Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 230-236

TISSUE DISTRIBUTION AND CHARACTERIZATION OF PEPTIDE C-TERMINAL α-AMIDATING ACTIVITY IN RAT

Junichiro SAKATA, Kensaku MIZUNO, and Hisayuki MATSUO

Department of Biochemistry, Miyazaki Medical College,
Kiyotake, Miyazaki 889-16, Japan

Received August 21, 1986

SUMMARY: The C-terminal α-amide formation of peptides is one of the most important events in prohormone processing. Recently, we developed a simple and sensitive assay for detecting α-amidating activity in tissues by using (^{125}I)-Ac-Tyr-Phe-Gly as a substrate. Using this assay method, we have determined the tissue distribution of α-amidating enzyme activity in adult male rat. High concentrations of α-amidating activity were found in pituitary, brain, thyroid, gastrointestinal tract, pancreas, heart, submaxillary glands and parotid glands. Alpha-amidating enzyme activities in all tissues examined exhibit very similar copper and ascorbate requirements, pH dependence, and behavior on gel-filtration.

A number of biologically active peptides identified in neural and endocrine tissues have an α-amide structure at their carboxyl termini. In most cases, the presence of the C-terminal α-amide structure is essential for biological activity. Recent elucidation of the nucleotide sequences of many precursors of α-amidated peptides suggests that biosynthesis of the C-terminal α-amide may involve the action of a specific enzyme which converts the peptide with C-terminal glycine residue to the corresponding des-glycine peptide α-amide. In 1982, Bradbury et al. first characterized the α-amidating activity which converts synthetic tripeptide D-Tyr-Val-Gly to D-Tyr-Val-NH_2, in extracts of porcine pituitary secretory granules (1). They also demonstrated that a C-terminal glycine in the substrate serves as a nitrogen-donor for α-amidation of the preceding amino acid residue (1). Since then, several investigators have characterized α-amidating enzyme activity in pituitary and other tissues including brain, thyroid, submaxillary glands and parotid glands (2-11). However, tissue distribution of the enzyme activity had not been clarified because of the low sensitivity and poor reproducibility of assays for α-amidating enzyme activity in crude tissue extracts. Recently, we developed a simple and sensitive assay for detecting α-amidating activity in tissues (7,8), and succeeded in purifying an α-amidating enzyme to homogeneity from Xenopus laevis skin (8). The present paper reports tissue distribution

0006-291X/86 $1.50

and characterization of α-amidating activity in adult male rat by using our assay.

MATERIALS AND METHODS

Materials: The radiolabeled tripeptide substrate ((^{125}I)-Ac-Tyr-Phe-Gly) and the corresponding α-amidated product ((^{125}I)-Ac-Tyr-Phe-NH_2) were synthesized and purified as reported in our previous papers (7,8).

Preparation of Tissue Extracts: Tissues from adult male rats (Wister) were dissected out immediately after decapitation. After weighing, tissues were homogenized with a Polytron mixer for 2 min in 5-50 volumes (v/w) of the extraction buffer (50 mM Tris-HCl buffer (pH 7.0) containing 10 mM N-ethylmaleimide, 1 mM DFP and pepstatin (10 μg/ml)). After centrifugation at 32,000 xg for 60 min, solid ammonium sulfate was added to the supernatant solution to a final concentration of 70% saturation. After stirring for 4 hr at 4°C, the precipitates obtained by centrifugation at 14,500 xg for 60 min were dissolved in the extraction buffer. Final volume of the extract was adjusted to 5-50 volumes (v/w) of the wet weights of tissues. The solution was frozen and stored at -80°C until used.

Enzyme Assays: The assay for α-amidating enzyme activity used in the distribution study was as follows (7,8): The enzyme solution prepared as above was incubated in a final volume of 250 μl with 4 nM (^{125}I)-Ac-Tyr-Phe-Gly (about 70,000-150,000 cpm), 50 μM CuSO4, 0.5 mM ascorbate, 100 μg/ml catalase (from beef liver, Boehringer) and 0.2 M Tris-HCl buffer (pH 7.0). The reaction was allowed to proceed for 2 hr at 37°C. Then 750 μl of 1 M Tris-HCl buffer (pH 7.0) and 2 ml of the organic phase of an ethyl acetate/water mixture was added. The two phases were mixed vigorously on a Vortex mixer. After centrifugation at 3000 rpm for 3 min, the organic phase thus separated was carefully transferred to another test tube. The radioactivity in organic and aqueous layers was each measured by a gamma scintillation counter. The yield of conversion was calculated from the ratio of the radioactivity in the ethyl acetate phase to the total radioactivity. The α-amidating activity in each tissue was determined in the linear range of conversion (0-70%) of substrate to product. One unit of activity is defined as the amount of enzyme that gives 50% conversion of substrate to product under the standard assay conditions (1 hr) described above. Protein concentration was estimated by the method of Lowry et al. (12).

RESULTS AND DISCUSSION

Assay for α-amidating activity

We have recently developed a simple and sensitive assay for detecting α-amidating activity in tissues by using (^{125}I)-Ac-Tyr-Phe-Gly as a substrate (7,8). The C-terminally α-amidated product (^{125}I)-Ac-Tyr-Phe-NH_2 can be separated by a simple procedure of ethyl acetate extraction from the substrate, which remains in the aqueous layer. By measuring the radioactivity in the organic and aqueous layers, the yield of conversion from the substrate to the α-amidated product was easily determined. The adequacy of this assay was confirmed by analyzing the reaction products on reverse-phase HPLC (Fig. 1). The radioactive material extracted in the ethyl acetate layer after the reaction was eluted as a single radioactive peak exactly identical with that of the authentic (^{125}I)-Ac-Tyr-Phe-NH_2, clearly indicating that the radioactivity extracted in the ethyl acetate layer was composed of the newly formed α-amidated peptide.

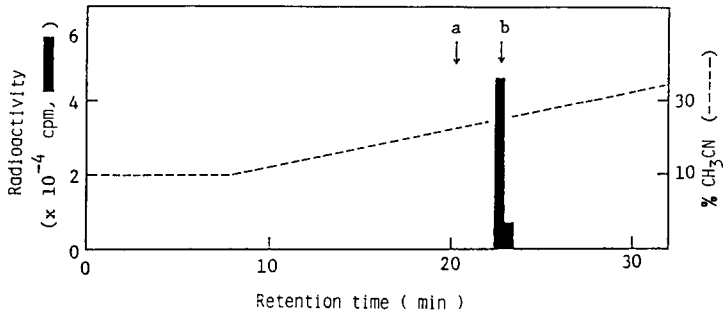

Fig. 1. Identification of (^{125}I)-Ac-Tyr-Phe-NH_2 produced from (^{125}I)-Ac-Tyr-Phe-Gly by the action of α-amidating activity in rat stomach. The radioactive materials extracted in ethyl acetate layer after the reaction was analyzed by reverse-phase HPLC on a column (0.39 x 30 cm) of μBondapak C-18 (Waters). Elution was carried out with a linear gradient (40 min) of CH_3CN concentration from 10 to 50% in 10 mM ammonium formate (pH 4.0) at a flow rate of 2.0 ml/min. Fraction size: 1.0 ml/0.5 min. Arrows indicate the elution positions of authentic peptides: a, (^{125}I)-Ac-Tyr-Phe-Gly; b, (^{125}I)-Ac-Tyr-Phe-NH_2.

Tissue distribution of α-amidating enzyme

Prior to the measurement of α-amidating enzyme activity in tissue, optimal assay conditions were determined for each type of tissue extract. As shown in Fig. 2 (O), α-amidating activity in the crude extract of tissue varied with the concentration of copper ion exogenously added. On the other hand, when tissue was extracted with buffer containing 10 mM N-ethylmaleimide

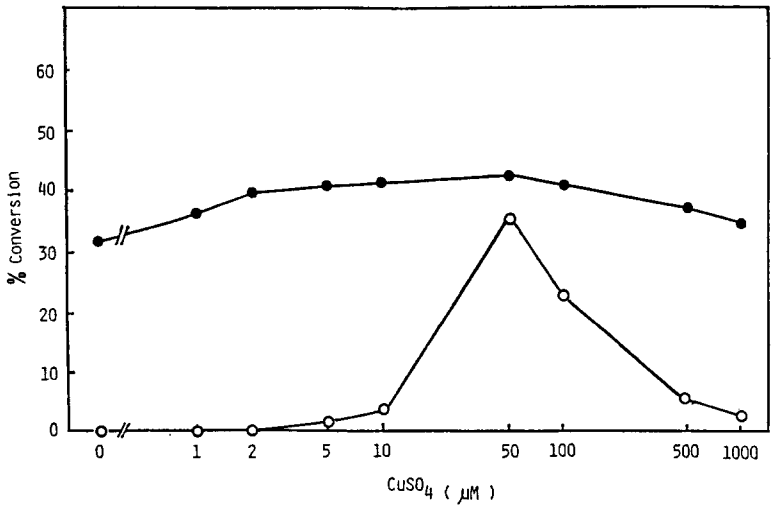

Fig. 2. Effect of copper ion concentration on α-amidating activity of rat stomach. The enzyme was prepared in two ways. (O); extraction with 50 mM Tris-HCl buffer (pH 7.0) containing 1 mM DFP and pepstatin (10 μg/ml). (●); extraction with the buffer containing 10 mM N-ethylmaleimide followed by ammonium sulfate precipitation as described in "MATERIALS AND METHODS". All assays were carried out by incubating the substrate for 2 hr with 80 μg protein of extract under conditions described in "MATERIALS AND METHODS" except for the copper concentration.

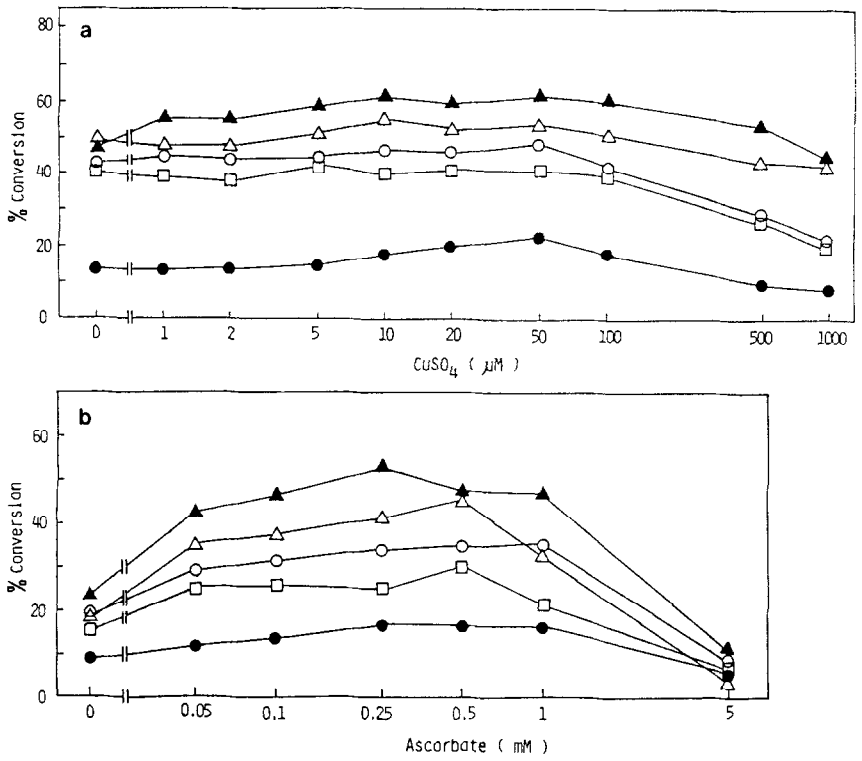

Fig. 3. (a) Copper dose response curve of α-amidating activity. Tissues were prepared as described in "MATERIALS AND METHODS". Extracts of gastric corpus (100 μg protein) (○), ileum (101 μg protein) (●), pituitary (12 μg protein) (△), submaxillary glands (38 μg protein) (▲) and atrium (21 μg protein) (□) were incubated for 2 hr with the substrate under conditions described in "MATERIALS AND METHODS" except for the copper ion concentration. (b) Ascorbate dose response curve of α-amidating activity. Tissues were prepared and incubated with the substrate as described above except for the ascorbate concentration exogenously added.

and the extract was submitted to ammonium sulfate precipitation, α-amidating activity in the tissue extract exhibited a constant value in the range of copper ion concentration between 2-100 μM (Fig. 2, ●). It seems likely that the addition of N-ethylmaleimide may overcome the inhibitory effects of endogenous thiol compounds (7,8). Thus, α-amidating activity in various tissues, such as ileum, pituitary, submaxillary glands and atrium were found to be accurately determined and compared, when the tissues were prepared by the procedures as above (Fig. 3a). In the present study, assays were routinely carried out in the presence of 50 μM $CuSO_4$. Fig. 3b shows the effects of ascorbate concentration on α-amidating enzyme activity. In the tissues examined, the optimal concentration of ascorbate for enzyme activity was found to be 0.25-0.5 mM. Assays for determining the distribution were performed in the presence of 0.5 mM ascorbate.

Under the conditions described above, the distribution of α-amidating activity in rat tissues was determined (Table 1). As a result, α-

Table 1. Distribution of α-amidation activity in rat tissues

Tissues	α-Amidation activity (units/mg protein)	Tissues	α-Amidation activity (units/mg protein)
Esophagus	0.220 ± 0.068	Brain(without cerebellum)	3.332 ± 1.118
Proventricle	0.392 ± 0.098	Cerebellum	0.286 ± 0.160
Gastric corpus	3.188 ± 0.800	Pituitary	45.824 ± 12.414
Gastric antrum	2.602 ± 0.366	Thyroid gl.	6.470 ± 0.744
Duodenum	1.950 ± 0.290	Submaxillary gl.	10.760 ± 2.488
Jejunum	1.160 ± 0.324	Parotid gl.	8.396 ± 0.826
Ileum	1.852 ± 0.494	Thymus	undetectable
Cecum	1.016 ± 0.210	Adrenal gl.	0.258 ± 0.036
Colon	1.996 ± 0.058	Lung	0.132 ± 0.020
Pancreas	0.838 ± 0.216	Kidney	0.034 ± 0.016
Heart	2.192 ± 0.554	Spleen	0.054 ± 0.006
Atrium	17.920 ± 0.836	Liver	undetectable
Ventricle	0.222 ± 0.063		

Data are means ± SD (n=3).

amidating activity was newly identified in gastrointestinal tract and pancreas, which are the sites responsible for the biosynthesis of many gastrointestinal hormones with C-terminal α-amide structure, such as gastrin, cholecystokinin, secretin, vasoactive intestinal polypeptide (VIP), pancreatic polypeptide and so on. Furthermore, substantial amounts of α-amidating activity were newly identified in heart. Among the tissues examined, pituitary had the highest specific activity. It was noted that heart, submaxillary glands and parotid glands, where the presence of α-amidating peptides had not yet been known, had the next highest level of α-amidating activity. In heart, most of the enzyme activity localized in atrium. A high level of activity was also observed in thyroid gland and brain. In gastrointestinal tract, gastric corpus and antrum had the highest specific activity. Taking into account the total mass of tissue, gastrointestinal tract was considered to be the major source of α-amidating enzyme activity in rat. Enzyme activity in cerebellum,

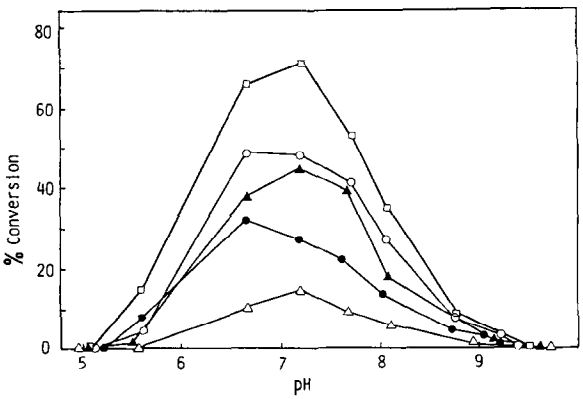

Fig. 4. Effect of pH on α-amidating activity. Extracts of gastric corpus (○), ileum (●), pituitary (△), submaxillary glands (▲) and atrium (□) were incubated with the substrate under conditions in "MATERIALS AND METHODS". The pH of the solution was adjusted with HCl or NaOH solution.

adrenal glands, lung, kidney and spleen could be detected even though in low levels. Enzyme activity in liver and thymus was not detectable under the present assay conditions.

Properties of α-amidating activity in tissues

Preliminary characterization of α-amidating activities in gastric corpus, ileum, pituitary, submaxillary glands and atrium was carried out. Alpha-amidating activity in each tissue was completely inhibited by the addition of 0.1 mM EDTA, but reverted under the presence of excess amounts (0.12 mM) of $CuSO_4$. As shown in Fig. 3b, α-amidating activity in each tissue was stimulated by exogenous ascorbate. Optimal pH of enzyme activity in each tissue was determined to be around 6.5-7.5 (Fig. 4). Fig. 5 shows patterns of gel-filtration of extracts obtained from rat pituitary, stomach, ileum, submaxillary glands, atrium and thyroid. In all cases, the major activity

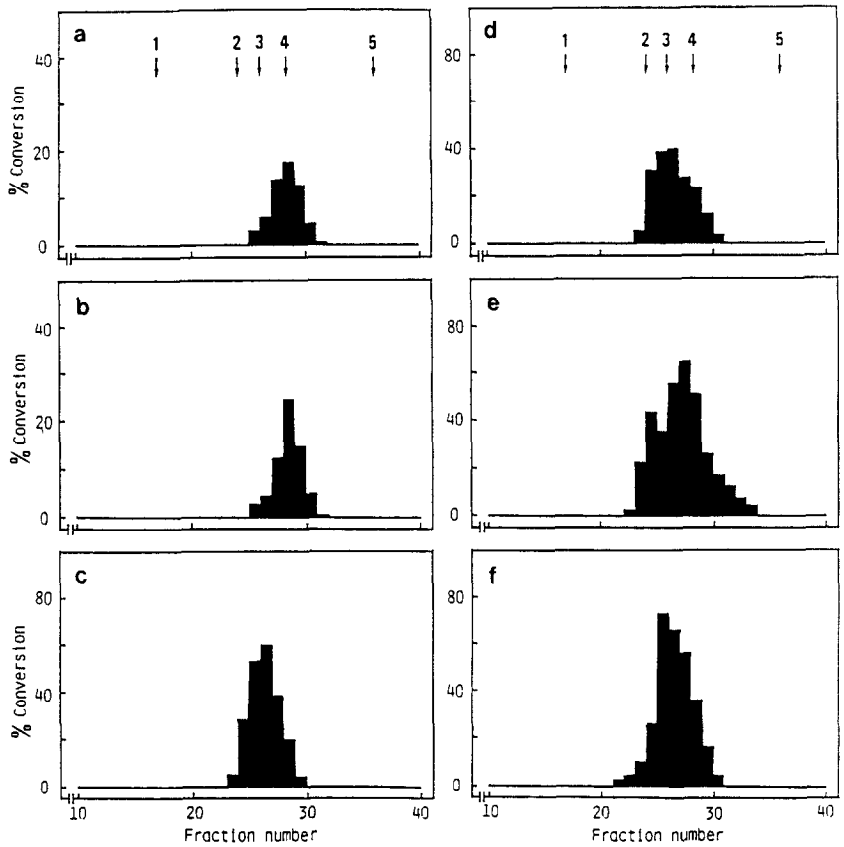

Fig. 5. Gel-filtration of rat tissue extracts. Extracts of tissues were chromatographed on a Superose 12 column (1.0 x 30 cm, Pharmacia), equilibrated with 50 mM Tris-HCl buffer (pH 7.0) containing 0.1 M NaCl and 2 μM $CuSO_4$. Flow rate: 0.5 ml/min. Fraction size: 0.5 ml/tube. (a) stomach , (b) ileum, (c) pituitary, (d) thyroid glands, (e) submaxillary glands, (f) atrium. Aliquots (100-150 μl) from each fraction were assayed as described in "MATERIALS AND METHODS". Arrows indicate the elution positions of thyroglobulin (1), bovine serum albumin(2), ovalbumin(3), soybean trypsin inhibitor (4) and cytochrome c (5), respectively.

emerged at an elution position corresponding to a molecular weight of about 30,000-60,000. Thus, α-amidating activities in rat stomach, ileum, pituitary, submaxillary glands and atrium all appear to exhibit very similar copper and ascorbate requirements, pH dependence, and behavior on gel-filtration. However, the relatively broad peaks of activity observed on gel-filtration may reflect the molecular heterogeneity of the α-amidating enzyme in each tissue.

To understand the mechanism and regulation of α-amide formation in neural and endocrine tissues, further purification and characterization of α-amidating enzyme in these tissues of mammals is now going on in our laboratory, utilizing the methods described for our previous purification of the enzyme in Xenopus laevis skin (8).

REFERENCES

1. Bradbury, A.F., Finnie, M.D.A. & Smyth, D.G. (1982) Nature 298, 686-688.
2. Eipper, B.A., Mains, R.E. & Glembotski, C.C. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 5144-5148.
3. Husain, I. & Tate, S.S. (1983) FEBS Lett. 152, 277-281.
4. Kizer, J.S., Busby, W.H., Cottle, C. & Youngblood, W.W. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 3228-3232.
5. Emeson, R.B. (1984) J. Neurosci. 4, 2604-2613.
6. Eipper,B.A., Myers,A.C., & Mains,R.E. (1985) Endocrinology 116, 2497-2504.
7. Mizuno,K., Kojima,M., Sakata,J. & Matsuo,H. (1986) Peptide Chemistry 1985, pp.351-356, Protein Research Foundation, Osaka.
8. Mizuno, K., Sakata, J., Kojima, M., Kangawa, K. & Matsuo, H. (1986) Biochem. Biophys. Res. Commun. 137, 984-991.
9. von Zastrow, M., Tritton, T.R., & Castle, J.D. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 3297-3301.
10. Murthy, A.S.N., Mains, R.E. & Eipper, B.A. (1986) J. Biol. Chem. 261, 1815-1822.
11. Kizer,J.S., Bateman,R.C., Miller,C.R., Humm,J., Busby,W.H. & Youngblood, W.W. (1986) Endocrinology 118, 2262-2267.
12. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. & Randall, J. (1951) J. Biol. Chem. 193, 265-275.