JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Synthesis of Morphiceptin (Tyr-Pro-Phe-Pro-NH_2) by Dipeptidyl Aminopeptidase IV Derived from *Aspergillus oryzae*

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Morphiceptin (Tyr-Pro-Phe-Pro-NH_2), tetrapeptide, was synthesized using dipeptidyl aminopeptidase IV (DP IV, EC 3.4.14.5) derived from *Aspergillus oryzae* RIB 915 as a catalyst. Tyr-Pro-OEt was incubated with Phe-Pro-NH_2 in the presence of DP IV under various conditions of temperature, concentrations of ethylene glycol, pH, reaction time, and others. Morphiceptin was obtained at 40% yield under the optimal reaction conditions: substrate, 4 mM Tyr-Pro-OEt·HCl and 20 mM Phe-Pro-NH_2·HCl; enzyme, DP IV, 0.275 nkat; solvent, 60% ethylene glycol containing 20 mM phosphate buffer at pH 7.0; amine, 4.2 mM diisopropylamine at 4 °C for 24 h. Amino group protection was unnecessary for synthesis of morphiceptin by DP IV.

KEYWORDS: Morphiceptin; opioid peptide; tetrapeptide; dipeptidyl aminopeptidase IV (DP IV); *Koji* mold (*Aspergillus oryzae*); enzymatic synthesis; proline-containing peptide

INTRODUCTION

Many physiologically active peptides derived from milk protein have been found and identified during the last 20 years. Among them are opioid peptides, opioid peptide antagonists, angiotensin-converting enzyme inhibitory peptides, macrophage-activating peptides, and platelet aggregation peptides (*1*−*5*). Morphiceptin is a physiologically active peptide obtained from bovine casein by enzyme digestion treatment and has the highest opioid activity among the β-casomorphins (*6*). Its amino acids are Tyr-Pro-Phe-Pro-NH_2. Furthermore, morphiceptin is highly selective for μ receptor; the structure of tetrapeptide amide is considered essential for opioid activity. Recently, morphiceptin has been reported to inhibit oxidation of the cytochrome P450 substrate and spontaneous firing of locus coeruleus (*7*, *8*).

However, many questions remain on the role of morphiceptin *in vivo*, detected in the intestine. To use morphiceptin as a food, we must examine its absorbability, stability, long-term intake effect, effective dose, and so on. A large amount of morphiceptin is required for these studies.

We have studied organic synthesis using enzymes (reaction in reverse direction from usual breakdown) (*9*). Many proteases activate peptide formation under a specific condition; thereby, the method of enzymatic synthesis of peptides has been established. However, the synthesis of proline-containing peptides is difficult because most proteases do not form a peptide bond for proline. Enzymatic synthesis of proline containing has been limited to di- or tripeptides such as Pro-Phe, Arg-Pro, Thr-Pro, Asp-Pro, and Phe-Pro-Gly (*10*−*13*) except for amidated enterostatin (Ala-Pro-Gly-Pro-Arg-NH_2) by the use of dipeptidyl aminopeptidase from *Lactococcus lactis* (*14*). Dipeptidyl aminopeptidase IV (DP IV, EC 3.4.14.5) is a serine enzyme that liberates X-Pro specifically. Because morphiceptin, tetrapeptide, includes -X-Pro- in its amino acid sequence, we assumed that morphiceptin may also be produced by DP IV. Recently, Tachi et al. reported that DP IV is produced easily by the *Koji* mold (*Aspergillus oryzae*) during the soy-sauce brewing process (*15*). This study screened *Aspergilli* with high DP IV activity, partially purified DP IV from *A. oryzae*, and investigated the optimal condition for morphiceptin synthesis using that DP IV.

MATERIALS AND METHODS

Materials. Tyr-Pro-OEt·HCl and Gly-Pro-*p*NA were obtained from Peptide Institute Inc. (Osaka, Japan). Morphiceptin and Phe-Pro-NH_2·HCl were purchased from Bachem AG (Bubendorf, Switzerland).

Cultivation. Six strains of *Aspergilli* isolated from various *Koji* specimens were used: *A. oryzae* 076 M, *A. oryzae* RIB 128, *A. oryzae* RIB 430, *A. oryzae* RIB 915, *A. sojae* 333 M, and *A. sojae* 337 M. Wheat bran *Koji* was prepared according to the method of Ichishima (*16*). Six strains of *Aspergilli* were inoculated on sterilized wheat bran and incubated at 30 °C for 72 h. Their enzyme activity was measured.

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10.1021/jf050420d CCC: $30.25
Published on Web 07/02/2005

Table 1. Activity of DP IV Isolated from *A. oryzae* and *A. sojae*

strain	DP IV (nkat/g of *Koji*)
A. oryzae 076M	0.48
A. oryzae RIB 128	0.67
A. oryzae RIB 430	0.36
A. oryzae RIB 915	0.81
A. sojae 333M	0.27
A. sojae 337M	0.27

Enzyme Assay. Hydrolytic activity of DP IV was determined by the method of Tachi et al. (*15*). The enzyme solution (0.1 mL), prepared as described below, was added to 0.5 mL of 0.45 mM Gly-Pro-*p*NA and incubated at 30 °C for 5 min. After the reaction was stopped by the addition of 0.1 M HCl (0.5 mL), the absorbance at 400 nm was measured. The activity of DP IV was shown by mol/s of *p*NA liberated at pH 7.5 at 30 °C, and that unit is 1 katal.

Partial Purification. Wheat bran inoculated with *Aspergilli* and cultured was added to a 10-fold volume of 20 mM phosphate buffer (pH 7.5) and kept at 4 °C for 3 h; the crude enzyme was obtained by filtration. Ammonium sulfate at 90% saturation was added to the crude enzyme solution and let to stand at 4 °C for 1 day. The precipitate obtained by salting out was collected and extracted again with 20 mM phosphate buffer (pH 7.5). The enzyme was purified partially with Sephacryl S-300 (Amersham Pharmacia) using 20 mM phosphate buffer solution (pH 7.5) as an eluent.

Synthetic Reaction. Ethylene glycol (0−80%), amines, 20 mM phosphate buffer (pH 6.0−7.5), and DP IV (0.250−0.375 nkat) were added to 50 μL of 40 mM Tyr-Pro-OEt·HCl and 25 μL of 400 mM Phe-Pro-NH_2·HCl in a 2 mL microtube to make a final volume of 500 μL. After the reaction for various times, peptides in 5 μL of the reaction mixture were examined by HPLC using 4.6 × 250 mm TSK-GEL ODS-80Ts column (Tosoh Corp., Tokyo, Japan). The column was eluted with a linear gradient of acetonitrile (0−60%) containing 0.1% TFA. The flow rate was 1.2 mL/min, and detection was made at 280 nm. Yields of morphiceptin and Tyr-Pro-OEt were calculated from the peak area of each component in comparison with that of authentic chemicals. After the synthesis reaction, the peak that coincided with morphiceptin was purified by MPLC using 0.1% TFA and acetonitrile as the eluent. The purified compund was analyzed by LC−MS. The ion trap mass spectrometer equipped with an electrospray ionization (ESI) source (Finnigan LCQ, Thermo Electoron Corporation, San Jose, CA) was used for analyzing the structure of the product.

RESULTS

Table 1 shows the activity of DP IV isolated from the six strains of *Aspergilli*. The highest activity was shown by DP IV from *A. oryzae* RIB 915, 0.81 nkat/g of *Koji*. This strain was selected for further experiments.

Table 2. Effect of Temperature on Morphiceptin Synthesis[a]

temperature (°C)	yield (%)	temperature (°C)	yield (%)
−20	12.4	30	0.9
4	13.8	40	0.5

[a] Reaction conditions: 40 mM Tyr-ProOEt·HCl, 50 μL; 400 mM Phe-Pro-NH_2·HCl, 25 μL; DP IV, 0.325 nkat; diisopropylamine, 2.0 μL; ethylene glycol, 55% in 20 mM phosphate buffer at pH 7.5; total volume, 500 μL; reaction time, 24 h.

The presence of four major components was confirmed in the products of the reaction between Tyr-Pro-OEt and Phe-Pro-NH_2 in the presence of partially purified DP IV. One of those products (rt = 26.0 min) was identified as morphiceptin by LC−MS (**Figure 1**). The two peaks at *m*/*z* 522 and 544 shown in the ESI mass spectrum of the product indicated two kinds of adducted molecules, a $[M + H]^+$ and a $[M + Na]^+$, respectively. The fragment peaks, *m*/*z* 408, 359, 262, and 261, generated by collision-induced dissociation of the protonated molecule (MS−MS), indicated b3, y3, y2, and b2, respectively. The fragment pattern was coincident with that of morphiceptin (*17*). From these results, the structure of the product was confirmed as morphiceptin. The morphiceptin yield was 13.8% at 4 °C but less than 1% at 30 or 40 °C (**Table 2**).

In the absence of ethylene glycol, Tyr-Pro-OEt was rapidly hydrolyzed. The morphiceptin yield increased with the increase of the ethylene glycol concentration. The yield was 19.5% in the presence of 60% ethylene glycol (**Figure 2**). The presence of amines such as triethylamine and 1,8-diazabicyclo-[5.4.0]-7-undecene (DBU) did not affect morphiceptin synthesis (data not shown). Application of 0.28 μL of diisopropylamine (final concentration of 4.2 mM) elicited a 31% augmentation in yield even though a further increase in the amount of diisopropylamine decreased the yield (**Figure 3**). The yield in the presence of 4.2 mM diisopropylamine at optimal pH (7.0) was 40.0% (**Figure 4**).

Figure 5 shows the effect of the Phe-Pro-NH_2 concentration on DP IV for morphiceptin synthesis. The increase in the Phe-Pro-NH_2 ratio to Tyr-Pro-OEt was followed by higher peptide synthesis; the optimum level was reached for Phe-Pro-NH_2/Tyr-Pro-OEt = 5:1. The morphiceptin yield increased with an

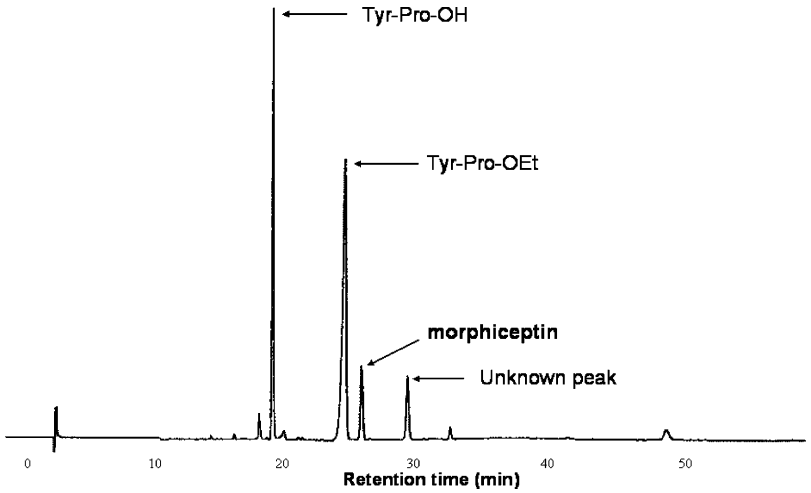

Figure 1. HPLC profile of the reaction mixture. We injected a reaction mixture catalyzed by DP IV (0.325 nkat/tube) using 50 μL of 40 mM Tyr-Pro-OEt·HCl and 25 μL of 400 mM Phe-Pro-NH_2·HCl as the substrate in 55% ethylene glycol containing 20 mM phosphate buffer (pH 7.5) at 4 °C. Conditions for chromatograms: column, TSK gel ODS-80Ts (5 μm); mobile phase, acetonitrile (0–60%) containing 0.1% TFA; flow rate, 1.2 mL/min; detector, UV 280 nm; sample, 5 μL.

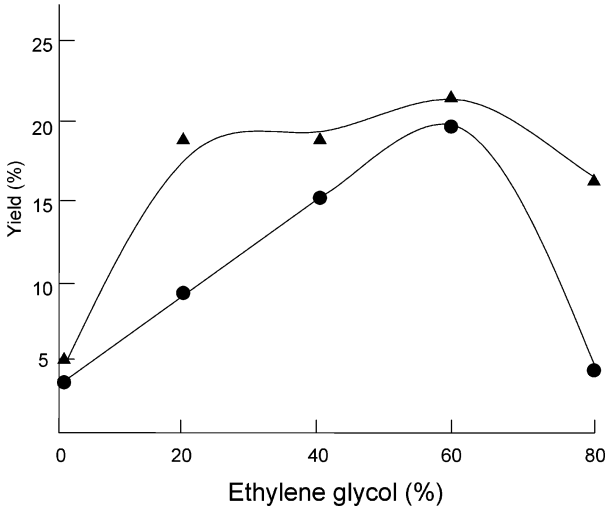

Figure 2. Effect of ethylene glycol on morphiceptin synthesis. Reaction conditions: 40 mM Tyr-Pro-OEt·HCl, 50 μL; 400 mM Phe-Pro-NH_2·HCl, 25 μL; DP IV, 0.325 nkat; pH 7.5, diisopropylamine, 2.0 μL; total volume, 500 μL; reaction temperature, 4 °C; reaction time, 24 h. Morphiceptin (●), Tyr-Pro-OEt (▲).

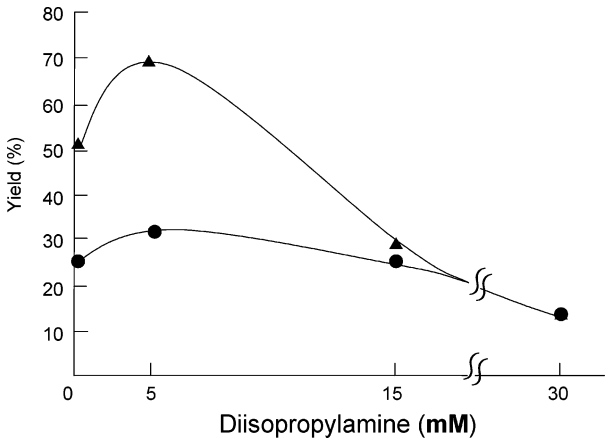

Figure 3. Effect of diisopropylamine on morphiceptin synthesis. Reaction conditions: 40 mM Tyr-Pro-OEt·HCl, 50 μL; 400 mM Phe-Pro-NH_2·HCl, 25 μL; DP IV, 0.325 nkat; ethylene glycol, 60% in 20 mM phosphate buffer at pH 7.5; total volume, 500 μL; reaction temperature, 4 °C; reaction time, 24 h. Morphiceptin (●), Tyr-Pro-OEt (▲).

increasing reaction time, reaching 40.0% at 24 h, but a further increase in the reaction time engendered only slight effects (**Figure 6**). The morphiceptin yield at this condition was 40.0%. The amount of DP IV within the range of 0.250−0.375 nkat only slightly affected the reaction, but the concentration of Tyr-Pro-OEt decreased with an increasing amount of DPIV (**Figure 7**). Therefore, we judged 0.275 nkat of DP IV addition to 500 μL of reaction mixture as optimal.

DISCUSSION

Enzymatic synthesis of peptides has been studied for a long time. Merits of this method are that (1) racemization does not occur during the reaction, (2) protection of the side-chain functional group is unnecessary, (3) side reactions are limited, and (4) the reaction proceeds under mild conditions (*13*). In fact, as shown in **Figure 1**, the reaction solution contained only a few byproducts other than the substrates and a product.

Generally, the peptide bond that can be formed by enzymatic synthesis is determined by the primary specificity of the enzyme.

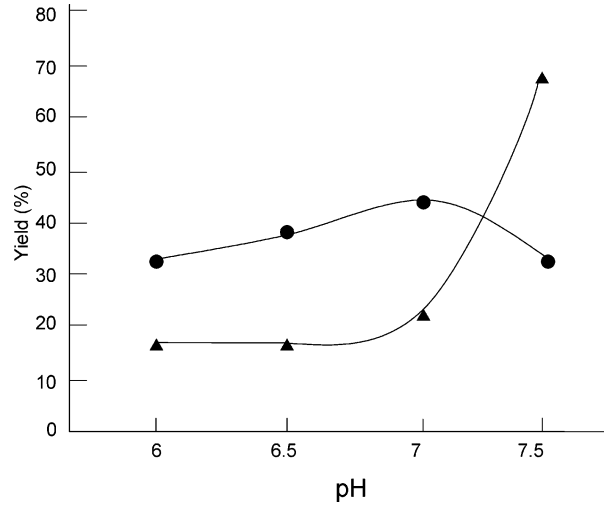

Figure 4. Effect of pH on morphiceptin synthesis. Reaction conditions: 40 mM Tyr-Pro-OEt·HCl, 50 μL; 400 mM Phe-Pro-NH_2·HCl, 25 μL; DP IV, 0.325 nkat; diisopropylamine, 0.28 μL; ethylene glycol, 60% in phosphate buffers of various pH; total volume, 500 μL; reaction temperature, 4 °C; reaction time, 24 h. Morphiceptin (●), Tyr-Pro-OEt (▲).

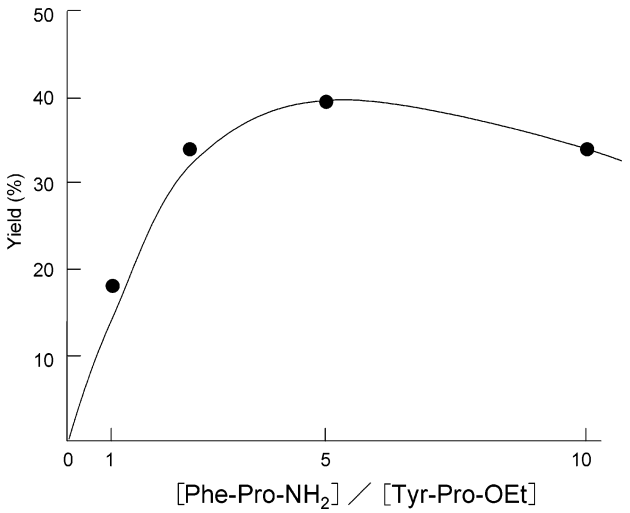

Figure 5. Effect of substrate ratio on morphiceptin yield. Reaction conditions: DP IV, 0.325 nkat; diisopropylamine, 0.28 μL; ethylene glycol, 60% in 20 mM phosphate buffer at pH 7.0; total volume, 500 μL; reaction temperature, 4 °C; reaction time, 24 h.

However, many proteolytic enzymes do not cut the peptide at the position of Pro. DP IV has been found in mammals, yeast, and bacteria (*18*−*20*); it is known to liberate dipeptide from the peptide having Pro at the second position from the amino terminal. We used *Koji* mold and screened strains having a high activity for specific separation of X-Pro. DP IV used in this study was a new enzyme isolated from mold. It had the same molecular weight as DP IV from hog kidney (*15*).

The DP IV used in this study hydrolyzes Gly-Pro-*p*NA and Arg-Pro-*p*NA specifically, but it does not hydrolyze substrates having a protective amino group such as benzyloxycarbonyl, e.g., Z-Gly-Pro-*p*NA (*15*). In this study, we used Tyr-Pro-OEt·HCl, which has no protective amino acid as an acyl donor. Morphiceptin has an amidized carboxyl group as well as gastrin (*21*), amylin (*22*, *23*), and galanin (*24*). The compound with $-NH_2$ seemed effective as the protective group of α-carboxyl for the synthetic reaction. We used Phe-Pro-NH_2 as a nucleophile.

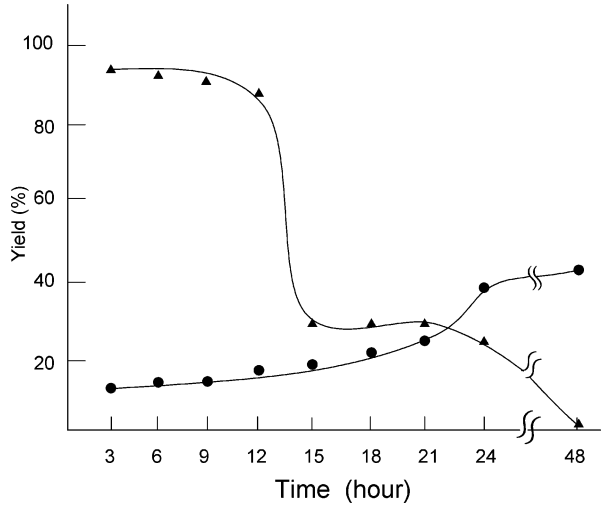

Figure 6. Effect of reaction time on morphiceptin synthesis. Reaction conditions: 40 mM Tyr-Pro-OEt·HCl, 50 μL; 400 mM Phe-Pro-NH_2·HCl, 25 μL; DP IV, 0.325 nkat; diisopropylamine, 0.28 μL; ethylene glycol, 60% in 20 mM phosphate buffer at pH 7.0; total volume, 500 μL; reaction temperature, 4 °C. Morphiceptin (●), Tyr-Pro-OEt (▲).

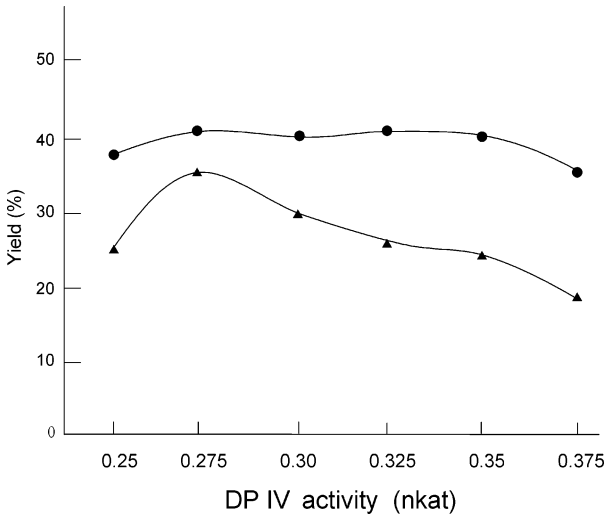

Figure 7. Effect of the amount of DP IV on morphiceptin synthesis. Reaction conditions: 40 mM Tyr-Pro-OEt·HCl, 50 μL; 400 mM Phe-Pro-NH_2·HCl, 25 μL; diisopropylamine; 0.28 μL; ethylene glycol, 60% in 20 mM phosphate buffer at pH 7.0; total volume, 500 μL; reaction temperature, 4 °C; reaction time, 24 h. Morphiceptin (●), Tyr-Pro-OEt (▲).

Tyr-Pro-OEt decreased rapidly in 12−15 h in this study (**Figure 6**). An unknown substance (rt = 29.5 min) increased as the reaction proceeded, and the decrement of Tyr-Pro-OEt coincided with the increment of the substance peak. We think the unknown substance is Tyr-Pro-Phe-Pro-OH because the reaction was monitored at 280 nm and DPIV has amidase activity (*25*).

For synthesis of peptides by protease, effective organic solvents are *N*,*N*-dimethyl formamide, dimethyl sulfoxide, glycerol, ethylene glycol, and 1,4-butanediol (*26*). We examined the effect of glycerol and ethylene glycol because the application of an excess amount of glycerol decreases the rate of decomposition (*10*). We used ethylene glycol as an organic solvent because ethylene glycol addition gave 1.3-fold more morphiceptin yield than glycol addition in our preliminary experiment.

In the present reaction system, the lower the temperature, the larger the amount of morphiceptin synthesized except for at −20 °C. Nilsson et al. suggested that the effect of low temperature is attributable to water activity but that mechanism is not yet known (*27*). However, in the absence of ethylene glycol, little Tyr-Pro-OEt was found in the reaction mixture. On the other hand, the higher the concentration of ethylene glycol, the larger the amount of morphiceptin synthesized. Ethylene glycol is considered to suppress the hydrolysis of Tyr-Pro-OEt to Tyr-Pro-OH and shift the reaction toward synthesis because Tyr-Pro-OEt remained in the presence of ethylene glycol.

Diisopropylamine was added to neutralize hydrochloride; it was effective. Nevertheless, the application of an extra amount of diisopropylamine suppressed the synthesis, probably affecting pH. In fact, the amount of remaining Tyr-Pro-OEt was influenced markedly by pH, suggesting the importance of pH in morphiceptin synthesis. The Phe-Pro-NH_2 concentration was 5 times higher than the Tyr-Pro-OEt in the optimum condition. The morphiceptin yield did not increase at the level Phe-Pro-NH_2/Tyr-Pro-OEt = 10:1. The lack of increase is attributable to the consumption of all Tyr-Pro-OEt within 24−48 h. Although the amount of enzyme (DP IV) added did not largely affect the percentage of synthesis, it affected the hydrolysis of Tyr-Pro-OEt. These results imply the following optimum condition for morphiceptin synthesis: substrate, Phe-Pro-NH_2/Tyr-Pro-OEt = 5:1 (20 mM/4 mM); enzyme, DP IV, 0.275 nkat; solvent, 60% ethylene glycol in 20 mM phosphate buffer at pH 7.0; diisopropylamine, 0.28 μL (4.2 mM); total volume, 500 μL; reaction temperature, 4 °C; reaction time, 24 h. Under this condition, the yield was about 40%.

The possibility of enzymatic synthesis of morphiceptin has been reported (*14*) but has not been demonstrated. We synthesized morphiceptin enzymatically in this study. A protective amino group such as the benzyloxycarbonyl group has been effective in many enzymatic approaches, but such a protective group is unnecessary for enzymatic synthesis of peptides using DP IV. This fact is an important merit of this method.

ACKNOWLEDGMENT

We thank Dr. Kenji Kobata, University of Shizuoka, for measuring the LC−MS data and Dr. Eiji Ichishima, Soka University, for advice and helpful discussion.

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Received for review February 23, 2005. Revised manuscript received June 3, 2005. Accepted June 7, 2005.

JF050420D