Use of Alumina Column High Performance Liquid Chromatography as Isolation Technique for Small Peptides

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The effective isolation of small peptides has been achieved by HPLC with alumina supports and hydrophilic eluents combined with a reversed phase HPLC. Of the four kinds of alumina supports tested, only Spherisorb A5Y showed the high column efficiency and a good reproducibility. Its elution mode appeared to be the same as the open column system, judging from the chromatograms of synthetic phyllolitorin and bradykinin. A mixture of the tryptic fragments of egg white lysozyme was separated by the combination of alumina and ODS columns, with a linear gradient elution of acetonitrile. The peaks which could not be isolated by one column were effectively separated by a second column; this was confirmed by determining their amino acid compositions. Spherisorb A5Y was found by the fluorescent X-ray analysis to be a complexed material of aluminum oxide and titanium oxide.

Keywords Alumina, HPLC, peptide, normal phase chromatography, fluorescent X-ray analysis

Experimental

Chromatography

The following alumina supports were tested in this work: Neosorb LC-XUP (Alumina I), Neosorb LC-N-1500 (Alumina II), Alox 60-D5 (Alumina III) and Spherisorb A5Y (Alumina IV). Alumina I was commercially available, and II was prepared by sintering at high temperature, 1500°C; both were kindly provided by Nishio Kogyo (Tokyo). Alumina III was obtained from Macherey-Nagel (Düren, G.F.R.) and IV from Phase Separation Ltd. (Queensferry, England). Each support, 5 µm, was packed in a stainless steel tube (150×4.0 mm I.D. or 150×4.6 mm I.D.).

A high performance liquid chromatograph with a computer-controlled dual pump (Model CCPD, Toyo Soda, Tokyo), a Toyo Soda gradient controller (Model GE–8000), a Shimamura sample injector with a 100 µl loop (Tokyo), a Shimadzu column oven (Model CTO-2A, Kyoto) and a Shimadzu variable wavelength UV detector (Model SPD-1) were used. Chromatography using an alumina column was performed with a linear gradient elution of acetonitrile–water with the descending mode of the organic solvent. 50 mM Phosphoric acid adjusted to pH 2.1 with triethylamine was used as the aqueous elution solvent. The reversed phase chromatography using ODS silica (Finepak Sil–C18, 250×4.6 mm I.D., 5 µm; JASCO, Tokyo) was performed in the usual manner by employing the linear gradient elution of the ascending mode of acetonitrile.
Preparation of the peptide mixture

Egg white lysozyme (Sigma, St. Louis, MO) was reduced with 2-mercatoethanol, according to the method of Canfield, and then carboxymethylated with iodoacetic acid. A 2.5 ml portion of the aqueous suspension of modified lysozyme (2.5 mg) was adjusted to pH 10 with NH₄OH and heated to 60°C for 15 min. After the pH was readjusted to 8 with HCl, 25 µg of bovine pancreas trypsin (Worthington Biochemicals, Freehold, NJ) was added and digested at 37°C for 120 min.

Chromatographic separation of the peptide mixture

The digested mixture of the peptide fragments derived from ca. 0.25 mg of lysozyme was chromatographed with either the reversed phase or the normal phase elution system; a sample from each peak monitored by the UV absorbance at 210 nm was collected in a small glass tube. The fractions were separated further with the other elution system and the resultant fractions were similarly isolated. An aliquot of each fraction was hydrolyzed in the usual manner with 6 M HCl at 110°C for 20 h to determine its amino acid composition.

Amino acid analysis was performed by the modified method of Benson and Hare using a Toyo Soda 805C amino acid analyzer.

All eluting solvents, including ethanol, acetonitrile or water were of HPLC grade; the reagents and other standard samples were of guaranteed grade.

Results and Discussion

Four kinds of alumina (I, II, III and IV) were investigated according to the criteria of column efficiency, reproducibility, and durability for repeated use with the solvent system of ethanol- or acetonitrile-aqueous phosphoric acid buffer.

The mixture of metanephrine and serotonin was chromatographed on each alumina column with an isocratic elution of 80% ethanol or acetonitrile. Table 1 shows the number of theoretical plates of each column for these amines, indicating that only alumina IV gave the high column efficiency for these amines. In addition, it showed a good reproducibility over more than 10-20 repeated runs. Other alumina supports Fig. 1 Elution profiles of synthetic phyllolitorin (A) and bradykinin (B) obtained by an alumina HPLC with the descending mode of gradient elution of acetonitrile, as shown in the figure. The sequence of phyllolitorin: Pyr-Leu-Trp-Ala-Val-Gly-Ser-Phe-Met-NHZ and of bradykinin: Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg. Column: Spherisorb A5Y, 150×4.0 mm I.D., 5 µm. Conditions: aqueous elution solvent 50 mM phosphoric acid adjusted to pH 2.1 with triethylamine, flow rate 1.0 ml/min, 30°C.

Table 1 Number of theoretical plates of four kinds of alumina columns

<table>
<thead>
<tr>
<th>Column*</th>
<th>Organic solventb</th>
<th>Theoretical plates</th>
<th>Metanephrine</th>
<th>Serotonin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alumina</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I Neosorb LC-XUP</td>
<td>EtOH</td>
<td>200</td>
<td>190</td>
<td></td>
</tr>
<tr>
<td>II Neosorb LC-N-1500</td>
<td>EtOH</td>
<td>270</td>
<td>340</td>
<td></td>
</tr>
<tr>
<td>III Alox 60-D5</td>
<td>EtOH</td>
<td>310</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td>IV Spherisorb A5Y</td>
<td>EtOH</td>
<td>2200</td>
<td>2500</td>
<td></td>
</tr>
<tr>
<td>Silica gel</td>
<td>V TSK-gel SIL-60</td>
<td>CH₃CN</td>
<td>4400</td>
<td>4300</td>
</tr>
</tbody>
</table>

a. Column size: I, II and IV, 150×4.0 mm I.D., 5 µm; III and V, 150×4.6 mm I.D., 5 µm. Column temperature: I and IV, ambient; II, III and V, 50°C.

b. Isocratic elution with 80% ethanol or acetonitrile-50 mM phosphoric acid adjusted to pH 2.1 with triethylamine. Flow rate: I, II and V, 1.0 ml/min; III and IV, 0.45 ml/min.

Fig. 2 Chromatogram of the crude methanol extract of the frog (Litoria infrafrenata) skin by the alumina HPLC. Injection size corresponded to 5 mg of dry skin. Column and conditions are the same as Fig. 1.
showed poor numbers of theoretical plates. Because of aqueous phosphoric acid buffer their supports may have lost their adsorption activities and been further solubilized along with the silica gel supports. Alumina II sintered specially at 1500°C was also not durable under this condition.

Synthetic phyllolitorin and bradykinin were chromatographed with the descending mode of gradient elution of organic solvent (Fig. 1). The chromatograms showed the presence of impurities in the synthetic peptides. However, they indicate that the hydrophobic peptide, like phyllolitorin, was eluted with the higher concentration of acetonitrile while the hydrophilic peptide, like bradykinin, was eluted with the lower concentration of the organic solvent. These apparent elution profiles were similar to those of the open column system of alumina for the separation of these peptides.

Figure 2 illustrates the chromatograms of the crude extract of the frog skin with alumina IV. The result suggests that this column is practically applicable for such separations.

**Combined chromatographic system with alumina and ODS columns**

A mixture of the tryptic fragments of lysozyme was separated by the combination of alumina and ODS columns. Figures 3 and 4 show the chromatograms of the same sample separated on two steps by switching the order of use of alumina and ODS columns. Figures 3A and 4A illustrate the first step of these chromatographies, while figures 3B and 4B show the second step. As shown in these chromatograms, the peaks which could not be isolated in one step of separation were effectively separated in the second step; this was confirmed by determining their amino acid compositions after hydrolyses of these peaks with 6 M HCl at 110°C for 20 h. Each peak in the second step corresponded to one of the peptide fragments of lysozyme. The elution orders of the peptide fragments were much different for each of these two columns. Roughly, alumina IV was normal phased and ODS-

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**Fig. 3** Separation of mixture of the tryptic fragments of egg white lysozyme by the combination of alumina (A) and ODS (B) columns. The second step treated only the shadowed fraction in (A). Alumina column: same as Fig. 1, ODS column: Finepak Sil-C18, 250x4.6 mm I.D., 5 µm.

**Fig. 4** Separation of the same mixture of the tryptic fragments of egg white lysozyme as Fig. 3 by the combination of ODS (A) and alumina (B) columns. The second step treated only the shadowed fraction in (A). Columns: same as Fig. 3, conditions: first step (A), same as Fig. 3 (B); second step, same as Fig. 1. Abbreviations of the peaks: same as Fig. 3.

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Additional details about the chromatograms and elution profiles are provided in the text, including the conditions used for each step and the identification of the peaks based on amino acid compositions.
silica was reverse phased. However, the orders were not necessarily according to their hydrophobicities. Other mechanisms might also contribute to the separation in each column.

Figures 5A and 5B are the spectra of the fluorescent X-ray analysis of two kinds of alumina: alumina for open column chromatography (A) and Spherisorb A5Y for HPLC (B). All other peaks except aluminium and titanium were produced by contamination or by the X-ray source itself.

This may be the reason why alumina IV was so stable to aqueous solvent.

In this respect, Spherisorb A5Y is actually Al₅Ti₅O₧ and not pure alumina. Such a mixed oxide matrix having favorable characteristics would be an ideal chromatographic support for the normal phase HPLC. The combination of this normal phase column and a reversed phase column will provide a more effective isolation of small peptides.

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References


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