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PEPTIDE SEPARATIONS ON SUBSTITUTED POLYSTYRENE RESINS

EFFECT OF CROSS-LINKAGE

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SUMMARY

The effect of copolymer cross-linkage on the resolution of soluble tryptic peptides of human globin (α- and β-chains) separated in columns containing substituted polystyrene resin classified to 11 ± 1 μm has been examined. With both the cation and anion exchange resins, polymers of lower cross-linkage provided better resolution; inferior resolution was obtained with 12% cross-linked resins. It was also observed that microparticle anion-exchange resins could be used in columns maintained at 55° instead of 35° as used traditionally. Resolution and yield with 20×1 cm resin beds were generally as good as or superior to much longer columns of crushed bead resin of the same chemical structure.

INTRODUCTION

The development of methods for isolating peptides in homogeneous form, often from quite complex mixtures, has played an important role in determination of the structure–function relationships of proteins. For example, primary structure elucidation and mapping of genetic changes at the phenotypic level require techniques for complete separation of peptides to insure that further characterization of the pure components can proceed. Among the variety of methods used, ion-exchange column chromatography, particularly with resins of substituted polysytrene, has been one of the most productive. These polymers have emerged as the best material for fractionating mixtures containing peptides of about 2 to 30 amino acid residues, the distribution usually encountered in enzymatic digests of proteins. They are less effective with larger fragments which require other types of ion-exchange material or the application of different separation techniques.

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Since there remains a constant need to resolve mixtures of peptides of all sizes, these resins continue to enjoy wide application. However, little systematic study has been made of the parameters affecting elution behavior, yields and resolution of peptides separated on them. In part, this may be attributed to the unique nature of each peptide mixture encountered. Unlike samples used for amino acid analysis which differ primarily only in the amount of each component present, peptide mixtures may be entirely different depending on their origin and method of production. This has resulted in the development of many variations in separation methods and has not enabled other workers, particularly those less experienced, to find the type of information they need to optimize the separation of their own particular mixtures.

In order to establish more useful guidelines for the use of substituted polystyrenes in the separation of peptides, we initiated studies to determine the effect on separation of such variables as temperature, eluent composition and polymer matrix. It has already been ascertained from the studies of Jones[1] that the more highly-sieved resins of smaller average diameter are superior for peptide separations, which has proven to be particularly true for microscale preparations[2]. However, these resins have been available only in a limited number of forms. The development of uniform particle size anion- and cation-exchange resins of this type with a range of cross-linkage has now provided an opportunity to examine the relationship between percent divinylbenzene present as a copolymer (or a "cross-linker") and the resolving properties of the resin. In particular, we examined the commonly held view that lower cross-linked resins were more suitable for peptide separations than higher ones. Parr[3] recommended 2 to 4% cross-linked resins for large ions and Schroeder[4] recommended 2% cross-linked cation- and anion-exchange resins for most peptide separations. An 8% cross-linked cation-exchange resin was used by Jones[1] and has seen wide application elsewhere[5–8]. However, 2% cross-linked anion-exchange resins continue to be used almost exclusively[9–12]. In this study, the cation-exchange resins DC-X8-11* and DC-X12-11 and anion-exchange resins DA-X2-11**, DA-X8-11 and DA-X12-11 were compared by separating on each a tryptic hydrolysate of normal human globin (α- and β-chains).

MATERIALS AND METHODS

All resins examined were prepared by and are available from Dionex Chemical Corp. (Sunnyvale, Calif., U.S.A.). Hemoglobin was prepared from blood freshly drawn from normal healthy adults by waterlysis. Heme was extracted by cold acetone–

* The resin nomenclature is: the first letter designates the polymer matrix ("D" represents divinylbenzene–polystyrene copolymer); the second letter denotes the type of ion-exchange resin ("C" represents a cation exchanger, "A", an anion exchanger); the number following the "X" is the percent cross-linking agent present; the final number represents the mean particle diameter in micrometers.

** Conventional polystyrene resins, such as Dowex 1-X2 contain 2% divinylbenzene, but the effective cross-linkage is actually increased to about 4% because methylene bridging occurs during polymerization. Dionex resins are devoid of methylene bridging because of the polymerization conditions used. Thus, although the designation "X2" in DA-X2-11 implies 2% cross-linkage, this resin actually contains about 4% divinylbenzene. Dionex elected to designate it as a 2% cross-linked resin because it is structurally similar to Dowex 1-X2 resins that have been widely used.

hydrochloride and the resulting globin recovered by lyophilization. Trypsin (TPCK) was obtained from Worthington Biochemicals (Freehold, N.J., U.S.A.). Pyridine was redistilled over solid ninhydrin (1 g/l). All other chemicals were reagent grade or the best quality available.

The tryptic hydrolysate was prepared by suspending globin (1 g) in 50 ml of water and adjusting the pH to 8.8. Trypsin (10 mg/ml in 0.001 *N* hydrochloric acid was added to a final concentration of 2% and the reaction allowed to proceed at 37° for 6 h. The pH was maintained by a pH stat (Radiometer Model TTT-11). The digestion was terminated by addition of 1 *N* HCl to pH 2.0 and the acidified mixture centrifuged at 10,000 *g* for 20 min; only traces of insoluble material were visibly present. The supernatant was subdivided into equal aliquots of about 3.5 ml containing approximately 2 μmol of peptides based on the combined molecular weight of the α- and β-chains. These samples were stored at −20°.

The column separations were carried out in jacketed glass columns of 0.9 cm I.D. In all instances, initial resin beds were 20 ± 0.5 cm. The lower column support was a wire screen resting on a PTFE disc (MER Chromatographic, Mountain View, Calif., U.S.A.). Resin, equilibrated in starting buffer (*vide infra*), was poured under nitrogen pressure (20 p.s.i.) and then pumped at a flow-rate of 30 ml/h for 3 h prior to loading. The slurries of anion-exchange resins were pre-heated to 55° before pouring while those of cation-exchange resins were poured at room temperature. Samples of the soluble tryptic peptides of globin were thawed and loaded onto the columns under nitrogen pressure. The columns were pumped at 30 ml/h for 10 min with starting buffer using a constant volume displacement pump and developed using a four chamber continuous gradient of pyridine acetate buffers. Two hundred ml of each buffer were used in the cation-exchange separations and 200 ml in the anion-exchange separations. Compositions of each gradient are listed in Table I. The final buffer was pumped through the column for an additional 1–3 h at the end of each separation.

TABLE I

ELUENT BUFFER COMPOSITIONS

The eluent buffer compositions were made up to 1.0 l with deionized–distilled water.

Resin	*pH*	*N**	*Acetic acid (ml)*	*Pyridine (ml)***
Cation-exchange				
Buffer 1	2.5	0.05	300	4.0
Buffer 2	3.1	0.2	278	16.1
Buffer 3	3.7	0.5	200	40.3
Buffer 4	5.0	2.0	143	161
Anion-exchange				
Buffer 1	9.0***	0.37	—	30
Buffer 2	5.5	0.5	6	40.3
Buffer 3	5.5	1.0	12	80.6
Buffer 4	5.0	2.0	143	161

* Normality of pyridine. The normality of acetate in the anion-exchange resin buffer solutions 1, 2, 3 and 4 is 0, 0.1, 0.2 and 2.4, respectively.

** Pyridine was redistilled after addition of solid ninhydrin (≈1 g/l) to the distillation flask.

*** This is the pH value for buffer 1 measured immediately after preparation with freshly distilled–deionized water. After standing, the pH of the buffer drops to 7.5–8.0 due to atmospheric carbon dioxide.

Ten percent of the column effluent were diverted into the detection system which monitored the ninhydrin reaction product spectrophotometrically at 570 nm of alkaline hydrolyzed effluent peptides[13]. By utilizing a stream divider and fraction collector at the column outlet, it would be possible to collect 90% of the separated peptides.

Although only small amounts of shrinkage occurred during the separations, resin was routinely removed from the column and washed on a sintered-glass funnel between each experiment.

RESULTS AND DISCUSSIONS

A tryptic digestion of an equimolar mixture of globin α- and β-chains would be expected to produce 27 unique peptides plus free lysine, ranging in size from 20 to 2 amino acid residues[14]. However, the peptides derived from residues α 1–7 and α 93–99 and from α 57–60 and β 62–65 are sufficiently similar that they probably would not be well resolved in a single chromatographic separation. Thus, the expected number of peaks for separation of this mixture is 26.

As shown in Fig. 1, the 8% cross-linked cation exchanger, DC-X8-11, yielded an elution profile with at least 21 peptides clearly discernable. As anticipated from past experience with this resin, peptides of more basic character that elute in the last half of the gradient are better resolved than those eluting in the first half. Separation of the same mixture on the 12% cross-linked resin was considerably less impressive. The entire elution profile demonstrated less delineated peaks, particularly in the early portion of the separation where larger peptides are known to elute. However, even smaller peptides eluting after free lysine (590–620 ml) show broader peaks, due to unfavorable interactions with the chromatographic medium. Tests with cation-

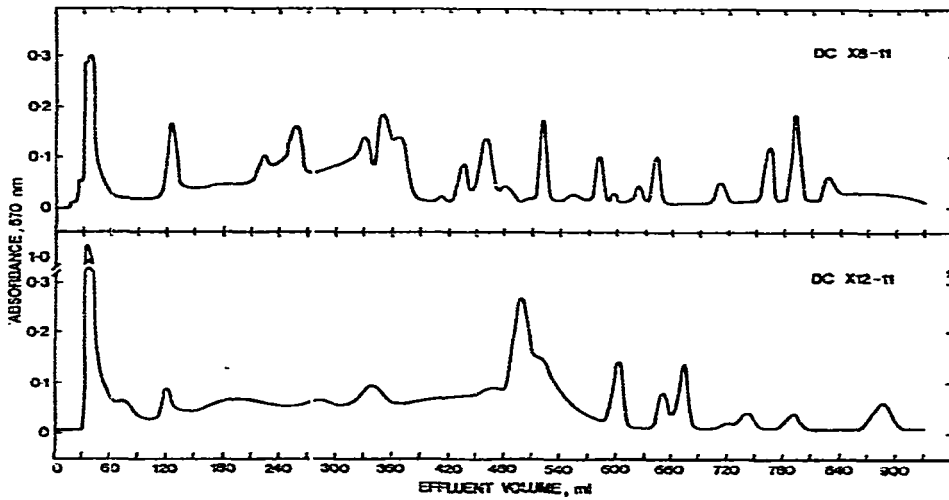

Fig. 1. Elution profiles of the separation of soluble tryptic peptides (2 μmol) of human globin (α- and β-chains) on cation-exchange resins DC-X8-11 and DC-X12-11. Resin bed dimensions: 0.9 × 20 cm; flow-rate: 30 ml/h; temperature: 55°; gradient: 4 chamber pyridine acetate (pH 2.5 to 5.0) (see Table I); monitoring: automatic analysis of ninhydrin color (570 nm) on continuously removed aliquot (≈10%)[13].

exchange resins of lower cross-linkage (2–4%) were unfeasible because the small particle diameter (11 ± 1 μm) contributed to pump pressures too high for practical applications. Separation of the same peptide mixture on the corresponding series of anion-exchange resins is shown in Fig. 2. Differences in elution profiles were not as great as those obtained with the two cation-exchange resins. However, resin with the lowest cross-linkage (DA-X2-11) produced slightly more symmetrical peaks and somewhat better definition than the 8% cross-linked resin. Both resins were clearly superior to the 12% cross-linked resin where some peptides that were separated on DA-X2-11 and DA-X8-11 were apparently excluded and appeared in the break-through peak (30 ml). This is particularly noticeable in the decrease in size of the peak emerging at 80 ml.

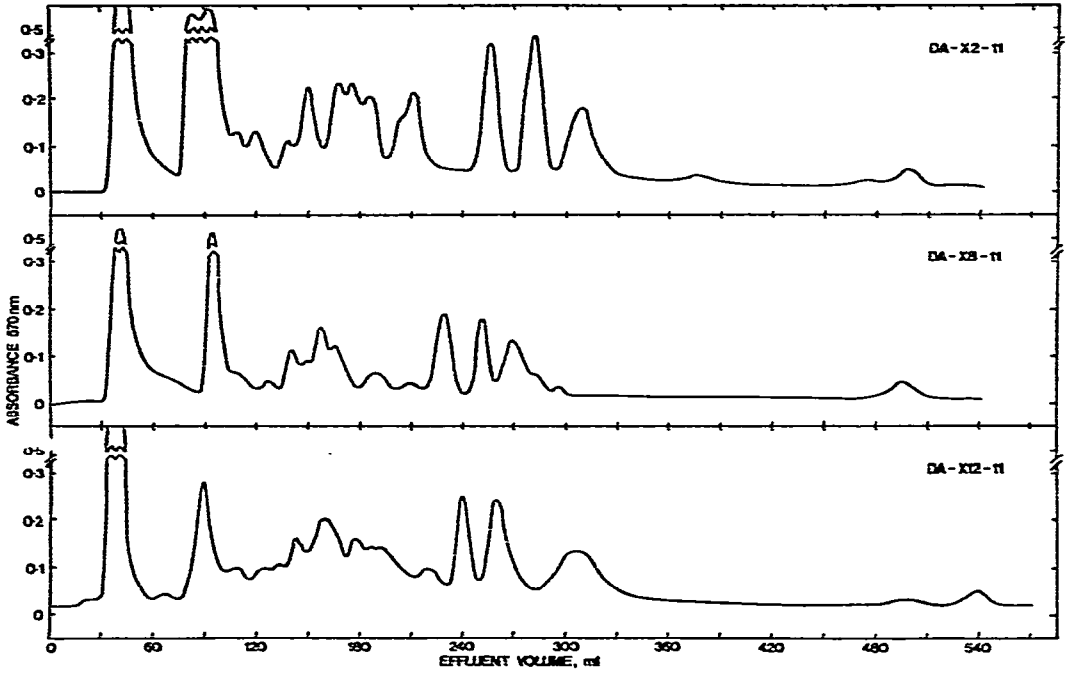

Fig. 2. Elution profiles of the separation of soluble tryptic peptides (2 μmol) of human globin (α- and β-chains) on anion-exchange resins DA-X2-11, DA-X8-11 and DA-X12-11. Gradient: 4 chamber pyridine acetate (pH 9.0 to 5.0) (see Table I); other conditions as in Fig. 1.

It should be noted that peptides found in the intermediate and later stages of the anion-exchange separations correspond to peptides emerging in the early stages of cation-exchange separations. Since they represent larger peptides with increased acidic character due to the initial pH of the gradient and since tryptic hydrolysates tend, because of the specificity of the enzyme, to distribute basic residues evenly, it is perhaps not unexpected that they are more effectively separated by anion-exchange resins of lower cross-linkage. It is possible that mixtures of peptides with a lower average size in which basic residues are not evenly distributed, such as those generated by thermolysin, will be more effectively separated on DA-X8-11. It does not appear

that 12% cross-linked resins in either series are particularly advantageous in peptide isolation work.

Two other observations emerging from these studies are worthy of note. First, all of the anion-exchange separations were carried out at 55°. In the past, substituted polystyrene anion exchangers of any particle size have been commonly used at 35° due to the mistaken impression that such resins are unstable at higher temperatures. The 20° temperature increase in all cases provided materially better resolution and significant increases in yield with most peptides[15]. Operation at the higher temperature also reduced operating pressures. With the three anion-exchange resins tested, pump pressures did not excees 40 p.s.i. throughout the separation (with the cation-exchange resins, about 80 p.s.i. were obtained). Second, anion-exchange separations were carried out in only 20-cm beds in contrast to the much longer (100 to 150 cm) beds traditionally used with Dowex 1-X2 resins of larger particle size (usually 200–400 mesh). It has already been noted that equivalent resolution is obtainable with shorter beds due to the smaller particle size of these resins[12].

Overall yield, although variable, is only marginally better with short beds than that obtained with the longer beds of crushed bead resins. Further, only minor differences in yield were found between the resins of different cross-linkage used in this study*. However, the effective yield in practical applications would be much higher for the better resolving resins since peaks would be confined to fewer fractions and less subfractionation would be required.

Although these experiments suggest that DC-X8-11, DA-X2-11, and occasionally DA-X8-11 are the resins of choice for separating peptides, it is not readily apparent which should be used for a particular mixture or in what order they should be utilized for initial and subfractionations. A predictive scheme that takes into account the anticipated peptide size distribution character based on the generating agent would greatly improve the basis for making such selections. Development of these guidelines is currently in progress.

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* The apparent differences in peptide yield from one separation to another are due primarily to differences in color yield of the various ninhydrin reagent batches. For this reason, the monitor is used only as a qualitative device for detecting effluent peptides.

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