Kinetic Analysis of Deactivation of Immobilized α-Chymotrypsin by Water-Miscible Organic Solvent in Kyotorphin Synthesis

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Abstract: Two different immobilized chymotrypsin derivatives were used to synthesize kyotorphin, using N-benzoyl-L-tyrosine ethyl ester and L-arginine ethyl ester as substrates, in water-DMF media. The first was adsorbed onto Celite particles and the second was multipoint covalently attached into polyacrylamide gel. In all cases, the conversion of the carboxyl substrate was carried out in first-order reaction conditions. For the adsorbed enzyme, the reaction kinetics deviated from first-order likely due to a fast irreversible inactivation of enzyme during the reaction time even at low DMF concentration (15–20% v/v). The covalent attachment of enzyme resulted in elimination of irreversible activity loss by organic solvent up to 60% (v/v) of DMF. The catalytic activity of the covalent derivative was conserved as appropriate for performing a synthetic reaction up to 60% v/v of DMF (in comparison to 30% v/v for the adsorbed derivative), showing a clear improvement in its stability against reversible denaturation by this solvent. The selectivity of the synthetic reaction was slightly enhanced (from 40–50%) with the increase in DMF concentration to 80% v/v, but it was significantly improved (to 80%) when L-argininamide was used as nucleophile. © 1999 John Wiley & Sons, Inc. *Biotechnol Bioeng* **65:** 170–175, 1999.
Keywords: kyotorphin; peptide synthesis; immobilized enzyme; enzyme deactivation; organic solvents

INTRODUCTION

The serine protease α-chymotrypsin catalyzes the transfer of an aromatic amino acid (acyl donor) to a wide range of nucleophile components, including water, offering excellent opportunities as catalyst for peptide synthesis (Kullmann, 1987). In this way, the presence of water-miscible organic solvents in the reaction media gives potential advantages, such as increased solubility of poorly water-soluble hydrophobic substrates and the shift of thermodynamic equilibrium of hydrolytic reactions to the synthetic way (Kise et al., 1990; Dordick, 1992). However, the increase in solvent concentration leads to reversible enzyme inactivation, caused by changes in protein tertiary structure (denaturation), while a more prolonged incubation results in irreversible inactivation (Khmelnitsky et al., 1991; Mozhaev et al., 1989; Tomiuchi et al., 1993). The use of immobilized derivatives allows to enhance the enzyme stability (Monsan and Combes, 1988) as well as to design the continuous process for industrial applications (Weethall and Pitcher, 1986).

However, the choice of rational immobilization design is a complicated task because many parameters of the catalyst have to be considered: activity and operational stability, resistance against irreversible inactivation, etc. (Guisán et al., 1991). In this work, two immobilized chymotrypsin derivatives were chosen to carry out the kinetically controlled synthesis of the analgesic dipeptide kyotorphin (Tyr-Arg) using activated substrates. α-Chymotrypsin, adsorbed onto Celite particles, commonly used as a catalyst for peptide synthesis in nonwater media (Adlercreutz, 1991; Lozano et al., 1995, 1996; Reslow et al., 1988a,b), was the first one. The other derivative was α-chymotrypsin, multipoint covalently attached to polyacrylamide gel by copolymerization method. This latter method has been suggested as a promising approach to obtain biocatalysts, stabilized against thermoinactivation (Martinek et al., 1977) and inactivation by organic solvents (Mozhaev et al., 1990). To compare the catalytic abilities of these catalysts, the kinetic analysis of synthetic reactions, catalyzed by both immobilized enzyme derivatives, has been performed.

MATERIALS AND METHODS

α-Chymotrypsin (CT) (EC 3.4.21.1) type II from bovine pancreas was purchased from Sigma Chemical Co. (St. Louis, MO). Methacryloyl chloride (Aldrich Chemical Co., Milwaukee, WI), acrylamide, N, N′-methylenbisacrylamide (Bis-acrylamide), N, N, N′, N′-tetramethylethylenediamide

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(TEMED) and ammonium persulfate (Bio-Rad, Richmond, CA) were used for enzyme immobilization. Pycrylsulfonic acid was from Sigma. Celite 545 (0.01–0.04 mm particle size) was obtained from Merck (Darmstadt, Germany). N-acetyl-L-tyrosine ethyl ester (ATEE), N-benzoyl-L-tyrosine ethyl ester (BTEE), L-arginine ethyl ester dihydrocloride (L-ArgOEt) and L-argininamide dihydrocloride (L-$ArgNH_2$) were obtained from Sigma. Triethylamine (TEA) was from Fluka (Milwaukee, WI). N,N′-dimethylformamide (DMF) of analytical grade (Merck) was used as organic cosolvent. 3-[N-morpholino]-propanesulfonic acid (MOPS) (Sigma) and other buffer components were of analytical grade.

Immobilization of α-Chymotrypsin in Polyacrylamide Gel

α-Chymotrypsin (CT) was immobilized into polyacrylamide gel (PAA) by using a slightly modified two-step copolymerization method proposed by Martinek et al. (1977).

Enzyme Modification Step

To 20 mL of enzyme solution (20 μ*M* in 0.1 *M* phosphate K buffer, pH 8.0), 0.1 mL of methacryloyl chloride dissolved in 0.5 mL of dioxane were added dropwise during 0.5 h of intensive stirring at 4°C. After each addition, the pH of the solution was adjusted to pH 8.0 by titration with 5 *m* KOH. Finally, the solution was incubated without stirring for 0.5 h at 4°C and then 1 h at room temperature.

Copolymerization Step

To 10 mL solution of modified CT, 3.3 g of acrylamide, 170 mg of BIS-acrylamide, and 15 μL TEMED were added. After cooling of the mixture to 0°C in an ice bath, 170 μL of 140 mg mL^{-1} ammonium persulfate solution in water were introduced. The polymerization mixture was rapidly replaced into thin-wall test tubes and kept overnight at 4°C. The resulting polymer was ground into a powder and washed stepwise with water, 0.2 *M* Na_2CO_3, 1 m*M* HCl, water again, and finally with 0.1 *M* MOPS buffer. The derivative was dried for 0.5 h by glass-filter and stored at 4°C.

The number of covalent bonds between enzyme and support (6 ± 1 bonds in our case) was estimated by titration with pycrylsulfonic acid of nonreacted amino groups of the protein after modification step (Panova et al., 1995). After immobilization, the CT-PAA derivative showed an esterase activity of 12–15 U g^{-1} support. One unit of esterase activity was defined as the amount of enzyme that hydrolyzes 1 mmol of ATEE/min under standard conditions of assay (pH 7.0, 40°C).

Immobilization of α-Chymotrypsin on Celite

Celite (3 g) was suspended in 5 mL of 10 m*M* MOPS buffer, pH 7.5. After 15 min of gentle stirring of suspension, the excess liquid was removed by decantation and moist Celite was mixed with 9 mL of 0.2 m*M* solution of α-CT in the same buffer. The mixture was stirred for 30 min at room temperature, then frozen at −30°C, and finally lyophilized. The residual activity of CT-Celite derivative was 4,000 U g^{-1} of support.

Kyotorphin Synthesis

The stock solution of substrates, containing 100 m*M* BTEE, 200 m*M* L-ArgOEt, and 400 m*M* of TEA in 50% v/v solution of DMF in 0.1 *M* aqueous MOPS buffer, pH 8.0, was prepared. Substrates stock solution (0.6 mL) was placed into a screw-cap test tube and diluted with 2.4 mL of DMF-aqueous buffer mixture of desired concentration. In all cases, the final substrate concentrations in assayed mixtures were 20 m*M* BTEE, 40 m*M* L-Arginine ethyl ester dihydrocloride, and 1% (v/v) TEA. The reaction was started by addition of 200 mg of CT-PAA or 60 μL of 10 mg mL^{-1} CT-Celite suspension in water, previously homogenized by magnetic stirring. Each aliquot of CT derivatives has identical esterase activity. The reaction mixtures were incubated at 30°C with gentle stirring. At regular time intervals, homogeneous aliquots of 100 μL were extracted and mixed with 300 μL of 50% (v/v) acetic acid to stop the reaction. Samples were then diluted with 800 μL of mobile phase B (see below), centrifuged (10 min at 2,800*g*), and finally analyzed by HPLC.

HPLC Analysis

Substrate and product concentrations were determined by HPLC using a Nova-Pack C-18 column (15 cm length and 3.9 mm internal diameter, 4 mm particle size and 6 nm pore size). Samples were eluted in linear gradient (Phase A: 40% v/v methanol with 0.5 m*M* SDS; Phase B: 90% v/v methanol with 0.5 m*M* SDS) at 1 mL min^{-1} flow rate. Elution profiles were monitored at 280 nm.

RESULTS AND DISCUSSION

Figure 1 presents the enzymatic mechanism of kinetically controlled peptide synthesis using an amino acid or peptide ester as acyl donor. This model involves the competitive distribution of the rapidly formed acyl-enzyme intermediate between water (hydrolysis) or another amino acid (aminoly-

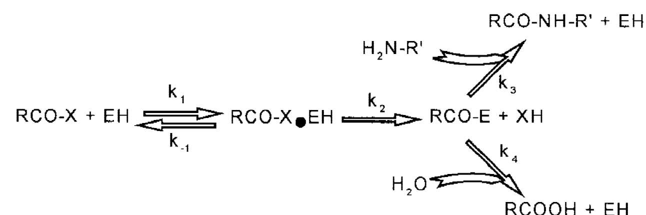

Figure 1. Simplified mechanism of the kinetically controlled peptide synthesis.

sis) as nucleophile acceptors (Bender et al., 1964). Thus, the efficiency in peptide synthesis can be expressed by two parameters: the rate of carboxyl substrate consumption, and the ratio between the peptide product yield and the overall consumed carboxyl substrate.

BTEE Consumption Kinetics

The half-conversion times of BTEE catalyzed by both CT-PAA and CT-Celite at different DMF concentration are presented in Table I. In all tested conditions, CT-PAA was the best catalyst. To gain better understanding of this effect, the kinetic analysis of BTEE consumption for both catalysts was carried out.

In the case of CT-PAA, it was observed that the time dependencies of BTEE consumption, presented by semi-logarithmic plots, were linear at all tested DMF concentrations (Fig. 2A). The reaction rate follows first-order equation:

$$-d[BTEE]/dt = k[BTEE] \quad (1)$$

which after integration can be expressed as:

$$\ln(BTEE/BTEE_0) = kt \quad (2)$$

where $BTEE_0$ is the BTEE concentration at t = 0

This observation is in agreement with Clapés et al. (1988), who reported that the apparent K_m values of chymotrypsin in organic media for N-protected amino acid esters greatly exceed the concentration range used in our case (up to 20 m*M*). So in $K_m >> [S]$ conditions, the substrate consumption follows a first-order reaction kinetics. The limiting step in kyotorphin synthesis was suggested as the slow formation of first encounter complex between enzyme and acyl substrate, determined by k_1 (when $k_2 >> k_1$, $k_2 >> k_{-1}$ and $k_3[NH_2R'] + k_4 >> k_1[RCOX]$ in Fig. 1). In this case, the BTEE conversion rate constant *k*, determined from slopes of Figure 2, is corresponding to the limiting step kinetic constant k_1 in Figure 1, and $k \sim k_1$ should be proportional to the active enzyme concentration. The observed linearity indicated that the enzyme activity was constant during the reaction time even at high DMF concentration (60–70% v/v), so neither inactivation processes nor kinetic phenomena like inhibition byproducts occurred during the process.

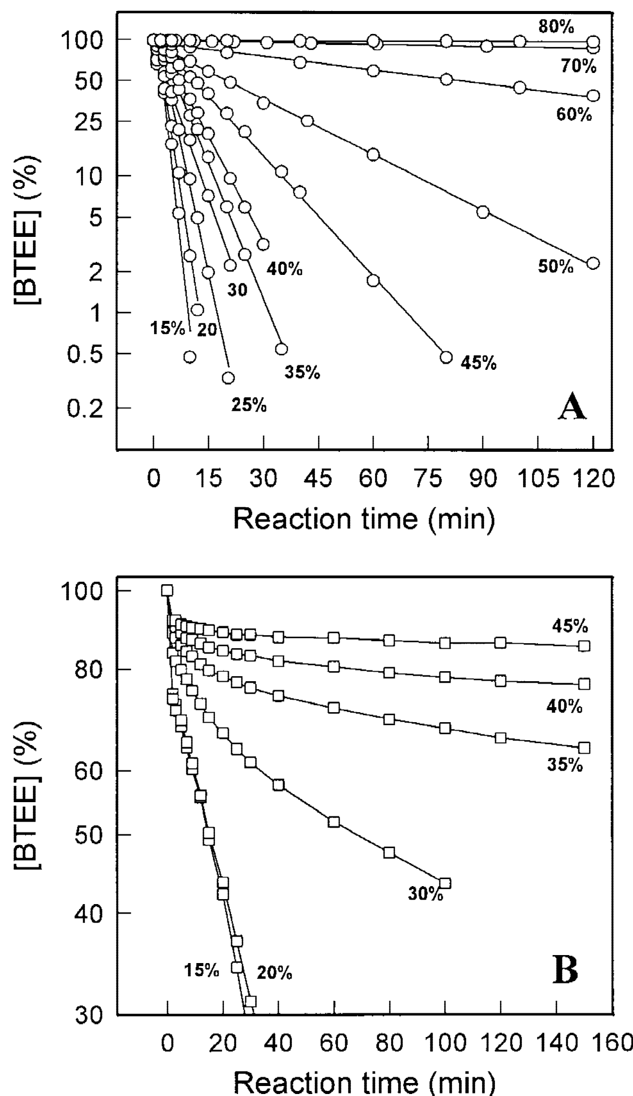

Figure 2. The BTEE conversion kinetics in semi-logarithmic plots of kyotorphin synthesis, catalyzed by CT-PAA (**A**) and by CT-Celite (**B**) at different DMF concentration at 30°C. Reaction conditions: 20 mM BTEE, 40 mM ArgEE, 80 mM TEA, 300 μl of 0.1 M MOPS (pH 8.0).

Table I. Half-conversion times of BTEE in kyotorphin synthesis, catalyzed by CT-PAA and CT-Celite at different DMF concentrations (for reaction conditions, see “Methods”).

[DMF] (% v/v)	CT-PAA (min)	CT-Celite (min)
15	2.0 ± 0.1	14.0 ± 0.2
20	2.8 ± 0.1	17.0 ± 0.5
30	3.7 ± 0.1	68.0 ± 2.0
35	6.4 ± 0.1	>1,200
40	7.2 ± 0.1	n.r.
45	10.7 ± 0.2	n.r.
50	19.0 ± 0.5	n.r.
60	85.0 ± 2.0	n.r.
70	1,350.0 ± 20	n.r.

n.r.: Not reached.
(The values represent the mean ± SD of triplicate determination.)

Figure 3 shows the dependence of the rate constant of BTEE consumption (k_1) from the DMF concentration. The CT-PAA derivative displayed a deactivation profile of classical threshold character, similar to profiles previously reported for aqueous-alcohols and diols solutions (Mozhaev et al., 1990). Such threshold dependencies have been explained by a reversible loss of enzyme activity by organic solvents (Mozhaev et al., 1989; Khmelnitsky et al., 1991). In our case, it was observed that an aliquot of CT-PAA (0.5 mL), being extracted from reaction media containing 60% v/v DMF after 6 h of reaction (corresponding to 88% of BTEE conversion, following the first-order reaction), dis-

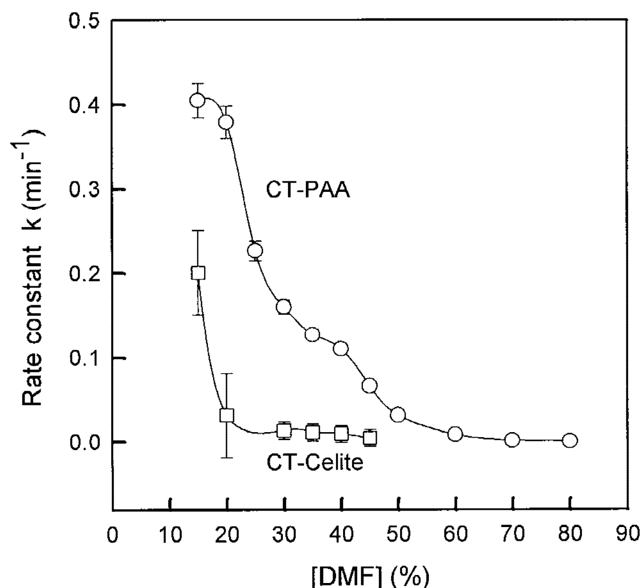

Figure 3. The evolution of the first-order rate constants k_1 of BTEE conversion reaction, catalyzed by CT-PAA (○) and CT-Celite (□), from DMF concentration. For reaction conditions, see Figure 1. The values represent the mean ± SD of triplicate determination.

played the same activity for ATEE hydrolysis as an aliquot of CT-PAA from aqueous solution. Even incubation of CT-PAA in 70% DMF during 24 h resulted in irreversible loss of only 10–12% initial activity. Thus, the decrease in activity of CT-PAA by DMF was related to fast reversible conformational transition of native enzyme into the denatured form. During the reaction time, both enzyme forms were stable against irreversible inactivation process.

With respect to the reaction catalyzed by CT-Celite, which has identical $K_m >> [S]$ conditions as the CT-PAA derivative (Clapés et al., 1992), the BTEE consumption kinetics were nonlinear in semi-logarithmic plots (Fig. 1B). This deviation from first-order kinetic can be related to changes in enzyme activity during the reaction time. It could be explained by reversible denaturation, followed by irreversible inactivation of enzyme, driven by the rate constant k_{in}. It has been shown (Lozano et al., 1995) that irreversible inactivation of CT-Celite by DMF can be adequately described by the series-type two-step deactivation model. However, the most important part of activity decay (from initial 100% to remaining 10–15%) can be satisfactorily described by the exponential function:

$$E = E_0 \exp.(-k_{in} t) \qquad (3)$$

where k_{in} is the first-order deactivation rate constant and E_0 and E are initial and residual concentrations of active enzyme.

In the case of CT-PAA, the contribution of irreversible inactivation process was negligible due to small values of k_{in}, while considering CT-Celite, the exponential deactivation term, resulted in deviation from first-order kinetics reaction (Fig 2B). It is important to note that the noncovalent enzyme-support interactions of CT-Celite can involve desorption of enzyme molecules into the medium, in contrast to covalently attached CT-PAA (Reslow et al., 1988a). In this work, soluble active enzyme was not detected in the bulk medium during reaction. However, the desorption of enzyme molecules from the support, followed by fast inactivation, could be the reason for fast irreversible loss of activity of CT-Celite derivative. Additionally, some kinetic effects and diffusion phenomena (i.e., inhibition or blockage of CT-Celite by products due to its elevated local concentration) could be another route of enzyme activity loss. To avoid the contribution of irreversible inactivation of CT-Celite, the initial BTEE consumption reaction rate was considered to calculate k_1 (Fig. 3). It can clearly be seen that CT-PAA was much more resistant against reversible denaturation by DMF than CT-Celite, which becomes fully inactivated when the DMF concentration was higher than 30% v/v.

Kyotorphin Yield

As a kinetically controlled process, the peptide synthesis occurs by a preferential nucleophilic attack on the acyl-enzyme intermediate by the R'-NH_2 rather than by water (Fig. 1). So the yield of dipeptide formation by the enzyme action could be adequately given in terms of selectivity of the reaction. This selectivity parameter is defined as the ratio between the rates of the peptide synthesis and acyl donor consumption (BTEE) (Lozano et al., 1994).

Figure 4 depicts the evolution of the selectivity parameters of kyotorphin synthetic processes catalyzed by both CT-PAA (Fig. 4A) and CT-Celite (Fig. 4B) derivatives during the reaction time at different DMF concentrations. In the case of CT-PAA, the selectivity continuously decreased due to the L-ArgOEt exhaustion. However, in the case of CT-Celite, the selectivity increased, showing saturation profiles (Fig. 4B). This fact could be explained by positive partition effects of the support, which could enhance the nucleophile concentration in the microenvironment of the enzyme (Adlercreutz, 1991; Reslow et al., 1988b).

Figure 5 shows the dependencies of maximal selectivity of reactions catalyzed by both derivatives on DMF concentration. The increase in DMF concentration produced a linear increase in selectivity of CT-PAA. As the same initial nucleophile concentrations were used, this result could be explained by enhanced reactivity of arginine nucleophile, due to a change in effective pK_b with decrease in the medium polarity (Semenov et al., 1988). Additionally, the affinity of α-chymotrypsin towards L-ArgOEt could be enhanced due to increasing ionic interactions between two aspartic residues (Asp-64 and Asp-38) of enzyme with a positively charged sidechain of substrate (Gololobov et al., 1992). In the case of CT-Celite, the decrease in maximal selectivity, which occurred at DMF concentration higher 30%, could be related to the irreversible inactivation of enzyme.

The high efficiency of CT-PAA to synthesize kyotorphin

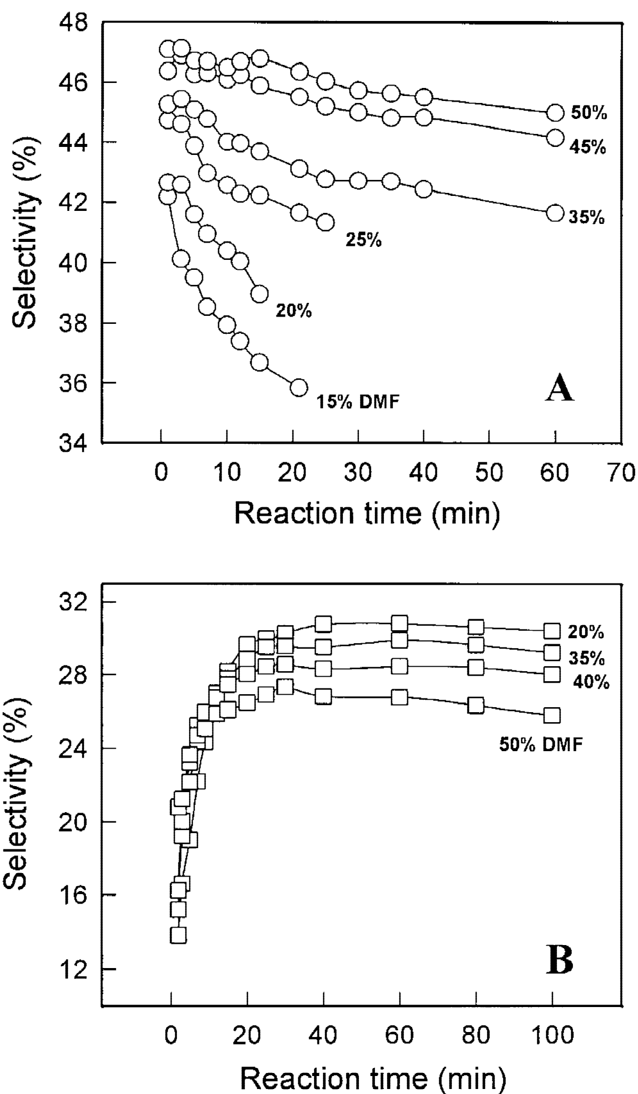

Figure 4. Evolution of selectivity of kyotorphin synthesis reaction, catalyzed by CT-PAA (**A**) and by CT-Celite (**B**), at different DMF concentration. Selectivity is presented as the ratio of kyothorphin yield to overall BTEE consumption during the reaction time.

in DMF media is presented in Table II, in which several standard kinetic approaches to enhance the yield of target product are shown. The kyotorphin yield was significantly enhanced (to 80%) when L-ArgNH_2 was used as nucleophilic substrate. Kullmann (1987) pointed out that amide nucleophiles are more compatible substrates with the geometry of the S'_1 or S'_2 site of CT than corresponding esters. Other reaction conditions also play an important role to optimize the peptide yield. TEA was used to neutralize hydrochlorides nucleophiles and to convert the main-chain amino group into a reactive deprotonated form (Reslow et al., 1988b; Semenov et al., 1988). The optimal yield was found at equimolarity of TEA with the overall concentration of amino groups. Either an increase or decrease in TEA concentration with respect to the optimal amount caused a decrease in selectivity (Table II). The deficiency of TEA could result in a shift of the equilibrium from the reactive

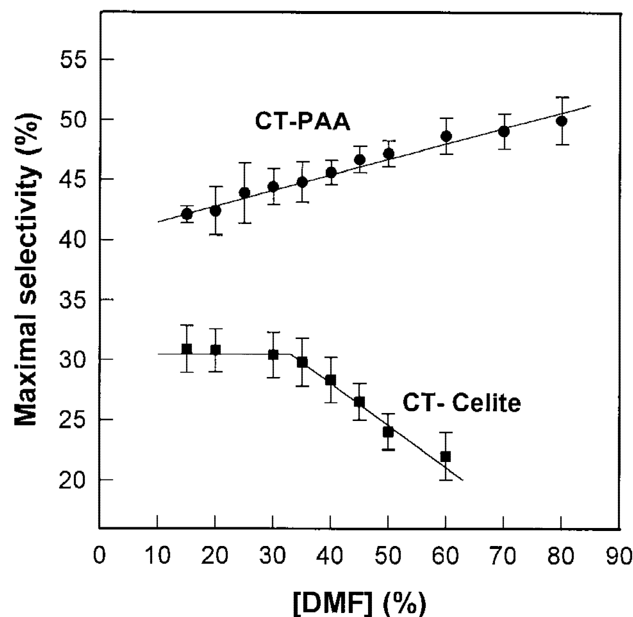

Figure 5. The dependence of maximal selectivity, achieved in kyotorphin synthesis reaction, catalyzed by CT-PAA (●) and CT-Celite (■) from DMF concentration. Selectivity is presented as the ratio of kyotorphin yield to overall BTEE consumption. The values represent the mean ± SD of triplicate determination.

deprotonated form of the main-chain amino group of substrate to the inactive one. The excess of TEA probably resulted in a change of effective pH in the enzyme microenvironment, with unfavorable effects for the activity of enzyme. This suggestion was confirmed by the decrease in the rate constant k_1 at elevated TEA concentrations.

CONCLUSIONS

The kinetically controlled kyotorphin synthesis in water-DMF is much more favorable by catalytic action of CT-PAA than by CT-Celite, due to significantly higher stability of CT-PAA against both reversible and irreversible inactivation and more favorable properties of the support, for example, its high aquaphilicity (Reslow et al., 1988b).

The immobilization design, based on multipoint covalent attachment of enzyme onto polyacrylamide support, allows one to obtain a catalyst with remarkable stability and high

Table II. The rate constant of BTEE conversion and selectivity parameters of kyotorphin synthesis, catalyzed by CT-PAA, at different conditions in 20% v/v DMF solution.

Nucleophile	[Nucleophile] (mM)	[TEA] (mM)	k_1 (min^{-1})	Selectivity (%)
	40	0	0.462	14
L-ArgOEt 2HCl	40	80	0.379	40
	40	160	0.165	34
	120	240	0.195	45
L-ArgNH_2 2HCl	40	80	0.560	81
	120	240	0.226	82

catalytic activity in water-organic media. These results could be perspectives to perform enzymatic reactions under conditions that require multiple, long-term incubations of catalyst in water-organic solutions, for example, in continuous-reactor peptide synthesis.

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