Journal of Chromatography, 206 (1981) 368–371
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,470

Note

Altered synthesis of carboxylacrylamide which offers improved resolution of venom proteins

T. P. A. KRUCK* and D. M. LOGAN
Biology Department, York University, Downsview, Ontario (Canada)
(Received October 27th, 1980)

Carboxylated polyacrylamide resins* (Bio-Rad Labs., Richmond, CA, U.S.A.) offer excellent resolution of protein mixtures but suffer from several operational disadvantages, *e.g.* variable bed volumes and flow-rates and difficulties with regeneration *in situ*. For these reasons Bio-Rad Labs. has ceased to manufacture CM-acrylamide gels and is offering other materials such as CM Bio-Gel A which have better mechanical properties. In preparing our own carboxyl polyacrylamide resin we have found that a modification of the technique used to prepare the commercial resins[1] offers improved resolution and more reproducible flow-rates. The details of this synthesis are reported here.

EXPERIMENTAL

Bio-Gel P30 (100–200) mesh was obtained from Bio-Rad Labs. Lyopholized crude snake venoms were obtained from Sigma (St. Louis, MO, U.S.A.). All other reagents were of analytical grade and were obtained primarily from Eastman (Rochester, NY, U.S.A.) and BDH (Poole, Great Britain).

Resin preparation

Bio-Gel P30 was hydrated prior to treatment by gentle agitation in an excess of double-distilled water (20 g resin in 500 ml of water) and was then partially deaminated by one of two techniques.

Resin A (commercial technique). A 500-ml volume of 1 *M* $NaHCO_3$–Na_2CO_3 buffer, pH 10.5, was added and the suspension was incubated at 65°C in a thermostatically controlled water-bath with continuous mixing. After 7.5 h the mixture was cooled to room temperature (20°C) by the addition of crushed ice. The resin was then washed once with 1 l of 1.0 *M* NaCl, followed by several washes with water until the pH was below pH 8. This produces the Na^+ form of the resin free from contaminating base. In the preparation of NH_4^+ form resins the NaCl wash was omitted.

* Although referred to as carboxymethyl acrylamide, these resins, prepared by the method of Inman and Dintzis[1] are actually deaminated polyacrylamide and should be more properly referred to as carboxyl polyacrylamide.

0021-9673/81/0000–0000/$02.50

Resin B (modified technique). The procedure for resin A was repeated but incubation was continued for only 3.5 h. The resin was then washed extensively with distilled water. The resulting resin slurry (1 l) was then diluted with 1 l of 1 *M* $NaHCO_3$–Na_2CO_3 buffer, pH 10.5 and incubated (with stirring) at 65°C for a further 4.5 h. The final resin was washed extensively with water and stored with 10^{-3} *M* sodium azide to prevent fungal growth.

Preparation of NH_4^+ form of resins (modified technique)

To 20 g of dry resin (original weight) in 1 l of water were added 135 ml of concentrated ammonium hydroxide with vigorous stirring (final concentration 1 *M* NH_4OH). After 1 h the resin was allowed to sediment, the supernatant was decanted and 1 *M* NH_4OH was added to give a final volume of 2 l. After a further hour of incubation the resin was washed repeatedly with water until the pH was less than pH 8.

Resin titration and column chromatography were carried out by conventional techniques. Details are included with the figure legends.

RESULTS AND DISCUSSION

Titration of resins

Resins A and B (Na^+ form) were titrated with 0.6 *M* HCl and the titration curves are shown in Fig. 1. Although the total number of titratable groups is essentially unchanged the p*K* values of the resins differ. Resins prepared by the method of Inman and Dintzis[1] have a pK_a of 4.5 while the modified procedure produces resins with a pK_a of 5.7 probably reflecting an altered distribution of titratable groups. In contrast, the capacities of the gels (mequiv./g) are essentially the same.

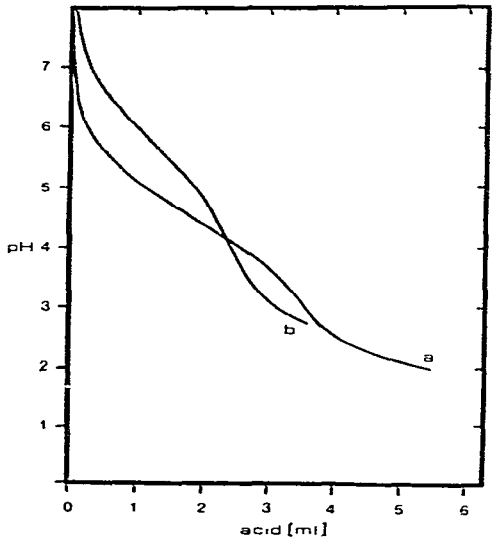

Fig. 1. Titration of carboxylated resins; titrant 0.6 *M* HCl. Resin A, curve a; resin B, curve b. Average pK_a values were calculated by simple curve fitting.

Chromatographic behaviour

Snake venom (*Bungarus fasciatus*) was fractionated on both resins and the results are shown in Fig. 2. Although the input in this case to the resin B column is lower the improved resolution is retained at inputs equal to that used for resin A. The major point is that under equivalent elution conditions resin B retains all proteins other than the initial 4 (peak 5 is guanosine) more strongly than resin A and resolves more completely those proteins (peaks 10–12) which are present in the largest amounts. Note in particular the reduced proportion of fraction 10 when freed from 11 and the appearance of a small peak between peaks 11 and 12 with resin B that cannot be detected with resin A.

With the removal of CM-30 from the commercial market we examined the synthesis of equivalent resins for our own use. Our modifications of the original

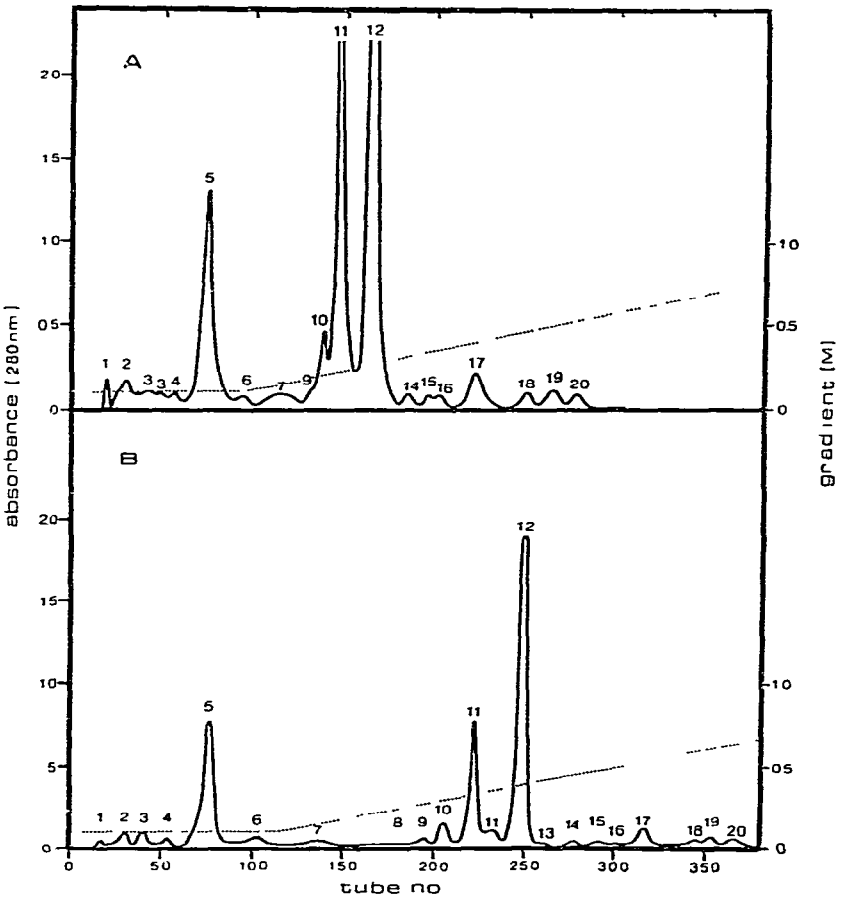

Fig. 2. Chromatography of *Bungarus fasciatus* venom on resins A and B. Column dimensions 2.5 × 80 cm. Elution conditions: initial elution with 0.1 *M* buffer ammonium acetate, pH 6.2, followed by a linear gradient of 0.1 *M* ammonium acetate, pH 6.2, (1 l) to 1.0 *M* ammonium acetate, pH 6.2, (1 l). A, resin A, B, resin B. ———, Protein concentrations; ———, gradient concentrations. Peaks 10, 11 and 12 represent the major α-neurotoxins.

synthesis (*i.e.* the deamination of polyacrylamide) although subtle have a substantial effect on the resin produced, increasing the pK_a values of the resin which we believe reflects an altered distribution of the titratable groups and in turn leads to improved chromatographic resolution by the resin. Most commercial resins are synthesized so as to randomize maximally the distribution of titratable groups (*i e.* random deamination). Our technique which produces resins of equivalent capacity appears however to produce a less random distribution of carboxyl groups. This is suggested by the titration of mono- and dicarboxylic acids in which the proximity of one titrated carboxyl group increases the pK of a second; *e.g.* propanoic acid pK_1 4.87 compared with malonic acid pK_{12} 5.69 and butanoic acid pK_1 4.82 compared with succinic acid pK_{12} 5.64 or maleic acid pK_{12} 5.79.

Thus the increased pK_a of resin B probably arises because of the proximity to the group being titrated of an ionized carboxyl group. In the case of random deamination such proximity would be much less probable.

If this interpretation of increased pK_a is correct (*i.e.* localized "patches" of carboxyl groups) then the enhanced retention and improved resolution of proteins is easily explained. The proteins being fractionated are basic, contain several titratable groups and react strongly with anionic binding sites on acetylcholine receptors. Under normal conditions (*i.e.* commercial resins) they bind to a limited number of groups and are eluted with relative ease. With resin B, however, the proximity of multiple groups produces enhanced binding and provides a wider titration range for elution, hence increased resolution. In terms of the extent of binding such a resin acts therefore as an intermediate between a randomly charged resin and an affinity resin in which one exploits the multiplicity of reactions between a protein and binding substrate. The synthesis of resins with local patches of high group density is thus an alternative to affinity resins where the appropriate ligand cannot be used for reasons of availability, purity or reactivity.

REFERENCE

1 J. K. Inman and H. M. Dintzis, *Biochemistry*, 8 (1969) 4074.