Covalent Chromatography for Chymotrypsin-like Proteases Using a Diphenyl 1-Amino-2-phenylethylphosphonate Derivative

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To establish a covalent chromatography system for purification of naturally occurring chymotrypsin-like serine proteases, a diphenyl 1-amino-2-phenylethylphosphonate derivative bearing Gly-Gly-Gly as a spacer was immobilized on the Sepharose 4FF gel. In this system, bovine chymotrypsin was selectively bound to the phosphonate immobilized on the gel and then released by the action of 2-pyridinealdoxime methiodide as the reactivated enzyme. Based on the study results for selective binding and reactivation conditions, chymotrypsin-like proteases from pancreatin (hog pancreas) were rapidly and highly purified within three hours.

Introduction

Diphenyl α-aminoalkylphosphonates are known to irreversibly and specifically inactivate serine proteases by forming a covalent bond as shown in Fig. 1 [1]. The phosphonates have been applied to several biological systems through their irreversible inactivation reactions. The utility of the phosphonates stems from their relatively increased stability in aqueous solutions when compared to organophosphorous compounds, such as diisopropylphosphofluoridate. Powers *et al.* succeeded in the synthesis of phosphonate conjugates with biotin or fluorescent compounds useful for the investigation of localization of intracellular serine proteases [2-5]. We also reported the selective removal of chymotrypsin

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Abbreviations
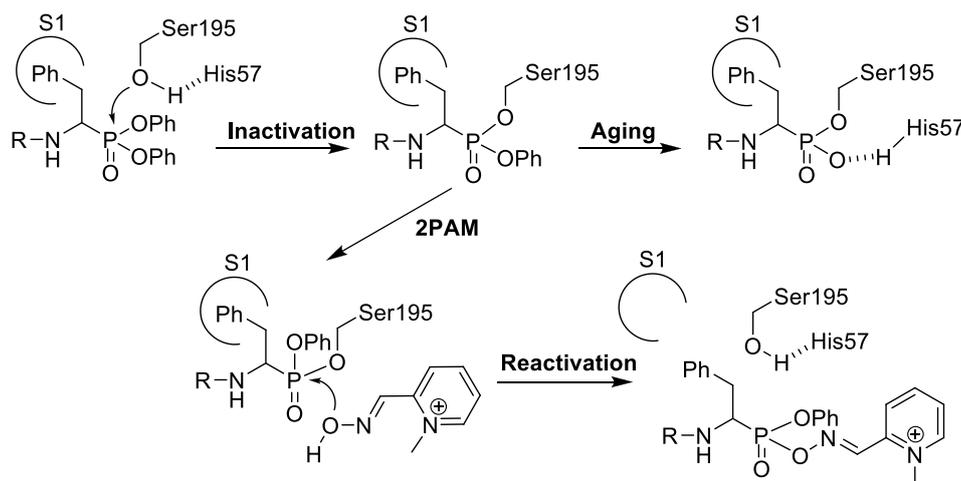2PAM, 2-pyridinealdoxime methiodide; Csin, chymotrypsin; Tsin, trypsin;

Figure 1. Inactivation reaction of the Phe type phosphonate against chymotrypsin and its reactivation by 2PAM.

(Csin) by using diphenyl 1-amino-2-phenylethyl-phosphonate (Phe type phosphonate) [6, 7].

The phosphonates can be also used for the purification of enzymes by making use of their reversible character. Ashani *et al*. immobilized 2-aminoethyl *p*-nitrophenyl methylphosphonate on Sepharose 4B (GE Healthcare, Little Chalfont, England) and purified acetylcholinesterase by using 2-pyridinealdoxime methiodide (2PAM) as an activator that broke and replaced the covalent bond between the phosphonate and enzyme [9]. Although purification was satisfactorily pursued, the time required for the reactivation of acetylcholin esterase was relatively long, e.g., 64−112 h with a 48−57% yield. The authors suggested the possibility of using the covalent chromatography system for purifying other serine proteases. However, to our knowledge, no effective purification system for serine proteases that uses the phosphonates has been reported.

In this study, to establish a covalent chromatography system for serine proteases that uses phosphonates, a Phe type phosphonate derivative (G3P) bearing Gly-Gly-Gly as a spacer
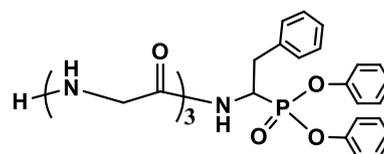

Figure 2: Chemical structure of the the Phe type phosphonate bearing Gly$_3$ spacer (G3P).

was synthesized (Fig. 2) and immobilized on Sepharose 4FF gel (GE Healthcare). The obtained gel was designated as G3P-gel. Previously, the Phe type phosphonate was immobilized on Sepharose 4B gel using 6-aminohexanoic acid as a spacer for the selective removal of Csin [6]. However, Csin captured by the gel could not be reactivated effectively by the addition of 2PAM (data not shown). The poor recovery of Csin might result from the hydrophobic character of the 6-aminohexanoyl group. Hence, we chose the Gly-Gly-Gly spacer which is more hydrophilic than 6-aminohexanoic acid, in order to enhance access of 2PAM to the phosphorous atom at the active site.

Using a mixture of bovine Csin and trypsin (Tsin), the selective binding ability of

G3P-gel against Csin and the reactivation behavior of Csin bound to the gel by 2PAM were examined. Finally, the availability of the G3P-gel was evaluated using a crude enzyme mixture from pancreatin that originated from the hog pancreas as a source of naturally occurring Csin-like serine proteases. The isolated product was characterized by enzymatic activity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and LC-MS/MS analysis.

Materials and methods

Materials

Pancreatin was purchased from Wako Pure Chemicals Industries Ltd. (Osaka, Japan). N^{α}-Benzoyl-L-arginine-*p*-nitroanilide (BAPNA) and N^{α}-benzoyl-L-tyrosine-*p*-nitroanilide (BTPNA) were purchased from Peptide Institute Inc. (Osaka, Japan). N^{α}-Tosyl-L-lysine chloromethyl ketone (TLCK)-treated Csin and *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated Tsin were purchased from Sigma Chemicals (St. Louis, MO, USA). Protein concentrations were determined with a bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific, MA, USA). Other chemicals were reagent grade.

Enzymatic activity was monitored on a Hitachi U-1800 spectrophotometer (Tokyo, Japan) equipped with a thermostated cell holder. Molecular ion masses of the synthesized compounds were determined with electrospray ionization time-of-flight mass spectrometer (ESI-TOFMS) on a Hitachi NanoFrontier LC-MS system (Tokyo, Japan). Reversed-phase HPLC analysis was performed using a Shimadzu HPLC system consisting of two LC-10ADvp pumps and an SPD-10Avp UV-Vis detector (Kyoto, Japan).

Synthesis of G3P

Thr Phe type phosphonate was synthesized according to the method proposed by Oleksyszyn [9]. An *N*-benzyloxycarbonyl derivative of the Phe type phosphonate was synthesized via the α-aminoalkylation of triphenyl phosphite with benzyl carbamate and phenylacetaldehyde in acetic acid. The obtained phosphonate was coupled with N^{α}-blocked tripeptide Boc-Gly-Gly-Gly and the deprotection of the Boc group gave the desired spacer-binding derivative G3P. The final product was purified by silica gel chromatography and identified by ^{1}H-NMR and ESI-TOFMS.

Immobilization of G3P on Sepharose 4FF gel

G3P (1 mg) was immobilized on the *N*-hydroxysuccinimide (NHS)-activated Sepharose 4FF gel (0.7 ml) in 0.1 M NaHCO$_3$ coupling buffer at a pH of 8.0 by rotation. To block the remaining active NHS-groups, the gel was incubated in 0.1 M Tris-HCl buffer for 3 h, followed by washing with 10 ml of the same buffer. The resultant G3P-gel was stored in 50 mM ammonium acetate buffer (pH 4.5) at 4 °C. The NHS-activated Sepharose 4FF gel was similarly treated with 0.1 M Tris-HCl buffer (pH 8.5) alone, to prepare a control gel without Phe type phosphonate as the reference.

The amount of the Phe type phosphonate introduced on the gel was determined by the quantitative analysis of the phenol released from the phosphonate during alkaline hydrolysis, using reversed phase HPLC.

Assay of enzymatic activity

Csin and Tsin activities were assayed using BTPNA and BAPNA as substrates,

respectively. For Csin activity, a dimethylsulfoxide solution (100 μl) of BTPNA was mixed with 880 μl of 40 mM Tris-HCl (pH 7.8) containing 16 mM CaCl$_2$ (Tris-buffer A) in a cuvette, and the mixture was incubated for 5 min at 37 °C. An aliquot (20 μl) of the sample solutions to be assayed was added to the mixture, giving a 1 ml total volume of the reaction mixtures. For Tsin activity, 10 μl of the sample solutions and 890 μl of Tris-buffer A were used.   The final concentrations of BTPNA and BAPNA were 0.2 mM and 0.7 mM, respectively. The production of *p*-nitroaniline was evaluated by monitoring the absorbance at 405 nm for 5 min. The enzyme unit (U) was expressed with the hydrolysis unit calculated using the following equation: U = A$_{405}$/min (*p*-nitroaniline molar extinction coefficient = 9920 [10]).

Inactivation and reactivation procedure for Csin from a model enzyme mixture by G3P-gel (batch)

To the model mixture (0.25 ml) containing bovine Csin (1 mg/ml) and Tsin (1 mg/ml) in Tris-buffer A, G3P-gel (50 μl) was added in the plastic column and the suspension was gently mixed by rotation at 25 °C for 15 min (contact step). This procedure was repeated three times.   Washing with 0.25 ml of 40 mM Tris-HCl (pH 7.8) containing 0.4 M NaCl (Tris-buffer B) was repeated five times. After discharge of the solution, the remaining Csin and Tsin activities in the effluent were measured. To the remaining gel, 0.25 ml of the reactivation solution (200 mM 2PAM in Tris-buffer A) was added and the suspension was gently mixed for 20 min at 25 °C to recover the components covalently attached to the gel (reactivation step). The reactivation step was repeated three times, and the effluents were collected individually to measure their residual

enzymatic activities.

Purification of Csin-like proteases from pancreatin

For the preparation of a crude extract from pancreatin, 750 mg of pancreatin was suspended in 15 ml of Tris-buffer A for 30 min at 4 °C. After centrifugation at 21,400 g and 4 °C for 15 min, the obtained supernatant was used as the crude extract from pancreatin. The crude extract (10 ml) was loaded onto 0.3 ml of G3P-gel in a column (0.6 × 5 cm).   The flow was circulated until no more decrease in Csin activity in the extract was observed. The gel was then washed with 6 ml of Tris-buffer B. The bound components were eluted by circulating 3 ml of the reactivation solution for 30 min at 25 °C. This elution was repeated again, and each effluent was subjected to gel-filtration chromatography using a HiTrap Desalting column (1.6 × 2.5 cm, GE Healthcare) to remove 2PAM. The fractions exhibiting Csin activity were collected and stored at 4 °C as the finally purified product. The crude extract was also treated with 0.3 ml of the gel in the batch method described above to purify Csin-like proteases.

Electrophoresis

SDS-PAGE was performed under non-reducing conditions, as suggested by Laemmli [11]. The gel was stained with the Coomassie brilliant blue (CBB) reagent (Atto Co., Tokyo, Japan). For fluorescence detection, the samples were incubated with the fluorescein-labeled Phe type phosphonate [3] in Tris-buffer A for 20 min at 25 °C before treatment with the SDS reagent. After electrophoresis, detection was directly performed using a Safe Imager 2.0 blue-light-transilluminator (Invitrogen, Carlsbad, USA) with emissions at around 460 nm, and the

gel was then stained with the CBB reagent.

Mass spectrometry

The two protein bands (29 and 28 kDa) were sliced into small pieces (~1 mm) and placed into 1.5 ml siliconized tubes individually. After washing with 25 mM ammonium bicarbonate containing 50% acetonitrile to remove CBB, the gel pieces were dehydrated and dried in a speed vacuum. To each tube, 100 μl of 25 mM ammonium bicarbonate containing 10 mM dithiothreitol (DTT) was added and incubated at 56 °C for 1 h. After washing with the ammonium bicarbonate buffer (100 μl), 100 μl of 55 mM iodoacetic acid in the same buffer was added. After reaction at 25 °C for 45 min, the gel pieces in the tubes were washed with the ammonium bicarbonate buffer, followed by dehydration. The obtained gel pieces were subjected to tryptic digestion at 37 °C overnight. The degraded fragments were extracted with 0.1% TFA and each extracted solution was desalted by ZipTipC_{18} (Millipore) before LC-MS/MS analysis. The prepared samples were analyzed in positive mode and MS/MS data was collected. MS/MS ion search was performed by MASCOT.

Results

Characterization of G3P-gel

The amount of the Phe type phosphonate immobilized on G3P-gel was estimated to be 1.2 μmol/ml of gel based on the amount of the phenol released from the gel. The gel was stored in 50 mM ammonium acetate at 4 °C. Even after storing for two months under these conditions, the gel showed effective binding ability against Csin.
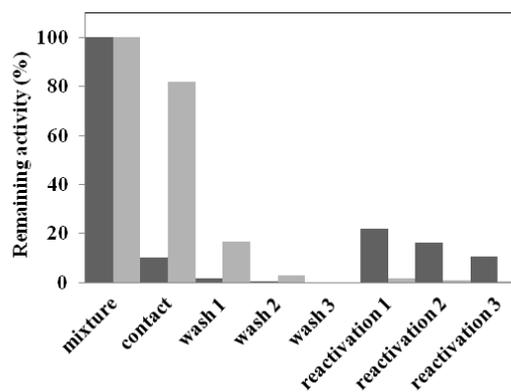
Selective inactivation and reactivation of Csin

Figure 3: Selective binding of bovine Csin (black) and Tsin (grey) in a model mixture to G3P-gel and reactivation of the bound Csin by 2PAM.

from a model mixture

The optimized conditions for purification of Csin using G3P-gel were determined in a model mixture system (Fig. 3). At first, the effect of the contact time (0–20 min) on the inactivation of Csin by the gel was examined. Since no change in the residual activities between 15 and 20 min of contact time was observed (data not shown), 15 min of contact time was chosen for further experiments. After the contact procedure was repeated four times, Csin was selectively bound to the gel (contact step). Secondly, non-specifically bound Csin and Tsin were almost removed by washing (3 times). From these results, the Csin binding capability was estimated to be 21 U/ml of the gel by using the hydrolysis unit against BTPNA. In addtion, the repeated 2PAM treatment (3 times) led to the 49% reactivation of Csin. On the other hand, less than 1% of Tsin activity was detected in the reactivated product. These results indicate that Csin can be selectively purified using G3P-gel in the batch method.

Purification of Csin-like serine proteases from pancreatin

Table 1. Purification of Csin-like serine proteases from pancreatin using G3P-gel.

purification steps	total protein (mg)	total activity[a] (U)	spedific activity (U/mg)	purity (fold)	yield (%)
[batch]					
crude	37	2.8	0.076	1	100
purified	0.27	0.34	1.3	17	12
[flow]					
crude	96	4.4	0.046	1	100
purified	0.57	0.77	1.4	30	18

[a]Csin-like enzymatic activity was assayed using BTPNA as a substrate. One unit of enzymatic activity was defined as the hydrolysis of 1 μmol of BTPNA per min, using 9920 $M^{-1}cm^{-1}$ as the extinction coefficient of *p*-nitroaniline at 405 nm.
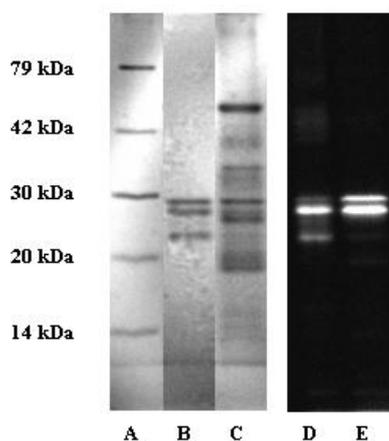
Figure 4: Non-reducing SDS-PAGE analysis of the final product (3 μg, B and D) and the crude extract (5 μg, C and E) with standard protein molecular weight markers (A). Lanes (A-C) were stained with CBB after lanes (D and E) were fluorescently detected.

To demonstrate the effectiveness of G3P-gel on the purification of Csin-like serine proteases, the crude enzyme extract from pancreatin was treated with the gel by two methods (batch and flow). As shown in Table 1, the specific activity of the Csin-like enzyme component (BTPNA hydrolyzing activity) by the batch method was estimated to be 1.3 U/mg,

A
29 kDa component: chymotrypsin-C-like [Sus scrofa]

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  1 MLGITVLAAL LAYASSCGVP SFPPNLSARV VGGENAIPHS WPWQISLQYL
 51 SGDTWKHTCG GTLITSTHVL TAAHCISNSR TYRVALGKNN LEVEDEEGSL
101 VVGVDSIFVH EKWNSLLIRN DIALIKLAEP VELSDTIQVS CLPEEGSLLP
151 QDYPCYVTGW GRLWTNGPIA AELQQGLQPV VDHATCSQRD WWGSTVRDTM
201 VCAGGDGVIS ACNGDSGGPL NCQAENGSWE VRGIVSFGSG LGCNTYKKPT
251 VFTRVSAYID WIDQKIQL
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B
28 kDa component: chymotrypsinogen B2-like isoform 2 [Sus scrofa]

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  1 MAFLWILSCF ALVGTAFSCG VPAIPPVLSG LSRIVNGENA VPGSWPWQVS
 51 LQDGTGFHFC GGSLISEDWV VTAAHCGVTT SDVVVAGEYD QASDAEDIQV
101 LKIAKVFKNP NFSLLTVRND ITLLKLATPA RFSRTVSAVC LPSASDDFPA
151 GTLCATTGWG KTKYTALKTP DKLQQAALPI VSSTVCKSYW GSKVTDVMIC
201 AGASGVSSCM GDSGGPLVCQ KNGAWTLVGI VSWGSSTCST TTPAVYARVT
251 ALIPWVQQIL ANN
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Figure 5: Identification of the 29 (A) and 28 kDa (B) components in the finally purified product by LC-MS/MS. The results of MS/MS analysis for the 29 and 28 kDa components matched the sequences of chymotrypsin-C-like protease (accession no. XP003127677) and chymotrypsinogen B2-like isoform 2 (accession no. XP003355797) from Sus scrofa, respectively. The peptide sequences detected by MS are underlined.

implying that the component was purified 17-folds from the crude extract in the batch method. Moreover, in case of the flow method, the final product was estimated to be 1.4 U/mg, implying that the Csin component was purified 30-fold. On the other hand, the Tsin-like enzymatic activity (BAPNA hydrolyzing activity) was undetectable. The total time for the purification was about 3 hours.

The SDS-PAGE analysis under non-reducing conditions showed that the final product contained three components with molecular masses of 29, 28, and 23 kDa (lane B in Fig. 4). These three components were also detected by the fluorescein-labeled Phe type phosphonate, which could selectively label Csin-like serine proteases in a manner similar to

that reported by Abuelyaman et al. [3], as shown in Fig. 4 (lane D). These findings indicate that the components corresponding to the three bands in the final product originate from Csin-like proteases. In particular, two components (29 and 28 kDa) seemed identical to the ones observed for the crude extract (lane E), showing that these two components would be Csin-like proteases from pancreatin. It has been known that there are several serine proteases in hog pancreas that can hydrolyze the Phe-X bond, *e.g.*, Csin-A, -B, -C, and elastase 2 [12]. In addition, the 23-kDa component may be a degradation product from the 29-kDa component because by comparison of the bands observed for the crude extract (lanes D and E), the fluorescence intensity of the 29-kDa band was reduced while the 23-kDa band was newly apparent. The LC-MS/MS analysis (Fig. 5) for the components of the final product suggested that the 29- and 28-kDa components would be Csin-C and Csin-B, respectively. Therefore, all these results demonstrated that the naturally occurring Csin-like proteases can be rapidly and highly purified from pancreatin by using G3P-gel.

Discussion

We demonstrated that the covalent chromatography system using a combination of G3P-gel and 2PAM was useful for purifying Csin-like serine proteases. Many Tsin-like serine proteases have been satisfactorily purified by affinity chromatography systems using benzamidine [13], aprotinin [14], soybean trypsin inhibitor [15], analogues of sunflower trypsin inhibitor-1 [16], and so on, as reversible ligands. Although some Csin-like serine proteases have also been isolated with Tsin-like ones, a lot of Csin-like proteases are still unknown, e.g., Csins

in fish. This is due to the limited affinity ligands for Csin-like proteases. In our covalent chromatography system, slight amounts of Csin-like proteases can be highly purified in one step. Even if the target proteases present in low concentration, G3P-gel can capture them by circulating the sample solutions (flow method). In addition, other dominant components can be easily removed by washing without loss of the target proteases because Csin-like proteases covalently fixed on the gel.

The system presented here can be also applicable to the purification of other serine proteases. Since diphenyl 1-aminoalkylphosphonates corresponding to various amino acids can be synthesized [1], replacement of the Phe type phosphonate moiety (P_1 position) by other phosphonates will lead to the preparation of gels available for the separation of serine proteases with desired substrate specificities. Moreover, the elongation of amino acid residues to the P_2 and P_3 positions in the phosphonate derivatives should be effective in improving the selectivity. This covalent chromatography system using a well-designed phosphonate derivative will enable the recoveries of highly purified serine proteases without excessive time-consumption.

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