Isolation of Tyrosine-Melanocyte-stimulating Hormone Release-inhibiting Factor 1 from Bovine Brain Tissue*

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Although Tyr-melanocyte-stimulating hormone release-inhibiting factor 1 (MIF-1) (Tyr-Pro-Leu-Gly-NH₂) can exert a number of biological actions in the brain and elsewhere, it has never been isolated from any tissue. Accordingly, we attempted to purify it from acetic acid extracts of bovine brain tissue by gel filtration chromatography and several different high performance liquid chromatographic systems. Peptide content was followed by a specific and sensitive radioimmunoassay with an antibody that was generated against synthetic Tyr-MIF-1. In each of the five applied high performance liquid chromatographic systems, the immunoreactive fractions coincided exactly with the elution time of synthetic Tyr-MIF-1 in the control runnings. The structure of the isolated peptide was identified by microsequence analysis as the tetrapeptide Tyr-Pro-Leu-Gly-NH₂ and shown to be biologically active.

*Tyr-MIF-1 (Tyr-Pro-Leu-Gly-NH₂) is a biologically active peptide that has been involved in the development of several novel concepts in the peptide field. For example, Tyr-MIF-1 is the first peptide for which a carrier-mediated transport system from brain to blood has been demonstrated (1-3). It also can act as an antiplatelet in several situations (4-9) including the immune system (10) and show multiple other extra-endocrine effects such as reversal of "behavioral despair" in an animal model of depression (11) and augmented binding of ß-aminobutyric acid-stimulated benzodiazepine receptors (12, 13).

Considerable evidence has been accumulated for the existence of this peptide in brain tissue by RIA supported by HPLC (5). Immunoreactive Tyr-MIF-1-like activity has been found in rat brain, particularly the hypothalamus (14), and shown to have a marked diurnal rhythm (15, 16). In addition, high affinity binding sites have been demonstrated for Tyr-MIF-1 in the brain (17, 18).

Despite this evidence for the existence and biological function of Tyr-MIF-1, it has never been isolated from brain or any other tissue. This paper describes the purification of Tyr-MIF-1 from bovine hypothalamic tissue and its identification as the tetrapeptide Tyr-Pro-Leu-Gly-NH₂.

MATERIALS AND METHODS

Tissue Extraction

Frozen bovine hypothalami (Pel-Freez Biologicals), an average of 14.8 g/tissue, were allowed to partially thaw on ice and then minced and put into 20 ml of 0.1 M acetic acid at 0 °C. This was placed in a boiling water bath for 4 min, and 130 ml of 0.1 M acetic acid was added. The suspension was chilled on ice, homogenized in an ice bath with a Brinkmann Polytron (setting 6) for 30 s, and centrifuged (29,000 × g, 20 min, 4 °C). The supernatant was removed, and the pellets were re-extracted with 0.1 M acetic acid and centrifuged as described above. The two supernatants were combined, centrifuged (50,000 × g, 45 min, 4 °C), and lyophilized.

HPLC

Five main HPLC systems were used in the purification of Tyr-MIF-1. All of these systems used the same pumps (Beckman Model 102A), controller (Beckman Model 421), injection valve (Altex Model 210A), UV absorbance detector (Beckman Model 165 variable wavelength detector), recorder (Kipp & Zonen), and fraction collector (Isco Retriever II), but utilized different columns and mobile phases as described below.

HPLC System 1—In this system, a preparative ODS reverse-phase HPLC column (25 × 2.11 cm; Spherisorb, Regis Chemical Co.) was used at a flow rate of 8 ml/min, with fractions collected at 1-min intervals. The linear gradient consisted of 0-60% methanol, 0.1% trifluoroacetic acid for 60 min and then 100% methanol, 0.1% trifluoroacetic acid for 19 min.

HPLC System 2—In this second system, a semipreparative reverse-phase HPLC column (25 × 1 cm; Spherisorb S5ODS2, Regis Chemical Co.) was used at a flow rate of 2 ml/min, with fractions collected at 1-min intervals. The linear gradient was of 0-100% methanol, 0.1% trifluoroacetic acid for 60 min.

HPLC System 3—In this third system, the same semipreparative column and flow rate were applied as in HPLC system 2, but the linear gradient consisted of 20-40% acetonitrile, 0.1% trifluoroacetic acid for 40 min.

HPLC System 4—in this system, an analytical C₁₈ column (25 × 0.46 cm; Vydac TPS 5 μ, Alltech Associates, Inc.) was equilibrated with 10% acetonitrile in 0.1% trifluoroacetic acid. The linear gradient was 10-25% acetonitrile, 0.1% trifluoroacetic acid for 30 min. The flow rate was 1 ml/min, and 1-ml fractions were collected.

HPLC System 5—in this system, the same column was used as in HPLC system 4, but with a different gradient. The column was
equilibrated with 30% solvent B (20% acetonitrile, 9.1% trifluoroacetic acid), and then the percentage of solvent B was raised to 60% for 30 min. The applied flow rate was 1 ml/min, and 1-ml fractions were collected. The gradient used to check the purity of the peptide after the fifth HPLC step contained two steps. The column was equilibrated with 30% solvent B; then, a linear gradient of 30–40% solvent B was applied for 30 min and followed by isocratic elution with 40% solvent B for 10 min.

**RIA**

The RIA described in 1980 (19) has been gradually modified (14, 16). Additional improvements, particularly in the antibody, are briefly described here. The antibody (number 801) generated in a rabbit against the tetrapeptide Tyr-MIF-1 was able to detect <10 pg of Tyr-MIF-1 by RIA, but did not cross-react at concentrations of 10,000 pg (<1 nm) with Tyr, Tyr-Pro, Tyr-Pro-Leu, Leu-Gly, Pro-Leu-Gly-NH₂ (MIF-1), Arg-vasopressin, mesotocin (Ile-oxytocin), Lys-vasotocin, isotocin (Ser₄,Leu⁴ oxytocin), corticotropin-releasing hormone, α-endorphin, ACTH-(1-39), delta sleep-inducing peptide, and prolactin. The derivatization and cleavage cycles were carried out on an Applied Biosystems 470A Protein Sequencer, and the amino acid derivatives from each cycle were then separated and assayed by an Applied Biosystems 130A Separation System and 900A Data Analysis Module.

**Peptide Preparation**

Tyr-Pro-Leu-Gly-free acid was prepared by solid-phase synthesis using tert-butyloxycarbonyl-Gly resin (Bachem) as starting material. The resulting peptide was removed from the resin by liquid hydrogen fluoride and purified on a preparative ODS reverse-phase column (25 × 2.11 cm; Regis Chemical Co.). It was eluted with a linear gradient of 10–40% acetonitrile, 0.1% trifluoroacetic acid for 40 min. Thin-layer chromatography of the purified peptide revealed single spots in the following three systems: 1-butanokacetic acidwater (4:1:1), ethyl acetate:acetic acidwater (30:20:6:1), and ethyl acetate:pyridine:acetic acidwater (30:20:6:11). Analytical reversed-phase HPLC showed a single peak.

**Biological Activity**

The antiopiate effect of isolated Tyr-MIF-1 was examined on human T-lymphocytes by an in vitro method recently used by us (10). In brief, total E-rosette formation was measured in human lymphocytes incubated overnight at 4 °C with sheep red blood cells at a ratio of 1:70. Based on our previous results with synthetic Tyr-MIF-1 (10), three groups were tested: media alone, morphine (10⁻⁶ M) alone, and morphine (10⁻⁶ M) + isolated bovine Tyr-MIF-1 (10⁻⁶ M). The results were compared by analysis of variance, followed by the Tukey test.

**RESULTS**

To collect sufficient material for sequence analysis of the Tyr-MIF-1-like immunoreactive peptide, 25 hypothalami were extracted (yield: 0.32 g of dry extract/tissue) and subjected to gel filtration. To determine the elution of Tyr-MIF-1 during this step, an identical column was used for calibration. Control Tyr-MIF-1 eluted in fractions 44–50, oxytocin in fractions 26–30, and MIF-1 in fractions 32–36. To ensure that all the fractions containing Tyr-MIF-1-like material or very closely related substances were collected, fractions 40–65 were pooled (Fig. 1). From the 25 hypothalami, 255 mg of dried material was obtained.

The relatively broad peak from gel chromatography was then fractionated on a preparative HPLC column. Fig. 2 shows a typical elution pattern of the Tyr-MIF-1-like immu-

![Fig. 1. Tyr-MIF-1-like immunoreactivity after gel filtration of bovine brain extract on Sephadex G-10 column (2.7 x 100 cm) in 0.02 M acetic acid, 0.002% sodium azide. V₀ is the void volume measured with blue dextran, and Vₖ is the total volume measured with sodium chloride.](image-url)

![Fig. 2. Tyr-MIF-1-like immunoreactivity after preparative HPLC of fractions 40–65 obtained from gel chromatography. The gradient is shown by the dashed line. % B represents methanol in 0.1% trifluoroacetic acid. The arrow shows the retention time of synthetic Tyr-MIF-1 during an identical experiment.](image-url)
Isolation of Tyr-MIF-1

**Fig. 3.** HPLC purification of Tyr-MIF-1-like immunoreactive material from previous step on reverse-phase HPLC column (25 X 1 cm). The dashed line shows % B (methanol in 0.1% trifluoroacetic acid) during separation. A$_{280}$ is shown by the solid line. Tyr-MIF-1-like immunoreactivity is indicated by the hatched columns. The arrow shows the elution of synthetic Tyr-MIF-1.

**Fig. 4.** HPLC purification of Tyr-MIF-1-like immunoreactive material from previous step on reverse-phase HPLC column (25 X 1 cm). The dashed line shows % B (acetonitrile in 0.1% trifluoroacetic acid) during chromatography. A$_{280}$ is shown by the solid line on a scale 10-fold smaller than in Fig. 3. Tyr-MIF-1-like immunoreactivity is indicated by the hatched columns. The arrow shows the elution of synthetic Tyr-MIF-1.

**Fig. 5.** HPLC purification of Tyr-MIF-1-like immunoreactive material from previous step on reverse-phase HPLC column (25 X 0.46 cm). The dashed line shows % B (acetonitrile in 0.1% trifluoroacetic acid) during chromatography. A$_{280}$ is shown by the solid line. A$_{215}$ is shown separately in the inset. Arrows show the elution of control Tyr-MIF-1. The hatched column indicates Tyr-MIF-1-like immunoreactivity.

Noreactive material after preparative HPLC. Fractions 41-46, with high immunoreactivity and identical elution time to synthetic Tyr-MIF-1 (Bachem), were pooled and lyophilized. About 14.25 mg of dried material was obtained at this stage from the 25 hypothalami.

The active fractions collected after preparative HPLC were further purified in two steps on a semipreparative HPLC column. This was followed by two consecutive runnings on the analytical column. In each of these four procedures, only one immunoreactive peak was present, and it coincided exactly with the elution time of synthetic Tyr-MIF-1 in the control runnings.

**Fig. 3** shows one of the chromatograms of the first separation on the semipreparative HPLC column. The only immunoreactive peak present eluted at the same position as synthetic Tyr-MIF-1 (fractions 39-40).
reactive material was about 10-fold smaller at 280 nm than during the previous HPLC step.

The fourth HPLC step was carried out on an analytical column. The amount of the Tyr-MIF-1-like material determined by RIA (about 100,000 pg in fraction 22) corresponded to the amount that was represented by the UV absorption at 280 and 215 nm (about 0.15 µg; fraction 22). To determine the amount of Tyr-MIF-1 by UV absorption, a calibration curve was used. Different amounts of the synthetic peptide were injected (0.05, 0.1, 0.25, 0.5, 0.75, and 1.0 µg), and the height of the peak was measured at 215 and 280 nm. Fig. 5 shows the results of the fourth HPLC step. In both Figs. 5 and 6, the UV absorption at 215 nm is also shown to provide more information about the peptide content of the sample during the last steps.

After the fifth HPLC step (Fig. 6), the UV absorbance of fraction 24 represented nearly 0.1 µg of Tyr-MIF-1 content, which correlated well with the amount determined by RIA (70,000 pg). This fraction did not show any additional peaks when it was checked in a different elution system (see "HPLC System 5" under "Materials and Methods"). The amount of the immunoreactive material finally collected from 25 hypotalami and determined by RIA was about 140,000 pg (310 pmol).

The material obtained after the fifth HPLC step was subjected to gas-phase microsequencing. Although a small amount of other phenylthiohydantoin material was detected, the major peaks were easily identified and indicated that the order of the amino acids was Tyr, Pro, Leu, and Gly. After the fourth cycle, the amount of the amine-containing side product was about 60% higher than during the previous cycles. This indicates an amidated C-terminal on the peptide.

The synthetic peptides Tyr-Pro-Leu-Gly-OH2 and Tyr-Pro-Leu-Gly-NH2 showed different elution times when they were checked in HPLC system 5. The free acid eluted much later (28.2 min) than the amide (23.6 min).

In the test for biological activity, analysis of variance revealed a significant effect of treatment ($F_{1,18} = 6.88; p < 0.01$). Morphine significantly ($p < 0.10$) reduced the number of total E-rosettes from 59.9 ± 1.1 to 45.9 ± 4.4%. This reduction of E-rosettes by morphine was significantly reversed by isolated bovine Tyr-MIF-1 (57.5 ± 1.9%; $p < 0.05$).

**DISCUSSION**

We used several procedures to isolate the tetrapeptide Tyr-Pro-Leu-Gly-NH2 from bovine hypothalamus. This tissue was selected because of its relatively high content of the peptide (14) and because of its size and availability.

The tissue was heated to inactivate enzymes, homogenized, and centrifuged at high speed to remove large substances. Other proteins were removed in the early fractions obtained from gel filtration of the extract on a Sephadex G-10 column (Fig. 1). Sephadex chromatography provided a major advantage in this purification since aromatic amino acids, especially tyrosine, strongly adsorb to the matrix. Although Tyr-MIF-1 is a larger molecule than Tyr, it is not large enough to overcome the effect of its aromatic ring so that it elutes later than small peptides not containing aromatic amino acids. This provided a good separation from substances like MIF-1 (Pro-Leu-Gly-NH2), which also has antiantipode and antidepresive activity but which elutes earlier from the column.

More than one area of Tyr-MIF-1-like immunoreactivity was seen after gel chromatography (Fig. 1). The antibody in the sensitive RIA used to follow Tyr-MIF-1-like immunoreactivity during the separation reacts only slightly with oxytocin, but not with MIF-1 or any of the Tyr-containing (N-terminal) fragments of Tyr-MIF-1. Therefore, it is possible that the two immunoreactive peaks eluting at approximately the positions of oxytocin and MIF-1 may represent a part of a precursor of Tyr-MIF-1 or even an aggregated form of the tetrapeptide (20). Immunoreactivity detected after V, could not represent either C-terminal fragments of Tyr-MIF-1 that would elute earlier or N-terminal fragments that would not be detected by our antibody.

The profile of UV absorption after the first HPLC step revealed the very high content of other materials still present in the peak containing Tyr-MIF-1 immunoreactivity (Fig. 2). We therefore anticipated that it would be necessary to perform several additional HPLC procedures.

Successive HPLC steps with different gradients of eluent resulted in progressive purification of the peptide, as reflected by UV absorption and RIA. Contamination by "shadowing" with standard (21) was ruled out after each use of standard by injection of diluent, followed by a washing procedure for at least six cycles and then by RIA of all tubes.

Figs. 3-6 show the chromatograms of the four consecutive purification steps that finally resulted in the pure peptide. In each of these four different steps, only one immunoreactive peak was detected; and in each case, its retention time was the same as that of control Tyr-MIF-1. The UV absorbance at 280 and 215 nm indicated the course of the isolation. After the fifth HPLC step, the peak that eluted at the same position as synthetic Tyr-MIF-1 contained the only immunoreactive material, and this did not contain any additional UV-absorbing material.

Gas-phase sequence analysis showed that the order of the amino acids was Tyr, Pro, Leu, and Gly. Although the analysis indicated the presence of an amide group, we synthesized the free acid to provide further evidence for the specific structure of the isolated peptide. Fig. 6 shows that the Tyr-MIF-1 immunoreactive material eluted exactly at the position of the amidated peptide, whereas the peptide containing the free...
acid eluted several fractions later. This result proves that the isolated material is identical to Tyr-Pro-Leu-Gly-NH₂.

Additional evidence that the isolated material is Tyr-MIF-1 was provided by its biological activity as an antiopeptide on human T-lymphocytes. It significantly reversed the suppression of total E-rosette formation induced by morphine, as has been reported for synthetic Tyr-MIF-1 (10).

Another immunoreactive peak different from that of Tyr-MIF-1 was detected by RIA during the first HPLC step (Fig. 2). The high specificity of the antibody generated against Tyr-MIF-1 makes it likely that this area represents one or more endogenous brain peptides very similar in structure to Tyr-MIF-1. Furthermore, the amidated form of Tyr-MIF-1 suggests that it is formed from a precursor molecule. These results support the idea of the existence of another endogenous family of peptides.

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REFERENCES


