

Limited Proteolysis of Antigens E and K from Ragweed Pollen¹

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The two ragweed allergens, antigens E and K, are present in pollen as single-chain polypeptides of about 38,000 daltons. During isolation the single-chain precursor of antigen E, or that of antigen K, can undergo limited proteolysis to give other isoelectric forms containing two noncovalently held chains of about 12,000 and 26,000 daltons. Carboxypeptidase A and B digestions of the different forms of antigen E or K indicate that the protease(s) responsible for these conversions have a trypsin-like specificity. Bovine pancreatic trypsin can also effect these conversions. A protease which in ragweed pollen catalyzes the hydrolysis of *N*, α -benzoyl-L-arginine *p*-nitroanilide was partially purified, but it did not catalyze these conversions. The single-chain and two-chain forms of antigen E did not show detectable differences in their antigenic activities in rabbits or in their allergenic activities in humans. This was the case also for the different forms of antigen K.

Ragweed pollen contains a number of protein components that are responsible for causing late-summer hay fever in humans (cf. (1)). Its two major allergenic components are antigens E and K which are acidic proteins of about 38,000 daltons. Both antigens can be isolated in multiple isoelectric forms which are designated as A, B, C, and D in the order of their increasing acidity (2, 3). Our early experiments showed that the relative yields of the isoelectric forms of antigens E and K varied with different experiments. Recently we had occasion to isolate ragweed antigens in large amounts. That work prompted us to study more closely the chemical significance of these isoelectric forms of antigens E and K.

The results to be presented below suggest that the different isoelectric forms of antigens E and K are generated on limited proteolysis of their respective A forms. The presence of proteases in ragweed pol-

len has been reported (4, 5). One such protease which catalyzes the hydrolysis of *N*, α -benzoyl-L-arginine *p*-nitroanilide (L-BANA)² (5) was partially purified and characterized in this work, but this protease was found not to be the agent responsible for the limited proteolysis of A form of antigens E and K.

MATERIALS AND METHODS

Short ragweed pollen was from Greer Laboratories (Lenoir, North Carolina); the pollen used in this study was collected in Pennsylvania in 1979. Specific rabbit anti-ragweed, anti-antigen E, anti-antigen K sera (2, 3), and affinity column-purified rabbit anti-antigen E antibodies (6) were described previously. Immunosorbent for isolation of antigen E-specific antibodies was prepared with B or C forms of antigen E and no difference in results was observed. Bovine pancreatic trypsin and α -chymotrypsin, carboxypeptidase A, and DFP-treated carboxypeptidase B were from Worthington Biochemical Corporation. Thermolysin was from Calbiochem Corporation. Pyroglutamyl aminopeptidase was from Boehringer Mann-

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² Abbreviation used: L-BANA, *N*, α -benzoyl-L-arginine *p*-nitroanilide.

heim GmbH. L-BANA was from Sigma Chemical Company. Denatured hemoglobin for protease assay was from ICN Pharmaceuticals.

Rocket immunoelectrophoresis was carried out according to a published procedure (7), with minor modifications. Electrophoresis was carried out in 7.5% polyacrylamide slab gels ($0.1 \times 10 \times 10$ cm) containing Tris buffer (8), or in 12.5% gels containing Tris-sodium dodecyl sulfate buffer (9).

Peptidase activity was followed spectrophotometrically at 405 nm by its hydrolysis of 0.1 mM L-BANA in 7.5 mM sodium phosphate buffer (pH 7.2) containing 150 mM NaCl. One unit of activity represents the hydrolysis of one micromole of substrate per minute at $25 \pm 2^\circ\text{C}$.

Carboxypeptidase A or B (1.5 nmol) digestions of antigens (18 nmol) were made in 450 μl of 20 mM sodium phosphate buffer (pH 7.9) at 25°C . At time intervals 100- μl aliquots of digests were diluted with 100 μl of 200 mM sodium citrate buffer (pH 2.2) for quantitation of released amino acids on an automatic amino acid analyzer. Carboxypeptidase B was dialyzed to remove contaminating amino acids before use.

Amino acid analysis of the samples (about 0.5 nmol) was done after hydrolysis in 100 μl of 6 N HCl at 110°C for 20 h.

Edman degradation of antigens (about 25 nmol) was carried out manually (10). About half the extract, corresponding to 10 nmol of antigen, was examined by high-pressure liquid chromatography on a Micropak MCH-10 column (0.45×20 cm) to identify the phenylthiohyantoin derivatives of released amino acids (11). The column was developed with a linear gradient from 15 to 35% acetonitrile in 14 mM 2-(*N*-morpholino)ethanesulfonate buffer (pH 6.5) for the first 28 min, then with 35% acetonitrile for another 20 min. The flow rate was 1 ml/min and the column temperature was 52°C . The effluent was followed by absorbance at 254 nm and the presence of 0.5 nmol of a single phenylthiohyantoin would have been detected. Pyroglutamyl aminopeptidase digestion of antigens was made as reported (12) and the activity of the enzyme was checked by its hydrolysis of L-pyroglutamyl-L-alanine.

Allergenic activity with antigens E and K was determined by histamine release assay with leukocytes from ragweed sensitive human donors (13).

Isolation of Antigens E and K and an L-BANA Hydrolyzing Protease from Ragweed Pollen

Antigens E and K were isolated as described previously (2, 3), with certain modifications for large-scale isolation. Pollen (5 kg) was defatted with ether, then extracted for about 18 h in 20 liters of 50 mM Tris + 30 mM HCl buffer (pH 7.95) containing 1.1 mM

L-cystine. Cystine was added to block the sulfhydryl group of antigen E (14) by a disulfide exchange reaction (15). The filtered extract together with wash (about 25 liters), after adjustment to pH 7 and 200 mM NaCl, was depigmented on stirring with Whatman DE-32 cellulose (0.6 kg dry weight) which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.95) containing 200 mM NaCl. The depigmented extract was concentrated to about 4 liters and dialyzed thoroughly against 12.5 mM Tris-HCl buffer (pH 7.95) in an Amicon DC2 hollow fiber dialyzer concentrator with a 2000 MW cutoff cartridge.

The concentrated extract was next separated by batchwise absorption to DE-32 cellulose (1.4 kg dry weight) which had been equilibrated with 12.5 mM Tris-HCl buffer (pH 7.95). One basic fraction was eluted with 12.5 mM Tris-HCl buffer (pH 7.95) and one acidic fraction with 25 mM Tris-HCl buffer (pH 7.95) containing 200 mM NaCl. The acidic fraction (about 11 liters) was separated by $(\text{NH}_4)_2\text{SO}_4$ precipitation into an antigen K- and an antigen E-rich fraction at 0-45% and 45-59% saturation, respectively (16). All operations up to this step were done at 4°C , while all subsequent ones were done at 25°C .

The two $(\text{NH}_4)_2\text{SO}_4$ precipitates were each dissolved in about 400 ml of 50 mM NH_4HCO_3 containing 400 mM $(\text{NH}_4)_2\text{SO}_4$ and applied to a Sephadex G-100 column (6.6×95 cm) at a flow rate of 200 ml/h. Rocket immunoelectrophoresis was used to detect antigens K and E, which were eluted at 2100-2500 ml and 2000-2400 ml regions, respectively. The 0-45% saturated precipitate also contained a protease with L-BANA hydrolyzing activity, which was eluted in the 1300- to 1700-ml region. After $(\text{NH}_4)_2\text{SO}_4$ precipitation, rechromatography, and dialysis, the crude antigens, each in about 100 ml of 50 mM Tris-HCl buffer (pH 7.95), were separated on a DE-32 column (4.8×20 cm). The column was eluted with a linear NaCl gradient from 0 to 80 mM in 3 liters of 50 mM Tris-HCl buffer (pH 7.95) at a flow rate of 200 ml/h. The different isoelectric forms of antigens E and K were pooled as indicated in Figs. 1A and 2. They were concentrated on ultrafiltration through Amicon UM-10 membrane, dialyzed, and rechromatographed. The yields of these antigens from four separate experiments are listed in Table I.

Ragweed antigen E was also isolated from pollen extract by affinity chromatography. The required affinity column (1.3×7.5 cm) was prepared by coupling rabbit anti-antigen E antibodies (20 mg) to CNBr-activated Sepharose 4B (2 g; Pharmacia Co.) in 25 ml of 0.1 M NaHCO_3 and 0.5 M NaCl for 2 h at 25°C . To block any unreacted sites, the Sepharose was treated with 50 mM glycylglycine in 0.1 M NaHCO_3 overnight. After applying the extract (3.4 g of pollen in 6 ml of buffer) to the affinity column, followed with extensive washing of the column with 50 mM Tris-HCl buffer (pH 7.95), the absorbed antigen E

(about 5 A_{280} units) was eluted with 50 mM glycine-HCl buffer (pH 2.9) at a flow rate of 30 ml/h and collected as 2-ml fractions in tubes containing 0.5 ml of 200 mM Tris-HCl buffer (pH 7.95). After dialysis, the crude antigen E was further purified by chromatography on a DE-32 column (0.9×20 cm) under conditions similar to those given earlier (Fig. 1B).

The crude protease fraction with L-BANA hydrolyzing activity, described earlier, was further purified by chromatographies on DE-32 cellulose and on arginine-Sepharose under the conditions given in Table II. The required arginine-Sepharose (17) was prepared by coupling L-arginine (0.6 mmol) and CNBr-activated Sepharose 4B (1 g) in 10 ml of 0.1 M NaHCO_3 and 0.5 M NaCl for 16 h at 25°C. The yields of this protease at different stages of purification are given in Table II.

RESULTS

Isolation of Isoelectric Forms of Antigens E and K

Crude antigens E and K were obtained from pollen extracts by batchwise adsorption to cellulose anion exchanger, followed by $(\text{NH}_4)_2\text{SO}_4$ fractionation and gel filtration on Sephadex G-100. The crude antigen concentrates were resolved into their separate isoelectric forms on cellulose anion-exchange chromatography. Two such experiments are shown for antigen E in Fig. 1A, and two for antigen K in Fig. 2. Immunodiffusion analysis with rabbit anti-ragweed serum showed the presence of antigen E in the region of 800–2800 ml of Fig. 1A, and the presence of antigen K in the region of 1100–3000 ml of Fig. 2. These results are not shown. The distribution of different isoelectric forms of antigens E and K varied in the two experiments, even though the crude antigens E and K were obtained under supposedly identical conditions.

The indicated A form of antigen E and the B and C forms of antigen K in Figs. 1A and 2 were rechromatographed to reduce contamination (results not shown) and the others were not. The yields for the A, B, and C forms of antigens E and K are given in Table I together with those from three other experiments. The combined yield of antigen E-A, -B, and -C forms was about 25% of the crude antigen E used, and that for antigen K was about 20%.

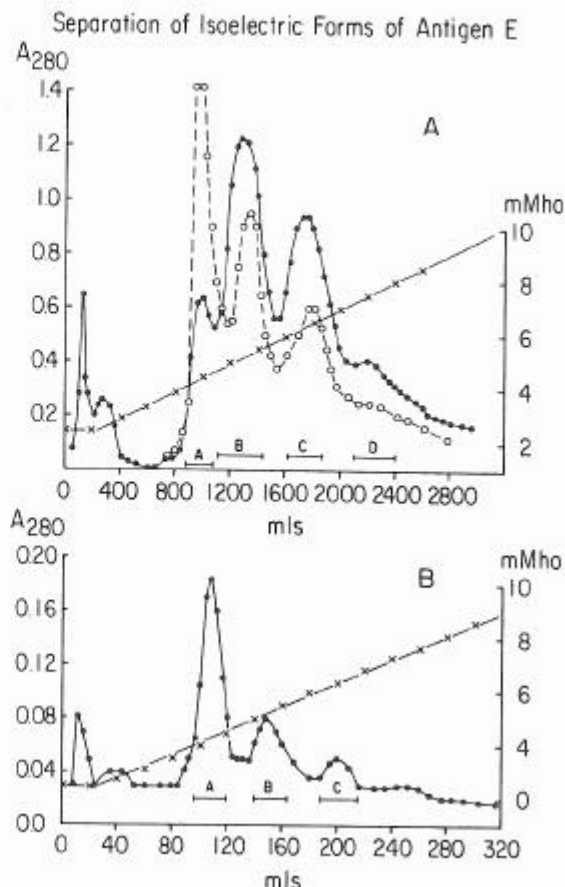


FIG. 1. Separation of isoelectric forms of antigen E on DE-32 cellulose. (A) The crude antigen E sample was isolated from 5 kg of pollen by a combination of fractionation procedures as described under Methods. The solid and the dashed curves are for experiments 1 and 2, respectively, listed in Table I; other experimental details are given in the text. (B) The antigen E sample was obtained from 6.8 g of pollen by affinity chromatography on an immunosorbent as described under Methods. The yields of antigen E-A, -B, and -C forms are, respectively, 2, 0.5, and 0.3 mg (experiment 5, Table I).

This poor recovery was partially because not all isoelectric forms of the two antigens were worked up, e.g., the D fraction of antigen E and the BC and D fractions of antigen K.

Crude antigen E was concentrated from a fresh pollen extract by affinity chromatography on an antigen E-specific immunosorbent. On ion-exchange chromatography (Fig. 1B), this sample contained mainly the A form of antigen E together with smaller amounts of B and C forms. When a pollen extract that had been stored at 4°C for about 4 months was examined, its content of the A form of antigen E had decreased slightly while those

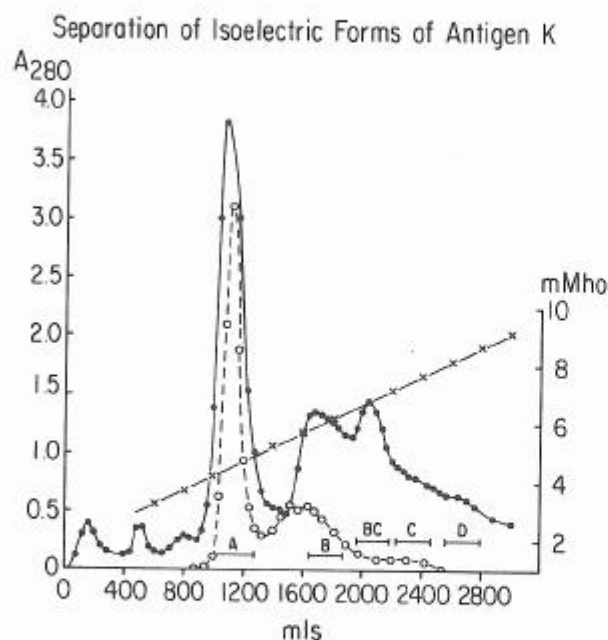


FIG. 2. Separation of isoelectric forms of antigen K on DE-32 cellulose. The antigen K sample was isolated from 5 kg of pollen, as described in the text. The solid and the dashed curves are for experiments 4 and 2, respectively, listed in Table I.

of the B and C forms had increased (results not shown). The overall yield of antigen E is better when affinity chromatography is used instead of the standard

chromatographic procedure. This is seen by a comparison of the yields listed in Table I.

Chemical Characterizations of Isoelectric Forms of Antigens E and K

The A, B, and C forms of antigen E differed in their electrophoretic mobilities on polyacrylamide gel electrophoresis in Tris buffer (Fig. 3A). The A forms of antigen E that were isolated by standard chromatographic procedure or by affinity chromatography showed identical electrophoretic mobility. On sodium dodecyl sulfate-polyacrylamide gel electrophoresis of freshly heated samples of antigen E, the B and C forms both showed mainly two bands of about 12,000 and 26,000 daltons, while the A form showed mainly one band of about 38,000 daltons (Fig. 3B).

When the A form (1 mg/ml) was treated with bovine pancreatic trypsin (20 μ g/ml) at pH 7.95 and 25°C for 2 h, it was rapidly converted to another form that was electrophoretically indistinguishable from its natural B form in charge (Fig. 3A) and in its two-chain structure (Fig. 3B). Similar

TABLE I
SUMMARY OF YIELDS OF ANTIGENS E AND K FROM 5 kg OF SHORT RAGWEED POLLEN

	Yields from separate experiments (g)				
	1	2	3	4	5 ^a
A. Antigen E					
Extract	11	7.2	8.5	13.2	
Acidic fraction	7.2	3.8	6.5	4.2	
Crude antigen E	2.0	2.1	2.4	2.5	3.58
Antigen E-A	0.06	0.19	0.11	0.03	1.47
Antigen E-B	0.19	0.17	0.36	0.23	0.37
Antigen E-C	0.13	0.11	0.21	0.25	0.22
B. Antigen K					
Extract	8.8	4.2	4.7	6.1	
Acidic fraction	4.1	3.5	2.8	4.6	
Crude antigen K	1.7	2.0	2.4	—	
Antigen K-A	0.03	0.49	0.26	0.49	
Antigen K-B	0.19	0.07	0.11	0.08	
Antigen K-C	0.14	<0.01	0.05	0.04	

^a The yield of antigen E given in experiment 5 was extrapolated from a workup of 6.8 g of pollen by affinity chromatography (Fig. 1B). The amounts of antigens E and K in the extract, in the acidic fraction and in crude antigens were measured by rocket immunoelectrophoresis.

treatment of the A form with α -chymotrypsin or thermolysin did not show such a change. The elution position of the trypsin treated A form from DE-32 cellulose was also indistinguishable from that of the natural B form (results not shown).

The above results confirm the previous reports (14, 18) that both B and C forms consist of two noncovalently held peptide chains. More interestingly, they show that the A form contains one chain and that it can be converted on limited proteolysis to a two-chain form.

In some preparations of the A form of antigen E, which was isolated by the standard chromatographic procedure, the sample contained two closely spaced bands on polyacrylamide gel electrophoresis in Tris buffer, and the additional band had a mobility which is intermediate to those of the normal A and B bands. Such a sample showed three bands of about 38,000, 26,000, and 12,000 daltons on sodium dodecyl sulfate-gel electrophoresis. The intensities of the 26,000- and 12,000-dalton bands with respect to that of the 38,000-dalton band appeared to be related to the relative concentrations of the two bands found by Tris gel electrophoresis. Following trypsin treatment, both bands in such a sample were converted to one single band with the same mobility as that of the B form. These results are not shown; they suggest that this additional band in some preparations of the A form of antigen E represents yet another isoelectric form.

In Fig. 4A are shown the Tris gel electrophoretic patterns of the A, B, C, and D forms and BC fraction of antigen K from Fig. 2, as well as the patterns of these same samples following trypsin digestion. Fraction BC is a mixture containing primarily the C form and another form, A', that has a mobility which is intermediate between those of the A and B forms. On trypsin digestion the A, B, and C forms were converted to a common main product with the same mobility as that of the D form, while the D and BC fractions were converted to two bands, one with the same mobility as that of the D form and the other with a faster mobility, which is designated as E form. The D and E forms

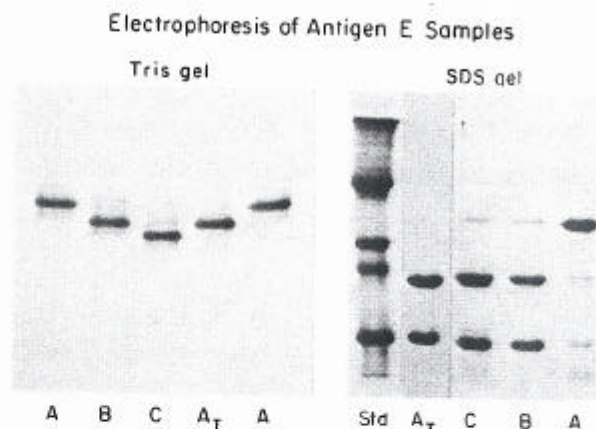


FIG. 3. Electrophoresis of antigen E samples. (A) Tris gel: from left to right, A, B, C, A_T, and A forms. A_T form represents A form (1 mg/ml) after 2-h digestion at pH 8 and 25°C with trypsin (20 μ g/ml). (B) SDS-gel: from left to right, protein standards of 67,000, 35,000, 28,000, and 13,000 daltons from top to bottom, trypsin-treated A form, C, B, and A forms. To promote dissociation of the peptide chains, the samples were heated at 60°C for 15 min in the sample buffer before electrophoresis.

were separable on cellulose anion-exchange chromatography and they were antigenically indistinguishable from the other forms of antigen K on immunodiffusion with rabbit anti-ragweed serum (results not shown). On reducing the concentration of trypsin used for the digestion, the sequential conversion of the A form of antigen K into its B, C, and D forms could be readily demonstrated (results not shown).

In Fig. 4B are shown the sodium dodecyl sulfate-gel electrophoretic patterns of heated samples of the A, B, and C forms of antigen K and its D form obtained on trypsin digestion. These results show that the A form of antigen K contains one chain of about 38,000 daltons, while the B, C, and D forms contain two noncovalently held chains of about 12,000 and 26,000 daltons.

Carboxypeptidase digestions of the A, B (natural and trypsin generated), and C forms of antigen E were carried out to determine their carboxyl terminal amino acid residue. The patterns of release of four selected amino acids from each form are given in Fig. 5A for carboxypeptidase A digestion at 2 and 6 h, and in Fig. 5B for carboxypeptidase B digestion at 0.05, 2, and 6 h. All four forms showed similar

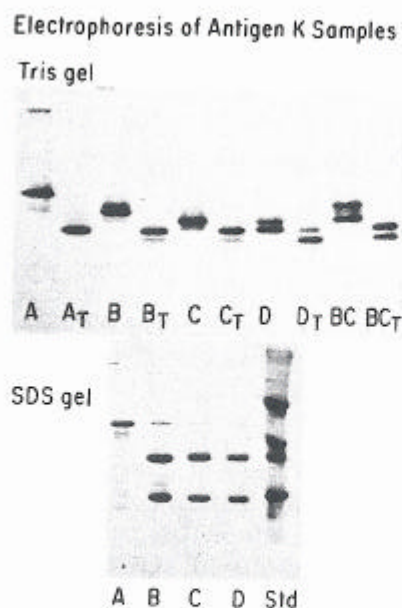


FIG. 4. Electrophoresis of antigen K samples. (A) Tris gel: from left to right, fractions A, B, C, D, and BC from Fig. 2 before and after trypsin digestion in alternate lanes. (B) SDS-gel: from left to right, A, B, C, and D forms, and protein standards. The samples were heated as described in Fig. 3B.

patterns of release of neutral amino acids on carboxypeptidase A digestion, but they differed in the release of basic and neutral amino acids on carboxypeptidase B digestion. The fastest amino acid released on carboxypeptidase A digestion was leucine; 0.55, 0.65, 1.1, and 0.88 equivalents were released, respectively, from the A, B (natural and trypsin generated), and C forms after 6 h of digestion. The fastest amino acid released on carboxypeptidase B digestion of the B (natural and trypsin generated) and C forms was lysine in amounts of 0.65, 1.3, and 0.55 equivalents, respectively, after 6 h, while similar digestion of the A form released only 0.22 equivalent of lysine. These results suggest that the A form has only one carboxyl terminal residue of leucine while the other forms contain two carboxyl terminal residues, one each of leucine and of lysine.

The data on carboxypeptidase A and B digestions of the A, B, and C forms of antigen K and its trypsin-generated D and E forms are given in Fig. 6A and B. The results suggest that the A form of antigen K has one carboxyl terminal residue of threonine (or glutamine) and all the other forms have threonine (or glutamine) and

arginine (or possibly lysine) as carboxyl terminal residues.

No amino terminal residue was detected when Edman degradation was carried out with the different forms of antigens E or K before or after pyroglutamyl digestion (11). The presence of 0.05 equivalent of an amino terminal residue would have been detected under the conditions used.

The B and C forms of antigen E are known to have indistinguishable amino acid compositions within experimental error (2). In the course of this work the amino acid compositions of the A, B, and C forms of antigen E, as well as that of the B form of antigen E which was obtained on trypsin digestion of the A form, were found to be indistinguishable from one another (results not shown). This was found to be the case also with the different forms of antigen K.

Immunological Studies of the Isoelectric Forms of Antigens E and K

Rabbit anti-sera specific for the A form and the natural and trypsin-generated B forms of antigen E were prepared. One rabbit was used for each form. When each of these sera was tested by immunodiffusion against the A, B (natural and trypsin-generated), and C forms of antigen E, identity of their precipitin lines was observed. Similarly, rabbit anti-sera specific for the A form and the trypsin-generated D form of antigen K were prepared. When each of these sera was tested by immunodiffusion against the A, B, C, and D (trypsin generated) forms of antigen K, again identical reactions were observed.

The allergenic activity of these antigen samples was tested by the histamine release method with leukocytes from six ragweed sensitive human donors. The concentrations of the A, B (natural and trypsin generated), and C forms of antigen E required for 50% release of histamine from the cells of each donor were identical within experimental error ($\pm 30\%$). The concentration range for 50% histamine release from the six donors was 0.22–1.2 ng/ml, with a geometric mean of 0.24 ng/ml. Similarly, the concentrations of the A,

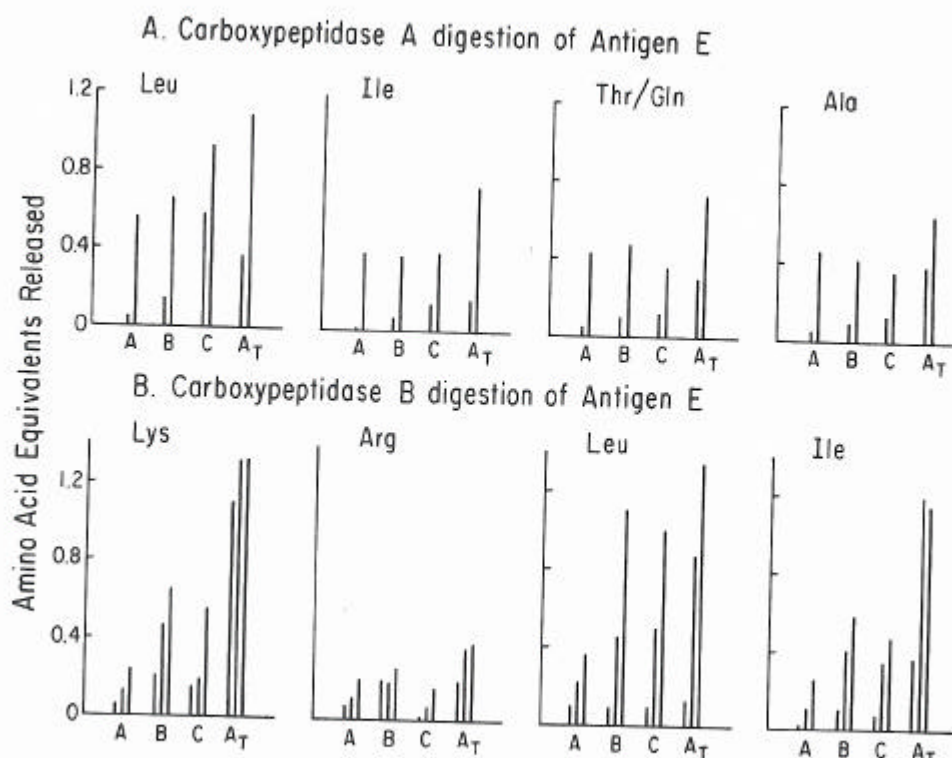


FIG. 5. Carboxypeptidase A and B digestions of antigen E forms A, B, C, and trypsin-digested A. For carboxypeptidase A digestion, the four fastest released amino acids at 2 and 6 h are given. For carboxypeptidase B digestion, lysine, arginine, and the two fastest released neutral amino acids at 0.05, 2, and 6 h are given. Under the conditions used for amino acid analysis, glutamine and threonine were not resolved.

B, C, and D (trypsin generated) forms of antigen K required for a 50% histamine release were identical for each donor; the concentration range for the six donors was 0.28–8.0 ng/ml, with a geometric mean of 0.69 ng/ml.

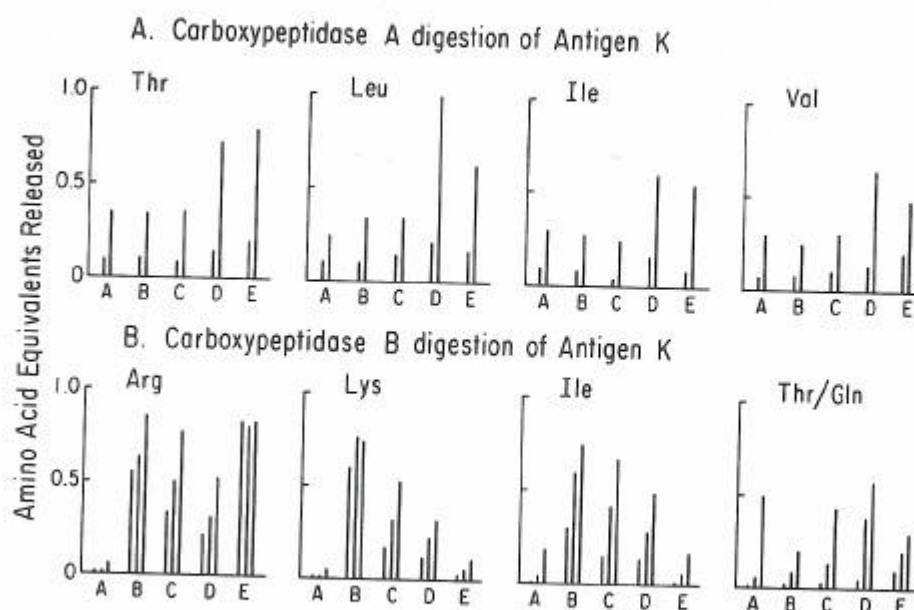


FIG. 6. Carboxypeptidase A and B digestions of antigen K forms A, B, C, D, and E. Forms D and E were obtained on trypsin digestion of fraction BC (Fig. 2) followed by chromatographic separation. Data are presented in the same manner as those given in Fig. 5.

TABLE II

SUMMARY OF YIELDS OF A RAGWEED POLLEN
PROTEASE AT DIFFERENT STAGES OF PURIFICATION

	Enzyme units	A_{280} units
Extract from 50 g of pollen ^a	11	—
Sephadex G-100 fraction	9	230
DE-32 fraction ^b	4	19
Arginine-Sephadex fraction ^c	1	0.9

^a The extract was processed as described under Materials and Methods. When isolation was done with 5 kg of pollen, the recovery of enzyme activity was usually less than one-half of that on small-scale isolation.

^b Separation was done with a (0.9 × 20 cm) column, using a linear NaCl gradient from 50 to 200 mM in 800 ml of 50 mM Tris-HCl buffer (pH 7.95) at a flow rate of 40 ml/hr. The protease was eluted as a broad peak at about 100 mM NaCl.

^c A (0.9 × 5 cm) column was eluted with a linear NaCl gradient from 0 to 100 mM in 160 ml of 50 mM Tris-HCl buffer (pH 7.95) at a flow rate of 30 ml/h. The protease was eluted at about 15 mM NaCl. A significant loss of activity occurred at this step during ultrafiltration and dialysis.

A Trypsin-Like Protease from Ragweed Pollen

The protease that catalyzes the hydrolysis of L-BANA was partially purified from the extract on $(\text{NH}_4)_2\text{SO}_4$ precipitation followed by chromatographies on Sephadex G-100 and on DE-32 cellulose. After final purification by affinity chromatography on arginine-Sephadex, the preparation was still heterogeneous on gel electrophoresis (results not shown). A summary of the yields of this protease at different steps of purification is given in Table II.

The chromatographic properties of the protease indicated it to be an acidic protein of about 100,000 daltons. It showed a high affinity for L-BANA with K_m of 6.6×10^{-6} M at pH 7.2, while trypsin had a K_m of 9.3×10^{-4} M for this substrate under the same conditions. The enzyme, at a concentration of 10–20 munit/ml in pH 7.95 Tris-HCl buffer, was inactivated to less than 1% activity by 20 μM N- α -tosyllysyl chloromethane or by 1 mM diisopropyl fluo-

rophosphate in less than 30 min. The enzyme was not inhibited by soybean trypsin inhibitor or aprotinin, both at 0.1 mg/ml, nor was the activity of this enzyme enhanced or inhibited in the presence of iodoacetamide, mercaptoethanol, or CaCl_2 .

The A form of antigens E or K (1 mg/ml) treated with this pollen protease (50 munit/ml) at pH 8 and 25°C for as long as 10 days did not show any detectable change when examined by gel electrophoresis. This pollen protease poorly digested denatured hemoglobin. Overnight incubation of a 1.0-ml solution of hemoglobin (10 mg) with pollen protease (14 munit) in pH 7 phosphate buffer at 25°C released 0.16 A_{280} unit of 6% dichloroacetic soluble peptides; under similar conditions of digestion with trypsin (5 μg), 1.2 A_{280} units of soluble peptides were observed.

DISCUSSION

The above findings strongly suggest that both antigens E and K are present in ragweed pollen as proteins containing a single peptide chain, and that during isolation the single-chain protein can undergo limited proteolysis to result in different isoelectric forms which contain two smaller peptide chains. Immunological studies did not detect any accompanying change in the antigenic or allergenic determinants of antigens E or K on limited proteolysis.

The present results demonstrate that trypsin digestion of the A form of antigen E can convert it into its corresponding B form, but not into its C form, while similar digestion of the A form of antigen K can convert it sequentially into B, C, and D forms. The exact chemical differences between these distinct forms of antigen E, or those of antigen K, remain to be determined. We are certain only that the limited proteolysis of antigen E or K is not accompanied by release of any large peptide fragments. This is indicated by the indistinguishable amino acid compositions of the different forms of antigen E or by those of the different forms of antigen K.

The findings on the carboxyl terminal residues of the different forms of antigens E or K, as well as the findings on trypsin

sensitivity of the A forms of antigens E and K, suggest that the putative ragweed protease responsible for the limited proteolysis of antigens E or K has trypsin-like specificity. Ragweed pollen is known to have a trypsin-like protease which catalyzes the hydrolysis of L-BANA (5). This protease was partially purified in this work, but it did not cause limited proteolysis of antigen E or antigen K. Therefore, identification of the responsible ragweed protease(s) remains to be made. Apparently this putative protease is not fully active in the ragweed pollen extract since an extract that had been aged for 4 months at 4°C still contained a significant portion of its antigen E in the single-chain form A. This fact suggests that the protease may be present in the extract in a precursor form and that it is activated during isolation and/or that the extract contains a specific inhibitor of the protease.

One useful application of the present findings is in the preparation of antigens E and K of high purity. These two antigens are difficult to resolve by conventional chromatographic procedures because of the similarity of their size and charge. They are separable on $(\text{NH}_4)_2\text{SO}_4$ precipitation, as was done in the present workup. Antigen E, which is isolated by the present procedure, is contaminated with antigen K (estimated to be about 5% for AgE-C form and much less for AgE-B form). This is because on $(\text{NH}_4)_2\text{SO}_4$ precipitation it is difficult to free the more soluble component (antigen E) from the less soluble one (antigen K). Preparations of B and C forms of antigen E can be freed of any contaminating antigen K by trypsin digestion followed by cellulose anion-exchange chromatography because of the larger difference in charge between the B or C forms of antigen E and the D or E forms of antigen K.

Protein antigens from other pollen sources, such as rye, timothy, and birch, are commonly isolated in multiple isoelectric forms (cf. (1) and (19)); therefore it

is likely that the present findings concerning ragweed pollen antigens may be applicable to antigens from other pollens.

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