Degradation of a Novel Tripeptide, *tert*-Butoxycarbonyl-Tyr-Leu-Val-CH_2Cl, with Inhibitory Effect on Human Leukocyte Elastase in Aqueous Solution and in Biological Fluids

Hiroyuki TOKUMITSU,* Yoshinobu FUKUMORI, Yuko TSUDA, Yoshio OKADA, Fumiyo KASUYA, Kazuo IGARASHI, and Miyoshi FUKUI

Faculty of Pharmaceutical Sciences, Kobe Gakuin University, Arise, Ikawadani-cho, Nishi-ku, Kobe 651-21, Japan.
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The stability of *tert*-butoxycarbonyl-Tyr-Leu-Val-CH_2Cl (YLV) with inhibitory effect on human leukocyte elastase was investigated in aqueous solution, α-chymotrypsin solution and biological media. In all cases studied here, the degradation was observed as a pseudo-first order reaction. The half-life for the degradation of YLV in an aqueous solution of pH 7.4 at 37 °C was 35.9 h. YLV was most stable at about pH 3.8—5.8 and the effect of temperature was explained by the Arrhenius equation. The activation energies of the degradation in aqueous solutions at pH 2.0, 4.8, and 7.4 were 24.6, 22.1 and 23.4 kcal/mol, respectively. The degradation products in aqueous solution were analyzed by HPLC-MS and were estimated as Boc-Tyr-Leu-Val-CH_2OH at pH 7.4 and H_2N-Tyr-Leu-Val-CH_2Cl at pH 2.0. In a bovine pancreas α-chymotrypsin solution at 37 °C, the half-life of YLV was 15 min at 25.6 μg/ml of α-chymotrypsin solution. In the rat plasma, the half-life of YLV was 42.4 min (YLV 26.7 μg/ml plasma), and in rat liver, lung and spleen homogenates, the degradation rate constants of YLV were 37.6, 10.3 and 23.5 times larger than that in plasma solution, respectively (all fluids containing 5 mg protein/ml). YLV was less stable than nafarelin acetate, secretin, adrenocorticotropic hormone (ACTH) and gonadorelin in an aqueous solution of pH 7.4.

Key words tripeptide; human leukocyte elastase inhibitor; degradation rate; aqueous solution; biological fluid; HPLC-MS

A new synthetic tripeptide, *tert*-butoxycarbonyl(Boc)-Tyr-Leu-Val-CH_2Cl (YLV) (Fig. 1) was found to have inhibitory effect on human leukocyte elastase (HLE).[1] Elastase and cathepsin G (chymotrypsin-like proteinase) from leukocytes participating in a fibrinolytic pathway[2] have attracted our interest in recent years due to their possible involvement in connective tissue turnover[3] and their responsibility for the tissue destruction that occurs in rheumatoid arthritis,[3] inflammation[4] and pulmonary emphysema.[5] We have been seeking peptides which might have the possibility of application in a pulmonary emphysema therapy.

In general, as peptides often show poor biomembrane penetration characteristics and rapid enzymatic degradation at the absorption sites,[6] it is first necessary to exactly evaluate their physicochemical and hydrolytic properties in order to select a route of administration and design a dosage form for optimization of pulmonary peptide delivery. In this study, the degradation kinetics and degradation products of YLV in aqueous solution with or without α-chymotrypsin, an endopeptidase, and in biological media were elucidated to obtain fundamental information for formulation studies. The results were compared with several other peptide drugs reported.

Experimental

Materials Boc-Tyr-Leu-Val-CH_2Cl (YLV) (Fig. 1) with the molecular weight (M.W.) of 525.5 and with three amino acids of the L-configuration was synthesized by Tsuda *et al.*[1] Inhibitory effects of the shorter analogs,[1] stereoisomers[1] and derivatives[7] of YLV on the amidolytic activity of HLE have already been studied. α-Chymotrypsin (39 units/mg solid, C-4129 type; from bovine pancreas) purified was obtained from Sigma Chemical Co., St. Louis, U.S.A. The other chemicals and solvents were of reagent grade.

Animals Male Wistar rats were obtained from Nippon SLC Inc., Hamamatsu, Japan. Those weighing 170—180 g were used to secure fresh liver, lung, spleen and plasma.

* To whom correspondence should be addressed.

Stability of YLV in Aqueous Solution YLV was first dissolved in polyethylene glycol 400 (PEG400) to make a 1.33 mg/ml solution and 400 μl was dissolved in 20 ml of several buffer solutions. These mixtures were incubated at 4, 20, 37, 52, 60 and 70 °C (0.1 °C precision) at 70 strokes/min (Taiyo Scientific Industries, Taitec Incubator Personal, Thermo Minder Mini-80). Sampling was carried out at predetermined time intervals and the samples were cooled to ice water temperature. The buffer solutions used and their pH values were as follows: HCl–CH_3COONa buffer (pH 2.0, 3.8, 4.8), phosphate (KH_2PO_4–Na_2HPO_4) buffer (pH 5.8, 7.4) and glycine–NaOH buffer (pH 9.8). Their ionic strength, μ, was adjusted to 0.1 with KCl. The pH was measured using a pH meter (Horiba Seisakusho, type F-12). When pH was checked both before and after the reaction, it was found unchanged. Fifty μl of each sample was injected directly onto a high performance liquid chromatographic (HPLC) column.

Hydrolysis of YLV in α-Chymotrypsin Solution The hydrolytic rate of YLV was determined at 37 °C in an isotonic phosphate buffer saline (PBS) of pH 7.4 containing α-chymotrypsin in the concentration range of 6.41—51.3 μg/ml. The YLV-PEG400 solution (1.33 mg/ml, 400 μl) was added to 20 ml of each α-chymotrypsin–PBS solution (pH 7.4) at 37 °C. These solutions were incubated at this same temperature (0.1 °C precision) and sampled at predetermined time intervals. At a concentration of 25.6 μg/ml of α-chymotrypsin, a solution containing twice the amount of YLV was also examined. Samples (350 μl) withdrawn were added to 350 μl of cold acetonitrile, mixed and cooled to ice water temperature to stop the reaction. The mixtures were filtered through a membrane (Ekicrodisc 25, Gelman Sciences Japan, Ltd.) and 50 μl of the filtrates were analyzed by HPLC.

Degradations of YLV in Plasma and in Liver, Lung and Spleen Homogenates Fresh plasma and fresh viscera were obtained by sacrific-

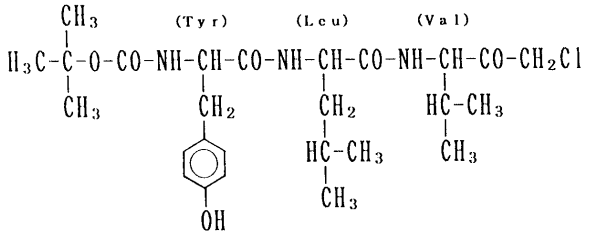

Fig. 1. Structure of YLV (M.W. = 525.5)

ing the rat. A 0.9% saline solution was first recirculated through liver and lung. The viscera were weighed and homogenized in an isotonic Tris–HCl buffer solution (pH 7.4) of 9 times the volume (ml) of their wet weight value. These viscera homogenates were then filtered through a triple gauze. After total protein concentration in the plasma and the homogenates were determined by the buret method, they were diluted by isotonic Tris–HCl buffer solution of pH 7.4 to adjust to 5 mg protein/ml. Stability measurements of YLV were performed in the whole plasma, in the plasma solution containing 5 mg total protein/ml and in the visceral homogenates containing 5 mg total protein/ml by incubation method, similar to the case of α-chymotrypsin solution. The YLV–PEG400 solution (1.33 mg/ml, 400 μl) was dissolved in 20 ml of the biological fluids at 37 °C except for the whole plasma: the measurement in the whole plasma was carried out at 1/2 scale of volume. In the whole plasma a two-fold concentration of YLV was also used. During incubation of these mixtures at 37 °C, 1 ml of sample in the whole plasma or 2 ml in the other biological fluids was withdrawn at predetermined time intervals and cooled in ice water. The sample (1 ml) withdrawn from the whole plasma mixture was added to 5 ml of cold acetonitrile, mixed and cooled to ice water temperature to stop the reaction and to remove protein. Samples (2 ml) withdrawn from the other biological mixtures were first added to 5 ml of cold methanol, then treated similarly. All samples were then centrifuged for 10 min at 3000 rpm (Kubota, type KN-30F). After 5 ml of the supernatant was diluted with 10 ml of distilled water, it was purified with a minicolumn (Sep-pak cartridges C_{18}, Millipore Co., Milford, U.S.A.). In brief, YLV was washed with water and eluted from the minicolumn by acetonitrile. The eluate was evaporated under reduced pressure below 20 °C, and the residue was dissolved in 700 μl of methanol and 300 μl of distilled water. Fifty μl of the final solutions were analyzed by HPLC. The recoveries of YLV in HPLC from whole plasma, liver homogenate, lung homogenate and spleen homogenate mixtures were 72, 70, 90 and 80%, respectively, and their measured values of YLV were defined as 100% of YLV remaining (%) at zero time. In all cases, the change in YLV recovery attended by decreases in YLV concentration in the mixture (YLV 26.7—2.67 μg/ml each homogenate containing 5 mg total protein, 53.3—2.67 μg/ml whole plasma) was not significant. All experiments were repeated three times.

HPLC Analysis The HPLC analysis was carried out with a Shimadzu system consisting of a LC-6A pump, a SPD-6A UV photometrical detector, a CTO-2A column oven and a C-R6A chromatopac, and a Rheodyne 7125 with a 50 μl loop. The chromatographic column was a Cosmosil packed column (type $5C_{18}$, 4.6 i.d. × 150 mm) purchased from Nacalai Tesque, Inc. (Kyoto, Japan). The mobile phase used was 2 : 1 (v/v) methanol–1/30 M phosphate buffer (pH 7.4). The flow rates were 0.4 ml/min for aqueous solution samples, 1.2 ml/min for α-chymotrypsin solution samples and 1.0 ml/min for biological medium samples, and the retention times of YLV were 20.4, 6.5 and 6.9 min, respectively. The wavelength for detection and the column temperature were 285 nm and 30 °C, respectively. All determinations were carried out by absolute calibration method. The correlation coefficient of YLV calibration curve showed as first-order equation was more than 0.9999 under all experimental conditions (YLV concentration range: 2.67—53.3 μm).

Degradation Products in Aqueous Solution Degradation product analysis was carried out by HPLC–atmospheric pressure chemical ionization-mass spectrometry (HPLC–APCI-MS, Hitachi model L-6200 and M-1000). pH 2.0 and 7.4 solutions containing YLV at 10 times the concentration used in the stability test were incubated at 70 °C to obtain degradation products. The degradation products in reaction solutions were separated and collected by HPLC. Prior to HPLC–APCI-MS analysis, the following pretreatments were carried out to remove phosphate from the collected solution containing a degradation product. With the reaction solution of pH 7.4, methanol was first evaporated and the residual solution was lyophilized. A small amount of methanol was added to the lyophilized sample to dissolve the degradation product and the mixture was filtered through a membrane (Ekicrodisc 13, Gelman Sciences Japan, Ltd.) to remove insoluble phosphate. With the reaction solution of pH 2.0, the collected solution was concentrated by evaporator and injected for HPLC with 3 : 2 (v/v) methanol–50 mM ammonium acetate as mobile phase. The solution collected by HPLC was concentrated again below 20 °C. HPLC–APCI-MS analysis was carried out under the following conditions based on previous study[8]: drift voltage, 115—123 V; vaporizer temperature, 210—230 °C; desolvent temperature, 390 °C; mobile phase, 7 : 3 (v/v) methanol–0.1% acetic acid for analysis of degradation product at pH 7.4 and 7 : 3 (v/v) methanol–50 mM ammonium acetate for analysis of degradation product at pH 2.0 condition; flow rate, 1.0 ml/min.

Results

Stability in Aqueous Solution Typical time courses of the disappearance of YLV at various temperatures at pH 7.4 (Fig. 2) and at various pHs at 37 °C (Fig. 3) followed the overall pseudo-first order kinetics. The calculated pseudo-first order rate constants, k_{obs} (degradation rate constants observed by the experiment), and the half-lives, $t_{1/2}$, are listed in Table 1.

Figure 4 shows the Arrhenius plots for the degradation of YLV in aqueous solution of pH 2.0—9.8. Effect of temperature, T, on the degradation rate constants, k_{obs}, of YLV in aqueous solution well fitted the Arrhenius equation. The calculated activation energies of degradation, E_a, at various pHs are also shown in Table 1; they exhibited no significant difference.

The relationship between k_{obs} and pH at 4—70 °C is plotted in Fig. 5. YLV was most stable in the buffer solution at about pH 3.8—5.8.

Degradation Products in Aqueous Solution The main degradation products of YLV in aqueous solution of pH 7.4 and of pH 2.0 were analyzed. Figure 6 shows the mass spectra of YLV and degradation products in pH 7.4 and

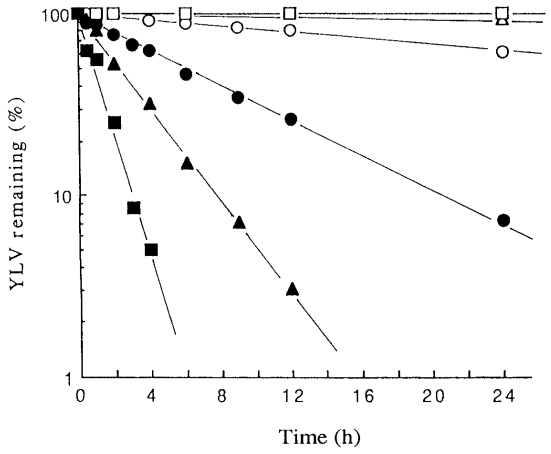

Fig. 2. First-Order Plots for the Degradation of YLV in Buffer Solution of pH 7.4

□, 4 °C; △, 20 °C; ○, 37 °C; ●, 52 °C; ▲, 60 °C; ■, 70 °C.

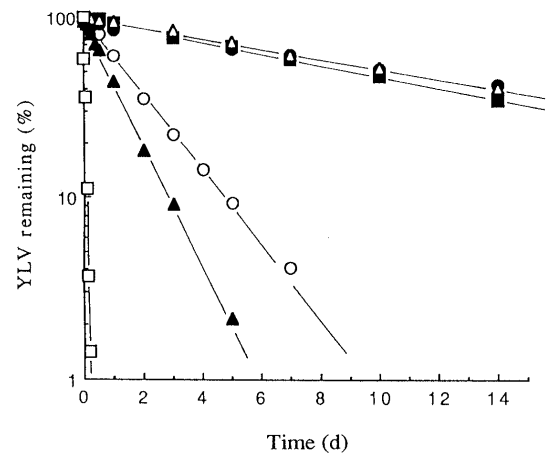

Fig. 3. First-Order Plots for the Degradation of YLV in Buffer Solution of pH 2.0—9.8 at 37 °C

▲, pH 2.0; ●, pH 3.8; ■, pH 4.8; △, pH 5.8; ○, pH 7.4; □, pH 9.8.

Table 1. Kinetic Parameters of Pseudo-First Order Reaction for the Degradation of YLV in Aqueous Solutions

pH	Buffer (μ=0.1)	°C	k_{obs} (h^{-1})	$t_{1/2}$ (h)	E_a (kcal/mol)
2.0	HCl·CH_3COONa	37	3.22×10^{-2}	21.5	24.6
		52	1.73×10^{-1}	4.00	
		60	5.49×10^{-1}	1.26	
		70	1.47	0.471	
3.8	HCl·CH_3COONa	37	2.47×10^{-3}	281	24.0
		52	1.37×10^{-2}	50.7	
		60	3.70×10^{-2}	18.7	
		70	1.06×10^{-1}	6.54	
4.8	HCl·CH_3COONa	37	3.14×10^{-3}	221	22.1
		52	1.42×10^{-2}	48.7	
		60	4.19×10^{-2}	16.5	
		70	9.51×10^{-2}	7.28	
5.8	Phosphate	37	2.70×10^{-3}	257	22.4
		52	1.09×10^{-2}	63.6	
		60	3.63×10^{-2}	19.1	
		70	8.37×10^{-2}	8.28	
7.4	Phosphate	4	2.26×10^{-4}	3070	23.4
		20	2.37×10^{-3}	292	
		37	1.93×10^{-2}	35.9	
		52	1.09×10^{-1}	6.36	
		60	2.93×10^{-1}	2.37	
		70	7.69×10^{-1}	0.901	
9.8	Glycine·NaOH	4	1.10×10^{-2}	63.0	24.1
		20	1.19×10^{-1}	5.82	
		37	1.07	0.650	
		52	6.71	0.103	

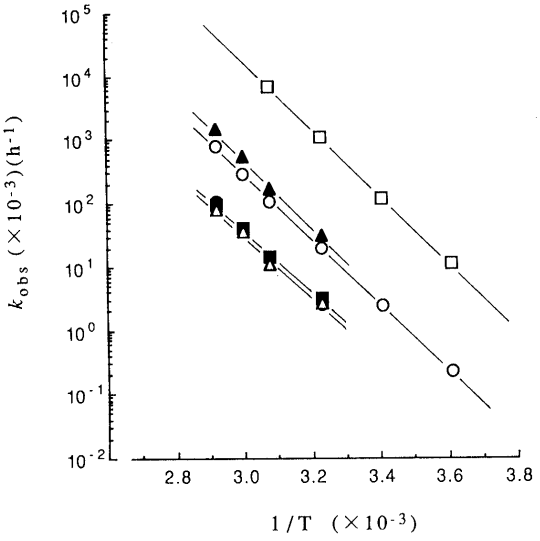

Fig. 4. Arrhenius Plots for the Degradation of YLV in an Aqueous Solution of pH 2.0—9.8

▲, pH 2.0; ●, pH 3.8; ■, pH 4.8; △, pH 5.8; ○, pH 7.4; □, pH 9.8.

pH 2.0, which yielded quasi-molecular ions at *m/z* 526, 508 and 426, respectively. The main degradation product of YLV at pH 7.4 was estimated as Boc–Tyr–Leu–Val–CH_2OH from molecular ions at *m/z* 508 (chloride isotope peaks not detected), 452 [–OCO–Tyr–Leu–Val–CH_2OH] and 408 [–Tyr–Leu–Val–CH_2OH] on the obtained mass spectra (Fig. 6B). The main degradation product of YLV at pH 2.0 was estimated as H_2N–Tyr–Leu–Val–CH_2Cl from fragment molecular ions at *m/z* 390 [H_2N–Tyr–Leu–Val–CH_2–], 277 [H_2N–Tyr–Leu–], 249 [H_2N–Tyr–NH–$CH(CH_2–CH(CH_3)_2)$–] and 136 [H_2N–

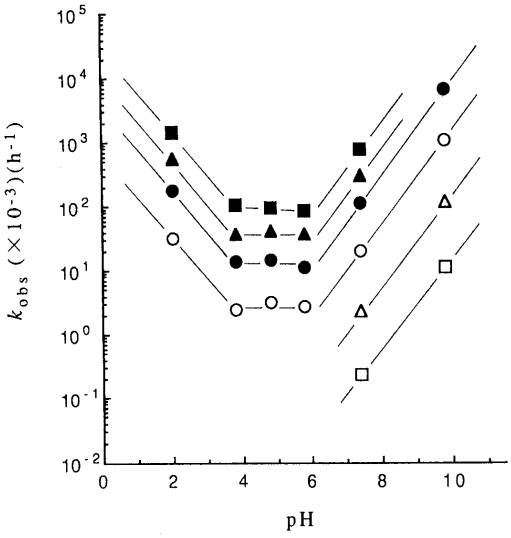

Fig. 5. The k_{obs}–pH Profile for the Degradation of YLV in an Aqueous Solution

□, 4 °C; △, 20 °C; ○, 37 °C; ●, 52 °C; ▲, 60 °C; ■, 70 °C.

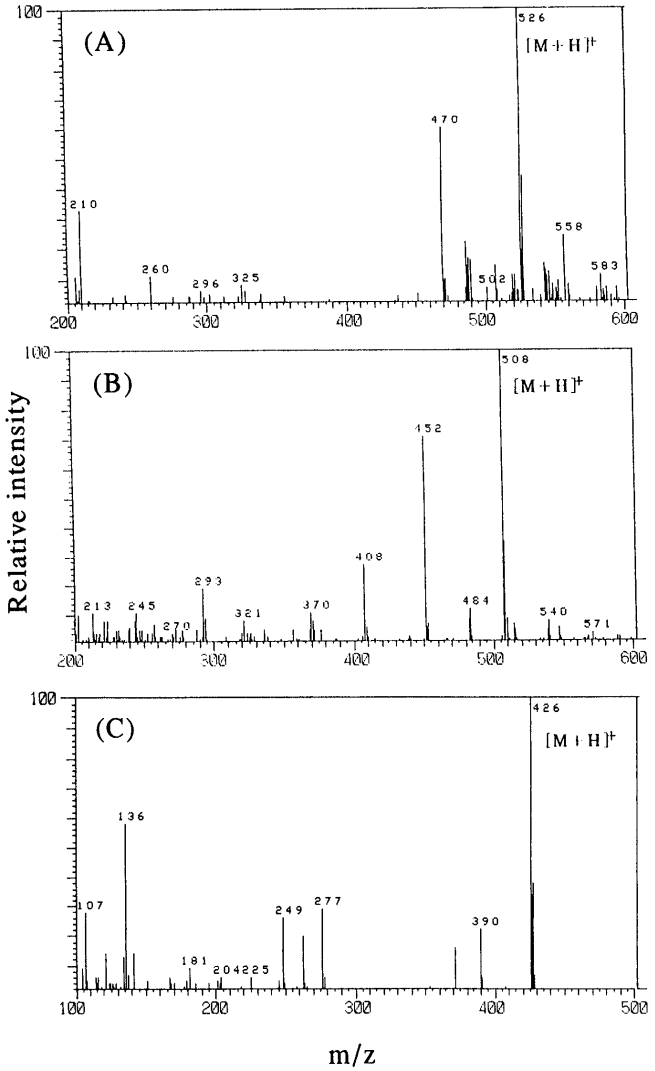

Fig. 6. Mass Spectra of YLV (A) and Degradation Products of YLV under pH 7.4 (B) and pH 2.0 (C) Conditions

$CH(CH_2–C_6H_4–OH)$–] (Fig. 6C).

In HPLC analysis, the degradation product of YLV at pH 2.0 exhibited a single peak, and was identified as

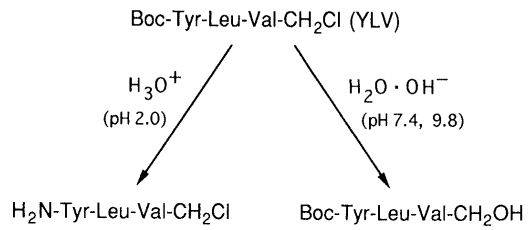

Fig. 7. Presumed (pH 7.4) and Identified (pH 2.0) Mechanisms of the Degradation of YLV

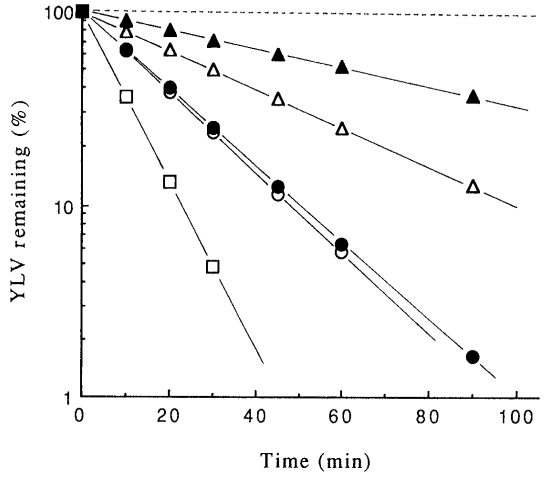

Fig. 8. First-Order Plots for the Catalyzed Hydrolysis of YLV in pH 7.4 Phosphate Buffer Saline Containing α-Chymotrypsin at 37 °C

α-Chymotrypsin concentration: □, 51.3 μg/ml; ○, 25.6 μg/ml; △, 12.8 μg/ml; ▲, 6.4 μg/ml; ---, 0 μg/ml (in pH 7.4 phosphate buffer, $\mu=0.1$) (26.7 μg YLV/ml); ●, 25.6 μg/ml (53.3 μg YLV/ml).

Table 2. Effect of α-Chymotrypsin Concentration and Substrate Concentration on Enzymatic Hydrolysis Rates of YLV

YLV amount (mg/ml)	Enzyme conc. (μg/ml)	k_{obs} (min^{-1})	$t_{1/2}$ (min)
26.7	0	3.22×10^{-4}	2150
	6.4	1.12×10^{-2}	61.9
	12.8	2.29×10^{-2}	30.3
	25.6	4.78×10^{-2}	15.0
	51.3	1.01×10^{-1}	6.86
53.3	25.6	4.59×10^{-2}	15.1

H_2N–Tyr–Leu–Val–CH_2Cl in comparison to a synthesized standard compound by LC-MS (Fig. 7). H_2N–Tyr–Leu–Val–CH_2Cl was stable at pH 2.0 and its inhibitory activity on HLE was 45% of YLV (data not shown).

α-Chymotrypsin-Catalyzed Hydrolysis The enzymatic hydrolysis of YLV was studied *in vitro* with bovine pancreas α-chymotrypsin at 37 °C. The time courses of the disappearance of YLV at various α-chymotrypsin concentrations and at different YLV concentrations are shown in Fig. 8. Table 2 summarizes the k_{obs} and $t_{1/2}$ of YLV in the α-chymotrypsin solution calculated from the figure. The degradation rate constants showed a significant dependence on enzyme concentration, while the rate constants were not decreased at a higher substrate concentration.

Degradation Kinetics of YLV in Plasma Hydrolysis study of YLV in plasma was performed *in vitro* with fresh rat plasma at 37 °C. The time courses of the disappearance

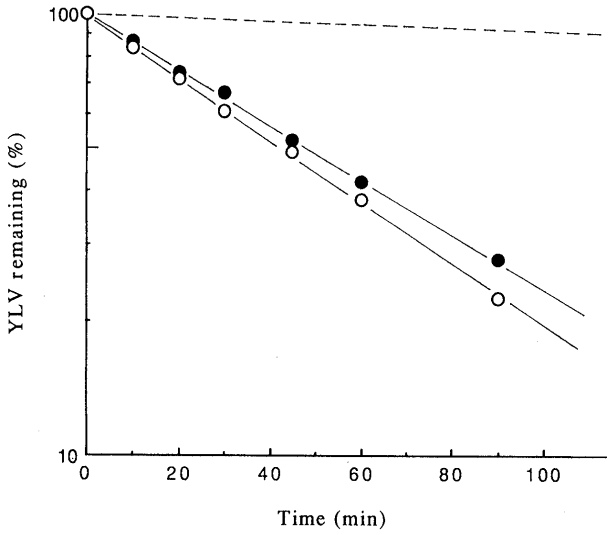

Fig. 9. First-Order Plots for the Degradation of YLV in Rat Plasma at 37 °C

YLV concentration: ○, 26.7 μg/ml plasma; ●, 53.3 μg/ml plasma; ---, 26.7 μg/ml pH 7.4 buffer solution ($\mu=0.1$).

Table 3. Pseudo-First Order Rate Constants (k_{obs}) for the Degradation and Half-Lives ($t_{1/2}$) of YLV in Biological Fluids and in Buffer Solution at 37 °C[a]

Biological fluid	YLV conc. (μg/ml)	k_{obs} (min^{-1})	$t_{1/2}$ (min)
Buffer solution[b]	26.7	3.22×10^{-4}	2150
Rat plasma	26.7	1.63×10^{-2}	42.4
	53.3	1.43×10^{-2}	48.4
Liver homogenate[c]	26.7	4.35×10^{-2}	15.9
Lung homogenate[d]	26.7	1.19×10^{-2}	58.1
Spleen homogenate[e]	26.7	2.72×10^{-2}	25.5
Plasma solution[f]	26.7	1.16×10^{-3}	598

a) Each result represents the mean of three experiments. *b*) pH 7.4 phosphate buffer solution ($\mu=0.1$). *c*) 1.8—2.0% rat liver homogenate containing 5 mg protein/ml (pH 7.4). *d*) 1.8—1.9% rat lung homogenate containing 5 mg protein/ml (pH 7.4). *e*) 2.0—2.1% rat spleen homogenate containing 5 mg protein/ml (pH 7.4). *f*) 9.0% rat plasma solution containing 5 mg protein/ml (pH 7.4).

of YLV at different YLV concentrations are shown in Fig. 9. The slopes of semilogarithmic plots of the percent of YLV remaining in plasma against time produced the k_{obs} and $t_{1/2}$, which are summarized in Table 3 together with the corresponding data in a buffer solution of pH 7.4. The $t_{1/2}$ for hydrolysis in rat plasma were 42.4 min (26.7 μg YLV/ml plasma) and 48.4 min (53.3 μg YLV/ml plasma). The k_{obs} in rat plasma was 50.6 times larger than that at 37 °C in aqueous solution of pH 7.4 (26.7 μg/ml), and YLV concentration only slightly affected k_{obs}.

Degradation of YLV in Liver, Lung and Spleen Homogenates Hydrolysis studies of YLV in the viscera and in plasma solution were performed *in vitro* at 37 °C with fresh biological fluids at 5 mg total protein/ml. Time courses of the disappearance of YLV in various biological fluids are shown in Fig. 10. The k_{obs} and $t_{1/2}$ of YLV calculated from Fig. 10 are summarized in Table 3. The k_{obs} in liver, spleen and lung homogenates were 37.6, 23.5 and 10.3 times larger than that in plasma solution, respectively. In this experiment, YLV was hydrolyzed

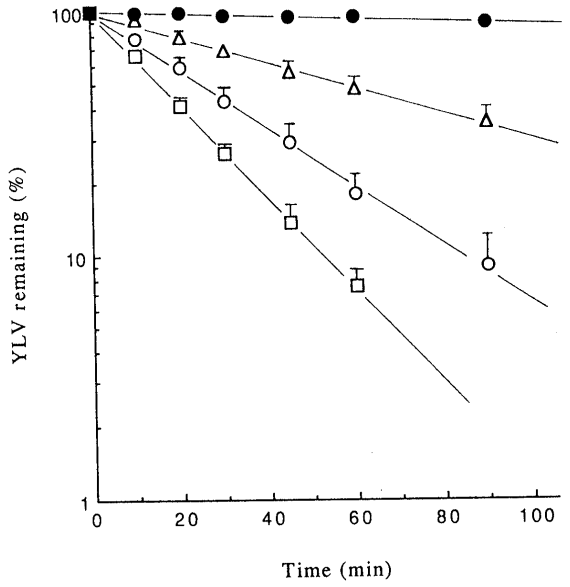

Fig. 10. First-Order Plots for the Degradation of YLV in Rat Visceral Homogenates and Rat Plasma Solution Containing 5 mg Protein/ml at 37 °C

□, liver homogenate; △, lung homogenate; ○, spleen homogenate; ●, plasma solution.

rapidly in spite of using homogenates of very low total protein concentration.

Discussion

When the stability of YLV in aqueous solution was compared with that of nafarelin acetate, an analog of luteinizing hormone releasing hormone (LH-RH), in aqueous solution,[9] both compounds exhibited similar U-shaped $\log k_{obs}$ *vs.* pH profiles (Fig. 5) with the maximum stability at pH 4—6 for nafarelin acetate (80 °C). But the pseudo-first order rate constants (k_{cal}: degradation rate constants calculated theoretically using k_{obs} values) of YLV in buffer solutions of pH 2.0, 5.8 and 7.4 at 80 °C, which were calculated from the Arrhenius plots (Fig. 4), were 4.03, 0.216 and 2.14, respectively, and were 130, 180 and 250 times larger than the k_{obs} of nafarelin acetate at 80 °C. The activation energies of YLV at pH 4.8 and 5.8 (Table 2), on the other hand, were nearly equal to that of nafarelin acetate at pH 5.4, which was reported to be 21 kcal/mol. Compared with the stability of porcine secretin in aqueous solution,[10] the k_{obs} of YLV were nearly equal to that of secretin in the pH region of maximum stability of YLV (pH 3.8—5.8). But at pH 2.0, 7.4 and 9.8, the k_{obs} of YLV were about 10 times larger than that of secretin. Compared with the stability of novel hexapeptide with neurotensin activity in aqueous solution reported by Tokumura *et al.*,[11] the activation energies of YLV (Table 2) were nearly equal to those of the hexapeptide at pH 6.1, 7.0 and 8.0, which were reported to be 22.2, 23.9 and 24.2 kcal/mol, respectively. But the k_{cal} of YLV at 80 °C were 450, 50 and 40 times larger than the k_{obs} of the hexapeptide at 80 °C at pH 2.0, 5.8 and 7.4, respectively. Additionally, in aqueous solution YLV was less stable than adrenocorticotropic hormone (ACTH)[12] and gonadorelin,[13] but might be more stable than most thyrotropin releasing hormone (TRH) derivatives.[14]

In the stability studies of YLV against α-chymotrypsin, it was found from the present results that YLV was rapidly catalyzed by α-chymotrypsin (Table 3). α-Chymotrypsin is an endopeptidase (serine protease) which catalyzes the hydrolysis of peptide bonds at the reactive carbonyl group belonging to the L-amino acids of tryptophan, tyrosine, phenylalanine and, to a lesser extent, leucine and methionine.[15] As expected from this substrate specificity, YLV was found to be readily cleaved at the bond between Tyr and Leu by α-chymotrypsin. The present results suggested a very rapid YLV degradation after oral administration (*p.o.*) because the normal concentration of α-chymotrypsin in the gut including the stool is about 0.5 mg/ml.[16] In contrast, the $t_{1/2}$ of [D-Ala^1]-peptide T amide (D-Ala–Ser–Thr–Thr–Thr–Asn–Tyr–Thr amide), an inhibitor of human immunodeficiency virus (HIV), was reported to be 1.1 and 0.6 min at 0.28 and 0.56 units/ml of α-chymotrypsin concentration, respectively (pH 7.4).[17] Since the $t_{1/2}$ of YLV was 30.3 min at 0.50 units/ml (12.8 μg/ml) of α-chymotrypsin concentration, YLV was more stable against α-chymotrypsin than [D-Ala^1]-peptide T amide.

The degradation of [D-Ala^1]-peptide T amide in 80% human plasma was not observed following incubation for 5 h at 37 °C,[17] while the $t_{1/2}$ of YLV at 45% rat plasma (25 mg protein/ml) was 85.8 min (data not shown) and approx. 10% of YLV was degraded during 5 h at 37 °C in pH 7.4 buffer solution (Fig. 2). This suggested that YLV might be less stable than [D-Ala^1]-peptide T amide in human plasma. In comparison with TRH, whose $t_{1/2}$ in human plasma was 9.4 min,[18] it was suggested that YLV might be more stable. However, determination of the YLV degradation rate in human plasma would be needed to compare with these peptides reliably and to advance YLV application for therapy.

The present results obtained by the stability tests using biological fluids indicated that YLV could be degraded rapidly in blood and especially in liver *in vivo*. Therefore, it would be difficult to obtain an efficient pharmacological effect at disease sites in lung after intravenous (i.v.) injection as a solution dosage form.

In order to apply YLV as a drug, it is essential that its stability be increased by a dosage form design and/or a molecular design. For example, it was reported that a drug could be stabilized physicochemically by forming a complex with cyclodextrin (CD)[19] and CD derivatives.[20] As a dosage form design, Niwa *et al.* reported that in an aqueous solution of pH 1.2 nafarelin acetate was stabilized by loading into D,L-lactide/glycolide copolymer nanosphere.[21] Further, in an effort to protect tyrosyl peptides against α-chymotrypsin, various bioreversible derivatives in the tyrosine phenol group of the peptide as prodrug were investigated by Kahns and Bundgaard.[22]

For pulmonary emphysema therapy under the existing YLV degradation property, studies on a dosage form which can efficiently deliver a drug at disease sites in lung[23] are needed. The particulate colloidal systems such as liposome, emulsion and nanoparticle have been applied widely as drug delivery systems; liposomes in particular have been researched briskly as devices for targeting to lung.[24] In recent years the long-circulating, reticulo-

endothelial system (RES) avoidable type of particulate colloidal devices have been explored actively. It is an especially attractive phenomenon that poly (methyl methacrylate) nanoparticles coated by poloxamer 407,[25] polysorbate 60,[25] polysorbate 80,[25] serum[26] or inactivated serum[26] escape from uptake by liver soon after i.v. injection and accumulate highly in lung for a long period, but by an as yet unknown mechanism. As another administration route than *p.o.* and i.v. injection, it might be suggested from the present results that direct administration to lung mucosa by inhalation of aerosols[24c,f,g,27] containing YLV might be more advantageous.

Conclusion

The stability of novel YLV with inhibitory effect on human leukocyte elastase was evaluated in aqueous solution, α-chymotrypsin solution and biological media. The degradation of YLV was observed as a pseudo-first order reaction in all cases. YLV was most stable between about pH 3.8—5.8 and the effect of temperature fitted into the Arrhenius equation. The degradation products analyzed by HPLC-MS were estimated as Boc–Tyr–Leu–Val–CH_2OH at pH 7.4 and H_2N–Tyr–Leu–Val–CH_2Cl at pH 2.0. YLV was less stable than nafarelin acetate, secretin, ACTH and gonadorelin in an aqueous solution of pH 7.4. The half-life of YLV in rat plasma was 42.4 min and YLV showed rapid degradation in α-chymotrypsin solution, in rat liver, lung and spleen homogenates. From these results, it was suggested that for treatment of disease sites in lung YLV must be stabilized and delivered with assistance of a dosage form design and/or a molecular design.

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References

1) Tsuda Y., Okada Y., Nagamatsu Y., Okamoto U., *Chem. Pharm. Bull.*, **35**, 3576—3584 (1987).
2) Nagamatsu Y., Okamoto U., Tsuda Y., Okada Y., *Throm. Haemostas.*, **51**, 243—247 (1984).
3) Tanaka T., Minematsu Y., Reilly C. F., Travis J., Powers J. C., *Biochemistry*, **24**, 2040—2047 (1985).
4) Janoff A., *Ann. Rev. Med.*, **23**, 177—190 (1972).
5) Senior R. M., Tegner H., Kuhn C., Ohlsson K., Starcher B. C., Pierce J. A., *Am. Rev. Respir. Dis.*, **116**, 469—475 (1977).
6) Humphrey M. J., Ringrose P. S., *Drug Metab. Rev.*, **17**, 283—310 (1986); Lee V. H. L., Yamamoto A., *Adv. Drug Deliv. Rev.*, **4**, 171—207 (1990).
7) Tsuda Y., Teno N., Okada Y., Nagamatsu Y., Okamoto U., *Chem. Pharm. Bull.*, **36**, 3119—3124 (1988).
8) Kasuya F., Igarashi K., Fukui M., Fukumori Y., Tokumitsu H., Fukuda T., Tsuda Y., Okada Y., *Biol. Mass Spectrom.*, **21**, 500—504 (1992).
9) Johnson D. M., Pritchard R. A., Taylar W. F., Conley D., Zuniga G., McGreevy K.G., *Int. J. Pharm.*, **31**, 125—129 (1986).
10) Tsuda T., Uchiyama M., Sato T., Yoshino H., Tsuchiya Y., Ishikawa S., Ohmae M., Watanabe S., Miyake Y., *J. Pharm. Sci.*, **79**, 223—227 (1990).
11) Tokumura T., Tanaka T., Sasaki A., Tsuchiya Y., Abe K., Machida R., *Chem. Pharm. Bull.*, **38**, 3094—3098 (1990).
12) Bhatt N. P., Patel K., Borchardt R. T., *Pharm. Res.*, **7**, 593—599 (1990).
13) Helm V. J., Müller B. W., *Pharm. Res.*, **7**, 1253—1256 (1990).
14) Bundgaard H., Møss J., *Pharm. Res.*, **7**, 885—892 (1990).
15) Hess G. P., "Chymotrypsin-Chemical Properties and Catalysis," The Enzymes, Vol. 3, ed. by Boyer P. D., Academic Press, New York, 1971, pp. 213—248.
16) Goldberg D. M., Campbell R., Roy A. D., *Biochim. Biophys. Acta*, **167**, 613—615 (1968); Dockter G., Hoppe-Seyler F., Appel W., Sitzmann F. C., *Clin. Biochem.*, **19**, 329—332 (1986).
17) Kahns A. H., Bundgaard H., *Int. J. Pharm.*, **77**, 65—70 (1991).
18) Møss J., Bundgaard H., *Pharm. Res.*, **7**, 751—755 (1990).
19) Hamada Y., Nambu N., Nagai T., *Chem. Pharm. Bull.*, **23**, 1205—1211 (1975).
20) Backensfeld T., Müller B. W., Wiese M., Seydel J. K., *Pharm. Res.*, **7**, 484—490 (1990).
21) Niwa T., Takeuchi H., Hino T., Kunou N., Kawashima Y., *J. Pharm. Sci.*, **83**, 727—732 (1994).
22) Kahns A. H., Bundgaard H., *Int. J. Pharm.*, **76**, 99—112 (1991).
23) Ranney D. F., *Biochem. Pharmacol.*, **35**, 1063—1069 (1986); Willmott N., *Adv. Drug Deliv. Rev.*, **5**, 133—148 (1990).
24) *a*) Hunt C. A., Rustum Y. M., Mayhew E., Papahadjopoulos D., *Drug Metab. Disposition*, **7**, 124—128 (1978); *b*) Takada M., Yuzuriha T., Katayama K., Iwamoto K., Sunamoto J., *Biochim. Biophys. Acta*, **802**, 237—244 (1984); *c*) Debs R. J., Straubinger R. M., Brunette E. N., Lin J. M., Lin E. J. Montgomery A. B., Friend D. S., Papahadjopoulos D. P., *Am. Rev. Respir. Dis.*, **135**, 731—737 (1987); *d*) Hughes B. J., Kennel S., Lee R., Huang L., *Cancer Res.*, **49**, 6214—6220 (1989); *e*) Maruyama K., Holmberg E., Kennel S.J., Klibanov A., Torchilin V.P., Huang L., *J. Pharm. Sci.*, **79**, 978—984 (1990); *f*) Kellaway I. W., Farr S. J., *Adv. Drug Deliv. Rev.*, **5**, 149—161 (1990); *g*) McCalden T. A., *ibid.*, **5**, 253—263 (1990).
25) Tröster S. D., Müller U., Kreuter J., *Int. J. Pharm.*, **61**, 85—100 (1990).
26) Borchard G., Kreuter J., *J. Drug Targeting*, **1**, 15—19 (1993).
27) Byron P. R., *Adv. Drug Deliv. Rev.*, **5**, 107—132 (1990); Niwa T., Takeuchi H., Hino T., Kawashima Y., *Yakugaku Zasshi*, **115**, 732—741 (1995).