High-Performance Liquid Chromatographic Separation of Histidine Oligopeptides

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Histidine oligopeptides which differ by one histidyl residue in length up to a total of 20 amino acid residues were separated by high-performance liquid chromatography on a commercially available strong cation-exchange column (TSK gel SP-2SW) using isocratic elution with a NaCl solution.

Keywords High-performance liquid chromatography, histidine peptides, cation-exchanger, silica gel

High-performance liquid chromatography (HPLC) is a useful means for separating and purifying biologically relevant polyelectrolytes. The electrolytes could be separated chromatographically on gel filtration columns in response to their molecular size and shape, and on ion-exchanger columns in response to the electrolytic charge. This paper describes an attempt to separate histidine oligopeptides, (His)_nGly ($n=2-19$), which were synthesized by a conventional solid-phase method, on various cation-exchanger columns. The histidyl side chain is the most important metal-coordinating group involved in the active site of metal enzymes.¹

Experimental

Reagents

(His)₄Gly and (His)₉Gly as internal reference were synthesized by a conventional liquid-phase method and identified by FBA mass spectrometry: (His)₄Gly; (M+H)⁺= m/z 622, and (His)₉Gly; (M+H)⁺= m/z 1310. (His)_nGly were synthesized by a solid-phase method with an LKB BIOLYNX 4175 (Bromma, Sweden). The starting materials were Fmoc-His(Boc)-OPFP, Fmoc-Gly-OPFP, and polyamide resin.

HPLC apparatus and columns

A TRI ROTAR SR2 HPLC system (JASCO Co.). The columns used were TSKgel SP-2SW (250×4.6 mm i.d.), SP-NPR (35×4.6 mm i.d.), and SP-5PW (75×7.5 mm i.d.) (TOSOH Co.). TSKgel SP-2SW is porous spherical silica gel whose surfaces are chemically bonded with sulfopropyl groups. TSKgel SP-NPR is a nonporous spherical hydrophilic resin, and TSKgel SP-5PW is a

porous spherical hydrophilic resin, both having sulfopropyl groups. Eluants were used which contained various concentrations (from 0.4 to 1.5 M) of NaCl; the pHs were adjusted by adding H₃PO₄-NaH₂PO₄. The final concentration of phosphate was 10 mM. The flow rate and column oven temperature were 1.0 ml/min and 50°C, respectively.

Results and Discussion

The peptides are required to have electric charges that are adequate to be adsorbed on ion-exchanger columns. Since the average pK_a of the imidazole residues in polyhistidine were shown to be 6.1², the pH of the eluant was set at 4.1, where the peptides might be positively charged.

Table 1 shows the capacity factors (k') of (His)₄Gly and (His)₉Gly for three different types of columns (TSK gel SP-2SW, SP-NPR, and SP-5PW). The TSKgel

Table 1 k' values of (His)₄Gly and (His)₉Gly on cation-exchange columns

	TSKgel		
	SP-2SW	SP-NPR	SP-5PW
(His) ₄ Gly	0.88	3.0	1.22
(His) ₉ Gly	8.0	200	30

The eluant used is 10 mM NaH₂PO₄ containing 1.0 M NaCl (pH 4.1). k' is related to retention time as $k'=(t_R-t_0)/t_0$, in which t_0 and t_R are retention time of a non-sorbed substance and apparent retention time, respectively.

Fig. 1 Relationship between k' and the NaCl concentration in the mobile phase. $\circ$, (His)₉Gly; $\bullet$, (His)₈Gly; Δ , (His)₇Gly; $\blacktriangle$, (His)₆Gly; $\square$, (His)₅Gly.

Fig. 2 Chromatogram of mixtures of (His)_nGly ($n=2-19$). The separation was performed on a TSKgel SP-2SW column with 1.0 M (A) and 1.5 M (B) NaCl in 10 mM NaH₂PO₄ at a flow rate of 1.0 ml/min and 50°C.

SP-2SW column appeared to adsorb the peptides loosely. Both the SP-NPR and SP-5PW columns tightly adsorbed high molecular weight peptides, probably due to strong hydrophobic interactions with the hydrophobic matrices. In an attempt to practically use an ion-exchanger column for separating high molecular weight peptides, it is recommended to use the TSKgel SP-2SW column.

The binding capacity of ion-exchanger to the charged species depended on the salt contained in the eluant.³ The effect of the NaCl concentration on the chromatographic separation of (His)_nGly mixtures was examined. Mixtures of (His)_nGly from $n=5$ to 9 adsorbed on the TSKgel SP-2SW column were eluted by various concentrations (*i.e.*, 0.75, 1.0, 1.25, and 1.5 M) of NaCl (pH 4.1 with 10 mM phosphate). Increasing concentrations of NaCl reduced the k' values, as is shown in Fig. 1. It is expected that lower molecular weight peptides can be separated by using an eluant of lower concentration, and high molecular weight peptides by an eluant of higher concentration. As expected, mixtures of the peptides from $n=2$ to 9 and from 10 to 19 were clearly separated by the difference of one residue using 1.0 M NaCl and 1.5 M NaCl, respectively. The chromatograms are shown in Fig. 2. The peaks were identified by internal references: (His)₄Gly and (His)₉Gly. The k' value of each peak increased with the number of histidyl residues, as is shown in Fig. 3.

In conclusion, mixtures of (His)_nGly ($n=2-19$) were separated on the ion-exchanger column TSKgel SP-

Fig. 3 Relationship between k' and n on the (His)_nGly ($n=2-19$). $\bullet$, (His)_nGly ($n=2-9$); $\circ$, (His)_nGly ($n=10-19$).

2SW according to the difference in the number of electric charges. Peptides with less than 10 histidyl residues were clearly separated on the column by isocratic elution with 1.0 M NaCl. Those peptides with more than 11 residues were successfully separated by 1.5 M NaCl. HPLC on a porous strong cation-exchanger is a useful means for separating and purifying oligopeptides with positive charges.

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References

1. W. H. Armstrong, in ACS Symp. Ser. No. 372, 1988, L. Que., Jr. ed., Ch. 1, p. 1 and references therein.
2. R. E. Wasylshen and J. S. Cohen, *J. Am. Chem. Soc.*, **99**, 2480 (1977).
3. C. T. Mant and R. S. Hodges, *J. Chromatogr.*, **327**, 147 (1985).

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