SEPARATION OF PEPTIDES ACCORDING TO CHARGE BY GEL FILTRATION IN THE PRESENCE OF IONIZED DETERGENTS

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1. Introduction

The molecular-sieving properties of cross-linked dextran (Sephadex) are commonly employed as a means of separating compounds on the basis of molecular size (for reviews, see [1]). This technique is often used to facilitate peptide separation in primary structure investigations. In this connection Dorner [2] included sodium dodecylsufate in the elution solvent to avoid peptide aggregation. During the course of our primary structure studies on carbonic anhydrase [3], we have used dodecylamine in the gel filtration solvent for peptide separation. To our surprise we found that peptides can change their elution volumes in an anomalous manner in the presence of a detergent containing an ionized group. For instance, two large fragments having 97 and 76 amino acid residues, respectively, are eluted in the expected order on Sephadex G-75 and G-50 using 1 M acetic acid as the solvent. However, when 2% dodecylamine is included in the solvent, the elution order of the two fragments is reversed. This finding led us to investigate the effect of detergents on the gel filtration properties of low-molecular weight compounds such as amino acids and dipeptides in order to shed light on the molecular basis for the phenomenon. The results are presented here and they show that the net charge of the amino acid or peptide markedly influences its gel filtration behaviour in the presence of an ionized detergent. The findings seem to extend the usefulness of gel filtration as a method for peptide fractionation and may also be applicable to charged species in general.

2. Materials and methods

Glass columns (100 × 1.0 cm) were packed with fine grade Sephadex G-25 or G-50 and a constant flow-rate was obtained with hydrostatic pressure. Fractions of 1.5 ml were collected every 20 min.

Dodecylamine or dodecanoic acid (Fluka A.G. puriss) were dissolved in water by addition of acetic acid or ammonia, respectively, to the desired pH. The highest possible pH without precipitation was 7.8 for 2% dodecylamine and the lowest possible pH for 2% dodecanoic acid was 9.3.

The following amino acids and peptides were dissolved in the elution solvents to a concentration of 20 μmole/ml: Asp, Glu, Pro, Gly, Ala, Phe, Tyr, Trp, His, Lys, Arg, Gly–Asp, Leu–Tyr and Gly–Phe. A 0.5 ml aliquot was applied to the column and the effluent was analyzed by applying 30 μl of each fraction on a Whatman 3 MM paper. After high voltage electrophoresis in pyridine–acetic acid buffer, pH 6.5, the neutral band was cut out and separated at pH 2. The papers were developed with ninhydrin reagent. The electrophoretic separations were performed in tanks employing organic solvents as coolants [4].

Fractions containing peptides which had to be isolated free of detergent were lyophilized and the dodecylammonium acetate or ammonium dodecanate were removed by liquid/solid extractions with heptane:n-butanol in the ratio 5:1. The procedure is conveniently carried out in a centrifuge tube. We recommend that the extraction be repeated three times followed by a final washing in pure heptane.

3. Results and discussion

Table 1 and fig. 1 show the elution behaviour of amino acids and dipeptides on Sephadex G-50 eluted with solvent containing 2% dodecylamine. Table 1 also contains the results obtained using the same column but eluting with 1 M acetic acid or with 0.5 M sodium acetate, pH 5.7, in the absence of detergent. The result with the latter two solvents is in accordance with the expected properties of Sephadex G-50. The small molecules with the exception of aromatic amino acids (cf. [5, 6]) do not separate but are eluted in the same volume as sodium chloride with a K_d-value about 1.0. In the solvent containing dodecylamine there is a marked distribution of K_d-values for the compounds listed in fig. 1 and table 1. Compounds having a positive charge have a K_d-value greater than 1.0 (i.e. more retarded than sodium chloride). Negatively charged molecules are eluted early from the column with K_d-values as low as 0.5, while the neutral compounds have intermediate K_d-values.

At pH 3 the α-carboxyl groups of peptides are partly protonated while the α-carboxyl groups of amino acids are considerable more ionized. The difference in net charge would account for the observation that the dipeptide Gly–Asp is more retarded than aspartic acid at pH 3. The opposite elution order is obtained at a higher pH which may be attributed to the difference in pK of the α-amino groups of the dipeptide and the amino acid. A similar difference in elution behaviour is encountered for the dipeptide Gly–Phe when compared with glycine and phenylalanine (see fig. 1).

Dodecylamine is a detergent forming micelles in water solution. The critical concentration for micelle formation for dodecylammonium acetate is 1.2×10^{-2} M [7] (2% solution corresponds to 1.1×10^{-1} M). The micelles formed are probably too large to penetrate the matrix of the gel which would mean that the total dodecylammonium ion concentration is greater in the liquid outside the gel particles compared with the solution inside. A Donnan effect obtained in this way would be expected to influence the partition of charged molecules and explain why a solute with a negative net charge has a tendency to appear outside the gel particles while a positively charged molecule would tend to appear inside. The K_d-values and the elution volumes presented in table 1 and fig. 1 reflect this phenomenon.

An anionic detergent would be expected to show the opposite effect. In fig. 2 one can see the behaviour of some amino acids and peptides on a Sephadex G-25 column eluted with 2% dodecanoic acid which had been titrated with ammonia to different pH values. Arginine and lysine are the only amino acids that have a positive net charge in the pH-range studied. They are

Table 1
Elution behaviour of amino acids and dipeptides on a Sephadex G-50 column (100 × 1.0 cm) in 2% dodecylamine–acetic acid.

	2% Dodecylamine						1 M HOAc	0.5 M NaOAc
	pH 2.9	pH 3.9	pH 4.3	pH 4.9	pH 5.9	pH 7.6		pH 5.7
Aspartic acid	0.97	0.84	0.72	0.64	0.59	0.60	0.97	0.97
Glutamic acid	1.00	0.93	0.81	0.67	0.59	0.60	0.98	0.97
Gly–Asp	1.03	0.91	0.78	0.60	0.53	0.50	0.98	0.95
Arginine	1.24	1.24	–	1.22	1.19	1.14	0.98	0.98
Histidine	1.19	1.19	–	1.16	1.10	1.00	0.98	0.97
Lysine	1.17	1.17	–	1.16	1.09	1.03	0.91	0.91
Phenylalanine	1.07	1.03	–	0.98	0.95	0.91	0.98	1.05
Tryptophane	1.22	1.10	–	1.05	1.00	0.97	1.29	1.41
Tyrosine	1.17	1.09	–	1.05	1.02	1.00	1.12	1.14
Gly–Phe	1.12	1.00	–	0.88	0.83	0.66	0.98	1.03
Leu–Tyr	1.09	0.98	–	0.86	0.79	0.60	0.98	1.05
Alanine	1.05	1.02	–	0.97	0.93	0.93	0.95	0.97
Glycine	1.09	1.03	–	0.98	0.97	0.91	0.97	0.98
Proline	1.02	1.00	–	0.95	0.93	0.93	0.98	0.97
Blue Dextran	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
NaCl							1.00	

The results are expressed K_d-values, defined as in the legend to fig. 1.

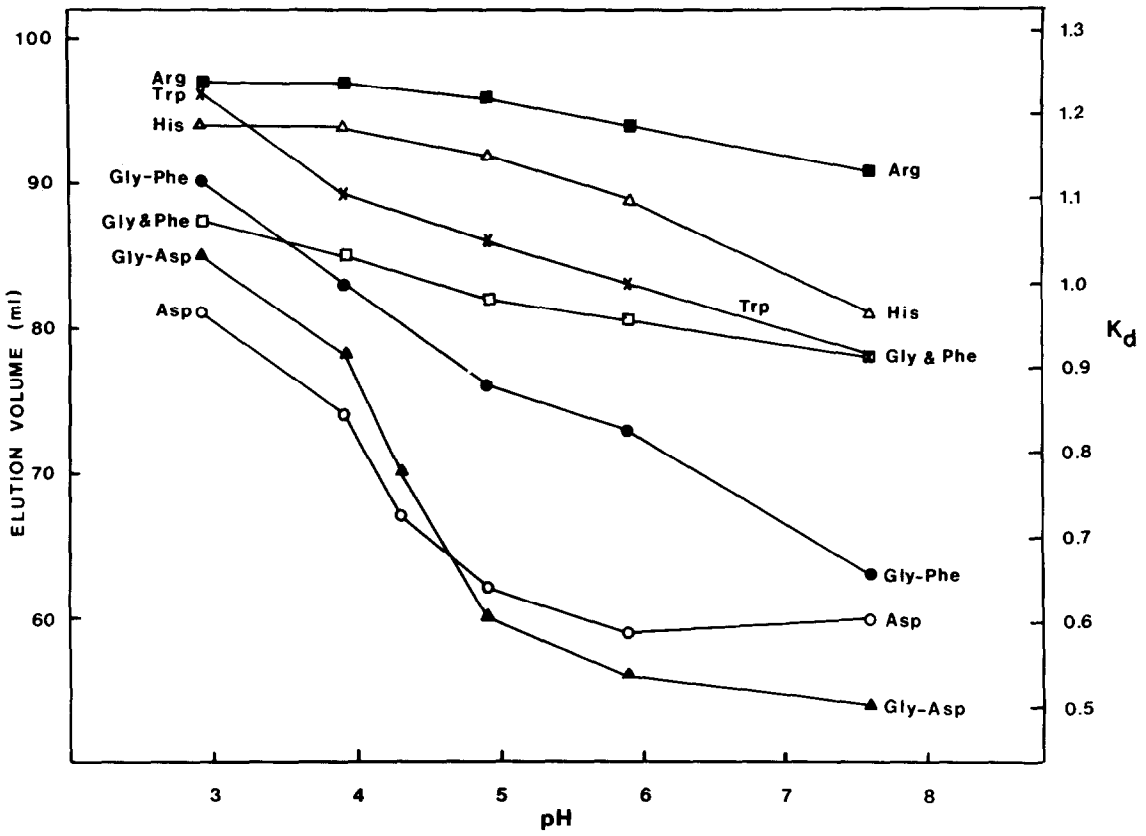
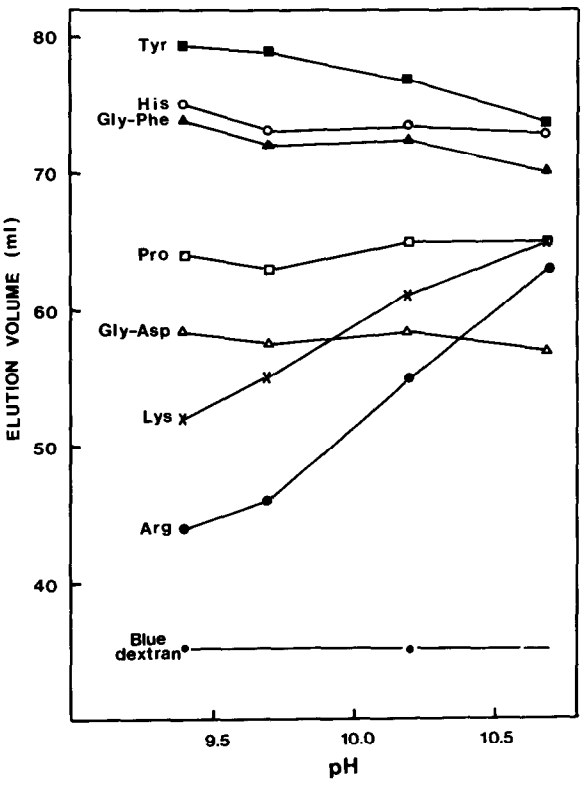


Fig. 1. Elution behaviour of some amino acids and dipeptides on a Sephadex G-50 column (100 × 1.0 cm) in 2% dodecylamine–acetic acid at different pH values. K_d is defined as $(V_e - V_0)/V_{NaCl} - V_0)$ where V_0 = elution volume for "Blue Dextran", V_e = elution volume for the amino acid or dipeptide, and V_{NaCl} = elution volume for NaCl.

also eluted earlier than the others at pH 9.4 and show increasing retardation at a higher pH.

One application of the method to peptide separation is illustrated in fig. 3. In carbonic anhydrase, there is a region of the polypeptide chain that from X-ray studies [8] is known to be involved in β-structure. This region of the enzyme is difficult to hydrolyze with proteolytic enzymes and often gives a complex mixture of peptides resulting from incomplete proteolytic digestion. In a tryptic hydrolysate a precipitate is formed at pH 5.7 which can be dissolved in 1 M acetic acid and fractionated on Sephadex G-25 as shown in fig. 3a. Fraction A was rechromatographed in 1 M acetic acid containing 2% dodecylamine, pH 3.7 (see fig. 3b). One fraction, B, was now retarded on the column. After lyophilizing and several extractions with heptane–n-butanol (5:1) the sequence analysis gave the following result: Leu–Phe–Gln–Phe–His–Phe–His–Trp–Gly–Ser–Thr–Asn–Glu–His–Gly–Ser–Glu–His–Thr–Val–Asp–Gly–Val–Lys. The peptide chromatographs in an approximately normal position in 1 M acetic acid (see fig. 3a) but is greatly retarded in the presence of dodecylamine at pH 3.7. At this pH the peptide should have a positive net charge of at least 2 but probably greater than 3. Its retardation in the dodecylamine solvent is therefore in agreement with the results obtained with the low-molecular weight model compounds.

Gel filtrations in 1 M acetic acid and in 1 M acetic acid containing 2% dodecylamine, pH 3.7, have in our hands been a powerful combination for peptide purification. The experiments described in this paper suggest



that a pH of 5–6 may be even better. One major advantage with the technique is that it may be applied to peptides that are insoluble in the pH range where separations according to charge by means of ion-exchange chromatography and paper electrophoresis are usually carried out. It would therefore be particularly useful in cases of "core" formation or where peptide precipitates must be separated. Dodecylamine and dodecanoic acid are conveniently removed from peptides as described in the Materials and methods section.

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Fig. 2. Gel filtration behaviour of some amino acids and peptides on a Sephadex G-25 column (100 × 1.0 cm) in 2% dodecanoic acid–ammonia of varying pH.

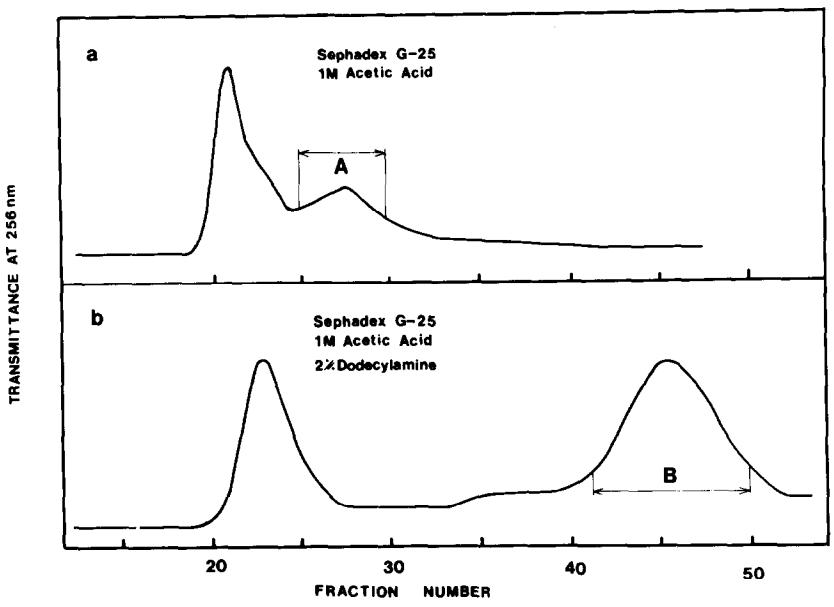

Fig. 3. a) Gel filtration of tryptic peptides from the region 77–173 in human carbonic anhydrase B. The peptides represent a fraction insoluble at pH 5.7. b) Rechromatography of fraction "A" from preceding chromatogram in the presence of detergent.

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