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# ENZYMATIC PEPTIDE SYNTHESES IN ORGANIC SOLVENT MEDIATED BY MODIFIED α-CHYMOTRYPSIN

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**Abstract** : Peptide couplings have been catalyzed in organic medium containing a slight amount of water (<0,5%) by PEG modified α-Chymotrypsin.

One of the most important question not yet well resolved in peptide chemistry remains the fragment coupling which is an unavoidable step in the convergent synthesis of large peptides. Owing to the risk of racemisation related to the peptidic nature of the N-bond of the carboxylic component and to the steric hindrance induced by both the size and conformation of the two peptidic moieties, chemical activation often gives unsatisfactory results.

To overcome this difficulty, enzymatic catalysis can be regarded as an alternative promising way.

According to the following equation, proteases are able to catalyze reversibly the hydrolysis and/or the synthesis of the peptide bond

$$\sim AA_n + AA_{n+1}\sim \rightleftharpoons \sim AA_n - AA_{n+1}\sim + H_2O$$

The amino-acid residues concerned have to be accepted by the enzyme and reaction conditions are choosen to shift this equilibrium to the right. For that purpose, two approaches based either under thermodynamic or kinetic control are generally used (1) . In the first case, the resulting peptide is excluded from the aqueous medium containing the protease either by precipitation or by solvent extraction. The syntheses under kinetic control are performed with proteases containing in their active site a serine or a cysteine residue able to form a covalent acyl enzyme complex from an ester derivative as the acyl donor component. The selectivity towards the peptide synthesis versus its hydrolysis depends on the difference in the rate constant of attack by the water or by the nucleophilic amino component.

In these procedures, the water side effects are minimized but not entirely suppressed and may lead to mixtures of truncated, deleted and even recombined peptides (2).

In our new stategy based on water exclusion from the medium, enzyme modification is needed in order to keep its catalytic activity and increase its solubility in organic solvents. These chemical modifications have to be done in aqueous buffer - in which the native enzyme is soluble - by using amphiphilic compounds able to bring the resulting adduct in organic medium. A good candidate for this purpose will be the monomethyl ether of polyethyleneglycol (MeO-PEG). This compound has been already used to modify several enzymes (3,4). Because of their low immunogenicity, PEG-Enzyme adducts have been studied as drugs (5); α-chymotrypsinogen has been modified by 2,4-(bis MeO-PEG) 6-chloro s-triazine which reacts on ε- aminolysyl groups laying at the protein surface. After tryptic activation, the modified α chymotrypsin has been generated (4)

Using different modifications of the lysyl residues which are known to have very few effect on the enzyme activity and are readily accessible, we have derivatized directly the native enzyme and applied the transformed bio catalyst to synthetize peptide bond in non aqueous medium. MeOPEGOH (Mw=5000) has been linked to α-chymotrypsin either (A) by cyanuric chloride activation (7) or (B) by an urethane group through p-nitrophenyl chloroformate activation (8) or (C) by an amide bond after carboxylic activation of MeO-PEGO-$CH_2$-$CO_2H$ (9) with DCC/ N-hydroxysuccinimide.

The modified enzymes with an increased molecular weight according to the substitution degree have been separated by gel filtration chromatography. The remaining esterase and amidase activity vs the substitution degree are given in Table 1.

Table 1

| | Cond.:Time(Hr)/Temp./ reagent excess[a)] | Subst. degree[b] % | Esterase[c] activity % | Peptidase[d]) activity% |
|---|---|---|---|---|
| Native α-chymo-trypsin (α-CT) | - | - | 100 | 100 |
| A-Modification | 1/40/75 | 95 | <5 | 0 |
| MeOPEG-s-triazine linkage to α-CT | 1/4/75 | 75 | 25 | 10 |
| B Modification MeOPEG-carbamate linkage to α-CT | 3.5/37/75 | 55 | 35 | 25 |
| C.Modification MeOPEG-amide linkage to α-CT | 6/4/50 | 75 | 50 | 30 |

a) Calculated on 15 lysyl residues per enzyme molecule ; b) TNBS Test (10); c) Acetyl tyrosine ethyl ester hydrolysis (11a); d) Glutaryl phenylalanine p-nitranilide hydrolysis(11b).

Enzyme peptide synthesis : Table 2 shows some results obtained with (C) modified enzyme which has both the best residual activity and the best chemical stability of the adduct.

In all these experiments, the enzyme concentration (determined by uv analysis) has been fixed at 15 M, those of ester and amino components at 15 mM. In contrary to often used conditions, it has to be pointed out that no excess of the nucleophile compound has been employed, except on one case (Table 2, entry 17).

Table 2

| Entry | Acyl Donor | Nucleophile | Reaction Time (days at 20°) | coupling[a] Yield | Hydrolysis of[a] starting ester |
|---|---|---|---|---|---|
| 1 | Z $PheOC_2H_5$ | $LeuNH_2$ | 4 | 40% | 8% |
| 2 | Z PheCam[b] | $LeuNH_2$ | 1,5 | 78% | 22% |
| 3 | BocTyrCam | $LeuNH_2$ | 1,5 | 80% | 20% |
| 4 | BocTyrN-MeCam | $LeuNH_2$ | 1,5 | 83% | 17% |
| 5 | Z PheOMem | $LeuNH_2$ | 4 | 50% | 7% |
| 6 | Z $PheOCH_2CF_3$ | $LeuNH_2$ | 1,5 | 85% | 15% |
| 7 | Z $PheOC_6H_5$ | $LeuNH_2$ | 4 | 35% | 9% |
| 8 | Z $PheOCH_2CN$ | $LeuNH_2$ | 1,5 | 90% | 10% |
| 9 | Z $PheOCH_2CF_3$ | LeuOMe | 4 | 8% | 14,5% |
| 10 | BocTyrCam | LeuOMe | 4 | 5% | 19% |
| 11 | Z$PheOCH_2CF_3$ | (D)$LeuNH_2$ | 2 | 63% | 14% |
| 12 | Boc(D)TyrCam | $LeuNH_2$ | 4 | 15% | 4% |
| 13 | Z PheCam | LeuOtBu | 4 | 30% | 23% |
| 14 | Boc$Tyr(Gly)_2$PheCam | $LeuNH_2$ | 2 | 70% | 17% |
| 15[c] | ZPheCam | $LeuNH_2$ | 4 | 0% | 6% |
| 16 | Z PheCam | $PheLeuNH_2$ | 2 | 67% | 21% |
| 17 | Z $PheOCH_2CF_3$[d] | $LeuNH_2$ | 20 Hours | 96% | 4% |
| 18[e] | Z $PheOC_2H_5$ | $LeuNH_2$ | 5 | 15% | - |
| 19[e] | Z PheCam | $LeuNH_2$ | 4,5 | 60% | - |

a) Determined by HPLC analysis ; b) Cam = Carboxamidomethyl, Mem = Methoxymethyl ;
c) Without enzyme ; d) conc = 6 mM.; e) with native, supended αCT in the same amount.

In these syntheses, the nature of the organic solvent plays a major role; the best medium is a mixture of t-amyl alcohol and benzene or trichloroethylene (50/50). A small water amount is absolutely needed (0,5%) to perform the synthesis (17) .For instance, in an hydrophilic medium like DMF, no synthesis occurs. That means that the enzyme must keep a certain amount of water molecules surrounding the chain to remain active (12a) and according to (12b) 150 water molecules are tightly bounded to the chymotrypsin molecule thus allowing a larger conformational flexibility. This requirement of a little amount of water in the organic medium has been already found in aminoacid esterification catalyzed by α-chymotrypsin in ethyl alcohol (13) or in peptide synthesis activated by α-chymotrypsin suspended in acetonitrile (14) or in t-amyl alcohol (15).The reaction rates

are generally smaller than in kinetic conditions (several days vs several hours) to achieve the coupling but we may notice a slight or no hydrolysis at all respectively concerning the starting ester or the newly formed peptide. The starting material hydrolysis can be nearly eliminated if the synthesis is performed with a lack of the acyl donor (6mM vs 15mM). Moreover in this case, the rate can also be significantly increased (compare entries 6 and 17).

The nature of the alcohol of the donor ester plays a major role since slight activated esters like Cam (16),N-methylCam or trifluoroethyl give better yields than the ethyl ester (compare entries 1 to 2-4-6-8). On the other side, the carboxylic protective group of the nucleophile is also important : Leu-amide is much better accepted than the corresponding methyl (entry 10) or t-butyl ester (entry 13) . Specificity towards α carbon chirality is not so important than for an unmodified enzyme in water medium, at least for the nucleophile component (entry 11). Conversely (entry 12), the carboxyl component is much less accepted .

For comparison, the heterogeneous catalysis performed with native α-chymotrypsin suspended in the same solvent has given lower coupling yields (compare entries 18, 19 to 1,2).

No secondary recombination or hydrolysis occurs in our system since the fragment coupling example (entry 16) shows that the only detected product is Z-Phe-Phe-Leu-$NH_2$ without any traces of the recombined peptide Z-Phe-Leu-$NH_2$ or of the hydrolysed on Z-Phe-Phe.This last example prompted us to apply this strategy to fragment coupling of the larger peptide Leu enkephalinamide (entry 14). Coupling of Boc Tyr Gly Gly Phe Cam and Leu $NH_2$ has been performed without any hydrolysis of the other sensitive bond already present situated after the OH-free tyrosyl residue.

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17) The water content (Karl Fischer) was 1.7% (w/w) for the lyophilised MeOPEG αCT and 0.2% for the organic solvent before reaction.


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