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Synthesis of Substrates for Rous Sarcoma Virus Protease

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Abstract

In order to obtain the fluorogenic substrates for Rous sarcoma virus protease (RSV-protease), some tryptophan containing heptapeptides [Phe-Gln-Trp-Tyr-Pro-Leu-Ala-$CONH_2$ (substrate I), Phe-Gln-Ala-Trp-Pro-Leu-Ala-$CONH_2$ (substrate II), Phe-Gln-Ala-Tyr-Trp-Leu-Ala-$CONH_2$ (substrate III)] were synthesized by a solid phase peptide synthesis. The obtained substrates and Phe-Gln-Ala-Tyr-Pro-Leu-Ala-OH (substrate IV), Phe-Gln-Ala-Tyr-Pro-Trp-Ala-OH (substrate V) were treated with RSV-protease. The substrates II, III and IV were cleaved, but the substrates I and V were not cleaved by RSV-protease.

Key words: Rous sarcoma virus protease, fluorogenic substrate, peptide synthesis

In all retroviruses, virus protein is initially synthesized as a large polyprotein precursor which consists of a single polypeptide chain *(1)*. The polyprotein undergoes proteolytic processing by the viral protease during virion assembly and maturation. This proteolytic processing of the precursors by the retroviral proteases is an obligate step in the formation of mature, infectious virions.

Therefore, if the proteolytic activity of retroviral proteases is inhibited, the multiplication of retrovirus must be prevented. Thus, inhibition of the viral protease has become an important target for the design of antiretroviral agents. In order to allow the exploration of potential inhibitors of retroviral proteases, it is necessary to obtain convenient assay methods for the proteases activities.

Rous sarcoma virus is a retrovirus causing Rous sarcoma of domestic fowls. Rous sarcoma virus polyprotein is processed by Rous sarcoma virus protease (RSV-protease) *(2)*.

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Abbreviation: Dns, dansyl; Boc, t-butyloxycarbonyl; Py, 4-pyridyl; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; DMSO, dimethylsulfoxide.

The assay method used in the characterization of RSV-protease was based on HPLC separation of products and substrate at several time intervals (*3*). However, this assay method requires much intension. Therefore, some fluorogenic substrates for retroviral proteases were synthesized (*4*, *5*).

Recently, we synthesized some tryptophan containing intramolecularly quenching fluorogenic substrates for pepsin which contained dansyl group and tryptophan residue (*6*, *7*). In this method, it is possible to determin the pepsin activity rapidly and simply. Therefore, we attempted to synthesis of fluorogenic substrates for RSV-protease. RSV-protease cleaves -Thr-Phe-Gln-Ala-Tyr-Pro-Leu-Arg-Glu- sequence of polyprotein at Tyr-Pro bond, then, the expected structure of the substrates were Phe-Gln- X - Y-Pro-Leu-Ala-NHNH-Dns or Dns-Phe-Gln-Ala- Tyr- X - Y -Ala-OCH_2Py. (X,Y=Trp or original amino acids). In order to estimate the moderate sequence for the cleavage by RSV-protease, some tryptophan containing heptapeptide amides were synthesized by a solid phase peptide synthesis.

Experimental Procedures

The substrates IV (Phe-Gln-Ala-Tyr-Pro-Leu-Ala-OH) and V (Phe-Gln-Ala-Tyr-Pro-Trp-Ala-OH) were synthesized at the laboratory of Oriental Yeast Industry by automated solid phase peptide synthesizer and purified by reversed-phase HPLC. All Boc-amino acids were synthesized by the literature (*8*). Chloromethylated polystyrene-divinylbenzene (2%) resin (0.66 mmol Cl/g) was purchased from Protein Research Foundation, Osaka, Japan. For amino acid analyses, peptides were hydrolyzed with 6 M HCl at 110 °C for 24 h and analyzed on a Hitachi amino acid analyzer KLA-5. Amino acid sequence was determined with a Applied Biosystems 477A Protein sequencer.

Boc-heptapeptide-resin. Boc-Ala-resin (430 mg, 0.1 mmol) was placed in a reaction vessel and swollen with AcOH (5 ml) for overnight. The following cycle was used for an addition of appropriate Boc-amino acid. The resin was rinsed with AcOH (5 ml × 3), shaken for 30 min in 1 M HCl in AcOH (5 ml), and rinsed successively with AcOH (5 ml × 3), EtOH (5 ml × 3), and DMF (5 ml × 3). The resin was shaken for 10 min in 10% NEt_3 in DMF (5 ml) and rinsed with DMF (5 ml × 3) and CH_2Cl_2 (5 ml × 3). The resin was suspended in CH_2Cl_2 (4 ml) containing 4-fold molar excess of Boc-amino acid and shaken for 10 min. DCC in equimolar amount with Boc-amino acid was added and shaking continued for 4 h. The coupling steps were terminated by rinsing with CH_2Cl_2 (5 ml × 3) and EtOH (5 ml × 3). After the incorporation of tryptophan residue to the resin, Boc- group was deblocked by 1 M HCl in AcOH containing 5% mercaptoethanol. After completion of the final coupling cycle, Boc-heptapeptidyl resin was washed with CH_2Cl_2, EtOH, AcOH, and EtOH (each 5 ml × 3), respectively.

Boc-heptapeptide amide. The Boc-heptapeptidyl resin was suspended in saturated ammonia in EtOH (5 ml). The mixture was shaken for 5 days at room temperature, and the

deprotected resin was filtered off and washed with EtOH (5 ml × 3). The combined filtrates were evaporated in vacuo and the residue was dissolved in 1 M HCl in AcOH containing 5% mercaptoethanol (2 ml), and left for 1 h at room temperature. The solution was evaporated in vacuo, and the residue (H-heptapeptide amide·HCl) was dissolved in 50% AcOH (3 ml) and hydrogenated in the presence of palladium black for 5 h. After removal of the catalyst, the filtrate was evaporated in vacuo, and the precipitate was collected by filtration with the aid of ether.

Enzymic Studies. The substrates (1 mg) were dissolved in a mixture of DMSO (1.59 ml) and 0.1 M phosphate buffer pH 6.0 containing 4 M NaCl (6.36 ml). Thirty μl of the RSV-protease solution (0.53 mg/ml) was added to the substrate solutions (255 μl) at 37 °C. After incubation for 24 h, the reaction mixtures (80 μl) were subjected HPLC assays. The resulted new peaks were collected and determined its amino acid compositions and the sequences.

Results and Discussion

The protected linear heptapeptide sequence was built up by stepwise addition of Boc-amino acid to the Merrifield resin, starting with L-alanine as the C-terminal amino acid. The obtained Boc-heptapeptidyl resin was treated with ammonia to give Boc-heptapeptide amide. The Boc-heptapeptide amide was then deblocked. The total yield of heptapeptide amides were 40-90% calculated from Boc-Ala-resin (Table I).

Table I. Yields and amino acid compositions of substrates

Substrate	Yield		Amino acid composition					
	mg	(%)	Phe	Glx	Ala	Tyr	Pro	Leu
I	36	39	0.9 (1)	1.0 (1)	1.0 (1)	0.9 (1)	1.0 (1)	1.1 (1)
II	78	94	0.8 (1)	1.0 (1)	2.0 (2)	—	0.9 (1)	1.0 (1)
III	67	75	1.0 (1)	1.0 (1)	2.3 (2)	1.0 (1)	—	1.2 (1)

* tryptophan content was not determined

As shown in Fig. 1-A, 2-A and 3-A, obtained peptide showed two peaks by HPLC analysis. The amino acid compositions of these two peaks were identified to the theoretical values of desired peptide amides. Presumably, the hydrolysis reaction was occurred at amidation and free peptides were made. Consequently, Boc-heptapeptidyl resins may obtained as pure form. As shown in Fig. 1-B, substrate I was not hydrolyzed by RSV-protease, but substrates II and III were hydrolyzed by the protease.

As shown in Fig. 2-B, the peaks of substrate II were decreased and two new peaks were appeared. The peaks **a** and **b** were collected and amino acid composition and amino acid sequence were determined. As shown in Table II, peak **a** was Pro-Leu-Ala and peak **b** was

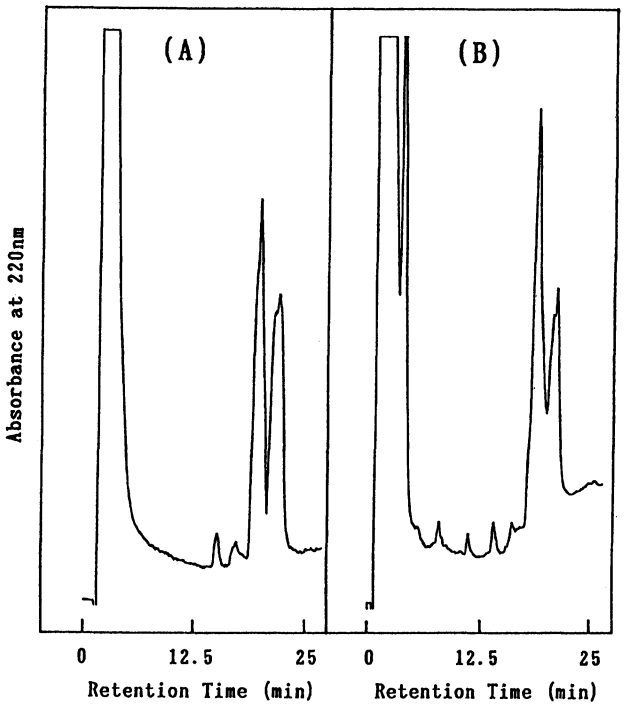

Figure 1.
HPLC trace of the substrate I and the reaction mixture. (A) Substrate I. (B) Reaction mixture of substrate I and RSV-protease. The reaction mixtures (80 μl) were applied to a C_{18} reversed-phase column (SPHERI-5 RP-18, 30×4.6 mm, Applied Biosistems) and eluted with a linear gradient of 0-90% 2-propanol/ acetonitrile/ water (7/3/4.3. v/v) containing 0.1% trifluoroacetic acid in 0.1% trifluoroacetic acid for 30 min at a flow rate of 0.5 ml/min.

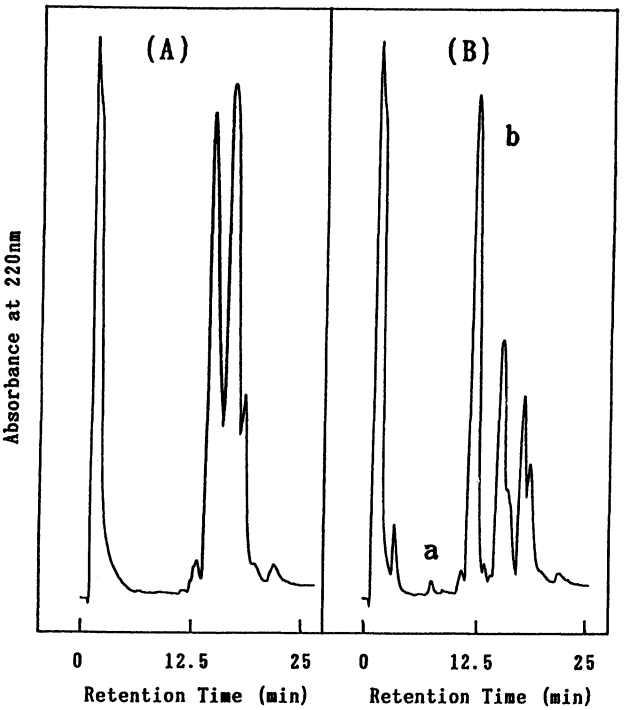

Figure 2.
HPLC trace of substrate II and the reaction mixture. (A) Substrate II. (B) Reaction mixture of substrate II and RSV-protease. The condition of chromatogarphy was same as described in Fig. 1.

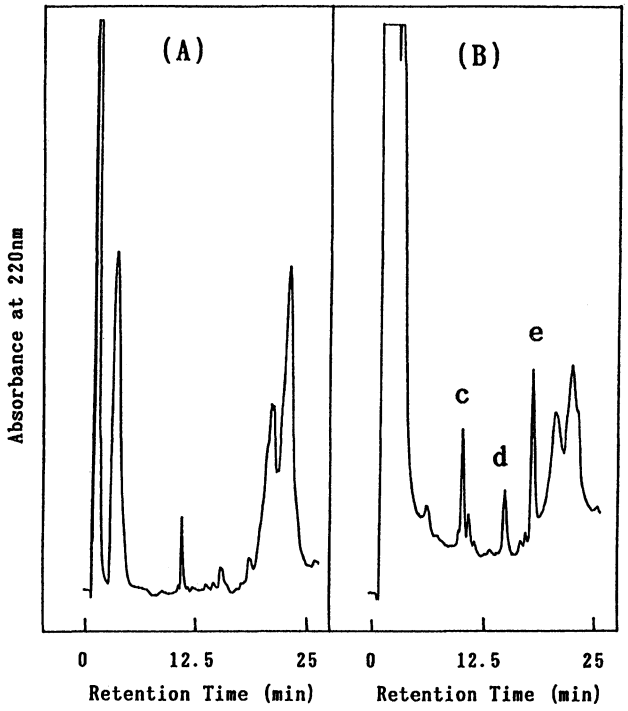

Figure 3. HPLC trace of the substrate III and the reaction mixture. (A) Substrate III. (B) Reaction mixture of substrate III and RSV-protease. The condition of chromatogarphy was same as described in Fig. 1.

Phe-Gln-Ala-Trp. Then, the substrate II was cleaved by RSV-protease at Trp-Pro bond.

Similarly, as shown in Fig. 3-B, the peaks of substrate III were decreased and three new peaks were appeared. The peaks **c**, **d** and **e** were collected and amino acid compositions and amino acid sequences were determined. As shown in Table II, peak **c** and **d** were Trp-Leu-Ala and peak **e** was Phe-Gln-Ala-Tyr. Then, the substrate III was cleaved at Tyr-Trp bond. The substrate I has Trp residue which has a bulky aromatic side chain at P_2 position, and the substrate II has Trp residue at P_1 position, substrate III has Trp residue at P_1' position.

Table II. Sequence of products

Peak	Amino acid composition						Sequence
	Phe	Glx	Ala	Tyr	Pro	Leu	
a	—	—	0.9	—	1.0	1.0	Pro-Leu-Ala
b	0.8	1.0	1.0	—	—	—	Phe-Gln-Ala-Trp
c	—	—	1.0	—	—	1.0	Trp-Leu-Ala
d	—	—	1.0	—	—	1.0	Trp-Leu-Ala
e	0.9	1.0	1.2	0.9	—	—	Phe-Gln-Ala-Tyr

Moreover, the substrate IV was cleaved but the substrate V which has Trp residue at P_2' position was not cleaved by RSV-protease (date was not shown). These data speak for a dislike for a large aromatic side chain at P_2 and P_2' position.

As shown in Fig. 2 and 3, the tryptophan containing substrates II and III were cleaved by RSV-protease, then, the dansylated fluorogenic substrates for RSV-protease may be obtained on the basis of the structures of the substrates II and III.

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