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NEW BIOCATALYSTS FOR PEPTIDE SYNTHESIS :GELS OF COPOLYMERIZED ACRYLIC DERIVATIVES OF α-CHYMOTRYPSIN AND POLYOXYETHYLENE

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Copolymers of acrylated derivatives of α-chymotrypsin and polyethylene glycol (PEG*) have been prepared and used as biocatalysts for the synthesis of model peptides in organic solvent. AcTyrLeuNH$_2$ is quantitatively obtained even after a dozen of cycles.

In peptide chemistry, segment coupling still remains a not completely explored area of research although this methodology is very attractive for the synthesis of large peptides.

Because of the possibility of racemization due to the peptidic nature of the α N-bond of the carboxylic component, and the steric hindrance resulting from the size and the conformation of the two moieties, chemical activation often leads to unsatisfactory results. These considerations and the fact that there is nearly no need of side chain protection, suggest the enzymatic catalysis as a promising alternative method.

Interest in enzymatic peptide synthesis has recently been spurred by the finding that enzymes can remain active in non aqueous media (1) and even display more enantioselectivity than in water (2). Moreover, enzymes modified by polyoxyethylene chains (PEG) become soluble in organic solvents and retain their catalytic activity under nearly anhydrous conditions (3-9).

In the well known enzymatic procedures, peptide synthesis is performed under thermodynamic or kinetic control (10). Water can induce deleterious side effects leading to mixtures of truncated and recombined peptides (11).

*Abbreviations used : PEG = polyethylene glycol; Ac = acetyl ; LeuNH$_2$: Leucine amide ; E.E. : ethyl ester, ATEE :NAcetyl tyrosine ethyl ester; A.A. =t.Amyl alcohol ; DCM : dichloromethane ; αCT = α-Chymotrypsin; Cam = carboxamidomethyl ester. HOBt : N-hydroxybenzotriazole. DCC= dicyclohexylcarbodiimide.TEMED =tetramethyl-ethylenediamine. TNBS = trinitrobenzenesulfonic acid.

In a course of our research on peptide segment condensation in organic solvent by a PEG- modified α-chymotrypsin (9), we improved the process by recovering and recycling more easily the biocatalyst. After some unsuccessful preliminary attempts to convalently link the already PEG modified enzyme to several supports, another approach has been followed, based on the copolymerization of acrylated derivatives of α-chymotrypsin and PEG chains. It was expected that, in that way, the PEG chains surrounding the enzyme molecule could maintain enzymatic activity in non-aqueous solvents. α-Chymotrypsin acrylated with acryloyl chloride on the ε-lysyl amino groups without loss of activity (12).The assumption was made that the polymerization step initiated by free radicals generated by TEMED/Ammonium persulfate would preserve the catalytic activity and increase the thermal stability (13). The resulting polymers were tested for their esterase activity in water (ATEE hydrolysis, see experimental section).

After drying, they were used as catalyst in the synthesis of Ac-Tyr-Leu-NH_2 in t-amyl alcohol solution. Preliminary experiments gave a wide array of results depending on operative conditions.

To rationalize this study and to assess the influence of the main factors on the preparation and utilization of the copolymer, an experimental fractional factorial design was constructed in order to save time and efforts (14,15). To get the maximum of information about a reaction with the minimum of experiments, BOX and HUNTER (14) have developed certain empirical sequential procedures in which the factors supposed to have some effect on the reaction can be modified between the two chosen levels limiting the experimental domain. For k factors, the complete design requires 2^k experiments, but, in a first step, if factorial interactions of higher order and, therefore, of less probable occurence, are neglected, only a part(2^{k-p}) of the experimental matrix can be utilized.

This strategy has been applied to the enzyme derivatization and copolymerization and to the utilization of the resulting copolymer for the synthesis of the model dipeptide, Ac-Tyr-Leu-NH_2, starting from equimolecular amounts of ATEE and Leu NH_2 and an enzyme/substrates molecular ratio of one thousandth. Six factors have been selected : 3 for the catalyst preparation and 3 for the peptide synthesis :

Factor A : To take into account the degree of freedom of the enzyme molecule inside the gel, the degree of substitution of α-chymotrypsin by acryloyl chloride was varied between 20% and 50% of the total lysyl -amino groups as judged by the TNBS test (21).

Factor B : PEG chain to α-chymotrypsin ratio : copolymers were prepared with a low (level +) or a large (level -) stoichiometric excess of PEG chains in order to modify the PEG surrounding of the enzyme in the copolymer. The same mixture (1/4 w/w) of monoacryloyl/bis-acryloyl PEG was used. Low and large PEG-content polymers are designed respectively, as R_a and R_b.

Factor C : This factor refers to a qualitative factor for polymer dehydration obtained either by lyophilization or by solvent exchange-procedure.

Factor D : The water content surrounding the enzyme seems to be important (3,6,9).Therefore, experiments have been done with or without added water (0,5% v/v).

Factor E : The polarity of the solvent also seems to be important; so either pure t-amylalcohol or a mixture 1/1(v/v) with dichloromethane was used.

Factor F : Owing to the expected thermal stability of the copolymerized enzyme, a temperature increase from 20° to 37° was used .

According to the reduced experimental matrix (table II), direct factorial effects are combined with the effects of some determined factorial interactions (i.e. A=BD=CE=CDF=BEF...).

Table I : Level of factors

Factor	Factor level	
	-	+
A	25%	50%
B	250	25
C	lyophilization	Solvent exchange
D	0	0.5%
E	AA	AA/DCM
F	20°	37°

Table II : Experimental Matrix

n° Experiment \ Factor level	A	B	C	D	E	F	* Y_S	* Y_H
1	-	-	-	+	+	+	12	6
2	+	-	-	-	-	+	18	9
3	-	+	-	-	+	-	11	2
4	+	+	-	+	-	-	33	4
5	-	-	+	+	-	-	77	23
6	+	-	+	-	+	-	41	59
7	-	+	+	-	-	+	92	8
8	+	+	+	+	+	+	67	17

* Y_S and Y_H : Yields of peptide synthesis and starting ester hydrolysis after 2 days reaction as determined by HPLC analysis.

Analysis of the results :

The eight experiments were performed in a statistical order and the yields corresponding to synthesis (Y_S) or hydrolysis (Y_H) are determined by HPLC analysis (table II).

The direct effect of the factors on these responses are calculated by means of the following equation:

$$L_i = 2/N \sum Y\{i,j...k\}$$

where L_i corresponds to the estimation of the direct effect of factor i confused with the associated interaction effects in the fractional design.

Table III : Estimated factor effect :

Effect	Y_S	Y_H
L_A	-8.2	+12.5
L_B	+13.5	-16.4
L_C	+50.5	21.3
L_D	+6.8	-7.2
L_E	-22.2	+10
L_F	+6.5	-11.9
Mean Average	44	16

As can be seen from table III, the most significant effects compared to the mean average are L_C for both Y_S and Y_H and L_B for Y_H.Let us consider first Y_S : L_C estimates the effect of factor C linked to the AE and BF interactions according to the fractional factorial design. As each of these factors does not have any effect higher than the mean average, it can be deduced that only factor C is important, at least in that experimental domain.

Factor C is concerned with the recovering mode of copolymers : either by lyophilization or by lyophilization followed by rehydration and drying by solvent exchange (water/t-amyl alcohol several times).

In spite of the insignificant effect of the 0,5% added water (factor D) a difference in the water retention inside the gels has been considered as a possible reason of the factor C effect. Karl-Fischer analysis has been used (table IV). It can be seen that actually, lyophilized polymers (level -) contain much less water than the solvent exchanged ones (level +).

Table IV : Polymer water content

Expn°	Polymer weight (mg)	Water quantity (mg)
1	166	11.8
2	377	26.3
3	21	3.4
4	42	6.4
5	166	171
6	377	381
7	21	21.4
8	41	37.8

Water content to polymer ratios are roughly one tenth for (C,level +,Exp n°1-4) than for (C,level-,Exp n°5-8). This remaining water content explains also why Factor C effect is again very high for the hydrolysis yield Y_H (table III : 21.3 vs 16 for mean average).

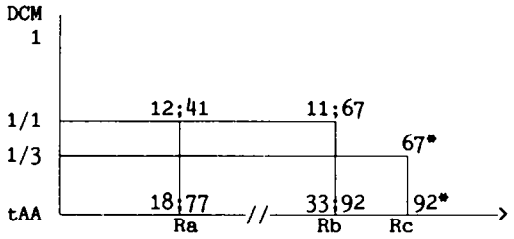
Besides L_C , L_B also appears significant for Y_H (L_B =-16.4). This means that hydrolysis is decreased when a polymer more loaded with α-chymotrypsin is used. Interestingly, this effect, although smaller, is reversed for the synthesis. Consequently, the synthesis could be increased by rising the enzyme content in the gel. This result could be explained by hydrophilicity of the PEG chains which are capable of striping off water molecules from the enzyme surface. It has been shown that water is needed for enzyme catalysis in organic solvents (16,17) and that the required amount varies significantly with the hydrophobicity of the solvent (18).

From this experimental design study, the following conclusions can be drawn :

- α-Chymotrypsin has to be weakly substituted
- High α CT/PEG ratio is needed.
- The solvent exchange procedure leads to better results due to the higher water content. This optimal water content will be discussed below.
- Pure t-amyl alcohol solvent is better than the mixture with dichloromethane.
- No temperature effect is detected at least between 20° and 37°, owing to the high thermal stability of the copolymerized enzyme (13). An experiment was done also at 50° without improving the results .

Taking into account these results, some additional experiments were done to optimize the Ac-Tyr-Leu-NH_2 synthesis by varying the α CT/PEG ratio and the organic medium used for the synthesis. Fig. 1 summarizes the results.

Fig. 1 : Ys with differents Gels Ri.



Ra = copolymers containing mono and bis acryloyl PEG in, respectively, 50 and 200 molar excess relative to the acrylated lysyl -amino groups of α CT.

Rb = 5 and 20 molar excess

Rc = 2.5 and 10 molar excess

*The experiments with Rc were done with a water content of 2,1% (v/v) during only one day at 20°. Results concerning Ra and Rb are drawn from the experimental factorial design (Table II).

Pure t-amyl alcohol was still better than a mixture containing 1/3 (v/v) of dichloromethane(67% vs 92% for Ys).

Polymer Rc containing twice more α CT than Rb has been prepared. This content corresponds to the maximum of enzyme retention. This modification drastically shortens the reaction time (22 h vs 48 h) to get the same excellent peptide yield (92%).

The most important factor determined by the factorial design has been carefully studied using the above conditions (table V)

With polymer Rc, it is clear that the best yield is obtained with 1%(v/v) water content . Moreover one can see the reproducibility of the results using the same catalyst over at least 6 cycles.

Replacement of water with formamide water mimic (of the same polarity) deeply decreases the yields. This compound could act as a competitive inhibitor during the acyl enzyme attack by the entering nucleophile (19).

An interesting remaining point concerns the stereoselectivity displayed by the biocatalyst toward the D-acyl donor (Boc D-Tyr Cam, table VI). Neither hydrolysis nor synthesis occurs. This is in contrast to the case of a D-nucleophile (D-Leu NH_2, Table VI) which is well accepted either by PEG-α-CT soluble in benzene (6), by PEG-α CT soluble in t-amyl alcohol - benzene (9) or by suspended enzyme (20).

The negative result obtained with D-Tyr as an acyl donor allowed us to eliminate the occurence of a chemical synthesis of the dipeptide ; moreover, this point has been verified with an enzyme inhibited by tosylphenylalaninechloromethylketone (see Table VI): a very low yield of AcTyr LeuNH_2 (8%) has been observed corresponding to a remaining esterase activity due to incomplete inhibition (7%).

Table V :

% Water* v/v		Ys** %	YH** %	Remaining starting** ester %
0,5		76	9	15
1	1°	97	2.3	0.7
	2°	97	2.7	0.3
	3°	99	1	0
	4°	97	2.6	0.4
	5°	98	1.8	0.2
	6°	97.5	2.3	0.2
2,5	1°	92.5	7.2	0.3
	2°	91,2	8.7	0.1
	5°	94	5.8	0.2
5		91.3	8.7	0
0.6+0.4 $HCONH_2$***		86	6.3	7.7
1% $HCONH_2$***		5	2	93

*% of water determined by Karl-Fischer analysis, calculated relatively to the volume of t-amyl alcohol .

Substrates concentrations = 45mM, E/S = 1 thousandth, at 20° in AA

** Yields determined by HPLC analysis after 22 hours reaction.

***Water has been partially or totally replaced by $HCONH_2$

Table VI : Stereoselectivity

aminoacid compounds	Ys	YH	YRSE*
Boc D-TyrCam + $LeuNH_2$	4	2.2	93
Boc-TyrCam + $LeuNH_2$	61	26	13
ATEE + D-$LeuNH_2$	85.1	14.5	0.4
ATEE** + $LeuNH_2$	8	5	87

* RSE = Yield of the recovered starting ester

** Synthesis performed with TPCK inhibited α CT with slight retention of esterase activity (7%).

CONCLUSION :

Copolymers of acrylated derivatives of α-chymotrypsin and polyethylene glycols have been prepared. They act as very efficient biocatalysts leading to a nearly quantitative yield of Ac-Tyr-Leu-NH_2 starting from equimolar amount of the amino acid components and an enzyme/peptide ratio as low as one thousandth. The best working conditions, namely degree of substitution of α CT , enzyme/PEG chains ratio and the

percent of needed water have been successfully estimated by means of an experimental fractional factorial design. This new biocatalyst will soon be used to prepare other dipeptides and to condense peptide segments of various length.

EXPERIMENTAL PART :

All solvents and starting materials were of reagent or analytical grade. Melting points were uncorrected and ^1H n.m.r. spectra were recorded on T.60, E.M. 390 Varian or 250 MHz BRUCKER spectrometers. Silica gel plates 60F254 were used for t.l.c. . H.P.L.C. analysis were done with Waters chromatograph equipped with a Nucleosil C-18 analytical column operating at 1ml/min. UV detection was made at 214 nm and peak integration was performed with a Delsi Enica 10 integrator . α-Chymotrypsin, PEG_{5000} monomethyl ether and PEG_{3400} and most of the amino acids components are commercially avaible except Cam ester which was prepared according to the published procedure (21).

Table VII : Physical constants of compounds

	Elution Volume (ml) HPLC	Rf t.l.c[c]	m.p°.
AcTyr	3.2[a]	0	-
ATEE	9.75[a]	0.55	-
$AcTyrLeuNH_2$	11.66[a]	0.29	242-243°
BocL(D)TyrCam	6.33[b]	0.48	82-85°
$Boc(D)TyrLeu\ NH_2$	9.75[b]	0.42	200°
$Boc\ Tyr\ Leu\ NH_2$	13.6[b]	0.42	155°

a and b respectively with 35% and 49% v/v $MeOH/H_2O$ eluent.
c eluent for t.l.c. = CH_2Cl_2 containing 10% v/v CH_3OH

- <u>Boc Tyr Cam</u> : Boc Tyr (20 mmol) is dissolved in 100 ml of a water/ethanol mixture (30/70 v/v). $CsHCO_3$ (30 mmoles) is added at 5°. After half an hour, the solution is evaporated under vacuum. The residue is further dried in a dessicator over P_2O_5 overnight.

This product is dissolved in anhydrous DMF (150 ml) containing α-chloroacetamide (30 mmol). The reaction is stirred during 36h at 20°. The solvent is evaporated under vacuum, the residue is dissolved with ethyl acetate (200 ml).The organic phase is washed with cold 5% $NaHCO_3$ solution, water, 10% citric acid solution and water, dried over Na_2SO_4 and evaporated under vacuum.

The product is purified by recrystallization in dichloromethane (Yield 65%)

^1H n.m.r.: DMSOd6, δ ppm, J_{Hz}; 1.34, Boc(9H)(s); 2.76, TyrβU(1H)^2JβUβD=-13.4, ^3JβUα=9.61,(dxd); 2.99, TyrβD(1H)^2JβDβU=-13.4, ^3JβDα=4.2,(dxd);

4.13-4.26, Tyr α(1H),(m); 4.45, CamCH₂(2H),(s); 6.67, TyrεE'(2H)³JE δ=8.35,(d); 7.05, Tyr δδ'(2H)³JE δ=8.35,(d); 7.36, TyrNH(1H)³JNα=7,(d); 7.37&7.4,CamNH₂(1H(s)+1H(s)); 9.25, TyrOH(1H),(s).
C, H, N, O, %=Calc. : 56.85, 6.56, 8.29, 28.3; found : 57.14, 6.77, 8.15, 27.74.

- Boc D Tyr Cam : the same process gives the D- enantiomer with the same yield.

- Boc Tyr Leu NH₂:

Leu-NH₂ hydrochloride (10 mmol) is dissolved in 50 ml of a mixture of DCM/DMF (1/1) at 0° with diisopropylethylamine (10 mmol). DCM (50 ml) containing Boc-Tyr (10 mmol) and HOBt (10 mmol)is added to this mixture . Then DCC (11 mmol) is added at 0° and the mixture is stirred 3H at 0° and overnight at 20°. After filtration to discard DCU, the solvent is evaporated under vacuum and the residue dissolved in ethyl acetate. The organic solution is washed with water, 1N HCl, water,5% $NaHCO_3$, brine ; dried over Na_2SO_4 and evaporated under vacuum. After washing with ethyl ether the dipeptide is isolated in 75% Yield - Recrystallization in ethyl acetate-hexane.
¹H n.m.r. : DMSOd6, δ ppm, J_{Hz}; 0.84, Leu δ U(3H)³J δ U'=6.35,(d); 0.875, Leu δ D(3H)³J δ D'=6.4,(d); 1.34, Boc(9H),(s); 1.39-1.62, Leuß'(3H),(m); 2.6-2.66, Tyrß U(1H)²JßUßD=-13.5, ³JßUα=10.1,(d); 2.82-2.87, TyrßD(1H)²JßDßU=-13.5, ³JßDα=4.3,(dxd); 4.04-4.08, Tyrα(1H),(m); 4.20-4.29, Leuα(1H),(m); 6.635, TyrεE'(2H)³J δε=8.23,(d); 7.015, Tyr δ δ'(2H)³J δ E =8.03,(d); 6.82,TyrNH(1H)JNα=8.2,(d); 6.95&7.19, LeuNH₂(1H,(s)+1H,(s)); 7.73, LeuNH(1H)JNα=8.23,(d); 9.09, TyrOH(1H),(s).
C,H,N,O, % = calc. : 61.12, 7.95, 10.69, 20.36; found : 61.17, 8, 10.43, 20.40.

- Boc D Tyr Leu NH₂ is identically obtained in 70% yield
¹H n.m.r. : DMSOd6, δ ppm, J_{Hz}; 0.72, Leu δ U(3H)³J δ U'=6.33,(d); 0.79, Leu δ D(3H)³J δ D'=6.34,(d); 1.34, Boc(9H),(s); 1.2-1.33&1.4-1.5, Leuß'(3H),(m); 2.65-2.8, TyrßU(2H)²JßUßD=-13.14,³JßUα=6.67,(dxq); 4-4.15, Tyr&Leuα(2H),(m); 6.63, TyrεE'(2H)³J δε =8.2,(d); 7.00, Tyr δ δ'(2H)³J δE=8.2,(d); 7.1, TyrNH(1H)³JNα=8.23,(d); 7.08&7.24, LeuNH₂(1H,(s)+1H,(s)); 8.04, LeuNH(1H)³JNα=8.26,(d); 9.18, TyrOH(1H),(s).

- Ac Tyr Leu NH₂ : (enzymatic kinetic synthesis) (11)

Ac-Tyr-OEt (1 mmole) and Leu-NH₂(HCl) (2 mmoles) were dissolved in 10 ml of dimethylformamide/ 0,2 M carbonate buffer (1:2) (v/v) (pH 9.9). After addition of 37 mg of α CT the reaction proceeded under vigorous stirring at 20° for 10-15 mn and was stopped by addition of 1N HCl to pH 2.8 . The resulting precipitate was removed by filtration and successively washed with water, 0.5M $NaHCO_3$, water, and dried over $MgSO_4$.
The compound was recrystallized from hot ethyl acetate in 65% Yield.
¹H n.m.r. : DMSOd6, δ ppm, J_{Hz}; 0.84, Leu δ U(3H)³J δ U'=6.25,(d); 0.89, Leu δ D(3H)³J δ D'=6.23,(d); 1.43-1.51, Leuß(2H)³Jß'=7; 1.51-1.61, Leu'(1H),(m); 1.78, CH_3C=O(3H),(s); 2.58-2.67, TyrßU(1H)²JßUßD=-13.5, ³JßUα=4,(dxd); 2.84-2.92, Tyrß D(1H)²JßDßU=-13.5, ³JßDα=4.8,(dxd); 4.21, Leuα(1H)JNα=8.24,(dxt); 4.36-4.47, Tyrα(1H),(m); 6.65, TyrεE'(2H)³J δ =8.5,(d); 7.05, Tyr δ δ'(2H)³J δE =8.5,(d);

7.02&7.2,LeuNH$_2$(1H,(s)+1H,(s)); 7.93, TyrNH(1H)JNα=8.31,(d); 8.05, LeuNH(1H)JNα=8.24,(d); 9.2, TyrOH(1H),(s).

C,H,N,O % = Calc. : 60.95, 7.52, 12.54, 19.10; found :60.92,7.83,12.71,18.54.

Copolymers preparation

- α CT derivatization :

To 1 μmole α CT acryloyl chloride is added either:

1) In low excess (10 μmol) in 4 ml borate buffer 0.05 M, pH 8.75 . pH is readjusted to 7.5 by adding 10N NaOH.

2) In large excess (430 μmol) added in 3 portions in 70 ml phosphate buffer 0.15 M, pH = 7.7.

The reaction mixtures are stirred for 4 hours at 4°.

The modified enzyme is purified by dialysis against 1 mM HCl at 4° during one day or more quickly by gel filtration (Sephadex G50, water eluent) and lyophilized. The number of acrylated lysyl groups is determined by TNBS test (21). In the first case.25% substitution has obtained(4 Lys) and in the second . 50% is obtained (7-8 Lys).

- Esterase activity : a 10 μl volume of the enzyme solution at 1 mg/ml is added to the U.V. cell containing 1.5 ml of a solution of ATEE (2mM) in a phosphate buffer 0.1M pH 6.8.

The decrease of absorbance, related to the hydrolysis rate, is recorded at 237 nm.

Relative to the native enzyme, low or high substituted α CT retains 90% or 75% of activity.

- Acryloyl PEG :

The acrylated PEG's are prepared as described previously (23) : PEG (OH)$_2$ M_w = 3400 (8.82 mmol) or MeOPEGOH M_w = 5000 (4.4 mmol) are dissolved in 100 ml benzene and TEA (distilled over ninhydrin) is added (154 or 38 mmol). The mixture is heated to 40°C to achieve complete dissolution, then acryloyl chloride (3.87 ml or 0.97 ml) is added in several portions. Stirring is maintained for one hour at 20°.The organic phase is washed with 3 x 10 ml distilled water, 1 x 10 ml 10% K_2CO_3 , water and dried over $MgSO_4$ and evaporated under vacuum.Modified PEG are precipitated 3 times by ethyl ether from DCM solution.Lack of Cl^- is ascertained by $AgNO_3$ test.

The number of vinyl groups fixed on the PEG chain has been determined by bromine titration (24). The degree of substitution is 80% for bis acryloyl PEG_{3400} and 75% for mono PEG_{5000}.

Copolymerization : Bisacryloyl PEG is a crosslinking agent whereas the monomethylether of monoacrylated PEG_{5000} serves as a matrix-agent :

The acrylated derivatives (enzyme and the two PEG) are dissolved in borate buffer 0.05 M, pH 8.75 saturated by N_2. Ammonium persulfate and TEMED are added in

catalytic amounts to produce free radicals. The mixture is stirred at 4° under nitrogen until polymerization takes place. The resulting gel is broken and washed several times with distilled water.

The enzyme retention can be estimated by a Bradford test (25) on the collected water fractions.

96 to 99% enzyme retention is observed with the above polymerization procedure either with the low or high substituted acryloyl α-chymotrypsin (Gel Ra or Rb).

Even for gel R_c, with a high enzyme content (Enzyme/PEG ratio of 12.5), the enzyme retention remains very high (90%). After lyophilization, gels of different loading degree are obtained :

Ra_4 = 6 µg α CT/mg gel

$Ra_{7.5}$ = 2.65 µg α CT/mg gel $\qquad$ Rc_4 = 93.6 µg α CT/mg gel.

Rb_4 = 46.8 µg α CT/mg gel

$Rb_{7.5}$ = 25 µg α CT/mg gel

Polymer inhibition by tosylphenylalanylchloromethylketone (TPCK) (26). The gel is swollen in phosphate buffer 0.1 M pH 6.8. TPCK, dissolved in MeOH is added (20 fold excess) and the contents shaken overnight at 20°.

7% residual esterolytic activity is observed.

Enzymatic synthesis.

As an example, a typical enzymatic synthesis is described below : ATEE (40µMol) and Leu NH_2 (40µMol) are dissolved in 1.2 ml t-amylalcohol.The gel containing 1 mg α-CT (40 nMol) is added, and the mixture is shaken at 20° for the required time (one or two days). Then 0.2 ml is withdrawn, t-amyl alcohol is evaporated under vacuum and the residue dissolved in CH_3OH is submitted to HPLC analysis (see physical constants table for elution volumes).

After each experiment, the biocatalyst is easily recovered by filtration, resuspended in fresh solvent i.e. t-amyl alcohol containing water (1% w/w). After shaking the vessel during 10 min, the washing solvent is discarded by filtration. The whole cycle is repeated three times afterwards the clean catalyst is ready for a next coupling reaction.

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