation of relevant allergen extracts.

Introduction

The genus *Chlorella* Beijerinck belongs to the order Chlorococcales and includes unicellular green algae. The *Chlorella* cells are spherical or ellipsoidal, 3–12 µm in size and have a vegetative life cycle. The genus is found in marine, limnic and terrestrial environments in all parts of the world. On land, *Chlorella* inhabit ephemeral waters, tree trunks, rocks and the upper parts of the soil from where they may become windborn [Schlichting, 1969]. *Chlorella* is also found in house dust [Bernstein and Safferman, 1970; Tiberg et al., 1984].

Chlorella has attracted attention as a potential allergen source, mainly because of its ubiquitous presence and high protein content. Clinical studies of *Chlorella* strains from different culture collections or isolated from air samples and house dust reported positive skin reactions in patients with respiratory allergy [Bernstein and Safferman, 1966, 1973; Benaim-Pinto, 1972; Mittal, 1979].

The *Chlorella* genus is diverse, probably including several unrelated species [for review see Kessler, 1984]. Our questions were the degree of heterogeneity

shown by the allergens and how representative an allergen extract preparation of a single *Chlorella* strain would be for the genus. In this study we have compared protein and carbohydrate content, allergen activity and IgE-binding capacity in allergen extracts from four species of different taxonomic groups within the genus. *Chlorella homosphaera* and different strains of *Chlorella vulgaris* and *Chlorella saccharophila* were selected to represent strains commonly occurring in outdoor environments in Sweden [Tiberg et al., 1984] while *Chlorella fusca* was chosen for showing distant serological relationships to the former [Kümmel and Kessler, 1980].

Material and Methods

Cultivation of Algae

C. vulgaris 211-11j, 211-81, 211-11f and 211-11p, *C. saccharophila* 211-9b and 211-1a and *C. fusca* 211-8c were obtained from the Sammlung von Algenkulturen, University of Göttingen, FRG. In addition, *C. homosphaera* Skuja, isolated from Lake Ekoln in Sweden [Claesson and Forsberg, 1978], was used. The eight *Chlorella* strains were cultivated axenically in 15 litres (batch cultures) of mineral medium [Staub, 1961] supplemented with 1 mg NaNO₃/l. The batches were aerated with 2–3% CO₂ in air with stirring and continuous light (10–50 µmol photons/m²/s outside the culture vessel) at 25°C. The algae were harvested after 3 weeks of cultiva-

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Table 1. Comparative properties of eight *Chlorella* strains regarding protein and carbohydrate content, RAST inhibition and number of IgE-binding components

Strain	Protein % of dry weight	Carbohydrate % of dry weight	RAST inhibition ¹ % relative potency	IgE-binding components (immunoblotting)
<i>C. homosphaera</i>	13.0	41	82	10
<i>C. vulgaris</i> 211-11j	24.5	30	73	8
<i>C. vulgaris</i> 211-81	28.9	47	70	10
<i>C. vulgaris</i> 211-11f	24.1	28	111	8
<i>C. vulgaris</i> 211-11p	22.7	25	105	10
<i>C. saccharophila</i> 211-9b	14.5	21	23	7
<i>C. saccharophila</i> 211-1a	21.9	30	38	13
<i>C. fusca</i> 211-8c	26.1	18	22	20

¹ The inhibition of a mixture of equal amounts of proteins from all 8 strains was defined as 100% relative potency in the RAST inhibition analysis.

tion, i.e. in late logarithmic growth phase ($OD_{560} = 1.0-1.5$), by centrifugation and stored after freeze-drying at -20°C . At harvest, the cultures were tested for bacterial contamination in trypton-yeast-glucose broth (5.0 g trypton, 2.5 g yeast extract and 10 g glucose dissolved in 1 litre distilled water) by incubating samples for several weeks at 25°C in darkness.

Preparation of *Chlorella* Extracts

Freeze-dried *Chlorella* cells (1.5 g) were extracted by ultrasonication in order to strip the cell wall components. The cells were suspended in an extraction buffer (0.125 M NH_4HCO_3 , pH 7.8, ratio 1:10 w/v) and ultrasonicated 4×1 min on ice with cooling intervals of 1 min in between. The suspensions were then centrifuged (18,000 rpm, 4°C) and the supernatants filtered (0.45 μm) after a onefold dilution in buffer. The crude extracts were freeze-dried and stored at 4°C .

Patient Sera

Three *Chlorella* RAST-positive sera were selected and pooled (3.6 Phadebas RAST units [PRU]/ml) to give specific IgE binding against as many extract components as possible in the immunoblotting experiment. One serum with RAST class 2 (1.7 PRU/ml) to *Chlorella* was used for the RAST inhibition assay. All sera were RAST positive to common inhalant and food allergens; one to pollen, cat and *Aspergillus*, one to pollen, *Candida*, *Alternaria* and several food allergens and the third serum to pollen, *Alternaria*, animal danders and hazelnut. The third serum donor was also positive in skin test with pollen and moulds.

Total Protein

The protein content of the *Chlorella* extracts was determined by amino acid analysis [Spackman et al., 1958].

Total Carbohydrate

The carbohydrate content of the extracts was determined with the anthrone method [Scott and Melvin, 1953] with glucose and mannose (1:1) as a standard.

Electrophoresis and Immunoblotting

The procedure given by Bengtsson et al. [1986] for polyacrylamide gel electrophoresis (PAGE) and immunoblotting was followed. In brief, the *Chlorella* extracts (7 mg protein/ml, 40 μl /well) were separated on a sodium dodecyl sulphate (SDS)-containing polyacrylamide gradient gel (7.5–20%) and transferred to 0.2 μm nitrocellulose membrane (Amersham) by electroblotting (0.5 A, 4 h) in a transfer buffer (25 mM Tris, 192 mM glycine, 10% [v/v] methanol). A 1:3 dilution of the serum pool and a 1:5 dilution of the ^{125}I -anti-IgE (Phadebas RAST[®] isotope; 200,000 cpm/ml) was used for the identification of allergens. Phadebas RAST[®] buffer with 0.5% (v/v) Tween 20 was used for blocking unbound nitrocellulose-binding sites and as dilution buffer. The X-ray film (Amersham HyperTM) was exposed for 96 h at -70°C . In immunoblotting control experiments, the patient serum was replaced by buffer, a negative serum and a negative serum with addition of 2,000 kU/l myeloma IgE.

RAST

An eight-strain mixture (equal parts protein) of *Chlorella* extracts was coupled to CNBr-activated paper discs analogously to commercial RAST discs. Circulating *Chlorella*-specific IgE antibodies in patient sera were determined in duplicate according to the standard procedure for Phadebas RAST[®] (Pharmacia AB). RAST inhibition was performed with the same discs according to the procedure described by Yman et al. [1975].

Results

Table 1 summarizes the protein and carbohydrate content, the relative allergen potency (RAST inhibition) and the number of IgE-binding components (immunoblotting) in the crude allergen extracts of *Chlorella*. The protein content, determined by amino

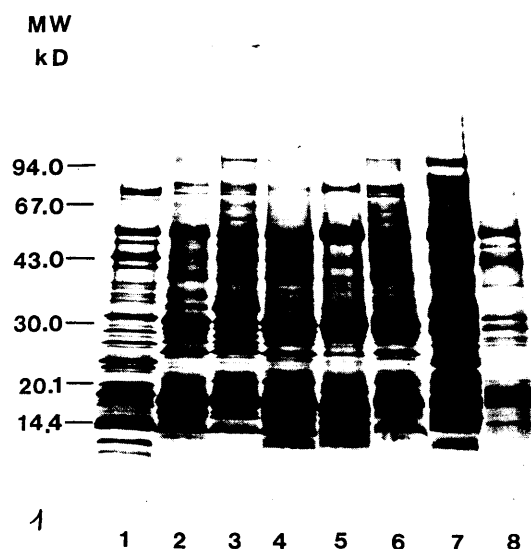


Fig. 1. SDS gradient PAGE of eight *Chlorella* extracts after staining with Coomassie brilliant blue. Lane 1, *C. homosphaera*; lane 2, *C. vulgaris* 211-11j; lane 3, *C. vulgaris* 211-81; lane 4, *C. vulgaris* 211-11f; lane 5, *C. vulgaris* 211-11p; lane 6, *C. saccharophila* 211-9b; lane 7, *C. saccharophila* 211-1a; lane 8, *C. fusca* 211-8c.

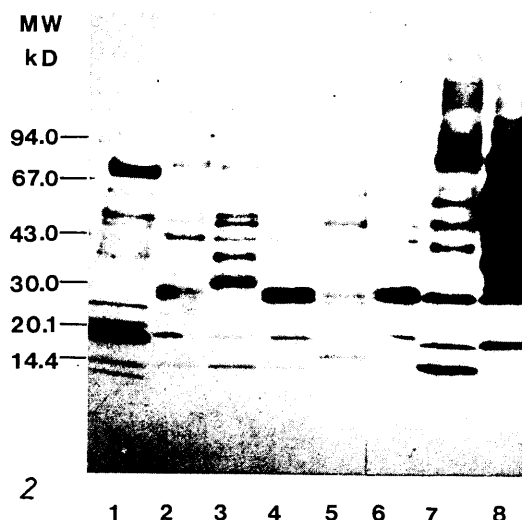


Fig. 2. Immunoblotting with isotope detection of IgE-binding components in eight *Chlorella* strains. Lane 1, *C. homosphaera*; lane 2, *C. vulgaris* 211-11j; lane 3, *C. vulgaris* 211-81; lane 4, *C. vulgaris* 211-11f; lane 5, *C. vulgaris* 211-11p; lane 6, *C. saccharophila* 211-9b; lane 7, *C. saccharophila* 211-1a; lane 8, *C. fusca* 211-8c.

acid analysis varied between 13 and 29% of the dry weight of the *Chlorella* extracts. The carbohydrate content, determined with a colorimetric method, ranged from 18 to 47% of the dry extract weight.

The total allergenic activity measured with RAST inhibition for the individual strains showed a potency variation of 22–111%. The inhibition of the 8-strain mixture extract was used as a reference and set by definition to 100%. Two distinct groups could be distinguished: one with high inhibition values, including *C. homosphaera* and the four *C. vulgaris* strains and one with low inhibition values, including the two *C. saccharophila* strains and *C. fusca* 211-8c.

The protein pattern of the eight *Chlorella* extracts is seen in figure 1. The molecular weights of the individual proteins varied from 13 to 94 kD. There were considerable differences in the protein-banding pattern between the four *Chlorella* species examined, although a few major bands were common to all strains. In particular, the protein pattern of the four *C. vulgaris* extracts showed similarities.

Using immunoblotting with a pool of patient sera, 7–20 IgE-binding components were identified in the individual extracts (table 1). The molecular weights of the majority of the radiostained bands ranged from

approximately 13 to 80 kD but a few with higher molecular weights were also noted (fig. 2). IgE-binding components in common to many strains were identified at 13, 17, 25, 46–50 and 72 kD, respectively (fig. 2), indicating similar patterns for *Chlorella* species. In the control experiments, faint radiostained bands appeared at 28, 50 and 72 kD. Figure 3 shows the result when a negative serum with added myeloma IgE was used. The unspecific binding was identical, though much weaker, when buffer or negative serum replaced the positive serum pool. No visible binding was detected using only nitrocellulose with different tracer-antibody concentrations. When examining the number of allergens in the individual extracts, two separate groups could be identified (table 1): *C. homosphaera*, the *C. vulgaris* strains and the *C. saccharophila* strains with 7–13 detectable allergens and *C. fusca* 211-8c with as many as twenty allergens. Comparing the allergen pattern (fig. 2, 4) of the individual strains showed that the species *C. homosphaera*, *C. vulgaris*, *C. saccharophila* and *C. fusca* formed four allergen groups but with the strain *C. saccharophila* 211-9b included in the *vulgaris* group. However, many allergens were in common to all strains. The strain *C. saccharophila* 211-9b shared all its seven

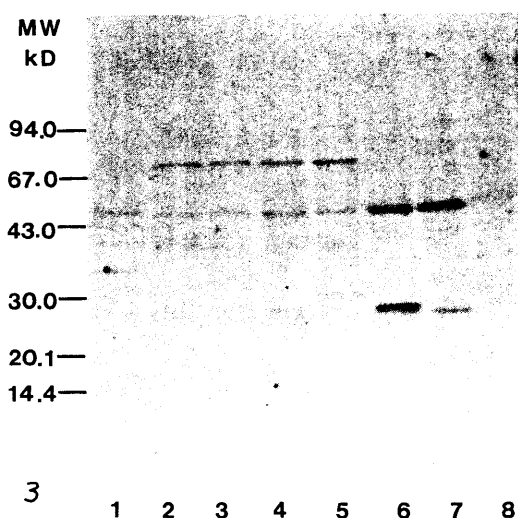


Fig. 3. Immunoblotting control experiment. A negative serum with addition of IgE myelom was used instead of the positive serum pol. The numbering of lanes is as in figure 2.

allergens with *C. vulgaris* 211-11j and 211-11f, while *C. saccharophila* 211-1a shared three allergens with *C. vulgaris* 211-11j, six with *C. fusca* 211-8c and two with *C. saccharophila* 9b.

Discussion

The present study demonstrates IgE-binding components in *Chlorella* extracts both with the RAST technique and immunoblotting, which confirm earlier clinical findings. IgE-mediated symptoms have previously been demonstrated in skin tests and conjunctival, nasal and bronchial provocation tests [Bernstein and Safferman, 1966, 1970, 1973; Benaim-Pinto, 1972; Mittal et al., 1979] with passively transferred antibodies [Bernstein and Safferman, 1966; Mittal et al., 1979; Benaim-Pinto, 1972] and leucocyte histamine release [Bernstein and Safferman, 1973]. However, the extracts used for those in vivo tests originated from different *Chlorella* strains and were not immunochemically characterized.

The total protein and carbohydrate content of the *Chlorella* extracts used in this study do not reflect their species or strain status. In general, protein and

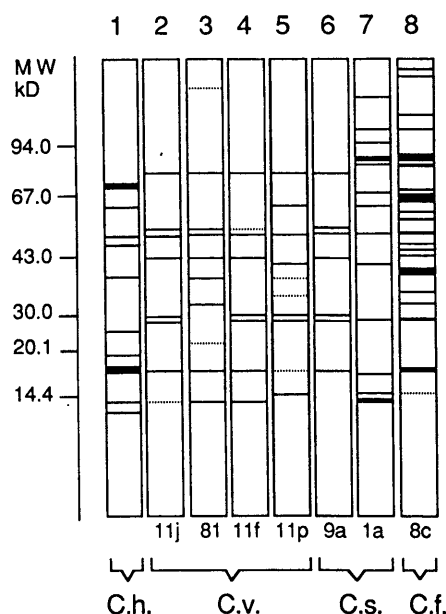


Fig. 4. Schematic drawing of the IgE-immunoblotting shown in figure 2. The numbering of lanes is as in figure 2. C.h. = *C. homosphaera*; C.v. = *C. vulgaris* strains 211-11j, 81, 11f, 11p; C.s. = *C. saccharophila* strains 211-9b, 1a; C.f. = *C. fusca* strain 211-8c.

carbohydrate values vary between different allergen extracts and this effect is especially pronounced for mould strains [Wallenbeck et al., 1984; Steringer et al., 1987].

The RAST inhibition results of the 'high-potency' group (*C. homosphaera* and *C. vulgaris*) correlated well with the number of IgE-binding components visualized in immunoblotting analysis (table 1). However, in the 'low-potency' group discrepancies were found. The extracts of *C. saccharophila* 211-1a and *C. fusca* 211-8c with 38 and 22% inhibition, respectively, revealed a high number of IgE-binding components (13 and 20, respectively). This effect is most likely a reflection of the allergens present on the RAST disc and the patient serum used for inhibition. An extract mixture of all strains, dominated by *C. vulgaris* (4 strains) was coupled to the RAST disc. According to the immunoblotting experiments, *C. saccharophila* 211-1a and *C. fusca* 211-8c contained additional allergens to those demonstrated in *C. homosphaera* and *C. vulgaris* (fig. 2). *C. saccharophila* 211-9b, with 23% inhibition and seven IgE-binding components, exhibited low allergenic potency demonstrating agreement between the RAST inhibition and blotting analysis.

Based on immunoblotting patterns, all strains could be classified into one of the following groups: *C. homosphaera*; *C. vulgaris* including *C. saccharophila* 211-9b; *C. saccharophila* 1a; *C. fusca* 211-8c (fig. 4). These groups show both consistent and specific patterns, confirming works by Kasatkina et al. [1973] and Sanders et al. [1971] where both strain- and species-specific antigens were found. The homogeneity of the *C. vulgaris* group is in agreement with Maruyama's [1977] immunological classification of *Chlorella* strains based on agglutination studies of cell surface antigens. Twelve of 17 *C. vulgaris* strains reacted with the same antiserum, while two *C. ellipsoidea* strains (reclassified as *C. saccharophila*) were heterologous [Maruyama, 1977]. According to our results, *C. saccharophila* 211-9b was homologous with *C. vulgaris* both regarding protein and IgE-binding spectrum. On the other hand, *C. saccharophila* 211-1a showed similarities both to *C. vulgaris* and to *C. fusca* 211-8c. The extracts of the two *saccharophila* strains also differed in the protein and carbohydrate content. The heterogeneity of the *saccharophila* taxon has recently been documented by comparison of DNA base composition and homology, where strain 211-1a showed almost no homology to any other *saccharophila* strain [Huss et al., 1987]. The *C. fusca* strain showed an entirely different allergen pattern as compared to the other seven *Chlorella* strains. For instance, the heavily stained bands (fig. 2) were reproducible. When staining the separation gel after electroblotting of the proteins onto the nitrocellulose membrane, a much more efficient transfer of the proteins was always evident as compared to the other *Chlorella* strains. Obviously, the proteins of *C. fusca* extract have different electrophoretic properties. This was not, however, reflected in the individual amino acid composition (data not shown). This species is, as well as *C. homosphaera*, physiologically and biochemically distant to the *C. vulgaris* group [Kessler, 1984]. Unspecific binding between the most common IgE-binding extract components and the anti-IgE tracer-antibody was found (fig. 3). This binding was strongly taxonomical and, contrary to the IgE-binding, it showed homology between the both *C. saccharophila* strains.

The agreement between our allergen grouping and other classifications applied to *Chlorella* show that the IgE-immunoblotting method defines the strain specificity well enough to be used as a tool for taxonomic studies. The fact that very few sera were used shows

the high variability of IgE, which makes it a particular useful marker.

Components binding IgE in extracts of the green alga *Chlorella* have been demonstrated in vitro both with RAST and immunoblotting. Our data demonstrate that selection of a strain is a crucial step for the production of an allergen extract from *Chlorella*. Because of the considerable variability in allergen composition, where the cell-surface allergens largely reflect the taxonomic status of the strains, a thoroughly characterized strain should be chosen. Alternatively, a mixture of representative strains of each species within the genus may also be used.

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