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Note

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A diphenyl α-aminoalkylphosphonate derivative, which is an irreversible inhibitor of chymotrypsin-like serine proteases, was immobilized on cyanogen bromide-activated Sepharose, and the selective binding of chymotrypsin to the obtained inhibitor-gel was evaluated using batch and column methods. Complete removal of chymotrypsin in an aqueous solution was done using the column method, while partial removal was done using the batch method.

Key words: covalent chromatography; diphenyl α-aminoalkylphosphonate; irreversible inhibitor of serine protease; selective removal of chymotrypsin

Isolation and purification of naturally occurring proteins and peptides are essential for studies on their structures and biological functions. In these processes, unexpected degradation of the target proteins or peptides by contaminating proteases often results in a decrease in yield. To prevent unexpected degradation, a cocktail of protease inhibitors has been used to inactivate contaminating proteases. However, the inactivated proteases still remain in the solution with the target products, and their separation from the target products is required in further processes. A technique to selectively remove the contaminating proteases would be useful for isolation and purification of the target products. This study was therefore designed to examine whether a covalent chromatographic technique using irreversible protease inhibitors is useful for removing some contaminating proteases (Fig. 1).

Diphenyl α-aminoalkylphosphonate derivatives have been reported to be selective and irreversible inhibitors of serine proteases.[1–11] The mechanism by which phosphonate derivatives inhibit serine proteases is similar to that proposed for diisopropyl fluorophosphates.[4] Since phosphonate derivatives can be easily synthesized and are fairly stable against hydrolysis, phosphonate derivatives can be coupled with amino acids and peptides to give the corresponding peptidyl phosphonate derivatives, which act as more potent and selective serine protease inhibitors than do phoshonate derivatives. Such properties of phosphonate derivatives are suitable for their immobilization on a chromatography gel. In fact, 2-aminoethyl p-nitrophenyl methylphosphonate was immobilized on Sepharose 4B (Pharmacia Biotech), and the obtained covalent affinity gel could be used for purification of acetylcholinesterase.[12] In this study, a phosphonate derivative corresponding to phenylalanine (Ahe-Phe-phosphonate, Fig. 2) was chosen as an irreversible inhibitor for chymotrypsin-like proteases as a model system for the selective removal of serine proteases using a covalent chromatographic technique (Fig. 1).

The Ahe-Phe-phosphonate derivative was synthesized as described previously.[13] First, a diphenyl phosphonate analog of *N*-benzyloxycarbonylphenylalanine, Z-Phe-phosphonate, was synthesized by α-amidoalkylation of triphenyl phosphite with benzyl carbamate and phenylacetaldehyde in acetic acid. Consequent cleavage of the benzyloxycarbonyl group with 25% HBr/acetic acid gave the corresponding hydrogen bromide salt of the Phe-phosphonate. Then 6-aminohexanoic acid was introduced into the Phe-phosphonate as a spacer, *i.e.*, the desired spacer-binding derivative (Ahe-Phe-phosphonate) was obtained as its trifluoroacetate salt by coupling of *N-tert*-butyloxycarbonylaminohexanoic acid to the Phe-phosphonate followed by deprotection of the *tert*-butyloxycarbonyl group with trifluoroacetic acid. The final product was purified by silica-gel chromatography and identified by IR, matrix-assisted laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF-MS), and ^{1}H-NMR.[14]

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Abbreviations: CNBr, cyanogen bromide; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry

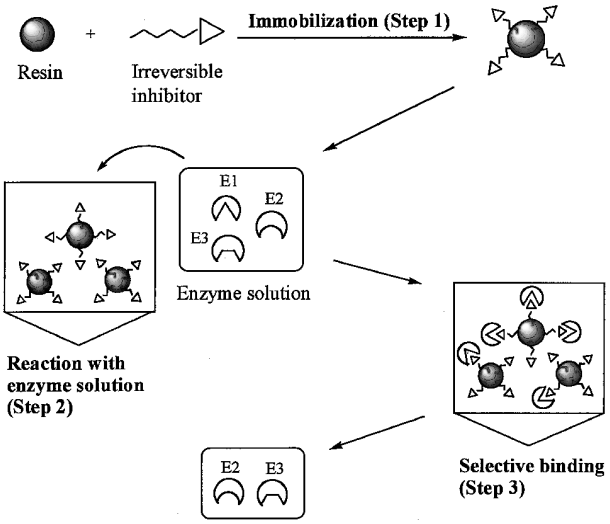
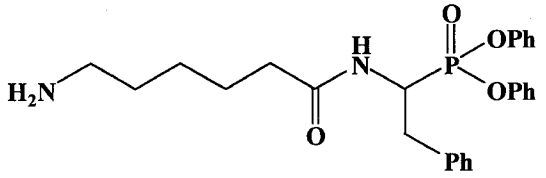


Fig. 1. Schematic Representation of Removal of Proteases by Covalent Chromatographic Technique Using Irreversible Protease Inhibitors.

Immobilization of irreversible inhibitors on a solid support for chromatography (step 1), reaction of the inhibitor-immobilized solid support with a sample solution containing contaminating proteases (step 2), and selective binding of contaminating proteases to the inhibitor-immobilized solid support to give a target product without contaminating proteases (step 3).



Ahe–Phe–phosphonate

Fig. 2. Spacer-Binding Phosphonate Inhibitor (Ahe-Phe-phosphonate) of Chymotrypsin-Like Serine Proteases.

As a spacer for immobilization, a 6-aminohexanoyl group (Ahe) was introduced into the phosphonate inhibitor (Phe-phosphonate).

The inhibitory activities of the obtained Ahe-Phe-phosphonate toward chymotrypsin and trypsin were evaluated before its immobilization. The Ahe-Phe-phosphonate irreversibly and time-dependently inactivated chymotrypsin with an inactivation rate constant, $k_{obs}/[I]$, of $43 \pm 5.0\ M^{-1}s^{-1}$, while it showed weaker inactivation ability toward trypsin with a $k_{obs}/[I]$ of $0.42 \pm 0.03\ M^{-1}s^{-1}$.[15] These results suggest that Ahe-Phe-phosphonate is able to selectively inactivate chymotrypsin-like serine proteases.

For preparation of the phosphonate inhibitor-immobilized gel, Ahe-Phe-phosphonate (5 mg) was immobilized on a commercially available affinity chromatography gel (cyanogen bromide (CNBr)-activated Sepharose 4B, 1 g) in 0.1 M $NaHCO_3$ coupling buffer containing 0.5 M NaCl at pH 8.3. For blocking the remaining active group, 2-ethanolamine (1 M, pH 8.0) was used. The volume of the obtained Phe-phosphonate inhibitor-immobilized gel (gel-I) was about 3.5 ml. The amount of the immobilized Phe-phosphonate inhibitor was estimated to be 4.8 μmol/ml of gel from the reduced amount of the Ahe-Phe-phosphonate derivative remaining in the coupling buffer after immobilization. Gel-I was stored in 40 mM Tris-HCl containing 16 mM $CaCl_2$ (pH 7.8) at 4°C.

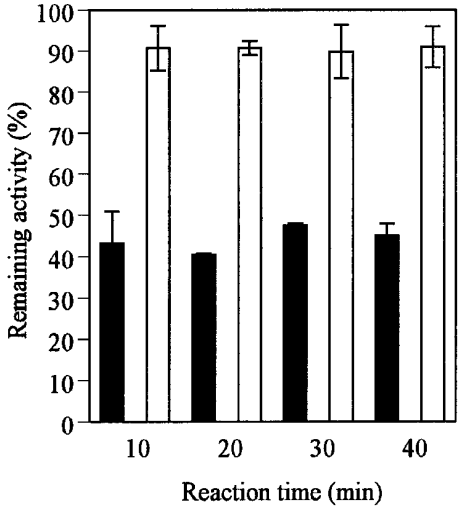


Fig. 3. Effects of Reaction Time on Changes in Enzymatic Activity of Chymotrypsin Caused by Treatment with Gel-I and the Control Gel by the Batch Method.

The remaining activities of chymotrypsin in the supernatants of the sample solutions after treatment with gel-I and the control gel were estimated using Bz-Tyr-pNA as a substrate. The amount of gel was 0.1 ml, and the reaction times were 10, 20, 30, and 40 min. Results are expressed as means ± SD of independent triplicate experiments. ■ gel-I, □ control gel.

For estimation of the amount of chymotrypsin binding to gel-I by the batch method, 0.5 ml of chymotrypsin solution (40 μM) was incubated with gel-I (0.1–0.3 ml) in a micro test tube at 25°C for 10–40 min using a rotator RT-50 (Titec). After centrifugation, portions of the supernatant were measured for the remaining activity using Bz-Tyr-pNA as a substrate. On the other hand, CNBr-activated Separose 4B gel treated with only 2-ethanolamine was put through the same binding experiment as the reference (control gel). Figure 3 shows the effects of reaction time (10–40 min) on the remaining activity of chymotrypsin when 0.1 ml of each of the gels (gel-I and control gel) was used. Regardless of the reaction time, the remaining activity of chymotrypsin was 40–44% after incubation with gel-I and 78–81% after incubation with the control gel. These results indicate that about 40% of chymotrypsin in the sample solution can bind to gel-I under these experimental conditions. Therefore, we chose 10 min as the reaction time for further experiments.

To determine the optimal amount of gel for the removal of serine protease by the batch method, we

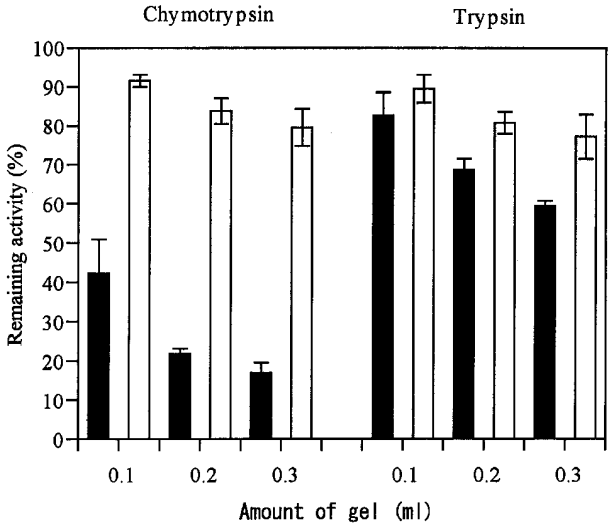


Fig. 4. Effects of Amount of Gel on the Changes in Enzymatic Activities of Chymotrypsin and Trypsin Caused by Treatment with Gel-I and the Control Gel by the Batch Method.

The remaining activities of chymotrypsin in the supernatants of the sample solutions after treatment with gel-I and the control gel were estimated using Bz-Tyr-pNA as a substrate. The reaction time was 10 min, and 0.1, 0.2, and 0.3 ml of gel-I or the control gel were used. Similarly, the remaining activities of trypsin were estimated using Bz-Arg-pNA as a substrate. Results are expressed as means ± SD of independent triplicate experiments. ■ gel-I, □ control gel.

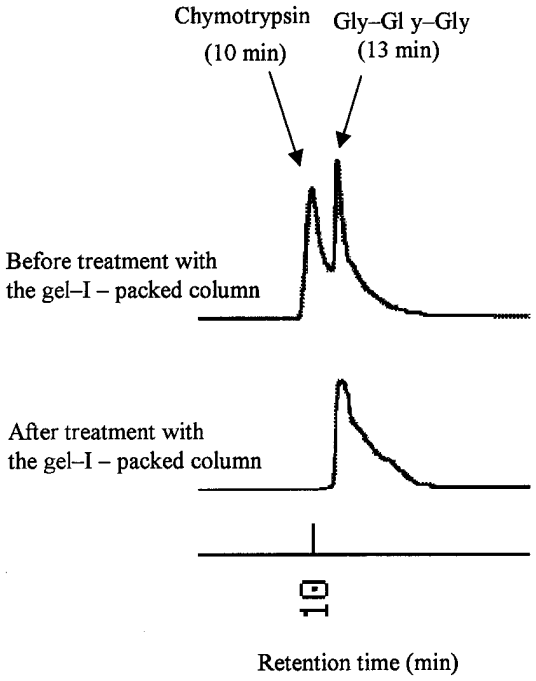


Fig. 5. Comparison of the Contents in the Sample Solution of Chymotrypsin before (A) and after (B) Treatment with a Gel-I-Packed Column (5 × 50 mm).

A sample solution (12 μM, 50 μl) of chymotrypsin containing a tripeptide, Gly-Gly-Gly (5 μM), as an internal standard was passed through the gel-I-packed column at a flow rate of 0.05 ml/min, and 40 mM Tris-HCl containing 16 mM $CaCl_2$ (pH 7.8) was used as the eluent. The eluted peak fraction was collected, and its composition was analyzed by a gel-filtration HPLC (Waters Protein-Pak60 × 2, 20 mM phosphate buffer (pH 7.0) containing 100 mM Na_2SO_4, 30°C, 0.5 ml/min). The elution profiles were expressed by monitoring at 280 nm

tested various amounts of gel-I (0.1–0.3 ml) and measured the remaining activity of chymotrypsin (Fig. 4). When the amount of gel was 0.1 ml, the remaining activities of chymotrypsin after incubation with gel-I and the control gel were 42 and 90%, respectively. Based on these findings, the net rate of chymotrypsin bound to gel-I, which we called the chymotrypsin removal rate, was estimated to be 48% by comparison of the remaining activities observed after incubation with gel-I and the control gel. The chymotrypsin removal rates by 0.2 and 0.3 ml of gel-I were 57 and 58%, respectively, suggesting that an increase in the amount of gel is not effective for increasing the chymotrypsin removal rate. On the other hand, the net rates of trypsin bound to 0.1, 0.2, and 0.3 ml of gel-I, which we called trypsin removal rates, were 2, 12, and 11%, respectively. A comparison of the chymotrypsin and trypsin removal rates by gel-I indicated that the most effective binding of chymotrypsin to gel-I can be obtained when 0.1 ml of gel-I is used. Under these experimental conditions, about 90 nmol of chymotrypsin was estimated to be removed by 1 ml of gel-I. All of these results indicate that chymotrypsin can be selectively, although only partially, removed by using gel-I and the batch method.

To try to complete the removal of chymotrypsin, gel-I was packed into a columm (5 × 50 mm), and a portion (50 μl) of chymotrypsin solution (12 μM) was passed through the gel-I-packed column. A tripeptide, Gly-Gly-Gly, was added to the chymotrypsin solution as an internal standard. The flow rate was 0.05 ml/min, and 40 mM Tris-HCl buffer containing 16 mM $CaCl_2$ (pH 7.8) was used as an eluent. The eluted fraction (30–50 min) was collected and analyzed using a gel-filtration HPLC system. Figure 5 shows the analytical gel-filtration HPLC profiles for the chymotrypsin solution before and after passing through the gel-I-packed column. A peak corresponding to chymotrypsin completely disappeared after the chymotrypsin solution had been passed through the gel-I-packed column, while a peak corresponding to the standard Gly-Gly-Gly still remained. Disappearance of the chymotrypsin peak was observed in nine further experiments using the same gel-I-packed column. In addition, enzymatic activity in the eluted fraction was also evaluated under conditions similar to those described in the legend of Fig. 4, and chymotryptic activity in the eluted fraction was found to decrease to an undetectable level (data not shown). These results demonstrated that chymotrypsin in a sample solution can be completely removed by using gel-I packed in a column.

To evaluate the selectivity in removal of

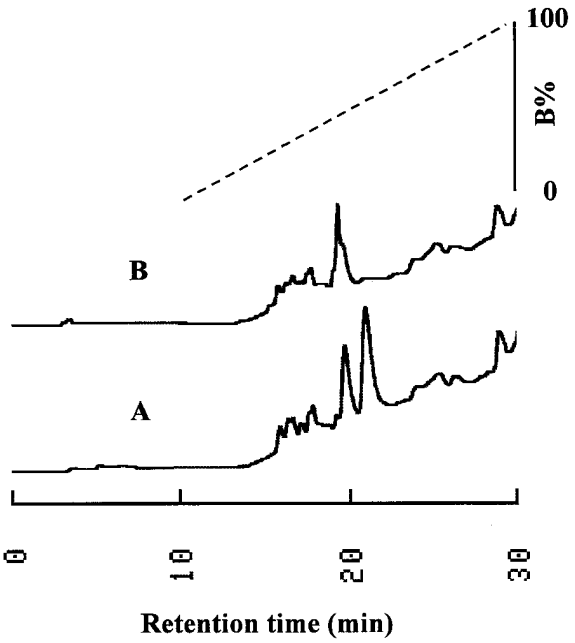


Fig. 6. Reversed-Phase HPLC Profiles of the Protein Compositions in the Eluted Fractions Passing through the Gel-I-Packed and Control Gel-I Packed Columns.

Reversed-phase HPLC analysis was done using an ODS-100s column (3.0×150 mm, Tosoh) with an acetonitrile-trifluoroacetic acid system as the mobile phase. The elution profiles were expressed by monitoring at 280 nm, and the flow rate was 0.3 ml/min.

chymotrypsin by the column method, a portion (50 μl) of mixed sample solution containing chymotrypsin and trypsin was passed through the gel-I-packed column under the same conditions as those described in the legend of Fig. 5. The eluted fraction was collected, and then the protein composition and enzymatic activity in the fraction were analyzed. The control gel was also packed into the column (5×50 mm), and the mixed sample solution was put through the obtained control gel-packed column as a reference. Figure 6 shows the reversed-phase HPLC profiles of the protein compositions in the eluted fractions passing through the gel-I-packed and control gel-packed columns. Two peaks corresponding to trypsin (19.6 min) and chymotrypsin (20.9 min) were observed in the eluted fraction from the control gel-packed column. On the other hand, the chymotrypsin peak disappeared but the trypsin peak still remained in the case of the gel-I-packed column. This result indicated that chymotrypsin was selectively removed by using the gel-I-packed column. Similar selective removal of chymotrypsin was also proved by comparison of the enzymatic activities in both eluted fractions (Table 1). After having been passed through the control gel-packed column, chymotrypsin and trypsin were eluted as a diluted fraction, but both chymotryptic and tryptic activities (5.7 and 7.7% compared to each activity in the mixed sample, respectively) still remained.

Table 1. Comparison of the Enzymatic Activities in the Eluted Fractions through the Gel-I-Packed and Control Gel-Packed Columns

	Relative Activity[a] (%)	
	Chymotrypsin	Trypsin
mixed sample[b]	100	100
gel-I[c]	ND[e]	6.2±0.6
control gel[d]	5.7±0.9	7.7±0.3

[a] Chymotryptic and tryptic activities in 100 μl of the eluted fractions through the gel-I-packed and control gel-packed columns were compared to those in 100 μl of the mixed sample solution. Each sample (100 μl) was mixed with 890 μl of 40 mM Tris-HCl buffer containing 16 mM $CaCl_2$ (pH 7.8), and then 10 μl of DMSO solution of Bz-L-Tyr-pNA or Bz-L-Arg-pNA was added. Final concentrations of Bz-L-Tyr-pNA and Bz-L-Arg-pNA were 10 mM. Relative activities are means± SD of triplicate experiments.

[b] Chymotrypsin and trypsin were dissolved in 40 mM Tris-HCl buffer, and the final concentrations (15 μM) of chymotrypsin and trypsin were measured by the UV absorption spectra ($A^{1\%}=20.5$ (278 nm) and $A^{1\%}=15.0$ (280 nm), respectively).

[c] The sample solution (50 ml) was passed through the gel-I-packed column at 25°C and the elution was monitored at 220 nm.

[d] The sample solution (50 ml) was passed through the control gel-packed column. Other conditions were the same as those for the gel-I-packed column experiment.

[e] Not detectable.

However, chymotryptic activity in the eluted fraction from the gel-I-packed column decreased to an undetectable level, while tryptic activity was detected at a level (6.2%) similar to that in case of the control gel-packed column. These results for the protein composition and enzymatic activity analyses demonstrated that chymotrypsin can be completely and selectively removed by the column method.

In this work, we prepared a Phe phoshonate derivative that is an irreversible and selective inhibitor of chymotrypsin and immobilized it on Sepharose gel. The obtained inhibitor-immobilized gel was demonstrated to be useful for removal of chymotrypsin in an aqueous solution by both batch and column methods. In conclusion, we demonstrated that a covalent chromatographic technique using diphenyl α-aminoalkylphosphonate derivatives is useful for selective removal of serine proteases.

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14) TFA·Ahe-Phe^{p}$(OPh)_2$; white solid; mp 223–225°C; IR ν_{max} (KBr) cm^{-1} 1593, 1545, 1491, 1261, 1205, 1186, 1137, 947; ^{1}H-NMR (400 MHz, CD_3OD) δ_H 0.96–1.09 (m, 2H), 1.29–1.45 (m, 4H), 2.01 (t, J= 7.0, 2H), 2.65 (t, J=7.8, 2H), 2.92–3.01 (m, 1H), 4.93–5.01 (m, 1H), 7.07–7.30 (m, 15H); MALDI-TOF-MS (m/z) 467.7 $(M+1)^+$.
15) Inactivation rate constants (k_{obs}/[I]) were measured using the incubation method under pseudo-first-order reaction conditions. For chymotrypsin, the Ahe-Phe-phosphonate derivative (5 μM) was incubated with chymotrypsin (0.5 μM) in 40 mM Tris-HCl buffer containing 16 mM $CaCl_2$ (pH 7.8) at 25°C. Portions, each of 300 μl, were withdrawn at 0.5, 5, 20, and 40 min after the start of incubation and mixed with the Tris-HCl buffer (2670 μl) and Bz-L-Tyr-pNA (30 μl, 60 μM) in DMSO to measure the remaining activities. For trypsin, the enzyme and Ahe-Phe-phosphonate concentrations were 0.4 μM and 320 μM, respectively, and Bz-Arg-pNA (100 μM) in DMSO was used as a substrate. Pseudo-first-order inactivation rate constants (k_{obs}) were obtained from plots of ln v_t/v_0 *vs.* time (triplicate experiments).