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The retardation of certain proteins during chromatography on tanned gelatin

Chromium–formalin tanned gelatin, the medium introduced by POLSON AND KATZ[1] for molecular exclusion chromatography, has the advantage that it is mechanically strong. This property makes it possible to maintain high flow rates over a large number of chromatographic runs, thereby making tanned gelatin ideally suited to preparative chromatography.

During a series of investigations in this laboratory an attempt was made to calibrate a tanned gelatin column, using the procedure of LAURENT AND KILLANDER[2] and ANDREWS[3,4], in order to use elution data as a rough guide to the molecular weight of protein fractions. It was observed, however, that certain proteins are retarded during chromatography on tanned gelatin and this paper reports on a preliminary investigation of this phenomenon.

Experimental

Unfractionated acid process pigskin gelatin (Gelrite type A, 275 bloom, Canada Packers Ltd., 60 Paton Road, Toronto 4, Canada) was made up at the required concentration in distilled water and tanned with Kromex chrome tanning salt (Chrome Chemicals (Pty) Ltd., Merebank Durban) as described by POLSON AND KATZ[1]. The tanned cubes were fragmented in a Waring blendor into granules which were packed into a column (4 × 45 cm) and washed with 0.14 *M* NaCl until the effluent was free of chromium. The 0.14 *M* NaCl was then replaced by formalin tanning solution, pH 9.0 (ref. 1), which was run through the column until formalin could be detected in the effluent. At this stage the gelatin was extruded from the column, suspended in formalin tanning solution, adjusted to pH 9.0 with 5 *N* NaOH and maintained at this pH for 2 h by the addition of further NaOH, prior to being set aside for 24 h at 4°. After this period the granules were repacked into the column, washed free of formalin with 0.14 *M* NaCl and equilibrated with buffer. The granules were then extruded from the column, homogenised for 3 to 5 min in a Waring blendor to yield finer fragments and finally, after temperature equilibration, were packed into a jacketed analytical column (1.75 × 125 cm). After packing the column was equilibrated with at least two column volumes of buffer before use.

The analytical column was packed and operated at 6–8°, except where the effect of running the column at 40° was investigated. In this case the gel was extruded from the column, equilibrated to 40° and repacked into the column, which was maintained at this temperature.

Protein mixtures, containing 5 mg of each protein species, were applied to the column in 1 ml of buffer. As a reference the mixture in successive chromatographic runs always had one component in common with the mixture used in the preceding run. The proteins used as standards were chymotrypsinogen A, trypsinogen, horse heart cytochrome C (grade II), horse skeletal muscle myoglobin (1 × cryst.), egg white lysozyme chloride (grade I) and yeast hexokinase (grade II) (Miles-Seravac Laboratories, Epping Industria, Cape Town); soybean trypsin inhibitor (Koch-Light Laboratories, Colnbrook, Bucks., Great Britain); bovine albumin (Cohn frac-

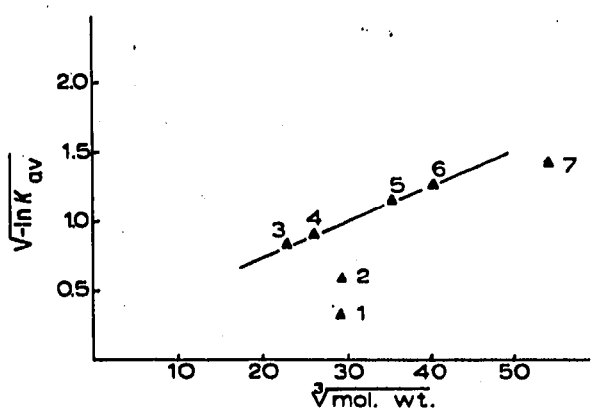
tion V) and bovine γ-globulins (Cohn fraction II) (Sigma Chemical Co., Mo., U.S.A.). Non-protein standards were Blue Dextran 2000 (Pharmacia, Uppsala, Sweden) and L-tryptophan (Sigma Chemical Co.).

Except where stated otherwise, the buffer system used consisted of 0.01 *M* Tris, pH 7.6 containing 0.15 *M* NaCl and 0.2% NaN_3. The effluent from the column was monitored at 280 nm in a Beckman DB spectrophotometer, equipped with a flow-through cell.

Results

Standard curves, based upon the theory of LAURENT AND KILLANDER[2] and according to the method of ANDREWS[3,4], relating elution volumes to molecular weights, are presented in Figs. 1 and 2, respectively. From these curves it is apparent that the proteins lysozyme, chymotrypsinogen A, trypsinogen and soybean trypsin inhibitor are retarded, relative to the other proteins tested, during chromatography on tanned gelatin under these conditions.

This retardation is an undesirable effect with regard to the estimation of molecular weights from elution volumes and therefore possible methods of eliminating the effect were investigated. Chymotrypsinogen A was selected for further study using an 8% chromium-formalin tanned gel. In successive experiments the effects of the following changes to the eluting buffer were investigated: (i) increasing the concentration of NaCl to 1.5 *M*, (ii) addition of 2-mercaptoethanol (1×10^{-3} *M*), and (iii) addition of EDTA (0.01 *M*). In each case the column was equilibrated with the



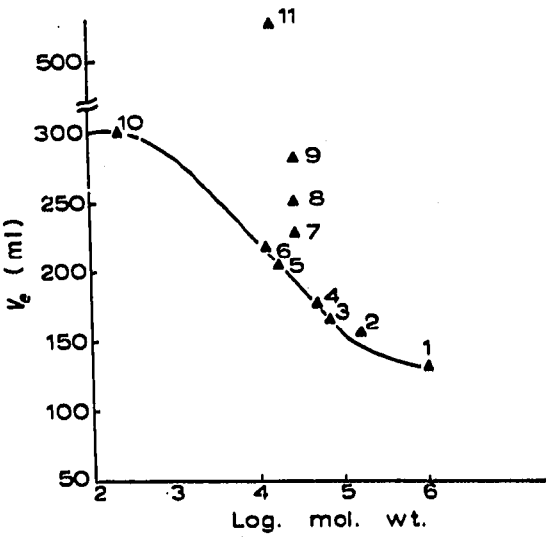


Fig. 1. Relationship between K_{av} (apparent) and molecular weight (mol. wt.) of a number of standard proteins chromatographed on 4% chromium-formalin tanned gelatin. No values are shown for lysozyme as this is eluted at a volume greater than V_{max} and the K_{av} value is thus meaningless. 1 = Chymotrypsinogen A; 2 = soybean trypsin inhibitor; 3 = cytochrome C; 4 = myoglobin; 5 = hexokinase; 6 = bovine serum albumin; 7 = γ-globulin.

Fig. 2. Relationship between the elution volume (V_e) and the molecular weight (mol. wt.) of a number of standard proteins chromatographed on 4% chromium-formalin tanned gelatin. 1 = Blue Dextran; 2 = γ-globulin; 3 = bovine serum albumin; 4 = hexokinase; 5 = myoglobin; 6 = cytochrome C; 7 = trypsinogen; 8 = soybean trypsin inhibitor; 9 = chymotrypsinogen A; 10 = tryptophan; 11 = lysozyme.

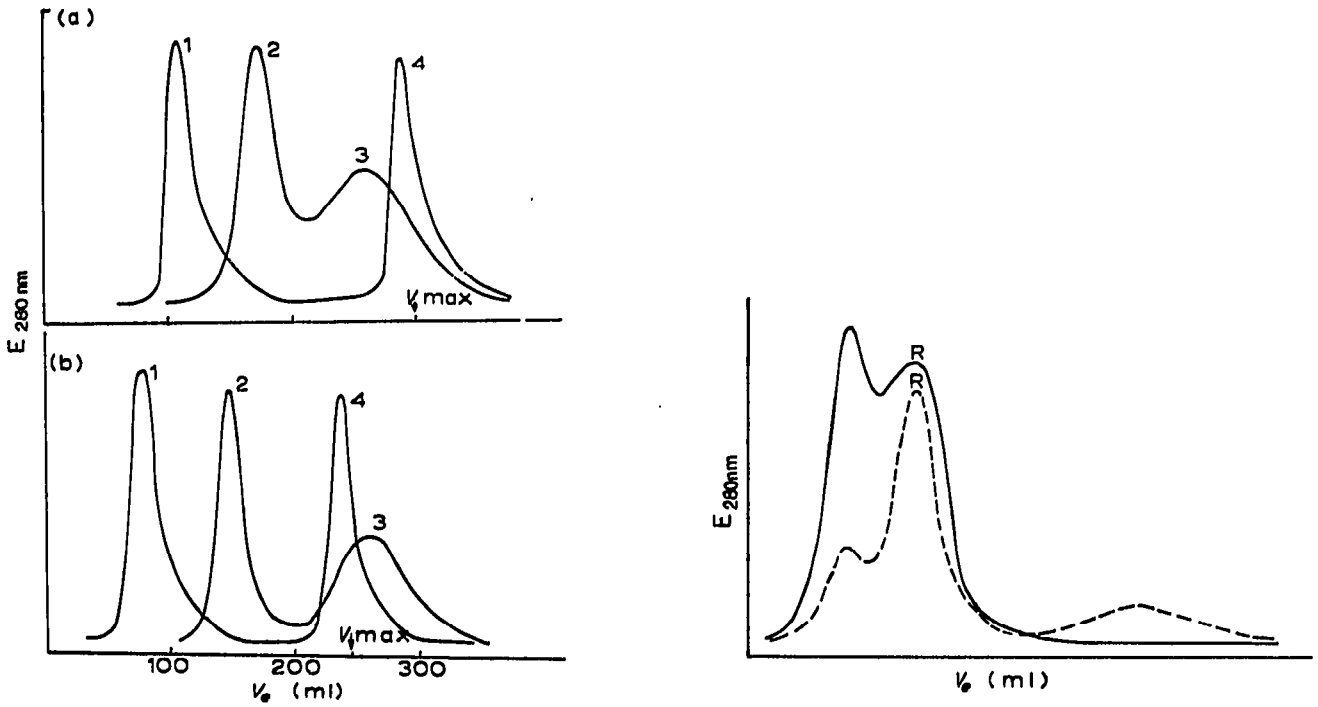


Fig. 3. The effect of a change in the operating temperature (a = 8°, b = 40°) upon the chromatography of chymotrypsinogen A on 8% chromium-formalin tanned gelatin. 1 = Blue Dextran; 2 = cytochrome C; 3 = chymotrypsinogen A; 4 = tryptophan.

Fig. 4. The effect of high urea concentrations upon the chromatography of chymotrypsinogen A on 8% chromium-formalin tanned gelatin. — — —, 6 *M* urea; ———, 8 *M* urea. R = cytochrome C reference peak.

new buffer before addition of the sample. As none of these agents reduced the retardation of the protein, it was concluded that retardation is probably not due to ion-exchange, sulphydryl interactions or co-ordinate bonding with the chromium. Furthermore, an indication that hydrophobic interactions are not operative is provided by the amino acid tryptophan, which is eluted at a volume corresponding to the calculated V_{max} of the column.

The effect of an increase in temperature from 8° to 40° and the effect of the addition of urea at two different concentrations were also investigated (Figs. 3 and 4). It is apparent from Fig. 3 that an increase in temperature from 8° to 40° causes the gel to shrink (as manifested by the decrease in V_{max}) although it does not affect the elution volume of chymotrypsinogen A. In the presence of 6 or 8 *M* urea, the gel completely lost its mechanical resilience, becoming soft and easily compacted. At the same time, in 6 *M* urea, the retardation effect was apparently reduced, resulting in the chymotrypsinogen A being distributed into two peaks while, in 8 *M* urea, the retardation effect was apparently completely eliminated as the chymotrypsinogen A was eluted as a single peak which lay in front of the cytochrome C reference peak.

Discussion

It is commonly accepted that the structure of gelatin results from the collapse of the more ordered hydrogen-bonded triple-helical structure of collagen. In the model of HARRINGTON AND VON HIPPEL[5], gelation of a hot solution of gelatin by

cooling is thought to be due to the reformation of poly-L-proline II type helices along regions of the individual peptide chains. These are thought to associate subsequently to regenerate local regions of triple helix, stabilised by interchain hydrogen bonds. An overall randomly ramified structure, composed of regions of triple helix linked together by single polypeptide chains (presumably in the poly-L-proline II configuration) is thought to result.

The possible role of hydrogen bonds in the stabilisation of the structure of tanned gelatin gels is indicated by the loss of gel strength occasioned by high concentrations of urea. Furthermore, as the retardation of chymotrypsinogen A is eliminated by high concentrations of urea, it may be concluded that retardation during chromatography, in the absence of urea, is either due to hydrogen bond interactions between the gel and the sample protein, or that it is a consequence of some unique three-dimensional aspect of the gel structure. That the Stokes' radius of chymotrypsinogen A is not markedly affected by high urea concentration is indicated by the chromatographic data of OLSON AND LIENER[6].

In the model outlined above, the regions comprised of single polypeptide chains would be particularly rich in potential (unpaired) hydrogen bonding groups which, during chromatography, could pair in a dynamic manner with complementary groups on the sample protein and thus cause its retardation. The degree of retardation experienced by a given protein would thus be a function of the number of potential hydrogen bonding groups arrayed upon its surface. It should be noted, however, that in the case of agarose, gelling is also commonly ascribed to intermolecular hydrogen bonding but agarose has not been found to retard proteins during chromatography in buffers of adequate ionic strength.

Although no conclusive explanation can be offered the observation that certain proteins are retarded during chromatography on tanned gelatin has the consequence that the molecular weights of proteins cannot be estimated confidently from their elution volumes on columns of this material. This does not detract from the usefulness of tanned gelatin as a separating agent, however (for example tanned gelatin would appear to be particularly suitable for the isolation of lysozyme), and, in view of its high strength and low cost, tanned gelatin should find widespread application, particularly in preparative chromatography.

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