Vol. 106, No. 1, 1982 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 14, 1982 Pages 186-193

AN ENKEPHALIN-GENERATING ENZYME IN BOVINE ADRENAL MEDULLA

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Received March 30, 1982

A trypsin-like enzyme has been partially purified from bovine adrenal chromaffin granules through the use of affinity chromatography. This enzyme preparation was able to generate met^5-enkephalin from endogenous substrate(s). Met^5-enkephalin production was not inhibited by sulfhydryl reagents such as p-chloromercuriphenyl sulfonate nor stimulated by dithiothreitol, suggesting that this enzyme is not a lysosomal enzyme such as cathepsin B. Enzymatic activity was strongly inhibited by several trypsin inhibitors including soybean trypsin inhibitor, aprotinin, and diisopropylfluorophosphate. These results imply that this adrenal enkephalin-generating enzyme is a serine protease.

INTRODUCTION

The adrenal medulla has recently been shown to contain large quantities of high molecular weight forms of enkephalin (1-6). There is strong evidence to suggest that at least some of these high molecular weight enkephalin-like peptides represent precursors to met^5- and leu^5-enkephalin. Recent studies suggest that adrenal met^5-enkephalin can be secreted into the bloodstream (7,8); plasma levels of this peptide increase following splanchnic nerve stimulation (8). Since adrenal enkephalin-like peptides are concentrated in chromaffin granules (6,9), it is probable that the enzymes responsible for the conversion of high molecular weight enkephalin-like peptides to the pentapeptides are present within the granule. Tryptic digestion of many of the high molecular weight enkephalin-containing peptides is able to release free met^5-enkephalin as well as arginine- and lysine-extended peptides (3). These results suggest that a trypsin-like enzyme may act to

Abbreviations: TLCK, tosyl lysyl chloromethylketone; STI, soybean trypsin inhibitor; DTT, dithiothreitol; met^5-enk, methionine^5-enkephalin; IR, immunoreactivity; DFP, diisopropylfluorophosphate; HPLC, high pressure liquid chromatography; PCMS, p-chloromercuriphenyl sulfonate; enk, enkephalin; arg^1 met^6 enk, arg-tyr-gly-gly-phe-met.

0006-291X/82/090186-08$01.00/0

generate met^5-enkephalin *in vivo*. We have previously shown that a soluble protein fraction prepared from chromaffin granules contains the enzymatic activity and the substrate(s) required for the production of met^5- and leu^5-enkephalin (10,11). This enzymatic activity was described as trypsin-like on the basis of the potent inhibition observed by trypsin inhibitors such as soybean trypsin inhibitor (STI) and aprotinin. In this report, we describe the partial purification of this enzyme and show that the partially purified enzyme retains the ability to produce met^5-enkephalin from endogenous high molecular weight substrate(s).

EXPERIMENTAL PROCEDURES

Enzyme preparation

Approximately fifty fresh bovine adrenal glands were obtained from a local slaughterhouse and kept chilled on ice until all the medullae were dissected. Chromaffin granules were prepared as described by Bartlett and Smith (12) and were lysed by freezing followed by thawing at 4°C in 5 ml of 5 mM Tris-HCl buffer, pH 7.4. Following centrifugation at 59,360 x g for 1 hr, proteins in the clear supernatant were precipitated by the addition of ammonium sulfate to 75% saturation. The membranes were reextracted by homogenizing with an additional aliquot of buffer and subjected to another freeze-thaw cycle. Following centrifugation, proteins in the supernatant were again precipitated with ammonium sulfate. The ammonium sulfate precipitates were combined by dissolving in 4-5 ml of 50 mM Tris-HCl, pH 7.4, and dialyzed overnight against 4 liters of the same buffer at 0-4°C to remove ammonium sulfate and endogenous low molecular weight enkephalin-immunoreactive substances (10). The dialyzed material was centrifuged at 20816 x g for 20 min to remove insoluble material and assayed for protein by the Lowry method (13). Typically, 70-100 mg protein were obtained from 50 adrenal glands. One to two hundred mg of protein were pumped through a 5.5 x 0.9 cm column of soybean trypsin inhibitor coupled to Sepharose-4B (Pierce Chemical Co.) at 0-4°C with a flow rate of 0.4 ml/min; 1.6 ml fractions were collected. The column was washed for approximately one hour with 50 mM Tris-HCl, pH 7.4. The material retained on the column was then eluted with 0.25 M acetic acid, pH 3.0. The pH of this eluate was immediately raised to approximately 7 with 1 M Tris, and the protein-containing fractions were pooled and concentrated by ultrafiltration at 4°C through an Amicon dialysis unit containing a PM-10 filter. The retained material was washed by dilution with 50 mM Tris-HCl, reconcentrated, stored frozen in aliquots, and used as the enzyme source. The material that was not retained by the STI column was pooled and concentrated by the addition of ammonium sulfate to 75% saturation. The precipitate was dialyzed overnight at 4°C against 50 mM Tris-HCl, pH 7.4, and the protein concentration measured (15-20 mg/ml). This fraction was stored frozen in aliquots and used as the substrate for the production of enkephalin-IR peptides. The purity of the chromaffin granule preparation was assessed by using acid phosphatase as a lysosomal marker (14); the granule pellet was found to contain 11% of the acid phosphatase activity recovered from the sucrose density gradient. Cathepsin B activity of the soluble protein fraction applied to the STI column was measured by the method of Barrett (15) using Bz-arg-napthylamide as substrate. Hydrolyzing activity was found to be at the limit of detection of the assay (< 5 nmol) even after a two-hour incubation of up to 600 μg protein.

Enzyme assay

The substrate fraction was heated at 95°C for 5 minutes before use to destroy possible enzymatic activity. Triplicate samples containing various amounts of

substrate and enzyme protein (as stated in table and figure legends) were incubated in a total volume of 100 µl of 0.1 M Tris-HCl, pH 7.5 at 37°C for 1-3 hours. Control samples containing the same amounts of substrate and enzyme protein were not incubated but heated immediately at 100°C for 5 minutes; controls with heated enzyme as well as without enzyme were carried out. Incubations were terminated by heating in a boiling water bath for 5 minutes. The samples were then chilled in an ice bath, frozen, and lyophilized. Low molecular weight enkephalin-immunoreactive peptides were extracted from the lyophilized reaction mixtures by vortexing or brief sonication with 1 ml of absolute ethanol. Following centrifugation at 4810 x g for 30 minutes, 0.8 ml of the clear supernatant was removed. This procedure results in the selective extraction of low molecular weight enkephalin-IR peptides (10). Aliquots of the ethanol extract were dried under vacuum in a Speed-Vac concentrator and resuspended in radioimmunoassay buffer (0.10 M sodium phosphate, pH 7.4, containing 0.1% each of bovine serum albumin, sodium azide, and 2-mercaptoethanol). Trypsin inhibitors were obtained from Sigma.

Radioimmunoassay

The met-enkephalin radioimmunoassay was carried out according to a previously published procedure (16) using [^{125}I]met^5-enk and antiserum raised in this laboratory to met^5-enkephalin-hemocyanin conjugate (17); the sensitivity of the assay was approximately 50 fmol. This antiserum exhibits total cross-reactivity with met^5-enk sulfoxide and arg^1-met^6-enk; however, the cross-reactivity to carboxyl terminal extended peptides such as arg^6-met^5-enk is extremely low (<0.5%).

HPLC

The dried ethanol extracts were dissolved in 350 µl of water containing 0.1% 2-mercaptoethanol and 200 µl were applied to a 250 x 4 mm reverse phase BioSil ODS-10 column (Biorad) equilibrated with 34% methanol in 0.1% trifluoroacetic acid. The column was eluted at a flow rate of 1 ml/min and 1 ml fractions were collected. Following 35 minutes of isocratic elution, the methanol concentration was raised to 60% in ten minutes and this concentration maintained for an additional ten minutes. The column was standardized by monitoring the absorbance at 220 nm of met^5-enk sulfoxide, arg^6-met^5-enk, met^5-enk, arg^1-met^6-enk, leu^5-enk, and arg^6-phe^7-met^5-enk (Peninsula). In addition, internal standards of [^{3}H]-met^5-enkephalin were also employed. Recovery of immunoreactivity, as estimated by the immunoreactivity in the sample applied to the column and the sum of the immunoreactive peaks eluted, was 80-100%.

Table 1

PURIFICATION OF ENKEPHALIN-GENERATING ENZYMATIC ACTIVITY

Step	Protein (mg)	Specific Activity (pmol met^5-enk-IR generated/mg prot/hr)	Total Activity (pmol met^5-enk-IR generated/hr)	Purification (fold)	Yield
Granule preparation	110	106	11660	--	--
STI-Sepharose	0.24	21920	5220	207	45%

Approximately 0.5 mg protein of granule preparation or 0.5 mg heated substrate fraction + 2.5 µg enzyme fraction were incubated in triplicate for 3 hrs at 37°C. Following termination of the reaction by boiling, low molecular weight enkephalin-IR peptides were extracted from the reaction mixtures using ethanol as described in the text. The mean value for control samples (reaction mixture heated immediately) was subtracted from the incubated samples to yield the total met^5-enk-IR generated.

Table 2

RECONSTITUTION OF AFFINITY-PURIFIED ENZYME AND SUBSTRATE[a]

Condition	Control	Incubated	Net Met^5-Enk-IR Production
Heated Substrate	9.7	4.1	0
Enzyme	3.2	3.0	0
Heated Substrate + Enzyme	11	100	89

[a]Values are expressed as pmol met^5-enk equivalents/3 hr incubation period; 500 µg of heated substrate protein and 2.6 µg protein of enzyme preparation were used.

RESULTS AND DISCUSSION

The purification of enkephalin-IR-generating activity achieved by affinity chromatography on STI-Sepharose, approximately 200-fold, is shown in Table 1. The most important accomplishment of this step is the separation of enzymatic activity from substrate (Table 2). When aliquots of the heated substrate fraction were

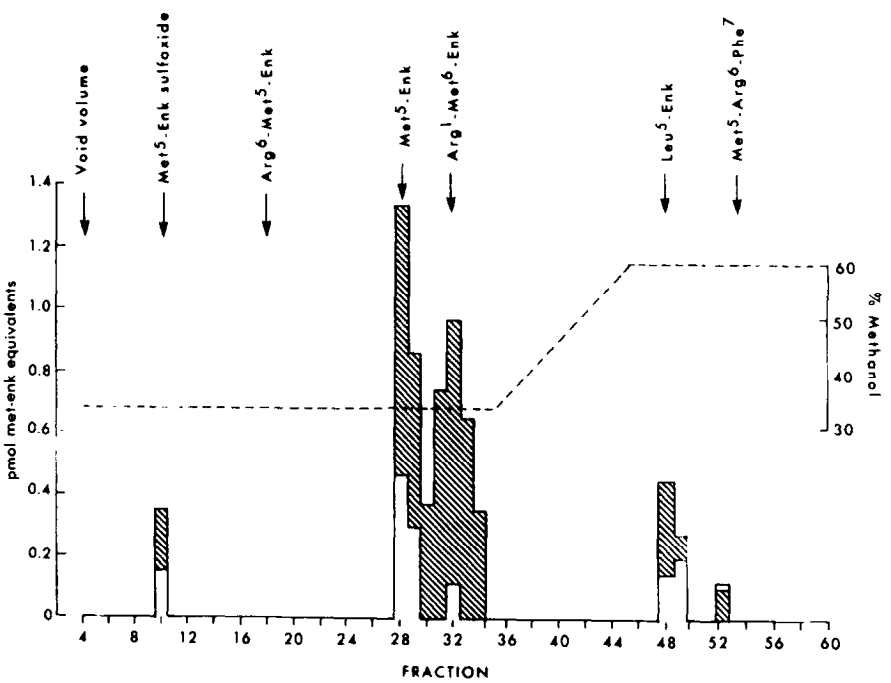

Figure 1. Reverse-phase HPLC of met^5-enk-IR generated by enzyme preparation. Heated substrate (200 µg protein) was combined with 1.3 µg protein from the enzyme fraction and the mixture incubated for 1 hr at 37°C (shaded bars) or heated immediately (open bars).

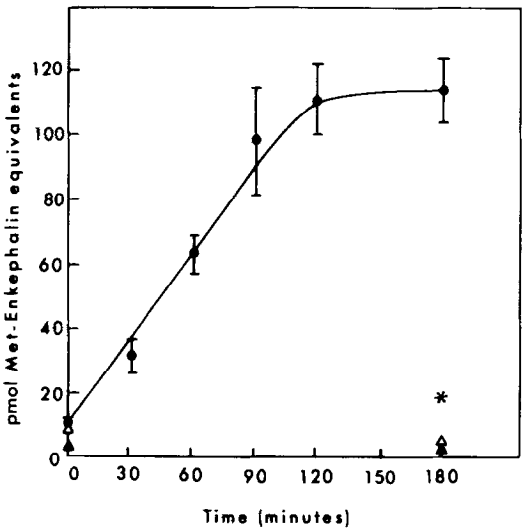

Figure 2. Time course of met^5-enk-IR production by enzyme preparation. ●——●, enzyme + heated substrate; △, substrate alone; ▲, enzyme alone; *, heated enzyme. Triplicate samples containing 0.5 mg heated substrate protein were incubated with the enzyme preparation (2.6 µg protein).

incubated in the absence of enzyme, no low molecular weight enkephalin-immunoreactive materials were produced. Similarly, incubation of enzyme alone did not result in an increase of immunoassayable enkephalin. However, when material from the two peaks obtained from the affinity column were incubated together, a ten-fold increase in met^5-enkephalin-IR was observed (Table 2). The immunoreactive material produced by the enzyme reaction was characterized by HPLC. As shown in Figure 1, immunoreactive peaks which were increased after incubation at 37°C for one hour included met^5-enkephalin and a peak eluting in the position of arg^1-met^6-enkephalin; further characterization of this later-eluting peak was not performed. These results indicate that the partially purified enzyme preparation is able to generate met^5-enkephalin from endogenous substrate(s). The enzyme preparation has little or no enkephalin-degrading activity; after a three hour incubation of [^{3}H]met^5-enk with the enzyme and substrate at 37°C, 88% of the radioactivity migrated at the position of met^5-enkephalin on HPLC (data not shown).

The time course of met^5-enkephalin-IR production is shown in Figure 2. Enkephalin-IR production reached a plateau at approximately 2 hours. A 60 minute incubation period was chosen to study the dependence of enkephalin production on

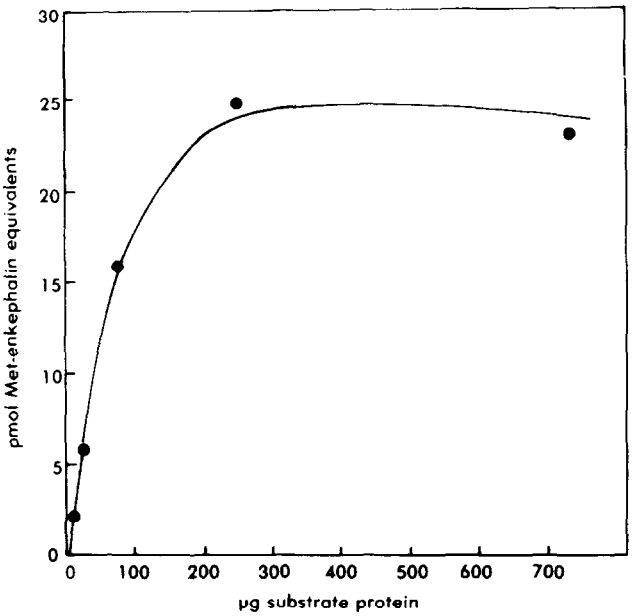

Figure 3. Dependence of met^5-enk-IR production on substrate concentration. Varying amounts of heated substrate and 1.7 µg enzyme were incubated in triplicate at 37°C for one hour; control samples were heated immediately. Results are expressed as the difference of the means of boiled and incubated samples; triplicates typically exhibited a variation of less than 15%.

substrate concentration (Fig. 3). Under these conditions, enkephalin-IR production was maximal using 200 µg protein of the substrate fraction. The dependence of enkephalin-IR production on substrate concentration and incubation time are compatible with an enzymatic production of met^5-enkephalin; the data given in Table 3 provide further support for this suggestion. As orginally found using a protein fraction prepared from whole granule lysate (10), STI was a highly potent inhibitor of enkephalin generation; at a concentration of 0.1 mg/ml, STI suppressed enkephalin-IR production by 80%. HPLC of reaction mixtures incubated in the presence of STI indicated inhibition of both the met^5-enk as well as the later-eluting peak. Since the addition of PCMS and leupeptin failed to suppress enkephalin-IR production (Table 3), the enzymatic activity described here is not likely to be due to a lysosomal enzyme such as cathepsin B (14). The fact that the levels of Bz-arg-napthylamide hydrolyzing activity in the granule lysate were extremely low further supports this conclusion. Since complete inhibition was not obtained using TLCK (10^{-3}M) and p-aminobenzamidine (10^{-3}M), this enzyme probably is not identical to

Table 3
INHIBITION OF MET^5-ENKEPHALIN-IR PRODUCTION[a]

Substance	Final Conc.	% Control
STI	1 mg/ml	0
	0.1 mg/ml	16.8
Aprotinin	1 mg/ml	22.6
TLCK	1 mM	60.0
p-Aminobenzamidine	1 mM	80.9
o-Phenanthroline	1 mM	87.4
Leupeptin	1 µg/ml	89.8
PCMS	1 mM	89.8
Dithiothreitol	2 mM	94.0
Calcium	2 mM	91.6
Sodium	20 mM	98.8
DFP[b]	1 mM	25.8

[a] Triplicate samples containing 200 µg of heated substrate protein and 1.7 µg of enzyme preparation were incubated for 60 minutes; results are expressed as the percentage of immunoreactive enkephalin generated in the absence of inhibitors.

[b] The enzyme preparation was preincubated with 5 mM DFP at 4°C for 10 minutes before addition to the reaction mixture.

trypsin (18,19). The inhibition observed in the presence of DFP indicates however that the enzyme falls into the class of serine proteases.

Prohormone converting activity has recently been described in rat pituitary secretory granules (20) as well as secretory granules prepared from anglerfish pancreatic islets (21). Unlike the enzymatic activity reported here, these arginyl proteases appear to be thiol-dependent and are strongly inhibited by p-chloromercuribenzoate and leupeptin (20,21). A protease specific for paired basic residues has been purified from pig pituitary glands (22). This enzyme exhibits some similarities to the enkephalin-generating activity described here in that it could be inhibited by DFP; however, partial inhibition by sulfhydryl reagents was also seen (22). It is of interest to note that similar paired-basic cleavage activity was observed in lysates prepared from beef anterior pituitary granules (22).

The results of these experiments thus provide evidence for a novel trypsin-like medullary enzyme which is capable of producing met^5-enkephalin from endogenous substrate(s). The existence of such an enzyme is further substantiated by a additional report describing the presence of trypsin-like enzymatic activity in bovine chromaffin granules (23). Previous workers have proposed a biosynthetic

pathway for met^5-enkephalin involving first a trypsin-like cleavage to produce arginine- and lysine-extended met^5-enkephalins, followed by carboxypeptidase B-like cleavage to yield free met^5-enkephalin (4,24). The results presented here indicate that at least *in vitro*, a portion of granule met^5-enkephalin may be synthesized with only trypsin-like enzymatic activity. Whether this medullary trypsin-like enzyme operates *in vivo* to generate met^5-enkephalin remains to be established; we are currently attempting to purify this enzyme and characterize its substrate(s) in an effort to answer this question.

ACKNOWLEDGEMENTS

I. Lindberg was a Pharmacology Research Associate supported by NIGMS, NIH.

REFERENCES

1. Kimura, S., Lewis, R.V., Stern, A.S., Rossier, J., Stein, S., and Udenfriend, S. (1980) Proc. Natl. Acad. Sci. USA 77, 1681-1685.
2. Lewis, R.V., Stern, A.S., Kimura, S., Stein, S., and Udenfriend, S. (1980) Proc. Natl. Acad. Sci. USA 77, 5018-5020.
3. Lewis, R.V., Stern, A.S., Kimura, S., Rossier, S., Stein, S., and Udenfriend, S. (1980) Science 208, 1459-1461.
4. Stern, A.S., Jones, B.N., Shively, J.E., Stein, S., and Udenfriend, S. (1981) Proc. Natl. Acad. Sci. USA 78, 1962-1966.
5. Mizuno, K., Minamino, N., Kangawa, K., and Matsuo, H. (1980) Biochem. Biophys. Res. Commun. 97, 1283-1290.
6. Hexum, T.D., Yang, H.-Y.T., and Costa, E. (1980) Life Sci. 27, 1211-1216.
7. Clement-Jones, V., Lowry, P.J., Rees, L.H., and Besser, G.M. (1980) Nature 283, 295-297.
8. Hexum, T.D., Hanbauer, I., Govoni, S., Yang, H.-Y.T., and Costa, E. (1980) Neuropeptides 1, 137-142.
9. Lewis, R.V., Stern, A.S., Rossier, J., Stein, S., and Udenfriend, S. (1979) Biochem. Biophys. Res. Commun. 89, 822-829.
10. Lindberg, I., Yang, H.-Y.T., and Costa, E. (1982) Adv. Biochem. Psychopharmacol., in press.
11. Lindberg, I., Yang, H.-Y.T., and Costa, E. (1981) Proc. Soc. Neurosci. 7, 91.
12. Bartlett, S.F., and Smith, A.D. (1974) Methods in Enzymology XXXI. Biomembranes, Part A, pp. 379-389, Academic Press, New York.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
14. Gianetto, R., and De Duve, C. (1955) Biochem. J. 59, 433.
15. Lindberg, I., Smythe, S.J., and Dahl, J.L. (1979) Brain Res. 168, 200-204.
16. Yang, H.-Y.T., Hong, J.S., and Costa, E. (1977) Neuropharmacology 16, 303-307.
17. Barrett, A.S. (1977) Proteinases in Mammalian Cells and Tissues, pp. 181-205, Elsevier/North Holland, Amsterdam.
18. Shaw, E., Mares-Guia, M., and Cohen, W. (1974) Biochemistry 4, 2219-2224.
19. Mares-Guia, M., and Shaw, E. (1965) J. Biol. Chem. 240, 1579.
20. Loh, Y.P., and Gainer, H. (1982) Proc. Natl. Acad. Sci. USA 79, 108-112.
21. Fletcher, D.J., Quigley, J.P., Bauer, G.E., and Noe, B.D. (1981) J. Cell Biology 90, 312-322.
22. Austen, B.M. (1982) Proceedings of the Seventh American Peptide Symposium, pp. 493-496, Pierce Chemical Co., New York.
23. Troy, C.M., and Musacchio, J.M. (1981) Proc. Sco. Neurosci. 7, 91.
24. Hook, V.H., Eiden, L.E.E., and Brownstein, M.S. (1982) Nature 295, 341-342.