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Note

Separations of amino acid homo-oligopeptides

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Peptides have been separated by numerous methods, *e.g.*, paper and thin-layer electrophoresis and chromatography, ion-exchange column and gas–liquid chromatography and counter-current distribution and their combinations[1]. Gel filtration has also been used for separation of peptides[2]. Little is known about the correlation of the molecular size of oligo-peptides with their chromatographic behavior on ion exchange resins or paper and thin-layer chromatograms, or their electrophoretic behavior on paper and thin layers. Knight[3] and Hamilton[4] reported the separation of some glycyl homo-oligopeptides by paper chromatography and by an amino acid analyzer, respectively. In this communication, we report the separation of a series of homologous glycyl or alanyl oligopeptides by cation exchange resin column chromatography and cellulose thin-layer chromatography (TLC), for comparison. We also discuss the correlation between molecular size of a series of homologous oligopeptides and their separation.

EXPERIMENTAL

Chemicals

Di-, tri-, tetra-, penta- and hexa-L-alanines, and tetra-, penta- and hexa-glycines were obtained from Sigma Chemicals (St. Louis, Mo., U.S.A.). TLC plates, cellulose precoated (0.1 mm) and silica gel 60 (0.25 mm), (20 × 20 cm) were purchased from E. Merck (Darmstadt, G.F.R.). Other chemicals were all domestic products of analytical grade and used without further purification.

Methods

Cation exchange resin column chromatography was carried out using a JEOL amino acid analyzer, model 5AH, according to the manual. The short column (125 mm × 8 mm I.D.) and the long column (600 mm × 8 mm I.D.) packed with JEOL resin LC-R-1 were equilibrated with 0.2 *N* sodium citrate buffer (pH 3.25) and eluted by the same buffer at 60°. The flow-rates were 1.22 ml/min, and 0.83 ml/min for the short and long columns respectively. The eluate was analyzed with ninhydrin reagent.

Thin layers were developed in ascending order with solvent mixtures as follows; solvent A, phenol–0.1% NH_3, 10:1[5]; solvent B, *n*-butanol–acetic acid–water, 4:1:5[6]; solvent C, *n*-butanol–pyridine–water, 6:4:3[7]; solvent D, collidine–pyridine–

water, 2:1:1[8]. After development, each spot was made visible by spraying with ninhydrin reagent[9].

RESULTS

A mixture of glycyl homo-oligopeptides was applied to the short column of an amino acid analyzer and eluted by the same buffer. As depicted in Fig. 1, five peaks were observed under these conditions. The peaks were identified by the elution time of an independent run of each peptide under identical conditions. The elution order was found to be free mono-, hexa-, penta-, tetra-, and tri- and di-glycines. Tri- and di-glycines were eluted in the same volume and could not be separated from each other. When the mixture was analyzed using the long column, the separation of tri- and di-glycines was still incomplete. A mixture of alanyl homo-oligopeptides was satisfactorily separated in the order of hexa-, penta-, tetra-, mono-, tri- and di-alanines using the long column and the same buffer used for the separation of glycyl homo-oligopeptides (Fig. 2). When the short column was used, the elution order of the five peptides remained unchanged, but free alanine was eluted in the same volume as tetra-alanine.

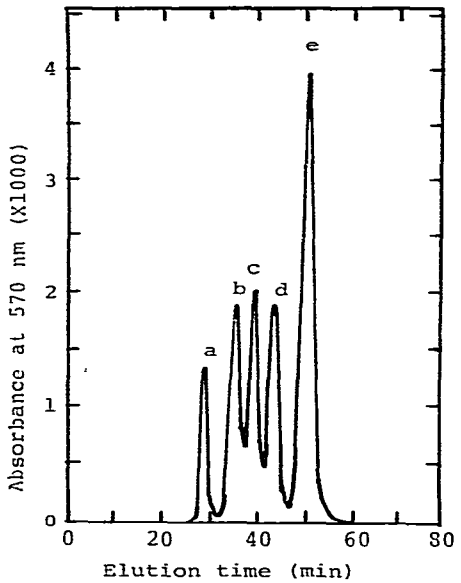

Fig. 1. Cation exchange chromatography of glycyl homo-oligopeptides using a short column of amino acid analyzer. A mixture of each peptide (0.2 μmole) and glycine (0.02 μmole) in 1.0 ml of a total volume was applied to the short column (125 mm × 8 mm I.D.) and eluted with 0.2 *N* sodium citrate buffer, pH 3.25 at 1.22 ml/min and 60°. (a) Glycine, (b) hexa-glycine, (c) penta-glycine, (d) tetra-glycine, and (e) tri- and di-glycines.

Elution time was plotted against logarithmic molecular weight of each glycyl and alanyl homo-oligopeptides using the long column (Fig. 3). A straight line was obtained in the case of alanyl homo-oligopeptides except for free alanine. Plots of di-

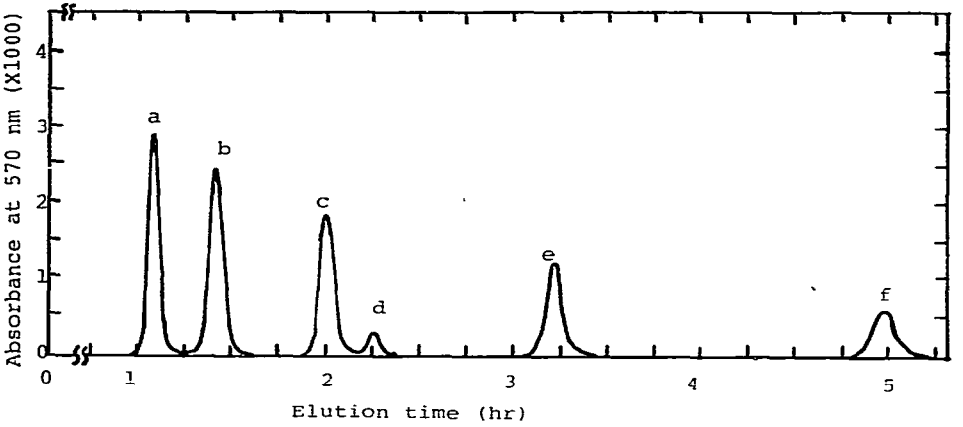

Fig. 2. Cation exchange chromatography of alanyl homo-oligopeptides using a long column of amino acid analyzer. A mixture of each peptide (0.2 μmole) and alanine (0.02 μmole) in 1.0 ml was applied to the long column (600 mm × 8 mm I.D.) and eluted with the same buffer as in Fig. 1 at 0.83 ml/min. (a) Hexa-alanine, (b) penta-alanine, (c) tetra-alanine, (d) alanine, (e) tri-alanine and (f) di-alanine.

and tri-glycines and free glycine deviated from the straight line formed by other glycyl homo-oligopeptides, *i.e.*, tetra-, penta- and hexa-glycines.

Investigations were then made to separate the homo-peptides on cellulose and silica gel thin layers. Compared with the cellulose plates, silica gel plates generally gave insufficient separation of each peptide by the use of the four solvent systems. Of the four solvents, solvent A gave the best separation of each glycyl and alanyl peptides

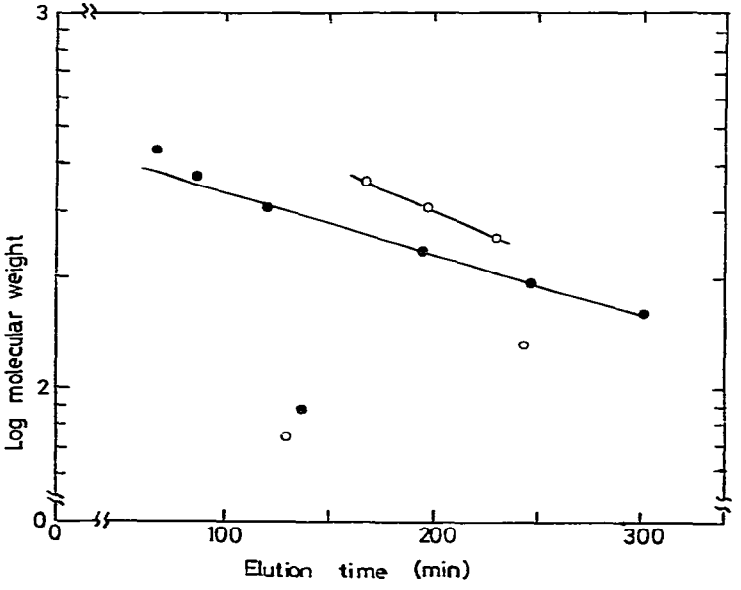

Fig. 3. Plots of molecular weight of peptides against elution time. Both glycyl and alanyl homo-oligopeptides were analyzed by the method described in the legend to Fig. 2. Elution time of each peak was recorded. (○) Glycyl peptides, (●) alanyl peptides.

TABLE I

THE R_F VALUES OF GLYCYL AND ALANYL HOMO-OLIGOPEPTIDES

Glycyl and alanyl homo-oligopeptides were chromatographed on cellulose thin-layer plate using solvent A and detected by ninhydrin spray[9].

Compound	R_F
Glycine	0.22
Di-glycine	0.21
Tri-glycine	0.26
Tetra-glycine	0.32
Penta-glycine	0.50
Hexa-glycine	0.63
Alanine	0.44
Di-alanine	0.56
Tri-alanine	0.69
Tetra-alanine	0.80
Penta-alanine	0.94
Hexa-alanine	—*

* Not migrated under the conditions used.

on cellulose plates. The alanyl homo-oligopeptides were completely separated from each other using solvent A, though hexa-alanine did not migrate under the conditions employed. The glycyl peptides were also satisfactorily separated with minor separation of free glycine from di-glycine. The R_F values produced with solvent A using cellulose plates are correlated in Table I. The R_F value increased with molecular weight of alanyl homo-oligopeptides, including free alanine through penta-alanine. The R_F values

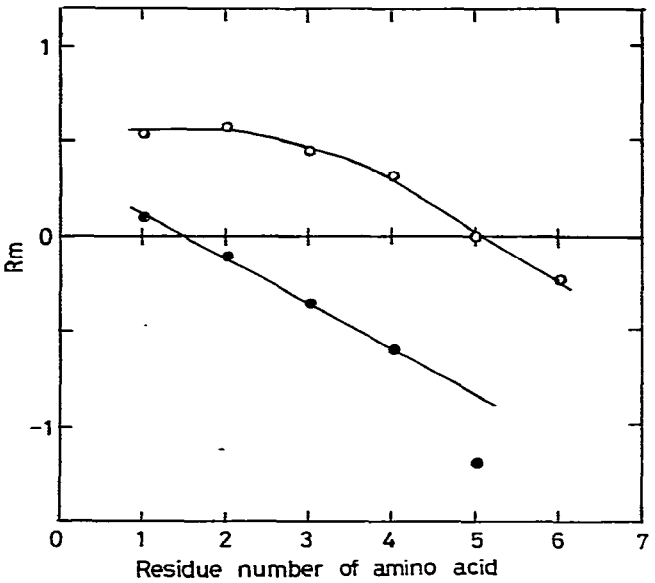

Fig. 4. Linear relationship between the R_M values and the residue number of amino acids. Each R_M value of glycyl and alanyl homo-oligopeptides was calculated from the R_F values presented in Table I. (○) Glycyl homo-oligopeptides, (●) alanyl homo-oligopeptides.

of glycyl homo-oligopeptides except for free glycine also increased with their molecular weights. The R_M calculated was plotted against residue number of each amino acid of glycyl and alanyl homo-oligopeptides (Fig. 4). A straight line was obtained with alanyl homo-oligopeptides except for penta-alanine which has too high an R_F value to yield a meaningful R_M value. In the case of glycyl homo-oligopeptides, only tetra-, penta- and hexa-glycines showed a linear relationship. The slopes of both lines were almost identical.

DISCUSSION

Amino acids and peptides were efficiently separated by ion-exchange chromatography. There are essentially two different types of interactions; coulombic forces between charged molecules and charged groups on the support; dispersion forces between the solute and the phase of the matrix. With regard to the former effect, Yanari *et al.*[10] reported the separation of mono-, di- and tri-glycines on DEAE-cellulose.

For amino acid analyses using Dowex 50, the elution volume correlated with the degree of cross-linking of the resin[4]. Peptides were usually fractionated by the low cross-linked resins due to the absence of peak broadening[11]. Little is known about the molecular sieving effect of ion-exchange chromatography on peptides. Only Schroeder[12] has described that peptides emerged more rapidly from the column of Dowex 50X8 than from that of Dowex 50X2 under the same conditions of elution.

We examined the molecular sieving effect of the cation exchange resin using polystyrene sulfonate resin as an amino acid analyzer. We selected glycyl and alanyl homo-oligopeptides as model peptides to minimize the charge difference and the partition effect of the peptides. Alanyl homo-oligopeptides emerged in the order of hexa-, penta-, tetra-, tri- and di-alanines (Fig. 2). The pK_a values reported were 2.34, 3.06 and 3.26 for mono-, di- and tri-glycines, and 2.34, 3.30, 3.39 and 3.42 for mono-, di-, tri- and tetra-alanines, respectively[13]. Compared with free alanine, di-alanine binds more tightly to the cation exchange resin at pH 3.25 due to the lower electronegativity of di-alanine at that pH. Though pK_a values of tri- and tetra-alanines are slightly higher than that of di-alanine, these peptides were eluted faster than di-alanine from the column. The elution order can not be explained solely by the dissociation constants of the peptides nor by a combined effect of partition and charge, because of the faster elution of hexa-alanine (having 6-methyl residue) than any other alanyl homo-oligopeptides. Since glycyl homo-oligopeptides were also eluted in the order of hexa-, penta-, tetra, and tri- and di-glycines, the elution order cannot simply be explained by either charge or partition effect of the peptides. It is consequently plausible that the separation of the alanyl or glycyl homo-oligopeptides arises from the molecular sieving effect of the cross-linkage of polystyrene sulfonate resin as well as coulombic forces.

Hamilton[4] reported the separation of a homologous series of glycyl peptides using an amino acid analyzer and a standard buffer system, whereas we were unable to separate completely the di- and tri-glycines under the conditions employed. The discrepancy between these results may be due to slightly different properties of the resin or use of a different buffer system.

The glycyl and alanyl homo-oligopeptides were also separated by cellulose thin-layer chromatography. The R_F values obtained increased with the molecular

weight of each series of both the glycyl and alanyl homo-oligopeptides, except for free glycine and hexa-alanine.

Whilst the R_M values of the alanyl homo-oligopeptides, except for penta-alanine, correlated with the residue number of the amino acid, those of mono-, di- and tri-glycines did not adhere to the straight line obtained from the other glycyl homo-oligopeptides tested. We cannot explain why R_M values of mono-, di- and tri-glycines were anomalous in this respect, though di- and tri-glycines also showed exceptional behavior in their elution times from the column of polystyrene sulfonate resin.

Knight[3] separated mono-, tri- and tetra-glycines by paper chromatography. Pardee[14] presented an empirical equation for the R_F values of amino acids and peptides. Our results obtained using mono-, di- and tri-alanines, and mono-, di-, tri- and tetra-glycines were consistent with his equation, but this was not so for other peptides. Since Consden *et al.*[9] stated that the R_F values of two compounds should differ by 10% to be separable, separation of mono- and di-glycines was not complete by cellulose thin-layer chromatography. Moreover, hexa-alanine could not be developed under our conditions.

The molecular sieving method using polystyrene sulfonate resin in a through-column chromatographic procedure may provide a novel and useful method for determination of the degree of polymerization of homo-oligopeptides.

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