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A Contribution to the Biochemical Characterization of Allergens in Pollen Extracts of *Secale Cereale*

N. Muić and N. Köhler-Kubelka

Department of Applied Biochemistry, »Andrija Štampar« School of Public Health, Faculty of Medicine, University of Zagreb and Institute for the Production of Sera and Vaccines, Zagreb, Croatia, Yugoslavia

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Buffer extracts of pollen from *Secale cereale* as used in therapy of pollinosis were prepared. All the extracts were found active upon intradermal test in pollen sensitive subjects. Electrophoretic and chromatographic analysis revealed that the extracts contain seven ninhydrin reacting components. Two amino acids, proline and lysine, and five others (very probably polypeptides) were found to be components of the extract. Two major peptide components (C_2 and *D*) were isolated and amino acids composition of acid hydrolyzates determined. Component C_2 contained Asp., Glu., Ser., Gly., Thr., Lys., Tyr., (Cys.?), and *D* component Asp., Glu., Ser., (Cys.?), Gly., Thr., Tyr., Val., Pro. In the original pollen extract's hydrolyzate His., Phen., Leu., not certainly Tryp., and Isoleu. were found in addition to the amino acids cited above. Of carbohydrates glucose, galactose and fructose were detected.

INTRODUCTION

In spite of great amount of experimental research on the chemical nature of pollen allergens in the past fifty years, there is no definite answer yet which group of chemical compounds from pollen extracts possesses the potency of allergizing.

The most favourable point of view is that the proteins from pollen cells are responsible for its allergenic capacity.

Formerly Prausnitz[1] had published that the only albumin portion from fractionated rye pollen proteins has showed the allergenic properties. Urbach[2,3] later on concluded in accordance with the results of his experimental investigations that the allergenic principle appeared to be associated with globulin and not albumin fractions in the pollen extracts of *Secale cereale*.

In general many investigations have recently established the fact again that the pollen allergens are proteinic in nature (Unger and co-workers[4], Roth and Nelson[5], Newel[6], Cohen *et al.*[7], Abramson, More and Gettner[8], Hampton, Johnson Mary and Frankel[9]). For the most part these studies are concerned about various ragweed allergens.

Timoty pollen allergens have been studied by Abramson, Engel and Moore[10], a preliminary report on fractionation of grass pollen has been published by Augustin[11], and Herbertson *et al.* have investigated allergens from alder pollen[12].

Because of variable success in the desensitation therapy of pollinosis with pollen extracts, prepared according to the conventional buffer elution method,

we supposed that native allergens occurring in pollen could be chemically changed during extraction procedures. The present communication is an attempt to acquire some evidence about the nature of pollen allergens from *Secale cereale* pollen in buffer extracts.

EXPERIMENTAL

Pollen

For the preparation of pollen extracts of *Secale cereale*, pollens were collected during summer 1958 in the region of Zagreb, Croatia, Yugoslavia. The pollen material was dried in a dessicator and was kept cool and dry.

Extraction

The preparation of buffer extract from pollen material was accomplished in such a manner that the crude material underwent as little chemical alteration as possible.

The defatting of pollen was carried out by washing it with successive portions of diethylether (pro narcosi). The ether was removed from defatted material merely by allowing it to evaporate in the air at room temperature.

Buffer saline, as used at the University laboratories of Michigan, was the extracting medium: NaCl 5 g.; KH_2PO_4 0.36 g.; Na_2HPO_4 7 g.; Phenol 4 g.; distilled water to make 1000 ml. Final *p*H of the buffer was close to 8. Adjustement was made using NaOH or HCl as needed.

Extracts of *Secale cereale* pollen were prepared by adding 1 g. of ether-defatted material to 49 ml. of extracting buffer. After extraction at room temperature (22°C) for 72 hours the extracts were filtered under sterile condition through a Seitz filter, aliquots (5 ml.) were rapidly frozen in ampoulles and lyophilized in order to preserve them sterile.

The total nitrogen content, determined by micro Kjeldahl method was 0.172 mg. per ml. of original extracts (1 g. of pollen in 49 ml. of buffer).

Biological investigations of buffer extracts from pollen of *Secale cereale* were performed by Dr. M. Mimica on pollen sensitive persons, by intradermal and scratch test at the Department of Medicine, University of Zagreb.

Paper chromatography

Both descending chromatography[13,14] for solvents phenol-water (4:1) and ascending chromatography[15] for *n*-butanol-acetic acid-water (4:1:1 and 4:1:5) were used for separation of pollen peptides and amino acids in hydrolyzates. In most experiments 0.02 ml. of conc. extract (0.1 g. of pollen in 0.2 ml. of water) or an adequate amount of hydrolyzed extract was applied on Whatman No. 1 paper strip.

The buffer extracts were desalted by dialysis against distilled water (48^h, 2°), the hydrolysates by electrodialysis in Consden apparatus.

For isolation of peptide fractions, the concentrated extract (0.06 ml.) was applied on a wider (11 cm.) strip of filter paper and developed one-dimensionally using phenol-water (4:1) as eluent.

The position of spots on air dried chromatograms were detected by employing minute drops of ethanolic ninhydrin along the paper strip.

Separated fractions were cut out from paper chromatogram and washed out from filter paper with warm water and hydrochloric acid (20%). Isolated fractions were hydrolyzed with 20% HCl in the sealed glass tubes for 24 hours at 105°C. The acid hydrolyzates were evaporated at 100°C and dried in vacuum desiccator over sodium hydroxide and calcium chloride.

The amino acids were detected by spraying of the developed paper chromatograms with 0.3% ninhydrin in ethanol. For the identification of amino acids a guide chromatogram with corresponding artificial amino acid mixture was simultaneously developed.

Paper electrophoresis

Zone electrophoresis was carried out according to the method published by Cremer and Tiselius[16], and Grassmann, Hannig and Knedel[17]. Veronal-veronal sodium buffer *p*H 8.6, ionic strength 0.05; Whatman No. 1, 5—10 V/cm; 1—2 mA; 4 hours, 0.02 ml. of conc. extract of *Secale cereale*. The proteins were fixed by drying at 105°C for 10 minutes and stained with 0.03% tetrabromophenolblue in 1% aqueous

mercurichloride[18], the peptides by spraying the electropherogram with 0.3% ethanolic ninhydrin.

Photometric evaluation

When dried the electropherograms were made translucent by immersing them into a mixture of paraffin oil and Cedax (Behring Werke, Bayer) in petroleum ether. The evaluation of transmittance of electropherograms and chromatograms was carried out in 1 cm. wide bands at intervals of 1 mm., with a modified Fischer electrophotometer and a green filter No. 525[18].

RESULTS AND DISCUSSION

All buffer extracts, made from pollen of *Secale cereale* and diluted with saline, were found to be skin reactive in high dilution upon intradermal test in *Secale cereale* pollen sensitive subjects. These extracts did not show some of the characteristic reactions for true proteins (mol. weight over 10 000). Concentrated pollen extract failed to precipitate with trichloracetic acid and coagulate by boiling. The components from extracts could not be fixed on filter paper in chromatographic procedures by heating them at 105°C, nor precipitated with mercuric chloride solution. When chromatographed, these components could be well stained on filter paper with bromophenolblue but they were badly fixed and could be washed out by water[18]. These results are in accord with prior experience of W. P. Dunbar with pollen extracts, as cited by Urbach[2] and Hansen[19]. Such properties are common with proteins having molecular weight less than 10 000. In fact Abramson[8] and Newel[20] and Augustin[11,21] have succeeded in isolating the biologically active fractions from ragweed and grass pollen extracts, with molecular weights between 5 000 and 13 000 respectively.

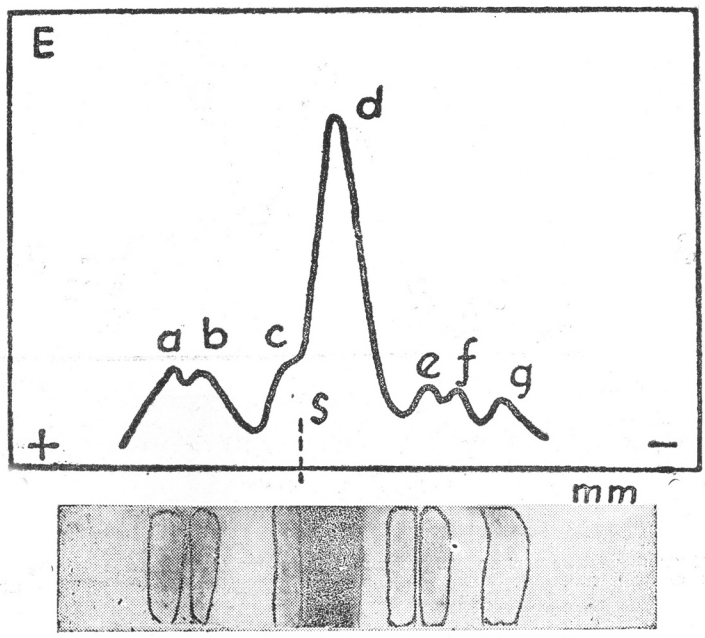

Fig. 1. Electropherogram with superimposed photometric evaluation. 0.02 ml. *Secale cereale* conc. pollen extract; veronal-veronal sodium buffer pH 8.6, ionic strength 0.05: 2.6 V/cm, 1.8 mA, 4 hours, 0.3% ninhydrin in ethanol, Whatman No. 1.

Electrophoretic separation of concentrated extract of *Secale cereale* pollen in alkaline (*p*H 8.6) veronal-veronal sodium buffer of satisfactory ionic strength (0.05) and potential gradient (8.6 V/cm.) was achieved (Fig. 1). The rye allergens were separated into three groups with at least seven components. Two components, *a* and *b*, migrated at *p*H 8.6 towards the anode not quite distinctly resolved, and four components migrated towards the cathode. The minor component *c* did not migrate at these experimental conditions. Because we cannot assume that a stoichiometric relationship exists between the denaturated proteins and the adsorbed dye, the photometric evaluation has only relative value of qualitative analysis. None of the components separated could be well fixed on paper fibres by heating; consequently the peptide components dyed with bromophenol blue, were washed out during the staining procedure.[18] Since the electropherograms were stained with ethanolic ninhydrin, we could not differentiate peptides from amino acids or exclude the overlapping of peptides and amino acids bands.

Paper chromatography was performed with various eluents. The application of consecutive elution[22] of the components from the paper, with salt solution in progressive concentrations, has not given satisfactory results. — Better separation of pollen peptides was achieved using *n*-butanol-acetic acid-water solvents.

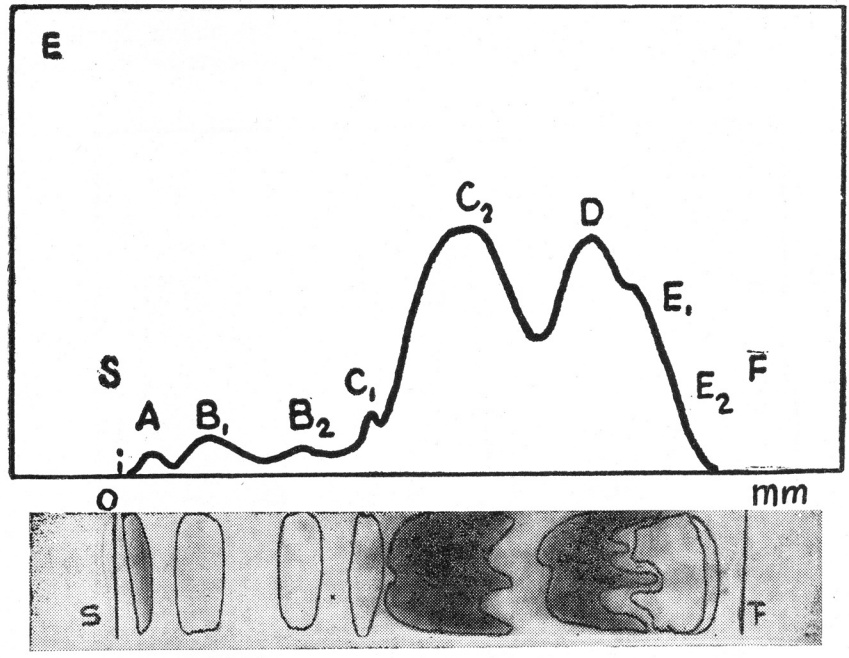

Fig. 2. Chromatogram with superimposed photometric evaluation; 0.015 ml. *Secale cereale* conc. pollen extract one-dimensionally developed, solvent phenol-water (4:1); 0.3% ninhydrin in ethanol. *S* = start, *F* = front.

The relative distances (*R*f) traveled by components were not however great enough for complete resolution of mixture. Spraying of the developed

chromatogram with ninhydrin revealed 5—6 polypeptide bands, from which two major bands could be well distinguished because of fairly different migration rate (*Rf* 0.14 and 0.31).

The third component reacted with ninhydrin producing yellow colour and with sodium salt of 1,2-naphthoquinone-4-sulphonic acid, red colour. This component, which showed the same migration rates on paper in all eluents as proline, should be considered as identical with it.

The chromatograms developed one-dimensionally with aqueous phenol revealed seven well separated components. Photometric evaluations of these chromatograms are presented in Fig. 2. The groups of components represented as peaks in this diagram cannot be identified with those in Fig. 1 because of a different distribution of the components during electrophoresis.

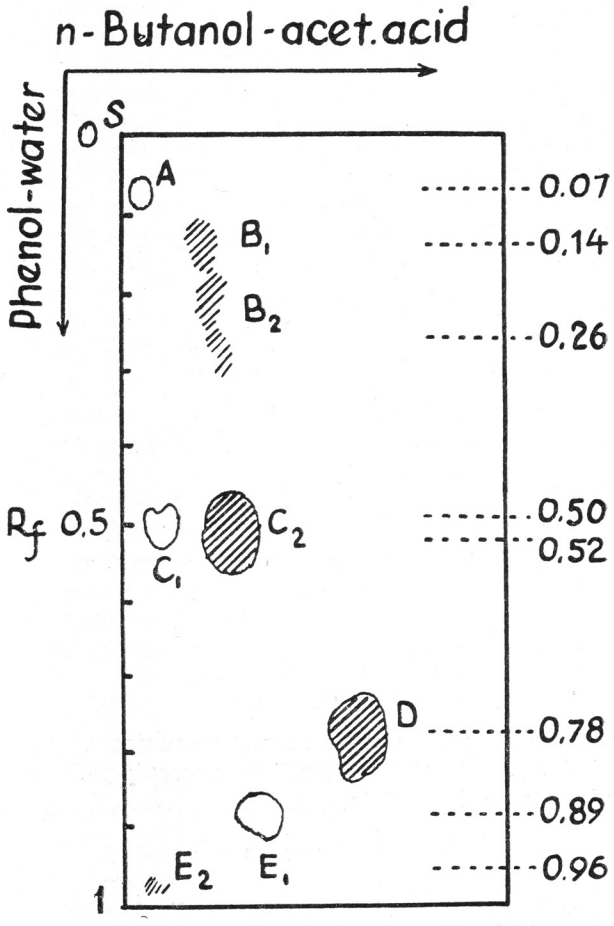

Fig. 3. Two-dimensional chromatogram of pollen extract (0.03 ml.) from *Secale cereale*. First direction, ascending: *n*-butanol-acetic acid-water (4:1:5); second direction, descending: phenol-water (4:1).

Most successful was two-dimensional chromatography of concentrated buffer extract from *Secale cereale* pollen. These chromatograms were developed in one dimension with butanol-acetic acid-water and in other with aqueous phenol. Fig. 3 shows the positions of components reacting with ninhydrin, on a two-dimensionally developed chromatogram. At least eight components could be well distinguished called *A*, B_1, B_2, C_1, C_2, *D*, E_1 and E_2. The *Rf* values corresponding to the components are added in the drawing. The components B_1 and B_2 are weakly separated and badly dyed with ninhydrin. It is evident from the drawing that the chief bands C_2 and *D* have satisfactory different relative migration rates (*Rf* values) in phenol-water solvent to be successfuly separated on one-dimensionally developed chromatogram. According to the results of the amino acids analysis component C_1 proved to be identical with lysine and E_1 with proline. All components in Fig. 3 denoted with capitals (*A*, B_1, B_2, C_1, C_2, *D*, E_1, E_2), correspond to those in Fig. 2.

For the preparation of components C_2 and *D* one-dimensional chromatograms with aqueous phenol were used. Larger sheets of paper (11 cm.) were streaked with 0.06 ml. of concentrated buffer extract from *Secale cereale* pollen (0.5 g. pollen per ml.) across the paper strip, thus a narrow and uniform band (0.3 cm./10 cm.) of substance was obtained. This sheet was then developed in the same manner as previously described, and the components from the chromatogram isolated. After isolation the substances C_2 and *D* were hydrolyzed and the amino acids composition of hydrolyzates accomplished by performing two-dimensional chromatograms. According to our results the component C_2 contains amino acids as follows: Asp., Glu., Ser., Gly., Thr., Lys., Tyr., (Cys.?) and *D* component Asp., Glu., Ser., (Cys.?), Gly., Thr., Tyr., Val., Pro.

In the acid hydrolyzate of the original pollen extract were found all amino acids cited above and some other more: His., Phen., Leu., (Isoleu.?), and in the alkaline hydrolyzate not certainly tryptophan.

For detection of carbohydrates concentrated pollen extract was chromatographed with phenol-water as solvent. Spraying of chromatograms with anilin-phosphate[23] revealed three spots with migration rates corresponding to glucose, fructose and galactose. Similar results have been found by Augustin[24] in grass pollen and Hampton *et al.*[9] in ragweed pollen extracts. From the results of our investigation it is evident that the favourable group of protein derivatives in buffer extracts from pollen of *Secale cereale* are polypeptides. Since it is known that pollen proteins show relatively high resistance to digestive enzymes[1,25] we can assume that native enzymes from pollen are responsible for the splitting of the proteins in rye pollen extract.

The fractions prepared chromatographically with butanol-acetic acid or phenol could not be used for skin test on sensitive subjects. Results of biological testing of suitable isolated fractions from *Secale cereale* pollen allergens will be published elsewhere at a later date.

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IZVOD

Prilog biokemijskoj karakterizaciji alergena ekstrakta peludi *Secale cereale*

N. Muić i N. Köhler-Kubelka

Ekstrakcijom peludi *Secale cereale* priređeni su alergeni, koji se upotrebljavaju u terapiji bolesnika osjetljivih na pelud. Ovi ekstrakti pokazivali su pozitivnu intradermalnu reakciju kod bolesnika osjetljivih na pelud *Secale cereale.*

Elektroforetska i kromatografska analiza pokazala je, da se ekstrakt sastoji iz sedam komponenata, koje su reagirale s ninhidrinom. Utvrđeno je, da su dvije komponente aminokiseline, prolin i lizin, a drugih pet su vjerojatno polipeptidi. Dvije veće, kromatografski dobro razdvojene komponente C_2 i *D*, bile su izolirane. U hidrolizatu ovih komponenata određene su kromatografski aminokiseline.

Komponenta C_2 sastoji se iz aminokiselina: asp., glu., ser., gli., treo., liz., tir., (cis.?); a komponenta *D* iz asp., glu., ser., (cis.?) gli., treo., tir., val., pro. U originalnom ekstraktu peludi nađeni su osim gore spomenutih aminokiselina još i histidin, fenilalanin i leucin, te tragovi triptofana i izoleucina. U koncentriranom ekstraktu peludi *Secale cereale* nađeni su kromatografijom ugljikohidrati glukoza, galaktoza i fruktoza.

ODJEL ZA PRIMIJENJENU BIOKEMIJU,
ŠKOLA NARODNOG ZDRAVLJA »ANDRIJA ŠTAMPAR«
MEDICINSKI FAKULTET, SVEUČILIŠTE U ZAGREBU
I
INSTITUT ZA PROIZVODNJU SERUMA I VAKCINA
ZAGREB

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