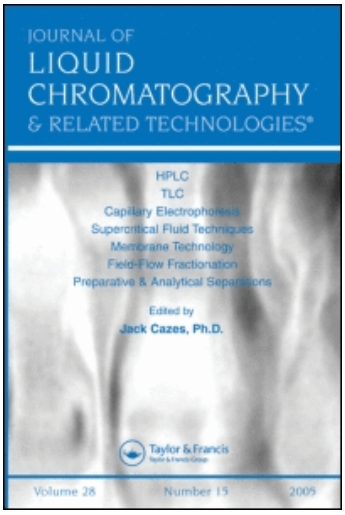

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IMPROVEMENT IN SELECTIVITY OF ANHYDROTRYPSIN-IMMOBILIZED DIOL SILICA COLUMN BY THE USE OF ELUENT CONTAINING CALCIUM ION

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ABSTRACT

The disadvantage of anhydrotrypsin-immobilized diol silica column, retention of basic peptides having no Arg or Lys at their C-termini and no retention of certain C-terminal Arg peptide, was overcome by using 10 mM acetate buffer (pH 6.0) containing 20 mM $CaCl_2$ as an eluent. The role of calcium ion was suggested to be attributable to masking of negatively charged sites which were present intrinsically on the diol silica and produced extrinsically on the support during activation and/or immobilization process.

INTRODUCTION

Since anhydrotrypsin (AHT), a catalytically inert derivative of trypsin in which the active site Ser residue is chemically converted to dehydroalanine residue, exhibits affinity toward peptides containing Arg or Lys at their C-termini, AHT-agarose is used for selective isolation of C-terminal peptide fragments from tryptic or chymotryptic digests of proteins

[1-5]. We recently prepared AHT-immobilized diol silica to improve the disadvantages of the AHT-agarose column such as slow separation speed and ligand leakage, and to use it as a precolumn of column-switching high-performance liquid chromatography (HPLC) system [6,7]. The AHT-diol silica column showed the expected excellent characteristics, but disadvantage in selectivity: a certain peptide was not retained on the column though having Arg at its C-termini, while half of peptides having no Arg or Lys at their C-termini were retained.

This paper describes the improvement in selectivity of AHT-diol silica column by the use of eluent containing calcium ion.

EXPERIMENTAL

Materials

Peptides used were purchased from the Peptide Institute (Osaka, Japan) and Sigma (St. Louis, MO, U.S.A.). Diol silica was prepared from Matrex silica beads (30-50 μm, 50 nm)(Amicon, Lexington, MA, U.S.A.) as described previously [8]. Other chemicals were of analytical-reagent grade. Deionized water (obtained with a Millipore RO-Q system) was used throughout this work.

Preparation of AHT column

The preparation method of AHT column was slightly modified as follows to immobilize larger quantities of AHT. Diol silica was activated with tresyl chloride (270 μl g^{-1}) for 20 min at room temperature. AHT was immobilized almost quantitatively by shaking the activated gel (300 mg) in 4 ml of 0.75 M phosphate buffer (pH 8.0) containing 15 mg AHT for 4 h at room temperature. The resulting gel was slurry-packed into a stainless-column (10 X 4.6 mm I.D.) after treatment with Tris-HCl buffer(pH 8.0) to remove excess active groups. The column was stored in 10 mM acetate buffer (pH 6.0) when not in use.

Evaluation of retention of peptides

The retention was evaluated as the percentage of peptide remaining on the AHT column after loading and washing, by using the column-switching system described previously [7]. The eluent for the loading and washing was 10 mM acetate buffer (pH 6.0) containing 20 mM $CaCl_2$ and delivered at a flow-rate of 0.5 ml/min. At 15 min after the injection of peptide solution (1 nmol/50 μl), a switching valve was changed for flushing the retained peptide from the AHT column onto an analytical column (Capcell Pak C_{18}; 150 X 4.6 mm I.D.). A straight-flushing mode was employed. The eluent for the flushing was CH_3CN-29 mM H_3PO_4 (3-25:97-75, v/v) containing 0.1 M $NaClO_4$, and delivered at a flow-rate of 1 ml/min. A typical chromatogram is shown in Fig. 1.

RESULTS

A preliminary study on the effect of calcium ion on selectivity of the AHT column prepared previously showed that it decreased non-selective adsorption of peptides having no Arg or Lys at their C-termini, but extinguished concurrently the retention of some of C-terminal Arg peptides and most of C-terminal Lys peptides [7]. We thought immobilization of larger quantities of AHT would be effective to overcome this disadvantage. A new column prepared in this study contained 50 mg AHT per gram, five times the amount immobilized on the previous column.

Table 1 shows the retentions of fifty peptides on the new AHT column. The peptides which had Arg or Lys at their C-termini and more than four or three amino acid residues, respectively, except Nos. 15 and 10, a C-terminal D-Arg peptide, were retained on the column when 10 mM acetate buffer (pH 6.0) was used as an eluent, and most of these retentions were nearly quantitative. However, five out of twenty-five peptides having no Arg or Lys at their C-termini were also retained on the AHT column. The retention of the five peptides to diol silica itself was fairly low except No. 37; 3% (No. 31), 62% (No. 37), 11% (No. 40), 7% (No. 41), 15% (No. 48).

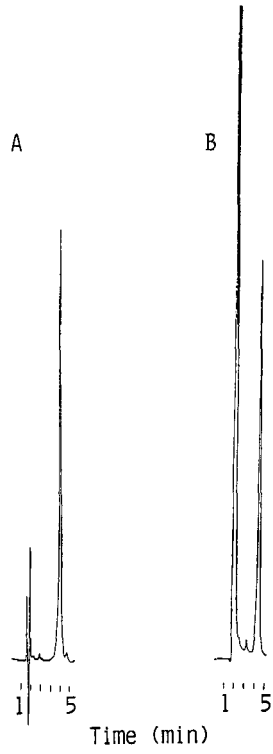

FIGURE 1. HPLC chromatograms of No. 7 obtained with (B) or without (A) the AHT precolumn.

These characteristics generally coincided with those of the previous AHT column [7].

The non-selective adsorption of peptides was suppressed completely by the addition of $CaCl_2$ (20 mM) to the eluent, while the retention of the C-terminal Arg peptides was hardly or only slightly affected. A C-terminal Arg peptide No. 15 became retained, though in small percentage, by the addition of $CaCl_2$. The retention of the C-terminal Lys peptides was generally reduced greatly by the addition of $CaCl_2$, but that of several peptides was hardly affected. Thus the selectivity of the AHT column was significantly improved.

TABLE 1
Retention of Fifty Peptides on the AHT Column

No.	Peptides[2]	Retention (%)[1] Carrier I[3]	Carrier II[3]
1	Tyr-Arg	0	0
2	Bz-Gly-Arg	0	0
3	Thr-Lys-Pro-Arg	96	100
4	Tyr-Ile-Gly-Ser-ArgNH_2	66	28
5	His-Leu-Gly-Leu-Ala-Arg	72	67
6	Tyr-Gly-Gly-Phe-Leu-Arg	89	86
7	Tyr-Gly-Gly-Phe-Leu-Arg-Arg	97	93
8	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Arg	96	92
9	Gly-Arg-Gly-Leu-Ser-Leu-Ser-Arg	87	68
10	Dnp-Gln-Gly-Ile-Ala-Gly-Gln-D-Arg	0	0
11	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	95	100
12	Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg	93	79
13	Phe-Ser-Trp-Gly-Ala-Glu-Gly-Gln-Arg	91	79
14	Tyr-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg	105	95
15	Ala-Asp-Ser-Gly-Glu-Gly-Asp-Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg	0	9
16	Bz-Gly-Lys	0	0
17	Lys-Trp-Lys	99	91
18	Thr-Pro-Arg-Lys	81	0
19	Pro-Phe-Gly-Lys	57	11
20	Tyr-Gly-Gly-Phe-Met-Lys	82	51
21	Tyr-Gly-Gly-Phe-Leu-Lys	79	13
22	Ser-Ile-Gly-Ser-Leu-Ala-Lys	99	97
23	Val-His-Leu-Thr-Pro-Val-Glu-Lys	58	5
24	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys	82	69
25	Tyr-Pro-Phe	0	-[4]
26	Tyr-Tyr-Phe	0	-
27	Glu-Val-Phe	0	-
28	Met-Leu-Phe	0	-
29	Val-Ala-Ala-Phe	0	-
30	Gly-Arg-Gly-Asp	0	0
31	Arg-Pro-Lys-Pro	40	0
32	Phe-Gly-Gly-Phe	0	-
33	Phe-Leu-Glu-Glu-Val	0	0
34	Tyr-Pro-Phe-Pro-Gly	0	0
35	Tyr-Gly-Gly-Phe-Leu	0	0
36	Tyr-Gly-Gly-Phe-Met	0	-
37	Arg-Ser-Arg-His-Phe	72	0
38	Arg-Lys-Asp-Val-Tyr	0	0
39	Lys-Val-Ile-Leu-Phe	0	0

(continued)

TABLE 1 (*continued*)

No.	Peptides[2]	Retention (%)[1] Carrier I[3]	Carrier II[3]
40	Arg-Val-Tyr-Ile-His-Pro-Phe	84	0
41	Arg-Val-Tyr-Ile-His-Pro-Ile	73	0
42	Tyr-Pro-Phe-Pro-Gly-Pro-Ile	0	0
43	Tyr-Gly-Gly-Phe-Met-Arg-Phe	0	0
44	Ser-Met-Glu-Val-Arg-Gly-Trp	0	-
45	Asp-Arg-Val-Tyr-Ile-His-Pro-Phe	0	0
46	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe	0	0
47	Arg-Pro-Pro-Gly-Phe-Ser-Pro-Leu	0	0
48	Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro	86	0
49	Trp-Ala-Gly-Gly-Asp-Ala-Ser-Gly-Glu	0	0
50	Arg-Arg-Leu-Ile-Glu-Asn-Ala-Glu-Tyr-Ala-Ala-Arg-Gly	0	0

1) Determined based on the peak area.
2) 1 nmole/50-μl injection.
3) I, 0.01 M acetate buffer (pH 6.0).
II, 0.02 M $CaCl_2$/0.01 M acetate buffer (pH 6.0).
4) Not determined.

To clarify how $CaCl_2$ could extinguish the non-selective adsorption, effect of its concentration on retention of selected peptides was investigated. As shown in Fig. 2, the retention of No. 40 was decreased rapidly with increasing concentration of $CaCl_2$, and extinguished at 1 mM. Nearly the same curve was also obtained by the addition of $MgCl_2$ (data not shown). The addition of NaCl was less effective for the suppression, the retention being extinguished at 30 mM. No. 15 became retained by the addition of $CaCl_2$, but no increase in retention depending on its concentration was observed. The addition of 20 mM $MgCl_2$ was also effective for the retention (data not shown). On the other hand, no retention was observed by the addition of NaCl at any concentrations. No substantial difference in effect between $CaCl_2$ and NaCl was observed in the case of No. 3, but its retention tended to decrease with increasing concentration of these salts. These results indicate that the suppression

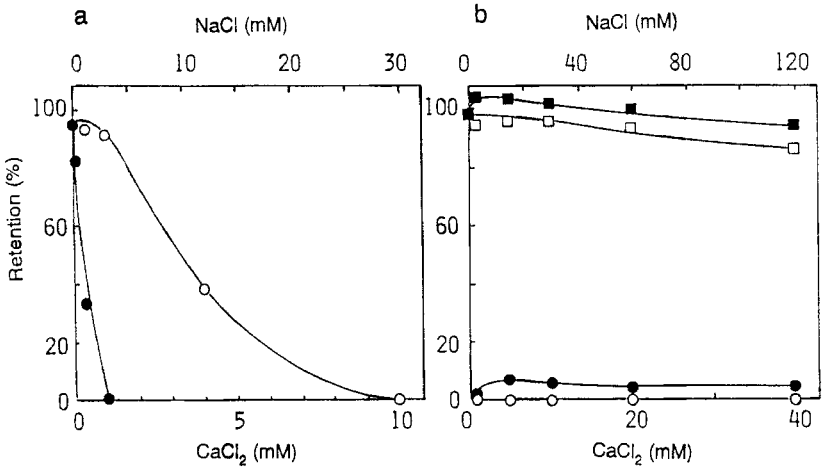

FIGURE 2. Effect of concentration of $CaCl_2$ (closed symbols) or NaCl (open symbols) on retention of peptides on the AHT precolumn. a, No. 40; b, No. 3 (□ ■), No. 15 (○ ●).

of the non-selective adsorption of peptides by calcium ion is not simply attributed to an increase in ionic strength.

Although the eluent containing 20 mM $CaCl_2$ was used in the above experiments, the disappearance of adsorption of No. 40 by using the eluent containing 1 mM $CaCl_2$, as shown in Fig. 1, reminded us that its concentration might be adequate to extinguish the non-selective adsorption of the other peptides and might result in an increase in the retention of the C-terminal Lys peptides. Actually, the retention of Nos. 31, 41 and 48 was 0-3% at 1 mM $CaCl_2$, and that of No. 18 was restored to 4%. The retention of No. 37, however, was 70%, indicating that the concentration of 20 mM was preferable for the complete suppression of the non-selective adsorption.

The new AHT column was satisfactorily stable throughout this study; no decrease in retention of No. 3 was observed after exposure for about 300 cycles to acidic eluents containing up to 25% acetonitrile during 6 months.

DISCUSSION

The data presented in this paper clearly indicated that the addition of $CaCl_2$ (20 mM) to eluent improved the selectivity of the AHT column, but decreased concurrently the retention of many C-terminal Lys peptides. Lower concentration of $CaCl_2$ increased the retention of the Lys peptides, but made impossible the complete suppression of the non-selective retention. The present AHT column, therefore, is not always usable for the selective isolation of C-terminal peptide fragments from tryptic or chymotryptic digests of proteins, though having effective selectivity as a precolumn of HPLC system. The use of arginylendopeptidase (mouse submaxillary protease) [9] instead of trypsin for digestion of proteins would compensate for the disadvantage of the AHT column. Kumazaki et al. reported that AHT prepared from *Streptomyces griseus* (S.G.) trypsin showed higher affinity than that from bovine trypsin for C-terminal Lys peptides [3]. Immobilization of AHT prepared from S.G. trypsin, therefore, would be another approach.

Among thirty peptides having no Arg or Lys at their C-termini, twelve were retained non-selectively in the previous study [7]. Five out of these twelve peptides were retained in this study. A careful investigation on amino acid composition of the twelve peptides showed that these were all basic peptides. We recently observed a similar phenomenon: two out of eighteen peptides having no aromatic amino acids at their C-termini were non-selectively adsorbed on an anhydrochymotrypsin (AHC)-immobilized column [10]. The two peptides are also basic. These facts suggest that some negatively charged site is present on the support or the ligands. As both AHT and AHC columns were prepared by using diol silica as a support and tresyl chloride as an activating reagent for immobilization of ligands, these common factors are more likely cause of the non-selective adsorption. The decrease in number of peptides adsorbed non-selectively by immobilizing larger quantities of AHT, as shown in this study, seems to support this assumption. However, the retention percentage of the five peptides to diol silica itself, except No. 37, was fairly low to explain the non-selective adsorption to the AHT column. Some change in property of the support, therefore, may occur during the

activation and/or immobilization process. In the case of No. 37, the adsorption is probably attributed to the diol silica itself. The presence of negative charge on diol silica is pointed out also by Schmidt et al. [11].

The difference in effect on suppression of the non-selective adsorption of peptides between calcium and sodium ion addition showed that the suppression by calcium ion was not simply attributed to an increase in ionic strength. The fact that magnesium ion shows nearly the same effect as calcium ion suggests that the non-selective adsorption is suppressed by divalent ions. These ions may mask specifically the negatively charged site as postulated above. The appearance of the retention of No. 15, an acidic C-terminal peptide, by the addition of calcium ion can be explained reasonably by the masking of the negatively charged site. At higher concentration, calcium ion may mask the negatively charged site which is present intrinsically on the diol silica, as shown by the example of No. 37. A diol silica showing less adsorption characteristics for basic peptides would be necessary for development of more excellent AHT column.

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