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Organic reactions catalyzed by insolubilized enzymes. Part III. Synthesis of peptides catalyzed by α-chymotrypsin immobilized on graft copolymers

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Abstract

α-Chymotrypsin (α-CT) has been covalently immobilized on polyethylene/2-hydroxyethyl methacrylate (PE/HEMA) graft copolymers, with loadings of 33–87 mg of immobilized protein/g copolymer. These enzymatic derivatives have been used in the kinetically controlled synthesis of dipeptides. The influence of the enzymatic loading, grafting and hydrolysis degree of the supports on the synthesis of the model dipeptide Bz–L-Tyr–L-Leu–NH_2 is discussed. Organic solvents with different log *P* values mixed in different proportions with an aqueous buffer have been used as reaction media The best yields with respect to peptide synthesis are obtained with ethyl acetate/buffer pH = 9.0 (2/1 v/v)). H–L-Leu–NH_2 seems to be a better nucleophile than H–L-Ala–NH_2, and Bz–L-Tyr–OEt seems to be a better acyl donor than Ac–L-Phe–OMe under the optimal experimental conditions.

Keywords: α-Chymotrypsin; Grafted copolymers; Immobilization; Peptide synthesis

1. Introduction

It is generally stablished [1,2] that immobilized proteases are useful in the synthesis of peptides; the more common insolubilization methodologies, such as hydrophobic modification via covalent attachment of trichlorotriazine-monomethoxy polyethylene glycol groups to the enzyme [3,4], microencapsulation in reverse micelles [5] or covalent coupling on different supports, e.g., agarose [6,7] Chitosan beads [8], controlled pore glass [9] etc., have been frequently used in the preparation of immobilized derivatives to catalyze the synthetic reactions.

One of the most important features of the insolubilized enzymes to be improved from a biocatalytical point of view is the mechanical behaviour [10]. Therefore, the use of synthetic polymers as supports for enzyme immobilization [11–13] is pertinent for those purposes. Immobilization of bovine pancreatic α-chymotrypsin (EC 3.4.21.1) on polyethylene-based graft copolymers has been described in the literature [14,15], yielding active

Abbreviations: α-CT = α-Chymotrypsin; HEMA = 2-Hydroxyethyl methacrylate; PE = Polyethylene; BTEE = Bz-L-Tyr-OEt (*N*-benzoyl-L-tyrosine ethyl ester); APEE = Ac–L-Phe–OEt (*N*-acetyl-L-phenylalanine ethyl ester); CMC = 1-Cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-*p*-toluensulphonate; DMF = *N*,*N*-Dimethylformamide

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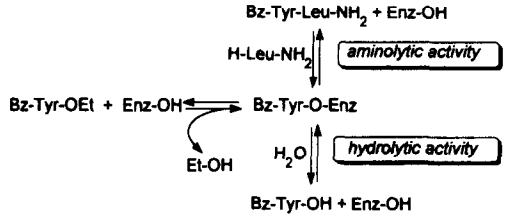
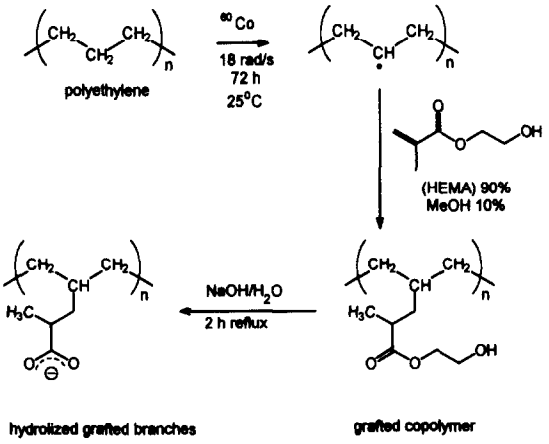


Scheme 1. Kinetically controlled peptide synthesis.



Scheme 2. Preparation of the PE/HEMA copolymers.

Table 1
Characterization of the copolymers

Polymer	Graft [a] %	% Hydrolysis	Activation [b]	Loading [c]	% Active [d]
C1	61.2	41.6	1.21	87.2	14.8
C2	47.6	89.0	2.20	33.3	1.2

[a] Calculated as described by Alves da Silva et al. [15].
[b] mmol COOH g^{-1} copolymer.
[c] mg protein g^{-1} copolymer (determined by the Lowry method) [16].
[d] Calculated by the ratio [(mg enzyme active)/(mg enzyme coupled)] × 100.

enzymatic derivatives for the hydrolysis of esters. In this paper we present the results obtained in the kinetically-controlled peptide synthesis in organic–aqueous biphasic media catalyzed by pancreatic α-chymotrypsin immobilized on those graft copolymers, as depicted in Scheme 1.

2. Materials and methods

2.1. Chemicals

The low-density polyethylene was obtained in powder form from Telcon Plastics, Ltd, UK. α-Chymotrypsin (α-CT) (EC 3.4.21.1.), type II, with a specific activity of 52 units per mg protein, 1-cyclohexyl-3(2-morpholinoethyl) carbodiimide metho-*p*-toluensulphonate (CMC) and hemoglobin were obtained from Sigma Química, Alcobendas, Madrid (Spain). All the other chemicals were obtained from Aldrich Chemical S.A., Alcobendas, Madrid (Spain).

2.2. Preparation of the immobilized enzyme

The graft copolymers were prepared as described elsewhere [14]. Two different graft copolymers, named C1 and C2 were obtained, with 61.2 and 47.6% of grafting, respectively. Subsequently, some of the ester groups of the poly(hydroxyethyl methacrylate) were hydrolyzed by refluxing the samples with 1 M NaOH for 2 h. The overall procedure is depicted in Scheme 2.

The enzyme was covalently coupled through the –COOH groups of the support after an activation step using CMC as previously described [15]: 2 g of copolymer were mixed with 400 mg of CMC in 20 ml of an enzyme solution (4 mg/g copolymer) in 0.1 M acetate buffer, pH = 5.00. The mixture was stirred for 20 h at 4°C, and the amount of enzyme immobilized determined by the Lowry procedure [16]. To check the enzymatic activity of the derivatives, named C1–CT and C2–CT, hemoglobin was used as substrate [17]. Table 1 shows the characterization of both copolymers (C1 and C2), and the loading and activity of the insolubilized derivatives C1–CT and C2–CT.

2.3. Peptide synthesis

The enzymatic synthesis of dipeptides was performed using 0.1 M Tris/HCl buffer, pH = 9.00 as aqueous medium, with different amounts of the organic consolvents with 1/4 (M/M) acyl donor/nucleophile ratio (acyl donor concentration, 10 mM). The reaction mixture (total volume = 20 ml) was placed in a thermostated water bath at 25°C and stirred magnetically for 5 min. Then, an

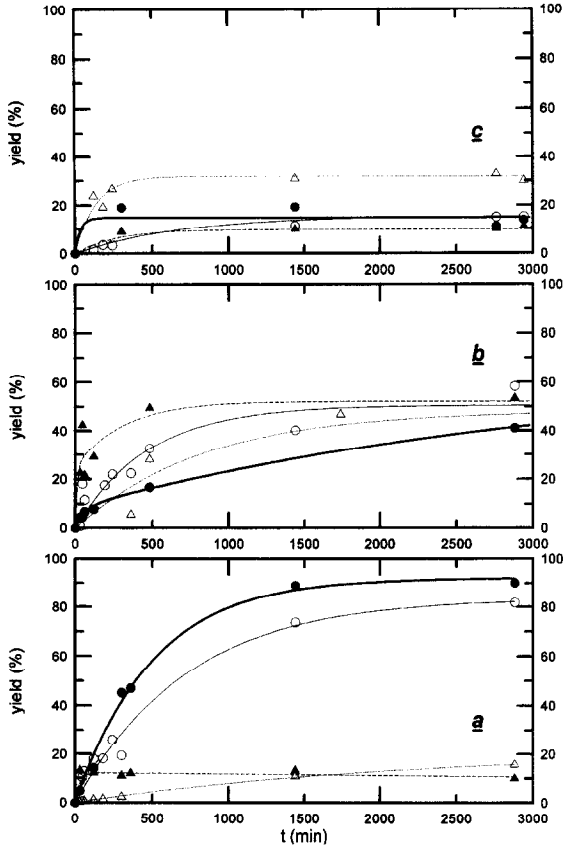


Fig. 1. Kinetically controlled peptide synthesis of Bz–L-Tyr–L-Leu–NH_2 using C1–CT and C2–CT derivatives in different media: (a) ethyl acetate/Tris buffer, pH = 9.00, 0.1 M 99/1, v/v. (b) ethyl acetate/Tris buffer, pH = 9.00, 0.1 M 80/20, v/v. (c) ethyl acetate/Tris buffer, pH = 9.00, 0.1 M 67/33 v/v. —●—Tyr–L-Leu–NH_2, C1–CT as catalyst; —○—Bz–L-Tyr–L-Leu–NH_2, C2–CT as catalyst; - - ▲ - -Bz–L-Tyr–OH, C1–CT as catalyst; · · · △ · · · Bz–L-Tyr–OH, C1–CT as catalyst.

Table 2
Kinetically controlled peptide synthesis of Bz–L-Tyr–L-Leu–NH_2 using the C1–CT derivative (55 mg, equivalent to 4.80 mg of native enzyme)

Solvent composition [a] (v/v)	Peptide yield/time (%/h)	[Peptide]/[Acid] ratio
2/1	(90 ± 8)/24	(6.8 ± 0.5)
4/1	(41 ± 4)/48	(0.8 ± 0.1)
99/1	(13 ± 1)/49	(1.2 ± 0.4)

Conditions as described in Section 2, Materials and methods.
[a] Ethyl acetate/Tris buffer, pH = 9.0 (0.1 M).

amount of immobilized enzyme was added. To analyze the increase in the reaction yield, samples of 0.05 ml were extracted at different reaction times, mixed with 0.1 ml of ethanol and 0.85 ml of the internal standard (naphthalene in acetonitrile, 0.47 mM) and stored at −15°C. The reaction was monitored by analytical HPLC on a LDC Analytical CM 4000 multiple solvent delivery system fitted with a 4 × 200 mm Nucleosil 128 (10 μ) column. Adequate resolution of samples was accomplished by isocratic dilution with a solvent system composed of a helium-degassed mixture of acetonitrile and deionised water in a proportion of 50/50. The flow rate was always maintained at 0.8 ml/min and analysis lasted 10 min. The elution was spectrophotometrically monitored at 270 nm. The amount of eluted substance was calculated by the internal standard method.

3. Results and discussion

3.1. Influence of the support

Figs. 12 and 1 shows the evolution of the kinetically-controlled synthesis of the model dipeptide (Bz–L-Tyr–L-Leu–NH_2), using amounts of C1–CT and C2–CT derivatives providing similar amount of free enzyme, according to the different loading of both derivatives (see Table 1). The reaction media composition (ethyl acetate/Tris buffer pH = 9.0 (0.1 M)) varied in proportions 2/1, 4/1 and 99/1 (v/v). Table 2 and Table 3 summarize the results, showing the peptide/acid ratio obtained in each case.

From these Tables we can observe how the best yields in peptide (90%) are obtained with the C1–

Table 3
Kinetically controlled peptide synthesis of Bz–L-Tyr–L-Leu–NH_2 using the C2–CT derivative (155 mg, equivalent to 4.82 mg of native enzyme)

Solvent composition [a] (v/v)	Peptide yield/time (%/h)	[Peptide]/[Acid] ratio
2/1	(72 ± 7)/24	(7.0 ± 1.0)
4/1	(58 ± 6)/48	(1.3 ± 0.2)
99/1	(12 ± 1)/49	(0.5 ± 0.1)

Conditions as described in Section 2, 'Materials and methods'.
[a] Ethyl acetate/Tris buffer, pH = 9.0 (0.1 M).

CT derivative, employing a reaction medium of 2/1 (v/v) composition. These results are better than those described by Clapés et al. [18], using 120 mg of α-chymotrypsin adsorbed on Celite, with water-saturated ethyl acetate as medium (93% peptide, 7% acid at 72 h), because similar yields can be obtained with this derivative, using lower amounts of the biocatalyst and shorter reaction times. On the other hand the use of hydrated organic media (biphasic conditions) renders better selectivities than those obtained with monophasic conditions e.g.: DMF/water using α-CT immobilized on agarose (20% DMF, 40 mg α-CT/ml, 42% acid, 53% peptide [6])). Under similar conditions, the use of C2–CT derivative leads to slightly lower peptide yields, although the maximum peptide/acid ratio obtained is similar to that obtained with C1–CT.

When the percentage of ethyl acetate is varied from 66% up to 99% (2/1 to 99/1 ratio), the yield of peptide synthesis diminishes for both derivatives, although the peptide/acid ratio seems to show a different behaviour, being higher for C2–CT in 4/1 conditions, and similar (considering the experimental error) for C1–CT in both 4/1 and 99/1 conditions.

In order to explain these results, we must consider that because of comparative reasons (conditions yielding similar amounts of coupled enzyme), and due to the different loading of both derivatives, the amount of derivative added into the reactor vessel in both cases is different. With the introduction by Reslow et al. [1] of the term aquaphilicity (described as the ability of the support to absorb water from water-saturated diisopropyl ether), the support must no longer be considered as inert, because of the competition established between the carrier and the enzyme for the water present in the medium. Therefore the hydrophilicity or hydrophobicity of the support will play an important role, specially when using media with low water percentages.

Alves da Silva et al. [19] described the relative high swelling capacity of these PE/HEMA copolymers, and its subsequent hydrophilic character. On the other hand, Ramos et al. [20] related the water absorption of the copolymers with the yield of grafting and the percentage of hydrolysis of the HEMA branches.

Table 4
Swelling capacity of C1 and C2 copolymers

Copolymer	% Uptake of H_2O	% Uptake of EtOAc
C1 (unhydrolyzed)	97.0 ± 4.8	10.2 ± 1.3
C1 (hydrolyzed)	95.8 ± 5.7	16.3 ± 2.0
C2 (unhydrolyzed)	128.9 ± 11.9	26.3 ± 1.6
C2 (hydrolyzed)	106.6 ± 10.9	18.2 ± 3.1

Conditions as described in Section 2, 'Materials and methods'.

Therefore, in order to achieve a better understanding of the support influence, some experiments were carried out to measure the swelling capacity of the copolymers in pure water and in ethyl acetate. Thus the copolymeric samples (100 mg) were immersed in distilled water or ethyl acetate (10 cm^3) at 298 K for 24 h. Excess solvent was removed by gentle filtration. The resultant moistened solid was, in each instance, weighed every two minutes over thirty minutes. The initial sorption capacity was obtained graphically after extrapolation at zero time. Each sample was dried to constant mass at 373 K. The % uptake of solvent is given by

$$\frac{W_i(w) - W_f(c)}{W_f(c)} \times 100\%$$

where $W_i(w)$ is the initial wet mass at zero time after filtration and $W_f(c)$ is the final dry mass corrected to allow for changes in blanks. Such changes were negligible. The results obtained are shown in Table 4.

Comparing the data presented in Table 1 and Table 4, we can observe how the C2 support retains higher amounts of solvent regardless of the hydrophobicity of the solvent. Therefore, the swelling capacity of both copolymers must be produced by their different grafting degree rather than by the hydrolysis percentage. Generally speaking, the behaviour of both derivatives is similar, considering the experimental error (5–10%). Nevertheless, as the enzymatic loading is lower in the case of C2–CT, in order to use the same amount

of enzyme in every set of experiments, greater amounts of C2–CT must be employed. Therefore, the influence of the swelling capacity will be greater for this derivative.

When there is a high percentage of aqueous solution in the reaction medium (2/1 conditions, Fig. 1a), the water molecules are distributed between the medium, the polymeric support and the enzyme. Due to the lower loading of C2–CT, and the higher amount used, the enzyme molecules must be surrounded by a greater number of water molecules, therefore working in a more hydrophilic microenvironment, and rendering lower initial rates and yields at 24 h in the peptide synthesis compared with the C1–CT derivative.

If the water amount is reduced (4/1 conditions, Fig. 1b), the competition between EtOAc, support and enzyme for the water molecules increases. For C1–CT, most of the interface medium-derivative water molecules are located around the enzyme, due to its higher loading, and for this reason the enzymatic microenvironment must be very hydrophilic, thus promoting the hydrolytic activity of the enzyme compared with the transferase activity. For C2–CT (lower loading) the water molecules on the interface may be distributed between the enzyme and the support, so that greater yields in peptide, compared with those of acid, are obtained.

When the reduction in the water content is much greater (99/1 conditions, Fig. 1c), the peptide yield is dramatically reduced. The high percentage of EtOAc is responsible for the elimination of most of the water molecules from the enzymatic microenvironment, and therefore the few molecules still surrounding the enzyme in the solid–liquid interface would be strongly retained by the support. Because of this, C2–CT leads to a greater hydrolytic activity compared to aminolysis, while C1–CT renders opposite results (more peptide than acid), showing a higher initial rate, although using both derivatives the final peptide yield is similar.

To conclude, the best peptide/acid ratio is obtained using EtOAc/buffer 2/1(v/v) in all cases.

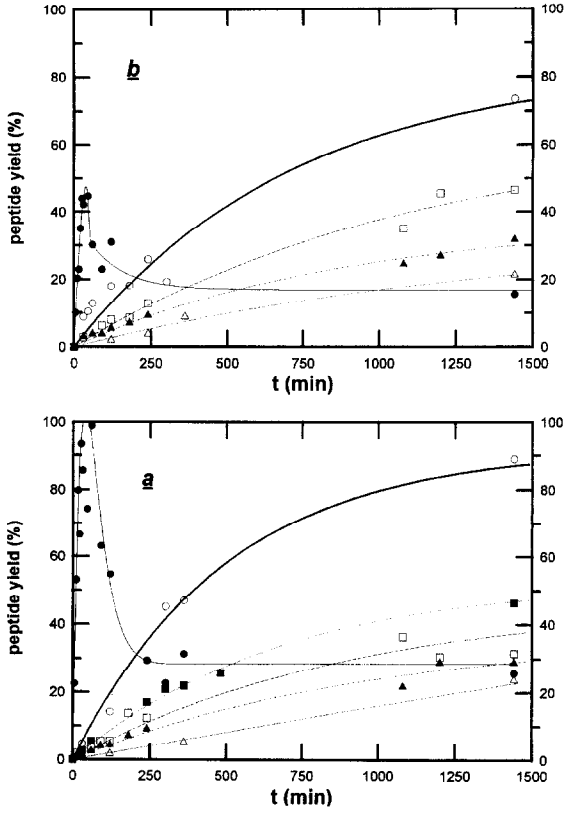


Fig. 2. Influence of the amount of derivative in the synthesis of the peptide Bz–L-Tyr–L-Leu–NH_2, using (a) C1–CT and (b) C2–CT derivatives. Medium, ethyl acetate/buffer pH = 9.0 (2/1 v/v). Conditions as described in Material and Methods. (a) C1–CT: —●— 110 mg (9.59 mg active enzyme); —○—55 mg (4.80 mg active enzyme); · · · ■ · · · 44 mg (3.84 mg active enzyme); - -□- -32 mg (2.79 mg active enzyme); — · ▲ — · 21 mg (1.83 mg active enzyme); — · · △ — · · 10 mg (0.87 mg active enzyme). (b) C2–CT: —●—290 mg (9.65 mg active enzyme); —○—145 mg (4.82 mg active enzyme); - -□- -86 mg (2.80 mg active enzyme); — · ▲ — · 56 mg (1.86 mg active enzyme); · · · △ · · · 26 mg (0.87 mg active enzyme).

In another set of experiments, different amounts of C1–CT and C2–CT were used. in the best reaction medium (ethyl acetate/buffer pH = 9.00, 2/1 (v/v)). The results are shown in Fig. 2. The maximum peptide yield and the peptide/acid ratio obtained using C1–CT and C2–CT are shown in Table 5 and Table 6.

Considering again the peptide/acid ratio as the parameter to be optimized for the synthetic reaction, we can observe that C1–CT presents the classical behaviour observed with α-chymotrypsin immobilized on agarose [6], where the greater the amount of active enzyme added, the greater

Table 5
Peptide synthesis catalyzed by C1–CT

Entry	C1–CT amount (mg)	Enzyme conc. (mg/ml)	Maximum yield (%)		Ratio	Time (h)
			Peptide	Acid		
1	110	0.479	94	6	15.7	0.5
2	55	0.240	90	10	9.0	24
3	44	0.192	47	21	2.2	24
4	32	0.139	32	20	1.6	24
5	21	0.092	29	19	1.6	24
6	10	0.043	24	24	1.0	24

Medium, ethyl acetate/buffer pH = 9.00, 2/1 (v/v). Conditions as described in Section 2, 'Materials and methods'.

the peptide/acid ratio (Table 5, entries 1 (5 mg of derivative, 7/25) and 2 (40 mg of derivative, 40/47)). The optimum amount of C1–CT to be used is 55 mg (entry 2), which yields a concentration of enzyme in the reactor of 0.24 mg ml^{-1}. In these conditions, the peptidase activity is not observed (Fig. 2a). Amounts of C1–CT higher than 55 mg of enzymatic derivative, also allow to obtain good yields (nearly 100%), even reducing the reaction time to half an hour (entry 1), but the amidase activity of the enzyme is highly promoted.

The behaviour of C2–CT is different from C1–CT. When using a large amount of derivative (290 mg, Table 6, entry 1) the amidase activity of the coupled enzyme is present at 0.5 h and therefore the maximum peptide yield obtained is only 45%, with a peptide/acid ratio of 1.3. Lowering the amount of derivative in the reaction vessel down to 145 mg (Table 6, entry 2) allows the elimination of the amidase activity, with a higher peptide/acid ratio, although the further reduction of the derivative amount does not improve the results.

The percentage of EtOAc was increased up to 80%, (4/1 conditions), and the amount of derivative added to the reaction medium was varied. The results are shown in Fig. 3.

In these experimental conditions, C2–CT derivative leads to peptide yields higher than those obtained with C1–CT, employing similar enzymatic loading, and the compared behaviour of C2–CT is even better for low derivative amount. This fact could be expected, because the polymer itself creates the hydrophilic microenvironment needed to allow the enzymatic activity, favouring the aminolytic activity over the hydrolytic activity. Nevertheless, when the percentage of EtOAc is increased to 99%, varying the amount of added derivative does not produce results better than those depicted in Fig. 1c.

3.2. *Influence of the nature of the solvent*

The physico-chemical properties of the organic solvents are known to dramatically influence the kinetically controlled peptide synthesis [18,21–24]. In this way, 1,4-butanediol (hydrophilic solvent, log $P = -1.72$ [25]) was chosen to constitute the organic phase in mixtures of different composition in volume with Tris buffer pH = 9.0, and compared with the previously used ethyl acetate (hydrophobic, log $P = 0.7$ [22]) in order to compare the synthetic behaviour of the C1–CT derivative with an insolubilized derivative of α-CT on agarose obtained by our group [6], the mixture N,N-DMF/buffer pH = 9.00 (20/80, v/v) was also tested as reaction medium. The results for the synthesis of the model dipeptide Bz–L-Tyr–L-Leu–NH_2 are shown in Table 7.

From this Table, we can observe that when the percentage of hydrophilic solvent 1,4-butanediol is increased the peptide yield remains constant although the acid yield decreases. This finding could be explained considering the hydrophilicity of the solvent (log $P > 0$). The greater the per-

Table 6
Peptide synthesis catalyzed by C2–CT

Entry	C2–CT amount (mg)	Enzyme conc. (mg/ml)	Maximum yield (%)		Ratio	Time (h)
			Peptide	Acid		
1	290	0.482	45	35	1.3	0.5
2	145	0.241	72	17	4.2	24
3	86	0.143	47	40	1.2	24
4	56	0.093	32	30	1.0	24
5	26	0.043	21	20	1.0	24

Medium, ethyl acetate/buffer pH = 9.00, 2/1 (v/v). Conditions as described in Section 2, 'Materials and methods'.

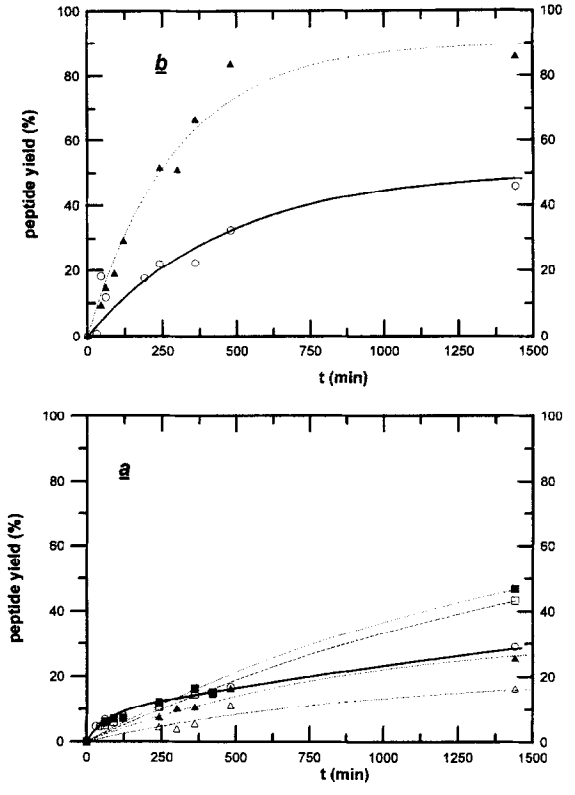


Fig. 3. Influence of the amount of derivative in the synthesis of the peptide Bz–L-Tyr–L-Leu–NH_2, using C1–CT (a) and C2–CT (b) derivatives. Medium, ethyl acetate/buffer pH = 9.0 (4/1 v/v). Conditions as described in Section 2, 'Materials and methods'. (a) C1–CT: —○—55 mg (4.80 mg active enzyme); · · · ■ · · · 44 mg (3.84 mg active enzyme); - -□- -32 mg (2.79 mg active enzyme); — · ▲ — · 21 mg (1.83 mg active enzyme); — · · △ — · · 10 mg (0.87 mg active enzyme). (b) C2–CT: —○—145 mg (4.82 mg active enzyme); — · ▲ — · 58 mg (1.94 mg active enzyme);

centage of 1,4-butanediol, the lower the amount of water the enzyme can use in the hydrolytic process and the lower the acid yield. Very high percentages of 1,4-butanediol (99/1) deactivate the enzymatic derivative. This behaviour is not observed with the hydrophobic solvent (EtOAc log $P = 0.7$, Table 2 and Table 3 and Table 5 and Table 6), which does not deactivate the enzyme completely, as could be expected from a solvent with log $P < 2$ [21]. This behaviour is well documented in the literature both with native or immobilized α-CT [6,21,23,26]. On the other hand, a very hydrophilic solvent such as DMF (log $P = -1.04$ [27] leads to low yield in peptide synthesis when employed at low percentages (DMF/H_2O = 20/80 v/v), favouring the hydrolysis of the ester. If we compare this result with the one reported by our group [6] using the same enzyme immobilized on agarose (20%, DMF 50% peptide, 50% acid at 20 min) we can conclude that the polyethylene grafted with hydroxyethyl methacrylate is less interesting than agarose for the synthesis of peptides in monophasic conditions using DMF as cosolvent. This

Table 7
Influence of the organic solvent in the synthesis of Bz–L-Tyr–L-Leu–NH_2 catalyzed by C1–CT (55 mg added)

Organic cosolvent	Ratio organic/aqueous [a] solvent % (v/v)	Yield (%)		Time (h)
		Peptide	Acid	
1,4-butanediol	66.7/33.3	30.7	67.3	22
1,4-butanediol	80/20	31.0	21.5	23
1,4-butanediol	99/1	– [b]	– [b]	–
N,N-DMF	20/80	2.9	32.0	60

Conditions as described in Section 2, 'Materials and methods'.
[a] Aqueous medium, Tris/HCl buffer pH = 9.0, 0.1 M.
[b] No reaction observed.

finding must be related to the different hydrophobicity of both supports. Probably, the greater hydrophobicity of the copolymer allows the DMF molecules to go around the enzyme in a higher degree than the water molecules, favouring the denaturation of the protein by DMF; this fact has already been reported in the literature [6,28]. In the case of the agarose, the enzyme remains active at the same DMF percentage because the support is very hydrophilic, and therefore there must be more water than DMF molecules in the microenvironment of the immobilized enzyme.

From the above, together with the results obtained with ethyl acetate as organic cosolvent (the greater the percentage of ethyl acetate, the lower the peptide yield, as can be expected from a chemical deactivation of the enzyme by the organic solvent, regardless of the characteristics of the copolymer), we can suggest that the organic solvent must directly interact with the molecules of the immobilized enzyme. The low peptide/acid ratios observed with high amounts of EtOAc (80/20 or 99/1 (Fig. 1)) should be related to the lipidic characteristics of the solvent. This solvents rejects water [29], and, as a consequence, the greater the percentage of EtOAc, the higher the percentage of water in the microenvironment of the immobilized enzyme and the greater the acid percentage obtained by hydrolysis of acyl donor ester. The increase in the hydrolysis percentage produced by an increase in the amount of water in the microenvironment has been reported by Clapés et al. [18], Noritomi et al. [30] and Sinisterra et al. [6].

3.3. Reutilization of the enzymatic derivatives

The reutilization of the biocatalysts is really important if we are intending to scale a reaction up to an industrial level. Thus, both enzymatic derivatives were compared in the same experimental conditions (ethyl acetate/ Tris buffer 4/1 v/v). The amount of derivative added is different: in each case (55 mg and 145 mg for C1–CT and C2–CT, respectively), although the enzyme concentration is similar in both cases, due to the different loadings of C1–CT and C2–CT. The reutilization was carried out by simply separating the biocatalyst from the reaction medium by filtration, careful washing (3×15 ml) with a solvent of identical composition as the reaction medium and drying. The results are shown in Fig. 4.

We can observe a different behaviour in both cases; C2–CT derivative quickly deactivates and so it less interesting from a synthetic point of view. This finding is explained by the great amount of water in the microenvironment of the enzyme, favouring its thermal deactivation. Therefore we need derivatives with high enzymatic loading on polymers with a high degree of grafting as supports, in order to obtain enzymatic derivatives active in peptide synthesis and able to be reused. In the case of the C1–CT derivative, the percentage of acid diminishes with the reutilization. This fact must be related to the utilization of the water molecules adsorbed on the polymer (which is not lyophilized) in the hydrolysis (first reaction) and a progressive dehydration of the polymer by the same reasons. Therefore in the first reaction, strong competition is observed between water and nucleophile molecules and a higher yield in acid is obtained. In the second reaction, there is a lower amount of water in the microenvironment and the kinetically controlled synthesis is promoted in favour of the the hydrolytic process. In the third reaction cycle the peptide synthesis slowly increases by the deactivation of the enzyme, probably because the removal of the water from the enzyme is now dramatic. These results agree with the reported data by Sinisterra et al. [31] in a study on the influence of the dehydration methodology of an agarose–CT derivative in the synthesis of peptides. From this finding we can conclude that the C1–CT derivative is more useful than C2–CT and that this derivative should be dehydrated before it can be used in the synthesis of peptides.

3.4. Influence of the structure of the substrates

The influence of the structure of the substrates in the peptide yield is well documented in the case of immobilized α-CT on agarose [6,26], Celite

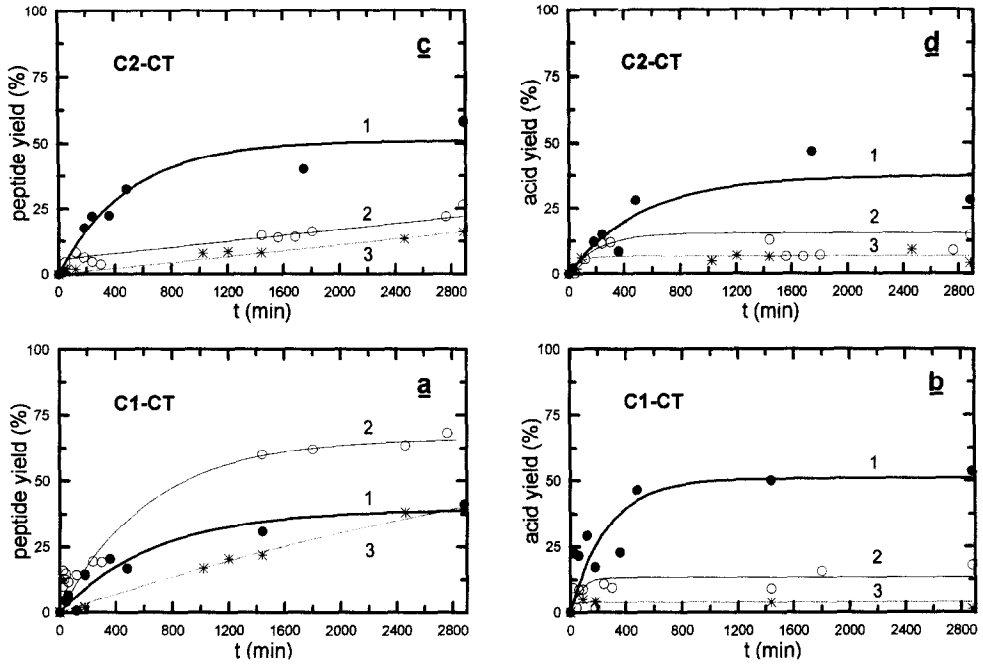


Fig. 4. Reutilization of C1–CT and C2–CT derivatives. Solvent, ethyl acetate/buffer Tris pH = 9, 80/20 (v/v). (a) Bz–L-Tyr–L-Leu–NH_2 yield using C1–CT; (b) Bz–L-Tyr–OH yield using C1–CT; (c) Bz–L-Tyr–L-Leu–NH_2 yield using C2–CT; (d) Bz–L-Tyr–OH yield using C2–CT; —●— first use; —○—second use; · · ·*· · · third use

[18], Chitosan [8], etc. and with PEG-chemically modified α-CT [3,28]. In order to analyze these effects in the case of the best derivative, C1–CT, and in the best experimental conditions for the model dipeptide (EtOAc/buffer) = 2/1, Ac–L-Phe–OEt (APEE) and Bz–L-Tyr–OEt were used as acyl-donors and H–L-Ala–NH_2 and H–L-Leu–NH_2 as nucleophiles. The maximum yields in peptide and acid are shown in Fig. 5.

We can observe that H–L-Leu–NH_2 is a better nucleophile than H–L-Ala–NH_2, and that Bz–L-Tyr–OEt is a better acyl-donor than Ac–L-Phe–OEt. A possible explanation of the low yields obtained in the synthesis of Ac–L-Phe–L-Ala–NH_2 and Bz–L-Tyr–Ala–NH_2 peptides could be the higher solubility of these peptides in water, which makes them very sensitive to the hydrolysis, as reported by Clapés et al. [18]. The observed chemoselectivity both in the acyl donor and in nucleophile agrees with the data reported in the synthesis of peptides in aqueous solution catalyzed by native [29] and immobilized enzyme [6], but disagrees with the results reported in slightly hydrated organic media, where H–L-Leu–NH_2 is a lesser nucleophile than H–L-Ala–NH_2 [18,23]. Kise et al. [23] have explained this different behaviour assuming that the specifity of the α-chymotrypsin for the nucleophile in the peptide synthesis is very similar to S'_1 specificity for the hydrolysis of peptides in aqueous medium. This selectivity is altered in organic media, where the conformation of the protein could be different. Besides, the higher hydrophilicity of H–L-Ala–NH_2 compared with that of H–L-Leu–NH_2 will favour the presence of the former in the water shield of the enzyme in those processes using slightly hydrated organic media, therefore increasing the synthesis when using H–L-Ala–NH_2 as opposed to H–L-Leu–NH_2. Due to the high percentage of aqueous phase present in our biphasic medium (33%), the situation is comparable to an homogeneous aqueous medium, and therefore H–L-Leu–NH_2 will be a better nucleophile.

On the other hand, the hydrolysis yields obtained are lower with Ac–L-Phe–OEt than with Bz–L-Tyr–OEt when Ala–NH_2 is used as nucle-

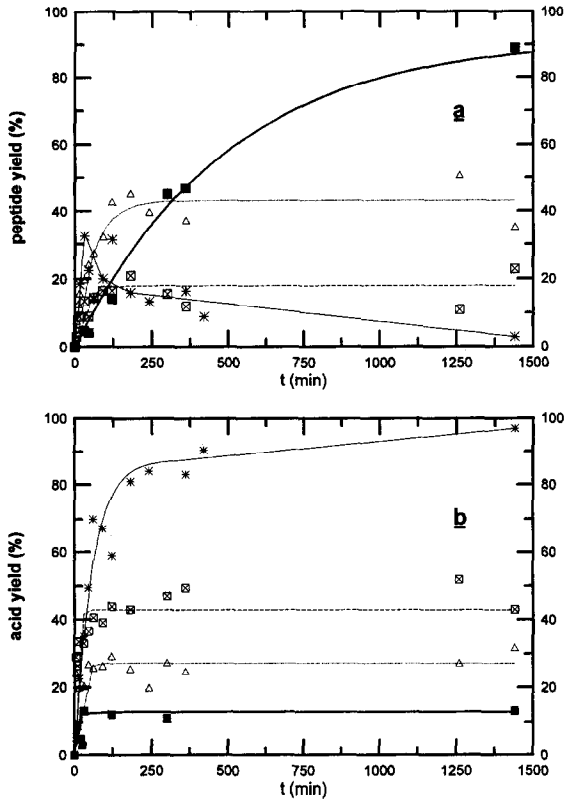


Fig. 5. Synthesis of different peptidic bonds catalyzed by the C1–CT derivative. Solvent, ethyl acetate/buffer Tris pH = 9.0, 2/1 (v/v). (a) AA_1–AA_2–NH_2 yield; (b) AA_1–OH yield. —■— Bz–L-Tyr–L-Leu–NH_2 —*— Bz–L-Tyr–L-Ala–NH_2; ···△··· Ac–L-Phe–L-Leu–NH_2; - -⊠- -Ac–L-Phe–L-Ala–NH_2.

ophile. This finding can be explained by two hypotheses:

i) The low reactivity –as a nucleophile –of H–L-Ala–NH_2 favours the hydrolysis of the acyl–enzyme complex.

ii) The stronger hydrophilic characteristics of alanine compared with leucine favours the arrival of water molecules (from the solvation environment of alanine) to the active site favouring the hydrolysis of the acyl–enzyme complex [32].

Therefore both processes will be more important in the case of the most reactive acyl donor Bz–L-Tyr–OEt

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