Syntheses of *N*-Acyl Dipeptide Derivatives by Metalloproteinases†

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X–Dipeptides (X=Z, Z(OMe), or Z(3Me)) have been catalytically synthesized by several microbial metalloproteinases. Among the enzymes used, Thermolysin showed the most remarkable ability in the coupling reactions of the esters or the amides of phenylalanine, leucine, isoleucine, and valine (and methionine in the case of the amide) with various X–amino acids. This method has been applied to the syntheses of ZPhePheOMe and ZPheIleOMe on a practical scale. Ammonium sulfate and sodium chloride had a marked effect on the condensed products, increasing the yield.

Since Bergmann and Fraenkel-Conrat[1] first demonstrated the papain catalyzed amide-bond formation between an acylamino acid and an amino acid anilide, the enzymatic syntheses of peptides have received much attention. However, only a few papers have appeared in which the substrates had the proper protecting groups for the elongation of the peptide chains, for example Z, Z(OMe), BOC, the methyl or ethyl ester, the non-sustituted amide, *etc.*[2,3] There appears to be two reasons for this. Firstly, the most investigations have used papain (thiol proteinase) or α-chymotrypsin (serineproteinase), both of which catalyze the hydrolysis of carboxylic esters comprising the primary alcohol or the non-substituted amide. Consequently the peptides containing those groups are not easily obtainable in high yields except the peptides which show extremely low solubilities in buffer solution.[2] When those peptides are once formed, they are barely subjected to hydrolysis of the C-terminal protecting groups. Secondly, many *N*-acylamino acid derivatives (acyl= Z, Z(OMe), BOC, *etc.*) are almost insoluble in water and troublesome on operating the enzymatic reaction. Recently, it has been reported that the peptides whose N- and C-terminal groups were protected by such groups were readily obtained in certain cases by several enzymes.[4]

In the course of the current study to extend the enzymatic syntheses of peptides, it has been found that several microbial metalloproteinases catalyze the formation of X–dipeptide methyl esters or amides (X=Z, Z(OMe), or Z(3Me)). The C-terminal groups in these dipeptides are not hydrolyzed by metalloproteinases, so it is anticipated that the syntheses of these peptides would be smoother using metalloproteinases than by thiol- or serineproteinases.

For inhibiting the action of serineproteinase contained in the crude metalloproteinases, potato inhibitor was used as reported previously.[4c]

Materials and Methods

Thin layer chromatography was performed on precoated TLC plates Silica Gel F_{254} (Merck) using the following solvent systems : AcOEt–$CHCl_3$ (10 : 1), AcOEt, *s*-BuOH–3% aqueous ammonia (8 : 3) (see Ref. 4c for other analytical procedures). Protease activity was assayed by the method of Tsuru *et al.*[5]

Potato Inhibitor. See Ref. 4c.

Proteinases. See Ref. 4c for Prolisin A (*Bacillus subtilis var. amyloliquefacience*), Thermolysin (*Bacillus thermoproteoliticus*), Thermoase (*Bacillus thermoproteoliticus*), and Tacynase N (*Streptomyces caespitosus*). Dispase I (*Bacillus polymyxa*, Godo Shusei Co., Tokyo, Japan, 4.93×10^6 PU/g) had little esterase action and was used without further purification. The partially purified metalloproteinase of *Bacillus subtilis* was obtained by treating Prolisin A with DEAE-Sephadex A-50[6] and freeze-drying. The protease activity was 5.3×10^6 PU/g and the esterase action was almost completely inhibited by the potato inhibitor in one tenth the weight of enzyme. Commercial Thermoase was purified by dissolving it in 0.01 M $Ca(OAc)_2$ aqueous solution, removing the insoluble materials and precipitating from acetone. The protease activity was 1.0×10^7 PU/g and the esterase action was suppressed almost completely by the potato inhibitor in one tenth the weight of enzyme.

Reagents. Z– or Z(OMe)–amino acids, amino acid methyl ester hydrohalogenides were all prepared by the ordinary methods. Z(3Me)–His(Bzl)–OH was prepared by the method previously reported.[4b]

Enzymatic Syntheses of Peptides (General Procedures). An enzyme and potato inhibitor, if necessary, were dissolved in a buffer solution and the insoluble materials filtered off. In a mixture of this solution, two appropriate substrates and 4 M NaOH aqueous solution were incubated and the product collected on a suction filter, washed thoroughly in succession with 1 M aqueous ammonia, 1 M HCl, and water, and dried *in vacuo* over P_2O_5 at room temperature. The product was recrystallized, if necessary, from organic solvents and weighed.

Results

Enzymatic reactions between X–AA_1–OH (X=Z, Z(OMe), or Z(3Me); AA_1=Ala, Val, Leu, Ile, Phe, Glu(OBzl), Asp(OBzl), Gln, Asn, Lys(Z), His(Bzl), Met, Cys(Bzl), Tyr, Trp, and Pro) and H–AA_2–OMe·HCl or H–AA_2–NH_2·HX (X=Cl or Br; AA_2=Ala, Val, Leu, Ile, Phe, Met, Cys(Bzl), Tyr, Trp, and Ser) have been investigated, although all combinations of both components were not examined for each enzyme. In many cases the high purity of the product from the reaction mixture was assured by physical constants and elemental analysis without further purification (Table 1). If necessary, the product was recrystallized

† All amino acids are of L configuration. Symbols and abbreviations are in accordance with the recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **247**, 977 (1971). Other abbreviations used are : PU=protease unit; *B. sub.*=*Bacillus subtilis*; Tris=tris(hydroxymethyl)methanamine; Z(3Me)=2,4,6-trimethylbenzyloxycarbonyl.

Table 1. Physical constants and elemental analyses of X-AA_1-AA_2-OMe and X-AA_1-AA_2-NH_2

Peptide	Mp (°C)	$[\alpha]_D^{25}$ a) (°)	Found (Calcd), % C	H	N	S
X-AA_1-AA_2-OMe						
ZPhePhe-	143—145	−18.0	70.21 (70.41)	6.12 (6.14)	6.09 (6.08)	
ZLeuPhe-	86—90	−22.2	67.47 (67.57)	7.03 (7.10)	6.74 (6.57)	
ZCys(Bzl)Phe-	96—100	−29.2	65.94 (66.37)	5.90 (5.98)	5.36 (5.53)	6.66 (6.33)
ZAlaPhe-	95—96	−12.0	65.69 (65.59)	6.17 (6.30)	7.53 (7.29)	
PGlnPhe-	179—180.5	−11.0	62.41 (62.56)	6.12 (6.18)	9.63 (9.52)	
ZCys(Bzl)Ile-	77—79	−24.2	63.29 (63.52)	6.74 (6.84)	5.62 (5.93)	6.88 (6.78)
ZPheIle-	100—101	−8.4	67.55 (67.57)	7.06 (7.10)	6.56 (6.57)	
Z(OMe)MetIle-	88—92	−9.8	57.12 (57.24)	7.29 (7.34)	6.38 (6.36)	7.32 (7.28)
ZLeuIle-	70—71	−24.0	64.18 (64.58)	8.17 (8.28)	7.13 (7.17)	
ZLys(Z)Ile-	120—122	−11.0	64.11 (64.41)	7.21 (7.28)	7.78 (7.77)	
Z(3Me)His(Bzl)Ile-	135—137	+6.0	67.05 (67.38)	7.18 (7.18)	10.23 (10.48)	
Z(OMe)Glu(OBzl)Ile-	70—71	−10.6	63.48 (63.61)	6.79 (6.88)	5.28 (5.30)	
ZSer(Bzl)Ile-	74—78.5	0	65.47 (65.76)	7.02 (7.08)	6.10 (6.14)	
ZAsnIle-	177—179	−12.8	57.20 (58.00)	7.00 (6.92)	10.61 (10.68)	
ZGlnIle-	154—155	−15.4	58.40 (58.94)	7.24 (7.19)	10.36 (10.31)	
ZCys(Bzl)Val-	70—72	−30.0	62.55 (62.85)	6.53 (6.61)	6.18 (6.11)	7.07 (6.99)
ZPheVal-	107—108	−16.0	66.57 (66.96)	6.79 (6.86)	6.70 (6.79)	
Z(OMe)Glu(Bzl)Val-	88—89	−17.9	63.06 (63.02)	6.67 (6.66)	5.44 (5.45)	
ZPheLeu-	111—113	−24.4	67.53 (67.57)	7.06 (7.10)	6.57 (6.57)	
Z(OMe)Glu(OBzl)Leu-	85—87	−21.5	63.69 (63.61)	6.83 (6.88)	5.30 (5.30)	
Z(OMe)Asp(OBzl)Leu-	76—78	−20.3	62.85 (63.01)	6.55 (6.67)	5.34 (5.44)	
X-AA_1-AA_2-NH_2						
ZAlaLeu-	186.5—187.5	−16.0	60.99 (60.87)	7.46 (7.51)	12.47 (12.53)	
ZPheLeu-	184—186	−19.5	66.93 (67.13)	7.00 (7.10)	10.40 (10.21)	
Z(OMe)Glu(OBzl)Leu-	141—142	−9.0	63.24 (63.14)	6.84 (6.87)	8.06 (8.18)	
Z(OMe)MetLeu-	195.5—196	−17.4	56.08 (56.45)	7.23 (7.34)	9.61 (9.86)	7.84 (7.54)
ZCys(Bzl)Leu-	169—170.5	−25.4	63.00 (62.99)	6.83 (6.83)	9.12 (9.18)	7.06 (7.01)
ZLys(Z)Leu-	183—185	−13.6	63.78 (63.86)	7.22 (7.27)	10.58 (10.64)	
ZProLeu-	185—186	−56.4	63.03 (63.14)	7.42 (7.53)	11.60 (11.63)	
ZSer(Bzl)Leu-	142—143	−3.4	65.43 (65.28)	7.11 (7.08)	9.67 (9.52)	
Z(OMe)LeuMet-	194.5—196	−14.5	56.47 (56.45)	7.42 (7.34)	9.61 (9.88)	7.49 (7.54)
Z(OMe)Glu(OBzl)Met-	153—155	−5.6	58.85 (58.74)	6.20 (6.26)	7.65 (7.90)	5.95 (6.03)
Z(OMe)MetMet-	183—184	−13.6	51.39 (51.44)	6.55 (6.59)	9.43 (9.47)	14.49 (14.46)
ZAlaPhe-	215—216	−19.9	64.82 (65.02)	6.24 (6.28)	11.49 (11.38)	
ZPhePhe-	232—235	−30.3	69.81 (70.09)	6.05 (6.11)	9.51 (9.43)	
Z(OMe)Glu(OBzl)Phe-	165—167	−18.0	65.24 (65.08)	5.97 (6.07)	7.60 (7.67)	
Z(OMe)MetPhe-	205—207	−24.7	59.87 (60.11)	6.30 (6.36)	9.27 (9.14)	
ZCys(Bzl)Phe-	181—182.5	−37.3	65.88 (65.96)	5.93 (5.95)	8.53 (8.55)	6.55 (6.52)
ZAlaVal-	258—263	+11.9	59.47 (59.79)	7.14 (7.21)	12.93 (13.08)	
ZPheVal-	243—246	−1.6	65.86 (66.48)	6·74 (6.85)	10.52 (10.57)	
Z(OMe)Glu(OBzl)Val-	190—197	+6.9	62.29 (62.51)	6.53 (6.66)	6.14 (6.53)	
Z(OMe)MetVal-	233—235	+2.2	55.30 (55.45)	7.07 (7.10)	10.31 (10.21)	7.82 (7.79)
ZCys(Bzl)Val-	117—118	−16.5	62.30 (62.28)	6.56 (6.59)	9.56 (9.47)	7.29 (7.23)
ZLeuVal-	233—235	+24.0 b)	62.28 (62.79)	7.91 (8.04)	11.37 (11.56)	

a) Conditions: for X-AA_1-AA_2-OMe; *c* 0.5, MeOH; for X-AA_1-AA_2-NH_2; *c* 1, DMF. b) *c* 1, AcOH.

from ethyl acetate and petroleum ether.††

Dipeptide Syntheses with Thermolysin. As shown in Table 2, the methyl esters of amino acids such as Phe, Val, Leu, and Ile condensed with various amino acids of the type X-AA_1-OH to give the desired products in good yields. Interestingly, the yields often increased, in some cases to a great extent, by the addition of 20% (wt/v) ammonium sulfate or sodium chloride (*vide infra*). In the cases of the methyl esters of tryptophan, tyrosine, and other amino acids the

†† The raw products were sufficiently pure in most cases, and if further purification was required, the simple removal of the materials insoluble in organic solvents was sufficient to give the pure products. However, the yields of the pure products were often considerably reduced by this procedure.

Table 2. Synthesis of X-AA_1-AA_2-OMe with thermolysin[a]

X-AA_1-AA_2-OMe	Yield (%)	X-AA_1-AA_2-OMe	Yield (%)
ZPhePhe-	76	Z(OMe)Glu(OBzl)Ile-	91.1[b]
ZLeuPhe-	47[b,c]	ZSer(Bzl)Ile-	87.4[b]
ZCys(Bzl)Phe-	65.6	ZAsnIle-	62.5[d]
ZAlaPhe-	61[d]	ZGlnIle-	65.0[d]
ZGlnPhe-	32.9[b,c]	ZCys(Bzl)Val-	80.7[b]
ZCys(Bzl)Ile-	84.7	ZPheVal-	75.0[b]
ZPheIle-	65.4[c]	Z(OMe)Glu(OBzl)Val-	80.2[b]
Z(OMe)MetIle-	78.2[b]	ZPheLeu-	56.8[b,c]
ZLeuIle-	82.8[b]	Z(OMe)Glu(OBzl)Leu-	71.6[b]
ZLys(Z)Ile-	51.7[b]	Z(OMe)Asp(OBzl)Leu-	70.6[b]
Z(3Me)His(Bzl)Ile-	53.9[b]		

a) A mixture of substrates (1 mmol each), Thermolysin (10 mg), 4 M NaOH aqueous solution (0.25 ml), and 0.2 M Tris·HCl (pH 8, containing 5×10^{-2} M $Ca(OAc)_2$, 10 ml) was incubated at 38—40 °C for 15 h. b) Ammonium sulfate (2 g) was added. c) After recrystallization from AcOEt-petroleum ether. d) Sodium chloride (0.8 g) was added and the buffer (4 ml) was used.

Table 3. Synthesis of X-AA_1-AA_2-NH_2 with thermolysin[a]

X-AA_1-AA_2-NH_2	Yield (%)	X-AA_1-AA_2-NH_2	Yield (%)
ZAlaLeu-	61.4[b]	ZAlaPhe-	78.2[c]
ZPheLeu-	86.0[c]	ZPhePhe-	90.7[c]
Z(OMe)Glu(OBzl)Leu-	81.6[c]	Z(OMe)Glu(OBzl)Phe-	84.6[c]
Z(OMe)MetLeu-	84.6[c]	Z(OMe)MetPhe-	79.4[c,d]
ZCys(Bzl)Leu-	68.8[c,d]	ZCys(Bzl)Phe-	90.7[c]
ZLys(Z)Leu-	77.3	ZAlaVal-	59.4[e]
ZProLeu-	52.8[c]	ZPheVal-	84.0[c]
ZSer(Bzl)Leu-	66.4	Z(OMe)MetVal-	79.7[c]
Z(OMe)Glu(OBzl)Met-	83.7[c]	Z(OMe)Glu(OBzl)Val-	66.3[c]
Z(OMe)MetMet-	81.8[c]	ZCys(OBzl)Val-	81.8[c]
Z(OMe)LeuMet-	82.0[c]	ZLeuVal-	79.8[c]

a) A mixture of substrates (1 mmol each), Thermolysin (10 mg), 4 M NaOH aqueous solution (0.25 ml), and 0.2 M Tris·HCl (pH 8, containg 5×10^{-2} M $Ca(OAc)_2$, 10 ml) was incubated at 38—40 °C for 16 h. b) Ammonium sulfate (0.8 g) was added and the buffer (4 ml) was used. c) Ammonium sulfate (2 g) was added. d) After recrystallization. e) The buffer (4 ml) was used.

condensed products were not produced even in the presence of ammonium sulfate or sodium chloride. Similarly the X–dipeptide amides were obtained in higher yields than those of the corresponding esters (Table 3). The amide of methionine, in contrast with the ester, also gave various dipeptides, except those of alanine and proline.

Effect of Ammonium Sulfate on the Yield of Peptides with Thermolysin. The effect of ammonium sulfate described above was examined in the following two cases. The reaction of Z–Phe–OH with either H–Leu–OMe·HCl or H–Val–OMe·HCl gave the expected products in 40 and 29% yields, respectively; in contrast, the yields of both reactions were increased by the addition of ammonium sulfate in 5—30% (wt/v), especially in the case of 20% (Fig. 1).

Dipeptide Syntheses with Metalloproteinases Other than Thermolysin. The peptides given by these proteinases in Table 4 were highly limited and other peptides were hardly obtained by each enzyme. These results demonstrate that Dispase I, Prolisin A, and Tacynase N are not such effective catalysts in the coupling reactions as Thermolysin. The amino acids appropriate for the amine components are all hydrophobic for the synthesis of peptides by using those proteinases.

Syntheses on a Practical Scale. The method was examined as to its suitability for practical peptide syntheses of the two peptides with two proteinases (Table 5). The Table shows that both peptides are easily obtained in good yields. Moreover, it was found that aqueous ammonium sulfate solution can be substituted for the buffer in the reaction with Thermoase and that the reaction can be conducted at room temperature (20 °C).

Discussion

In general, microbial metalloproteinases specifically catalyze the "hydrolysis" of the peptide bond with a hydrophobic amino acid residue at the amino side of the linkage.[7] However, the "hydrolysis" of the bond with tyrosine or tryptophan is catalyzed at rather a slow rate.[8] In the present study, all the amino acids of the amine components which readily formed the peptide bond with various X–amino acids were

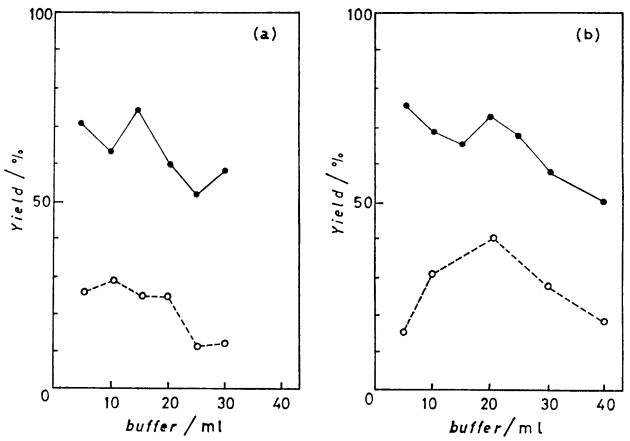

Fig. 1. The effect of ammonium sulfate on the yield of peptide synthesis with Thermolysin. a) ZPheOH+HValOMe·HCl; b) ZPheOH+HLeuOMe·HCl. A mixture of substrates (1 mmol each), Thermolysin (10 mg), 0.2 M Tris·HCl (pH 8, containing 5×10^{-2} M $Ca(OAc)_2$, 5—40 ml), ammonium sulfate (20% wt/v for the buffer), and 4 M NaOH aqueous solution (0.25 ml) was incubated at 40 °C for 16 h and treated under the general procedures. $(NH_4)_2SO_4$: ●—● present; ○--○ absent.

hydrophobic, while the derivatives of tyrosine or tryptophan were practically inert to the coupling reaction. Consequently, the most scissile bond is the most suitable point for the condensation. It is clearly seen in Table 2 that the isoleucine methyl ester in its amine component is the most suitable, and the susceptibility of the methyl esters of phenylalanine, valine, and leucine are successively decreasing in that order. For the carboxyl component, various amino acid derivatives except those of valine, isoleucine, proline, and tryptophan are applicable and there appears to be no difference in susceptibility towards the metalloproteinases used. Better results were obtained in the syntheses of the *N*-acyl dipeptide amides (Table 3) and the methionine amide was also found appropriate to this reaction. The different sensitivity of the enzymes towards the ester or the amide is attributed to the properties of the enzymes as endopeptidases.†††

Table 4. Synthesis of Z-AA_1-AA_2-OMe with several enzymes[a]

AA_1AA_2	Enzyme	Yield (%)	Mp (°C)
PheLeu	Dispase I	37.5	105—107
PhePhe	Dispase I	62	134—137
Cys(Bzl)Leu	Dispase I	94[b]	69—71
PheIle	Prolisin A	72.7	99—102
Cys(Bzl)Ile	Prolisin A	78	72—74
PheTyr	Tacynase N	44[c]	131—132

a) A mixture of substrates (1 mmol), enzyme, and 0.2 M Tris·HCl (pH 8, containing 5×10^{-2} M $Ca(OAc)_2$, 10 ml) was incubated at 38—40 °C for 15 h; each enzyme used: Dispase I (15 mg); Prolisin A (20 mg) with potato inhibitor (60 mg); Tacynase N (20 mg) with potato inhibitor (20 mg). b) Elemental analysis, Found: C, 63.23; H, 6.71; N, 5.81; S, 6.70%. Calcd for $C_{25}H_{28}N_4O_5S$: C, 63.52; H, 6.84; N, 5.93; S, 6.78%. c) Elemental analysis, Found: C, 67.77; H, 6.04; N, 6.16%. Calcd for $C_{27}H_{28}N_2O_6$: C, 68.04; H, 5.93; N, 5.88%.

The dissimilarities in catalytic ability in the syntheses of dipeptides by Thermolysin and the other enzymes are interesting when compared to those in the hydrolysis of the peptide bond.[9] Morihara *et al.*[9b] reported that Z–Gly–Leu–NH_2 is hydrolyzed by Thermolysin and also by *B. sub.* metalloproteinase at approximately the same rate and that Z–Gly–Phe–NH_2 is hydrolyzed by Thermolysin 10 times faster than by *B. sub.* metalloproteinase. Feder and Shuck[9d] reported that the most pronounced difference between Thermolysin and *B. sub.* metalloproteinase at the dipeptide level was observed when phenylalanine is the amino acid donating the amino group to the cleaved bond. Accordingly, in the "hydrolysis" of the peptide bond, there is only

††† An endopeptidase catalyzes the hydrolysis of the peptide bond containing the C-terminal residues (whose carboxyl group is free or protected by the ester group) either very slowly or not at all.

Table 5. Synthesis in large scale[a]

Peptide (mmol)	Enzyme (mg)	Solvent (ml)	Additive (g)	Yield (%)	Mp (°C)
ZPhePheOMe (30)	Th[b] (600)	buffer[d] (600)	—	73	133—135
	Th[b] (600)	buffer[d] (600)	$(NH_4)_2SO_4$ (120)	70	133—135
	Th[b] (600)	H_2O[e] (450)	—	(60)[f]	110—119
	Th[b] (600)	H_2O[e] (450)	$(NH_4)_2SO_4$ (120)	80	133—135
ZPheIleOMe (50)	*B. sub.*[c] (700)	buffer[d] (500)	—	76	96—98

a) A mixture of substrates, enzyme, solvent, additive, and 4 M NaOH aqueous solution of equimolar quantity with the amine component was stirred in a three-necked flask at 20 °C for 16 h and treated under the general procedures. b) Partially purified Thermoase and the potato inhibitor (60 mg). c) Partially purified metalloproteinase of *B. sub.* and the potato inhibitor (70 mg). d) 0.2 M Tris·HCl (pH 8, containing 5×10^{-2} M $Ca(OAc)_2$). e) With continuous control of the pH at 7.2 by the addition of 4 M NaOH. f) Pure product was not obtained.

a slight difference in efficiency between the two enzymes. As shown *B. sub.* metalloproteinase (Prolisin) was far less capable of catalyzing the peptide "synthesis" than Thermolysin.

An examination of the variation of the Thermolysin activity caused by ammonium sulfate or sodium chloride, shows an increment in the peptidase activity,[10] in contrast to the remarkable decrease in protease activity.[10] This effect, coupled with the salting out effect of the salt, seems to play a major role in the increased yield of the product.

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