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Report

A Novel Method for the Synthesis of Kyotorphin, Tyr-Arg, and ^{3}H-Tyr-Arg, Catalyzed by Tyrosyl-tRNA Synthetase from *Bacillus stearothermophilus*

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A novel method of dipeptide synthesis is described that can be carried out in aqueous solution and does not require complicated protecting and deprotecting procedures. An analgesic neuropeptide named kyotorphin, H-Tyr-Arg-OH, was synthesized from unprotected tyrosine and arginine in a new enzymatic reaction catalyzed by immobilized tyrosyl-tRNA synthetase from *Bacillus stearothermophilus*. The reaction could be a useful tool in the syntheses of radioisotope-labeled oligopeptides to be used in receptor binding assays. ^{3}H-Kyotorphin was prepared by this method at a yield of 72% and could be used in receptor binding assays after a single chromatographic separation.

KEY WORDS: kyotorphin; tyrosyl-tRNA synthetase; *Bacillus stearothermophilus;* peptide synthesis.

INTRODUCTION

Kyotorphin (H-Tyr-Arg-OH) is an analgesic neuropeptide, which was originally isolated from bovine brain by Takagi *et al.* (1). Recent studies revealed that kyotorphin may play a physiologically significant role as a neurotransmitter/neuroregulator (2–6). There are increasing demands for radioisotope-labeled kyotorphin of a high specific activity to study the histochemical localization of kyotorphin receptors in the brain. However, since chemically synthesized ^{3}H-labeled kyotorphin was unstable and possessed a relatively low specific activity, the use of the labeled compound has been restricted. Recently, enzymatic peptide synthesis and, specifically, protease-catalyzed condensation of amino acid derivatives have been intensively investigated (7). We have been interested in peptide synthesis catalyzed by aminoacyl-tRNA synthetase (ARS) (8):

$$AA_1 + AA_2 + ATP \rightarrow AA_1\text{–}AA_2 + AMP + PP_i$$

where AA is amino acid and PP_i is inorganic pyrophosphate.

In a previous paper (9), we have reported general properties of ARS-catalyzed peptide synthesis, and a variety of dipeptides was prepared by this method. One of the problems in the application of this reaction is the commercial unavailability of the enzyme. Furthermore, during the peptide synthesis reaction, inactivation of the ARS was detectable. Therefore, it is desirable to immobilize ARS on water-insoluble material and to use this immobilized catalyst repeatedly.

In this paper, synthesis of the analgesic peptide, kyotorphin, catalyzed by immobilized tyrosyl-tRNA synthetase (TyrRS) is reported. Application of this method to the synthesis of radioisotope-labeled kyotorphin is also reported.

MATERIALS AND METHODS

Reagents. Tyrosyl-tRNA synthetase was purified from a thermophile, *Bacillus stearothermophilus*, NCA-1503, according to the procedure reported elsewhere (10). CNBr-activated Sepharose 4B was from Pharmacia. Authentic kyotorphin (L-Tyr-L-Arg) and the diastereomer (L-Tyr-D-Arg) were purchased from Sigma Chemical Co. and ^{3}H-tyrosine was from New England Nuclear. All other reagents were of the highest quality commercially available.

Assay of TyrRS. Activity of TyrRS was assayed by amino acid hydroxamate formation or with the aminoacyl-tRNA method (11). One unit of TyrRS was defined as the amount of enzyme necessary to produce 1 nmol of tyrosine hydroxamate at 40°C or tyrosyl-tRNA at 30°C in 10 min. All units are determined with the hydroxamate assay unless indicated otherwise.

Immobilization of TyrRS. A stock solution of TyrRS in 50% glycerol was dialyzed against 50 m*M* potassium phosphate buffer, pH 8.0. To 20 ml of this dialyzed solution (6700 U/ml), 2 g of CNBr-activated Sepharose 4B which was previously treated with 1 m*M* HCl was added and shaken mildly for 30 min at 30°C. After filtration, the resin was further treated with 50 ml of 100 m*M* Tris solution (pH 9.0) to de-

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stroy the unreacted CNBr groups. The resulting immobilized enzyme (3.4 g) had a specific activity of 11,000 U/g wet resin, and the activity yield was 28%.

Kyotorphin Synthesis by Immobilized Enzyme. To a solution (20 ml) of 100 m*M* arginine, 50 m*M* Bicine (pH 8.5), 20 m*M* magnesium chloride, 20 m*M* ATP, and 5 m*M* tyrosine was added 3.4 g of immobilized enzyme and the resulting solution was shaken gently at 45°C. In a typical run, kyotorphin was produced at a concentration of 2 m*M* (40% yield) after 2 days. Immobilized enzyme was filtered and used repeatedly. The progress of the reaction was monitored on a Shimadzu HPLC LC-4A equipped with a UV detector (275 nm), employing a Shimadzu ISC-07/S1-504 cation-exchange column (4 × 150 mm). The mobile phase consisted of (A) 0.117 *M* sodium citrate, 0.05 *M* sodium borate, and 0.348 *M* sodium chloride, pH 9.4, and (B) 0.067 *M* sodium citrate, pH 4.25. The elution was isocratic for 10 min at 50% A and by linear gradient from 50 to 90% A in 10 min at a flow rate of 1 ml/min.

Isolation and Purification of Kyotorphin. Kyotorphin was purified according to the method of Yajima *et al.* (12). The reaction mixture from four runs (80 ml) containing 54 mg of kyotorphin was combined and concentrated in a rotary evaporator to 20 ml and the resulting solution was applied on 270 ml of a CM-cellulose column (45 × 170 mm) which was previously equilibrated with 5 m*M* ammonium bicarbonate (pH 8.0). Kyotorphin was eluted with the same buffer by the isocratic mode for 7.5 hr at a flow rate of 4.5 ml/min and then by linear gradient from 5 to 100 m*M* ammonium bicarbonate solution in 7.6 hr. Kyotorphin fractions were combined (450 ml), freeze-dried, and further purified by reversed-phase semipreparative HPLC, employing a YMC-Pack, S-343 column (20 × 250 mm) and 5% acetonitrile/0.01 *M* HCl as eluent. Kyotorphin fractions were combined and evaporated to dryness and the product was reprecipitated from methanol and ether. The purity of the product (19.1 mg) was 96.4% by HPLC employing a Bondapak C_{18} column (3.9 × 300 mm) and elution with 0.01 *M* HCl at a flow rate of 1 ml/min. The product had a retention time of 8.6 min and was free of the diastereomer, L-Tyr-L-Arg (retention time, 10.4 min). The purification yield was 35%.

Biological Assay. The analgesic effect of intracisternal administration (13) of kyotorphin in mice was analyzed by the tail-pinch method as reported by Takagi *et al.* (14).

Radioactive Kyotorphin Synthesis. ^{3}H-Tyr-Arg synthesis was run according to the procedure described above except that 0.5 mCi of ^{3}H-tyrosine (72.5 Ci/mmol) was used as the starting material, and the total volume of the reaction mixture was 0.5 ml. ^{3}H-Tyr-Arg was analyzed and isolated by HPLC employing a Cosmosil $_5C_{18}$ column (4.6 × 250 mm) and 5% acetonitrile/0.1% trifluoroacetic acid–NH_4OH (pH 7.0) as eluant.

N-Terminal Modification of Oligopeptides. A solution (100 μl) of 2000 U/ml TyrRS, 5 m*M* tyrosine, 50 m*M* oligopeptide, 10 m*M* ATP, 10 m*M* $MgCl_2$, and 50 m*M* Bicine (pH 8.5) was incubated at 40°C for 130 hr. Tyrosyl-oligopeptides were analyzed with HPLC employing a Water's Novapak C_{18} column (8 × 100 mm). The mobile phase was a linear gradient from 10 to 30% acetonitrile in 20 min in 5 m*M* tetrabutylammonium bromide and 10 m*M* potassium phosphate solution (pH 8.0) for the analysis of Leu-enkephalin and from 20 to 30% acetonitrile in 10 min in 0.01 *M* HCl for Tyr-bradykinin. The yield was based on the amount of tyrosine.

Stability of TyrRS Under the Peptide Synthesis Condition. The stability of immobilized TyrRS was examined under the kyotorphin synthesis condition described above. After filtration, immobilized enzyme was repeatedly used in the same reaction. Activity was expressed as the amount of kyotorphin synthesized in 24 hr. The stability of free TyrRS was determined in a $TyrLeuNH_2$ synthesis reaction. A solution (1 liter) of 11 m*M* Tyr, 20 m*M* $MgCl_2$, 100 m*M* $LeuNH_2$, and 4000 U/ml TyrRS was incubated for 4 days at 45°C. Periodically, an aliquot of 2 μl was withdrawn and analyzed for its TyrRS activity by tRNA assay.

RESULTS AND DISCUSSION

Tyrosyl-tRNA synthetase was isolated from a thermophile, *B. stearothermophilus,* and was characteristic for its excellent thermal stability compared with that of a mesophile, *Escherichia coli.* Furthermore, tyrosyl-tRNA synthetase was immobilized on CNBr-activated Sepharose 4B. The immobilization yield was 28% and the activity of the resulting immobilized enzyme was 11,000 U/g wet resin. On immobilization of the enzyme onto Sepharose 4B, the thermal stability of the enzyme was further increased by approximately 7°C, which is shown in Fig. 1.

A typical reaction mixture for kyotorphin synthesis contained 5 m*M* tyrosine, 100 m*M* arginine, 20 m*M* magnesium chloride, 20 m*M* ATP, 50 m*M* Bicine (pH 9.5), and 2000 U/ml immobilized enzyme. The reaction mixture was incubated at 45°C for 2 days. Under this condition, kyotorphin accumulated up to a concentration of 2 m*M* (40% yield based on tyrosine).

Kyotorphin was isolated by CM-cellulose column chromatography, followed by reversed-phase chromatography. Crude kyotorphin was reprecipitated from methanol and ether. The purity of the product was 96.4% based on HPLC analysis. The main impurity of the product was arginine, and no peak was detectable with a retention time identical to that of the kyotorphin diastereomer (L-Tyr-D-Arg). Immobilized

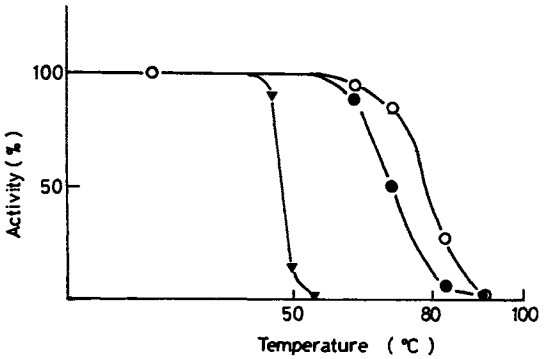

Fig. 1. Heat stability of immobilized TyrRS. The TyrRS preparation was heated for 15 min in a solution of 50 m*M* Hepes and 2 m*M* mercaptoethanol. Residual activity was assayed by the tyrosine hydroxamate formation method at 40°C. ○, Immobilized TyrRS on Sepharose 4B; ●, free TyrRS from *Bacillus stearothermophilus;* ▼, free TyrRS from *Escherichia coli.*

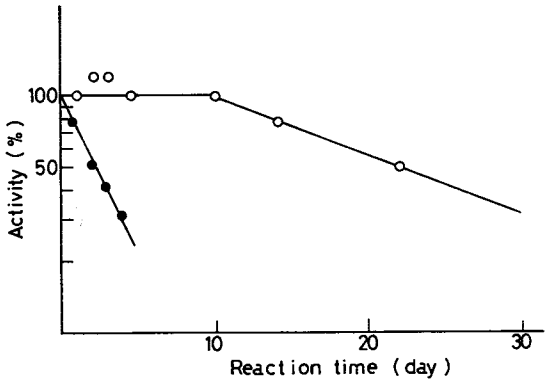

Fig. 2. Long-term stability of immobilized TyrRS. ○, Immobilized TyrRS on Sepharose 4B. The activity of immobilized TyrRs was based on the amount of kyotorphin produced after 24 hr. ●, Free TyrRS. The activity of free TyrRS was monitored by tRNA assay in Tyr-$LeuNH_2$ synthesis reaction.

TyrRS was recovered from the reaction mixture by filtration and repeatedly used for peptide synthesis. The change of activity of the immobilized enzyme during the repeated use is summarized in Fig. 2. The operational stability of the immobilized TyrRS was superior to that of the free enzyme under similar conditions.

Kyotorphin thus obtained had a ^{1}H-NMR spectrum identical to that of the authentic sample. For the analysis of the purity of this product, we examined its analgesic effect by intracisternal administration in mice (Fig. 3). The ED_{50} value of the enzymatically synthesized kyotorphin was 8.7 μg/mouse, which is similar to that of chemically synthesized kyotorphin (9.0 μg/mouse). Furthermore, the fact that the administration of 0.1 mg/kg sc naloxone completely inhibited the analgesic effect provided additional confirmation (3).

Employing ^{3}H-tyrosine as the starting material, enzymatic synthesis of radioactive kyotorphin was examined. As shown in Fig. 4, all of the radioactivity was found in either

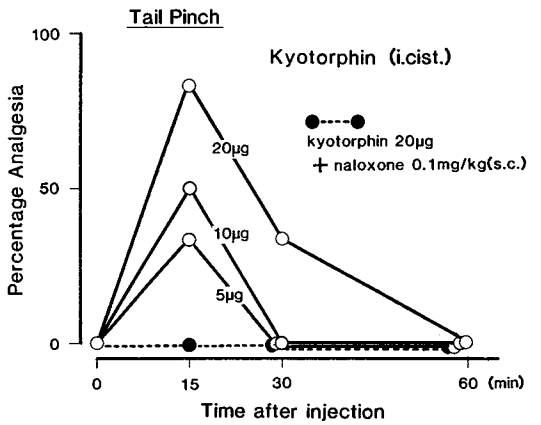

Fig. 3. Analgesic potency of enzymatically synthesized Tyr-Arg in the tail-pinch test. The doses of kyotorphin (i. cisternal) are shown in the figure. ○——○, Naloxone at 0.1 mg/kg sc was given before the i. cisternal injection of kyotorphin. Analgesic activities were determined with the tail-pinch test. At least six mice were used for each group.

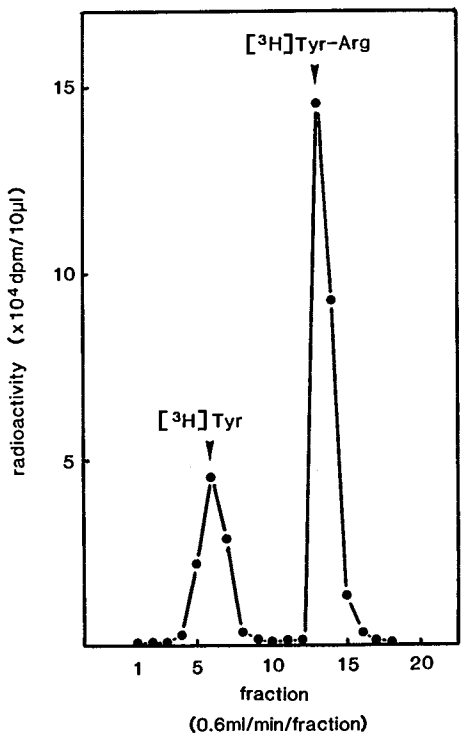

Fig. 4. Synthesis of ^{3}H-Tyr-Arg by the catalysis of TyrRS: a solution (500 μl) of 13.8 μ*M* ^{3}H-tyrosine (72.5 Ci/mmol), 100 m*M* arginine, 20 m*M* ATP-$MgCl_2$, 50 m*M* Bicine (pH 9.5), and 200 μl of immobilized TyrRS was incubated at 45°C for 2 days. The reaction mixture was analyzed directly with HPLC employing a Cosmosil $_5C_{18}$ (4.6 × 250-mm) column and 5% acetonitrile/0.1% trifluoroacetic acid–NH_4OH (pH 7.0) as eluant.

tyrosine or kyotorphin, and 72.2% of the total radioactivity was found in ^{3}H-Tyr-Arg by HPLC employing a Cosmosil $_5C_{18}$ column. After isolation by HPLC using the same condition, ^{3}H-Tyr-Arg exhibited the same activity as Tyr-Arg in the receptor binding assay using rat brain synaptic membranes (15) (K_d, 12.8 ± 3.8 n*M*; B_{max}, 1.00 ± 0.35 nmol/mg protein).

The reaction could be carried out under mild conditions, i.e., at 40°C in aqueous solution, because of the endoergonic nature of the reaction consuming ATP as an energy source. As compared to the conventional chemical (16) and enzymatic methods (17), kyotorphin synthesis catalyzed by ARS requires no protection of amino acids. The specificity of ARS for the amino acid substrate is so strict that none of the protected amino acids was activated by the enzyme.

The application of ARS-catalyzed peptide synthesis to the N-terminal modification of oligopeptides was also examined. In Table I, the results of the synthesis of two biologically active oligopeptides were compared to that of a dipeptide (tyrosylphenylalaninamide) synthesis. Leucine-enkephalin and tyrosylbradykinin were prepared by this method in comparable yields, indicating that the reaction is not restricted to dipeptide synthesis.

Since the catalyst of the reaction, ARS, is not commercially available as a pure preparation and it requires multiple

Table I. N-Terminal Tyrosylation of Oligopeptides by the Catalysis of TyrRS

Oligopeptide	Product	Yield (%)
GlyGlyPheLeu	Leu-enkephalin	86
Bradykinin	Tyr-bradikinin	64
$PheNH_2$	$TyrPheNH_2$	54

chromatographic steps for its preparation, it is necessary to use this catalyst repeatedly. As shown in Fig. 2, it was possible to use this immobilized enzyme at least seven times or for 20 days while retaining more than 50% of the initial enzyme activity. The half-life of the enzyme increased by an order of magnitude upon immobilization on Sepharose 4B.

As demonstrated in radioactive kyotorphin synthesis, ARS-catalyzed peptide synthesis could be a useful tool in introducing a radioactive amino acid to the N-terminal end of oligopeptides. Thus, radioisotope-labeled oligopeptides, which are frequently used in receptor binding experiments, are synthesized in a single reaction from free amino acid and oligopeptide by this method.

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