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IMPROVED SEPARATION OF BASIC PEPTIDES IN ANION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

A method is described for the separation of peptides on anion-exchange-resin columns with particular emphasis being given to the resolution of basic arginine and basic lysine peptides. The addition of N-methylpiperidine, pK_a 10.08, to the solvent systems used either with stepwise or gradient separations improves the resolution in the high pH range compared to methods previously used.

INTRODUCTION

Purification of peptides is a fundamental step in the determination of the amino acid sequence of a protein. Separation on ion-exchange resins, first introduced by Moore and Stein[1] for the amino acid analysis of proteins, is now widely used for peptide purifications. An important improvement when using such resins for this purpose was the introduction of volatile organic solvents as buffers that could be used in combination with the ninhydrin reaction[2–6]. The procedure most commonly used is to separate peptides of varying charge on a cation-exchange resin (Dowex 50) followed by rechromatography of poorly resolved components on an anion-exchange resin (Dowex 1). With the procedures described in the literature, however, both types of resins have disadvantages with respect to the separation of basic peptides. On cation-exchange resins, these peptides are often so strongly bound that drastic elution conditions are required such as high ionic strength or high pH. On anion-exchange resins, on the other hand, they may be bound too weakly and be eluted with the solvent front.

It was previously observed in this laboratory that certain basic arginine and lysine peptides could be separated on Dowex 1 using N-methylpiperidine acetate, pH 11.2, as a starting solvent[7]. The aim of the present investigation was to improve the resolution of basic peptides in anion-exchange chromatography utilizing the properties of N-methylpiperidine. Since this solvent is a ninhydrin-negative, volatile amine with a high pK_a value (10.08), chromatography on Dowex 1 can be initiated at a relatively high pH and the separation of basic arginine peptides from basic lysine peptides may readily be achieved.

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MATERIALS AND METHODS

Buffers

The buffer constituents used are listed in Table I.

TABLE I

BUFFER CONSTITUENTS USED IN THE CHROMATOGRAPHIC EXPERIMENTS

All of the compounds except dimethylallylamine were refluxed for 1 h with ninhydrin prior to distillation at the temperature given.

Compound	*Origin and grade*	*Boiling point (°C)*	*pK_a*	*Ref.*
N-Methylpiperidine	Fluka (Buchs, Switzerland), practical grade	107	10.08	9
N,N-Dimethylallylamine	Eastman (Rochester, NY, U.S.A.)	–	8.72	9
N-Ethylmorpholine	Eastman, practical grade	139	7.70	9
2-Methylpyridine (α-picoline)	Merck (Darmstadt, G.F.R.), chromatographic grade	129	5.97	10
Pyridine	Mallinckrodt (St. Louis, MO, U.S.A.) AR	116	5.17	10

Peptide mixtures

Human carbonic anhydrase B was prepared according to Henderson and Henriksson[8]. After heat-denaturation (90°C for 10 min at pH 8.0) the enzyme was hydrolyzed overnight at pH 9.0 with trypsin or chymotrypsin (Worthington, Freehold, NJ, U.S.A.) using a weight ratio of enzyme to substrate of 1/50. The pH was then lowered to 6 with acetic acid and insoluble material was removed by centrifugation. The soluble portion was lyophilized and used for the chromatographic experiments.

Procedures

Dowex 1-X2 (200–400 mesh, Cl^-) (Baker, Phillipsburg, NJ, U.S.A.), was converted into the acetate form[6], suspended in water and deaerated by suction. Columns were packed by the slurry technique. Deaerated water was then run through overnight before adjusting the resin to the desired length. Prior to use, the column was equilibrated overnight with the first solvent using a soda lime trap to avoid CO_2 absorption from the air.

All column operations were carried out at 20°C. Approximately 4 μmoles of a peptide mixture, dissolved in 1.5 ml of the starting buffer and adjusted to pH 11.7 with NaOH, were applied to the column. The flow-rate of 9 ml/h was kept constant by a peristaltic pump and 3-ml fractions were collected. Further experimental details are given in the captions to Figs. 1–3.

Arginine peptides were located by spotting 15 μl of each fraction onto a paper strip which was either developed with Sakaguchi reagents[11] or the phenanthrenequinone test[12]. Aliquots of 50 μl from each fraction were also applied at 1.5-cm intervals to a Whatman No. 3MM paper and the peptides were separated by high-voltage electrophoresis at pH 6.5[13] after which the paper was dipped in cadmium–ninhydrin reagent[14]. In some cases, peptides containing histidine were identified by means of the Pauly reagent[11].

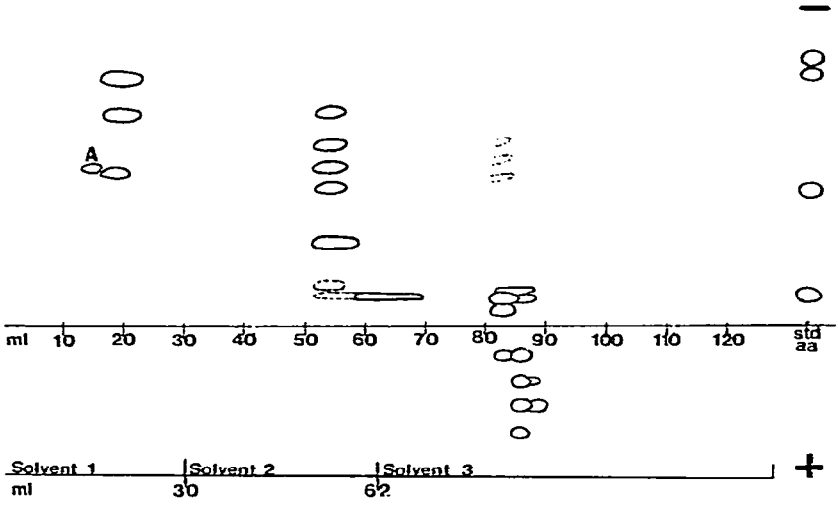

Fig. 1. Group separation of tryptic peptides from human carbonic anhydrase B obtained by stepwise elution from Dowex 1. Column: 25 × 0.9 cm. Aliquots of the fractions were spotted on Whatman No. 3MM paper and subjected to high-voltage electrophoresis at pH 6.5 followed by cadmium–ninhydrin staining. The positively charged arginine peptides emerged 19 ml after the start using solvent 1 (0.1 *M* N-methylpiperidine adjusted to pH 11.2 with acetic acid). Positively charged lysine peptides emerged at 54 ml, or 24 ml after the introduction of solvent 2 (0.1 *M* N-methylpiperidine and 0.1 *M* N-ethylmorpholine adjusted to pH 8.0 with acetic acid). Neutral and negatively charged peptides were eluted with 1 *M* acetic acid (solvent 3). Traces of incompletely eluted positively charged lysine peptides also emerged with solvent 3. Spot A preceding the arginine peptide front is a peptide containing both arginine and lysine residues (*cf.*, Fig. 3).

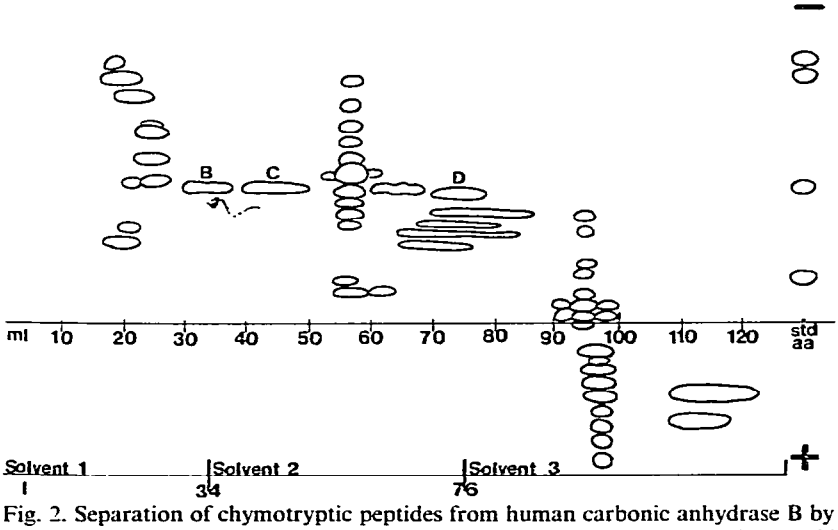

Fig. 2. Separation of chymotryptic peptides from human carbonic anhydrase B by stepwise elution from Dowex 1. For experimental details see Fig. 1. The positively charged arginine peptides emerged after 19 ml of solvent 1. Positively charged lysine peptides were eluted 24 ml after the introduction of solvent 2. Neutral and negatively charged peptides emerged after applying solvent 3. The peptides marked B and C were found to contain arginine and phenylalanine. The peptides marked D following the lysine peptide front were found to contain histidine by means of the Pauly test. The last two spots in the chromatogram are tryptophan-containing peptides with a net charge of −3 derived from the acetylated amino-terminus of human carbonic anhydrase B[15].

RESULTS

Figs. 1 and 2 show a stepwise elution on Dowex 1 carried out on tryptic and chymotryptic peptides, respectively, from human carbonic anhydrase B. The elution was carried out in three steps and the compositions of the solvents used are given in the caption to Fig. 1. It is apparent that this procedure results in a quick and reproducible separation of the peptides into three main groups: the positively charged arginine peptides, the positively charged lysine peptides and the neutral and negatively charged peptides. Each group emerges from the column with its particular solvent front. Some chymotryptic arginine peptides containing phenylalanine were found to be slightly retarded, and some chymotryptic histidine-containing peptides were eluted between the lysine and the neutral–acidic peptide fronts.

The chromatography of tryptic peptides on Dowex 1 using a pH gradient is shown in Fig. 3. The compositions of the solvent systems are described in the legend. Basic peptides containing arginine were eluted with the starting buffer, and were well

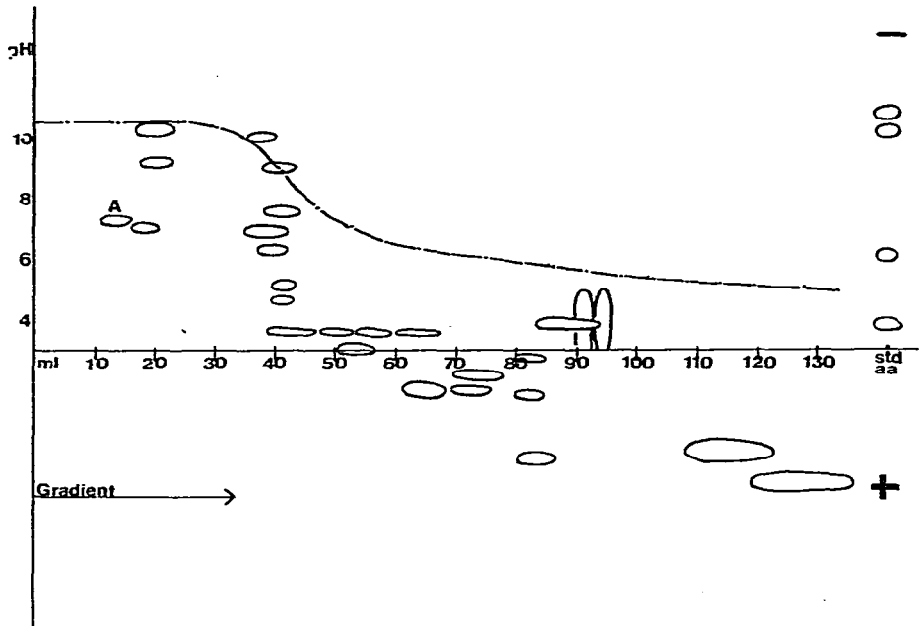

Fig. 3. Separation of tryptic peptides from human carbonic anhydrase B by gradient elution from Dowex 1. Column: 35 × 0.9 cm. The column had been equilibrated with a mixture of 0.1 *M* each of N-methylpiperidine, N-ethylmorpholine, α-picoline and pyridine adjusted to pH 11.2 with acetic acid. A gradient was arranged by placing this solvent in a constant-volume mixing chamber of 100 ml. The buffer in the upper vessel was the same mixture adjusted to pH 4.7 with acetic acid. The pH curve in the diagram was derived from pH measurements of alternating fractions. The peptides emerging at about 20 ml after the start of the gradient are the positively charged arginine peptides. A peptide marked A, always preceding the arginine peptide front, was found to contain both arginine and lysine residues. Elution of the positively charged lysine peptides commenced at an effluent volume of 36 ml at a pH of 10.0 and was completed 8 ml later after a drop in pH to 8.0. The neutral and negatively charged peptides were distributed in the subsequent fractions. Two peptides, recognized as being derived from the acetylated amino-terminal portion of human carbonic anhydrase B[15], emerged at about 110 ml and were completely eluted at an effluent volume of 150 ml at pH 5.0. They contain tryptophan residues and have a net charge of −3.

separated from the lysine-containing basic peptides and from the neutral and acidic peptides. Some attempts were made to achieve a better resolution within the lysine peptide group by using less steep gradients. Variations included the use of longer columns and a higher concentration of N-methylpiperidine (0.3 *M* instead of 0.1 *M*) or addition of 0.1 *M* N,N-dimethylallylamine to the solvent. The use of a less steep pH gradient was accompanied with pronounced tailing that negated the benefits obtained by the somewhat improved resolution of the lysine peptides.

DISCUSSION

The aim of this study was to improve the resolution of basic peptides in anion-exchange chromatography. For that purpose we have utilized a ninhydrin-negative heterocyclic amine, N-methylpiperidine, as a constituent of the solvent systems. Since N-methylpiperidine is a stronger base (pK_a 10.08) than the compounds usually employed in separations of this kind[3,5,6], buffering capacity is obtained at a more alkaline pH and the chromatography can be started at a higher pH.

If a peptide is to be retarded on an anion-exchange resin, the pH of the buffer used should preferably be such that it renders the peptide negatively charged. The side-chain of arginine has a p*K* of 11.6–12.6 and the ε-amino group of lysine a p*K* of 9.4–10.6 depending upon the adjacent amino acid residues in the peptide. An application of this method is shown in Figs. 1 and 2. Chromatography is started with a buffer at pH 11.2. The positively charged arginine peptides emerge either with the solvent front or are slightly retarded if aromatic amino acids are present. Lysine peptides, on the other hand, remain bound to the resin and do not emerge even with extended elution at this pH (Fig. 6B in ref. 7). Stepwise elution with a buffer at pH 8 and acetic acid, respectively, results in a group separation of basic lysine peptides from the neutral and acidic peptides present in the mixtures.

A pH gradient elution is also presented (Fig. 3) which provides a similar resolution of basic peptides into arginine and lysine peptides to that described above. As the gradient proceeds the other peptides in the mixture are successively eluted. Our gradient system has certain features in common with the procedure for Dowex 1 chromatography described by Schroeder[6] who utilized N-ethylmorpholine, α-picoline and pyridine as buffering substances. The gradient system used here, however (see caption to Fig. 3), contains N-methylpiperidine in addition. In this way, an initial pH of about 11 was achieved, as compared to 9.4 in the system used by Schroeder where the buffering capacity at this pH is accounted for by N-ethylmorpholine (pK_a 7.70).

The gradient elution system (Fig. 3) has been applied to the tryptic peptides from human carbonic anhydrase B. Since each protein may pose different problems with respect to peptide separation, the shape of the gradient used here may not be the ideal one for every case. Different shapes should be easy to obtain by varying the volume of the mixing chamber and the pH or the composition of the buffers. It may also be advantageous in some cases to utilize the conditions given by Schroeder[6] after an initial elution covering the pH range 11.2–9.4 with a buffer containing N-methylpiperidine.

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