

The effect of time and extraction buffers on residual protein and allergen content of extracts derived from four strains of *Alternaria*

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Background: A series of studies was performed to identify optimal elution conditions for production of desired *Alternaria* allergens with simultaneous reduction of undesired ones.

Methods: Proteins and allergens from four strains of *Alternaria* extracted for differing time intervals and in different buffers were analyzed by one- and two-dimensional electrophoresis, as well as by immunoglobulin E enzyme-linked immunosorbent assay inhibition and immunoblotting.

Results: The amount of protein and carbohydrate released varied with each time interval but was consistent between buffers. Extracts from longer time intervals tended to contain more carbohydrate. Electrophoresis of the four strains demonstrated many similar proteins; however, the concentrations of these proteins showed considerable interstrain difference. Comparison of extraction times for single strains by immunoblotting showed that certain allergens are preferentially released during specific time intervals. Some allergens were seen to be most prevalent in a 24-hour extract, whereas others were most prevalent in a 1-hour extract. Two-dimensional electrophoresis resolved bands into discrete spots. The major shared elements of the four strains could be easily identified. The appearance and disappearance of individual protein elements with time was seen.

Conclusions: Elution conditions have a significant impact on quantities of specific glycoproteins contained in extracts of *Alternaria* and must be controlled and optimized when such extracts are produced for allergen purification. (*J ALLERGY CLIN IMMUNOL* 1993;91:930-8.)

Key words: *Alternaria*, extracts, immunoblotting, two-dimensional electrophoresis, ELISA inhibition, mold allergy

Allergenic extracts from mold of the genus *Alternaria* prepared in the laboratory or obtained commercially have been examined by many investigators. Strong interstrain differences in allergen content have been demonstrated with the use of techniques such as crossed immunoelectrophoresis,^{1,2} RAST,²⁻⁵ and western blot.⁶⁻⁸ Few studies have examined the extent to which these differences are determined by the extraction buffer or time of elution. A recent study examined

Abbreviations used

ELISA:	Enzyme-linked immunosorbent assay
Ig:	Immunoglobulin
PMSF:	Phenylmethyl sulfonyl fluoride
RAST:	Radioimmunosorbent assay
SDS-PAGE:	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TBS:	Tris-buffered saline

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the effect of vigorous physical measures to break up the mold and the presence of protease inhibitors or phenol-binding agents on the allergen yield. Although these authors did not achieve complete inhibition of proteases, their procedures produced extract with higher overall protein content but did not result in significantly greater release of a specific allergen.⁸ They speculated that the reported 31 kd allergen is either a surface protein or a quickly released cytoplasmic protein.

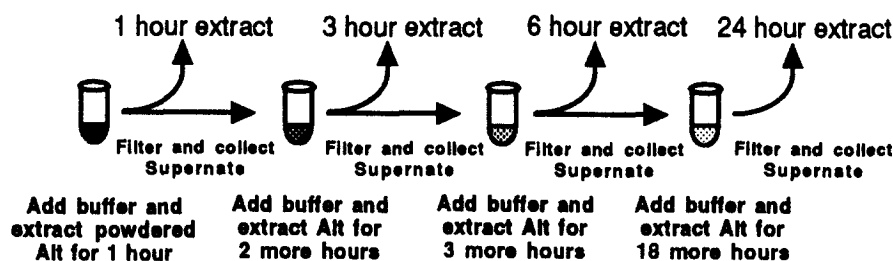


FIG. 1. Procedure for interval extraction of *Alternaria* (Alt).

We have been interested in purification of major *Alternaria* allergens and have found significant differences in the content of these allergens for different strains eluted under various conditions. To determine whether it is possible to optimize conditions for production of desired allergens with simultaneous reduction of undesired allergens, the following series of studies was performed with four strains of *Alternaria alternata*.

METHODS

Human volunteers

All clinical studies were performed after approval was obtained from the Pediatrics Institutional Review Board of the University of Missouri-Kansas City. Written consent was obtained from each volunteer before any experimental procedure was performed. Volunteers were tested by the prick skin test method with a commercial *Alternaria* preparation (Hollister-Stier, Spokane, Wash.). Those individuals with a wheal size greater than 5 mm after 20 minutes were considered to be *Alternaria*-sensitive. Blood samples were obtained from these *Alternaria*-sensitive individuals with standard techniques, and sera that contained anti-*Alternaria* IgE as determined by direct ELISA were pooled. The final serum pool that was used for these studies contained serum samples from five individuals.

Source materials

Four strains of *Alternaria alternata* (designated A, B, C, and D) were obtained from Harriet Burge, PhD at the University of Michigan and grown at 24° C for 4 weeks on a chemically defined medium of minimum salts and sucrose as described previously.⁹ (These strains can be obtained by contacting Robert Esch, PhD at Greer Pharmaceuticals in Lenoir, N.C.) The cultures were exposed to 12-hour periods of alternating light and dark during their growth. Fungal mats were inspected by light microscopy to ensure the presence of both spores and mycelia. These mats were then washed with distilled water, air dried for 24 hours at 37° C, and powdered with a mortar and pestle.

Extraction

Two extraction methods were used for this study. To evaluate the effect of extraction buffer, the powdered mixtures of spores and mycelia were extracted at 4° C in four different buffers at a concentration of 10 gm dry weight per

100 ml buffer. These buffers included 50 mmol/L sodium acetate with 0.15 mol/L NaCl (pH 5.0), 50 mmol/L Tris with 0.15 mol/L NaCl (pH 7.4), 50 mmol/L sodium bicarbonate with 0.15 mol/L NaCl (pH 8.6), and deionized water (pH 8.0). To determine the time period that gives the highest apparent yield of particular allergens, a residual extraction method was used (Fig. 1). For this procedure, each of the four strains was extracted for 1 hour, and the mixture was then centrifuged at 600 g. All of the supernatant was removed and replaced with a volume of deionized water equal to the amount removed. The pellet was resuspended and the extraction was allowed to proceed. This procedure was repeated at 3, 6, and 24 hours. These same materials were also extracted continuously for 1 hour and for 24 hours without removal of liquid. All extracts were cleared through 0.45 µm filters (Millipore, Bedford, Mass.), dialyzed three times against 50 mmol/L ammonium bicarbonate (pH 8.6) and lyophilized. Lyophilized preparations were stored at -20° C until used.

Measurement of protein and carbohydrate

Protein concentrations were measured by the Lowry method¹⁰ with bovine serum albumin as the calibration standard (Sigma, St. Louis, Mo.). Carbohydrate concentrations were determined by a phenol-sulfuric acid method with 0.5 mg/ml glucose as the standard.¹¹

One-dimensional electrophoresis

Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the extracts was performed on 8% to 18% gradient vertical slab gels with the method of Laemmli¹² and compared with prestained molecular weight markers obtained from BioRAD (Richmond, Calif.) to determine molecular weights. Lyophilized samples were reconstituted to a concentration of 10 mg/ml, and 10 µl of each sample, diluted in 30 µl of Tris-SDS (pH 6.8) with mercaptoethanol (reduced) or without mercaptoethanol (unreduced) and 25% glycerol, was applied to each lane.

Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed according to the method of O'Farrell¹³ Isoelectric focusing of the *Alternaria* extracts was performed in 20 cm tubes that contained 3.8% acrylamide, 0.2% N,N'-methylene-bis-acrylamide, 2% Nonidet P-40, 8 mol/L urea, and 2% Bio-

TABLE I. pH Values of four extraction buffers before *Alternaria* is added (initial) and during continuous extraction for 0, 1, and 24 hours

Time (hr)	Deionized water	Sodium acetate	TBS	Sodium bicarbonate
Initial	8.0	5.0	7.4	8.6
0	8.0	5.4	7.7	7.4
1	7.7	6.4	7.7	7.4
24	7.3	6.5	7.4	7.0

lytes (equal amounts of pH 3-10 and pH 3-5, BioRAD) on a BioRAD Protean II apparatus. After prefocusing for 1 hour at 200 V, *Alternaria* samples that contained 500 µg dry weight per tube were dissolved in 8 mol/L urea, 2% Nonidet P-40, 2% Biolytes (pH 3-10), and 5% β-mercaptoethanol and applied to the tubes. Electrophoresis was performed at 500 V for 10 hours and then at 800 V for 1 hour. After isoelectric focusing was performed, tubes were extruded with 25% glycerol and ultrathin extrusion needles (BioRAD). The tubes were briefly washed in distilled water and placed in Tris-SDS (pH 6.8) with mercaptoethanol and 25% glycerol for 30 minutes. If the second dimension was run immediately, tubes were layered onto the top of 1.5 mm 8% to 18% gradient SDS-polyacrylamide gels, and electrophoresis was performed as described above for SDS-PAGE. Tubes were stored at -70° C until used, if they were not run immediately. One tube representing each antigen was stained with 0.1% Coomassie Brilliant Blue. Both the one-dimensional SDS-PAGE and the second-dimension slab gels were silver stained according to the method of Wray et al.¹⁴

Immunoblotting

Proteins from SDS-PAGE gels were transferred electrophoretically to nitrocellulose membranes (BioRAD TransBlot) in 25 mmol/L Tris and 192 mmol/L glycine buffer, pH 8.8, which contained 20% methanol at 30 V overnight. Nitrocellulose membranes were then blocked overnight with 5% nonfat dry milk (Carnation Inc., Los Angeles, Calif.) in Tris-buffered saline (TBS). These blots were subsequently probed overnight with 1:10 of the pooled human serum. The blots were next incubated for 2 hours with 1:1000 alkaline phosphatase-conjugated goat anti-human IgE and developed with alkaline phosphatase substrate until bands were well visualized.

Inhibition ELISA

Aliquots of a 10 µg/ml solution (200 µl/well) of each *Alternaria* extract in 0.15 mol/L bicarbonate buffer pH 9.6 were adsorbed to wells of polystyrene microtiter plates (Immulon 2, Dynatech, Chantilly, Va.) at 37° C overnight. Coated plates were then washed with phosphate-buffered saline-Tween 20 that contained 0.01% sodium azide and 0.1% gelatin. Serial half-log dilutions of the inhibiting antigen were added to a final volume of 100 µl per well. An equal volume of pooled sera, which was diluted in phosphate-buffered saline, Tween-20, and azide buffer, from

Alternaria-sensitive individuals was then added to each well for a total volume of 200 µl per well and allowed to incubate at room temperature for 4 hours. The plates were washed, and 100 µl per well of 1:1000 alkaline phosphatase-conjugated goat anti-human IgG (Sigma) or 1:1000 rabbit anti-human IgE (Sigma) was added overnight. The latter was followed by addition of 100 µl per well 1:1000 alkaline phosphatase-conjugated goat anti-rabbit IgG. All plates were washed in phosphate-buffered saline, Tween-20, and azide buffer between steps. After addition of 1 mg/ml p-nitrophenyl phosphate, the color was allowed to develop until an internal standard read 1.0 absorbency units as measured at an optical density of 405 nm with a Titertek Multiscan (Flow Labs, McLean, Va.). The results were expressed as percent inhibition of the optical density at 405 nm versus inhibitor concentration.¹⁵

Determination of enzyme activities

Enzyme activities of extracted *Alternaria* preparations were detected with a commercial kit (ApiZym, Analab Products, Plainview, N.Y.). We followed the procedure as recommended by the manufacturer. Antigens were applied at a concentration of 10 mg dry weight per milliliter, and the reactions were read with the use of a color chart supplied with the kit. The scale ranged from 0 to 5, with 0 representing no color change and 5 representing a strong color change, which indicated the most active preparations.

Total proteolytic activities

Total endoprotease activity of extracted materials was determined by their ability to degrade fibrin mats. Soluble fibrinogen and thrombin were purchased as a kit (Boehringer Mannheim, Indianapolis, Ind.). Soluble fibrinogen was activated to fibrin with thrombin and placed in a Petri dish to a depth of 1 mm. After the opaque fibrin mat had formed, 5 µl aliquots of the *Alternaria* extracts were placed on the surface. In addition, 5 µl aliquots of serial dilutions of a commercial pronase (Sigma) preparation with known international units per milliliter was applied to provide a standard curve. The dishes were then incubated at 37° C in a humidity chamber for 1 hour. Diameters of the lysis areas for the pronase control within the fibrin mats were measured and plotted against the log of the known enzyme activities. The diameters of the unknown preparations were next compared with the standard curve and their activities, expressed as international units per milliliter, were calculated by linear regression.

TABLE II. Extractable dry weight per gram of dry mycelium, protein, carbohydrate, and carbohydrate to protein ratio of eluted material from *Alternaria* extracted continuously in four buffers for 1 and 24 hours

Buffer	Extraction time	Extractable weight per gram of mycelium (mg/gm)	Carbohydrate (mg/ml)	Protein (mg/ml)	CHO/PRO
Sodium acetate	1 hr	13	4.5	2.5	1.8
	24 hr	12	4.1	1.3	3.2
TBS	1 hr	15	4.7	2.3	2.0
	24 hr	19	4.8	2.1	2.3
Sodium bicarbonate	1 hr	15	4.4	2.0	2.2
	24 hr	22	4.5	2.6	1.7
Deionized water	1 hr	21	5.1	4.5	1.1
	24 hr	17	4.3	2.2	2.0

CHO/PRO, Carbohydrate to protein ratio.

TABLE III. Enzyme activities of preparations extracted in four buffers for 1 or 24 hours measured with an ApiZym kit

Buffer	Extraction time	Alkaline phosphatase	Acid phosphatase	Leu amino peptidase	α -galactosidase	N-acetyl glucosaminidase
Sodium acetate	1 hr	3	5	2	3	3
	24 hr	3	4	3	2	3
TBS	1 hr	4	4	3	3	4
	24 hr	5	5	3	3	3
Sodium bicarbonate	1 hr	4	5	3	3	4
	24 hr	3	5	2	3	3
Deionized water	1 hr	3	4	3	2	3
	24 hr	5	5	3	4	4

Results are expressed on a scale from 0 to 5 and are based on a concentration of 10 mg dry weight of extract per milliliter.

RESULTS

Effect of extraction buffer, elution time, and protease inhibitors

Table I shows the pH of each buffer during extraction. The final concentration of electrolytes for each extract approximated the initial values of the buffers alone. Distilled water extracts contained undetectable amounts of sodium, 35 mmol/L of potassium, 43 mmol/L chloride, and an osmolality of 189 mOsm. Though the initial pH values differed over a range of 5.0 to 8.6 among the buffers, the final pH values after 24 hours of continuous extraction exhibited a narrower range of 6.5 to 7.4 (Table I). The dry weight of extractable material was least for the acetate buffer and ranged from 15 to 22 mg/gm for the other three buffers. There was no consistent pattern of extractable weight (Table II). In addition, the carbohydrate to protein ratios ranged from 1.1 to 3.2 for extractable materials from the four buffers. The sodium acetate and TBS extracted the most carbohydrate, whereas sodium bicarbonate and deionized water tended to

extract more protein. ApiZym enzyme profiles were similar in that each extract had high activities of alkaline and acid phosphatases, leucine amino peptidase, α -galactosidase, and N-acetyl glucosaminidase (Table III).

SDS-PAGE and IgE immunoblot of *Alternaria* extracted in the four solutions for 1 and 24 hours are shown in Fig. 2. There are no clear differences for glycoproteins in the low molecular weight range; however, high molecular weight proteins were less abundant when extracted in TBS and in deionized water for 24 hours. Also, a 50 kd band is only seen in acetate after 1 hour and not after 24 hours. A 30 kd band, which is more prominent at 1 hour, is seen in all IgE blots. There was also an allergen at about 140 kd in the bicarbonate extract and a 70 kd allergen in the TBS. The presence of phenylmethyl sulfonyl fluoride (PMSF) (a protease inhibitor) did increase the number of high molecular weight moieties, as shown in Fig. 3. In particular, two groups of glycoproteins at pH 7.0 with molecular weights of 90 and 65 are visible

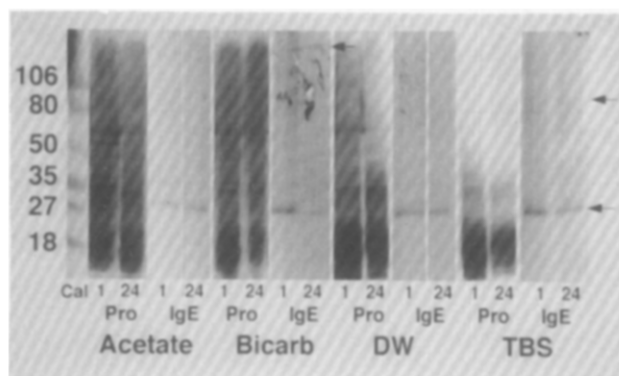


FIG. 2. *Alternaria* extracted in sodium acetate (pH 5.0), sodium bicarbonate (pH 8.6), TBS (pH 7.4), and deionized water for 1 and for 24 hours continuously.

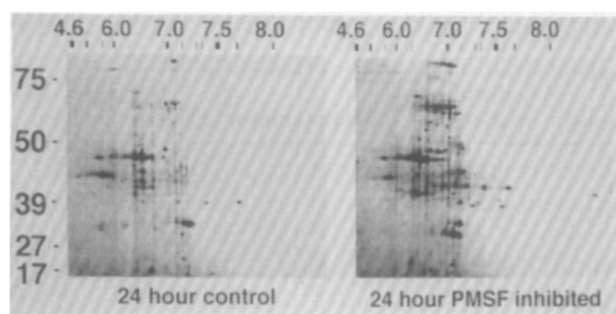


FIG. 3. Silver stain of two-dimensional electrophoresis of *Alternaria* strain A extracted with and without PMSF protease inhibition.

at 24 hours with PMSF and not in the control without the protease inhibitor. This was true for all four strains after 1 and 24 hours, though it was more pronounced at 24 hours. Strain A at 24 hours is shown in the Fig. 3 because it is representative of the other patterns and most clearly shows the differences.

Interval (residual) extraction

The carbohydrate to protein ratio tended to decrease between 1 and 6 hours for strains A and B, to remain the same for strains C and D, and to increase at 24 hours for all of the strains (Table IV). The highest carbohydrate to protein ratio was found in all of the 24-hour preparations. There was no consistent relationship between protein or carbohydrate content and extraction time, though the protein did drop at 24 hours for strains A and D. Carbohydrate content was consistently the highest in 24-hour preparations, with the exception of strain D. The total dry weight eluted was greatest during the first hour and was least between 6 and 24 hours, which suggests that most of the extractable material is eluted early. The elutable

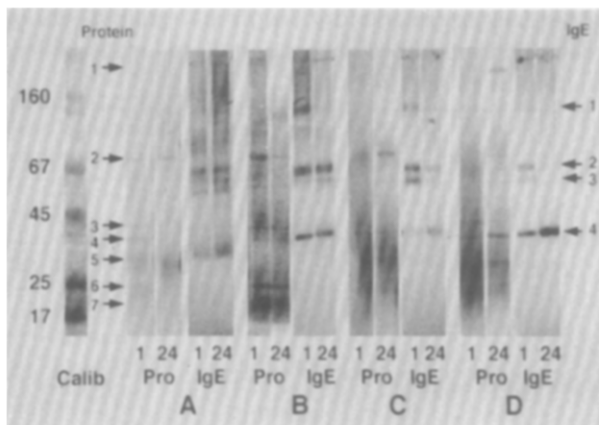


FIG. 4. Protein and IgE immunoblot of SDS-PAGE of extracts eluted for 1 and 24 hours.

dry weight decreased with time when compared with protein and carbohydrate content, which suggests that substances that are not measured by either of these assays (e.g., chitin) tend to be eluted early.

Total proteolytic activities of the continuously extracted preparations were not consistent. It should be noted that though proteases were inhibited by PMSF before the preparations were desalted and concentrated, the inhibitory action did not remain after these procedures were completed. Inhibition of enzymes during extraction may have resulted in lower total protein content for these extractions and possibly a less potent extract. The lowest proteolytic activity was in the 24-hour fractions. As shown in Table IV, protease activity did not correlate with total protein ($r^2 < 0.01$; $p = \text{NS}$), carbohydrate ($r^2 = 0.17$; $p = \text{NS}$), or dry weight ($r^2 < 0.01$; $p = \text{NS}$) by linear regression.

One-dimensional protein electrophoresis and IgE immunoblots (Fig. 4) showed a remarkable and consistent difference between protein and IgE-recognized bands. As a rule, bands that were detected by silver stain were not detected by IgE, and conversely, IgE bands did not appear on the protein stains. In addition, clear differences in protein composition and IgE binding patterns for each strain were seen at different times. As can be seen in Fig. 4, protein bands (arrows to the left) were present at >160 kd (arrow 1) in strains B and D; 70 kd (arrow 2) in strains A, B, and C; 42 and 40 kd (arrows 3 and 4) in strains B, C, and D; 31 kd (arrow 5) in strains A, C, and D; and 25 and 22 kd (arrows 6 and 7) in strains A and B. The darkness of these bands varied considerably for the different time periods. For example, the prominent 70 kd protein band in strains A and C is minimally observed in the 1-hour sample but quite evident in the 24-hour one. Just the opposite is seen in strain B.

TABLE IV. Dry weight of total extractable material (starting with 10 gm dried mold per fraction eluted in deionized water), carbohydrate, protein, carbohydrate to protein ratio, and protease activity determined by fibrin digestion

Strain	Extraction time	Extractable dry weight (mg)	Carbohydrate (mg/ml)	Protein (mg/ml)	CHO/PRO	Protease activity (IU/ml)
A	1 hr	212	3.0	4.0	0.8	0.2
	3 hr	83	2.7	4.0	0.7	0.3
	6 hr	82	2.1	4.6	0.5	0.3
	24 hr	40	3.4	2.9	1.2	0.1
B	1 hr	430	3.6	2.9	1.2	0.4
	3 hr	180	2.9	3.5	0.5	0.7
	6 hr	98	2.9	5.5	0.5	1.5
	24 hr	75	3.8	3.6	1.1	0.2
C	1 hr	262	3.3	2.7	1.2	0.2
	3 hr	108	3.1	2.4	1.3	1.1
	6 hr	98	2.6	2.5	1.1	2.6
	24 hr	75	4.1	2.6	1.6	0.1
D	1 hr	360	3.5	3.4	1.0	0.6
	3 hr	164	3.8	3.4	1.1	0.1
	6 hr	106	4.0	3.2	1.2	0.1
	24	75	3.5	1.8	2.0	0.0

CHO/PRO, Carbohydrate to protein ratio.

Staining of the 42 kd band is increased between the 1-hour and 24-hour extractions of D, whereas it decreases in strain A. The 25 and 22 kd bands are only visible in strain A at 1 hour, whereas strain B exhibits these bands at 1 and 24 hours.

The IgE immunoblot strips that were probed after 1 and 24 hours of elution demonstrated clear differences in extracted allergens (Fig. 4, *arrows to the right*). A dark band was seen at 140 kd (*arrow 1*) for strains B and C, though this band was more prominent at 1 hour. Two bands at 67 and 65 kd (*arrows 2 and 3*) were seen in all of the strains at 1 hour; however, in the 24-hour extracts they were attenuated in strains C and D. Strains B and D had a very strong band at about 42 kd (*arrow 4*), which was darker at 24 hours. There was very little IgE binding to glycoproteins below 31 kd.

Two-dimensional electrophoresis of strain A (Fig. 5) was able to resolve the extracted proteins into over 50 discrete spots. The majority of spots for all four strains are located below 25 kd. There is a general trend for the number of individual proteins to be greatest in the 6-hour extracts and to be somewhat diminished in the 24-hour samples. A strongly acidic protein at 21 kd is seen in all of the timed extracts. There are also specific quantitative differences in protein content between the timed extracts. In strain D (Fig. 6) when the 6-hour extract is compared with the 24-hour extract, the general trend is for the spots to increase in intensity. A notable exception is the acid region spot

at 67 kd, which is more intense in the 24-hour sample. The interstrain similarities are also remarkable, with at least five major spots clearly recognizable in all of the time intervals for all of the strains. Quantitative differences between strains are also clearly seen not only in the appearance of minor elements but also in the relative amounts of the major common elements.

IgE immunoblot from a two-dimensional electrophoresis of strain A reveals three recognized spots (Fig. 7). These are located at 70, 45, and 17 kd. Because these gels are more strongly reduced, the 17 kd may represent the reduced form of a 31 kd allergen reported previously, which does reduce to 17 kd.⁸ The 70 kd spot also appears to correspond to an allergen described previously.⁹ It is interesting that despite the large number of protein spots, only a very limited number are recognized by human IgE.

ELISA inhibition with IgG and IgE from allergic patients revealed remarkably little difference between the fractions for each time interval (Fig. 8) even though significant qualitative differences were seen with electrophoresis. There was also very little difference between the IgG and IgE ELISA inhibition curves. The fractions all inhibited IgE to essentially the same degree.

DISCUSSION

We performed these experiments in part to determine whether an "optimal" extraction time and buffer can be identified for preparing extracts of *Alternaria*.

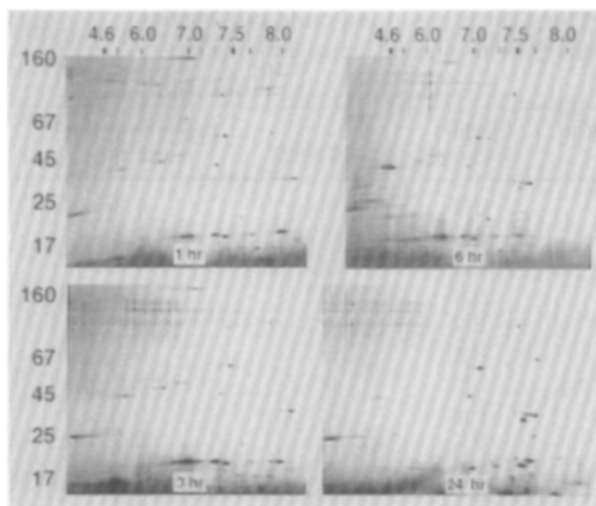


FIG. 5. Protein stain of two-dimensional electrophoresis of strain A at different elution times.

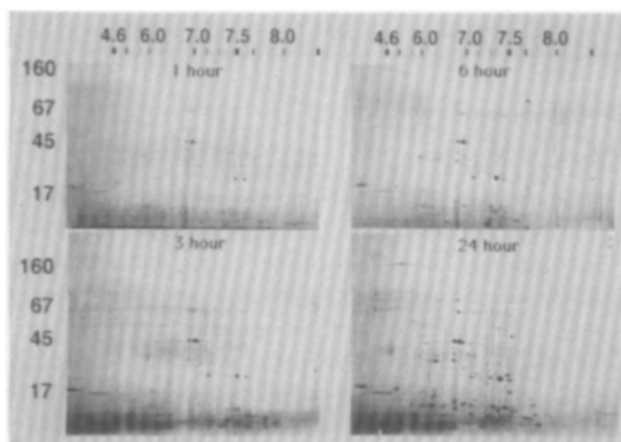


FIG. 6. Protein stain of two-dimensional electrophoresis of strain D at different elution times.

In doing so, we found that there is a specific optimal time of extraction for individual allergens; however, we could not determine a single best time of extraction for all allergens or for whole biologic activity. Extraction buffer concentration and pH do not seem to make much difference except that the low pH buffer resulted in a lower yield. In fact, deionized water appears to work as well as the more traditional bicarbonate extraction buffer. This is confirmed by the fact that each of the major allergens could be extracted by deionized water, given the right extraction time. Because individual allergens did not appear in extracts during continuous elution, production of individual allergens was better accomplished by the interval or residual extraction method, which did result in preparations enhanced for individual allergens.

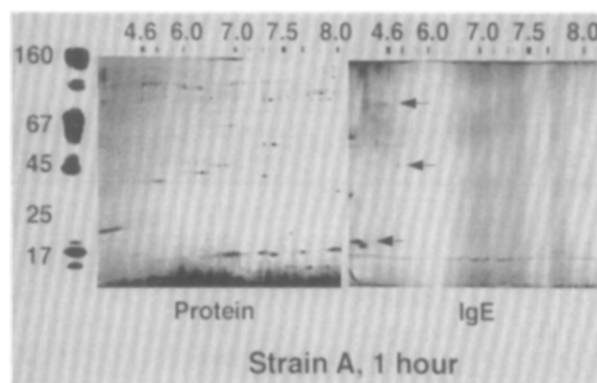


FIG. 7. Two-dimensional electrophoresis of four timed extracts of strain A revealing total protein and IgE recognized glycoproteins by immunoblot. The three arrows correspond to a low pH 70 kDa dot, a 45 kDa dot, and a low molecular weight doublet at approximately 29 kDa. One or more of these three dots appeared in the majority of two-dimensional IgE immunoblots that were performed.

Esch¹⁶ suggested that active proteases that remain in the *Alternaria* extracts for significant periods of time after extraction might have an effect on elution of glycoproteins. Inhibition by PMSF did result in greater numbers of high molecular weight proteins, but none of them appeared to be major allergens of *Alternaria*. Paris et al.⁸ noted no change in the 31 kd allergen eluted with differing extraction media or in the presence of protease inhibitors. Though they did conclude that the best extraction method utilized a carbonate buffer that contains additives to prevent precipitation and enzymatic hydrolysis of proteins, the reasons for their conclusion were not completely clear. Though we did not use proteolytic enzyme inhibitors for most of the extracts in this study, we do assume that one of the reasons for differing protein elution with time is the varying exposure to fungal proteases.

Immunologic activities were similar for each of the timed fractions as measured by both IgE and IgG ELISA inhibition. This suggests that total allergen content of all extracts is sufficient to bind a similar quantity of IgE from allergic individuals despite the fact that qualitative differences are demonstrable by IgE immunoblotting. Since these qualitative differences are present, measurement of total IgE activity directed toward *Alternaria* for standardization might be inappropriate, since individuals might recognize only one of the allergens.

Our one-dimensional SDS-PAGE patterns for whole *Alternaria* were similar to those of Paris et al.,⁸ Wahl et al.⁵ and Kroutil and Bush.¹⁸ This was particularly true for proteins that range from 15 to 100 kd. The silver staining technique that we used does tend to reveal a larger number of bands than the results that

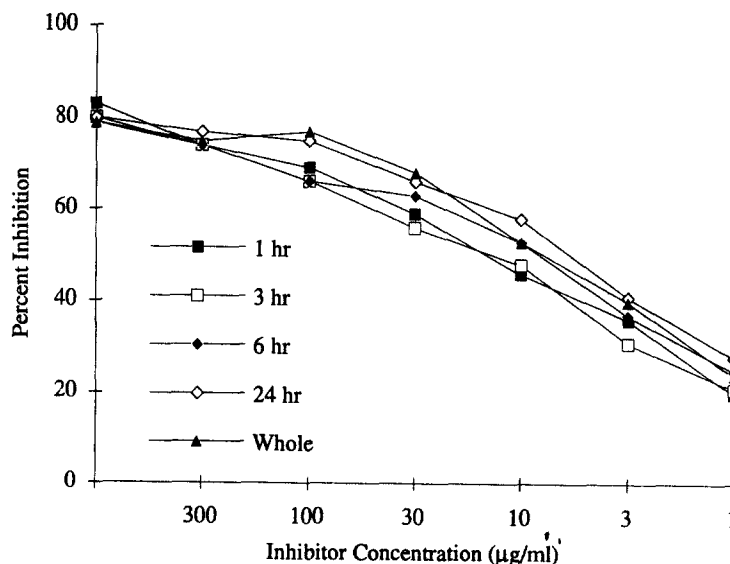


FIG. 8. IgE and IgG inhibition ELISA for strain A. The other three strains produced virtually identical patterns of inhibition.

were obtained with Coomassie blue by other investigators; however, the general protein patterns are quite similar. The IgE blotting pattern we obtained is also consistent with the results of Paris et al.⁸ in that allergens of varying sizes are found especially in the molecular weight range above 30 kd. A major low molecular weight allergen seen in this work does appear to be above the 31 kd allergen of Paris et al.⁸ in three of our strains, but in one strain the major low molecular weight allergen is at about 31 kd. Our observations also differ from theirs in that a second major allergen in the 70 kd range was observed in many of the extracts. This seems to be consistent with the IgE binding region reported by Kroutil and Bush.¹⁸ Our blots detected allergens of higher molecular weight than those of Karlsson-Borga et al.¹⁹ who eluted materials for a shorter time of 1 hour with column desalting as opposed to Paris et al.⁸ and Kroutil and Bush¹⁸ who extracted for more than 12 hours and then performed overnight dialysis. Our results indicate that for some strains, there is greater production of high molecular weight material in the shorter extraction periods.

Yunginger et al.²⁰ described a 31 kd allergen that was heterogeneous according to both SDS-PAGE and thin-layer electrofocusing. We found that the IgE binding material in this molecular weight range appears to be homogeneous for three strains and heterogeneous for strain A. This same material was essentially a smear by SDS-PAGE detected by silver stain. We were able to resolve this material into two lower molecular weight spots with two-dimensional electrophoresis.

Many of the *Alternaria* extracts displayed a high background and produced few distinct bands when examined by one-dimensional electrophoresis. Two-dimensional electrophoresis was able to resolve this "background" into discrete protein components, which makes the technique almost essential for evaluation of these extremely complex mixtures. With two-dimensional SDS-PAGE the major shared elements of the four strains could be identified. Also, the appearance and disappearance of protein elements, probably as a result of proteolytic action,¹⁷ could be followed.

Two-dimensional electrophoresis appears to be a good method for resolving complex extracts into discrete components. Comparison of the one- and two-dimensional IgE immunoblots indicates that the 30 to 33 kd allergen found on the one-dimensional blot seemed to be replaced with two bands in the 17 kd range on the two-dimensional blots. Also, the 70 kd allergen could be one or more of as many as six individual spots. These observations for *Alternaria* are consistent with those of Olsen et al.²¹ who demonstrated that a single band on one-dimensional SDS-PAGE could be resolved into multiple IgE-recognized spots on the second dimension for *Poa p* allergens.

It seems likely that extraction conditions could be optimized for production of a particular glycoprotein. For example, an IgE-recognized 67 kd band was more prominent in the 1-hour extracts than in preparations eluted for longer times, which suggests that it is eluted rapidly (Fig. 4). To produce quantities of this glycoprotein, one would therefore start with strain B or C and elute for 1 hour. On the other hand, to produce

quantities of the 42 kd allergen, one would start with strain B or D and collect the 24-hour elution. The 140 kd allergen can only be obtained from strain B extracted for 1 hour. Of course, these observations are based on electrophoretic data and not on quantitative measurement of the specific allergens. A more exact determination of the ideal conditions for production of specific glycoproteins must therefore wait until accurate and sensitive assays for their measurement are available. The main point is that conditions can be optimized for their production.

CONCLUSION

Extraction time, strain differences, and to a much lesser degree, extraction buffers, do qualitatively and quantitatively affect the contents of *Alternaria* preparations. Total biologic activity does not seem to vary as much as the concentrations of individual glycoproteins. Because standardization of allergenic extracts is ultimately a goal of this type of research, it is essential that qualitative differences be considered when biologic activities of "standardized" extracts are measured. Use of specific elution conditions, which must be determined empirically, may be useful for production of certain desired glycoproteins. In addition, quantitative methods for assaying individual glycoproteins in *Alternaria* must be developed to assist in optimization of these conditions.

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