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β-NEO-ENDORPHIN, A NEW HYPOTHALAMIC "BIG" LEU-ENKEPHALIN OF PORCINE ORIGIN : ITS PURIFICATION AND THE COMPLETE AMINO ACID SEQUENCE

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SUMMARY : A new hypothalamic "big" Leu-enkephalin of porcine origin, designated as β-neo-endorphin, has been isolated from a side fraction, obtained in our previous isolation of α-neo-endorphin and PH-8P (dynorphin[1-8]). The complete amino acid sequence has been elucidated to be : Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro. The sequence has been confirmed by the comparison of natural β-neo-endorphin with a synthetic peptide. In addition, β-neo-endorphin exhibits potent opioid activity in guinea-pig ileum assay.

In 1979, we proposed the first evidence for the presence of "big" Leu-enkephalin by isolating and characterizing α-neo-endorphin from the porcine hypothalamus (1,2,3). Later on, dynorphin was also identified as the second form from the porcine pituitary by the group of Goldstein (4). However, further information about endogenous peptides indicative of precursor-intermediate-product relationship is required, in order to clarify the biosynthetic feature of Leu-enkephalin, which still remains unclear.

As a tool for finding out unknown pro-Leu-enkephalins related to α-neo-endorphin and dynorphin in the tissue extracts, we have recently developed a sensitive radioimmunoassay for [Arg^6]-Leu-enkephalin, which is a common tryptic fragment of the above "big" Leu-enkephalins known so far (5). It has been demonstrated in our recent isolation of PH-8P, a hypothalamic opioid peptide related to dynorphin, that the radioimmunoassay mentioned above made it feasible to monitor and purify pro-Leu-enkephalins which release immunoreactive [Arg^6]-Leu-enkephalin upon trypsinization (6). As previously reported, high performance liquid chromatography (HPLC) on a reverse phase column at the final purification step of PH-8P afforded another immunoreactive peak (peak #4), along with peak #7, from which PH-8P was purified (6).

From peak #4, we have isolated another new opioid nonapeptide, designated as β-neo-endorphin, and determined its complete amino acid sequence to

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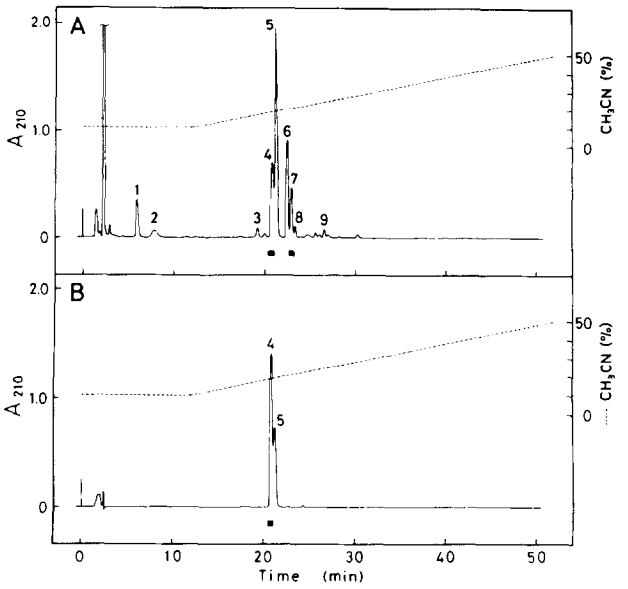

Fig. 1. Final purification steps of β-neo-endorphin.
(A) The immunoreactive fraction (3.21 mg), obtained by CM-Sephadex C-25 chromatography in our previous purification of PH-8P (Fig. 2 in ref. (6)), was loaded on a μ-Bondapak C-18 column (3.9 x 300 mm) and was eluted at 2 ml/min with 50 mM KH_2PO_4-H_3PO_4 (pH 2.0) using a linear gradient of CH_3CN from 10% to 50%. Black bars indicate the immunoreactive fractions against anti-[Arg^6]-Leu-enekphalin antiserum upon trypsinization. Peak #7 had been identified with PH-8P (dynorphin[1-8]).
(B) Rechromatography of peak #4 obtained above (Fig. 1A). Chromatographic conditions used were quite same as above. The immunoreactivity of [Arg^6]-Leu-enkephalin after trypsinization was observed at the black bar area.

be Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro, as the present paper describes. The proposed sequence was verified by the chromatographic comparison of natural β-neo-endorphin with synthetic peptide. The amino acid sequences of tryptic peptides were also determined and confirmed by their syntheses. This paper also describes opioid activity of β-neo-endorphin, which was measured by guinea-pig ileum assay.

We have very recently established the complete amino acid sequence of α-neo-endorphin, as will be reported in the succeeding paper (7), and β-neo-endorphin is revealed to be the C-terminal deleted peptide from α-neo-endorphin.

MATERIALS AND METHODS

Purification : The starting material for the present isolation was a side fraction, obtained in our previous isolation of α-neo-endorphin (1). A small and basic fraction, obtained from the acid extracts of 30,000 porcine hypothalami by gel filtration on Sephadex G-25 , followed by batch-wise treatment with SP-Sephadex, was chromatographed on SP-Sephadex C-25 (cf. Fig. 1.

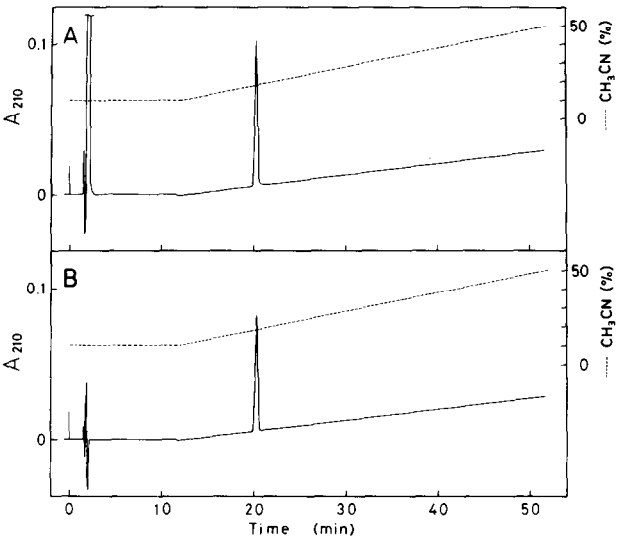

Fig. 2. Identification of natural β-neo-endorphin with the synthetic peptide. (A) Peak #4 in Fig. 1B was further purified to homogeneity by HPLC (μ-Bondapak C-18) with 10 mM ammonium formate (pH 4.0) using a linear gradient of CH_3CN from 10% to 50%. One nanomole of natural β-neo-endorphin, that purified above, was loaded on a μ-Bondapak C-18 column (3.9 x 300 mm) and eluted with the same conditions as in Fig. 1. (B) One microgram of the synthetic β-neo-endorphin was chromatographed under exactly the same conditions as above.

in ref. (1)). This chromatography yielded two major immunoreactive fractions, designated as fraction G (tubes #115-129) and fraction I (tubes #149-160); from the latter, α-neo-endorphin had been purified. Lyophilized material from fraction G was then submitted to successive chromatographies on Sephadex G-25, CM-cellulose (CM-52) and CM-Sephadex C-25, to yield a single immunoreactive peak (cf. Figs. 1, 2 in ref. (6)). The immunoreactive material thus obtained (3.21 mg) was separated into two immunoreactive peaks (peaks #4 and #7) by the first HPLC on a μ-Bondapak C-18 column, as shown in Fig. 1A (cf. Fig. 3 in ref. (6)). PH-8P (dynorphin[1-8]) had been purified from peak #7, as reported in our previous paper (6).

Lyophilized material from peak #4 was submitted to the present purification, performed by repeated HPLC on a μ-Bondapak C-18 column (3.9 x 300 mm: Waters Associates Inc.) with a linear gradient of either 50 mM KH_2PO_4-H_3PO_4 (pH 2.0)-CH_3CN or 10 mM ammonium formate (pH 4.0)-CH_3CN system. Aliquots of all the fractions were trypsinized and then submitted to the radioimmunoassay utilizing the C-terminal directed anti-[Arg^6]-Leu-enkephalin antiserum by the method, as described in our previous papers (5,6). In the radioimmunoassay at the final stage of purification, a few μg of bacitracin was added to the radioimmunoassay tube to prevent the adsorption of the sample to the tube surface. Fractions that showed the immunoreactivity were pooled, lyophilized and submitted to the next step. Further experimental conditions are shown in the figure legends.

Structural analyses : All analyses were performed in a nanomole scale in a similar way as for PH-8P (6). Amino acid analysis was carried out on a Hitachi #835 amino acid analyzer after hydrolysis of the peptide (ca. 2.5 μg) in 4M methanesulfonic acid containing 0.2% of tryptamine (Pierce) at 110°C for 20 hr.

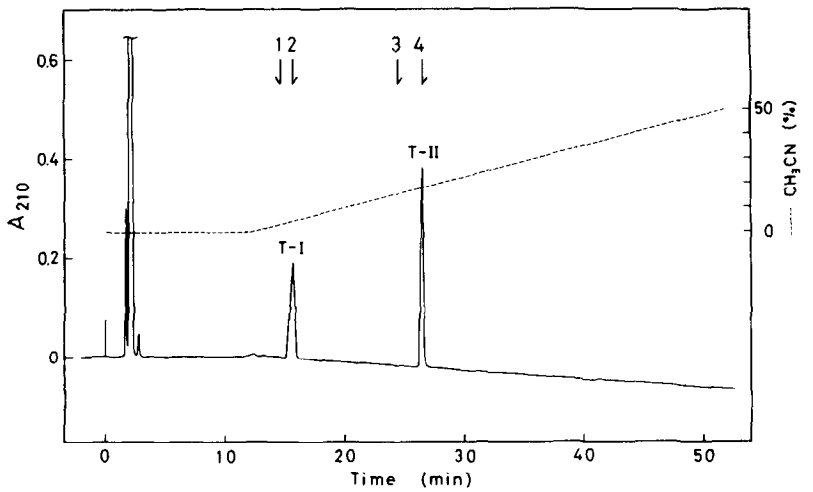

Fig. 3. Reverse phase HPLC of tryptic digests of β-neo-endorphin. Tryptic digests of the purified β-neo-endorphin (ca. 10 nmole) were loaded on a μ-Bondapak C-18 column and eluted at 2 ml/min with 10 mM ammonium formate (pH 4.0) using a linear gradient of CH_3CN from 0% to 50%. This column had been calibrated with 1) Lys-Pro-Tyr, 2) Lys-Tyr-Pro, 3) [Arg^6,Lys^7]-Leu-enkephalin and 4) [Arg^6]-Leu-enkephalin. T-I and T-II showed the retention times identical with 2) and 4), respectively.

Tryptic digestion of the peptide (10 nmole) was carried out with 200 ng of TPCK-trypsin (Worthington) in 1% of aq. ammonium bicarbonate (pH 8.0) at 37°C for 2 hr. Tryptic digests thus obtained were directly applied to reverse phase HPLC and eluted with 10 mM ammonium formate (pH 4.0)-CH_3CN system (Fig. 3). The tryptic peptides (T-I and T-II) purified above were submitted to amino acid analyses and micro-sequencing in the same manner as the parent β-neo-endorphin. Five nanomoles of β-neo-endorphin and its tryptic peptides were used for the sequence analysis, which was performed by the dansyl-Edman procedure, mainly according to the method of Bruton and Hartley (8). The dansyl amino acids were identified by TLC on a polyamide sheet. In all analyses, control experiments were carried out under the same conditions, except that only the sample peptide was omitted.

Syntheses of the peptides : β-Neo-endorphin and one of its tryptic peptides (T-I: Lys-Tyr-Pro) were synthesized on the basis of the proposed sequence by the solution method. For the calibration of HPLC column, tripeptide (Lys-Pro-Tyr) was also synthesized by the same method. Details of the syntheses will be reported separately. Physical constants of the peptides are determined as follows;

β-neo-endorphin : $[\alpha]_D^{20.5}$=-33.5° (c: 0.49, H_2O); Rf^1=0.66, Rf^2=0.80

Lys-Tyr-Pro (T-I) : $[\alpha]_D^{20.0}$=-18.1° (c: 0.44, H_2O); Rf^1=0.44, Rf^2=0.55

Lys-Pro-Tyr : $[\alpha]_D^{20.0}$=-21.8° (c: 0.44, H_2O); Rf^1=0.40, Rf^2=0.47

Rf values were measured by TLC on a cellulose plate (Merck), developed by the following two solvent systems.

Rf^1 : 1-butanol:CH_3CO_2H:H_2O = 4:1:5 (v/v) (upper phase)

Rf^2 : 1-butanol:CH_3CO_2H:H_2O:pyridine = 15:3:12:10 (v/v)

Homogeneity of the peptides was confirmed by HPLC as well as the above TLC.

Table 1. Amino Acid Composition of β-Neo-endorphin and Its Tryptic Peptides

Amino Acid	Total	T-I	T-II
Pro	0.98 (1)	1.01 (1)	
Gly	2.01 (2)		1.98 (2)
Leu	1.01 (1)		0.97 (1)
Tyr	1.98 (2)	1.06 (1)	0.94 (1)
Phe	1.07 (1)		1.12 (1)
Lys	0.97 (1)	0.94 (1)	
Arg	0.99 (1)		0.98 (1)

Purity of the peptides was further verified by elemental analyses, amino acid analyses, which showed good agreement with theoretical values. Syntheses of [Arg^6]-Leu-enkephalin (T-II) and [Arg^6,Lys^7]-Leu-enkephalin have been previously reported (2,3).

Opioid activity : Opioid activity was measured by the use of electrically stimulated myenteric plexus-longitudinal muscle from guinea-pig ileum in the reported manner (9). Opioid activity of Leu-enkephalin was also determined to compare with that of β-neo-endorphin.

RESULTS AND DISCUSSION

The present purification of β-neo-endorphin was a part of our systematic survey for pro-Leu-enkephalins related to α-neo-endorphin and dynorphin, in the acid extracts of 30,000 porcine hypothalami. Pro-Leu-enkephalin fractions were monitored in every fractionation step by the aid of the radioimmunoassay for [Arg^6]-Leu-enkephalin, which was assumed to be released from pro-Leu-enkepahlin upon trypsinization. In our previous isolation of PH-8P (dynorphin[1-8]) from fraction G, which was eluted ahead of α-neo-endorphin on SP-sephadex C-25 chromatography, the bulk of the immunoreactivity contained in fraction G was separated into two fractions, peak #4 and peak #7, by reverse phase HPLC at the final purification step of PH-8P (Fig. 1A). PH-8P had been isolated from peak #7, as reported (6). The present purification of peak #4 was performed by repeated HPLC on a μ-Bondapak C-18 column. Figs. 1A and 1B represent progressive patterns of purification of peak #4. Finally, on the third HPLC, a new opioid nonapeptide, designated as β-neo-endorphin, has been purified from peak #4 to homogeneity, as shown in Fig. 2A, in a yield of 30 nmole starting from 30,000 porcine hypothalami.

On the basis of its amino acid composition, as listed in Table 1, β-neo-endorphin was considered to be a nonapeptide, consisting of Pro_1, Gly_2, Leu_1,

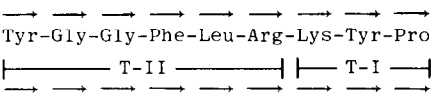

Fig. 4. Amino acid sequence of β-neo-endorphin.
(⟶) : Determined by the dansyl-Edman procedure.

Tyr_2, Phe_1, Lys_1, Arg_1. Sequence analysis of the peptide on a nanomole scale was successfully performed up to the carboxyl end by the dansyl-Edman procedure, as shown in Fig. 4. The results provided by micro-analyses of the tryptic peptides, as listed in Table 1 and Fig. 4, are also in good agreement with the sequence established above. Tryptic digestion of β-neo-endorphin afforded only two tryptic fragments, T-I and T-II, which were well separated by HPLC on a reverse phase column, as seen from Fig. 3. T-I and T-II thus isolated were submitted to amino acid analyses as well as Edman-degradation. From the data listed in Table 1 and Fig. 4, it was concluded that T-I is Lys-Tyr-Pro, while T-II is [Arg^6]-Leu-enkephalin. The structures of T-I and T-II were confirmed by the chromatographic comparison on HPLC with synthetic Lys-Tys-Pro and [Arg^6]-Leu-enkephalin, respectively, as seen from Fig. 3. The data mentioned above revealed that tryptic cleavage took place specifically at the linkage of Arg^6-Lys^7 and no other fragmentations were detectable. Furthermore, definite confirmation of the proposed structure of β-neo-endorphin was provided by comparing natural β-neo-endorphin with the synthetic peptide, synthesized by the solution method according to the sequence determined above. As seen from Figs. 2A and 2B, the chromatographic behavior of synthetic β-neo-endorphin on a reverse phase column was exactly identical with that of natural β-neo-endorphin.

Thus, the complete amino acid sequence of β-neo-endorphin was unambiguously established to be Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro.

In the structure established for β-neo-endorphin, the Leu-enkephalin structure is followed by a characteristic basic amino acid pair of Arg-Lys, which is usually observed in pro-hormone sequences. This fact suggests that β-neo-endorphin may be an intermediate in the biosynthetic pathway of Leu-enkephalin in brain. However, it should be noted that β-neo-endorphin has a proline residue at its carboxyl end. This indicates the possibility that there may exist in brain a processing enzyme that cleaves specifically the linkage of Pro-X, which is usually resistant to proteolysis.

In addition. β-neo-endorphin has been proved to show very potent opioid activity, 7.6 times as potent as Leu-enkephalin in the guinea-pig ileum assay, in which its IC_{50} value was determined to be 15.4 nM. Its potent opioid

activity and unique structural feature suggest that β-neo-endorphin may not serve only as an intermediate to Leu-enkephalin, but also may have its own physiological function.

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