

Studies on the Atopic Allergen in Ripe Tomato Fruits

I. Isolation and Identification of the Allergen

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Introduction

In recent years much attention has been given to the chemistry of inhalant allergens. The allergens hitherto studied proved to be complex mixtures of carbohydrate-protein conjugates with characteristic chemical properties. The remarkable finding of N-glycosidic linkages (3, 4, 13, 11) between peptide moiety and sugar constituents, moreover, largely explains the unusual properties of purified atopic allergens (2).

Since in patients with an atopic disease positive skin tests to extracts of foodstuffs are of frequent occurrence, the isolation and identification of the deleterious agents in foods leading to hypersensitivity phenomena in atopic patients is of considerable importance. Furthermore, the literature provides evidence that foodstuffs may not only contain substances giving rise to hypersensitivity phenomena triggered off by the interaction of atopic allergens and reagins (IgA-class antibodies), but may also contain compounds responsible for delayed type reactions mediated by sensitized cells as well as antigens inducing immediate type anaphylactic reactions involving IgM or IgG-class antibody (7). The purification and characterization of the substances in foods responsible for these different hypersensitivity phenomena may probably lead to a better understanding of the underlying immunochemical mechanisms and of the relevant clinical manifestations of food allergy. Until now very little is known about the chemical nature of these various agents.

Confining ourselves to food allergy in atopic patients we started in 1963 with the purification and chemical identification of

the atopic allergens in foods. A preliminary investigation by BERRENS and YOUNG (5) on the allergen in ripe tomato fruits indicated the probable glycoprotein nature of the specific allergen. An extension of this work, proving the active fraction from ripe tomato fruit to be a polydisperse mixture of carbohydrate-protein complexes will be reported here.

Materials and Methods

Tomatoes. The allergen was extracted from whole red ripe tomatoes. Extraction and purification procedures were carried out in the cold (4° C), unless otherwise stated.

Analytical Methods

Protein nitrogen was determined by the microKjeldahl method, total hexoses by the orcinol-sulphuric acid method (9) and uronic acid by the Dische-carbazole-method (10).

Agar gel electrophoresis was carried out on microscope slides as outlined by WIEME (15) at 220 Volts and 20 mA per slide (15 min) using a veronal buffer pH 8.6, $\mu = 0.04$; fixation in ethanol : acetic acid : water (90:5:5 v/v); stains: Naphtol Blue Black for protein, periodate-fuchsin Schiff for carbohydrate. Sedimentation studies were performed with 1% solutions in 1% NaCl in a Spinco Model E analytical ultracentrifuge. Pictures were taken after the rotor had reached full speed (59,780 r.p.m.); temperature 16° C, angle 50°. Ultraviolet absorption spectra were determined of 0.05% solutions in 0.01 M-phosphate buffer at pH 7 and room temperature against the same buffer as a blank, using a Zeiss spectrophotometer type PMQ II equipped with quartz cells of 1 cm light path.

Hydrolysis and Chromatography

The constituent monosaccharides of the carbohydrate moiety of the allergen fraction were identified after hydrolysis on cation exchange resin (Dowex 50W $\times$ 8, H⁺ form) in aqueous suspension at 100° C for 16 hours. The resin was removed by filtration and washed twice with water. The supernatant and washings were combined and taken to dryness under vacuum over NaOH pellets in a desiccator. One-dimensional chromatographic separation of sugars was effected on Whatman No. 1 paper using two different solvent systems, viz. ethylacetate-pyridine-water (8:2:1 v/v) and *n*-butanol-acetic acid-water (4:1:5 v/v upper layer). The position of the sugars on the chromatograms was detected with aniline hydrogen phthalate; glucose and xylose were used as reference standards. The overall amino acid composition was determined after hydrolysis with 6 N.HCl in sealed tubes at 100° C during 24 h in an automatic recording apparatus of the type described by SPACKMAN, STEIN and MOORE (12).

Column Chromatography

DEAE-Sephadex (A 50 medium grade) was prepared for use by repeated washings with 0.5 M.NaOH and 0.5 M.HCl and subsequent rinses with water, during which the fines were decanted. The resin was packed into a column provided with a cooling jacket (20 $\times$ 1 cm) and washed with phosphate buffer 0.01 M., pH 7. A solution of the allergen (2% in the same buffer) was then applied to the column and elution was performed at 4° C with a phosphate buffer incorporating increasing amounts of NaCl. Fractions of 5 ml were collected at a rate of about 60 ml/h, and the extinction of the eluted fractions was read at 275 nm; for the determination of carbohydrates 0.5 ml of each fraction was withdrawn and analyzed for hexoses, applying the orcinol-sulphuric acid method (9).

Isolation and Purification Procedure

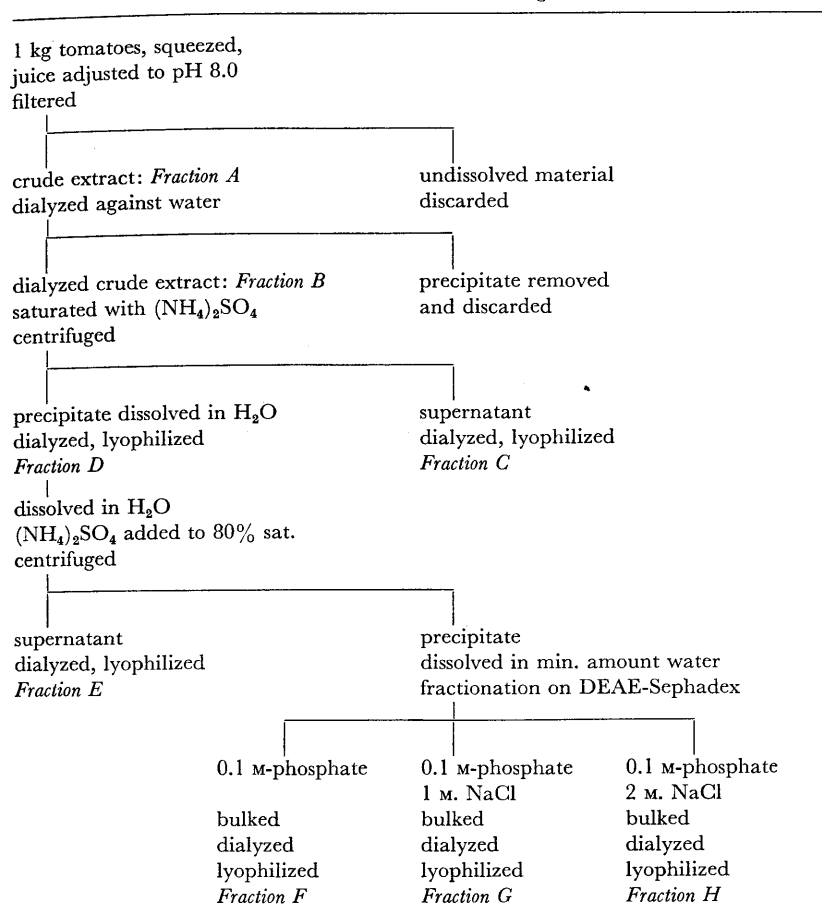
Red ripe tomatoes were washed with water, cut into 4–8 pieces and homogenized in a Waring-Blendor for 10 minutes. The tomato juice was transferred to glass containers, stirred thoroughly and the pH raised from about 4.5 to 8 with 4 N NaOH; a few ml of toluene were added for preservation. The squeezed tomatoes were then filtered overnight through cheese-cloth; additional filtration over filter aid (Hyflo Supercel) on a Buchner funnel proved necessary for the separation of large quantities of pectine material from the allergen-containing extract. The opalescent yellow filtrate (A) was dialyzed against repeated changes of water in the cold. After two days a grey white precipitate had formed that became denser upon prolonged dialysis. Dialysis was therefore continued for two other days and the precipitate of (eu)globulins and hemicelluloses was removed by centrifugation. Neither the precipitate, nor the dialyzate, encompassing the low-molecular weight material (salts, amino acids, sugars, peptides) contained allergenic material as revealed by skin tests in patients with an atopic hypersensitivity to tomatoes (see section 4); these fractions were discarded. The supernatant (B) was chilled to 4°, the pH adjusted to 6, and solid $(\text{NH}_4)_2\text{SO}_4$ was added with stirring to a degree of 100% saturation. The mixture was allowed to stand in the refrigerator overnight, when the bulk of the protein material had precipitated. The dense brown precipitate was separated by centrifugation and the clear supernatant was kept for further dialysis (C). The precipitate was dissolved in 0.1 M-phosphate buffer pH 7 and dialyzed until free of salt. The solution was then centrifuged to remove a minimal amount of precipitated material and dried by lyophilization; it yielded 100 mg/kg tomatoes of an easily soluble brown substance (D), i. e. about 0.7% by weight of the total amount of material dissolved (A).

Fraction D proved to be still inhomogeneous; agar electrophoresis revealed at least four protein bands: together with two not clearly separated diffuse bands on the positive side of the slide, three bands on the negative side (Fig. 1a). Preceding chemical studies indicated that besides the (allergenic) glycoprotein this fraction also contained free carbohydrates and globulins. Fraction D was therefore subjected to several purification procedures, e. g. fractionation by precipitation with cetyltrimethylammoniumbromide, ammonium sulphate, acetone and ethanol and by precipitation at the isoelectric point (pH 3–4). All these methods proved to be far from selective. By repeating the precipitation with $(\text{NH}_4)_2\text{SO}_4$ and subsequent chromatography on DEAE-Sephadex, however, the skin-reactive glycoprotein could be separated from contaminating protein and carbohydrate.

The following procedure was used routinely: 250 mg of D was dissolved in 250 ml of water. The solution was chilled to 4° C, the pH adjusted to 6 and some insoluble material was removed by filtration; solid $(\text{NH}_4)_2\text{SO}_4$ was then added with vigorous stirring to 80% saturation. The brown precipitate was separated after 16 h by centrifugation, dissolved in a minimum amount of distilled water, dialyzed against several changes of water for 24 h and subsequently against 0.01 M-phosphate buffer pH 7 for 16 h: the retentate was then applied to a 20 cm column packed with DEAE-Sephadex. The column was pretreated with 20 ml buffer 0.01 M. pH 7. Fractionation was performed by stepwise elution with 75 ml of phosphate buffer 0.1 M. pH 7, 100 ml of buffer + sodium chloride (1 M.) and 50 ml of buffer containing 2.0 M. NaCl respectively. The plots of u. v.-absorption at 275 nm and of 505 nm absorption for hexose content has been shown in Fig. 2. The bulk of brown material is eluted from the column at 1 M. NaCl; some residual brown substance remained on the column and could not be completely removed. Fractions were bulked according to the peaks, dialyzed and freeze-dried.

The substance eluted in the first peak (F, 60 mg) consisted of globulins and unbound neutral carbohydrate, while the second peak (G, 60 mg), eluted with 1 M. NaCl, contained the active allergenic glycoprotein (compare Fig. 2c); some residual brown

Table I
Fractionation of tomato allergen.



(inactive) material was eluted in the third peak. The yield of active material (G) is 20–30 mg of allergen per kg tomatoes, i. e. about 0.15% by weight of the total amount of extractable solids and 4% of the soluble high molecular weight material. Attempts at further purifications of G designed for the isolation of an electrophoretically more homogeneous compound, or of a more active fraction, were unsuccessful.

The fractionation procedure, which was guided by skin tests, has been summarized in Table I.

Clinical Results

The skin-reactive potency of the isolated fractions was assayed in nine atopic patients with a positive intracutaneous immediate-type reaction to a commercial tomato extract (Bencard). These

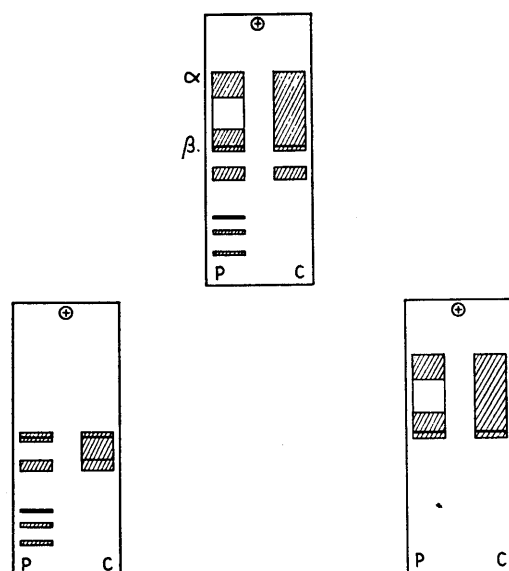


Fig. 1. Schematic representation of the results of agar electrophoresis of the fractions: D top (1a); F bottom-left (1b); and G bottom-right (1c). C Periodate-fuchsin Schiff stain, P Naphtol Blue Black stain.

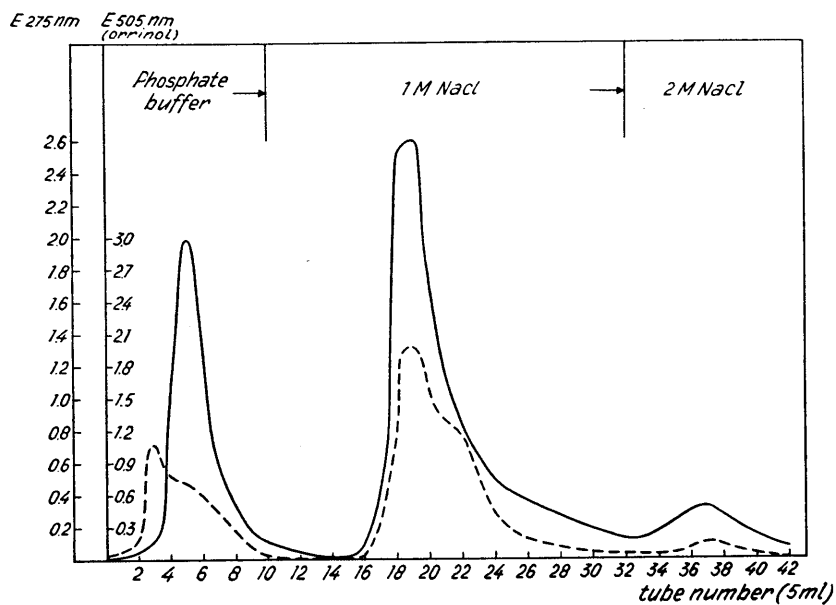


Fig. 2. Chromatography of fraction D, on a column of DEAE-Sephadex (20 x 1 cm) using a phosphate buffer (0.1 M., pH 7) with increasing concentrations of sodium chloride. Smooth curve: u. v.-absorption at 275 nm. Dotted curve: 505 nm absorption for hexose content.

patients also gave positive skin reactions with a number of inhalant and food allergens. They had an atopic disease, viz. angioedema, rhinitis, urticaria, atopic dermatitis or bronchial asthma. The data of these patients are recorded in Table II.

Skin tests were performed as previously described (6): sterilized stock solutions containing 1 mg of freeze-dried material in 1 ml of a pH 7 physiological buffer were diluted serially and 0.1 ml of an appropriate solution was injected by the intracutaneous route. Reactions were read after 20 min by skilled independent persons and have been graded $\pm$ through 3+. In order to enable a comparison of the relative potency of these fractions on a (semi) quantitative basis the activity was expressed in units per mg of dry weight. A unit of allergenic activity was defined to be that amount of substance giving an average reaction of 1+. Furthermore, there exists an experimentally established semi-logarithmic relationship between apparent activity (or allergen concentration) and skin reaction. Therefore, if a 1+ reaction is defined to be caused by 1 unit, a 2+ reaction will be obtained by the injection of 10 units, a 3+ reaction by the injection of 100 units of activity. The activity per mg was calculated by multiplying the average activity obtained for the standard dilution with the dilution factor and, 0.1 ml being injected, with a factor of ten. The relative activities and the yields of the fractions are recorded in Table III.

Table III illustrates that the specific allergen is concentrated in fraction G eluted from the column at 1 M. NaCl; the other fractions (e. g. E, F and H) contain only residual activity. The recovery of activity is about 60%. A considerable loss of activity occurs during column chromatography, probably because of inactivation by oxidation on the surface of the Sephadex particles (c. f. 1). Column fractionation performed at room temperature led to even more pronounced inactivation. The purified tomato allergen fraction is 375 times more active than the undialyzed extract and about 20 times more active than the extractable high molecular weight material. A relatively large amount of aspecific material (pectines, enzymes, irritant substances) is being removed during the fractionation process.

Table II
Clinical data of patients, tested for skin-reactivity with various tomato fractions

Sex	Age	Clinical symptoms of atopy	Blood eosinophils number per mm ³	Family history of atopy	Inhalant allergens	positive skin-reactions to	Remarks
E. J. v. A.	F 13	angioedema rhinitis	344	+	Human dandruff, house dust, pollen, kapok	Food allergens*** tomatoes, bananas, oranges	angioedema after eating tomatoes and oranges
A. L. S.	F 24	angioedema urticaria	11	—	Human dandruff, house dust, pollen, kapok	fruits*, flour, vegetables	angioedema after eating fruits
A. Q.	M 18	rhinitis atopic dermatitis	187	+	Human dandruff, house dust, moulds, feathers	fruits*, flour, vegetables, fish	positive skin reactions also to tomato pollen. Rhinitis and conjunctivitis during work in tomato-hothouses
E. v. T.	F 16	angioedema urticaria	187	—	Human dandruff, house dust, pollen, moulds, kapok	fruits*, flour, vegetables, fish, shell-fish, egg, chocolate	angioedema after eating various foodstuffs
J. S.	F 12	atopic dermatitis	88	+	Human dandruff, house dust, moulds	tomatoes, spinach, potatoes	
A. J.	M 26	atopic dermatitis	264	—	Human dandruff, house dust, pollen, kapok	tomatoes, flour, fish, spinach, pork, milk, egg, chocolate	
A. J. M.	M 42	angioedema urticaria	178	+	Human dandruff, house dust, pollen, moulds, kapok, feathers	fruits*, flour, vegetables, shell-fish, chocolate	angioedema after eating various foodstuffs
A. H. V.	M 36	atopic dermatitis rhinitis	363	+	Human dandruff, house dust, pollen, moulds, kapok **	tomatoes, oranges	itching after eating tomatoes, positive skin reactions also to tomato pollen
A. V.	M 45	rhinitis bronchial asthma	297	+	Human dandruff, house dust, pollen, moulds	tomatoes, flour, vegetables	

* tomatoes included

** other foodstuffs not tested

*** where the presence of positive skin reactions to fish, shell-fish, fruits, flour and vegetables is indicated patients had positive skin reactions to most kinds of fish, fruits etc.

Table III

Fraction	Yield in mg/kg tomatoes	Skin reactivity U/mg	Total activity
A	16.000	20	320.000
B	600	450	270.000
C	450	none	—
D	100	2.700	270.000
E	30	20	600
F	25	150	3.750
G	25	7.500	187.500
H	2	20	40

Table IV
Analytical data.

Fraction	Protein N %*	Hexose %*	Uronic acid %*	Precipitate with 10% TCA** saturated picric acid	
A	1.2	20.0	3.9	±	±
B	5.3	28.1	10.2	+	+
C	1.0	36.0	10.0	—	—
D	8.5	22.5	7.5	+	+
E	2.3	30.0	10.0	—	—
F	10.2	12.6	1.5	+	+
G	8.5	21.0	8.0	—	(±)
H	not done	2.6	0.2	—	—

* Average values of four separate isolations ($\pm 20\%$)** — no precipitate, $\pm$ weak reaction, + precipitate*Chemical Results*

A. Examination of the Fractions

For the chemical characterization of the allergen the various fractions obtained were subjected to a number of analytical procedures and precipitation reactions. The results are collected in Table IV. All fractions contained protein material as well as bound uronic acids and hexoses.

The fractions B, D and G were obtained by lyophilization as pale brown powders which dissolved easily in buffer solutions. Fraction A, a brown hygroscopic substance, also dissolved in water. Fractions C and E were white powders, which after lyophilization were very difficult to dissolve in buffer solutions. Insoluble matter could be brought into solution by the addition of a few drops of 1 N. NaOH to aqueous suspensions. The latter two fractions largely contained carbohydrate material only (pectines and hemicelluloses).

As observed from Table IV large quantities of carbohydrate substances are removed during the fractionation process, being retained in the C- and E-fractions.

Fraction F, a grey-white substance, dissolved in buffer solutions but not in distilled water. Whereas, the preceding allergen-containing fractions A, B and D gave positive precipitation reactions with TCA and with piric acid, fraction G did not. Obviously, most of the protein material precipitating with these reagents remains in fraction F, containing 10.2% N, 12.6% hexoses and 1.5% uronic acids. Therefore, fraction F also contains carbohydrate, apart from the globulins mentioned before (Fig. 1b). The material is probably largely unbound to protein, as evident from the observation of the first carbohydrate peak in the elution pattern on DEAE-Sephadex (Fig. 2) not coinciding with the plot of 275 nm absorption.

B. Identification of the Active Allergen

Contrary to fraction F, the active allergen fraction G had all the characteristics of a glycoprotein. In aqueous medium the allergen gave mostly negative reactions typical of a protein solution; no precipitation was observed upon the addition of Esbach's reagent, 10% TCA, sulphosalicylic acid or two volumes of acetone. It may readily be precipitated from borate solutions at pH 9.5 with cetyltrimethylammonium bromide and with lead acetate. The allergen is remarkably stable at neutral pH. Solutions of fraction G may be heated for more than four hours at 100° before a slight precipitate appears. In alkaline solutions (pH 12) stability is much less pronounced. Solutions of G are weakly colloidal between pH 3 and 4.5. Above pH 5 clear pale yellow solutions are the result. The yellow colour deepened upon the addition of NaOH.

The isolated allergen, moreover, had a typical ultraviolet absorption spectrum that differed from those of other (glyco)-proteins: there is a marked absorption at wavelengths over 300 nm, the characteristic tryptophan-tyrosin maximum at 278 nm evident in the u. v. spectra of other proteins was not observed. These characteristic differences from normal spectra may become obvious from Fig. 3 where the u. v.-absorption curves have been given of fractions F and G; F has an almost normal spectrum.

The apparent deviation of these curves allows the deduction of other chromophores being present. However, we have as yet not

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been able to detect any bound plant pigments in the purified allergen preparations. An explanation for these findings will be given in a separate paper (8).

Fraction G contained 7–10% protein N. In Table V the amino acid composition is given for the two fractions F and G. If the encounter of all of the commonly occurring amino acids is taken as a criterion of completeness, the allergen seems to be constituted of a full protein. Slight differences may be observed in the molecular ratio between the amino acids of the two fractions: fraction G possesses more lysine, histidine and threonine and significantly less alanine and valine than fraction F.

Fraction G contained about 8.5% bound uronic acids and 21% hexoses. The following constituent monosaccharides could be identified by paper chromatography of resin hydrolysates: arabinose 4+, galactose 3+ and xylose 1+, together with galacturonic acid and/or glucuronic acid. The chromatograms were often very difficult to interpret because of tailing, and because large quantities of carbohydrate material remained at the point of application. Therefore, prolonged treatment with resin at 100° C for more than 24 h was attempted with additional hydrolysis of the water washings in 6 N. HCl at 100° C for 16 h. The same sugars were detected

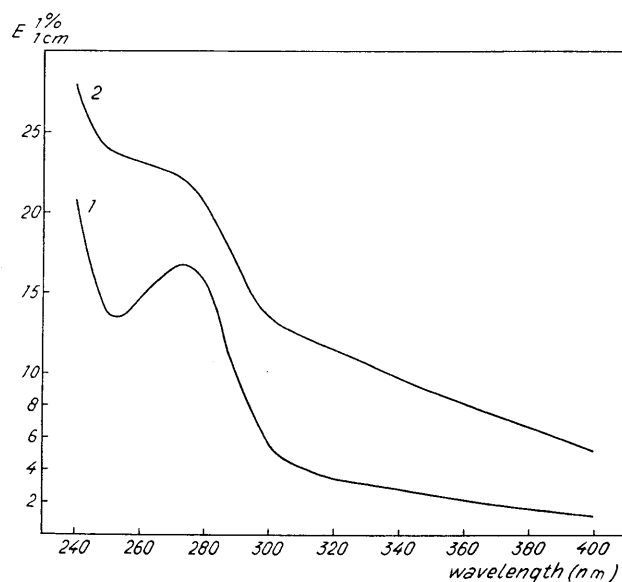


Fig. 3. Ultraviolet absorption spectra of fraction F (1) and fraction G (2) in phosphate buffer (0.01 M, pH 7).

Table V
Amino acid composition of fractions F and G (in mol. %).

Amino acid	F*	Fraction G*
Alanine	6.5	7.4
Arginine	2.5	2.3
Aspartic acid	13.3	12.6
Cysteine/cystine	+	+
Glutamic acid	10.7	10.2
Glycine	10.6	9.9
Histidine	0.65	1.3
Isoleucine	5.3	5.3
Leucine	9.6	8.9
Lysine	4.6	6.0
Phenylalanine	4.4	4.4
Proline	6.5	6.7
Serine	8.5	8.3
Threonine	8.2	6.2
Tryptophan	+	+
Tyrosine	3.4	3.3
Valine	5.1	7.3

* + present, assayed separately

in addition to minor traces of two sugars which could be identified as glucose and mannose. Even after treatment with resin for 30 h, hydrolysis was not complete and in the protein material absorbed to the resin – after elution with 3 N. NH_3 and subsequent hydrolysis with 6 N. HCl – sugars could still be detected. Furthermore, the periodate Schiff reaction was only very weak in spite of a high carbohydrate content. It seems likely therefore that the carbohydrate moiety of the allergens is highly branched.

The homogeneity of fraction G was examined by electrophoresis and sedimentation studies. Agar electrophoresis at pH 8.6 reveals the allergen to migrate towards the anode in two diffuse, not clearly separated bands. In two of the four preparations only one broad protein band was observed, extending from the point of application to the approximate position of the α -globulins of normal human serum subjected to electrophoresis under identical conditions. If stained with the carbohydrate-periodate-Schiff reagent only one diffuse band became visible overlapping the NH_2 -protein stainable material. Pretreatment with 4 M urea prior to electrophoresis did not affect the separation of the protein and carbohydrate moiety, which may therefore be concluded to be firmly

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Fig. 4. Sedimentation pattern of fraction G (1%) in 1% NaCl solution. Picture taken 51 min after reaching full speed (59,780 r.p.m.). Temp.: 16.0° C. Angle: 50°. Direction of sedimentation from right to left.

attached by covalent bonds. Under the conditions of electrophoresis on paper or in starch gel the allergen also migrates towards the anode in one band. On these electropherograms no distinct separate bands indicating admixture with protein impurities could be detected. However, the diffuse bands suggested the active fraction to be far from homogeneous. Apparently, the allergen preparation is a polydisperse mixture of closely related molecules with slightly different electrophoretic mobilities.

In the ultracentrifuge the allergen sedimented as a single peak and again, no contamination with compounds of a different molecular weight was found. The sedimentation peak was rather flat, suggestive of a fair degree of polydispersity (Fig. 4). The pictures were not sharp because of light absorbance by the allergen solution and it is noteworthy that the boundary between light and dark moved with the top of the curve, indicating that the chromophores are firmly attached to the allergen molecules.

The average sedimentation constant of fraction G was calculated at $S_{20} = 1.5\text{--}2.0$.

Discussion

Apparently, the main allergenic constituent (fraction G) of ripe tomato fruit has been isolated free of distinct impurities. The possibility should of course be considered of contamination with non-reactive molecules masking the active allergen, but in view of the reactivity of the allergen fraction relative to that of other atopic allergens this alternative is considered unlikely just now. It has, in fact, not been possible to carry the purification of fraction G, in terms of the removal of inactive molecules, to a further stage.

The analytical results and the chemical properties of the active fraction indicate the allergen in tomatoes to be a protein-carbohydrate complex. This view is supported by the results of electrophoresis and sedimentation studies, and is further sustained by the fact that, during rechromatography on DEAE-Sephadex, the peaks of u. v.-absorption coincide with the maxima in the carbohydrate plot. Despite the failure to detect distinct impurities the active allergen fraction proved to be heterogeneous, as judged from the patterns obtained during electrophoresis and ultracentrifugation. Consequently, the inference may be drawn that the purified atopic allergen, in fact, consists of a polydisperse mixture of closely related glycoproteins, rather than of one single molecular species; these closely similar glycoproteins mutually differ in net charge and in the degree of carbohydrate binding.

Remarkably, the chemical nature of the skin-reactive allergen from ripe tomato fruits closely resembles that of purified inhalant allergens from widely different sources, viz. ipecacuanha (4), cotton seed (13), ragweedpollen (11) or house dust (3). These inhalant allergens have also been found to be complex mixtures of protein-carbohydrate conjugates; many features of the physicochemical behaviour of these allergens have now also been encountered in the atopic allergen from tomatoes. Moreover, the sedimentation constant of the latter is of the order of magnitude expected for purified atopic allergens (14).

Although the characterization of the tomato allergen within the same class of protein-sugar compounds as described for inhalant allergens is considered of some importance, the discovery in itself does not seem to offer an explanation for cross-sensitization to inhalant and food allergens observed in atopy (Table II). However, on close examination of the active allergen molecules, the tomato allergen has been found to incorporate N-glycosidic linkages, which have been suggested to be a common link between atopic allergens in general (2). Proof for the incorporation of N-substituted 1-amino-1-deoxy-2-ketoses within the molecular structure of the allergenic glycoprotein in tomato fruits will be reported separately (8).

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Authors' add

Summary

A description is given of the purification of the specific atopic allergen in ripe tomato fruits. The purified allergen, containing 8.5% protein N, 21.0% combined hexoses and 8.0% uronic acids, was found to liberate all the commonly occurring amino acids upon hydrolysis, together with galacturonic acid, arabinose, galactose and xylose. Electrophoresis and sedimentation studies indicated the preparation to be a polydisperse mixture of closely related allergenic glycoproteins. The sedimentation constant was calculated at $S_{20} = 1.5-2.0$. The chemical nature of the skin-reactive allergen from ripe tomato fruits closely resembles that of purified inhalant allergens.

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