Rapid Purification of Arrowhead Proteinase Inhibitors by High Performance Hydrophobic Interaction Chromatography on a PEG Bonded Phase Column

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A new hydrophobic interaction HPLC column is used for the rapid purification of proteinase inhibitors isolated from arrowhead. The inhibitors, partially purified by DEAE-cellulose column chromatography, are resolved into three components with a mobile phase gradient of decreasing salt concentration from 1.1 M ammonium sulfate in 0.01 M phosphate buffer to phosphate buffer alone. This new HPLC column is found to be very useful for rapid, semipreparative purification of hydrophobic protein and sample loading of up to 1.6 mg of inhibitors can be fully resolved on an analytical column.

INTRODUCTION

Two new proteinase inhibitors (A and B) have been isolated from arrowhead (*Sagitteria sagittrifalia* J) by using stepwise elution on a DEAE-cellulose column (Zhang *et al.*, 1979). Both A and B are able to inhibit the activities of trypsin and chymotrypsin as well as kallikrein. The primary structure of inhibitor B has been elucidated and consists of 141 amino acid residues (Chi *et al.*, 1985). Owing to similarity in their chromatographic behaviour, inhibitor B obtained by this method is usually contaminated with a small amount of A and vice versa. Relatively pure inhibitors are therefore difficult to obtain on this column and the purification procedure is also time consuming and labour intensive.

High performance hydrophobic interaction chromatography (HPHIC) is a rapidly developing technique for purifying or analysing proteins, that complements ion exchange chromatography, size exclusion chromatography and affinity methods. In HIC, proteins are induced to adsorb to weakly hydrophobic matrices by high salt concentration mobile phases, and are selectively desorbed during a descending salt gradient. The descending salt gradient weakens the hydrophobic interaction and allows the protein to be eluted from the column. Under these conditions, the tertiary structure generally is kept and protein denaturation in the separation process is minimized.

Various packing materials have been developed and employed in HPHIC of proteins (Kato *et al.*, 1983; Gooding *et al.*, 1984; Miller *et al.*, 1985; Fausnaugh *et al.*, 1984; Ingram *et al.*, 1985). Recently, we developed a new HIC packing material, polyethylene glycol (PEG) bonded phase, for protein separation (Chang *et al.*, 1985; 1987). This material exhibited retentive properties weaker than those commercially available for HIC.

In this paper we demonstrate the application of the PEG bonded phase columns to the HIC separation and purification of proteinase inhibitors of arrowhead and compare the retentive behaviour of the PEG column in protein separation with the BioGel TSK Phenyl 5 PW column.

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EXPERIMENTAL

HPLC apparatus. The gradient liquid chromatograph consisted of a model 6000A and an M45 pump controlled by an M-720 System Controller [Waters Associates, Milford, MA, USA], a 7125 injection valve [Rheodyne Inc., Cotati, CA, USA], a Model 165 variable wavelength detector [Beckmann Instruments Inc., Berkeley, CA, USA], and a XWT-200 dual pen strip chart recorder (DaHua Metering Co., China).

Columns. PEG column (100×5 mm ID): slurry packed with 10 μm PEG-1500 bonded phase with an average pore size of 500 Å. The material was made in our laboratory, in 2-propanol–methanol (3:1). BioGel TSK Phenyl 5 PW column: obtained from BioRad (Richmond, CA, USA).

All chemicals and reagents were of analytical grade without further purification. All standard proteins were purchased from Sigma (Cleveland, WI, USA). The arrowhead proteinase inhibitors were gifts from Prof. Cheng-wu Chi (Shanghai Institute of Biochemistry, Shanghai, China).

Amino acid composition analysis. Amino acid analysis was performed on a few micrograms of protein after 24 h of hydrolysis at 110 °C in a sealed tube in 5.7 N HCl containing 0.1% mercaptoethanol and phenol. After hydrolysis, HCl was removed by vacuum evaporation and the amino acids were separated and determined on an LKB 4400 Amino Acid Analyzer using a column packed with Ultropak 8 cation exchange resin. Three buffers with pH values of 3.25, 4.25 and 6.25 were successively used for stepwise elution.

Electrophoresis. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the modified Laemmli method (Laemmli, 1970). Samples were heated in a boiling water bath for 5 min in 25 mM Tris–HCl buffer pH 6.8 with 0.5% bromophenol blue before being loaded onto the gel. Electrophoresis was carried out in a Tris–glycine buffer solution containing 0.1% SDS at 80 V for about 1 h, and then at 130 V for 3 h.

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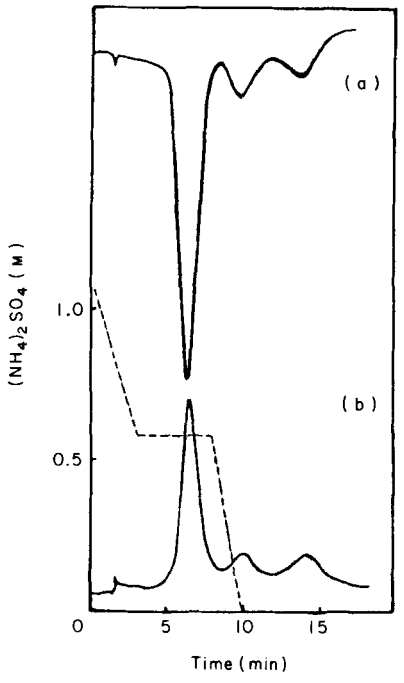

Figure 1. Separation of arrowhead proteinase inhibitor B purified by DEAE-cellulose column chromatography. Column, 100×5 mm ID PEG-1500 bonded phase; Mobile phase: solvent A, 1.1 M ammonium sulfate in 0.01 M phosphate buffer pH 7; solvent B, 0.01 M phosphate buffer pH 7. Flow rate 1 mL/min. Elution, 30 min. Detection wavelength: (a) 230 nm; (b) 280 nm.

Chromatography. 200 μL (containing 1.6 mg) of the sample was injected into the PEG column (100×5 mm) and chromatographed at a flow rate of 1 mL/min with a 30 min gradient of descending salt concentration from 1.1 M ammonium sulfate in 0.01 M phosphate buffer pH 7 to 0.01 M phosphate buffer alone. The eluted fractions of the sample were separately collected as indicated by the line in Fig. 3, and concentrated by dialysis against saturated ammonium sulfate solution. The precipitate of each component was then dissolved in water, dialysed against water and lyophilized.

RESULTS AND DISCUSSION

Figure 1 shows the HPHIC separation of arrowhead proteinase inhibitors on the PEG column after purification by DEAE-cellulose column chromatography. The elution condition consisted of a 30 min mobile phase gradient of decreasing salt concentration from 1.1 M ammonium sulfate in 0.01 M phosphate buffer to 0.01 M phosphate buffer alone at a flow rate of 1 mL/min. Inhibitor B purified by the DEAE-cellulose column was resolved into three components on the PEG column. To check the purity of eluted fractions, dual wavelength detection at 230 and 280 nm was used. It can be seen that both elution profiles (Fig. 1) were identical, indicating that each peak in the profile is a single component.

The gradient shape had a significant effect on the chromatographic behaviour of inhibitors. Figure 2 illustrates the dramatic differences caused by different gradient shapes in separation. When a 10 min linear gradient of decreasing salt concentration was used only

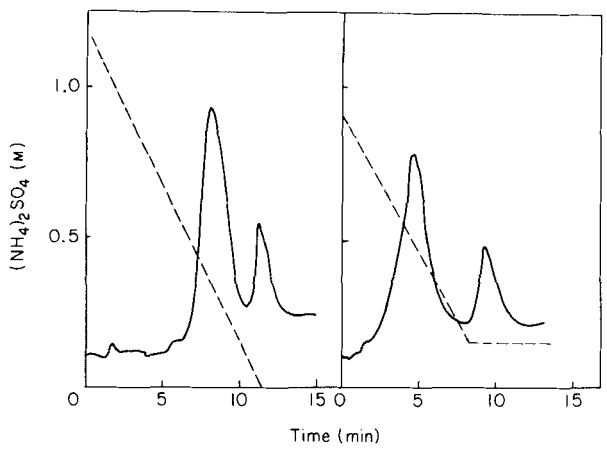

Figure 2. Effect of initial salt concentration and gradient shape on the separation of arrowhead proteinase inhibitors. Chromatographic conditions as in Fig. 1. Detection wavelength: 280 nm.

two peaks appeared on the chromatogram; even when different gradient slopes were used. The early eluted major peak may be proteinase inhibitor B. However, baseline separation of the three peaks can be obtained by optimizing the gradient shape as shown in Fig. 1.

Figure 3 shows the semipreparative separation of the inhibitors on the same analytical PEG column by applying about 1.6 mg of sample under overload conditions. The eluted fractions of peaks 1, 2 and 3 were collected

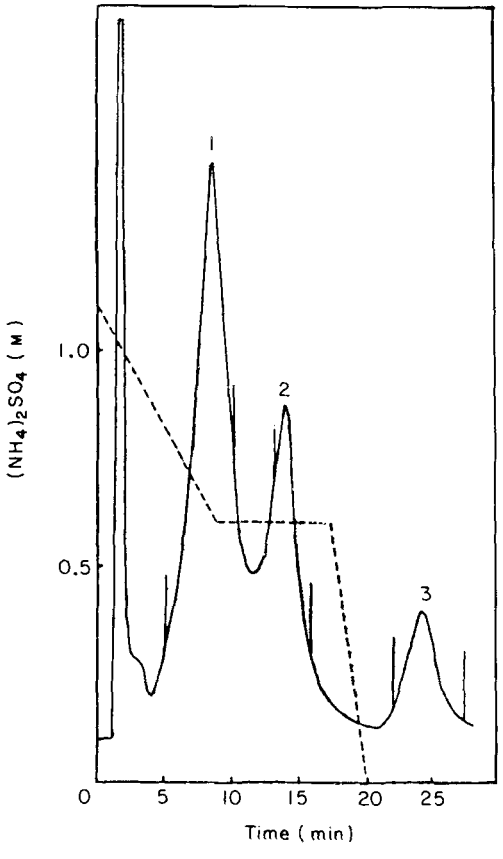

Figure 3. Semipreparative separation of arrowhead proteinase inhibitors on an analytical PEG column. Chromatographic conditions as in Fig. 1 except a 1.6 mg sample in 200 μL was injected and a 20 min gradient was used. The line indicates collection of protein fraction 1, 2 and 3.

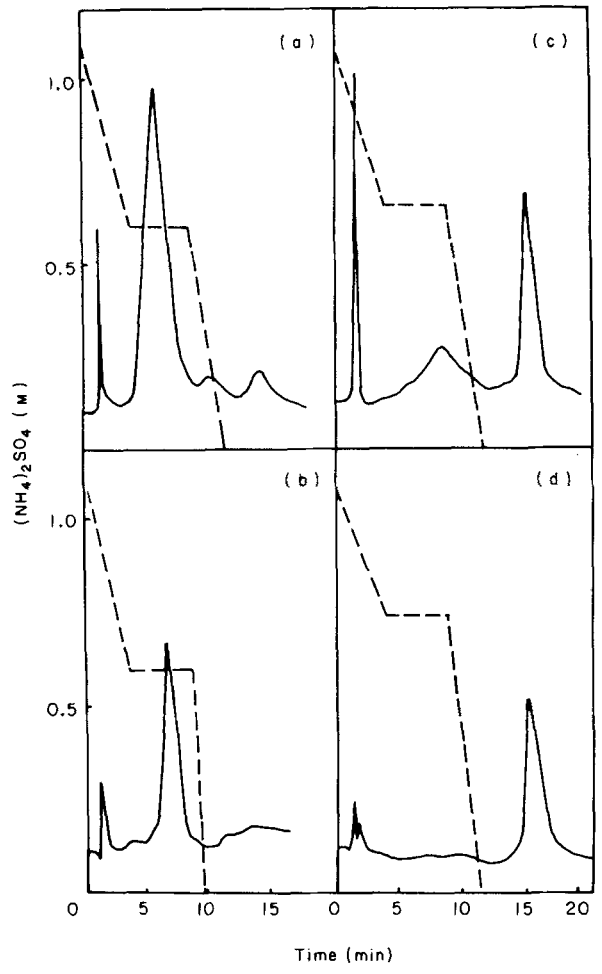

Figure 4. Analysis of arrowhead proteinase inhibitors. Chromatographic conditions as in Fig. 1, except a 12 min gradient time and a 280 nm detection wavelength were employed. (a), (c), Before purification by PEG column. (b), (d), After purification.

and processed as described in the methods section. Isolated component 1 shows a single peak (Fig. 4(b)) having identical retention time to the major peak of the partially purified inhibitor B (Fig. 4(a)). It is thus most likely to be pure inhibitor B and, similarly, the isolated component 2 should be proteinase inhibitor A Fig. 4(c, d). An unexpected result was observed when the collected fraction of component 3 was rechromatographed. A peak matched in retention time with component 1 (inhibitor B) appeared. The characteristics of component 3 and its transformation during the separation process require further investigation.

Confirmation of the purity and identity of components 1 and 2 were provided by SDS-PAGE and amino acid analysis. Both components 1 and 2 purified by the

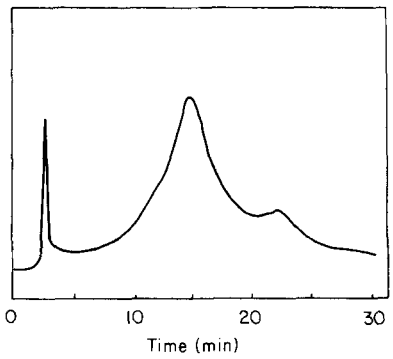

Figure 5. Chromatographic profile of arrowhead proteinase inhibitor B on BioGel TSK Phenyl 5 PW. Column, 75×7.5 mm ID. Mobile phase: 0.01 M phosphate buffer pH 7. Flow rate 1 mL/min. Detection wavelength 280 nm.

PEG column showed a single band in SDS-PAGE electrophoresis, and the results of amino acid analysis indicated that the compositions correspond with that of relatively pure inhibitor A and B, respectively, which was reported by Zhang *et al.* (1979).

A comparison between PEG bonded phase column and a commercial HIC column, BioGel TSK Phenyl 5 PW, for the separation of the proteinase inhibitors has also been carried out. When the TSK column was employed to separate the same sample as shown in Fig. 1, the inhibitors could not be eluted from the column by isocratic elution using 0.1 M phosphate buffer pH 7. The elution was performed under the strongest elution condition. A poor chromatographic profile was obtained on this column, but only by isocratic elution with 0.01 M phosphate buffer (Fig. 5). These results indicate that for strongly hydrophobic proteins such as arrowhead proteinase inhibitors, the PEG column gives better resolution than the Phenyl 5 PW column. The difference was attributed to the weaker hydrophobicity on the surface of the PEG bonded phase.

The above conclusion has been verified by comparing the resolving power of both columns for the separation of standard proteins. The results showed that for hydrophilic proteins such as cytochrome *c* and myoglobin, separation was better on the TSK Phenyl column than on the PEG column, but for hydrophobic proteins α-chymotrypsin and α-chymotrypsinogen, the PEG column is superior.

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