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# ENZYMATIC SYNTHESIS OF ASPARTYL-CONTAINING DIPEPTIDES

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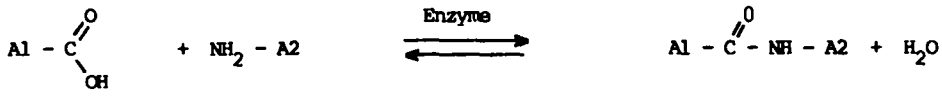
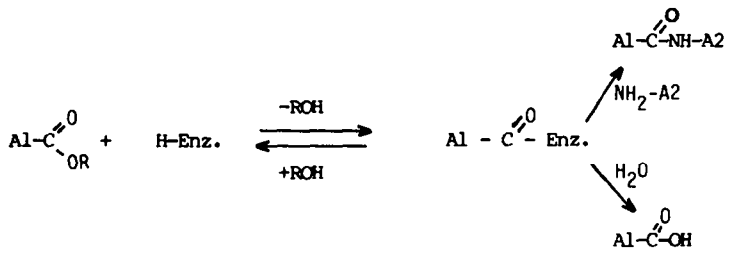
Abstract - We show that the ability of thermolysin to catalyze the coupling of aspartyl residue with other aminoacids is restricted to phenylalanine. Esterification of the two carboxylic groups of N-protected aspartic acid allows chymotrypsin and papain to catalyze the desired synthesis.

The use of proteases to catalyze peptide bond formation is an interesting alternative to chemical methods. The main advantage is to avoid the risk of racemization, and, although yields are not always excellent, many studies have been reported in the last few years[1]. According to the nature of enzymes and substrates, the syntheses will run under thermodynamic or kinetic control. In the former case, the peptide bond is formed by the reverse process of the hydrolysis (scheme I) : the problem is to displace the equilibrium towards synthesis. In the second case (scheme II), for serine and thiol proteases, the "acyl donor" substrate is an ester, the covalent acyl-enzyme intermediate is deacylated competitively by water and the "nucleophilic" aminoacide. It is then possible to obtain a high concentration of peptide, after a short time, then the reaction can go back and reach the equilibrium position[1b,2,3].

Scheme I



Scheme II



In most cases, the enzyme will exhibit its specificity for both substrates, but this specificity can be very narrow for the "acyl-donor" aminoacid[1a,4] while various aminoacids, even D aminoacids[3] or other compounds like alcohols, hydroxylamine[5] aromatic amines or hydrazines[6,7] will act as nucleophiles. Generally, coupling reactions are realized between lipophilic aminoacids (Phe, Leu, Ile). The reasons are that the resulting dipeptide is insoluble in the reaction mixture, and, moreover, that many proteases exhibit their maximum activity with lipophilic "acyldonor" aminoacids. For example, chymotrypsin, papain and thermolysin are good catalyst for the synthesis of dipeptide like Phe(Tyr, Trp)-X[2-4,8-11].

An important exception is the synthesis of Z-Asp-PheOCH$_3$, precursor of the artificial sweetener "aspartam", catalyzed by thermolysin[12]. This is the only known synthesis of unprotected aspartic acid-containing dipeptide. For such a synthesis however, due to the polyfunctionality of aspartic residue, enzymatic catalysis should be particularly useful.

In this paper, we first show that in the thermolysin-catalyzed synthesis of Z-Asp-PheOMe, the specificity of the enzyme for Phe (and not for Asp) is the important feature, so that no other aspartic acid-containing dipeptides can be easily synthetized. We then describe how a minor and reversible structural modification of aspartic acid allowed chymotrypsin and papain to catalyze the synthesis.

## RESULTS AND DISCUSSION

### Thermolysin-catalyzed synthesis

Our results and some from the litterature are reported in Table I.

Table I : Thermolysin catalyzed syntheses[a]

| Acyl-donor Aminoacid( mM) | Nucleophilic Aminoacid (mM) | % Yield (our results) | % Yield (litt.) |
|---|---|---|---|
| Z-Asp (100) | PheOCH$_3$ (400) | 70 | 96[12] |
| Z-Asp (100) | PheNH$_2$ | 70 | |
| Z-Asp (100) | LeuOCH$_3$ (400) | 0 | |
| Z-Asp (100) | IleOCH$_3$ (400) | 0 | |
| Z-Asp (100) | LeuNH$_2$ (400) | 0 | |
| Z-Asp (100) | Leu(NH$_2$)[b] (200) | 20 | |
| Z-Asp(OCH$_3$)(100) | PheOCH$_3$ (100) | 52 | |
| Z-Phe | LeuOCH$_3$ | | 57[13a] |
| Z-Leu | IleOCH$_3$ | | 82[13a] |
| Z(OMe)Asp(OBzl) | LeuOCH$_3$ | | 71[13a] |

a - syntheses were realized in 5 ml 200 mM Tris buffer, pH 6,8 containing 50 mM calcium acetate and 5 mg thermolysin at 40°C for 20 hours. Yield is calculated for the substrat present in lower concentration.

b - 200 mM L-PheOCH$_3$ was added as a cosubstrate. No synthesis of Z-Asp.PheOCH$_3$ was observed.

When Z-Asp. was the acyl donor, no synthesis was observed with $LeuOCH_3$, $IleOCH_3$ or $LeuNH_2$, although these aminoacids were good nucleophiles in other thermolysin-catalyzed synthesis[13]. It appears that, in the particular case of the synthesis of Z-Asp-$PheOCH_3$, the presence of $Phe-OCH_3$, makes the reaction possible. One of the substrates, presumably Z-Asp, which first reacts with the enzyme[13], might involve a strict specificity for the other one, or $PheOCH_3$ may act as an allosteric substrate, giving the enzyme the adequate conformation for accomodation of Z-Asp. The last hypothesis is suggested by the following result : when $PheOCH_3$ and $LeuNH_2$ were present at the same concentration, some coupling of $LeuNH_2$ has been observed.

- Chymotrypsin-catalyzed synthesis

Peptide-bond formation catalyzed by chymotrypsin is widely investigated[9-11]. It is a cheap and stable enzyme, useful for synthetic purpose[16]. However, it is specific for lipophilic acyl donor aminoacids[1a,17] and is not suitable for aspartic acid coupling.

Chymotrypsin also catalyzes the hydrolysis of many esters[18], among them the α ester of N-acetylaspartic acid dimethylester[19]. Since the enzyme catalyzes peptide bond formation according to scheme II[17], such a compound could be a good substrate in coupling reactions.

As N-acetyl protecting group is not convenient in peptide synthesis, we prepared the N-benzyloxycarbonyl aspartic dimethyl ester (Z-$Asp(OCH_3)OCH_3$) and diethylester (Z-$Asp(OC_2H_5)OC_2H_5$) and N-acetoacetylaspartic dimethylester ($CH_3-CO-CH_2-CO-Asp(OCH_3)OCH_3$) and measured the activity of chymotrypsin for their hydrolysis. The results are reported in Table II. All compounds were hydrolyzed at comparable rates. The activities of the enzyme were of the same range than for acetyl aspartic acid dimethyl ester[19].

Table II : Enzymatic hydrolysis of aspartic acid derivatives[a].

| | Activities (μmole/min/mg of protein) | |
|---|---|---|
| | Chymotrypsin | papain |
| Z-$Asp(OCH_3)OCH_3$ | 1.25 | 0.4 |
| Z-$Asp(OCH_3)OCH_3$ | 0.5 | 0.6 |
| N-acetoacetyl-$Asp(OCH_3)OCH_3$ | 2.0 | - |

a - 10 mM substrate was incubated in 20 ml 100 mM NaCl, EtOH 5 % with 20 mg enzyme and the pH adjusted to 7.8 for chymotrypsin and 5.0 for papain by continuous addition of NaOH.

We studied the use of these derivatives in peptide bond formation. The results are presented in table III. In a water/dimethylformamide medium 40/60 (wg), pH 9 and 40°C, with concentrations of 150 mM for both substrates, coupling with phenylalanine derivatives occured with good yields. The synthesis was observed also with leucine and arginine derivatives, generally good nucleophiles in chymotrypsin-catalyzed synthesis.

Table III : Chymotrypsin catalyzed syntheses[a].

| | % Yield | | % Yield |
|---|---|---|---|
| Z-$Asp(OCH_3)$-Phe-NH-NH-ϕ | 54 | N-acetoacetyl-$Asp(OCH_3)$-Phe-NH-NH-ϕ | 53 |
| Z-$Asp(OCH_3)$-Phe-$NH_2$ | 59 | Z-$Asp(OCH_3)$-Leu-NH-NH-ϕ | 40 |
| Z-$Asp(OC_2H_5)$-Phe-NH-NH-ϕ | 6 | Z-$Asp(OCH_3)$-Phe-OBzl | 17 |
| Z-$Asp(OC_2H_5)$-Phe-$NH_2$ | 54 | Z-$Asp(OCH_3)$-Arg-$OCH_3$ | 20 |

a - Syntheses were realized in 5 ml of 100 mM sodium carbonate buffer, pH 9/DMF (60/40 wg) containing both aminoacid derivatives (100 mM) and chymotrypsin (100 mg) at 40°C.

Various parameters were studied for the synthesis of Z-Asp($OCH_3$)Phe-NH-NH-$\phi$ :

- Addition of organic solvents is generally a favorable feature as it decreases water activity[19]. This aspect of enzyme technology is especially well documentated for chymotrypsin[3]. Among the solvents more often used, three were tested (table IV) : although diethylacetate and dimethylsulfoxide could be used, in this particular case, 60 % dimethylformamide gave the best results.

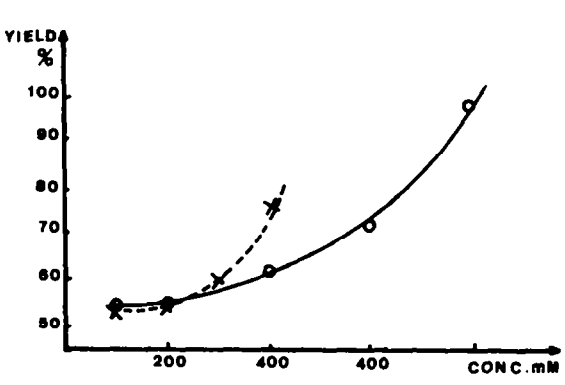
Table IV :Utilization of organic solvents on the chymotrypsin-catalyzed synthesis of Z-Asp($OCH_3$)-Phe-NH-NH-$\phi$[a].

| Solvent | % | Yield |
|---|---|---|
| DMSO | 50 | 30 |
| Butanediol | 80 | 16 |
| DMF | 36 | 35 |
| DMF | 60 | 54 |
| DMF | 80 | 43 |

a - Experimental conditions, except the nature and percent of the organic solvent, are as in table III.

- Effects of the concentration of substrates are indicated on figure 1. According to scheme II, an increase in nucleophilic aminoacid is favorable. The effect of the increase in concentration of aspartic acid derivative was rather unexpected, as no saturation effect was observed. This cannot be explained by an important competition of the hydrolysis reaction : after synthesis, HPLC analysis of the residual material showed that the concentration of Z-Asp($OCH_3$) (resulting of hydrolysis) is very low. We suppose that one of the reaction product is competitive inhibitor of Z-Asp($OCH_3$)$OCH_3$.

Chymotrypsin-catalyzed synthesis of Z-Asp($OCH_3$)-Phe-NH-NH-$\phi$



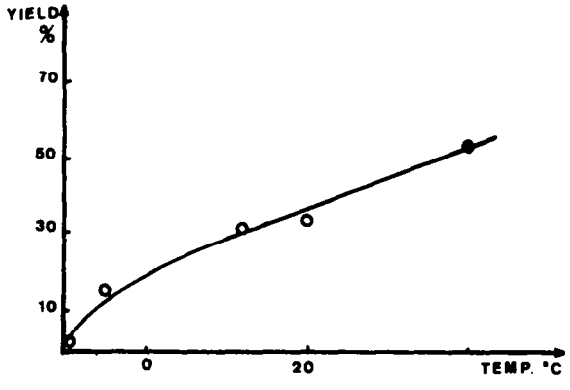




Figure 1 : Influence of substrate concentration on yield : (O O O : Variation of the concentration of aspartic acid dimethylester, X X X : Variation of the concentration of phenylalanine phenylhydrazide. Yield is calculated for the substrat present in lower concentration. (All experimental conditions are as in table III).

Figure 2 : Influence of temperature on yield : (all other experimental conditions are as in table III).

- The temperature effect is illustrated on figure 2. The dipeptide yield decreases at temperatures lower than 40 °C. It has been suggested[3,16b] that, when competing with hydrolysis, aminolysis yield rather increases at low temperature, due to a tighter binding of the nucleophilic aminoacid. Our results show that hydrolysis is not competing. Decrease in dipeptide yield may be due to the longer reaction time required that leads to the enzyme denaturation.

Papain catalyzed synthesis

Like chymotrypsin, papain is often used in peptide synthesis. Coupling is generally realized under thermodynamic control (scheme I)[7,9,10,20]. However, an acyl-enzyme intermediate is formed in papain-catalyzed reactions and some dipeptide syntheses by ester or amide aminolyses are known[1a,21]. Thus we tried to use this enzyme in place of chymotrypsin.

Table II reports the activity of papain in hydrolysis of Z-aspartyl dimethyl and diethyl esters. The activities seem to be in the same range as for chymotrypsin but it is difficult to compare, as a crude preparation of papain was used. Table V presents some dipeptide syntheses : ethyl acetate or ethanol as cosolvent gave the best results. Yields were generally lower than with chymotrypsin. The reaction was slow, product precipitation was observed up to 20 hours. It is possible that, in this particular case, hydrolysis of the ester occurs first and that synthesis continues under thermodynamic control.

Table V : Papain catalyzed syntheses[a].

| | 75 % ethyl acetate | 40 % ethanol |
|---|---|---|
| Z-Asp($OCH_3$)-Phe-NH-NH-$\phi$ | 27 | 41 |
| Z-Asp($OCH_3$)-Phe-$NH_2$ | 20 | 13 |
| Z-Asp($OC_2H_5$)-Phe-NH-NH-$\phi$ | 37 | 30 |
| Z-Asp($OC_2H_5$)-Phe-$NH_2$ | 30 | 10 |
| N-acetoacetyl-Asp($OCH_3$)-Phe-NH-NH-$\phi$ | 21 | 39 |

a - Syntheses were realized in 10 ml Mc Ilvaine buffer pH 5.5/organic solvent, containing 100 mM mercaptoethanol, 100 mM aspartic acid derivative, 200 mM phenylalanine phenylhydrazide and 600 mg papain.

CONCLUSION

We shown that $PheOCH_3$ plays a special role in Z-Asp-$PheOCH_3$ synthesis. Its presence is required to synthesize Z-Asp-Leu-$NH_2$. Thus, thermolysin-catalyzed synthesis of aspartic acid-containing dipeptide is restricted.

Both chymotrypsin and papain catalyze the coupling reaction between dimethyl or diethyl esters of N-protected aspartic acid and other aminoacid derivatives like phenylhydrazide aminoacids. These reactions display a great interest in peptide synthesis. It is not necessary to protect selectively the β carboxylic function of aspartic acid. The diester is easy to prepare by action of HCl in ethanol or methanol. Phenylhydrazide protection can be introduced by papain-catalyzed reaction[22]. The deprotection is realized by oxidation with ferric chloride or N-bromosuccinimide to give the acid[10] or methyl ester[23], or to activate the carboxylic function for further chemical coupling[23,24].

## EXPERIMENTAL SECTION

### Thermolysin catalyzed syntheses

Acyl-donor aminoacid (100 mM) and nucleophilic aminoacid (400 mM) were dissolved in 5 ml 200 mM Tris buffer, pH 6.8 containing 50 mM calcium acetate. The reaction was started by addition of 5 mg of thermolysin. The reaction mixture was shaken for 20 hours at 40°C and stopped with 1N HCl. The residue was filtered, washed successively with 0.5 N HCl, water, 5 % $NaHCO_3$ and water and dried on anhydrous $Na_2SO_4$.

### Chymotrypsin catalyzed syntheses

In 5 ml of a mixture of 100 mM sodium carbonate buffer pH 9 and DMF (60/40 wg) were dissolved starting aminoacid derivatives (usually 150 mM). The reaction was started by addition of 200 mg chymotrypsin and the mixture was shaken for 10 min. at 40°C and stopped with 1N HCl. The residue was filtered, washed successively with 0.5 N HCl, water, 5 % $NaHCO_3$, water and dried on anhydrous $Na_2SO_4$.

### Papain catalyzed syntheses

600 mg papain was dissolved in 10 ml of a mixture of Mc Ilvaine buffer pH 5,5 and ethyl acetate (25/75 wg) or EtOH (60/40 wg) containing 100 mM mercaptoethanol, 200 mM aspartate derivative and 200 mM phenylalanine. Coupling proceeded with agitation for 20 hours. The residue was filtered, washed successively with 0.5 N HCl, water, 5 % $NaHCO_3$ and water, then dried on anhydrous $Na_2SO_4$.

### Analytical data :

* Z-Asp-PheOCH$_3$ : consistent with literature data[12,16d].

* Z-Asp(OCH$_3$)-PHe-NH-NH-$\phi$ m.p. = 178-180°C $(\alpha)_D^{20}$ = -15° (C 1, MeOH) ; $^1$H NMR (300 MHz) ($CD_3CN$), $\delta$ (ppm) = 7.4 (m, 10H, aromatic) ; 5.1 (s, 2H, Z-$CH_2$) ; 4.65 (m, H, CH) ; 4.45 (m, H, -CH) ; 3.6 (s, 3H, O-$CH_3$) ; 3.2 (m, 2H, $CH_2$) ; 2.9 (m, 2H, -$CH_2$).

* Z-Asp(OCH$_3$)-PHe-NH2 m.p. = 192-193°C $(\alpha)_D^{20}$ = -16° (C 1, MeOH) ; $^1$H NMR (300 MHz) ($CD_3CN$), $\delta$ (ppm) = 7.5 (m, 10H, aromatic) ; 5.2 (s, 2H, Z-$CH_2$) ; 4.7 (m, H, CH) ; 3.7 (s, 3H, O-$CH_3$) ; 3.1 (m, 2H, $CH_2$) ; 2.7 (m, 2H, -$CH_2$).

* Z-Asp(OC$_2$H$_5$)-PHe-NH2 m.p. = 160-161°C $(\alpha)_D^{25}$ = -14° (C 1, MeOH) ; $^1$H NMR (300 MHz) ($CD_3CN$), $\delta$ (ppm) = 7.5 (m, 10H, aromatic) ; 5.2 (s, 2H, Z-$CH_2$) ; 4.7 (m, H, CH) ; 4.4 (m, 5H, O-$CH_2$) ; 2.9 (m, 2H, $CH_2$) ; 2.7 (m, 2H, -$CH_2$) ; 1.5 (t, 3H, $CH_3$).

* Z-Asp(OC$_2$H$_5$)-LPHe-NH-NH-$\phi$ m.p. = 230°C $(\alpha)_D^{25}$ = -11° (C 1, MeOH) ; $^1$H NMR (300 MHz) ($CD_3CN$), $\delta$ (ppm) = 7.5 (m, 10H, aromatic) ; 6.9 (m, 5H, aromatic rings) ; 5.1 (s, 2H, Z-$CH_2$) ; 4.6 (m, H, CH); 4.2 (m, 5H, O-$C_2H_5$).

* Z-Asp(OCH$_3$)-PHeOCH$_3$ m.p. = 94°C $(\alpha)_D^{25}$ = -10° (C 1, MeOH) ; $^1$H NMR (60 MHz)($CD_3CN$), $\delta$(ppm) = 8.4 (m, 10H, aromatic) ; 7.1 (m, 1H, NH) ; 7.0 (m, 1H, NH) ; 5.03 (s, 2H, Z-$CH_2$) ; 4.5 (m, 1H, CH); 4.4 (m, 2H, O-$CH_2$) ;3.6 (m, 6H, O-$CH_3$) ; 2.6 (m, 2H, -$CH_2$) ; 3.0 (m, 2H, -$CH_2$) ; 1.5 (t, 3H, $CH_3$).

* Z-Asp-Leu-NH2 m.p. = 210°C $(\alpha)_D^{25}$ = -10° (C 1, MeOH) ; $^1$H NMR (300 MHz) (DMSO), $\delta$ (ppm) = 7.9 (m, 1H, NH) ; 7.6 (m, 1H, (Asp)NH) ; 7.4 (m, 5H, aromatic) ; 5.1 (s, 2H, (Z)-$CH_2$) ; 4.3 (m, H, (Asp)CH) ; 4.2 (m, H, (Leu)CH) ; 3.5 (s, 3H, $OCH_3$) ; 2.7 (m, 2H, (Asp)-$CH_2$) ; 2.4 (m, 2H, $CH_2$) ; 1.5 (m, 6H, $(CH_3)_2$).

* Z-Asp(OCH$_3$)-Leu-NH-NH-$\phi$ m.p. = 177°C $(\alpha)_D^{25}$ = 0 (C 1, MeOH) ; $^1$H NMR (60 MHz) ($CD_3CN$), $\delta$ (ppm) = 7.4 (m, 5H, aromatic) ; 6.9 (m, 5H, aromatic (NH-NH-f) ; 5.0 (s, 2H, $CH_2$) ; 4.8 (m, H, CH) ; 4.6 (m, H, CH) ; 3.4 (s, 3H, $CH_3$) ; 2.8 (m, 2H, $CH_2$) ; 2.9 (m, 2H, $CH_2$) ; 1.0 (m, 6H, $(CH_3)_2$).

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