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# Kinetically Controlled Enzyme-Catalyzed Synthesis of Kyotorphin

## An Optimization Study

P. CLAPÉS*, G. VALENCIA, F. REIG, AND J. M. GARCÍA ANTÓN

*Laboratory of Peptides, Biological Organic Chemistry Department, Centro de Investigación y Desarrollo, CSIC, Jorge Girona Salgado 18-26, 08034 Barcelona, Spain*

AND J. MATA

*Chemical Engineering Department, Faculty of Chemistry, University of Barcelona, Diagonal 647, 08028 Barcelona, Spain.*

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## ABSTRACT

Regarding the α-chymotrypsin-catalyzed synthesis of Z-Tyr-Arg-$NH_2$, the effect on the reaction yield of the following experimental factors is discussed: DMF/buffer proportions, reaction temperature, and donor/nucleophile ratios. The experimental design shows that relatively better yields are obtained by increasing the reaction temperature and lowering the cosolvent proportion while maintaining a slight excess of nucleophile. The ascensional line reaches an optimal response surface very flat voided of a well-defined maximum.

**Index Entries:** Kinetic control, of kyotorphin synthesis; enzyme catalysis, of kyotorphin synthesis, synthesis, of kyotorphin; kyotorphin, synthesis of; optimization, of kyotorphin synthesis.

*Author to whom all correspondence and reprint requests should be addressed.

## INTRODUCTION

The dipeptide Tyr-Arg (kyotorphin) has been isolated from the bovine brain and has probed to induce analgesia when injected intracisternally into the rat brain. However, it has been found to produce weak inhibitory activity toward the electrically induced contraction of the longitudinal muscle of the guinea pig ileum (GPI) (*1,2*). Although the details of the mechanism still remain to be determined, it does not bind to specific opioid receptors, suggesting that the naloxone-reversible analgesic effects are mediated by enkephalin release (*3*).

This peptide sequence, supporting different protective groups, has been already synthesized by both chemical and enzymatic means (*4–6*). However, our current interest in small opioid peptides and enzymatic synthesis has led us to study some aspects of the coupling reaction between Tyr and Arg to yield kyotorphin as follows:

$$\text{R-Tyr-OMe} + \text{Arg-R}' \xrightarrow{\alpha\text{-chymotrypsin}} \text{R-Tyr-Arg-R}' + \text{R-Tyr-OH}$$

On the basis of a controlled kinetic approach, the choice for a catalyst was α-chymotrypsin, and, for an organic cosolvent, DMF. The donor ester (R-Tyr-OMe) was used as the benzyloxycarbonyl derivative and the nucleophile (Arg-R′) as the amide form. The reaction yielded as condensation products the highly water-soluble dipeptide Z-Tyr-Arg-$NH_2$ and the byproduct Z-Tyr-OH resulting from substrate hydrolysis.

Despite the considerable research effort dedicated to the study of the use of α-chymotrypsin in peptide synthesis (*7,8*), a clear picture of the best reaction conditions has yet to emerge. As a result of this situation, a great dispersion in reaction conditions is currently in use. To throw light on this point, the present study deals with the optimization of three important parameters which greatly affect the reaction course. Thus, we have examined the effect on the reaction yield of cosolvent/buffer proportion, reaction temperature, and donor/nucleophile ratio. A Box-Wilson method has been selected using a $2^3$ factorial experimental design with four replicated center points to estimate the experimental error (*9,10*). For these purposes, a first set of reactions was carried out in a carbonate buffer-DMF (45, 50, and 55%) solutions containing 1:0.7, 1:2, and 1:3.3 (*M/M*) ratios of Z-Tyr-OMe to Arg-$NH_2$ at temperatures of 10, 15, and 20°C.

To confirm the first set of data a second, more reduced design has been conducted. At a fixed substrate ratio of 1:1.5, experiments at 10, 20, and 30°C and 45, 55, and 65% DMF have been performed.

Finally, in light of previous results and to approach as closely as possible the optimal reaction conditions, five more experiments have been conducted at a substrate ratio of 1:1.5 and cosolvent proportions of 30, 35, 40, 45, and 50% DMF, at temperatures 15, 22.5, 30, 37.5, and 45°C.

## MATERIALS AND METHODS

The donor ester Z-Tyr-OME (mp = 91–92°C; $[\alpha]_D^{20} = -32.6$, c = 2 DMF) (*11*) (mp = 92–93°C; $[\alpha]_D^{23} = -32.7$, c = 2 DMF) was prepared in our laboratory by standard procedures. Ammonolysis of Arg-OMe·2HCl (mp = 195–196°C; $[\alpha]_D^{20} = 19.1$, c = 2.5 MeOH) (*12*) (mp = 196°C; $[\alpha]_D^{17} = 21.7$, c = 2.5 MeOH) by MeOH/$NH_3$ yielded the nucleophile, Arg-$NH_2$·2HCl·$H_2O$ ($[\alpha]_D^{20} = 13.5$, c = 1 $H_2O$) (*13*) ($[\alpha]_D^{24} = 13.8$, c = 1 $H_2O$). Bovine α-chymotrypsin (45 mU/mg) was obtained from Merck. Other chemicals were of the purest commercial grade available.

Enzymatic reactions were performed in a carbonate buffer (0.2*M*, pH = 9)-dimethylformamide mixtures containing 45, 50, 55, or 65% DMF, 30 mM Z-Tyr-OMe with various donor ester/nucleophile ratios (1:0.7, 1:2, 1:3.3, 1:1.5) and 10 μM α-chymotrypsin. Four sets of reaction temperatures (10, 15, 20, and 30°C) were assayed. After trial experiments, the whole set of reactions was monitored during 20 min. The yields reported are those reached after 10 min when a complete reaction conversion was achieved or the reaction rate was close to zero.

Frozen samples were analyzed by HPLC on a Perkin-Elmer series 2 system fitted with a 250 × 4 mm Spherisorb ODS2 (10 μ) column eluted isocratically with an ACN/phosphate buffer (0.02*M*, pH = 7) (50:50 v/v) at a flow rate of 0.8 mL/min and ultraviolet detection at 230 nm. Under such conditions capacity factors were k′ (Z-Tyr-Arg-$NH_2$) = 1.56 and k′ (Z-Tyr-OMe) = 2.13.

Characterization of Z-Tyr-Arg-$NH_2$ was performed after purification by gel filtration chromatography used Sephadex G-25 and further semi-preparative HPLC on a $C_{18}$ column (10 μ) and ACN/water (50:50 v/v) as the eluting system, by means of amino acid analysis, $^1H$ NMR spectroscopy, and FAB-MS.

## RESULTS AND DISCUSSION

The experimental design and the observed results presented in Table 1 show the influence of the factors examined upon the reaction yield. Condensation product yields have been calculated by a ratio between the amount of condensation products and either Z-Tyr-OMe or Arg-$NH_2$, depending on which is in minor proportion in the reaction media. Byproduct (Z-Tyr-OH) yield is only referred to as Z-Tyr-OMe.

To quantitatively assert how the three experimental factors affect the reaction yield we have fitted the experimental values to a first-order equation. The resulting calculations and an F-test for the experimental data are presented in Table 2. It should be noted from Eq. (1)

$$r = 87.83 + 3.46\left(\frac{\text{SR-2}}{1.3}\right) + 3.25\left(\frac{T\text{-}15}{5}\right) - 3.25\left(\frac{\%\text{C-}50}{5}\right) \quad [1]$$

Table 1
Experimental Design Presenting the Conditions Assayed and the Reaction Yields of Aminolysis (Z-Tyr-Arg-$NH_2$) and Hydrolysis (Z-Tyr-OH) Obtained[a]

| Substrate ratio | Temp., °C | DMF, % | Yield Z-Tyr-Arg-$NH_2$, % | Yield Z-Tyr-OH, % |
|---|---|---|---|---|
| 1/3.3 | 10 | 45 | 97 | 6 |
| 1/3.3 | 20 | 45 | 90 | 7 |
| 1/3.3 | 10 | 55 | 87 | 6 |
| 1/3.3 | 20 | 55 | 90 | 6 |
| 1/0.7 | 10 | 45 | 84 | 33 |
| 1/0.7 | 20 | 45 | 92 | 37 |
| 1/0.7 | 10 | 55 | 69 | 12 |
| 1/0.7 | 20 | 55 | 91 | 24 |
| 1/2 | 15 | 50 | 88 | 10 |
| 1/2 | 15 | 50 | 90 | 9 |
| 1/2 | 15 | 50 | 89 | 8 |
| 1/2 | 15 | 50 | 87 | 8 |

[a]Yields of condensation products have been calculated by a ratio between the amount of Z-Tyr-Arg-$NH_2$ and either Z-Tyr-OMe or Arg-$NH_2$, depending which is in minor proportion in the reaction media. Yields of byproducts are referred to only as Z-Tyr-OMe.

that $r$, chemical yield of condensation product given as percentage; SR, molar substrate ratio of Z-Tyr-OMe to Arg-$NH_2$; $T$, reaction temperature (°C); %C, organic cosolvent percentage in the reaction media.

The extent of the contributions of the three factors is equally balanced. However, while substrate ratio and temperature exert a positive effect, cosolvent percentage negatively perturbs the yield values. Nonetheless, the fit is quite good, since the observed $F$-value for $P = 0.01$ is below the predicted.

On the other hand, data can be examined as two separate groups considering experiments with high or low substrate ratios. When similar equation fitting and statistic tests are applied to both sets of data the resulting calculated values summarized in Table 2 are obtained. Experiments with low substrate ratios present good fit, as the F-test demonstrates. Therefore, it can be drawn from Eq. (2)

$$r = 86.26 + 7.50\left(\frac{T\text{-}15}{5}\right) - 4\left(\frac{\%C\text{-}50}{5}\right) \qquad [2]$$

that $r$, chemical yield of condensation product given as percentage; SR, molar substrate ratio of Z-Tyr-OMe to Arg-$NH_2$; $T$, reaction temperature (°C); %C, organic cosolvent percentage in the reaction media.

When temperature increases and cosolvent percentage declines reaction yield rises. On the contrary, the influence of the factors on high substrate ratio experiments is not much greater than the experimental error. We can then reject the resulting equation and no quantitative differences can be outlined. This is in fair accord with expectations. However, de-

Table 2
Statistical Analysis of the Results Observed at the Following First-Order Experimental Designs: (a) Whole Set of Points, (b) Low-Substrate Ratio Level Points, (c) High Substrate Ratio Level Points

| Exptl. design | Crude sum of squares | Regression sum of squares | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Source of variation | | | | | | | |
| | | $b_0$ | $b_1$ | $b_2$ | $b_3$ | Residual | Error | Lack of fit | $F_{exp.}$ |
| (a) | 93074 | 92576 | 96 | 85 | 85 | 232.5 | 5 | 227.5 | 27 |
| (b) | 59896 | 59513 | 225 | 64 | | 94.5 | 5 | 89.5 | 27 |
| (c) | 64518 | 64518 | 4 | 25 | | 42 | 5 | 18.2 | 11 |

spite the high yields obtained, economical considerations and downstream purification makes these conditions impractical.

In order to assess the results obtained at the low substrate ratio, a second experimental design has been devised. A $2^2$ factorial design was carried out varying the temperature (10, 20, and 30°C) and the cosolvent percentage (45, 55, and 65%) while maintaining a slight excess of nucleophile (1:1.5). This choice of substrate ratio has been done on the basis of the byproduct yields resulting from the first set of experiments. Although Z-Tyr-OH yields presented for the low substrate ratio level are not very realistic due to the excess of Z-Tyr-OMe, it seems very likely that such excess could be partly responsible for the increase of byproduct yield. For this reason, in order to keep byproduct yields down to an acceptable level and not to go far from the economical (equimolar) substrate proportions, a slight excess of nucleophile (1:1.5) has been chosen.

The results, showing strikingly different yields, are presented in Table 3. Due to such differences a statistical analysis could not be applied. The most important difference is the low yield obtained on experiments performed at 30°C and 65% cosolvent. Under such conditions the enzyme is ineffective in promoting the synthesis because DMF inhibits the hydrolysis of the substrate ester. On the contrary, the highest yield corresponds to the experiment conducted at 30°C and 45% DMF, as the tendency of the previous set of data predicted.

In addition, at the lowest temperature assayed (10°C), better yields are obtained by using low cosolvent percentage (45%). This is in good agreement with Schellenberger and Jakubke (*14,15*). References cited therein reported that contrary to equilibrium-controlled peptide synthesis, organic solvents do not increase the yield of peptides in kinetically-controlled synthesis.

As a conclusion and despite the lack of decisive influence by the cosolvent, the results given above already show that relatively better yields are obtained when high temperature and low DMF proportions are assayed. Moreover, in this system, a slight excess of nucleophile leads to more successful experiments. Finally, five more experiments were conducted through the ascensional line. The ascensional rate was

Table 3
Second Experimental Design Showing the Influence on the Reaction Yield of Temperature and Cosolvent Percentage at a Fixed Substrate Ratio of 1:1.5

| Temperature, °C | DMF, % | Yield Z-Tyr-Arg-$NH_2$, % | Yield Z-Ty-OH, % |
|---|---|---|---|
| 30 | 65 | 9 | 2 |
| 30 | 45 | 91 | 9 |
| 10 | 65 | 41 | 3 |
| 10 | 45 | 89 | 9 |
| 20 | 55 | 86 | 8 |
| 20 | 55 | 88 | 7 |

Table 4
Experiments Conducted at a Fixed Substance Ratio of 1:1.5 Throughout the Ascensional Line Following the Reaction Yield Tendency Shown by Eq. (2)

| Temperature, °C | DMF, % | Yield Z-Tyr-Arg-$NH_2$, % | Yield Z-Tyr-OH, % |
|---|---|---|---|
| 15 | 50 | 87 | 11 |
| 22.5 | 45 | 85 | 14 |
| 30 | 40 | 88 | 12 |
| 37.5 | 35 | 86 | 13 |
| 45 | 30 | 36 | 7 |

deduced from the tendency followed by the reaction yield, as expressed by the design Eq. (2). One unit decrease of cosolvent percentage leads to a 1.5 unit increase of temperature. Thus a reduction of 5% cosolvent percentage implies approximately 7.5°C temperature increase. Since the yields presented in Table 4 are very similar, the optimal response surfaces have to be very flat. Consequently, a unique optimal point cannot be defined and only a wide range of maximum points are accessible.

Unfortunately, an important limitation of any optimization attempt of an enzymatic synthesis is the few experimental conditions and factors that can be studied at once. Since there is a large number of factors influencing these reactions in very wide intervals, the information retrieved could be considered of a limited use. Although this is a very controversial point, we do believe that such a work is of great value. Indeed, the increasing interest in the industrial applications of enzymatic reactions will require much more such work in scaling up processes. Finally, we want to point out that in applications of these results to other enzymatic syntheses we recommend several other factors be screened, in order to show whether these or others are important or critical as well.

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