Development of Substrate for Carboxypeptidase-B by Employing Thiaarginine Peptides

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Carboxypeptidase-B (CPB) is involved in the biosynthesis of numerous peptide hormones and neurotransmitters. CPB catalyzes hydrolysis of the basic amino acids from the C-terminal position in polypeptides during posttranslational prohormonal processing. Various peptides containing thiaarginine residue at C-terminal position were synthesized and tested for their hydrolysis by CPB. A colorimetric assay, employing Ellman's reagent to detect the thioguanidine released upon hydrolysis of the dipeptide substrates, showed that thiaarginine is a suitable mimetic for arginine. Kinetic studies on the four substrates, Z-L-Ala-DL-thia-Lys, Z-L-Ala-DL-thia-Arg, Z-L-Lys-DL-thia-Arg, and Z-L-Lys(Boc)-DL-thia-Arg, gave K_m (mM) of 0.66, 5.08, 0.024, and 0.006 and k_{cat} (min $^{-1}$) of 340, 5200, 151 and 335, respectively.

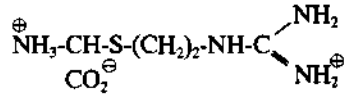
Introduction

The very recent and significant discovery[1-7] of peptide analogs containing nucleophilic substituents attached to the α-carbon of a glycine residue has many uses.[8-13] Such α-substituted glycine containing peptide analogs were first developed in 1984 by Gilvarg *et al.*,[1] who were examining novel ways to deliver impermeant molecules into bacteria using their peptide transport systems. Peptide transport systems which allow utilization of peptides from external environment have been found in many microorganisms including *E. coli*.

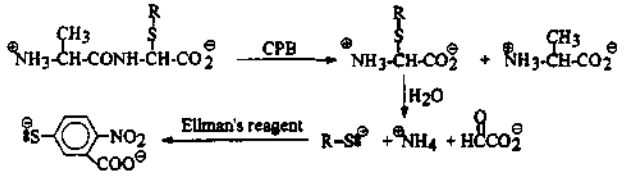
The α-substituted glycine peptides-where the α-carbone is attached to a nucleophilic moiety such as nitrogen, oxygen or sulfur- are stabilized by the delocalization of the lone pair nitrogen electrons in the peptide bond. When the peptide bond of an α-substituted glycine residue is released as shown in Scheme 1 and it is broken down to yield a nucleophilic substituent along with ammonia and glyoxylate. If the substituent had been linked to the glycine residue through sulfur, decomposition yields a compound with free sulfhydryl group. Its appearance can be monitored spectrophotometrically in the presence of Ellman's reagent, which reacts rapidly and quantitatively with free sufhydryl groups to form a highly coloured anionic species that absorbs at 412 nm.[14]

Carboxypeptidase B (EC 3.4.2.2) has been characterized as an enzyme that selectively catalyzes the release of the carboxyl terminal basic amino acids, such as arginine and lysine, from proteins and peptides.[15,16] The enzyme has also been found to possess esterase activity which is related to the metal content of the enzyme.[17,18] A number of substrates and a variety of assay procedures have been proposed for the determination of the enzyme; activity; these have included uv spectrophotometry[19,20] to directly monitor the cleavage of the peptide bond and both colorimetric[21,22] and fluorometric methods[23] to measure the amino acid released from the C-termnus. The most common assay for CPB uses hippuryl-Arg (Bz-Gly-Arg) as a substrate and measures the increase in absorption at 254 nm resulting from the release of hippuric acid.[24] *N*-Acyl dipeptide substrates also have been employed in the characterization of the specificity of the enzyme. CPB shows considerable activity toward neutral peptides of this type[25-29] and toward the corresponding depsipeptide.[30,31] A novel colorimetric assay employing urethane hydrolase I and lactase has also been reported by Matsumura *et al.*[21] This method can be applied to contaminated samples such as culture media and sera. Another approach involves separation of the product from a substrate, followed by quantitation of the product. Separation methods used include HPLC,[32] thin-layer chromatography,[33] and solubilization in organic solvents.[23,34,35] Recently, a sensitive radiometric assay using ^{125}I-acetyl-Tyr-Ala-Arg as a substrate has been developed to detect CPB in cultured cells.[36]

As arginine is the most frequently occurring amino acid at the endopeptidase cleavage site,[37-45] we have designed four peptides containing thia-arginine as the mimetics for the natural compound and as the substrates in a colorimetric assay for CPB based on the principle of Scheme 1. This paper focuses on the study for the suitability of thiaarginine peptide in order to provide a sensitive and specific assay which characterize the CPB involved in protein hormonal processing at basic sites.[13,37,38,46] Thia-arginine is a mimetic for the arginine and the group attached to the glycine resembles the side chaine of arginine as shown below.



Hippuryl-L-arginine, Bz-Gly-Arg, was known as best sub-



Scheme 1. Cleavage of peptide bond and subsequent breakdown of α-substituted glycine residue.

strate for CPB due to the fairly low K_m and high k_{cat} so far.[20] Based on this compound, we desigined several Z-X-thia-Arg analogs as detector peptides, where X is basic (Lys) or non basic (Ala) amino acid.

Experimental

A purified preparation of carboxypeptidase-B isolated from pancreatic cells was purchased from Sigma. Ellman's reagent was from Pierce, and S-2-aminoethyisothiouronium bromide hydrobromide was from Aldrich Chemical Corp. Benzyloxycarbonyl-L-lysine (ε-*t*-butoxycarbonyl)*p*-nitrophenylester, Z-Lys(Boc)-ONp, was from BACHEM Corp. All substrates were synthesized by solution method. Benzyloxycarbonyl-L-alanyl-α-DL-acetoxyglycine, Z-L-Ala-DL-Gly (α-OAc), was prepared as described by Hong *et al.*[7] ^{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 chromtography (tlc) was carried out on precoated (0.25 nm) Merk 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*-butanol-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 Merk column (25×0.4 cm) packed with LiChrospher 100 RP-18 (10 μm) and methanol-water solvent system.

Benzyloxycarbonyl-L-alanyl-DL-thiaarginine, Z-L-Ala-DL-S-Arg (1). S-(2-Aminoethyl)isothiouronium bromide hydrobromide (56 mg, 0.2 mmol) and triethylamine (6 mL, 0.4 mmol) were combined in 5 mL of dimethylformamide (DMF) and stirred for 5 min. Into the reaction mixture, Z-L-Ala-DL-Gly(α-OAc) (41 mg, 0.2 mmol) was added and stirred for 12 h. The solvent was removed by evaporation *in vacuuo*, and the resulting pale yellow syrup was dissolved in small volume of $CHCl_3$/DMF (9/1, v/v). Purification by flash chromatography, eluting with 20% (v/v) methanol in dichloromethane gave the title compound as a white solid (80 mg, 68%), mp 122-127 ℃, Rf(D) 0.61. ^{1}H NMR (DMSO-d_6) δ_H 1.2 (d, 3H, alanyl CH_3), 2.8 (t, 2H, CH_2), 3.1 (m, 2H, CH_2), 4.4 (q, 1H, alanyl CH), 5.1 (s, 2H, benzylic CH_2), 5.6 (m, 1H, glycyl CH), 7.3 (s, 5H, aromatics).

Benzyloxycarbonyl-L-alanyl-DL-thialysine, Z-L-Ala-DL-S-Lys (2). This compound was synthesized by the same method of 1 from Z-L-Ala-DL-Gly(α-OAc) (41 mg, 0.2 mmol) and 3-mercaptopropyl amine hydrochloride (32 mg, 0.2 mmol). Yield (42 mg, 63%), mp 130-133 ℃, Rf (D) 0.60. ^{1}H NMR (DMSO-d_6) δ_H 1.2 (d, 3H, alanyl CH_3), 2.8 (t, 2H, CH_2), 3.0 (m, 2H, CH_2), 3.3 (t, 2H, CH_2), 4.4 (q, 1H, alanyl CH), 5.1 (s, 2H, benzylic CH_2), 5.6 (m, 1H, glycyl CH), 7.3 (s, 5H, aromatics).

Benzyloxycarbony-L-lysine(α-*t*-butoxycarbonyl) amide, Z-L-Lys(Boc)-NH_2 (3). Dry ammonia gas was led in a gentle stream to the stirred solution of Benzyloxycarbonyl-L-lysine(ε-*t*-butoxycarbonyl)-*p*-nitrophenyl ester (1 g, 2 mmol) in tetrahydrofurane (50 mL). One hour after dry ammonia gas was passed, the flask was stoppered and kept at room temperature overnight. The solvent was concentrated to give crude product. Purification by flash chromatography, eluted with 5% methanol in dichlomethane gave the compound as a white crystalline solid (710 mg, 92%), mp 141-142 ℃, Rf (A) 0.60. ^{1}H NMR (DMSO-d_6) δ_H 1.2 (s, 9H, Boc), 1.5-1.6 (m, 6H, CH_2), 2.9 (m, 2H, CH_2), 3.9 (q, 1H, CH), 5.0 (s, 2H, benzylic CH_2), 6.8 (s, 2H, NH_2), 7.3 (s, 5H, aromatics).

Benzyloxycarbony-L-lysine(ε-*t*-butoxycarbonyl)-α-DL-hydroxyglycine, Z-L-Lys(Boc)-DL-Gly(α-OH) (4). A mixture of glyoxylic acid monohydrate (92 mg, 1 mmol) and **3** (395 mg, 1 mmol) in 50 mL of acetone was refluxed at 65 ℃ for 12 h. Methylene chloride (50 mL) was added and the reaction mixture was stored in a refrigerator overnight. The resulting solid was filtered to give 200 mg of white solid. The filtrate was allowed to stand for 5 h at room temperature to give an additional 170 mg of solid product; yield (79%), mp 62-67 ℃, Rf(A) 0.13, ^{1}H NMR (DMSO-d_6) δ_H 1.2 (s, 9H, Boc), 1.4-1.6 (m, 6H, 3CH_2), 2.8 (m, 2H, CH_2), 4.0 (q, 1H, CH), 5.0 (s, 2H, benzylic CH_2), 5.3 (m, 1H, CH), 8.4 (b, 1H, OH), 7.3 (s, 5H, aromatic), 8.6 (d, 1H, glycyl NH).

Benzyloxycarbony-L-lysine(ε-*t*-butoxycarbonyl)-α-DL-acetoxyglycine pyridinium salt, Z-L-Lys(Boc)-DL-Gly(α-OAc)·Py (5). Z-Lys(Boc)-DL-Gly(α-OH) (469 mg, 1 mmol), catalytic amount of *N,N*-diemthylaminopyridine (DMAP) was suspended to 20 mL of acetic anhydride and cooled to 0 ℃. Then 50 mL of pyridine was added and the reaction mixture was stored at 5 ℃ for 1 h. Concentration of the reaction mixture produced a residue that was triturated with a mixture of petroleum ether/Et_2O (1 : 1, v/v) to afford a white solid; yield 297 mg (78%), mp 78-83 ℃, Rf (B) 1.4. ^{1}H NMR (DMSO-d_6) δ_H 1.2 (s, 9H, Boc), 1.4-1.6 (b, 6H, CH_2), 2.0 (s, 3H, acetyl CH_3), 2.8 (m, 2H, CH_2), 4.0 (q, 1H, CH), 5.0 (s, 1H, benzylic CH_2), 6.2 (d, 1H, glycyl CH), 7.25 (s, 5H, aromatic).

Benzyloxycarbony-L-lysine(ε-*t*-butoxycarbonyl)-DL-thiaarginine, Z-L-Lys(Boc)-DL-S-Arg (6). This compound was synthesized by the same method of 1 from Z-L-Lys(Boc)-DL-Gly(α-OAc)·Py (100 mg, 0.2 mmol) and S-(2-aminoethyl)isothiouronium bromide hydrobromide (56 mg, 0.2 mmol). Yield 78 mg (52%), mp 96-103 ℃, Rf(D) 0.68, ^{1}H NMR (DMSO-d_6) δ_H 1.3 (s, 9H, Boc), 1.4-1.6 (m, 6H, CH_2), 2.6 (q, 2H, CH_2), 2.8 (m, 2H, CH_2), 3.0 (q, 2H, CH_2), 4.0 (q, 1H, lysyl CH), 4.9 (q, 1H, arginyl CH), 5.1 (s, 2H, benzylic CH_2), 6.7 (d, 1H, NH), 7.4, 7.5, 7.6, 7.9 (d, 1H, NH), 8.1 (m, 2H, NH_2).

Benzyloxycarbony-L-lysine-DL-thiaarginine, Z-L-Lys-DL-S-Arg (7). The protected peptide **5** (76 mg, 0.1 mmol) was dissolved in 5 mL of trifluoroacetic acid (TFA) and stirred for 30 min at room temperature. TFA was removed by evaporation *in vacuuo*, and the resulting pale yellow syrup was dissolved in small volume of $CHCl_3$/DMF(9/1). Purification by flash chromatography, eluting with 30% (v/v) methanol in dichloromethane gave the title compound as a white solid (68 mg, 87%). Final purification was done by HPLC, eluting with MeOH/H_2O (20-60% MeOH in H_2O). Fractions containing the product were combined, evaporated, and lyophilized to give pure product. mp 134-139 ℃, Rf(D) 0.59. ^{1}H NMR (DMSO-d_6) δ_H 1.4-1.6 (m, 6H, CH_2), 2.7 (q, 2H, CH_2), 2.8 (m, 2H, CH_2), 3.1 (q, 2H, CH_2),

4.1 (m, 1H, CH), 4.9 (q, 1H, CH), 5.1 (s, 2H, benzylic CH_2), 7.3 (s, 5H, aromatic).

Activity measurement. Substrates hydrolysis was monitored by following the increase in the absorbance at 412 nm with a Carl Zeiss model PM Q II spectrophotometer. For a typical assay, a 100 mM Tris-HCl buffer, pH 7.5, was adjusted to be 0.07 mM in substrate and 0.5 mM Ellman's reagent. One milliliter was placed in a 1-cm light path cuvette and an initial spectrophotometric reading was taken. Reaction was initiated by the addition of CPB and the absorbance was read at 412 nm.

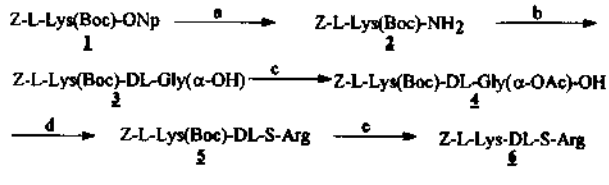
Results

Synthesis. Synthesis of Z-L-Lys(Boc)-DL-S-Arg was accomplished as outlined in Scheme 2. Ammonolysis of Z-L-Lys(Boc)-ONp with dry ammonia gas in THF produced Z-L-Lys(Boc)-NH_2 **2**. Addition of **2** to glyoxylate produced dipeptide **3**. Subsequent treatment of **3** with catalytic amount of DMAP in acetic anhydride led to the peptide acetate **4**. Rearrangement of S-(2-Aminoethyil)isothiouronium bromide hydrobromide in triethylamine followed by bimolecular substitution reaction with acetoxy peptide **4** gave Z-L-Lys(Boc)-DL-S-Arg **5**. Removal of the Boc-protecting group by TFA afforded the required dipeptide as a mixture of DL-diastereoisomers **6**.

It is generally recognized that peptides containing D-amino acids at C-terminal are not substrates for CPB.[47] Therefore, it is important to measure the exact amount of all L-isomer in the product. In an attempt to prove the relative ratio between LL and LD-isomers, Z-L-Lys(Boc)-DL-S-Arg was hydrolyzed with CPB, leading to the liberation of thioguanidino group from the peptide substrate. The amount of thioguanidino group released was measured with Ellman's reagent. Reaction rate was determined by an increase in absorbance at 412 nm resulting from the degradation of Ellman's reagent. The result showed that Z-L-Lys(Boc)-DL-S-Arg contained 42% of LL-isomer.

Biological activity. The time course of hydrolysis of Z-L-Lys(Boc)-DL-S-Arg by CPB was examined as a function of enzyme concentration. As can be seen in Figure 1, absorbance increases proceed linearly with time. Figure 1 also reveals that the assay can quantitate enzyme concentration in the range of 1.489 to 5.96×10^{-1} μg/mL.

Moreover, the rate of hydrolysis, over this range of enzyme concentration, is directly proportional to the concentration (Figure 2). Incidentally it can be concluded that the low concentration of Ellman's reagent used in these assays (0.5 mM) is adequate to provide that the detection of



Scheme 2. Synthesis of Z-L-Lys-DL-S-Arg substrate. (a) NH_3 in THF, 1 hr, 96%; (b) glyoxylic acid hydrate in acetone, 65 ℃, 12 hr, 79%; (c) Ac_2O, DMAP, 2 hr, 78%; (d) S-(2-Aminoethyil) isothiouronium bromide hydrobromide, Et_3N, DMF, 12 hr, 56%; (e) TFA, 0.5 hr, 87%.

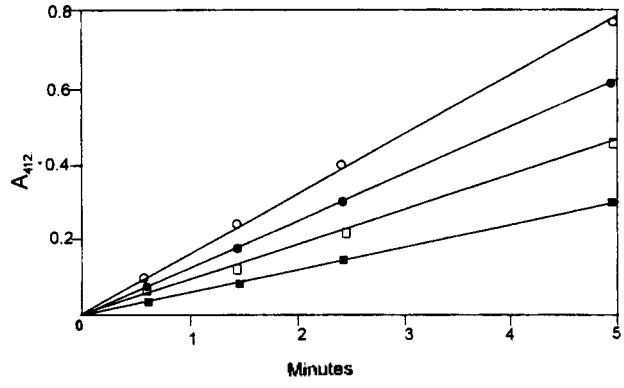


Figure 1. Time Course of hydrolysis of Z-L-Lys(Boc)-DL-S-Arg by carboxypeptidase-B. Temperature 23 ℃. Enzyme concentration (μg/mL): 1.489 (○), 1.191 (●), 0.893 (□), 0.596 (■). See materials and method for conditions.

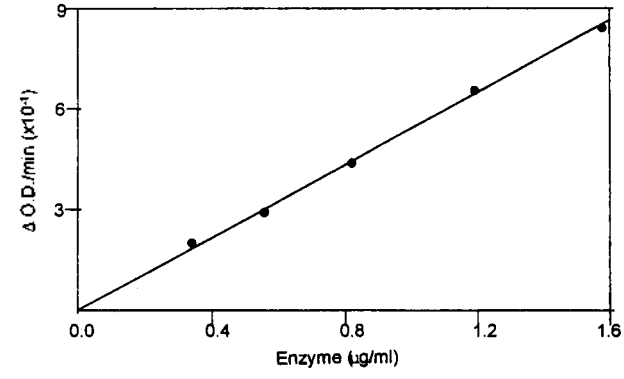


Figure 2. The rate of Z-Lys(Boc)-S-Arg hydrolysis as a function of CPB concentration. Initial concentration of Z-Lys(Boc)-S-Arg, 0.07 mM.

released sulfhydryl is not a rate-limiting step. A comparison of rates of carboxypeptidase-B catalyzed hydrolysis of the peptide substrates, Z-L-Lys(Boc)-DL-S-Arg, Z-L-Lys-DL-S-Arg, and Z-L-Ala-DL-S-Arg are shown as a function of substrate concentration in the doulbe-reciprocal plots in Figure 3. Kinetic parameters for the hydrolysis of the peptides by CPB are given in Table 1. In terms of the kinetic power (k_{cat}/K_m), Z-L-Lys(Boc)-DL-S-Arg is the best substrate for

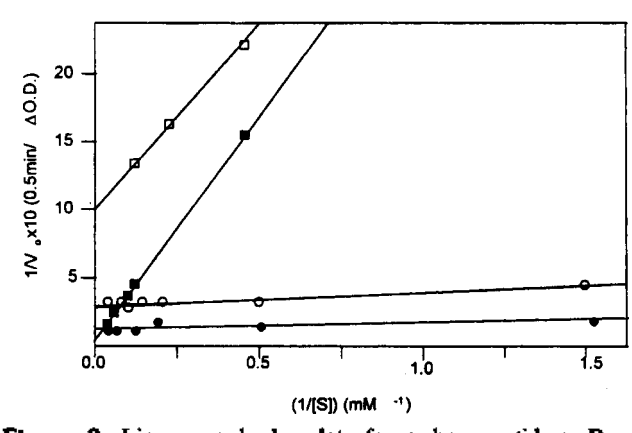


Figure 3. Lineweaver-burke plots for carboxypeptidease-B catalyzed hydrolysis of four peptide substrates. Z-Lys(Boc)-S-Arg (●), Z-Lys-S-Arg (○), Z-Ala-S-Arg (■), Z-Ala-S-Lys (□).

Table 1. Kinetic constants of various substrates for CPB

No.	Substrates	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
1	Z-Lys(Boc)-S-Arg	0.0066[b]	335	5.08×10^4
2	Z-Lys-S-Arg	0.024	151	6.29×10^3
3	Z-Ala-S-Arg	5.08	5,200	1.02×10^3
4	Z-Ala-S-Lys	0.66	340	5.15×10^2
5	Z-Ala-Arg[a]	1.4	15,600	1.14×10^4
6	Bz-Gly-Arg[a]	0.2	6,300	3.15×10^4

[a]Data were taken from reference 38. [b]Values given are the mean ±S.E.M. of three determinations.

CPB. This comparison refers to the LL isomer as the LD isomer was not recognized by the enzyme.

Discussion

The results demonstrate different kinetic behaviour of carboxypeptidase B catatalyzed hydrolysis of *N*-blocked basic (Lys) and non-basic (Ala) dipeptide substrates containing α-substituted glycine residues (Table 1). Z-L-Lys(Boc)-DL-S-Arg (k_{cat}/K_m=50,757 min^{-1} mM^{-1}) **1**, proved to be better substrate than typical substrate, Bz-L-Gly-L-Arg (k_{cat}/K_m=31,500 min^{-1} mM^{-1}) **6**, in terms of kinetic power (k_{cat}/K_m). The enormous improvement in K_m by changing the penultimate residue from alanine to ε-butoxycarbonyl lysine suggests the importance of a adjacent binding of CPB toward substrate. This site appears to bind tightly to large aliphatic side chains, while having moderate affinity for small aliphatic side chains. Although the K_m of Z-L-Lys(Boc)-DL-S-Arg (K_m=6.6×10^{-3} mM, k_{cat}=335 min^{-1}) **1**, is greatly improved from that of the Z-L-Ala-DL-S-Arg (K_m=5.08 mM, k_{cat}=5,200 min^{-1}) **3**, its k_{cat} is decreased by approximately a factor of ten. This observation maybe interpreted as transition-state analog theory. According to the theory, Z-L-Lys (Boc)-DL-S-Arg **1**, binds to CPB more tightly than Z-L-Ala-DL-S-Arg **3**, in the enzyme-substrate complex formation, because low K_m value of **1** means that **1** fit in the active site better, namely, form more interactions with CPB than **3**.

The K_m of the thiaarginine compound, Z-L-Ala-DL-S-Arg (K_m=5.08 mM) **3**, is about three times worse than the K_m of the natural compound, Z-L-Ala-L-Arg (K_m=1.4 mM) **5**. This may be due to the longer S-C bond compared to the natural C-C bond. The K_m for the thialysine compound, Z-L-Ala-DL-S-Lys (K_m=0.66 mM) **4**, is about ten times better than the thiaarginine compound, Z-L-Ala-DL-S-Arg (K_m=5.08 mM) **3**. This is probably due to a lower K_m for the natural lysine compared to the natural arginine compound.[34] The thialysine compound, Z-L-Ala-DL-S-Lys **4**, containing a S-C bond, has two times lower K_m than the natural arginine compound, Z-L-Ala-L-Arg **5**. This finding is probably not due to a S-C bond substitution on lysine side chain, but due to the different side chain stucture of two residues which exert different mode of binding on CPB. The k_{cat} for the thia compound **4** compared to the k_{cat} for the thiaarginine **3** is 15 times lower. This finding indicates that among the basic amino acids at C-terminal position, arginine plays a best role in degradation of substrate. Although the k_{cat} is not known for the natural compound of thialysine **4**, we can speculate that hydrolysis rates of thia-substrates are not so greatly affected by thia-substitution. From the results of analogs modified at C-termianl (Z-L-Ala-X) **3-5**, the kinetic powers (k_{cat}/K_m) indicate that the natural arginine compound, Z-L-Ala-L-Arg **5**, has the greatest binding energy which means the best CPB substrate.

Although Z-Lys(Boc)-S-Arg **1** is split about 1.7 times more faster than Bz-Gly-Arg **6** in terms of catalytic efficiency, this efficiency can be doubled by higher sensitivity of the detection method of the released thioguanidine using Ellman's dianion (α=13,600) whose extinction coefficient is 6.8 times greater than that generated by the hydrolysis of Bz-Gly-Arg **6**. This gives an assay with approximately twelve times higher sensitivity than the one based on the cleavage of Bz-Gly-Arg **6**. Another advantage stems from the fact that absorbance increase of Z-lys(Boc)-S-Arg **1** can be monitored from a low base line, because Z-Lys(Boc)-S-Arg itself gives no absorbance at the observed wave length. Whereas, in case of Bz-Gly-Arg **6**, substrate itself absorbs very strongly in the region of 254 nm where splitting is followed, therefore there is relative decrease in absorbance to be measured. The product of Bz-Gly, has 79% of the absorbance of substrat of Bz-Gly-Arg, severely limiting the absorbance change over which the reaction can be monitored.

Consequently, it has been shown that the thiaarginine compound is a fairly suitable substrate for CPB. We can speculate on the possible kinetic constants of these substrates with the actual processing enzymes. Because the hormonal processing enzymes are very specific, they depend only on k_{cat}/K_m and not at all on the strain model as trypsin does.[48] Strain increases the ratio of k_{cat}/K_m while induced fit decreases the ratio. It can be predicted that the ratio of k_{cat}/K_m for the processing enzymes are therefore greater than those for CPB, but only future studies with the actual processing enzymes can reveal more about the constant. Our study shows that thiaarginine peptide can be used as more approachable substrate in characterizing the CPB in protein hormonal processing.

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