SEPARATION OF PEPTIDES ON CELLULOSE PHOSPHATE

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Cellulose phosphate cation exchange powders have received little attention as a means for the column chromatographic isolation and purification of proteins and peptides. MARSHALL *et al.*[1] used cellulose phosphate for the isolation of carbamyl phosphate synthetase from frog livers with succinate buffer as the eluent. MITZ AND SCHLEUTER[2] used this ion exchange material for the adsorption and storage of proteolytic enzymes. Several polysaccharide hydrolases from fungi have been separated by MCCLENDON AND KREISHER[3] on cellulose phosphate by means of a pH gradient that was produced by citrate and acetate buffers. MERIGAN *et al.*[4], CANFIELD AND ANFINSEN[5] and BRAUN[6] have reported the separation of peptide mixtures on cellulose phosphate. Using ammonium acetate and pyridine acetate buffers and a gradient in both the pH and the ionic strength of the eluent, CANFIELD AND ANFINSEN successfully separated the peptides from the peptic and chymotryptic hydrolysates of egg white lysozyme.

We have modified the method of CANFIELD AND ANFINSEN and applied it to the purification of tryptic peptides from the α and $\beta\beta^{A_{1c}}$ polypeptide chains from human hemoglobin A_{1c}[7–9] after their preliminary separation on Dowex-50 X2 by the method of SCHROEDER *et al.*[10]. A partial correlation of peptide structure with chromatographic behavior in this system will be discussed.

EXPERIMENTAL

Preparation of column

Whatman Cellulose Phosphate Powder P70 was sized into a 250–325 mesh fraction by dry sifting. Approximately 10 g of this fraction was suspended in an equal volume of water, filtered onto a Buchner funnel, and allowed to drain under slight suction. After it had been washed successively with 100 ml of 1 *F* NaOH, 200 ml of H_2O, 100 ml of 1 *F* HCl, and 300 ml of H_2O, the material was suspended in twice its volume of water and allowed to settle; the fine particles were decanted. The suspension and decantation of fines was repeated a second time. Finally, the settled cellulose phosphate was suspended in three times its volume of buffer B (Table I), and a 0.9 × 100 cm chromatographic column was poured in a single section with this suspension.

Equilibration and loading of column

The column was equilibrated at 28° with 250 ml of buffer B. The mixture of peptides, which contained up to 50 μmoles of each of several peptides (the maximum

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TABLE I

BUFFER COMPOSITIONS FOR CELLULOSE PHOSPHATE CHROMATOGRAPHY

Buffer	*pH*	*Ionic strength*[a]	*Pyridine (ml/l)*	*HOAc (ml/l)*
A	3.93	0.025	2.00	8.42
B	3.93	0.05	4.05	15.00
C	3.93	0.10	8.10	30.00
G	6.12	0.20	166.0	11.38
H	6.12	0.45	390.0	25.70

[a] Calculated from pK' values.

capacity of the column may be larger), was dissolved in 3 ml of buffer A, applied to the column, and rinsed in with three 1-ml portions of buffer B.

Development of column

The column was developed at 28° with a two-vessel gradient device[11]; the mixer, which had twice the area of the reservoir, was filled with 232 ml of buffer B (Table I) and the reservoir with 116 ml of buffer C. A flow of 50 ml/h was maintained and 4.4-ml fractions were collected. At the 80th fraction, any buffer in the gradient device was removed, and the mixer and reservoir were filled with 440 ml and 220 ml of buffers C and G, respectively. At fraction 220, the gradient was discontinued, and development was completed to fraction 260 with buffer H. Peptides were detected in the effluent by reaction with ninhydrin after alkaline hydrolysis as described elsewhere[12].

Re-equilibration of column

The column was re-equilibrated with buffer B immediately after a chromatogram was finished, because, on prolonged standing in buffer H, the cellulose phosphate tended to decompose. This was evidenced by an irreversible shrinkage of the column as well as by an increase over the normal operating pressure of about 10 p.s.i. during subsequent chromatograms. Even with this precaution, a column eventually developed sufficient back pressure (70 p.s.i.) so that it was desirable to discard it and pour a new column with freshly prepared cellulose phosphate. It is possible that the useful life of a column could be extended by operating it at a lower temperature. The shrinkage as well as the increasing back pressure seemed to have no deleterious effect upon the separations.

RESULTS

Fig. 1 shows a typical separation, while Fig. 2, which was composed from 18 individual chromatograms, summarizes the chromatographic behavior on cellulose phosphate of several tryptic peptides from the α and β chains of human hemoglobin A_{Ic}*. Because of slight variations between chromatograms the actual position of emergence of a particular peptide may vary somewhat from the position shown in

* The nomenclature in these figures follows that of BAGLIONI[13]. The detailed primary amino acid sequence of any particular peptide can be found in the review by SCHROEDER AND JONES[14].

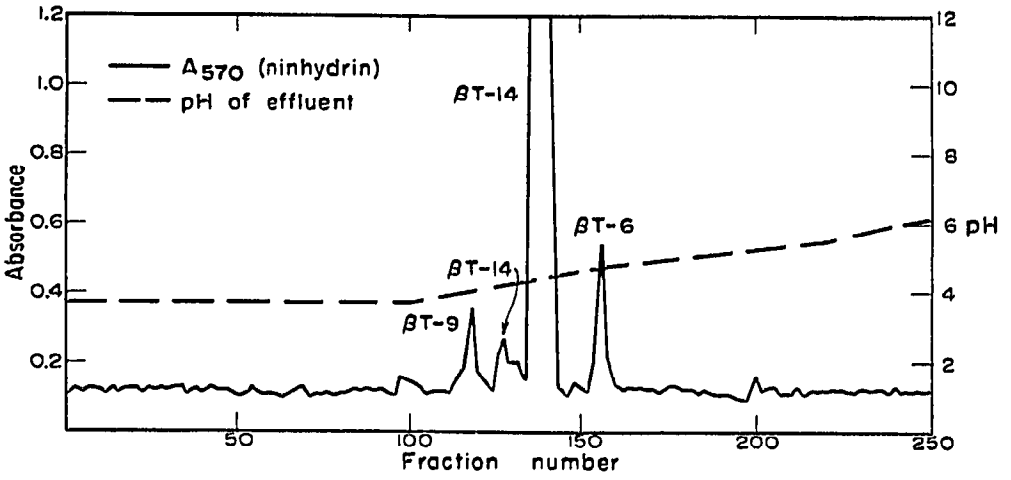

Fig. 1. Separation of a mixture of the tryptic peptides βT-9, βT-14 and βT-6 by chromatography on cellulose phosphate. Column size: 0.9 × 100 cm. Fraction size: 4.4 ml.

Fig. 2. Although examination of Fig. 2 would suggest a poor separation of most of the peptides, this is not the case. In the first place, the α and β chains themselves had been separated so that α and β peptides in identical positions do not interfere with each other. In the second place, an initial separation had been made by chromatographing on Dowex-50 X2 and only a relatively simple mixture was chromatographed on cellulose phosphate. Thus, αT-2, αT-4, αT-8 and αT-10 which have virtually identical behavior under these conditions were present in widely separated zones on Dowex-50 X2. Nevertheless, by chromatography on cellulose phosphate each of these separated from other peptides with which they were coincident on Dowex-50. If desired, a more gradual gradient could be used to separate peptides that are difficult to separate under the conditions given.

A correlation between the length and ionic charge of the peptide and its position of emergence is given in Table II. With some exceptions, the longer more negative peptides emerge prior to the shorter more positive ones. More particularly, peptides

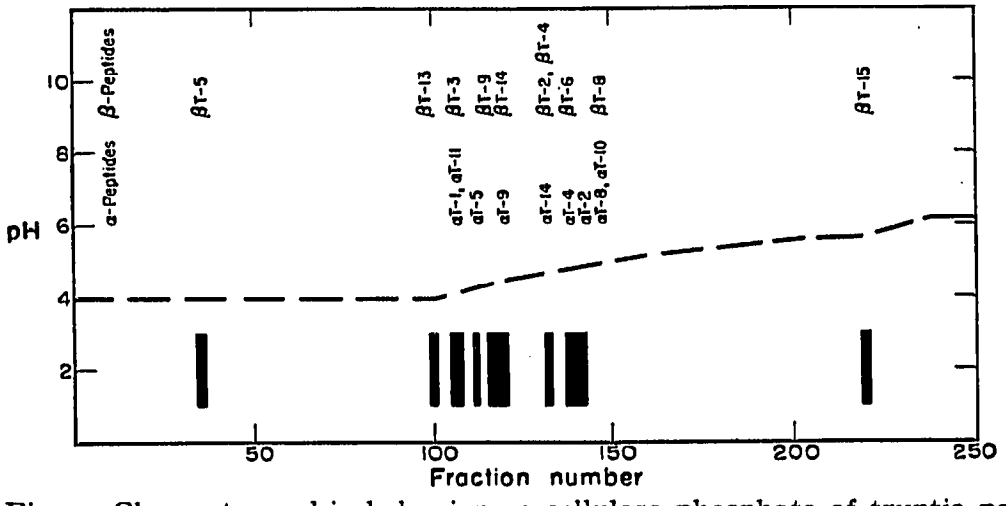

Fig. 2. Chromatographic behavior on cellulose phosphate of tryptic peptides from the α and β chains of human hemoglobin A_{Ic}. The shaded boxes show the approximate position of emergence of the peptides listed above them. See text for discussion.

TABLE II

CORRELATION BETWEEN PEPTIDE LENGTH, CHARGE AND CHROMATOGRAPHIC MOBILITY ON CELLULOSE PHOSPHATE

Peptide[a]	*L*[b]	*Q*[c]	*Peptide*[a]	*L*[b]	*Q*[c]
			βT-5	19	—2
			βT-13	12	0
αT-1, αT-11	7	0	βT-3	13	—2
αT-5	9	1			
αT-9	29	—1.5	βT-9	16	—0.5
			βT-14	12	1.5
αT-14	2	1	βT-4	10	1
			βT-2	9	1
αT-4	13	—1.5			
αT-2	4	1	βT-6	2	1
αT-8	1	1	βT-8	1	1
αT-10	2	1			
			βT-15	2	0.5
			βT-7	4	1.5

[a] The peptides are arranged from top to bottom, in descending order of their chromatographic mobilities and are spaced approximately as in Fig. 2.

[b] *L* is the number of amino acid residues in the peptide.

[c] *Q*, a measure of ionic charge, was calculated by assigning a value of —1 to glutamic acid and aspartic acids, +1 to lysine and arginine, +0.5 to histidine, and 0 to all other amino acids.

which contain more than 6 amino acid residues and an ionic charge no greater than 2 emerge prior to pH 4.7 irrespective of the sign of the ionic charge. Peptides that are less than 7 amino acids in length and which bear a net positive charge emerge after pH 4.7. Some peptides which contain histidine appear to be anomalously retarded: αT-9, αT-4, and βT-7, for example. βT-7 is an extreme case. This peptide did not emerge from the column under the chromatographic conditions employed; however, after the column had been used for several successive chromatograms, βT-7 appeared in the effluent. The sequence of this peptide is Ala–His–Gly–Lys; its excessive retardation may be due to the combination of its short length, large positive charge, and the presence of histidine.

DISCUSSION

The correlation of peptide structure and chromatographic behavior found here is very similar, but not identical, to that found for Dowex-50 chromatography[10]. Even the anomalies occur for the same peptides. This similarity suggested that peptides which are difficultly separable on Dowex-50 by the method of SCHROEDER *et al.*[10] might be purified on that resin by rechromatography with appropriate modifications of the buffers of Table I. This has indeed been found to be the case[8,9,15].

Two difficulties encountered with cellulose phosphate chromatography were "double zoning" and increased resistance to flow in the column as it was repeatedly used. The latter effect has already been discussed. The former was manifested as in Fig. 1 in which a single peptide (βT-14) is detected in two zones. As further examples, the major portion of βT-13 and βT-9 was detected in the position designated in Fig. 2 but small amounts have been found in the vicinity of βT-5, and *vice versa*. Although

double zoning has been observed on Dowex-1[10], it has been our experience that the problem is more troublesome in the case of cellulose phosphate.

One advantage of cellulose phosphate chromatography is the lack of contamination of the effluent by cellulose phosphate itself. In fact, resin contaminants in the residue from the effluent from Dowex-50 chromatograms were to a large extent removed by subsequent chromatography on cellulose phosphate.

The purity and yields of individual peptides provide some measure of the overall usefulness of a chromatographic method. Amino acid analysis of the individual peptides demonstrated that they were usually free of contamination by other peptides. However, the yields of the purified peptides were less satisfactory. The average yield was 53 %, but the yield for different peptides was highly variable: βT-9 and βT-7, for example, were obtained in yields of 92 % and 0 % respectively. Generally the better yields were obtained with acidic peptides and the poorer yields with basic peptides.

In one instance, an application of cellulose phosphate chromatography has been found to be particularly helpful. Because of the hydrolytic lability of the imine double bond in Schiff bases, these compounds can sometimes be difficult to isolate. Such a Schiff base is found in a tryptic peptide from hemoglobin A_{Ic}[7–9]. However, by a slight modification[7–9] of the methods described here (essentially, a more gradual gradient over a narrower pH range), this peptide has been successfully isolated and purified in better than 90 % yield (based on amount applied to the cellulose phosphate column).

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SUMMARY

A chromatographic method is described whereby peptides can be isolated and purified by column chromatography on cellulose phosphate with volatile pyridine–acetic acid buffers. The chromatographic behavior of these peptides has been correlated with their length and ionic charge. Attention is drawn to the fact that chromatography on cellulose phosphate cannot only be a useful adjunct to other methods of purifying peptides, but may be of particular usefulness in isolating and purifying certain types of labile Schiff base compounds.

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