CHROM. 6002

The subunits of antigens of ragweed pollen

A previous report from this laboratory[1] described an antigenic correlation between two purified antigens of ragweed pollen: antigen E (ref. 2) and BPA-R (Basic Protein Antigen-Ragweed). While current immunodiffusion experiments have shown that antigens E and BPA-R possess common antigenic determinants, antigen E appears to contain additional determinants which are absent in BPA-R. Further experiments were designed to investigate the structural basis of this relationship.

The analysis of the subunits of totally reduced proteins by gel chromatography in guanidine hydrochloride (GuHCl) have been reported from several laboratories[3-5]. The present report deals with the use of the above method for resolving the subunit composition of ragweed pollen antigens.

Experimental

Reference proteins and reagents. The following reference proteins and their molecular weight assignments[4] were used in this study: ovalbumin (43,000), chymotrypsinogen (25,700), β-lactoglobulin (18,400) and cytochrome C (12,400) were purchased from Calbiochem, San Diego, Calif. Hemoglobin (15,500) was obtained from Mann Research Laboratories, New York, N.Y. Rabbit γG-globulin was prepared from normal rabbit sera by the method of LEVI AND SOBER[6]. The molecular weights assigned to the heavy and light chains of γG-globulin were 49,000 and 23,500, respectively. Guanidine hydrochloride was obtained from the Eastman Co., Rochester, N.Y. and Heico Inc., Delaware Water Gap, Pa. Iodoacetic acid and 2-mercaptoethanol were Eastman Co. products.

Reduction of proteins. For the reduction of reference proteins we used the method of EDELMAN AND POULIK[7] with some modifications. The proteins (7–17 mg) were dissolved in 0.65 ml of 6 *M* GuHCl buffered with Tris, pH 8.6, and containing 0.1 *M* 2-mercaptoethanol. The sample was incubated for 6 h at 37°. Iodoacetic acid (0.35 ml of 0.3 *M* in 6 *M* GuHCl, buffered with Tris, pH 8.3) was mixed with the sample and the reaction was allowed to proceed for approximately 10 min. Blue Dextran (6% solution in GuHCl), DNP-leucine (0.5% solution in GuHCl) and solid sucrose (100 mg) were added to the sample and a volume of 0.25 ml was applied to the agarose column. Antigen samples (3.0–4.0 mg) were treated in a similar manner except that the reagent volumes were scaled down to limit the final volume to about 0.25 ml.

Column preparation. GuHCl was dissolved in aqueous Bio-Gel A-5m (nominal 6% agarose) to provide a final concentration of 6 *M* salt. After incubation at room temperature overnight the gel slurry was de-aerated under vacuum and poured in a Pharmacia K15/90 column. The gel bed length was 85 cm and a constant solvent pressure of 20 cm was used throughout the study.

Sampling technique. The effluent was monitored at 280 mμ with the LKB Uvicord II absorptiometer equipped with a recorder. Fractions of about 1.0 ml were collected by drop count technique with the LKB Ultrorac apparatus. Weight determinations were made on the effluent samples by measuring the tube weights before and after sample collection with a Mettler top loading balance type K71. A conversion

to volume was made by dividing the individual sample weights by a specific gravity factor determined for the 6 M GuHCl solvent used in the chromatographic experiment. Supplemental readings were made on the samples (diluted three times with water) at 280 mμ on the Beckman DU spectrophotometer to obtain exact absorbance values for the UV peaks.

Ragweed pollen antigens. Fraction D-IV and antigen E (IV-C antigen) were prepared from ragweed pollen by the method of KING *et al.*[2]. The BPA-R was prepared as reported previously[1]. The antigen E and BPA-R were homogenous by gel chromatography on Sephadex G-100 and by ion-exchange chromatography. As further evidence of purity the antigens formed single precipitin bands against their respective homologous antisera. The purified antigens were dialyzed against distilled water and freeze dried prior to reduction experiments.

Treatment of chromatographic data. The distribution coefficient K_d, as defined by GELOTTE[8] and applied to the treatment of data by FISH *et al.*[4] dealing with gel chromatography of proteins in dissociating solvents, was used in the present study. The formula for K_d is as follows:

$$K_d = \frac{V_e - V_0}{V_t - V_0}$$

where

V_e = elution volume of protein as measured by the maximum absorbance at 280 mμ.

V_0 = elution volume of an unretarded macromolecule as exemplified by Blue Dextran.

V_t = the total volume within and without the gel particles as determined by the elution volume of DNP-leucine.

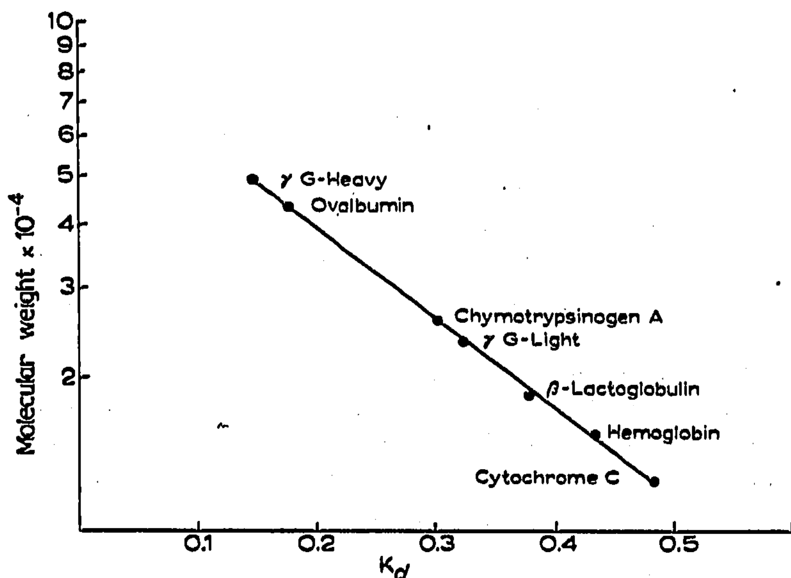
Results and discussion

As noted in Fig. 1 the plot between the logarithm of the molecular weight and K_d (the distribution coefficient) for the reference proteins is expressed by a linear relationship in agreement with other studies[3,5]. The effective range of the calibration plot was fully adequate for determining the molecular weights of the protein antigens.

The results of the gel chromatographic studies on the fully unfolded and reduced proteins of ragweed pollen are shown in Fig. 2. Similar chromatographic UV profiles were obtained for antigen E and the D-IV fraction, although the latter contained two "early" components which were negligible in the antigen E pattern.

The estimated molecular sizes of the subunits of antigen E represented by the components 1 and 2, Fig. 2, were 23,000 and 15,000, respectively. The UV extinction coefficient for antigen E (ref. 2) was used to estimate the weights of components 1 and 2. The weights were roughly proportional to the molecular weights determined for components 1 and 2. The summation of the molecular weights of the subunits was in agreement with the reported value of 38,000 determined by equilibrium ultracentrifugation for antigen E (ref. 2).

The D-IV fraction of ragweed pollen was shown by KING *et al.*[2] to be composed of impurities and a mixture of isomers, one of which, upon separation from the mixture, was designated as antigen E. The occurrence of components 1 and 2 (Fig. 2)



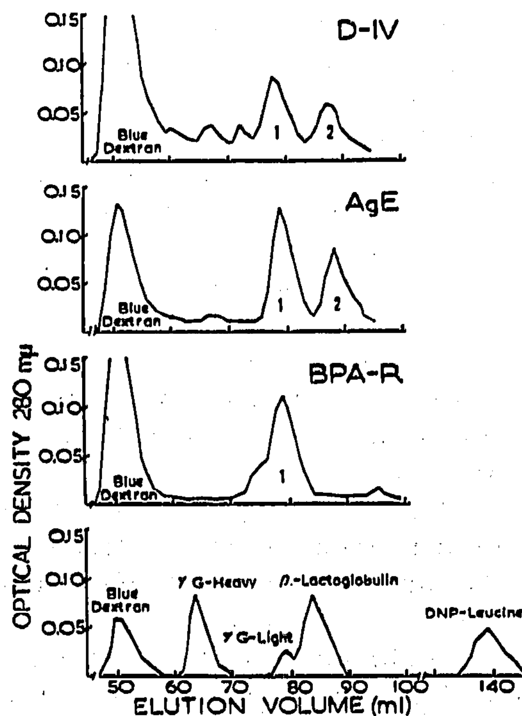


Fig. 1. Calibration graph for the estimation of molecular weights of reduced and alkylated proteins by gel filtration on agarose in 6 *M* GuHCl. The linear relationship between K_d and molecular weight was determined by the method of least squares analysis.

Fig. 2. Elution diagrams of reduced and alkylated protein antigens of ragweed pollen on agarose in 6 *M* GuHCl. The lowest diagram outlines the elution profile of a group of reference proteins chromatographed together with Blue Dextran and DNP-leucine; DNP-leucine was tested together with the antigen samples (profiles omitted in the diagram). The optical density measurements were made on all of the samples diluted three times with water.

as prominent peaks in the D-IV fraction suggests that the isomers might possess a fairly uniform composition of heavy and light chain subunits.

The demonstration of one main chromatographic component in the BPA-R sample (Fig. 2) which eluted in the same position as component 1 of antigen E indicates that the BPA-R is composed of a single polypeptide chain of similar molecular dimensions, *i.e.* 23,500 as noted in Table I as the heavy chain of antigen E. The molecular weight of the intact BPA-R was determined in an earlier study[1] to

TABLE I

K_d AND MOLECULAR WEIGHT DETERMINATIONS FOR THE SUBUNITS OF RAGWEED POLLEN ANTIGENS

Antigen	*Chromatographic fraction*[a]	K_d	*Estimated mol. wt.*
AgE	Component 1[b]	0.331[b]	23,000
	Component 2[b]	0.438[b]	15,000
BPA-R	Component 1	0.326	23,500
D-IV	Component 1	0.314	—
	Component 2	0.423	—

[a] See Fig. 2 for origin of chromatographic components.
[b] Average of duplicates.

be approximately 28,000. A source of error in the latter estimate may be due to anomalous behaviour of the protein as a function of the shape of the molecule[9]. This presumed error is probably negligible in the present molecular weight estimate of the BPA-R, since upon reduction in concentrated GuHCl the reference and unknown proteins adopt a similar random coil conformation[10].

The study of proteins by the foregoing chromatographic technique is useful for the determination of the total numbers and molecular sizes of polypeptides making up the protein structure. Reduction in the presence of GuHCl results in the complete unfolding of the polypeptide chains[3,10] with a concomitant loss of biologic potency. Consequently, tests performed on the antigen E subunits with specific anti-AgE serum were negative. Studies are currently in progress to prepare biologically active subunits of antigen E and to elucidate the nature of the bonding between the subunit components.

The foregoing investigation forms part of a comprehensive study on the standardization of the antigens of ragweed pollen which attempts to clarify the nature and extent of allergenic components contained in crude extracts of ragweed. The detection of a probable common polypeptide chain in the BPA-R and antigen E and the elucidation of the subunit structures of the antigens are regarded as fundamental advances in the above study.

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