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Note

Amino acid separation on SE-Sephadex C-25

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Protein hydrolysates and amino acid mixtures may be fractionated by cation-exchange chromatography on various supports. Sulfonated polystyrene-based resins, *e.g.* Dowex 50, have found the widest application since they have a high capacity and are chemically stable over the complete pH range. They are available with varying degrees of crosslinking and resins of greater than 8% crosslink exhibit little water regain and bed volume change. The principal disadvantage of sulfonated polystyrene resins is their pronounced affinity for aromatic substrates, which necessitates the operation of the column at elevated temperature to minimize Van der Waals' adsorption. However, substances with very pronounced aromatic character *e.g.* trityrosine, are not easily eluted from Dowex 50 even at elevated temperature. Conventional fractionation of protein hydrolysates on sulfonated polystyrene has almost certainly led to disappearance, by adsorption, of novel crosslinking species specifically being sought in elastic proteins[1,2]. In suitable situations cellulose-based ion exchangers can be used, *e.g.* cellulose phosphate was required to isolate dityrosine and trityrosine from resilin[3,4]. For the systematic fractionation of protein hydrolysates, however, these cellulose exchangers have serious disadvantages. Cellulose phosphate has two pK values[5] and does not retain acidic and neutral amino acids. Theoretically, SE-cellulose should be a more suitable alternative, since the pK value of its sulfonic acid group is similar to that of Dowex 50, and it would thus be expected to retain acidic amino acids. However, like cellulose phosphate, the material dissolves in strong alkali, and its exchange capacity is very low, of the order of 0.2 mequiv./g (ref. 5). Furthermore there is often very wide variation in the quality and exchange capacity of cellulose exchangers, even when derived from the same manufacturer[6]. Two batches of SE-cellulose were titrated in my laboratory and proved to have negligible exchange capacity, which indicated inadequate substitution at the manufacturing stage. For these reasons SE-Sephadex C-25 was examined as a possible suitable alternative for cation-exchange chromatography of amino acids. The material is relatively stable and, unlike cellulose exchangers, can be recycled through 1.0 M acid and alkali without serious alterations in flow-rate and without risk of degradation. Compared to cellulose exchangers it also has a relatively high exchange capacity, of the order of 2.3 mequiv./g (ref. 7), and appears to be manufactured to consistent quality. It suffers from the disadvantage of having a low crosslinking density, with

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consequent high water regain, and a bed volume which varies with ionic strength. These volume changes dictate that it cannot be recycled within a column, or operated by gradient elution techniques more than once without repouring the column. Single-buffer systems of constant pH, analogous to the conventional two-column procedure of Moore and Stein[8], gave reproducible results that were much superior to those obtained with cellulose exchangers. The procedure offers a more satisfactory alternative to the use of polystyrene or cellulosic exchangers and facilitates the separation of aromatic substrates at room temperature.

EXPERIMENTAL

SE-Sephadex C-25 (Pharmacia, Montreal, Canada), 40–120 μ, was sieved in the dry state and particles smaller than 300 mesh were collected. The material was washed extensively and recycled through 1.0 *M* HCl and 1.0 *M* NaOH to remove soluble polysaccharides. After the final alkali wash the material was washed with water and then with the appropriate buffer, poured into a column, and final equilibration achieved within the column. For the separation of acidic and neutral amino acids, the buffer used was 0.04 *M* pH 3.25 sodium citrate and the column dimensions were 1.2×100 cm. For the separation of basic amino acids the buffer used was 0.5 *M* acetic acid with 0.16 *M* NaCl and the column dimensions were 1.2×50 cm. In order to resolve dityrosine from histidine, 0.5 *M* acetic acid with 0.16 *M* KCl was used as eluant. Elution of the desmosine isomers required 0.5 *M* acetic acid with 0.4 *M* NaCl. Aliquots (5 μmoles) of stock solutions of amino acids were mixed before loading onto the column and elution carried out at a rate of 12 ml/h. Fractions of 2 ml were collected. Ascorbic acid (5 μmoles) was incorporated in each mixture to serve as an internal marker detected by UV absorption. UV absorption was continuously recorded at 280 nm using a flow-cell of pathlength 0.2 cm (Instrument Specialities, Lincoln, Nebr., U.S.A.). Ninhydrin analyses of 1-ml aliquots were carried out by the method of Rosen[9], except that the propanol diluent was substituted by ethanol. The peaks were identified by relative height when simple mixtures were used with individual amino acid concentrations differing by at least 100%, and also by paper chromatography after evaporation, with butan-1-ol–acetic acid–water (60:15:25), as developer. The desmosines were obtained from elastin as previously described[10].

RESULTS AND DISCUSSION

Elution profiles of amino acid separations are illustrated in Figs. 1 and 2. Acidic and neutral amino acids eluted from the long column (Fig. 1) were eluted in the general order of their pK_a values. Tyrosine was eluted with alanine and before glycine.

Basic amino acids were eluted from the short column (Fig. 2A–C) in a sequence which corresponded to their elution from Dowex 50, with the exception of the aromatic amino acids tryptophan and dityrosine and also the desmosines. When eluted with 0.5 *M* acetic acid that contained 0.16 *M* NaCl (Fig. 2A) dityrosine was not resolved from histidine at room temperature. Dityrosine was separated from histidine, and all amino acids were accelerated, when the column was eluted with

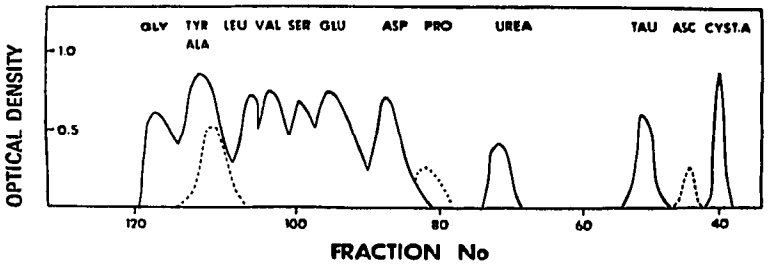

Fig. 1. Elution profiles of acidic and neutral amino acids from a 1.2 × 100 cm column of SE-Sephadex C-25 eluted with 0.04 *M* sodium citrate. Details are listed in the text. Cyst. A = Cysteic acid; Asc = ascorbic acid; Tau = taurine. The profile reads from right to left. ———, O.D. at 570 nm; · · · ·, O.D. at 440 nm; – – – –, O.D. at 280 nm.

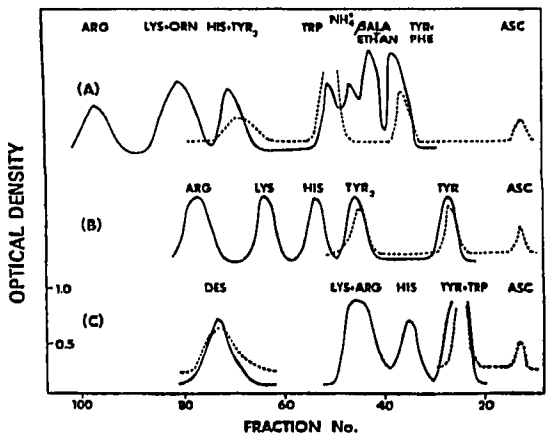

Fig. 2. Elution profiles of aromatic and basic amino acids from a 1.2 × 50 cm column of SE-Sephadex C-25. (A) The eluant was 0.5 *M* acetic acid that contained 0.16 *M* NaCl. (B) The eluant was 0.5 *M* acetic acid that contained 0.16 *M* KCl. (C) The eluant was 0.5 *M* acetic acid that contained 0.4 *M* NaCl. Asc = Ascorbic acid; Tyr_2 = dityrosine; Ethan = ethanolamine; Des = desmosines. The profile reads from right to left. ———, O.D. at 570 nm; – – – –, O.D. at 280 nm.

0.5 *M* acetic acid that contained 0.16 *M* KCl. The desmosines behaved unusually and necessitated a high Na ion concentration (0.4 *M*) to elute them. They were eluted after arginine (Fig. 2C).

The separation method reported here represents a distinct advantage over those based on cellulosic cation exchangers. In the reported fractionation of a resilin hydrolysate[3,4], the acidic and neutral amino acids were probably not retained by cellulose phosphate, since they were not retained in experiments carried out in my laboratory under similar conditions. The elution positions of amino acids other than tyrosine and dityrosine were not recorded and hence the resolution of any other amino acids that were retained cannot be evaluated. All amino acids were retained on the SE-Sephadex column. In the experiments reported here the much reduced adsorption of aromatic substrates by SE-Sephadex, compared to Dowex 50, is well illustrated in the elution position of tyrosine, which is eluted symmetrically, at room temperature, in the neutral amino acid range and before glycine (Fig. 1). This may

be compared to the elution of tyrosine and phenylalanine from Dowex 50 with citrate buffer which required elevation of column temperature to 75°, after the neutral amino acids had emerged at 37° and 50° (see ref. 8). Also, in the elution from Dowex 50 at 50° with 4 *N* HCl, phenylalanine was eluted after arginine and tyrosine not eluted at all[11]. That aromatic adsorption is nevertheless still present on SE-Sephadex is indicated by the elution position of dityrosine, which is eluted late, with the basic amino acids, and similarly by the relative elution position of tryptophan. Dityrosine is eluted at room temperature, both from SE-Sephadex and from cellulose phosphate. It cannot readily be eluted from Dowex 50 at room temperature, and when eluted at 60° it emerges between tyrosine and histidine, and at 40° near to arginine[12]. The very late elution of the desmosines is of interest. Since the pyridinium nuclei of the desmosine isomers are positively charged in acetic acid, they cannot be considered to be aromatic relative to tyrosine or tryptophan in the generally accepted meaning of the term[13]. The desmosines have an isoelectric pH falling between the neutral and basic amino acids[14] and they are eluted in the expected position, between phenylalanine and lysine, on Dowex 50 at 60° with gradient elution on an automated system. Unlike typical aromatic substrates, their elution position on this latter resin is relatively independent of temperature[12]. Furthermore, the desmosines, unlike aromatic amino acids, are not adsorbed on charcoal[2] and are not retarded on polyacrylamide gel[13]. The pK_a value of the ring nitrogen of the desmosines will approximate that of pyridine (5.23) and hence a formal positive charge will be present under the conditions of elution reported here. Since this charge is not in close proximity to the partial negative charge of the carboxyl residues, it might be expected to exert greater electrostatic affinity towards the negative sulfonate charge on the exchanger. This increased affinity for the support would be expected of a pentabasic amino acid, and it might be exploited in an analytical procedure for these amino acid isomers.

Since SE-Sephadex C-25 has a comparatively low degree of crosslinking, which corresponds to Sephadex G-25, the material has considerable water regain (G-25= 2.5 ml/g). The experiments reported here would indicate that a more highly crosslinked exchanger corresponding to *e.g.* G-10 or G-15 would be of greater value for similar cation-exchange chromatography of small molecules, particularly since gradient elution could be used without excessive changes in bed volume. Such a material would be even more superior to SE-Sephadex C-25 when compared to ion-exchange celluloses. It would also have a greater exchange capacity on account of its smaller bed volume per unit weight. It likely would have a greater resolving power since the diameter of the hydrated beads would be smaller than those of SE-Sephadex C-25. Either form of Sephadex cation exchanger would appear to be useful for examining hydrolysates of proteins that are suspected to contain novel aromatic residues. The use of the C-25 form has made possible the fractionation of a variety of hitherto unreported aromatic chromophores from hydrolysates of elastic proteins[15].

CONCLUSIONS

A two-column procedure has been developed in which SE-Sephadex C-25 may be used for the examination of protein hydrolysates. Fair resolution of acidic and neutral amino acids, and good resolution of basic amino acids was obtained.

The method affords a more preferable alternative to polystyrene- or cellulose-based exchangers for the examination of mixtures containing strongly aromatic cations. The results indicate that a more highly crosslinked Sephadex exchanger could find useful application in this field.

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