0, 1600.0, 1555.7 cm^{-1}; MS (70 eV) m/z: 346 (M^+); HRMS (M^+) calcd for $C_{19}H_{30}O_2Si_2$ 346.1784, found 346.1787.

8: ^1H NMR ($CDCl_3$, 300 MHz) δ_H 0.16 (9H, s, -Si(CH_3)$_3$), 0.28 (6H, s, -Si(CH_3)$_2$), 1.26 (3H, s, methyl), 1.57 (3H, s, methyl), 4.59 (2H, dt, J=4.9, 1.6 Hz, -CH_2CH=CH_2), 5.28 (1H, dq, J=10.6, 1.6 Hz, -CH_2CH=CH_2), 5.50 (1H, dq, J=17.2, 1.6 Hz, -CH_2CH=CH_2), 6.06 (1H, m, -CH_2CH=CH_2), 6.82-6.90 (2H, m, ArH), 7.24 (1H, td, J=7.1, 1.8 Hz, ArH), 7.42 (1H, dd, J=7.1, 1.8 Hz, ArH); ^{13}C NMR ($CDCl_3$, 50 MHz) δ_C −2.52, −2.08, 30.1, 69.5, 77.6, 97.9, 103.7, 112.7, 117.6, 120.9, 130.0, 133.4, 134.3, 159.9, 162.7; UV (CH_2Cl_2) λ_{max} 306, 298, 261 nm; FT-IR (NaCl) 2953.7, 2151.8, 1488.6, 1246.5 cm^{-1}; MS (70 eV) m/z: 346 (M^+); HRMS (M^+) calcd for $C_{19}H_{30}O_2Si_2$ 346.1784, found 346.1785.

Acknowledgment. The authors would like to thank Miss Kang Hee Seo for helping the preparation of compounds, Mr. Bang Duk Kim at KAIST for NMR experiments. This investigation was supported by Organic Chemistry Research Center-KOSEF and the Korea Advanced Institute of Science and Technology.

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The chemical shifts for the ethynyl carbons of (**2**) and (**8**) appear at (δ 97.49, 103.30) and (δ 97.9, 103.7), respectively. From this results, it is thought that the C(1)-C(2) triple bond of (2) remains intact in this photoreaction.

Synthesis of Carbobenzoxy-alanyl-thiaarginine (thialysine) benzyl ester and Kinetic Studies with Trypsin

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Carbobenzoxy-alanyl-thiaarginine benzyl ester and carbobenzoxy-alanyl-thialysine benzyl ester were synthesized in solution. Kinetic studies were carried out using three different analytical methods, semi-classical method, progress curve analysis and competitive spectrophotometry. In competitive spectrophotometry, carbobenzoxy-valyl-glycyl-arginyl-*p*-nitroaniline was used as a detector. Kinetic constants such as K_m and V_{max} measured by competitive spectrophotometry are almost the same as those values measured by semi-classical method. Colorimetric Ellman's assays showed the thio-peptido mimetics to be a suitable substrates for trypsin. Kinetic studies with trypsin gave K_m of 2.33 mM and k_{cat} of 1.50×10^5 min^{-1} for carboxy-alanyl-thiaarginine benzyl ester and K_m of 3.41×10^{-3} mM and k_{cat} of 520×10^2 min^{-1} for carbobenzoxy-alanyl-thialysine benzyl ester, respectively. Kinetic constants (K_m=2.04×10^{-2} mM, K_{cat}=4.42×10^3 min^{-1}) for natural substrate, carbobenzoxy-alanyl-lysine benzyl ester, were also evaluated by competitive spectrophotometry in order to compare the mode of binding on trypsin.

Introduction

The breakdown of the alpha-substituted glycine[1-5] peptides yields a nucleophilic substituent along with ammonia and glyoxylate. If the substituent had been linked to the glycine residue through sulfur, decomposition lead to a compound with free sulfhydryl group. Its appearance can be

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monitored spectrophotometrically in the presence of Ellman's reagent,[1,16] which reacts rapidly and quantitatively with free sulfhydryl groups to form a highly colored anionic species monitored at 412 nm. In this manner, the alpha-substituted glycine peptide attached to the glycine residue by sulfur can be applied to enzymatic assays[1-4] in which the hydrolysis rate can be followed at 412 nm. Several examples of these detector peptides have already been explored and are used in the assay for leucineaminopeptidase, a cytosolic exopeptidase,[6] in the assay for pancreatic carboxypeptidase A in blood serum,[7] carboxypeptidase B[1,3] and transport system.[3-5,8-15]

The most frequent cleavage sites of proprotein processing has been found to occur at pairs of basic amino acids and most commonly at Lys-Arg and Arg-Arg sites.[17-25] A trypsin-like enzyme is believed to be the endopeptidase which initially nicks the pair on the carboxyl side of the second amino acid in recognition.[26] Arginine is known to occurs most commonly at the endopeptidase's cleavage site over lysine residue, that is, arginine residue than lysine is preferred on the carboxyl side of the base pair. Therefore, the finding of a sensitive and specific assay method will allow possible detection and characterization of the trypsin-like enzyme involved in proprotein processing at basic sites. In order to develop a novel assay for trypsine, previously designed thia-arginine,[1,2] mimetic for arginine, and thia-lysine, mimetic for lysine, were incorporated into the peptide substrate by adding alanine to the C-side of the thia-arginine or thia-lysine; Z-Ala-S-Arg-OBn and Z-Ala-S-Lys-OBn.

Trypsin catalyzes the hydrolysis of only those peptide bonds in which the carbonyl group is contributed by either lysine or arginine residue. The structure of the enzyme-substrate complexes for the trypsin was well studied with the use of polypeptide inhibitors which bind tightly to the enzymes as a result of being locked into a conformation which a normal flexible substrate can assume on binding.[34] Trypsin is known to have a pocket for side chain binding, called subsite S1 (Schechter and Berger nomenclature),[37] which is 10-12 Å deep and 3.5-4 Å by 5.5-6.5 Å in cross section.[33] Since one methylene group is approximately 4 Å in diameter, the lysine or arginine side chains fit well into the S1 pocket.[35] The peptide substrates containing thia-arginine or thia-lysine we have designed as the mimetics for the natural compound have little different side chain structures, that is, sulfur is smaller (1.8 Å) than a methylene and the S-C bond is longer than C-C bond which is replaced by S-C bond in the thia-analogs. Therefore, the part of our study is to verify how the longer size of the thia-substituted side chains affect the enzyme's function which is dependent on substrate structure. Our previous paper reported simply that thia-substituted peptide was a fairly suitable substrate for carboxypeptidase B.[1] In this paper, we will examine more carefully how side chain differences affect kinetic constants by comparing the trypsin susceptibility of two substrates, thia-substituted analog and its natural analog.

Kinetic studies were made to compare the kinetic constants with these synthetic substrates. Three methods of analyses were used. First one was the initial rate method of Lineweaver-Burke that provide the initial velocity values at different concentrations of the substrate.[30] Second one was progress curve analysis of the integrated Michaelis-Menten equation.[31] Third one was competitive spectrophotometry,[2,27] in which one could determine the kinetic constants for an alternative substrate, competitor, by monitoring its perturbation of the attack on chromogenic substrate, detector. The experiment was carried out using trypsin with Z-Val-Gly-Arg-pNA as detector peptide in the presence of Z-Ala-Lys-OBn as competitive substrate. Although two substrates compete to bind on trypsin, only detector substrate is detected spectrophotometrically. The undetected competitor, Z-Ala-Lys-OBn, change the progress curve for the detector, Z-Val-Gly-Arg-pNA, in a manner which is influenced by the V_{max} and the K_m of the competitor substrate. Michaelis-Meten equation (semi-classical method), the progress curve equation and the competition equation are listed below.

1) Semi-classical method equation: $1/V_o=K_m/V_{max} \cdot 1/[S]+1/V_{max}$
2) Progress curve equation: $1/t \cdot \ln[S_o/(S_o-y)]=-1/K_m \cdot y/t+V_{max}/K_m$
3) Competitive equation: $B_o/V_o[1-(S_o-y/S_o)^n]=\Delta t$,

where Δt is the lag time. S_o, B_o, y are the initial concentration, kinetic parameter and certain substrate concentration at time t, respectively. The value of n, ratio of kinetic power, can be calculated from the equation; $n=K_a/V_a \cdot V_b/K_b$, where a and b are the concentration at time t of substrate A and B, respectively. V_a and V_b are the maximum velocities and K_a and K_b are the Michaelis constants observed when the enzyme is acting on A or B alone. It is assumed that the Michaelis constants represent affinity constants.

Experimental

Trypsin was purchased from Sigma. Ellman's reagent[16] was purchased from Pierce. S-2-aminoethylisothiouronium bromide hydrochloride and benzylglyoxylate were purchased from Aldrich Chemical Corp. Z-Val-Gly-Arg-pNA (Chromozym TRY) was purchased from Boehringer Mannheim Corp. Z-Ala-Lys-OBn was purchased from Aldrich Corp. Kinetic studies were carried out with Hitachi U-2000 spectrophotometer. Benzyloxycarbonyl-L-alanylamide, Z-Ala-NH_2, was prepared as described by Hong.[1,10] ^{1}H NMR spectra were recorded on a General Electric GN-260 spectrometer using tetramethylsilane as internal standard (δ scale). Flash chromatography was performed on Merk silica gel 60 (0.040-0.063 nm) using nitrogen pressure. Analytical thin-layer chromatography (tlc) was carried out on precoated (0.25 nm) Merck silica gel F-254 plates. Rf values of tlc and purity were determined in the following solvent systems: A, $CHCl_3$-MeOH (9/1); B, $CHCl_3$-MeOH (9/2); C, $CHCl_3$-MeOH-AcOH (7/2/1); D, n-BuOH-AcOH-H_2O (4/1/1). Compounds were visualized by uv, ninhydrin and KI/starch. Reversed phase HPLC was performed on LiChrograph system utilizing a Merck column (25×0.4 cm) packed with LiChrospher 100 RP-18 (10 μm) and methanol-water solvent system.

Benzyloxycarbonyl-alanyl-(-hydroxyglycine benzylester, Z-Ala-Gly((-OH)-OBn (1). This compound was synthesized by the same manner as described by Hong[1,10] from Z-L-Ala-NH_2 (2.5 g, 11.2 mmol) and benzylglyoxylate (1.2 g, 12 mmol); Yield 3.2 g, (80%), mp 109-121 °C, Rf (C) 0.62, ^{1}H NMR (DMSO-d_6) δ_H 1.2 (d, 3H, -CH_3), 4.1 (m,

1H, alanyl CH), 5.0 (s, 2H, benzylic -CH_2-), 5.1 (s, 2H, benzylic -CH_2-), 5.4 (d, 1H, glycyl -CH-), 6.3 (b, 1H, -OH), 7.1 (s, 5H, aromatic), 7.2 (s, 5H, aromatic).

Benzyloxycarbonyl-alanyl-(-acetoxyglycine benzylester, Z-Ala-Gly((-OAc)-OBn (2). This compound was synthesized by the same manner as described by Hong[1,10] from **1** (320 mg, 1 mmol) and acetic anhydride/pyridine (10 mL/10 mL); yield 380 mg (93%), mp 98-101 °C, Rf (C) 0.76, ^{1}H NMR (DMSO-d_6) δ_H 1.2 (d, 3H, -CH_3), 2.0 (s, 3H, acetyl-CH_3), 4.3 (q, 1H, alanyl CH), 5.0 (s, 2H, benzylic -CH_2-), 5.1 (s, 2H, benzylic -CH_2-), 6.2 (d, 1H, glycyl -CH-), 7.1 (s, 5H, aromatic), 7.2 (s, 5H, aromatic).

Benzyloxycarbonyl-alanyl-thialysine benzylester, Z-Ala-S-Lys-OBn (3). This compound was synthesized by the same method as described by Hong[1,10] from **2** (45 mg, 0.2 mmol) and 3-mercaptopropyl amine hydrochloride (32 mg, 0.2 mmol). Crude product was further purified by Cellex-P column. Cellex-P (diameter: 1.1 cm, length: 7.5 cm) is a cation exchange cellulose powder containing phosphonate functional group (0.87 meq. per g) in the sodium form. One grams of Cellex-P was used to make up the column. Column was washed by 0.5 M triethylamine (10 mL) and then the excess triethyamine was washed off with water. Small portion of each final crude product (500 μL of the 155 mM) of **3** or **4** was charged and then column was washed with 25 mL of water. Ten mL of 0.1 M calcium acetate solution was used to elute product then water was used to help elute compound. The Sakaguchi assay[28] was done to monitor product elution. Product fractions were collected and lyophilized; yield (50 mg, 63%), mp 119-124 °C, Rf (D) 0.82, ^{1}H NMR (DMSO-d_6) δ_H 1.2 (d, 3H, -CH_3), 2.8 (t, 2H, -CH_2-), 3.0 (m, 2H, -CH_2-), 3.3 (t, 2H, -CH_2-), 4.0 (q, 1H, lysyl-CH-), 4.4 (q, 1H, alanyl CH), 5.0 (s, 2H, benzylic -CH_2-), 5.1 (s, 2H, benzylic -CH_2-), 5.6 (d, 1H, glycyl -CH-), 7.1 (s, 5H, aromatic), 7.2 (s, 5H, aromatic).

Benzyloxycarbonyl-alanyl-thiaarginine benzylester, Z-Ala-S-Arg-OBn (4). This compound was synthesized by the same method as the preparation of Z-Ala-S-Lys-OBn **3** using Z-Ala-Gly(α-OAc)-OBn (45 mg, 0.2 mmol) and S-(2-aminoethyl)isothiouronium bromide hydrobromide (56 mg, 0.2 mmol); yield 86 mg (53%), mp 84-87 °C, Rf (D), 0.73, ^{1}H NMR (DMSO-d_6) δ_H 1.2 (d, 3H, -CH_3), 1.4-1.6 (m, 4H, 2.8 -CH_2-) 2.6 (m, 2H, -CH_2-), 4.4 (q, 1H, alanyl -CH-), 4.9 (q, 1H, arginyl -CH-), 5.0 (s, 2H, benzylic -CH_2-), 5.1 (s, 2H, benzylic -CH_2-), 5.6 (d, 1H, glycyl -CH-), 7.1 (s, 5H, aromatic), 7.2 (s, 5H, aromatic).

Kinetic study with Z-Val-Gly-Arg-pNA. For the kinetic run, assay mixture contained 0.2 mM of Z-Val-Gly-Arg-pNA, 100 mM of Tris-HCl (pH 8.0) and 4.2 μM of trypsin in final volume of 1 mL. Reaction was started by addition of enzyme and the initial velocity was measured spectrophotometrically by monitoring the release of the free nitroaniline at 406 nm.

Competitive spectrophotometry of Z-Val-Gly-Arg-pNA with Z-Ala-Lys-OBn. In competitive spectrophotometry, to 1 mL of cubette were added 0.1 mM Z-Val-Gly-Arg-pNA as a detector, 1 mM of Z-Ala-Lys-OBn as a competitor, 100 mM Tri-HCl (pH 8.0), and 4.2 μM of trypsin. The release of the free nitroaniline was recorded at 406 nm for the inhibited reaction. For the uninhibited reaction, Z-Ala-Lys-OBn was excluded in the reaction mixture. In direct measurements, absorbance of a mixture of 0.5 mM of Z-Ala-Lys-OBn, 5 mM Tris-HCl (pH 8.0) and 4.2 μM of trypsin was recorded at 220 nm.

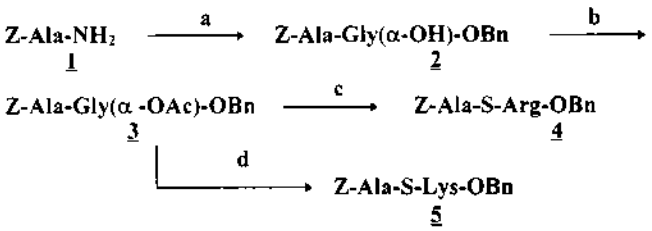
Competitive spectrophotometry of Z-Val-Gly-Arg-pNA with Z-Ala-S-Lys-OBn. Standard assay mixture contained 0.1 mM Z-Val-Gly-Arg-pNA, 0.1 mM Z-Ala-S-Lys-OBn, and 100 mM Tris-HCl (pH 8.0) in a final volume of 1 mL. Reaction was started by the addition of 4.2 μM of trypsin. The release of free nitroaniline was recorded at 406 nm for the inhibited reaction. For the uninhibited reaction, Z-Ala-S-Lys-OBn was excluded in the standard assay mixture. In semi-classical measurements, varying concentrations of Z-Ala-S-Lys-OBn, 0.5 mM Ellman's reagent, 100 mM Tris-HCl (pH 8.0) and 0.146 μM of carboxypeptidase B were placed in cuvette and absorbance change was recorded at 412 nm after addition of 4.2 μM of trypsin.

Semi-classical study with Z-Ala-S-Arg-OBn. Varying concentrations of Z-Ala-S-Arg-OBn were added to 100 mM Tris-HCl (pH 7.5) and 0.5 mM Ellman's reagent mixture in cuvette. Reactions were started by addition of 0.146 μM of carboxypeptidase B and 21 μM of trypsin. Absorbance changes were recorded at 412 nm.

Assay for L-L to L-D ratio of Z-Ala-DL-S-Lys-OBn and Z-Ala-DL-S-Arg-OBn. The assay followed the method described by Hong and Park.[10] Ellman's assay was used to determined the percentages of the L-L isomer of the final products.[16] Assay mixture contained 100 mM Tris-HCl (pH 7.5), 0.5 mM Ellman's reagent and 0.05 mM final product. Reaction was started by addition of 5.8×10^{-2} μM of carboxypeptidase B and 0.42 mM of trypsin. Resulted p-nitrobenzothioate was monitored at 412 nm.

Results

Synthesis. Synthesis of Z-Ala-S-Lys-OBn and Z-Ala-S-Arg-OBn were accomplished as outlined in Scheme 1. Nucleophilic addition of Z-Ala-NH_2 **1** to benzyl glyoxylate produced dipeptide **2** in 80% yield. Subsequent treatment of **2** with catalytic amount of *N,N*-dimethylaminopyridine (DMAP) in acetic anhydride produced acetoxy peptide **3** in 93% yield. Rearrangement of S-2-aminoethylisothiouronium bromide hydrobromide to 2-mercaptoethylguanine at neutral pH,[29] followed by bimolecular substitution reaction with acetoxy peptide **3** gave Z-Ala-S-Arg-OBn **4**. In the same manner, Z-Ala-S-Lys-OBn **5** was synthesized from 3-mercaptopropyl amine hydrochloride and acetoxy peptide **3**. Purification by Cellex-P column chromatography afforded the



Scheme 1. Synthesis of Z-Ala-S-Arg substrate. [a] Benzylglyoxylate in acetone, 65 °C, 12 hr, 80%. [b] Ac_2O, DMAP, 2 hr, 93%. [c] S-(2-Aminoethyl) isothiouroniumbromide hydrobromide, Et_3N, DMF, 12 hr, 53%. [d] 3-mercaptopropylamide hydrochloride, Et_3N, DMF, 12 hr, 63%.

required dipeptide mimetic as a mixture of diastereomer; L-L and L-D. Peptide substrate containing D-amino acids are known for poor substrates for trypsin. Therefore, it needs to assess the content of only L-L stereoisomer in the products. Unfortunately, we were not able to discriminate the relative ratios of stereoisomeric mixture from chemical shift assignment of products. Then, we turned to enzyme analysis in an attempt to prove the relative ratio of L-L to L-D isomer. The final product, Z-Ala-S-Arg-OBn, was hydrolyzed by the simultaneous treatment of carboxypeptidase B and trypsin, leading to the liberation of thioguanidino group from peptide substrate. The quantity of thioguanidino group released was measured by Ellman's test.[16] Resulted *p*-nitrothiobenzoate (ε=13,600) was determined by monitoring an increase in absorbance at 412 nm. The results showed 47 % of the L-L form for Z-Ala-S-Arg-OBn and 45% of the L-L form for Z-Ala-S-Lys-OBn, respectively.

Kinetic study with Z-Val-Gly-Arg-pNA. Figure 1 shows the progress curve for Z-Val-Gly-Arg-pNA. Although the substrate has fairly good K_m (1.09×10 mM) and k_{cat} (3.84×10^3 min^{-1}), the substrate seems to be worse for trypsin than thia-derivatives in terms of kinetic power (Table 1).

Competitive study of Z-Ala-Lys-OBn with Z-Val-Gly-Arg-pNA. Figure 2 shows the plot of the absorbance in optical density (OD) units versus time in second for the hydrolysis rate of Z-Val-Gly-Arg-pNA with Z-Ala-Lys-OBn as a competitor. Curve one is the uninhibited hydrolysis reaction and curve two is the inhibited hydrolysis reaction. The various time differences (Δt) and product concentration increment per unit time (y) are determined from comparison of two curves.

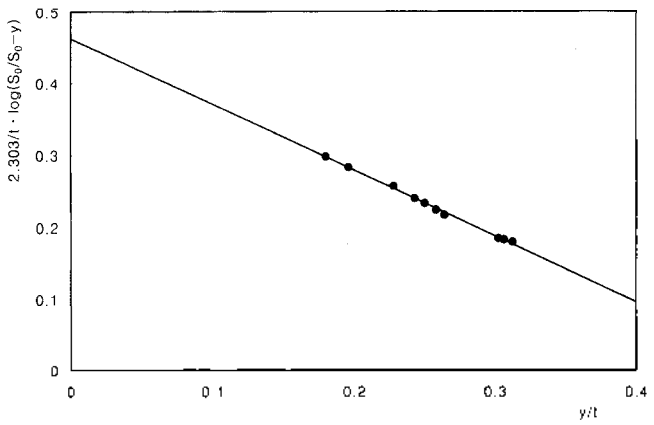


Figure 1. Direct linear plot for determination of kinetic properties from the method of progress curve equation using Z-Val-Gly-Arg-pNA as trypsin substrate.

Table 1. Kinetic Constants of Various Substrates

Substrates	K_{cat}/K_m (min^{-1}/mM^{-1})	K_m (mM)	V_{max} (μmol/min·μg enzyme)	K_{cat} (min^{-1})
Z-Val-Gly-Arg-pNA	3.52×10^4	1.09×10	1.1×10^{-1}	3.84×10^3
Z Ala-Lys-OBn[a]	2.21×10^5	2.04×10^{-2}	1.3×10^{-1}	4.42×10^3
Z-Ala-S-Lys-OBn[a]	1.42×10^5	3.62×10^{-3}	1.5×10^{-2}	5.10×10^2
Z-Ala-S-Lys-OBn[b]	1.47×10^5	3.41×10^{-3}	1.3×10^{-2}	5.20×10^2
Z-Ala-S-Arg-OBn[a]	6.32×10^4	1.92	4.7	1.20×10^5
Z-Ala-S-Arg-OBn[b]	6.50×10^4	2.33	4.4	1.50×10^5

[a] Kinetic studies were done by competitive spectrophotometry. [b] Kinetic studies were done directly using Ellman's reagent.

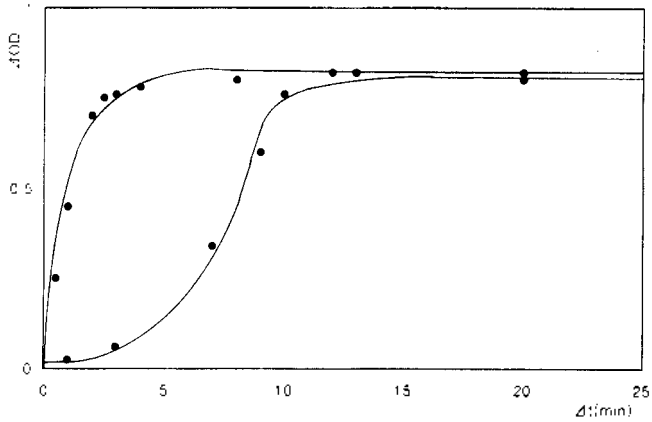


Figure 2. Competition between Z-Ala-Lys-OBn and Z-Val-Gly-Arg-pNA with trypsin. For the uppercurve, the reaction mixture contained only Z-Ala-S-Lys-OBn. For the lower curve, the condition was identical but in addition Z-Val-Gly-Arg-pNA was persent.

Figure 3 shows the best n value (6.25) for fitting a straight line to the date from Figure 2. Figure 3 represents the following equation graphically: $[(S_o\text{-}y)/S_o]^n = -(V_b/B_o)(\Delta t+1$. This line has a slope of $-(V_b/B_o)$, an intercept of (B_o/V_b) on the Δt axis. The intercept on the abscissa permits the immediate calculation of V_b. Using V_b and the newly determined n and the previously evaluated K_a and V_a, K_b can be calculated from the equation: $n=(K_a/V_a)\cdot(V_b/K_b)$. The K_m is five times lower than that of Z-Val-Gly-Arg-pNA and also kcat is quite high (4.42×10^3 min^{-1}). Z-Ala-Lys-OBn is the best substrate for trypsin studied in this paper in terms of k_{cat}/K_m (2.21×10^5 min^{-1}/mM^{-1}).

Competitive study of Z-Ala-thia-Lys-OBn with Z-Val-Gly-Arg-pNA. Figure 4 shows the plot of absorbance in OD units versus time in minutes for the hydrolysis rate of Z-Val-Gly-Arg-pNA with Z-Ala-S-Lys-OBn as a competitor. Curve one is the uninhibited reaction and curve

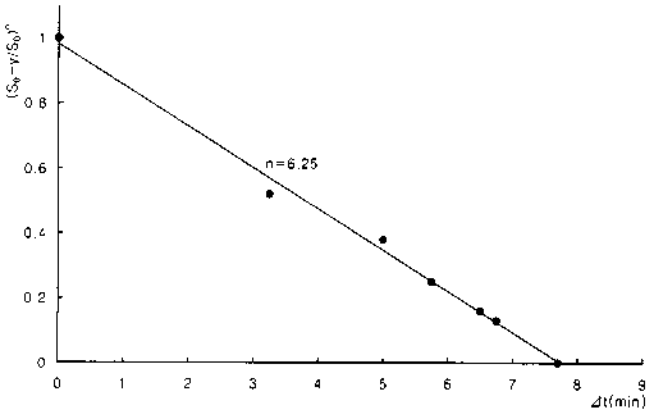


Figure 3. Evaluation of the ratio of kinetic powers of Z-Ala-Lys-OBn and Z-Val-Gly-Arg-pNA. The additional time required to achieve the same extent of hydrolysis of Z-Val-Gly-Arg-pNA due to the presence of Z-Ala-Lys-OBn was plotted as a function of the raction of the reaction completed.

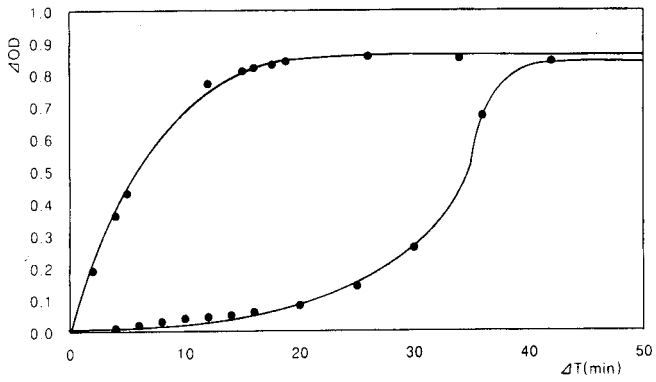


Figure 4. Competition between Z-Ala-S-Lys-OBn and Z-Val-Gly-Arg-pNA with trypsin. For the uppercurve the reaction mixture contained only Z-Ala-S-Lys-OBn. For the lower curve the condition were identical but in addition Z-Val-Gly-Arg-pNA was present.

two is the inhibited hydrolysis reaction. Figure 5 shows the best n value (4.0) for fitting a straight line to the plot of $[(S_o\text{-}y)/S_o]^n$ versus Δt. The K_m is low (3.62×10^{-3} mM) and the k_{cat} is also low (5.10×10^{2} min^{-1}), indicating good binding to trypsin but, slow splitting. The ratio of k_{cat} to K_m is 1.42×10^{5} min^{-1}/mM^{-1}. Z-Ala-thia-Lys-OBn works as good substrat as natural peptide, Z-Ala-Lys-OBn.

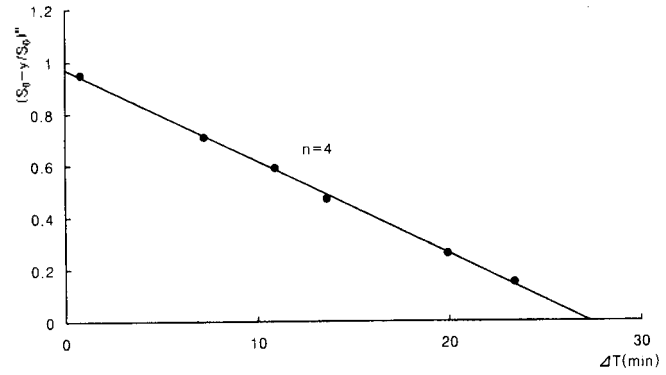


Figure 5. Evalulation of the ratio of kinetic power of Z-Ala-S-Lys-OBn and Z-Ala-S-Lys-OBn. The additional time required to achieve the same extent of hydrolysis of Z-Val-Gly-Arg-pNA due to the presence of Z-Ala-S-Lys-OBn was plotted as a function of fraction of the reaction completed.

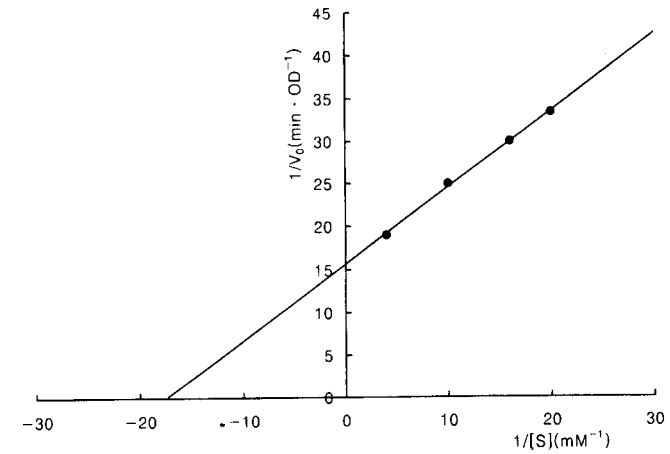


Figure 6. Lineweaver-Burke plot for Z-Ala-S-Lys-OBn as a trypsin substrate.

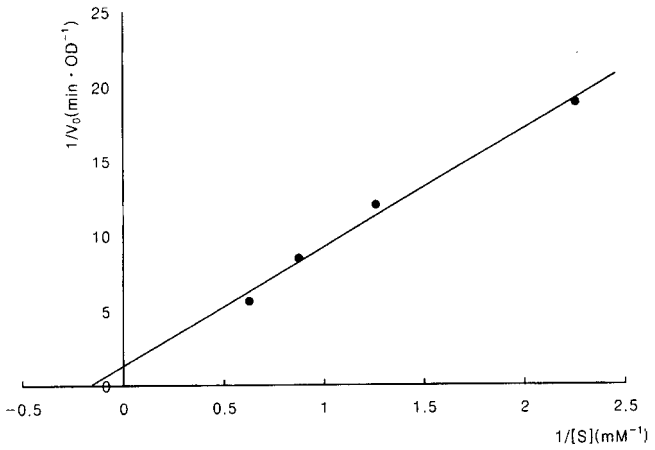


Figure 7. Lineweaver-Burke plot for Z-Ala-S-Arg-OBn as a trypsin substrate.

Semi-classical study of Z-Ala-S-Lys-OBn. Figure 6 shows the semi-classical curve in which $1/V_o$ was plotted versus 1/[S]. Direct measurement with Ellman's reagent yielded almost same results as competitive study.

Semi-classical study of Z-Ala-S-Arg-OBn. Figure 7 shows the semi-classical curve of double reciplocal plot. The K_m was found to be 2.33 mM and the k_{cat} was 1.50×10^{5} min^{-1}. The substrate has poor binding as compared to the thia-lysine compound and also slow splitting. Z-Ala-S-Arg-OBn was not so good as Z-Ala-S-Lys-OBn in terms of catalytic power.

Discussion

Kinetic studies on two different chromogenic peptide substrates, or nonchromogenic natural peptide substrate were made to compare the kinetic constants. Three methods of analyses were used in this experiment. First one was the initial rate method of Lineweaver-Burke[30] to determine the initial velocities at different concentrations of α-thia-substituted substrate. Second one was progress curve analysis of the integrated Michaelis-Menten equation[31] to determine the kinetic constants of Z-Val-Gly-Arg-pNA. This method was turned out to be advantageous over the semi-classical method because of following reasons. In the Lineweaver-Burke plot, the accurate measurement of a series of initial velocities was difficult. Since velocity was decreasing with time, the initial velocity must be extrapolated from the later velocities. This estimate becomes more deteriorative when substrate concentration are exceptionally low. Furthermore, for the accurate plot of a series of velocities versus substrate concentration, the same amount of enzyme must be added to each experiment. Thus pipetting accuracy is greatly required for the precision of the kinetic study. In the integrated Michaelis-Menten equation, since all the kinetic data is obtained from one experiment independently on initial velocities, this equation is not affected by such drawbacks. But the one major drawback is that this equation yields linear plots only when the initial concentration of sub-

strate is around or above its K_m value.[32] This limitation does not apply to competitive spectrophotometry, because it represents the ratio of kinetic constants that is of importance. Another advantage of the competitive spectrophometry is on the fact that it provides convenient way of measuring the kinetic constants of nonchromogenic substrates whose rates cannot be measured by initial rate method or progress curve analysis. Since, in case of Z-Ala-Lys-OBn, the rate of hydrolysis could not be measured by either progress curve analysis or initial velocity method directly, competitive spectrophotometry was used for kinetic run. In the experiment, Z-Val-Gly-Arg-pNA was used as detector to measure the progress curve in the presence of Z-Ala-Lys-OBn as a competitor. The presence of the competing substrate, Z-Ala-Lys-OBn, changed the progress curve for the Z-Val-Gly-Arg-pNA in a manner that is influenced by the kinetic constants of Z-Ala-Lys-OBn.

The fact that the K_m value for Z-Ala-S-Lys-OBn is approximately five times lower than that for Z-Ala-Lys-OBn indicates that the substitution of a methylene group by sulfur does not disrupt fitting P1 side chain into trypsin S1 pocket in a negative way. The fact that the k_{cat}/K_m values for Z-Ala-Lys-OBn is 1.6 times higher as compared with Z-Ala-S-Lys-OBn indicates that S-C substitution contributes negatively to yield the full binding energy of the transition state resulted in slow rate of hydrolysis of thia-lysine compound.

Since the breakdown of acyl-enzyme intermediate which is formed by nucleophilic addition of the Ser-195 to the peptide benzyl ester bond is rate-limiting step, the low kcat value (5.10×10^2 min^{-1}) for the Z-Ala-S-Lys-OBn is mainly attributed to the changing of the rate-limiting step. As the binding energy of the S1' site associated with the leaving group binding promote a tetrahedral distortion at the carbonyl carbon of the substrate bond being cleaved, the lowered k_{cat} value could be explained in part by incorrect pyramidalization of the terminal proton-acceptor atom of the leaving group caused by the S-C substitution.[38]

From the results of analogs modified at C-terminal, Z-Ala-S-Lys-OBn (k_{cat}/K_m=1.47105 $min^{-1}/mmol^{-1}$) and Z-Ala-S-Arg-OBn ($k_{cat}/K_m=6.50\times 10^4$ $min^{-1}/mmol^{-1}$), the kinetic powers indicate that the Z-Ala-S-Lys-OBn has greater binding energy. The fact that the K_m of the Z-Ala-S-Lys-OBn ($K_m=3.41\times 10^{-3}$ mM) is about 640 times better than that of Z-Ala-S-Arg-OBn indicates that P1 site of the substrate, first amino acid from the scissile bond, plays a significant role in the mode of binding on S1 site. Although Z-Ala-S-Lys-OBn shows greater binding affinity, 300 times worsened kcat for Z-Ala-S-Lys-OBn compared to Z-Ala-S-Arg-OBn could be interpreted by the looser binding of the tetrahedral intermediate caused by different mode of binding of P1 side chain into S1 pocket. This explanation can be possible by the model for the complementarity of enzyme and substrate called strain model which is characteristic of trypsin.[36]

Kinetic constants such as K_m or V_{max} measured by competitive spectrophotometry are almost same as those values measured directly using Ellman's reagent. Consequently, it has been shown that the thia-analogs exert as fairly suitable substrate for trypsin and competitive spectrophotometry can be applied even when either the monitored substrate or the competitive substrate form a product that significantly inhibits the enzyme.

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