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Enkephalin biosynthetic pathway: Proteins of 8000 and 14,000 daltons in bovine adrenal medulla

(high-pressure liquid chromatography/chromaffin granules/opioid peptides/trypsin)

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ABSTRACT **Two [Met]enkephalin-containing polypeptides have been purified from extracts of bovine adrenal chromaffin granules. One is 8000 daltons in size and contains the enkephalin sequence at the carboxy terminus. The other (14,000 daltons) has two internal enkephalin sequences and a third sequence at the carboxy terminus. All of these sequences can be released by trypsin, indicating a possible enkephalin precursor role for the polypeptides.**

In previous papers (1–5) we reported the detection and isolation of peptides and proteins from extracts of bovine adrenal medulla that yield enkephalin on treatment with trypsin. These peptides and proteins show no relationship to pro-opiocortin, β-lipotropin, or β-endorphin (1, 2). The largest of these enkephalin-containing proteins (approximately 50,000 daltons) contains a ratio of seven [Met]enkephalin sequences to one [Leu]enkephalin sequence within its primary structure (5). Many of the intermediate-size peptides also contain multiple enkephalin sequences, including one with a [Met]enkephalin sequence at both the amino and carboxy termini and another with both a [Met]enkephalin and a [Leu]enkephalin sequence (2). Hexapeptides and heptapeptides composed of the enkephalin sequence with extensions of basic amino acids at the carboxy terminus have also been isolated (unpublished data). We have now identified two larger enkephalin-containing polypeptides from adrenal extracts and have purified them to homogeneity. Data on the partial chemical characterization of these peptides are presented.

MATERIALS AND METHODS

The methods used for isolation of bovine adrenal chromaffin granules, acid extraction of the granules, and Sephadex G-75 chromatography have been described (1, 2). The pooled material in the area corresponding to 5–10 kilodaltons (kDal) was pumped directly onto a Lichrosorb RP-18 high-pressure liquid chromatography (HPLC) column (Ace Scientific, Edison, NJ). Further HPLC was done with an Ultrasphere ODS column (Rainin Instrument, Ridgefield, NJ). The pooled material in the 10- to 17-kDal area was treated in the same manner, but with an additional HPLC step on a Spherisorb CN column (Lab Data Control, Riviera Beach, FL). The diphenyl column used was made as described (6). Material was eluted from all HPLC columns with gradients of 1-propanol in 0.5 M formic acid/0.4 M pyridine, pH 4.0. Column fractions were monitored by a fluorescamine detection system (7).

All fractions were treated with trypsin (1) and carboxypeptidase B (5) and then assayed with a radioligand-binding assay by use of neuroblastoma–glioma hybrid cells (8). Protein was measured by direct injection into the fluorescamine detection system with purified peptides used as standards. $NaDodSO_4$ slab-gel electrophoresis (9) and amino acid analysis (10) were conducted as described. Further experimental details are provided in the figure legends.

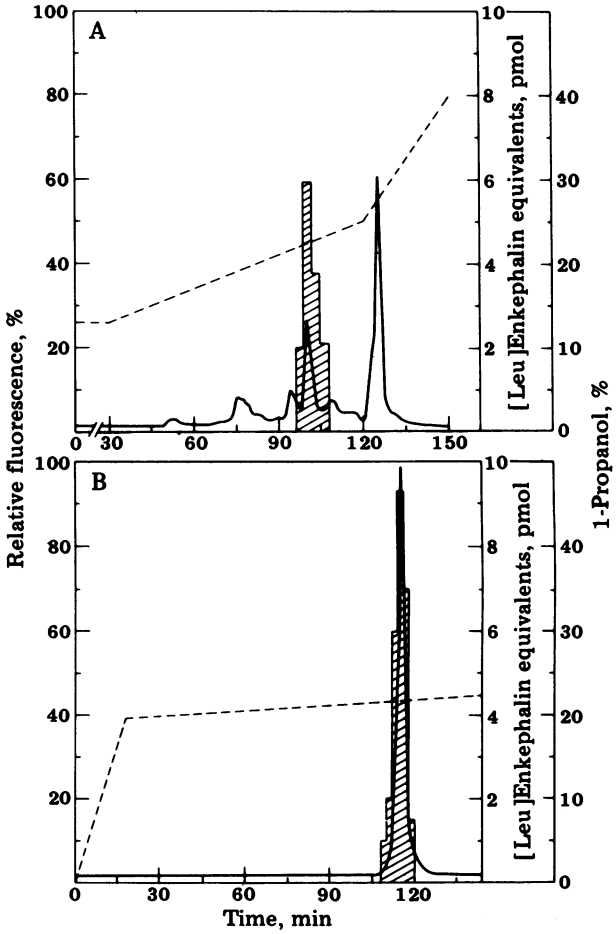

FIG. 1. Final purification steps of the 8-kDal peptide. (*A*) Pooled fractions from Sephadex G-75 chromatography were pumped onto a Lichrosorb RP-18 column (4.6 × 250 mm) at 80 ml/hr. The column was washed with 20 ml of starting buffer (0.5 M formic acid/0.4 M pyridine, pH 4.0) and peptides were eluted with a gradient of 1-propanol (- - -) in the same buffer at 20 ml/hr. Six percent of the column effluent was diverted to the detection system. Aliquots (5 μl) of each fraction (3 min) were digested with trypsin and assayed. (*B*) The active fractions for *A* were pooled and lyophilized. The peptides were redissolved in the above starting buffer containing 4 M urea (1 ml) and were then injected onto an Ultrasphere C_{18} column (4.6 × 250 mm). Peptides were eluted and assayed as in *A*. ▨, [Leu]Enkephalin equivalents; —, relative fluorescence.

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Abbreviations: kDal, kilodalton(s); HPLC, high-pressure liquid chromatography.

Table 1. Purification of 8-kDal and 14-kDal peptides

Procedure	Vol, ml	Activity,* nmol	Protein,† mg	Specific activity, nmol/mg	Recovery, %	Purification, -fold
			8-kDal peptide			
Original material	50	167	430	0.39	100	1
Sephadex G-75	174	75.2	4.2	17.9	45	46
Lichrosorb RP-18	5.5	64.2	0.63	102	38	262
Ultrasphere C_{18}	4.0	56.8	0.45	124	34	318
Diphenyl	4.0	55.0	0.43	128	33	328
			14-kDal peptide			
Original material	50	203	430	0.47	100	1
Sephadex G-75	182	107.7	6.7	16	53	34
Lichrosorb RP-18	5.5	72.8	1.74	42	36	89
Ultrasphere C_{18}	4.2	61.2	0.93	66	30	140
Spherisorb CN	4.0	59.1	0.76	78	29	166
Diphenyl	4.0	56.2	0.72	78	28	166

* Receptor-binding activity was determined after trypsin digestion and represents only the carboxy-terminal [Met]enkephalin.

† Protein was assayed by injection of 5-μl aliquots into the fluorescamine detection system. The standards used were the purified proteins.

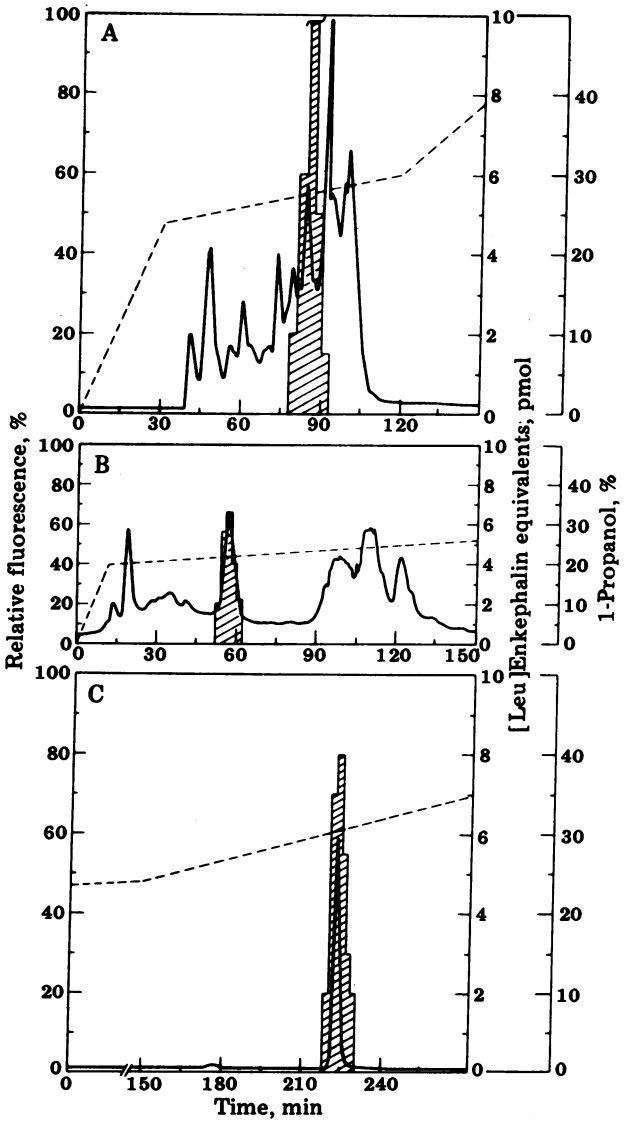

FIG. 2. Final purification steps of the 14-kDal peptide. The chromatographic conditions used were as described in Fig. 1. (*A*) Lichrosorb RP-18; (*B*) Ultrasphere C_{18}; (*C*) Spherisorb CN columns. All columns were 4.6 × 250 mm. ---, 1-Propanol gradient; —, relative fluorescence; ▨, [Leu]enkephalin equivalents.

RESULTS

Chromatography of extracts of adrenal medullary chromaffin granules on Sephadex G-100 separated several distinct areas of opioid peptide containing material varying in size from 500 to 50,000 daltons. Two of these, the 8-kDal and 14-kDal peptides, were purified to homogeneity by the schemes presented in Table 1. Each represents an appreciable proportion of the acid-soluble chromaffin granule protein, 0.3% for the 8-kDal and 0.8% for the 14-kDal peptide. Excellent yields were obtained, approximately 30% in both cases.

Two steps of HPLC were sufficient for purification of the 8-kDal peptide (Fig. 1). Three HPLC steps were necessary to achieve homogeneity for the 14-kDal peptide (Fig. 2). Evidence for their homogeneity was obtained by gel electrophoresis (Fig. 3). Additional evidence of homogeneity is demonstrated in Table 1, in which it is apparent that the specific activity of both proteins had attained essentially a constant value even after additional HPLC under entirely different conditions.

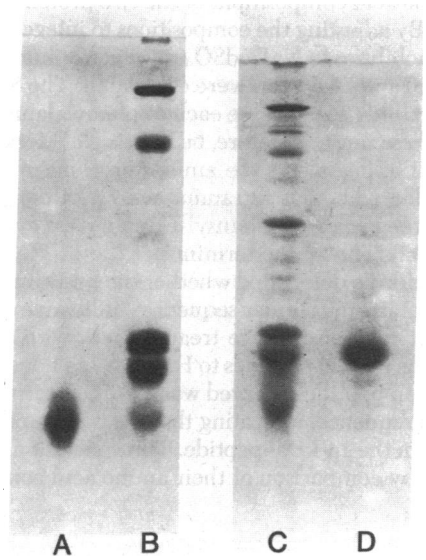

FIG. 3. Electrophoresis on polyacrylamide gels (12%) containing 0.1% $NaDodSO_4$. (*A*) 8-kDal peptide (20 μg); (*D*) 14-kDal peptide (20 μg). Mixtures of standard proteins: (*B*) 10 μg and (*C*) 5 μg. Standard proteins used for comparison were RNase (13.4 kDal), β-lactoglobulin (18 kDal), ovalbumin (45 kDal), and bovine serum albumin (68 kDal).

Table 2. Amino acid composition of the 8-kDal and the 14-kDal peptides

Amino acid	8-kDal peptide Value ± SEM*	8-kDal peptide Residues	14-kDal peptide Value ± SEM*	14-kDal peptide Residues
Asx	4.12 ± 0.14	4	6.80 ± 0.10	7
Thr	5.26 ± 0.16	5	7.06 ± 0.13	7
Ser	4.32 ± 0.11	4	5.95 ± 0.13	6
Glx	9.27 ± 0.13	9	18.58 ± 0.20	19
Pro	9.00 ± 0.35	9	6.54 ± 0.14	7
Gly	3.24 ± 0.07	3	10.14 ± 0.08	10
Ala	5.63 ± 0.20	6	7.31 ± 0.10	7
Val	0.2 ± 0.10	0	2.26 ± 0.08	2
Met	0.81 ± 0.05	1	4.71 ± 0.07	5
Ile	0.20 ± 0.10	0	0.10 ± 0.05	0
Leu	14.54 ± 0.35	15	17.57 ± 0.08	18
Tyr	1.93 ± 0.04	2	5.00 ± 0.02	5
Phe	1.00	1	3.00 ± 0.02	3
His	1.11 ± 0.02	1	1.11 ± 0.02	1
Lys	6.26 ± 0.12	6	10.95 ± 0.07	11
Arg	2.35 ± 0.05	2	4.19 ± 0.07	4
Cys	3.37 ± 0.16	4	5.42 ± 0.29	6
Trp	0.96 ± 0.07	1	0.00	0
Total		73		118
Molecular weight		8032		14,190

* Averages from four preparations with three analyses of each. The peptides were hydrolyzed for 24 hr at 104°C in constant boiling HCl containing 0.1% thioglycolic acid.

The two intact polypeptides were devoid of opiate receptor-binding activity; however, on treatment with trypsin, they yielded peptides with considerable activity. The active tryptic fragments were identified and quantitated by amino acid analysis after their separation by HPLC. The 8-kDal peptide produced one equivalent of [Met]enkephalin; the 14-kDal peptide yielded one equivalent of [Met]enkephalin and two equivalents of [Met]enkephalin-Arg^6.

The amino acid compositions of the two proteins are shown in Table 2. By adjusting the compositions to integer values and using the mobilities in $NaDodSO_4$ gel electrophoresis, precise masses of 8.0 and 14.3 kDal were calculated. The 8-kDal peptide contains only one residue each of phenylalanine and methionine. These must, therefore, be in a single [Met]enkephalin sequence. It appears that the amino terminus of the 8-kDal peptide is blocked because no amino acids were detected by the Edman (three cycles) and dansyl-Edman (two cycles) procedures (11, 12). The amino terminus of the 14-kDal protein is glutamic acid. To determine whether the 8-kDal and 14-kDal proteins contain any similar sequences indicative of a precursor–product relationship, we treated them with trypsin and subjected the tryptic peptides to HPLC (data not shown). The only common peptide observed was a COOH-terminal [Met]-enkephalin sequence, indicating that the 8-kDal peptide is not derived from the 14-kDal peptide. This conclusion was further confirmed by comparison of their amino acid compositions.

DISCUSSION

In this report we have characterized two intermediate-size polypeptides in what is, apparently, the pathway of enkephalin biosynthesis. We have studied the properties of purified hexapeptides and heptapeptides (unpublished data) and of two larger peptides of 3900 and 4700 daltons (2). All the hexapeptides and heptapeptides are extended at the carboxyl end by a lysyl or an arginyl residue, indicative of the action of a trypsin-like protease on larger precursors. Each of the two larger peptides contains two copies of enkephalin per mole, which are also released by trypsin. Pulse–chase studies have further indicated a precursor–product relationship between these peptides and the enkephalins (5).

In contrast to those two peptides, the 8-kDal peptide contains only a single [Met]enkephalin sequence located at the carboxy terminus. Whether the rest of this polypeptide chain is of biological interest remains to be seen, but a trypsin-resistant core suggests this as a possibility. Three copies of [Met]enkephalin are present in the 14-kDal peptide, with one at the carboxy terminus. The sequences of the 3.9-kDal, 4.7-kDal, and 8-kDal peptides cannot be present in the 14-kDal peptide. Our preliminary studies indicate that these and other enkephalin-containing peptides are released from perfused bovine adrenal glands in proportion to their concentration in the chromaffin granules. Thus, the relatively high concentrations of both the 8-kDal and the 14-kDal peptides indicate that they may represent more than just enkephalin precursors. Perhaps they contain sequences that directed them to specific target tissues or they may possess unique biological activities of their own.

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