

Variability in Different Strains of *Cladosporium herbarum* with Special Attention to Carbohydrates and Contents of Two Important Allergens (Ag-32 and Ag-54)

M. SWÄRD-NORDMO,¹ T.-L. ALMELAND² and L. AUKRUST³

¹Institute of Pharmacy, University of Oslo, ²Allergy Unit, Pediatric Research Institute, The National Hospital, University of Oslo, and ³Biochemistry Department, Nyegaard & Co. AS, Oslo, Norway

Ten different strains of *Cladosporium herbarum* cultivated and extracted under identical experimental conditions were compared with respect to: 1) carbohydrates quantified by methanolysis and gas-liquid chromatography, 2) contents of two important *Cladosporium* allergens Ag-32 and Ag-54 estimated by rocket radioimmuno-electrophoresis, 3) protein content determined by the Lowry and Bradford methods, respectively, and 4) allergenic activity as measured by RAST. The allergen extracts contained 45-73% carbohydrate material which mainly consisted of mannose, galactose and glucose. The Ag-54 content increased with increasing mannose and decreasing glucose concentration. No linear correlation between carbohydrate content and allergenic activity was found. The strains differed extensively in immunological composition. One of the strains totally lacked Ag-32 and Ag-54. To be clinically relevant, allergen preparations of *C. herbarum* should be based on a representative pool of several *C. herbarum* strains.

Key words: allergy; carbohydrates; *Cladosporium*; mould.

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CLINICAL ASPECTS

A randomly selected *C. herbarum* strain may not function in a clinical situation due to the lack of important allergens.

Cladosporium herbarum is an important cause of clinical allergy, but immunotherapy with moulds has been highly controversial (12). One possible reason for the restricted clinical value of mould extracts may be the great variability in antigen and allergen composition and the possible lack of important allergens in the various preparations used.

Studies on *Alternaria alternata* demonstrated substantial differences between culture filtrates from different isolates and between different batches of the same isolate (19). Wallenbeck et al. revealed biochemical and immunological differences between *Aspergillus fumigatus* strains grown and processed under identical condi-

tions (23). Aukrust found considerable immunological differences when comparing two extracts prepared identically from *C. herbarum* obtained from two different sources, and grown under non-identical conditions (2).

Little attention has been paid to the presence of carbohydrates in allergen extracts, although the immunogenicity in man of several polysaccharides is well established. Antigenic peptidoglycans have been found in several fungi including *Trichophyton* (5) and *Cladosporium* (13, 21). Further, ovalbumin, which is a well-characterized glycoprotein (15) has been classified as a major allergen in hen egg white (10) and one important allergen in rye-grass has been char-

acterized as a glycoprotein (9). In addition several less characterized glycoproteins have been identified as allergens. Based on these facts it is relevant to investigate the possible role of the carbohydrate moiety in the IgE binding reaction of allergens.

The aim of the present work was to see if any connection could be found between carbohydrate and allergen contents in a preliminary study, to compare 10 different strains of *C. herbarum* with respect to immunological and biochemical differences, and to estimate the content of two important allergens Ag-32 and Ag-54. The strains were grown and processed under identical experimental conditions.

MATERIAL

Cladosporium herbarum

The strains of *C. herbarum* were obtained from Allergon AB (Engelholm, Sweden) as lyophilized mycelium and conidia. The strains were cultivated on synthetic culture medium under identical experimental conditions similar to those used by Allergon AB in routine growth of *C. herbarum*. The 10 strains were harvested after a growth period of 2 weeks.

Rabbit antisera

Two pools of rabbit antisera to *C. herbarum* were prepared as described by Aukrust (2) using the *C. herbarum* material generally available from Allergon AB (anti-Ch-E) and ALK (Allergologisk Laboratorium A/S, Copenhagen) (anti-Ch-77). The anti-Ch-E contained precipitating antibodies to Ag-32 but not to Ag-54, while the anti-Ch-77 contained precipitating antibodies to Ag-54 and not to Ag-32, detectable in the CIE reference patterns of *C. herbarum*. Both reference patterns gave rise to approximately 60 precipitates.

Human sera

The presence of the *Cladosporium* allergens Ag-32 and Ag-54 was demonstrated in radio-

immunoelectrophoretic experiments with two individual sera, TdM and TN, containing high amounts of specific IgE to only Ag-32 and Ag-54, respectively (2).

A patient serum pool was prepared from equal amounts of serum from 30 individuals with suspected allergy to moulds, as confirmed either by Phadebas RAST® (Pharmacia Diagnostics, Uppsala, Sweden) or by skin prick test with at least one of five commercially available *C. herbarum* extracts (1).

METHODS

C. herbarum extracts

Extraction of 10 different strains of *C. herbarum* was performed according to Aukrust (2). A 10% (w/v) suspension of lyophilized mycelium and conidia in 0.125 mol/l ammonium bicarbonate – 0.015 mol/l sodium azide buffer (pH 7.8 25°C) was gently agitated for 24 h at 6°C, filtered through Whatman No. 1 filter paper and centrifuged. The supernatant was dialyzed twice at 6°C, using 0.005 mol/l ammonium bicarbonate – 0.015 mol/l sodium azide for 20 h and distilled water for 4 h. The retentate fraction was lyophilized and stored at 6°C.

A reference extract of *C. herbarum* had been prepared in an analogous way from fungal material grown under the same experimental conditions.

Quantification of Ag-32 and Ag-54

1. *Rocket immunoelectrophoresis.* Estimation of the content of selected allergens was achieved by rocket immunoelectrophoresis essentially as described by Axelsen et al. (4). Ag-32 and Ag-54 contents of the different *C. herbarum* strains were compared with the reference strain. The experiments were carried out on glass plates (5 × 7 cm) using 1% agarose gel (Litex, Glostrup, Denmark) containing a buffer of 0.073 mol/l Tris, 0.024 mol/l barbital, 0.006 mol/l calcium lactate and 0.003 mol/l sodium azide (pH 8.6 25°C). Dimensions of the gel sections were 7 ×

3.5×0.11 and $7 \times 1.5 \times 0.15$ cm respectively, denoted from the anode. The antigens were run overnight at 2 V/cm into a gel containing 150 μ l anti-Ch-E or 200 μ l anti-Ch-77. The plates were pressed, washed and dried as described by Axelsen et al. (4), taking care to avoid heating the plates, which were stored dry at 6°C until used for autoradiography.

2. *Autoradiography.* Rocket immunoelectrophoresis preparations were incubated with selected patient sera for detecting individual allergens as described by Aukrust & Borch (3), basically by incubation with 400 μ l patient serum diluted to 7 ml with incubation buffer (0.5 mol/l phosphate buffer pH 7.4 containing 0.9% sodium chloride and 0.2% Tween 20) for 24 h at 25°C, followed by incubation with approximately 250,000 cpm ^{125} I-anti-IgE (Phadebas® RAST Isotope Reagent) in 7 ml total volume for another 24 h. The plates were washed, dried and placed on X-ray photographic film for autoradiography (2). The quantification of Ag-32 and Ag-54 was based on measuring the height of the precipitates.

Crossed immunoelectrophoresis (CIE)

Crossed immunoelectrophoresis was carried out in accordance with previously described procedures (2) basically as described above. The dimensions of the gel sections on the plates were $5 \times 3.5 \times 0.11$, $5 \times 1 \times 0.13$, $5 \times 1 \times 0.15$, and $5 \times 1.5 \times 0.13$ cm, respectively, denoted from the anode. The first dimension electrophoresis was performed at 10 V/cm for 30 min. The number of visible precipitates was recorded for each strain using 100 μ l anti-Ch-77 rabbit anti-serum in the cathodic and 300 μ l in the anodic gel sections.

Radioallergosorbent test (RAST)

The allergen extracts were coupled to CNBr-activated paper discs (Whatman No. 20) and RAST was performed basically as described by Yman et al. (24). The results were expressed as uptake of ^{125} I-anti-IgE in per cent of the total amount applied.

Determination of protein

Protein content was estimated by a modification of the method of Lowry et al. (14) as described by Bensadoun & Weinstein (6) and by the method of Bradford (7) using human serum albumin as reference.

Determination of carbohydrates

The monosaccharides were quantified by methanolysis and gas-liquid chromatography of the trimethylsilylated methylglycosides, basically as described by Reinhold (17). The dried sample (1 mg) was heated at 80°C for 24 h with 1 mol/l HCl in anhydrous methanol (1 ml) using mannitol as internal standard. The methanolysate was dried under a stream of N₂ gas, re-N-acetylated with acetic anhydride-pyridine (1:1, 0.2 ml, 25°C) for 3 min and evaporated to dryness. The simultaneous partial O-acetylation was reversed by heating the sample at 65°C for 1 h with 0.5 mol/l HCl in anhydrous methanol (1 ml). After solvent evaporation the sample was derivatized with pyridine:hexamethyldisilazane:trimethylchlorosilane (TMSi) (5:2:1) for 30 min at 25°C. The TMSi ethers of the methylglycosides were chromatographed on a glass column (0.2 $\times$ 390 cm) packed with 3% SE-52 on Varaport 30 with N₂ (30 ml/min) as carrier gas. The chromatograph (Carlo Erba 4200) was equipped with a flame-ionisation detection system. Peak areas were determined by disc-integration (LDC 308/9 computing integrator).

Blanks for the carbohydrate analysis were prepared by treating the culture medium in exactly the same way as were the individual *C. herbarum* strains. The medium was agitated with buffer, filtered, centrifuged, dialyzed and lyophilized as described for the preparation of *C. herbarum* extracts, before carbohydrate analysis was performed as described above.

RESULTS

Results from the carbohydrate analysis are given in Table 1. Fig. 1 shows the correlation between Ag-54 and mannose content and the

corresponding relation between Ag-54 and glucose content as measured by linear regression analysis. Estimation of protein contents is summarized in Table 2.

RAST experiments using the 30-patient serum pool are summarized in Table 2. Preliminary dose-response experiments for each extract indicated 5 mg/ml dry weight substance of extract to be the optimal concentration for coupling. At this concentration a plateau was reached where further increase of extract concentration did not significantly alter the degree of isotope uptake obtained. Triplicate analyses were then performed using 5 mg/ml solutions. Variation in CIE precipitates is shown in Table 2.

Ag-32 and Ag-54 were quantified by means of rocket radioimmuno-electrophoresis and the results are given in Table 2. Rocket immuno-electrophoresis of different strains of *C. herbarum* is demonstrated in Fig. 2A. Fig. 2B shows the corresponding X-ray film from autoradiography experiments with selected individual patient serum containing high amounts of specific IgE to Ag-54 only.

In a separate experiment one strain of *C. herbarum* was cultivated as described above and harvested after 2, 3, 4, 5, 6 and 8 weeks growth. The fungal material was extracted and processed as described above. Contents of Ag-32, Ag-

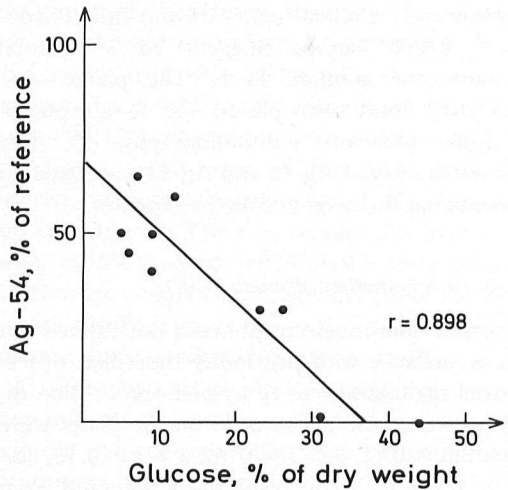
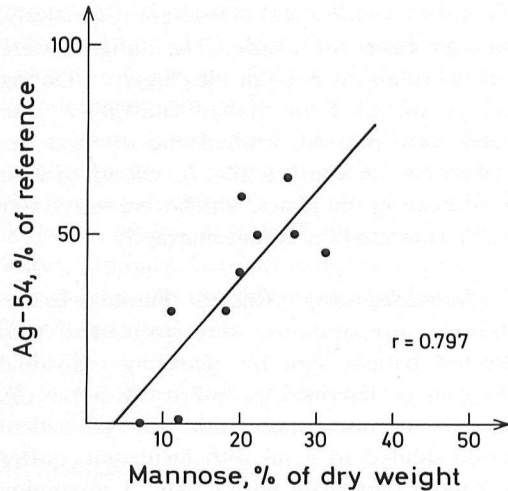


Table 1

Carbohydrate analysis of extracts from 10 different strains of *C. herbarum*. The monosaccharide contents were determined by gas-liquid chromatography of the trimethylsilylated methylglycosides

Strain	Per cent of dry weight			Total
	Mannose	Galactose	Glucose	
1	26	24	7	57
2	22	22	5	49
3	12	17	31	60
4	20	18	12	50
5	20	23	9	52
6	31	31	6	68
7	18	19	26	63
8	11	11	23	45
9	27	37	9	73
10	7	9	44	60

Fig. 1. The correlation between Ag-54 content and the mannose and glucose concentrations respectively. r = correlation coefficient from linear regression analysis.

54 and the total allergenic activity were compared with a reference as shown in Fig. 3.

DISCUSSION

The different strains of *C. herbarum* showed a high degree of immunological and biochemical variation. The carbohydrate composition of the 10 extracts differed considerably, especially with respect to glucose, which in strain No. 10 made

Table 2

Variability of extracts from 10 different strains of *C. herbarum* as compared with a reference extract

Strain	Protein % of dry weight		No. of prec. in CIE	Ag-32 % of ref.	Ag-54 % of ref.	RAST % uptake
	Mod. Lowry	Bradford				
Ref.	14	10	30	100	100	36
1	9	14	16	1000	65	35
2	9	24	22	100	50	26
3	2	3	8	30	1	21
4	5	11	23	30	60	18
5	4	8	30	100	40	18
6	3	13	24	5	45	18
7	3	8	22	120	30	17
8	3	7	24	30	30	14
9	4	8	12	100	50	13
10	1	2	5	0	0	6

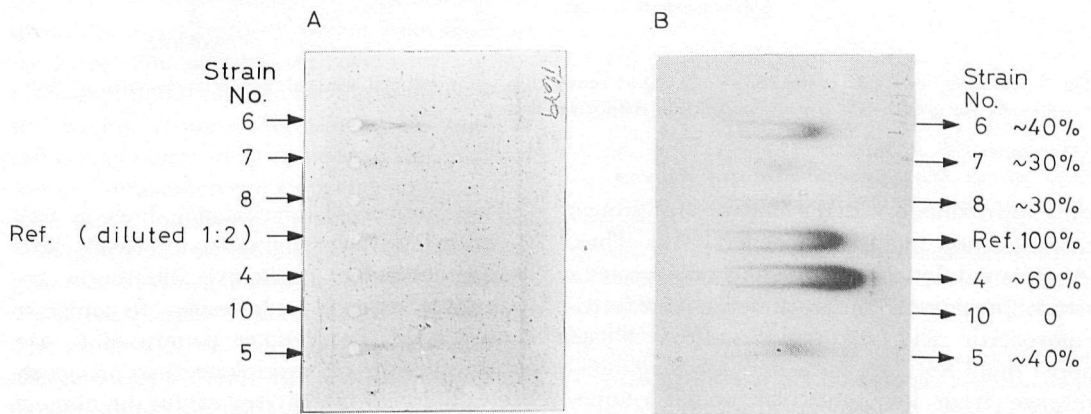


Fig. 2. Quantification of Ag-54 in 6 different strains of *C. herbarum* expressed as % of a reference. A: Rocket immunoelectrophoresis plate using $8.2 \mu\text{l}/\text{cm}^2$ anti-Ch-77. B: Corresponding autoradiography experiment using selected patient serum containing high amounts of specific IgE to Ag-54 only.

up as much as half of the material. The culture medium contained glucose as the only carbohydrate in significant quantities. However, the blanks introduced in the carbohydrate analysis described above demonstrated that the glucose did not originate from the culture medium or the filtration process during extraction.

Though the number of strains investigated was small, a clear connection could be seen between the contents of Ag-54 and two of the monosaccharides. The Ag-54 content increased linearly with increasing mannose ($r = 0.797$)

and decreasing glucose ($r = 0.898$) concentrations. Linear correlation could not be found between Ag-32 content and carbohydrates or between total carbohydrate concentration and allergenic activity ($r < 0.5$). The data were not investigated for non-linear correlation between these parameters.

Protein and carbohydrate contents did not amount to 100% in these experiments. This may be due in part to the presence of lipids, since no defatting step was included in the extraction procedure. The protein values are

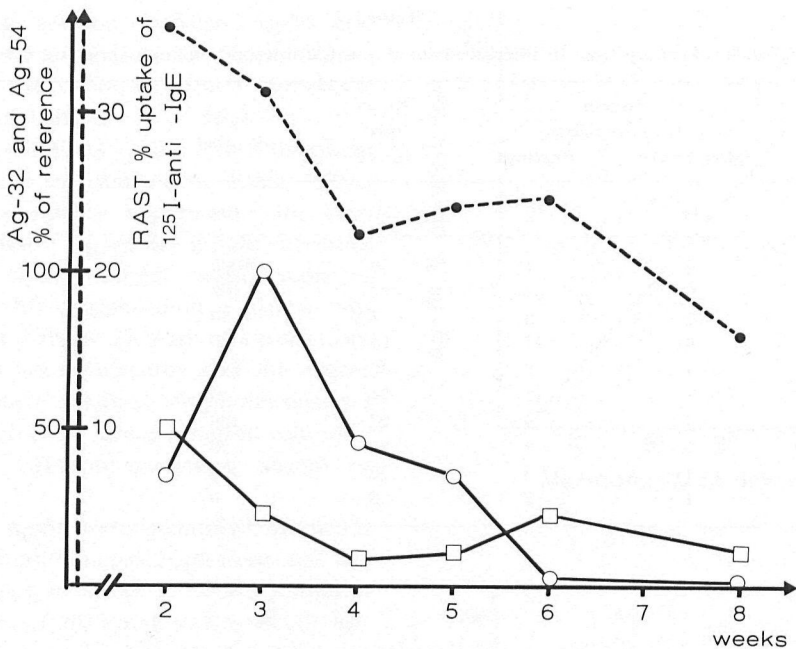


Fig. 3. Variability of Ag-32 content (○—○), Ag-54 content (□—□) and total allergenic activity as measured by RAST (-----) during growth of *C. herbarum* on synthetic culture medium.

only approximate, with the limitations of protein quantification methods in general (16). Thus, in the modified Lowry method only proteins which precipitate in the presence of 6% trichloroacetic acid, 0.3 mol/l Na-deoxycholate and 1 mol/l NaCl are measured, avoiding interference from low molecular weight components. There is always a possibility that glycoproteins may fail to precipitate with trichloroacetic acid, particularly when containing large carbohydrate moieties (11). Fungal material in general is known to contain glycoproteins (20). This is consistent with findings in *C. herbarum* (18) and related fungi (13, 25). Carbohydrate-rich glycoproteins will most probably be extracted under the conditions used in this study (22). Consequently, the estimates of protein content by the modified method of Lowry given in this paper are probably too low, and the results of the Bradford method should be regarded as the most reliable. It is probable, however, that differences in protein contents reflect differences between the strains even if the exact amounts are inaccurate.

The number of precipitation lines in CIE varied extensively, ranging from 5 to 30, clearly demonstrating qualitative differences between the extracts with respect to antigenic content. A CIE reference pattern of *C. herbarum* showing 62 precipitates has previously been published (2). In view of this the number of precipitation lines in the present CIE experiments may seem surprisingly low. However, this may be explained by the fact that none of the strains were run against their matching antisera. A drastic reduction in the number of precipitation lines has previously been demonstrated (2) when using cross-combinations of *Cladosporium* antisera and allergen extracts.

Immunological variation was confirmed by the RAST experiments, which showed that the highest isotope uptake that could be achieved varied from 6 to 35%.

Little information was obtained from the rocket immunoelectrophoresis pattern as such, and the use of tracer patient sera and autoradiography technique was necessary in order to identify and quantify the individual allergens.

Rocket radioimmuno-electrophoresis revealed considerable differences in the Ag-32 and Ag-54 content between strains, most clearly reflected by the Ag-32 concentration which varied by a factor of 1000. Strain No. 10 was remarkable for its lack of both Ag-32 and Ag-54.

The studies of antigen content during cultivation of *C. herbarum* demonstrated that growing conditions such as time are of importance for the immunological quality of the fungal material. Of particular interest may be the fact that Ag-32 could scarcely be detected after 6 weeks' growth in this particular strain, although it was clearly present after 2–5 weeks' growth.

The possibility exists that experimental growth conditions, though identical, are not necessarily optimal for all strains in question. When growing *Cladosporium*, one cannot expect to find the same degree of germination or growth at a fixed growth period from batch to batch (8). This may be one reason for the immunological and biochemical variation between the strains. However, certain strains may be inferior to others in all respects as raw material sources for allergen extract production.

In conclusion the experiments presented demonstrated that different strains of *C. herbarum*, grown and extracted under identical conditions, show considerable immunological variability. The contents of the two important *Cladosporium* allergens Ag-32 and Ag-54 vary extensively. Certain strains do not contain these important allergens at all. The results show that identical production routines are no guarantee for making allergen extracts containing sufficient amounts of relevant allergens. Obviously, it is practically impossible to characterize every single *C. herbarum* strain with respect to the individual patients in a clinical situation. Consequently, the allergen extracts should be based on a representative pool of several *C. herbarum* strains. That seems to be the only way to increase chances that all important allergens are present. This approach should also be advantageous with respect to keeping batch-to-batch variation to a minimum. The use of a mixture of several strains does, however, lead to a possible dilution of components present in a few strains only. Hopefully this is only a minor

disadvantage since it is unlikely that components present in only a few strains will turn out to be allergologically important for the majority of patients.

A striking feature for all of the 10 mould extracts investigated is that the percentage of carbohydrate is several times that of protein. Furthermore, there seems to be a possible correlation between the sugar composition and the content of the important allergen Ag-54. It may be worthwhile to further investigate the possible importance of carbohydrates for the allergenic activity.

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Address:

Marit Swärd-Nordmo
Research and Development Section
Nyegaard & Co. AS
P.O. Box 4220, Torshov
Oslo 4
Norway

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